A CYCLOPHILIN-DEPENDENT MITOCHONDRIAL PORE.

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ABSTRACT

The immunosuppressant Cyclosporin A (CsA) has been shown to protect against cell injury caused by ischaemia and reperfusion. A mitochondrial lesion in the form of a Ca\(^{2+}\) sensitive non-selective inner membrane pore is reputed to contribute to the progression of this form of injury and the pore is blocked by CsA. This study was aimed at resolving the CsA receptor of the pore to establish whether this protein was novel or whether it was already recognised. Mitochondrial CsA binding proteins were identified using a tritiated derivative of CsA, \(^{3}\)H[PA-CS], which contains a photoactive diazirine group at position 8 of the molecule. Many components were photolabelled. In order to identify the relevant protein use was made of the fact that CsA binding to mitochondrial membranes was potentiated by ADP and depressed by Ca\(^{2+}\). Heart mitochondria were photolabelled in the presence of ADP (2mM) or Ca\(^{2+}\) (>100\(\mu\)M). Fractionation of mitochondrial membranes extracted in detergent revealed components between 11 and 25kDa exhibiting ADP/ Ca\(^{2+}\)-sensitive labelling. ADP increased \(^{3}\)H[PA-CS] labelling of this fraction whilst Ca\(^{2+}\) reversed it. The ADP/ Ca\(^{2+}\)-sensitive labelled component was purified to a single band on SDS-PAGE migrating at approximately 22kDa. Identical proteins were purified from sheep heart and rat liver mitochondria. The 22kDa protein displayed peptidylprolyl cis trans isomerase (PPIase) activity suggesting it belonged to the cyclophilin (CyP) family of proteins. The protein displayed a \(K_I\) of 8nM for CsA and a \(k_{cat}/K_m\) of 5.8\(\mu\)M\(^{-1}\) s\(^{-1}\). This agrees well with other known cyclophilins. Partial amino acid sequencing of the protein revealed sequence similarity with human cyclophilin D (CyPD). CyPD was prone to proteolytic attack during purification. However removal of the outer mitochondrial membrane with low concentrations of digitonin and the subsequent treatment of the mitoplasts with 0.5M [NaCl] resulted in increased stability with an almost 10 fold increase in protein yield. The localisation of CyPD was investigated using fractionated mitochondria. This showed that it is localised in the matrix space. Though CyPD is photolabelled in an ADP/ Ca\(^{2+}\)-sensitive manner, the \(^{3}\)H[CsA binding capacity of CyPD was unaffected by ADP [2mM] or Ca\(^{2+}\) [>100\(\mu\)M]. Also the PPIase activity of the protein was not influenced by high concentrations of ADP or Ca\(^{2+}\). This, as well as photolabelling studies, suggested that CyPD binds to another component in intact mitochondria that is sensitive to ADP and Ca\(^{2+}\), and that this interaction confers the same sensitivity to CyPD-CsA interaction. The occasional elution of the ADP/ Ca\(^{2+}\) sensitive component at 29kDa, rather than 22kDa, suggests that the target might be a low molecular weight protein. However a CyPD affinity column failed to retain any specifically interacting target protein from inner membrane extracts. These findings provide firm evidence that CyPD interacts with the Ca\(^{2+}\) sensitive pore and that it might be involved in pore regulation.
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The work presented produced the following publications;

Involvement of cyclophilin D in the activation of a mitochondrial pore by \( \text{Ca}^{2+} \) and oxidant stress.

Evidence for the involvement of membrane-associated cyclosporin-A-binding protein in the \( \text{Ca}^{2+} \)-activated inner membrane pore of heart mitochondria.

On the nature of cyclosporin A binding component of the mitochondrial \( \text{Ca}^{2+} \)-dependent pore.

A cyclophilin D dependent mitochondrial pore and its role in cell death.
## Contents

List of Figures and Tables. 1

### Chapter 1: Introduction.

- [1.1] Pore History. 5
- [1.2] $\text{Ca}^{2+}$ regulation in heart. 6
- [1.3] Mitochondrial $\text{Ca}^{2+}$ regulation. 7
- [1.4] $\text{Ca}^{2+}$ regulation during Ischaemia and Reperfusion. 12
- [1.5] Pore Characteristics. 15
- [1.6] Effectors of the pore. 16
  - [1.6.1] Inducers: 16
    - [1.6.2] $\text{Ca}^{2+}$. 16
    - [1.6.3] Phosphate. 17
    - [1.6.4] Oxidative Stress. 18
    - [1.6.5] Thiol reagents. 21
    - [1.6.6] Inhibitors of the ADP/ATP translocase. 22
    - [1.6.7] Fatty acids. 23
- [1.7] Pore Inhibitors. 23
  - [1.7.1] Adenine nucleotides. 24
  - [1.7.2] Free radical scavengers. 24
  - [1.7.3] Cations. 25
[1.7.4] Inhibitors of the adenine nucleotide translocase. 26
[1.7.5] pH 26
[1.7.6] Phospholipase A2 inhibitors. 27
[1.7.7] Cyclosporin A. 27
[1.7.8] Other inhibitors of the pore. 29

[1.8] Cyclosporin A and Cyclophilins. 29
[1.8.1] Cyclosporin A (CsA). 29
[1.8.2] Cyclophilins and FKBPs. 30
[1.8.3] Other CsA binding proteins. 31

[1.9] Immunosuppression and cyclophilins. 32
[1.9.1] Calcineurin. 33
[1.9.2] Involvement of calcineurin in T-cell activation. 34

[1.10] Localisation and function of cyclophilins. 34
[1.10.1] Cyclophilins A, B, C, and D. 34
[1.10.2] Role of cyclophilins. 36
[1.10.3] Cyclophilin and the Ca²⁺-activated membrane pore. 40

[1.11] Other models of the Ca²⁺-activated pore. 41
[1.11.1] Membrane impairment hypothesis. 41
[1.11.2] The mitochondrial megachannel (MMC). 43
[1.11.3] Mitochondrial benzodiazepine receptor (mBzR). 44
[1.11.4] Ca²⁺ release without the induction of the membrane pore
(prooxidant - induced Ca²⁺ release). 45

[1.12] The role of the pore in ischaemic/reperfusion injury. 46
[1.13.1] Cell Death;  


Chapter 2: Materials and Methods.  

[2.1] Preparation of rat heart and liver mitochondria.  

[2.2] Preparation of Sheep heart mitochondria.  


[2.4.1] Introduction.  
[2.4.2] Photolabel.  
[2.4.3] Protocol for photolabelling.  
[2.4.4] Preparation of photolabelled Submitochondrial Particles (SMPs).  

[2.5.1] Use of CHAPS as detergent.  
[2.5.2] The determination of $^3$H[PA-CS] binding to mitochondrial phospholipids.  

[2.6] Calibration of gel permeation columns  


[2.8] Inhibition of liver mitochondria carboxylesterase activity using substituted trifluoromethylketones.
[2.8.1] Carboxylesterase assay. 59

[2.9] Measurement of Peptidylprolyl cis trans isomerase (PPIase) activity. 59

[2.9.1] Rationale of assay. 59

[2.9.2] PPIase activity. 63

[2.10] Measurement of $[^3$H]CsA binding using Sephadex LH-20 minicolumns. 64

[2.11] Preparation of mitochondrial subfractions 66

[2.11.1] Preparation of PPIase from mitochondrial subfractions. 68

[2.12] SDS-PAGE analysis of purified proteins. 70

[2.12.1] Staining of gels. 75

[2.13] CyP22-affinity chromatography. 78

[2.13.1] PPIases and their interactions 78

[2.13.2] Attempts to isolate CyP22 binding proteins using affinity chromatography. 78

[2.14] Isoelectrofocusing (IEF). 79

[2.14.1] Carrier Ampholytes. 80


[2.15] Attempt at immobilising CyP to activated Sepharose column. 82

Chapter 3: Results.

[3.1] Identification of mitochondrial CsA binding proteins by photolabelling using $[^3$H][PA-CS]. 91

[3.1.1] Introductory: Identification of CsA binding proteins. 91

[3.1.2] Introductory: Mitochondrial CsA binding proteins. 92

[3.1.3] Introductory: The effects of ADP and Ca$^{2+}$ on pore function. 96
[3.1.4] Introductory: How might photolabelling to the relevant CsA binding component of the pore be effected by ADP and Ca$^{2+}$.

[3.1.5] The photolabelling of mitochondria in the presence of Ca$^{2+}$ and ADP.

[3.1.6] Resolution of photolabelled SMP components on a Superose 12 gel filtration column connected in dries with a Superdex 75 gel filtration column.


[3.1.8] Inhibition of mitochondrial carboxylesterase activity by substituted trifluoromethylketones.


[3.1.10] LH-20 and PPIase activity measurements also demonstrate the existence of a cyclophilin between 11-22kDa in size.

[3.1.11] Comparison of the ADP/ Ca$^{2+}$ sensitive labelled PPIase with CyP18.

[3.1.12] Examination PPIase binding to Ca$^{2+}$ and ADP.


[3.2] Purification of the ADP/ Ca$^{2+}$ sensitive labelled PPIase from mitochondria.

[3.2.1] Analysis of solubilised SMPs from photolabelled mitochondria on ion exchange chromatography.
[3.2.2] A photolabelled component with a high molecular weight was also fractionated on Mono S cation exchange column.

[3.2.3] The major peak observed on Mono S column eluted at the same position as CyP18 on Superdex 75 gel filtration column.

[3.2.4] The ADP/ Ca$^{2+}$ sensitive photolabelled PPIase displays a molecular weight between 22-23kDa.

[3.2.5] The single radioactivity peak observed on Mono S is identical to the CsA binding protein that displays ADP/ Ca$^{2+}$ sensitive photolabelling.

[3.2.6] Measurement of $K_{cat}/K_m$ of CyP22 and its $K_d$ for CsA.

[3.2.7] The location of CyP22 in SMPs.

[3.2.8] The salt-dissociable PPIase and the CHAPS extracted PPIase are the same proteins.

[3.2.9] Investigation into the stability and localisation of CyP22 in mitochondria.

[3.2.10] Purification of each PPIase activity from the fractionated mitochondria.

[3.2.11] Separation of CyP22 and CyP18 on the basis of overall charge.

[3.2.12] Overall summary.

[3.3] Analysis of CyPD (CyP22) binding partner(s) that are responsible for ADP/ Ca$^{2+}$ sensitive photolabelling of the PPIase in intact mitochondria.

[3.3.1] Preservation of PPIase activity under different conditions.
[3.3.2] The use of iodinated CyPD to investigate potential interacting partners.

[3.3.3] The use of Bolton and Hunter reagent (BHR) to modify CyPD.

[3.3.4] Isoelectrofocusing of modified CyPD.

[3.3.5] The effects of ADP and Ca\(^{2+}\) on the salt dissociable PPIase activity from rat liver mitoplasts.


[3.4.] The dissociation of CyPD from the inner membrane is potentiated by Ca\(^{2+}\) but unaffected by other pore ligands.

[3.4.1] Introduction.

Chapter 4: Discussion.

[4.1] Photolabelling of mitochondrial membranes is ADP/ Ca\(^{2+}\) sensitive.

[4.2] ADP and Ca\(^{2+}\) binding sites in mitochondria.

[4.3] A 22kDa PPIase (CyP22) that is identical to CyPD photolabelled in an ADP/ Ca\(^{2+}\) dependent manner.

[4.4] CyPD (CyP22) contains no intrinsic ADP or Ca\(^{2+}\) binding sites.


[4.6] CyPD is found in the matrix and inner membrane fraction of mitochondria.


[4.8] The association of CyPD with the membrane pore.
[4.9] The effect of pore ligands on the association and dissociation of CyPD with the membrane

[4.10] Cyclophilin and the membrane pore in SMPs.


[4.12] Further studies

Bibliography
List of Figures and Tables.

Fig. 1. Ca$^{2+}$ regulation in a typical heart cell.

Fig. 2. Schematic representation of Cyclosporin A.

Fig. 3. Reaction catalysed by peptidylprolyl cis trans isomerase.

Fig. 4a. A Schematic representation of $^3$H[PA-CS].

Fig. 4b. Procedure for the subfraction of mitochondria.

Fig. 5. Modification of lysine residues by Bolton and Hunter reagent.

Fig. 6. Reagent (6-aminohexanoic acid N-hydroxysuccinimide ester) used to construct a CyPD affinity column.

Fig. 7a Elution pattern of $^3$H]CsA in different concentrations of CHAPS.

Fig. 7b. The elution pattern of $^3$H]CsA in 6% CHAPS/ pH 7.2 on a LH-20 minicolumn equilibrated in 0.6% CHAPS.

Fig. 8. Calibration of Superose 12 and Superdex 75 (Pharmacia) gel permeation columns at pH 7.2.

Fig. 9. A wide range of membrane components (13-66kDa) are covalently labelled by $^3$H[PA-CS].

Fig. 10. The soluble fraction of mitochondria does not demonstrate ADP/ Ca$^{2+}$ sensitive photolabelling.

Fig. 11a. Membrane components with approximate mol.wt 13kDa are covalently labelled in an ADP/ Ca$^{2+}$-sensitive manner.

Fig. 11b. The effect of ADP and Ca$^{2+}$ on the photolabelling of heart mitochondrial membranes.

Fig. 12a. Gel filtration analysis of the influence of ADP, Ca$^{2+}$ and EGTA on membrane labelling of liver mitochondria.

Fig. 12b. The effect of ADP and Ca$^{2+}$ on the photolabelling of Liver mitochondrial membranes.
Fig. 13. Fractionation of \(^3\)H[PA-CS] labelled rat heart mitochondria on Superose 12/ Superdex 75 series column demonstrates components (approximately 15kDa in size) that covalently label in an ADP/ Ca\(^{2+}\)-dependent manner.

Fig. 14a. Gel filtration analysis of rat heart mitochondrial membranes revealed a component between 26-29kDa that photolabelled in ADP/ Ca\(^{2+}\)-sensitive manner.

Fig. 14b. Gel filtration analysis of rat heart mitochondrial membranes revealed a photolabelled component between 26-29kDa.

Fig. 15. The photolabelling of mitochondrial phospholipids.

Fig. 16. The elution pattern of free \(^3\)H[CsA mixed with membrane extract on Superose 12 gel filtration column.

Fig. 17. Inhibition of carboxylesterase activity by substituted trifluorolketones.

Fig. 18. \(^3\)H[PA-CS] labelling pattern is unaffected by the addition of 4,4,4 Trifluoro-1, 3-phenyl-1,3-butanedione (TFPB).

Fig. 19. SDS-PAGE analysis of the Ca\(^{2+}\) -sensitive and ADP -sensitive photolabelling of heart mitochondria.

Fig. 20. Mitochondrial binding proteins are excluded in the void volume.

Fig. 21. Membrane component expressing ADP/ Ca\(^{2+}\)-sensitive photolabelling also display peptidylprolyl cis trans isomerase activity and \(^3\)H[CsA binding capacity.

Fig. 22. Elution profile of CyP18 on a Superdex 75 (Pharmacia) gel permeation column.

Fig. 23. Fractions exhibiting \(^3\)H[CsA binding and PPIase activity correlate well with ADP/ Ca\(^{2+}\) sensitive photolabelling profile on series column.

Fig. 24. \(^3\)H[CsA binding capacity of 22kDa PPIase from rat heart mitochondria is unaffected by pore ligands Ca\(^{2+}\) and ADP.

Fig. 25. Fractionation of photolabelled membranes reveals a single \(^3\)H peak between 75-100mM [NaCl] on a Mono-S cation exchange column (1ml, Pharmacia).

Fig. 26. Two radioactivity peaks are revealed on cation exchange chromatography.

Fig. 27. The tighter binding component (figure 26 (II)) is probably the high molecular weight contaminant observed in the soluble fraction of mitochondria.
Fig. 28. A single radioactivity peak corresponding to a molecular weight of 18kDa is resolved on narrow range gel filtration column.

Fig. 29. A single protein peak corresponding to 18kDa is observed with contamination at high and low molecular weights.

Fig. 30. A single $^3$H[PA-CS] is observed at 100mM NaCl concentration.

Fig. 31. $^3$H[PA-CS] activity peaks at a molecular weight around 18kDa.

Fig. 32. The labelled protein from rat heart mitochondria demonstrates a molecular size of 22-23kDa.

Fig. 33. SDS-PAGE reveals a single peak corresponding to a molecular weight 22kDa.

Fig. 34. The elution profile of photolabelled SMPs on Mono-S chromatography correlates well with $^3$H[CsA] binding profile of extracted proteins on Mono-S cation exchange column.

Fig. 35. A single PPIase exhibiting in rat heart mitochondria is developed between 100-125mM [NaCl] on Mono-S.

Fig. 36. CyP18 and CyP22 are observed as two different proteins on SDS-PAGE analysis.

Fig. 37a. Peptidylprolyl cis trans Isomerase activity of the heart 22kDa protein.

Fig. 37b. Data from Fig 31a plotted according to the relationship $\ln (A_t/A_m) = -kt$ where $A_t$ is the absorbance change that occurred subsequent to any time $t$ and $A_m$ is the maximal absorbance change recorded.

Fig. 37c. The ratio $k_{cat}/K_m$ (from Fig 37b) was determined at the different CsA concentrations.

Fig. 38. CyP22 is dissociated from membranes in high salt media.

Fig. 39. The detergent extracted PPIase displays a molecular weight around 26-27kDa on SDS-PAGE.

Fig. 40. Salt dissociable PPIase is sensitive to proteolysis.

Fig. 41. Silver staining followed by Blue toning of salt dissociable and CHAPS extracted PPIases.

Fig. 42. CHAPS significantly affects the migration of the PPIase.

Table. 43a, b, c, and d. Purification of salt dissociable and CHAPS extractable 22kDa PPIase from sheep and rat heart mitochondria.
Fig. 44. The purification of PPIases from different fractions of rat liver mitochondria.

Fig. 45. Distribution of PPIase activity in rat liver mitochondria.

Fig. 46. Amino acid sequencing of CyP22

Fig. 47. The partial fractionation of CyP18 and CyP22 using cation exchange chromatography.

Fig. 48. High pH, PMSF and protease inhibitors are needed to preserve PPIase activity of CyP22.

Fig. 49. The PPIase activity of CyP22 is stable in 50% Glycerol.

Fig. 50. Total activity PPIase activity before and after treatment with Bolton and Hunter reagent.

Fig. 51. Peptidylprolyl cis trans isomerase activity of CyP22 is retained after modification with Bolton and Hunter reagent.

Fig. 52. Isoelectrofocusing of modified CyP22 revealed 2 major bands between pH 7.9-8.3 on a denaturing gel.

Fig. 53. ADP Ca^{2+} -sensitive binding partner of CyP22 is not found attached to the outside of the mitochondrial inner membrane.

Fig. 54. Non-specific interactions of inner membrane proteins with Sepharose-4B matrix activated with 6-aminohexanoic acid N-hydroxysuccinimide ester are minimised at high pH.

Fig. 55. Cyp22 affinity column is unable to retain any specifically interacting component from the mitochondrial inner membrane.

Fig. 56. Almost all PPIase activity associated with the inner membrane is displaced by [50mM] NaCl.

Fig. 57. Ca^{2+} potentiates the dissociation of CyP22 from rat liver inner membranes whilst NAD^+, NADP^+, Na_2HPO_4 and tertiary Butylhydroperoxide are ineffective.

Table 58. NAD^+, NADH, NADP^+, and NADPH do not effect the association/dissociation of CyP22 with the inner membrane.
Mitochondrial oxidative phosphorylation using membrane potential requires the inner membrane to be permeable only to solutes that possess specific transport systems. The loss of inner membrane impermeability can occur through processes that damage the integrity of the membrane bilayer or through the incorporation of pore forming molecules in the membrane. Other than these mechanisms mitochondria become non-specifically permeable to solutes only if loaded with Ca\(^{2+}\) and presented with one of a huge number of different inducing agents. The detrimental effects of Ca\(^{2+}\) on the permeability of mitochondria have been known for sometime. Early swelling studies [Lehninger, (1967)] showed Ca\(^{2+}\) and phosphate to uncouple mitochondrial respiration. This dysfunction was prevented by adenine nucleotides and potentiated by pyridine nucleotide-oxidising agents (e.g. oxalacetate, acetoacetate) [Lehninger et al, (1978)]. It was not until the late 70s that heralded the landmark work of Hunter and Haworth [Haworth and Hunter, (1979); Hunter and Haworth, (1979a/b); Haworth and Hunter, (1980)] in which the pore model was introduced and explained the Ca\(^{2+}\) dependent permeabilisation of the inner membrane. These workers first proposed the idea of a Ca\(^{2+}\) - sensitive, hydrophilic proteinaceous channel or pore that allowed non-specific permeation of solutes through the inner membrane. Rather than depending on the nature of the molecules, i.e. charge, molecular type, the passage of components through the pore was reported to be dependent upon their molecular size (i.e. approx. 1500Da).

Using solute entrapment techniques Crompton and colleagues demonstrated the reversibility of the Ca\(^{2+}\) activated permeability of the inner membrane [Al-Nasser and Crompton, (1986a/b)]. Later, employing pulse flow solute entrapment techniques, the same group went on to study the kinetic properties of the pore and estimate its diameter [Crompton and Costi, (1990)].

Following Fournier’s earlier finding that the cyclic undecapeptide immunosuppressant Cyclosporin A (CsA) (fig. 2) could adversely delay Ca\(^{2+}\) induced permeabilisation of mitochondria [Fournier, (1987)], Crompton [Crompton et al, (1988)] and Pfeiffers’ groups [Broekemeier et al, (1989); Broekemeier and Pfeiffer, (1989)] went on to report the inhibition of mitochondrial permeabilisation by CsA at nanomolar concentrations. CsA binding at
60pmol/ mg of mitochondrial protein was sufficient to completely inhibit Ca\(^{2+}\) and phosphate induced pore opening. With the discovery of such high binding affinity, the use of CsA to investigate components of the pore was begun.

The presence of Ca\(^{2+}\) was sufficient to induce pore opening, however pore opening was accelerated by the addition of an inducer, for example phosphate. A huge variety of inducers that potentiate the opening of the Ca\(^{2+}\) sensitive pore in mitochondria have been demonstrated. Similarly, a wide array of inhibitors of the pore have also been documented which largely counteract the effects of pore inducers. These findings suggested a common site through which both inhibitors and inducers of the pore exerted their effects.

Recent electrophysiological experiments in which mitoplast membranes were patched clamped revealed the existence of high conductance channels on the inner membrane [Sorgato et al, (1987)]. The conductance of these channels was inhibited by Cyclosporin A, ADP and Mg\(^{2+}\) and stimulated by Ca\(^{2+}\) [Szabo and Zoratti, (1992)]. Due to these characteristics, the high conductance channels of the membrane were postulated to represent the Ca\(^{2+}\) activated pores of membranes.

Based on the fact that CsA inhibited the peptidyl prolyl cis \textit{trans} isomerase activity (PPIase) (fig. 3) of a family of proteins called cyclophilins [Fischer et al, (1984); Takahashi et al, (1989)] a cyclophilin was hypothesised to play a critical role in pore function [Halestrap and Davidson, (1990); Halestrap, (1994)]. Recent studies have shown a PPIase activity intimately involved in regulating the release of Ca\(^{2+}\) into the cytoplasm from the sarcoplasmic reticulum of muscle cells [Timerman et al, (1993); Brillantes et al, (1994)]. Therefore, based on this and other evidence, it was possible to suggest that a PPIase was also involved in modulating the channel activity of the membrane pore.

[1.2] \underline{Ca\(^{2+}\) regulation in heart.}

Since the pore is believed to play a critical role in Ca\(^{2+}\) induced mitochondrial dysfunction, it would be useful to briefly look at Ca\(^{2+}\) fluxes in beating heart cells and mitochondria, and relate this to Ca\(^{2+}\) changes in ischaemia/ reperfusion. A large [Ca\(^{2+}\)]
gradient exists across the sarcolemmal membrane of heart cells (approx. $10^5$). The extracellular free $[\text{Ca}^{2+}]$ of the cell is around 1mM whilst intracellular free $[\text{Ca}^{2+}]$ transiently varies between 0.1μM to 2μM during each beat of the cell. Under normal conditions of contraction-relaxation the depolarisation of the sarcolemmal membrane causes the voltage dependent $\text{Ca}^{2+}$ channel to allow $\text{Ca}^{2+}$ to enter the cell. This influx of $\text{Ca}^{2+}$ mediates the release of $\text{Ca}^{2+}$ from the sarcoplasmic reticulum (SR) stores (via $\text{Ca}^{2+}$ induced $\text{Ca}^{2+}$ release) and both then stimulate contraction of the cell. The sudden rise in cytosolic free $[\text{Ca}^{2+}]$ (up to or greater then 1μM) is rapidly removed by the ATP dependent $\text{Ca}^{2+}$ pumps located in the SR phospholipid bilayer and the sarcolemmal membrane. Besides ATP dependent $\text{Ca}^{2+}$ extrusion, $\text{Ca}^{2+}$ is removed more efficiently by the Na$^+$/Ca$^{2+}$ exchanger located in the sarcolemmal membrane [Reuter and Seitz, (1968)] (fig. 1). The exchanger makes use of the downhill energy gradient of Na$^+$ into the cell to extrude Ca$^{2+}$ out of the cell. Na$^+$ entering the cell in this manner would then be expelled from the cell by the ATP dependent Na$^+$/K$^+$ pump (fig. 1). The role of the ATP dependent pumps in heart cells is therefore the rapid and periodic removal of $\text{Ca}^{2+}$ from the cytosol so that the relaxation of the contractile proteins can occur.

[1.3] Mitochondrial $\text{Ca}^{2+}$ regulation.

Mitochondria possess an elaborate cycle for transport of $\text{Ca}^{2+}$ across the inner membrane. The cycle consists of three mechanisms that show carrier or gated pore kinetics and perhaps a fourth mechanism in the form of a $\text{Ca}^{2+}$ activated inner membrane pore. The mitochondrial $\text{Ca}^{2+}$ cycle is slow and unregulated [Crompton, (1990)] and therefore mitochondrial free $[\text{Ca}^{2+}]$ changes follow the cytosolic free $\text{Ca}^{2+}$ transients, albeit rather more slowly. Mitochondria have a physiological requirement for $\text{Ca}^{2+}$ since it serves to integrate cytosolic energy demands with mitochondrial metabolism. Initially it was considered that mitochondrial free $\text{Ca}^{2+}$ served to buffer the increase in extramitochondrial $[\text{Ca}^{2+}]$ and played a role in regulating cytoplasmic free $[\text{Ca}^{2+}]$ [Nicholls, (1978)]. However, studies by Denton and McCormack presented evidence to suggest that mitochondrial $\text{Ca}^{2+}$ enhanced the activity of key enzymes involved in controlling the overall rate of mitochondrial oxidative metabolism
Fig. 1. Ca^{2+} regulation in a typical heart cell.
Fig. 2. Schematic representation of Cyclosporin A.
Fig. 3. Reaction catalysed by peptidylprolyl cis trans isomerase activity.
Intramitochondrial pyruvate dehydrogenase, NAD⁺-linked isocitrate dehydrogenase, oxoglutarate dehydrogenase and presumably ATP synthase are all controlled by the matrix concentration of free Ca²⁺. An intramitochondrial [Ca²⁺] between 0.2-3μM is needed to activate these enzymes. As a result it appears that the messenger role of Ca²⁺ is used to co-ordinate oxidative metabolism in mitochondria with the energy requirement of the cytoplasm of the cell. Therefore an increase in heart muscle contractility results in an increase in cytosolic [Ca²⁺]. The increase in cytosolic [Ca²⁺] is consequently relayed to the mitochondrial matrix where it increases the rate oxidative metabolism and the rate of ATP synthesis [Denton and McCormack, (1985)]. Hormones such as adrenaline that increase the rate of heartbeat increase the cycling of Ca²⁺ into the cytoplasm (through protein kinase A activity). This results in an increase in the rate of mitochondrial NADH formation via the Ca²⁺ dependent dehydrogenases leading to an increase in the rate of oxidative phosphorylation.

Ca²⁺ is transported into the mitochondrial matrix down its electrochemical gradient through the Ca²⁺ uniporter located in the inner membrane. Ca²⁺ transport into the matrix is an energy dependent process that requires a membrane potential. By incubating mitochondria with different concentrations of FCCP (p-trifluoromethoxy carbonyl cyanide phenylhydrazone, membrane potential dissipater), graded levels of Ca²⁺ accumulation were demonstrated [Rottenberg and Scarpa, (1974)]. As the transporter is present in very small amounts (1pmol/mg of mitochondrial protein) [Reed and Bygrave, (1974)] it has an extremely high V_max (in excess of 1200nmol Ca²⁺/mg protein/ per min at 22°C). This feature of the uniporter allows very fast removal of extramitochondrial Ca²⁺ when the cytosolic free [Ca²⁺] rises above 0.5-1μM. The Ca²⁺ uniporter is a difficult component to isolate and purify and has not been clearly identified. Recently though, proteins have been purified from mitochondria that display Ca²⁺ transport kinetics similar to the Ca²⁺ uniporter [Panfili et al, (1980); Miranova et al, (1982); Zazueta et al, (1994)].

Two efflux mechanism are responsible for transporting Ca²⁺ out of the matrix. The Na⁺/Ca²⁺ exchanger of the inner membrane [Jung et al, (1995)] exchanges 2 or more Na⁺ for Ca²⁺ and displays a V_max of about 18 or 3nmol Ca²⁺/mg protein/min in heart and liver.
respectively. The $H^+$/Ca\(^{2+}\) exchanger substitutes 2 or more $H^+$ for a Ca\(^{2+}\) (fig. 1), exhibits a $V_{max}$ of 2nmol Ca\(^{2+}\) / mg protein/ min and is an energy requiring process (since the inner membrane pH gradient is dissipated to a certain extent).

[1.4] **Ca\(^{2+}\) regulation during Ischaemia and Reperfusion.**

In normal myocardial function, the transient increases in cytosolic [Ca\(^{2+}\)] are quickly removed by Ca\(^{2+}\) ATPases located on the sarcolemmal membrane and SR phospholipid bilayer (fig. 1) and by the Na\(^+\)/Ca\(^{2+}\) exchanges on the sarcolemmal membrane. Therefore mitochondria are never exposed to high concentrations of free cytosolic Ca\(^{2+}\) for long periods of time. Since the mitochondrial Ca\(^{2+}\) transport cycle is low and unregulated, it never has sufficient time to respond to the transient increases in cytosolic [Ca\(^{2+}\)]. Since mitochondrial Ca\(^{2+}\) levels follow the [Ca\(^{2+}\)] changes in the cytosol very slowly, the transmission of transient [Ca\(^{2+}\)] changes to the matrix is largely damped out. As a result mitochondrial matrix [Ca\(^{2+}\)] changes during each heart beat are very small. As already mentioned the slow nature of the Ca\(^{2+}\) transport system in mitochondria allows it to regulate key enzymes involved in oxidative metabolism. Myocytes loaded with the Ca\(^{2+}\) indicator Indo I were used to demonstrate the response of mitochondrial Ca\(^{2+}\) transport cycle to changes in cytosolic [Ca\(^{2+}\)]. After high frequency electrical stimulation of single heart cells, mitochondrial [Ca\(^{2+}\)] increased after about one minute. This suggested that single cytosolic Ca\(^{2+}\) transients were insufficient to induce mitochondrial [Ca\(^{2+}\)] change. Rather, a succession of cytosolic [Ca\(^{2+}\)] transients over a period of time caused the mitochondrial Ca\(^{2+}\) transport cycle to readjust to a higher steady state and the mitochondrial free [Ca\(^{2+}\)] to increase [Miyata et al, (1991)]. It appears that if cytosolic free [Ca\(^{2+}\)] is maintained (non-transient) at raised levels than mitochondria will have sufficient time to adjust to the elevated cytosolic free [Ca\(^{2+}\)] and, as a result, accumulate massive amounts of Ca\(^{2+}\). Elevated levels of non-transient cytosolic free [Ca\(^{2+}\)] do not occur under normal metabolic conditions but they do exist in ischaemia. In this situation mitochondrial Ca\(^{2+}\) overload is inevitable. During ischaemia the resting cytosolic free [Ca\(^{2+}\)] in heart cells is elevated and remains so beyond 1μM [Chiens and Engler, (1990)]. Although the mitochondrial Ca\(^{2+}\) transport cycle is slow and unregulated it now has sufficient time to
follow the elevated $[\text{Ca}^{2+}]$ of the cytosol. The transport cycle continues to take up $\text{Ca}^{2+}$ into the matrix and the $\text{Ca}^{2+}$ efflux mechanisms become completely saturated. This ultimately leads to mitochondrial $\text{Ca}^{2+}$ overload (intramitochondrial free $[\text{Ca}^{2+}]$ greater than 25$\mu$M). It is the elevated level of free mitochondrial $[\text{Ca}^{2+}]$ that activates the non-specific $\text{Ca}^{2+}$-sensitive inner membrane pore.

The drop in ATP levels is the underlying cause of high mitochondrial free $[\text{Ca}^{2+}]$. The most studied condition that causes an increased mitochondrial free $[\text{Ca}^{2+}]$ is ischaemia/reperfusion injury. If after a certain length of ischaemia heart cells are oxygenated, two processes can occur; the cells may return to preischaemic rates of respiration, termed reversible injury, or there is accelerated cell death, termed irreversible injury or reperfusion injury. Myocardial energy demands are very high. They are met by high rates of oxidative phosphorylation (providing almost 90% of total ATP) and glycolysis (providing the remaining 10% of total ATP) which together maintain a [ATP] greater then 5mM. A large number of biochemical changes occur in heart cells during ischaemia/reperfusion injury. The rate of ADP phosphorylation (i.e. ATP synthesis) decreases considerably whilst high energy phosphate (creatine phosphate) pools are exhausted [Jennings and Steenbergen, (1985)]. ATP is needed to maintain ionic homeostasis in heart cells. ATP is hydrolysed to sustain ionic gradients of different ions between different compartments of myocytes. During periods of ischaemia/reperfusion severe ionic imbalances occur [Pierce and Czubryt, (1995)]. These play a critical part in the development of tissue injury during ischaemia and reperfusion. The depletion of ATP leads to loss of active $\text{Ca}^{2+}$ extrusion via $\text{Ca}^{2+}$-ATPases of sarcolemmal membrane and active removal from the cytoplasm by ATP dependent $\text{Ca}^{2+}$ pump of SR. This leads to elevated levels of free $[\text{Ca}^{2+}]$ in the cytosol. Besides the loss of the $[\text{Ca}^{2+}]$ gradient across the sarcolemmal membrane other ionic gradients are also lost. Intracellular $\text{Ca}^{2+}$ efflux is dependent upon the $[\text{Na}^+]$ gradient across the plasma membrane. This gradient is maintained by ATP dependent $\text{Na}^+/\text{K}^+$ exchange of the plasma membrane. With the depletion of ATP level cytosolic free $[\text{Na}^+]$ is elevated [Tosaki et al, (1989)]. This inevitably leads to increased cytosolic free $[\text{Ca}^{2+}]$ since $\text{Ca}^{2+}$ efflux exchange is now inoperative. The $[\text{Na}^+]$ gradient is also important in regulating the intracellular pH through the $\text{Na}^+/\text{H}^+$ exchange [Mahensmith and Aronson, (1985)], since $\text{H}^+$ is extruded on the $\text{Na}^+$ gradient across the sarcolemmal
membrane. Elevated levels of free cytosolic free $[Na^+]$ lead to increased intracellular $[H^+]$ (acidosis). Elevated intracellular $[H^+]$ protects against mitochondrial $Ca^{2+}$ induced damage (see section 1.7.5) Severely depressed ATP levels with elevated levels of $Na^+$, $Ca^{2+}$, $H^+$ are conditions that persist during ischaemia and reperfusion.

Cytosolic $[Ca^{2+}]$ variations were investigated during global ischaemia by NMR using $^{19}$F-BAPTA (as $Ca^{2+}$ indicator) [Steenbergen, (1990)]. These studies showed that ATP depletion (as indicated by cell shortening) preceded the extensive rise in intracellular free $[Ca^{2+}]$. They reported resting cytosolic free $[Ca^{2+}]$ to increase beyond 1μM before irreversible injury occurred on reperfusion. In similar studies, myocytes microinjected with aequorin as a $Ca^{2+}$ indicator [Allshire et al (1987)] showed cytosolic free $[Ca^{2+}]$ to rise after cell shortening (i.e. severely depleted ATP levels). Analogous experiments in which luciferase loaded cells superfused with luciferin [Bowers et al, (1993)] showed a fall in the luciferase signal (between 4-15 minutes) in cells that coincided with cell shortening. Taken together, ATP levels have to be severely depressed (loss of around 90%) before rises (>1-2μM) in intracellular free $[Ca^{2+}]$ are observed. As expected ADP levels are briefly raised in these cells since the nucleotide is rapidly converted to nucleosides and bases (primarily inosine and xanthine) [Jennings and Steenbergen, (1985)]. Resting cytosolic free $[Ca^{2+}]$ rises in single heart cells after prolonged anoxia but is promptly restored to low levels after reoxygenation provided a critical limit around 2μM in not exceeded. If this value is exceeded then reoxygenation fails to restore $Ca^{2+}$ homeostasis [Allshire et al, (1987)]. As briefly mentioned earlier, an acutely lowered ATP level in conjunction with raised cytosolic free $[Ca^{2+}] >2μM$ may be a prerequisite for irreversible injury (reperfusion injury).

Elevated levels of free intracellular $[Ca^{2+}]$ during ischaemia/ reperfusion are relayed to mitochondria [Allen et al, (1993)] where they induce $Ca^{2+}$-sensitive mitochondria uncoupling via the putative membrane pore. Opening of the $Ca^{2+}$ induced inner membrane pore would result in the loss of membrane impermeability. Since ATP synthase is coupled to respiration via a $H^+$ circuit, then the introduction of an alternative route for $H^+$ influx (the pore) would uncouple oxidative phosphorylation and potentiate ATP hydrolysis. This would lead to more
Ca\(^{2+}\) entering the cell resulting in further stimulation of pore opening with continual ATP hydrolysis. Essentially the cell would be driven into a vicious cycle of energy deprivation.

Some researchers have ruled out the involvement of mitochondrial dysfunction in ischaemia/reperfusion injury. Siegmund et al [Siegmund et al, (1993)] used single myocytes to demonstrate a rise in the cytosolic free [Ca\(^{2+}\)] with severely depleted ATP levels. They stated that contractile proteins were sensitised by the increased free [Ca\(^{2+}\)] so that, upon reoxygenation (ATP synthesis), the cells hypercontracted which led to cytoskeletal damage. They implied that under conditions of irreversible injury (reperfusion) mitochondria remain functionally competent.

[1.5] **Pore Characteristics.**

Upon the activation of the Ca\(^{2+}\)-sensitive membrane pore mitochondrial membrane potential becomes dissipated and mitochondria become permeable to solutes (i.e. swelling). Mitochondria do not appear to display gradual permeabilisation but are either permeable or impermeable to solutes (this would rule out membrane perturbation as a possible cause of permeability, see section 1.11.1) [Al Nasser and Crompton, (1986a/b)]. These findings suggested that pore opening is an all-or-nothing event with no semi-open or closed states [Hunter et al, (1976); Al Nasser and Crompton, (1986a/b). Permeabilisation-resealing techniques used by Cromptons’ group further supported the existence of a non-specific pore. These workers showed that \(^{14}\)C sucrose entry into Ca\(^{2+}\) permeabilised mitochondria was immediately stopped by the addition of EGTA [Crompton and Costi, (1988); Al- Nasser and Crompton (1986a)]. Labelled sucrose and other components that had already entered mitochondria were trapped inside and no further entry took place as long the mitochondria remained in this state. If permeabilisation was due to uncharacteristic behaviour of the phospholipid bilayer then labelled sucrose would still have continued to enter mitochondria. These findings disagreed with the studies conducted by Pfeiffers’ group which suggested that lipid bilayer dysfunction was the cause of Ca\(^{2+}\) sensitive mitochondrial permeabilisation [Pfeiffer et al (1979); Beatrice et al, (1980); Broekemeier et al, (1985)] (see section 1.11.1).
Using entrapment techniques with fast time resolution Cromptons' group demonstrated that when the Ca\(^{2+}\) sensitive pore was induced by Pi (phosphate), the same amount of \(^{14}\)C sucrose entered the permeabilised mitochondria irrespective of whether the sucrose was present throughout the permeabilisation procedure, or added 4 seconds before resealing (using EGTA). This indicated that mitochondria were either in a completely permeable or a completely impermeable state, with the change from the former to the latter occurring with a \(t_{1/2}\) of 860ms [Crompton and Costi, (1988)]. Measuring relative rates of mannitol, sucrose and arsenazo III permeation the same group estimated the putative pore diameter to be approximately 2.5nm in diameter. Earlier experiments conducted by Haworth and Hunter (1979b) using various sized polyethylene glycol (PEG) clearly demonstrated a discrete cut-off at 1500Da. This correlated well with a pore diameter of 2.5nm. The hydrodynamic radii of PEG 1500 was estimated to be around 1.2nm [Ginsberg and Stein, (1987)]. Of other aqueous pores, porin from mitochondrial outer membrane has pore diameter of 1.7nm [Ludwig et al (1986)] and gap junction pores with 1.6 - 2.0nm [Loewenstein, (1985)].

[1.6] Effectors of the pore.

[1.6.1] Inducers:

[1.6.2] Ca\(^{2+}\).

The accumulation of Ca\(^{2+}\) by mitochondria is a prerequisite for pore opening. As briefly discussed earlier (section 1.3) mitochondria in heart cells with severely depressed ATP levels will take up huge amounts of Ca\(^{2+}\) with impunity. The need for Ca\(^{2+}\) is absolute since the addition of Ruthenium Red (RR) to block the Ca\(^{2+}\) uniporter, before Ca\(^{2+}\) accumulation, abolished permeability changes [Hunter and Haworth (1979a); Vercesi et al (1988)]. Mn\(^{2+}\) or Sr\(^{2+}\) will not substitute for Ca\(^{2+}\) dependent Ca\(^{2+}\) efflux, mitochondrial swelling or membrane depolarisation [Pfeiffer et al (1979); Hunter and Haworth (1979a); Beatrice et al, (1980)]. The degree of permeabilisation in a heterogeneous population of mitochondria is dependent upon the extent of the Ca\(^{2+}\) load. Some mitochondria are more susceptible to induction by Ca\(^{2+}\).
than others [Hunter et al, (1976)] and will therefore accumulate Ca\(^{2+}\) more rapidly than their more resilient counterparts. Evidence to support this has come from studies in which mitochondria were allowed to take up a quantity of Ca\(^{2+}\) known to be sufficient to lead to complete permeation of all organelles. On the addition of RR to depress further Ca\(^{2+}\), only part of the mitochondrial population was permeabilised. It was proposed that in the absence of RR vulnerable mitochondria released their Ca\(^{2+}\) load upon pore opening, the released Ca\(^{2+}\) was then taken up by the more resistant mitochondria until they were fully permeabilised. In this way all mitochondria will eventually become permeabilised after a certain length of time [Beatrice et al (1982); Riley and Pfeiffer, (1985)]. Ruthenium Red would protect the more resilient mitochondria from taking up the Ca\(^{2+}\) wave.

In the presence of RR Ca\(^{2+}\) influx into mitochondria was inhibited. However, upon the addition of a Ca\(^{2+}\) ionophore, ionomycin, the inhibition was relieved [Vercesi et al (1988)]. This indicated that Ca\(^{2+}\) induced permeabilisation was due to some matrix located Ca\(^{2+}\) binding sites [Bernardi et al (1992)].

1.6.3 Phosphate.

As mentioned earlier the effects Ca\(^{2+}\) on mitochondrial permeabilisation (i.e. swelling, membrane depolarisation) were potentiated by phosphate (Pi) [Drahota et al, (1965); Lotscher et al (1980); Crompton and Costi (1988)]. Inorganic phosphate stimulates both Ca\(^{2+}\) uptake and Ca\(^{2+}\) release [Roos et al, (1980)]. However, Pi had no effect on Ca\(^{2+}\) uptake or release when mitochondria were treated with N-ethylmaleimide [Beatrice et al, (1980)]. N-ethylmaleimide is postulated to modify sites involved in permeabilisation- see later, section 1.6.5. This suggested that Pi interacted with some matrix facing site of the pore. In place of phosphate, lactate depressed mitochondrial swelling and the loss of preaccumulated Ca\(^{2+}\) in liver mitochondria in which pyridine nucleotide pools had been oxidised by acetoacetate [Wolkowicz and McMillin, (1980)] (see section 1.6.4). This suggested that Pi triggered Ca\(^{2+}\) uptake, and efflux was an event independent of the redox state (NADH/ NAD) of mitochondria. Coupled mitochondria maintain a constant phosphorylation potential (ATP/ADP). Increase in extramitochondrial [Pi] will cause an increase in the ATP/ADP ratio as
ADP is transported out in exchange for Pi. A high extramitochondrial [Pi] was shown to induce the depletion of matrix adenine nucleotides via operation of the Pi/ATP-Mg\(^{2+}\) or Pi/HADP\(^{2-}\) antiporter [Hutson et al, (1989); Lapidus and Sokolove, (1994)]. Since ADP and ATP are known inhibitors of the pore (see section 1.7.1), loss of these agents would clearly potentiate Pi induced Ca\(^{2+}\) release through the Ca\(^{2+}\) sensitive membrane pore.

[1.6.4] Oxidative Stress.

Oxidative stress is capable of inducing Ca\(^{2+}\) release from mitochondria loaded with Ca\(^{2+}\). Mechanisms producing oxidative stress are complicated and not completely understood. In its simplest form biological oxidative stress might be defined as any condition that disturbs the prooxidant - antioxidant balance toward favouring the former.

Mitochondria continuously produce oxygen free radicals (superoxide anions) at two sites along the respiratory chain. The first site is the ubiquinone to cytochrome b step which passes through the intermediate ubisemiquinone [Turrens et al, (1985)]. Ubisemiquinone is capable of reducing oxygen to superoxide which dismutates to form hydrogen peroxide. This is catalysed by superoxide dismutase (SOD). The second site of superoxide anion formation is the NADH dehydrogenase [Turrens and Boveris, (1980)] which appears quantitatively less active than the ubiquinone-cytochrome b site as O\(^{2-}\) generator.

Hydrogen peroxide formed from superoxide anions is further metabolised by glutathione peroxidase and glutathione reductase [Bellomo et al, (1984); Frei et al, (1986)]. Glutathione (GSH) is oxidised to the disulphide form (GSSG) by glutathione peroxidase with the formation of water from the peroxide. The regeneration of reduced glutathione depletes the pools of reduced pyridine nucleotides (see below).

The oxidation of glutathione leads to pore induction. This strongly suggested that the oxidation of reduced pyridine nucleotide pools (oxidative stress) was intimately involved in pore opening [Wolkozwic and McMillin, (1980)]. However, some workers in the field
attribute mitochondrial permeabilisation (pore induction) directly to the redox states of the GSH pools [Beatrice et al, (1984); Traber et al, (1992)]. Interestingly, studies conducted by Hoek and Rydstrom [Hoek and Rydstrom, (1988)] in which GSG reductase activity was inhibited by BCNU (bischloroethyl-nitrosourea which potentiates oxidation of GSG pools) did not lead to Ca\(^{2+}\) release from mitochondria. This suggested that pyridine nucleotide oxidation was the cause of pore opening.

\[
\text{GSH peroxidase} \\
H_2O_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2H_2O
\]

\[
\text{GSG reductase} \\
\text{GSSG} + \text{NAD(P)H} \rightarrow 2\text{GSH} + \text{NAD(P)}
\]

Besides the production superoxide anions in mitochondria, superoxide anions are also produced from ATP catabolism. During ischaemia and reperfusion (Jennings and Steenbergen, (1985)] ATP is degraded into hypoxanthine and xanthine. These form substrates for xanthine oxidase. Xanthine oxidase activity produces oxygen derived free radicals [McCord, (1985); Huizer et al, (1986)]. Again, neutralisation of these radical species exhausts the GSG pool with the consequent oxidation of reduced pyridine nucleotide pools. The depletion of reduced pyridine nucleotides has been implicated to be involved in mitochondrial damage [Takeyama et al, (1993)].

It is the extensive exhaustion of reduced pyridine nucleotides by oxidising agents that triggers the release of preaccumulated Ca\(^{2+}\) from mitochondria. During normal mitochondrial oxidative metabolism, levels of oxygen free radicals sufficient to cause damage are never reached since they are kept low by high levels of glutathione. The maintenance of glutathione levels is an active process requiring ATP. Therefore, under aphysiological conditions in which ATP levels are severely depressed i.e. ischaemia and reperfusion, levels of oxygen free radicals increase and overwhelm the antioxidant capacity of mitochondria. The subsequent exhaustion
of reduced glutathione levels can lead to widespread oxidation damage e.g. protein inactivation as a result of SH group oxidation.

Enzymatic reduction of acetoacetate and oxalacetate to \( \beta \)-hydroxybutyrate and malate respectively by \( \beta \)-hydroxybutyrate and malate dehydrogenase leads to the oxidation of reduced pyridine nucleotides. The depletion of reduced pyridine nucleotides by these oxidising agents has also been demonstrated to induce \( Ca^{2+} \) release from overloaded mitochondria [Lehninger et al, (1978); Hunter and Haworth, (1979a); Wolkowicz and McMillin, (1980); Beatrice et al, (1980)]

Lipid hydroperoxides are generated during normal metabolism but their levels are considerably increased during oxidative stress, ischaemia/ reperfusion. Tertiary butylhydroperoxide (t-BuOOH) is a short chain analogue of lipid hydroperoxides. Tertiary BuOOH is metabolised by 2 distinct pathways; (i) cytochrome P-450 metabolism which produces toxic peroxy and alkoxy radicals [Davies, (1989)] and (ii) oxidation of glutathione which produces L-butanol. The lipid analogue has been routinely used to study the detrimental effects of oxygen free radicals in mitochondria [Crompton and Costi, (1988); Crompton et al, (1988); Broekemeier et al, (1989)] and in cells [Imberti et al, (1993); Nieminen et al, (1995)]. The hydroperoxide oxidises glutathione and pyridine nucleotides via the GSH peroxidase, GSG reductase and the energy linked transhydrogenase. Tertiary-BuOOH had no effect if glutathione reductase was selectively inhibited by selenium [Traber et al, (1992)]. Interestingly, t-BuOOH induced \( Ca^{2+} \) release from mitochondria was inhibited by Phospholipase A inhibitors nupercaine and trifluoperazine, [Beatrice et al, (1980); Broekemeier et al, (1989)]. This led Pfeiffers’ group to suggest a role for lipid peroxidation in the development of mitochondrial permeability changes (see section 1.11.1). In contradiction to this suggestion studies by Erdahl et al [Erdahl et al, (1991)] proposed that t-BuOOH - induced mitochondrial permeabilisation was unrelated to a lipid peroxidation dependent perturbation of membrane lipid. Rather, the products of lipid hydrolysis i.e. lipid peroxides, acted on the pore.

The effects of oxidative stress on mitochondrial permeabilisation can be prevented by oxygen free radical scavengers. Butylhydroxytoluene (BHT), a free radical scavenger (see
section 1.7.2), was shown to prevent t-BuOOH [Carbonnera and Azzone, (1988)] as well as phosphate [Novgorodov et al, (1987), Novgorodov et al, (1989)] and Adriamycin (antineoplastic agent) [Sokolove, (1994)] induced Ca^{2+} release from mitochondria.

Clearly the oxidation of reduced pyridine nucleotides by free radical generating agents is a major factor contributing to the opening of the inner membrane Ca^{2+} -sensitive pore. Therefore, factors that can prevent or delay the oxidation of nucleotide pools (redox state) tend to protect mitochondria from the damaging effects of Ca^{2+} induced permeabilisation.

[1.6.5] Thiol reagents.

The hydrophobic SH crosslinking reagent phenylarsine oxide (PhAsO) was shown to induce Ca^{2+} efflux from Ca^{2+} loaded mitochondria [Novgorodov et al, (1987)]. Cd^{2+} [Rasheed et al, (1984)] and PhAsO cause the oxidation of dithiol groups which implies that SH groups are involved in pore function. Treatment with N-ethylmaleimide (NEM, also a SH crosslinking reagent) also causes Ca^{2+} release from mitochondria with the dissipation of the membrane potential and mitochondrial swelling. The oxidising effects of NEM are somewhat confusing. At low concentrations (10\mu M) it offers protection against the deleterious effect of Ca^{2+} efflux [Petronilli et al, (1994a)] whilst at high concentrations (50-100\mu M) it potentiates Ca^{2+} release from mitochondria [Pfeiffer et al, (1979); Beatrice et al, (1980); Broekemeier et al, (1985)]. Bernardi’s group have confirmed both the protective (at low concentrations) and inducing effects (at high concentrations) of NEM [Petronilli et al, (1994b)]. They have identified two classes of SH groups. Crosslinking at the first group prevents pore opening whilst substitution at the second group induces pore opening. The authors suggest that two cysteinyl groups are present on the pore forming complex. Oxidation of both residues is needed for pore opening. Oxidation of one of the SH groups to a disulphide with low concentration of NEM prevents reaction of the second SH group. The oxidation of one SH group maintains the closed pore state. However at higher concentrations of NEM both residues are oxidised leading to pore opening. The effects of NEM confirm that thiol groups are involved in pore activity [Beatrice et al, (1980); Rizzuto et al, (1987); Vercesi et al, (1988); Petronilli et al, (1994b)].
Formation of co-ordination complexes between heavy metal divalent cations e.g. Cd\(^{2+}\), Hg\(^{2+}\) or Zn\(^{2+}\) and the two SH groups on the pore might also be responsible for inducing the membrane pore [Rasheed et al, (1984); Chavez et al, (1985)].

[1.6.6] **Inhibitors of the ADP/ATP translocase.**

Ca\(^{2+}\) induced permeabilisation of mitochondria is promoted by impermeant inhibitors of the translocase. Both carboxyatractylate and atractylate stabilise the nucleotide binding site on the cytoplasmic side (c-state) of the ADP/ATP translocase [Le Quoc and Le Quoc, (1988); Novgorodov et al (1990); Novgorodov et al, (1991); Macedo et al, (1993)]. Bongkrekic acid (a membrane permeable inhibitor of the translocase) prevents Ca\(^{2+}\) induced permeabilisation by stabilising the transporter in the m-configuration (matrix facing). Besides carboxyatractylate, pyridoxal -5- phosphate and palmitoyl CoA are also capable of pore induction. Palmitoyl CoA in the absence of carnitine prevents ADP binding to the translocase whilst pyridoxal -5- phosphate reacts with amino acids, specifically lysyl residues, on the cytoplasmic face of the translocase to induce permeabilisation [Bognor et al, (1986)]. Together they stabilise the c-configuration of the translocase and promote Ca\(^{2+}\) induced permeabilisation of mitochondria.

As briefly mentioned earlier, the addition of acetoacetate to Ca\(^{2+}\) loaded mitochondria results in the release of Ca\(^{2+}\) in conjunction with the oxidation of reduced pyridine nucleotides. Bongkrekic acid and ADP can protect against acetoacetate induced Ca\(^{2+}\) efflux [Le Quoc and Le Quoc, (1985)] which suggests that the effects of pyridine nucleotide oxidation may somehow involve the translocase. The conformation of the ADP translocase (c or m state) must therefore play a critical role in Ca\(^{2+}\) induced membrane permeabilisation.

Interestingly, acyl CoAs are also able to interact with the cytoplasmic and the matrix face of the translocase to influence permeabilisation [Klingenberg, (1977); Palmer and Pfeiffer, (1981); Paulson and Shug, (1984)]. By binding to the cytoplasmic face of the translocase acyl CoAs can cause permeabilisation.

Fatty acids act as classical uncouplers. They enter mitochondria in a protonated electroneutral form and leave as anions in an electrogenic process mediated by the ADP/ATP translocase [Andreyev et al., (1989); Skulachev, (1991)]. A direct interaction of fatty acids with the pore forming units has been suggested [Petronilli et al., (1993)].

The agents covered above are the principle inducers of the membrane pore. The range of different pore inducers is large. Their modes of actions are not fully understood. However, one point is clear, rather than a single mechanism accounting for pore induction a number of overlapping processes are responsible for causing Ca$^{2+}$ sensitive membrane permeabilisation. Inhibitors of the pore, like the inducers, are also variable in action. Any general mechanism proposed to explain pore operation in intact mitochondria must therefore be able to cope with the paradox of a specific pathway present (the pore) that is apparently modulated by a number unspecific effectors.

[1.7] Pore Inhibitors.

The accumulation of Ca$^{2+}$ by mitochondria is an absolute requirement for pore opening (mitochondrial permeabilisation). Agents that prevent Ca$^{2+}$ accumulation or minimise matrix free [Ca$^{2+}$] can protect against the detrimental effects of Ca$^{2+}$ on membrane permeability.

Similar to inducing agents, the array of inhibitors of Ca$^{2+}$ induced membrane permeabilisation is large and diverse. Since inducing agents of the pore overlap in their mode of action, the same is also true for inhibitory factors. For example, permeabilisation of mitochondria by acetoacetate [Le Quoc and Le Quoc, (1989)] has been reported to be inhibited by dithiothreitol, pyruvate, bongkrekic acid and ADP. This implicates the simultaneous involvement of SH groups, reduced pyridine nucleotide pools and the ADP/ATP translocase respectively in the mitochondrial pore. Overlapping mechanisms were also demonstrated by Macedo et al [Macedo et al., (1993)] and Carbonerra and Azzone.
Carbonerra and Azzone, (1987)]. Tertiary-BuOOH triggered Ca\(^{2+}\) release from Ca\(^{2+}\) loaded mitochondria was depressed by dithiothreitol (DTT), BHT and ADP. However, suppression by ADP was far more effective if Ca\(^{2+}\) release was triggered by phosphate rather than hydroperoxide. Overlapping mechanisms involving membrane peroxidation (see section 1.11.1) are also reported [Beatrice et al, (1980)] in which phospholipase A\(_2\) inhibitors delayed Ca\(^{2+}\) release (inhibition of swelling) induced by phosphate.

[1.7.1] Adenine nucleotides.

The protective effects of ADP on Ca\(^{2+}\) induced permeabilisation was originally thought to be due its conversion to ATP [Chappel and Greville, (1961); Azzi and Azzone, (1965]. However, it was later discovered that ADP was in fact more efficient then ATP at preventing Ca\(^{2+}\) release [Haworth and Hunter, (1979); Lotscher et al, (1980); Vitorica and Satrustegui, (1985); Crompton and Costi, (1988)]. ATP and AMP were found to be less potent at restoring Ca\(^{2+}\) induced dissipation of membrane potential than ADP.

ADP increased the rate of pore closure of permeabilised mitochondria by 10-fold [Crompton and Costi, (1990)]. This suggested that ADP competed with Ca\(^{2+}\) to close the pore. By binding to the pore ADP depressed the effects of Ca\(^{2+}\). NADH and NAD\(^{+}\) have similar effects on pore closure as ADP but are only as effective at much higher concentrations (200 and 2000 fold higher than ADP respectively). NADH behaves synergistically with ADP to close the pore [Hunter et al, (1976)]. Similarly, ADP behaves synergistically with CsA [Andreeva and Crompton, (1994); Novgorodov et al, (1994)] to close the pore. This suggested the involvement of co-operative binding sites in the activation of the membrane pore.

[1.7.2] Free radical scavengers.

BHT (butylhydroxytoluene) is the most common agent used to protect against the deleterious effects of Ca\(^{2+}\) induced permeabilisation initiated by either phosphate or hydroperoxide. At concentrations around 50\(\mu\)M BHT can completely prevent the dissipation
of membrane potential when permeabilisation is induced by either phosphate [Novgorodov et al, (1987)] or crosslinking reagents e.g. diamide or PhAsO [Carbonerra and Azzone, (1988)].

GSH levels are depressed (i.e. reduced pyridine nucleotide pools are exhausted) in mitochondria in which permeabilisation (Ca\(^{2+}\) efflux) is triggered by diamide or hydroperoxide but not by phosphate [Carbonerra and Azzone, (1988)]. The effect of both inducers is alleviated by BHT. As suggested earlier, these findings reflect the involvement of more than one mechanism in pore induction.

[1.7.3] Cations.

Sr\(^{2+}\), Mn\(^{2+}\) and Ba\(^{2+}\) substituted for Ca\(^{2+}\) are unable to act as inducers of mitochondrial permeabilisation [Haworth and Hunter, (1979a,b); Jung and Brierley, (1981)]. However, they were found to be effective inhibitors of Ca\(^{2+}\) induced membrane permeabilisation. Mn\(^{2+}\) and Sr\(^{2+}\) were shown to exert their inhibitory effects from the matrix side [Saris and Bosch, (1988); Bemardi et al, (1993)]. Mn\(^{2+}\) and Sr\(^{2+}\) competed with Ca\(^{2+}\) for the matrix side binding site. The binding of Ca\(^{2+}\) to this site activates the opening of the membrane pore. Bernardi's group also showed that an inhibitory site for the divalent cations (including Ca\(^{2+}\)) also exists on the cytoplasmic facing side of membrane [Bernardi et al, (1993)]. Ca\(^{2+}\) induced permeabilisation (reported by patch clamp studies in which conductance of mitoplast membranes was measured) revealed the existence of regulatory Ca\(^{2+}\) binding sites [Szabo et al, (1992); Bernardi et al, (1992)] on the matrix facing side of the inner membrane. These studies also revealed Mg\(^{2+}\), Sr\(^{2+}\) and Mn\(^{2+}\) binding sites on the cytoplasmic facing side of the inner membrane. No Mg\(^{2+}\) sites on the matrix facing side were found. This was expected since the Ca\(^{2+}\) uniporter of the inner membrane is able to transport Sr\(^{2+}\), Ba\(^{2+}\) and Mn\(^{2+}\) into the matrix but not Mg\(^{2+}\).

Besides divalent cations, monovalent and trivalent cations have also been reported to behave as competitive inhibitors of the Ca\(^{2+}\) activated pore. Monovalent cations are less effective than divalent cations at inhibiting the pore whilst trivalent cations are more effective.
than divalent cations at inhibiting the pore [Haworth and Hunter, (1979)]. Lanthanides (La$^{3+}$) were also effective at inducing pore closure [Haworth and Hunter, (1979)].

[1.7.4] Inhibitors of the adenine nucleotide translocase.

Carboxyatractylate and atractylate induce Ca$^{2+}$ dependent permeabilisation. These agents bind to the translocase and lock it into the c-configuration (nucleotide binding site facing the cytoplasm) [Le Quoc and Le Quoc, (1988)]. Bongkrekic acid and matrix acyl CoAs bind to the carrier from the matrix side and secure it in the m-configuration.

[1.7.5] pH.

A major determinant of pore opening in depolarised mitochondrial is matrix pH. A low matrix pH favours pore closure [Bernardi et al, (1992); Petronilli et al, (1993)]. Studies conducted by Haworth and Hunter [Haworth and Hunter, (1979)] reported that protons inhibited mitochondrial permeabilisation in a Ca$^{2+}$ competitive manner and that the K_m for activation by Ca$^{2+}$ decreased with increasing pH. Decreasing the pH prevented Ca$^{2+}$ induced mitochondrial swelling which suggested that the pore remains closed under a low pH [Al-Nasser and Crompton, (1986a)]. Lowering the pH does not decrease the Ca$^{2+}$ uptake by mitochondria but rather appears to compete with Ca$^{2+}$ to inhibit the pore [Halestrap, (1991)]. Competition between Ca$^{2+}$ and a low pH to respectively open and close the pore was supported by electrophysiological studies. Ca$^{2+}$ activated conductance of the mitochondrial megachannel (MMC, see section 1.11.2) was depressed by lowering the pH of the media. The inhibitory effects of low pH on membrane conductivity were overcome by increasing the [Ca$^{2+}$] [Szabo and Zoratti, (1991); Szabo et al, (1992)]. The binding sites involved in these effects were predicted to be located on the matrix side of the membrane [Bernardi et al, (1992)]. Inhibition of the pore by low pH was substantially eliminated with diethlypyrocarbonate (DPC). Ca$^{2+}$ loaded mitochondria failed to undergo permeabilisation at acidic pH values unless they were first treated with DPC. This implied the involvement of protonatable regulatory sites in pore activity. DPC prevents protonation at histidyl residues.
This suggested that reversible protonation of one or more histidyl residues on the matrix side of mitochondria played a critical regulatory role in pore opening [Nicolli et al, (1993)].

[1.7.6] Phospholipase A\textsubscript{2} inhibitors.

Perturbations in phospholipid deacylation-reacylation cycling involving inner membrane phospholipids was suggested to induce Ca\textsuperscript{2+} specific permeabilisation [Pfeiffer et al, (1979); Beatrice et al, (1980); Erdahl et al, (1991)]. Inhibitors of the mitochondrial phospholipase A\textsubscript{2} an enzyme that plays a critical role in phospholipid deacylation-reacylation, see section 1.11.1, were reported to prevent Ca\textsuperscript{2+} release from intact mitochondria loaded with Ca\textsuperscript{2+} [Broekemeier et al, (1985)]. Trifluoperazine, dibucaine and quinacrine strongly inhibit phospholipase activity. Trifluoperazine protects against t-BuOOH induced Ca\textsuperscript{2+} release from intact mitochondria [Broekemeier et al, (1989)] and hepatocytes [Imberti et al, (1993); Nieminen et al, (1995)]. Presumably, phospholipase inhibition prevents the accumulation of lysophospholipids and fatty acids which induce membrane permeability change, or less likely, metabolites of these components may contribute to membrane damage (see section 1.11.1).

[1.7.7] Cyclosporin A.

Submicromolar concentrations of the immunosuppressant Cyclosporin A (CsA) delays Ca\textsuperscript{2+} induced permeabilisation of mitochondria [Fournier et al, (1987); Crompton et al, (1988); Broekemeier et al, (1989); McGuinness et al, (1990); Halestrap and Davidson, (1990)]. Besides partitioning into the phospholipid bilayer, 2 classes of CsA binding sites exist in rat liver mitochondria [McGuinness et al, (1990)]. A low affinity, high capacity site (K\textsubscript{d} 100nM, 60pmol/ mg of mitochondrial protein) and a high affinity, low capacity site (K\textsubscript{d} 5-12nM, 6pmol/ mg of protein). Mitochondrial permeabilisation was inhibited by CsA with a K\textsubscript{d} similar to that of the high affinity CsA binding sites of the membrane thus implicating their involvement in pore function [McGuinness et al, (1990)]. CsA can prevent the dissipation of the membrane potential and mitochondrial swelling induced by Ca\textsuperscript{2+} and a variety of inducing agent that include Pi and t-BuOOH [Crompton et al, (1988); Broekemeier and Pfeiffer,
(1989); Solem and Wallace, (1993)]. However, studies in liver mitochondria by Pastorino et al [Pastorino et al, (1993)] showed CsA to be incapable of preventing pore opening, unless supplemented with the phospholipase A\textsubscript{2} inhibitor butacaine, when the Ca\textsuperscript{2+} activated pore was induced by phenylarsine oxide and cyanide. Experiments using cultured hepatocytes showed CsA and L-carnitine to protect against cell death caused by anoxia and rotenone but not by anoxia and cyanide unless butacaine was also added. It was concluded that CsA and L-carnitine prevented cell death by delaying mitochondrial permeabilisation [Pastorino et al, (1993)]. The use of a phospholipase A\textsubscript{2} inhibitor to potentiate the CsA effect again suggests the involvement of overlapping mechanisms in mitochondrial pore induction.

Electrophysiological studies involving patch clamp techniques established the competition between Ca\textsuperscript{2+} and CsA to block the mitochondrial megachannel (MMC). Inhibition of the megachannel by CsA could be relieved by increasing the [Ca\textsuperscript{2+}]. Based on a large number of similarities, the megachannel was postulated to correspond to the non-specific inner membrane pore (see section 1.11.2) [Petronilli et al, (1989); Szabo and Zoratti, (1992)].

CsA inhibition of the pore was potentiated by ADP and Mg\textsuperscript{2+} [Novgorodov et al, (1992); Andreeva and Crompton, (1994)]. This implied the existence of co-operative binding sites on the pore. Interestingly, synergy between the phospholipase inhibitor trifluoperazine and CsA to block the Ca\textsuperscript{2+} activated pore was demonstrated by Pfeiffer’s group [Broekemeier and Pfeiffer, (1989)]. This further stressed the involvement of overlapping mechanism in pore activation.

CsA potently inhibits mitochondrial permeabilisation induced by N-ethylmaleimide, Hg\textsuperscript{2+}, WY-14643, oxalacetate, ruthenium red and rhein [Broekemeier et al, (1989)]. Again these findings also support the involvement of overlapping mechanisms in pore induction.

Cyclosporin A binds tightly to a family of proteins called the cyclophilins. Their properties and a possible role in pore function are discussed section 1.8.2.
[1.7.8] Other inhibitors of the pore.

The inhibitory effects of polyamines on mitochondrial permeabilisation are well documented but are not completely understood [Lapidus and Sokolove, (1993); Evtodienko et al, (1993); Lapidus and Sokolov, (1994)]. It was suggested that spermine potentiated the effects of matrix ADP to close the pore by increasing the affinity of the ADP binding site on the pore [Lapidus and Sokolov, (1994)].

[1.8] Cyclosporin A and Cyclophilins.

[1.8.1] Cyclosporin A (CsA).

Cyclosporin A (CsA) is a naturally occurring secondary metabolite produced by Norwegian soil fungus, *Tolypocladium inflatum* [Borel, (1986)]. Since it was approved for clinical use in the 1980s, CsA (molecular mass 1203) has revolutionised organ transplant through its widespread use in the prevention of graft rejection. However, better known for its clinical and immunosuppressive applications, CsA was also employed as a biochemical tool to probe signalling pathways involved in immunosuppression. The structurally related compounds FK506 (molecular mass 822) and rapamycin (molecular mass 915) are being evaluated for immunosuppressive capacity since, unlike CsA, they appear not to cause nephrotoxicity [Ryffel et al, (1988)]. Since the effectiveness of CsA in immunosuppression was recognised attempts were made to synthesise the peptide and its derivatives [Wenger, (1984); Galpin et al, (1988)].

The immunosuppressive activity of CsA has been associated with a large part of the molecule. Variations at most residues render the drug immunosuppressively incompetent. Only few positions allow modifications without considerable loss of potency [Quesniaux et al, (1987); Durette et al, (1988); Sigal et al, (1991)]. This is important in the design of photoactive derivatives used in labelling exercises. Interestingly the cyclic peptide adopts two types of conformations depending on its environment. NMR studies have shown that in a typically apolar condition the hydrophobic side groups of the molecule are extended away from the backbone. In contrast, in an aqueous environment they are folded back over the
peptide [Lautz et al, (1992)]. This is an important feature of the molecule since the same hydrophobic groups that extend away from the backbone are involved in protein-drug interactions.

[1.8.2] Cyclophils and FKBPs.

Due to its high abundance in the cytosol of thymocytes (0.1-0.4% of total protein) a 18kDa CsA-binding protein was considered to be the relevant CsA receptor [Handschumacher et al, (1984); Harding et al, (1986); Haendler et al, (1987)]. This was named cyclophilin. The purification and subsequent sequencing of a porcine peptidyl prolyl cis trans isomerase (PPIase) enzyme showed it to be identical to the previously identified cyclophilin [Fischer et al, (1989); Takahashi et al, (1989)]. Cyclophilin displays PPIase activity that is inhibited by CsA (K_d 0.2 - 20nM) in the nanomolar range depending on the nature of the measuring media. Since these findings, different isoforms of the protein have been isolated from prokaryotes and eukaryotes. Interestingly, unlike many other proteins that undergo large structural changes upon binding their ligands, cyclophilins change very little upon binding CsA. Rather the cyclic peptide changes conformation to inhibit the PPIase activity [Theriault et al, (1993), Pflugl et al, (1993)]. By means of isotope-edited NMR techniques it was shown that the 9, 10 peptide bond of CsA was in the trans configuration when bound to cyclophilin and in the cis configuration when in solution. The change from cis to trans configuration was a time-dependent process [Fesik et al, (1990)]. This is probably consistent with recent kinetic studies that demonstrated the time-dependent inhibition of PPIase activity by CsA involved slow cis-trans isomerisation of imidic bond in the molecule [Kofron et al, (1992)]. This explains why when measuring inhibition of PPIase activity the cyclic peptide has to be incubated for approx. 4 minutes with the PPIase; this gives the peptide sufficient time to undergo cis-trans isomerisation.

Inhibition of PPIase activity is not exclusive to CsA. FK506 (a more potent immunosuppressant with reduced toxicity) also inhibits PPIase activity (K_d of 0.4nM) but not of cyclophilins. FK506 (isolated from Streptomyces tsukubaensis ) binds to FKBPs (FK506 binding proteins) which belong to a family of PPIases that differ considerably from
cyclophilins [Siekierka et al, (1989); Harding et al, (1989); Galat et al, (1993); Peattie et al, (1992)]. FKBPs are also ubiquitous proteins found in prokaryotes and eukaryotes. As with CsA-cyclophilin interaction, FK506-FKBP interaction is also time-dependent [Zarant et al, (1995)]. Rapamycin also binds FKBPs ($K_d$ 0.2nM) to inhibit PPIase activity but has no immunosuppressive activity. In this way it antagonises the effects of FK506 [Dumont et al, (1990)].

[1.8.3] Other CsA binding proteins.

Proteins other than cyclophilins have been demonstrated to bind CsA. Calmodulin binds both immunosuppressive and non-immunosuppressive derivatives of CsA through a hydrophobic pocket shared by a number of calmodulin antagonists [LeGrue et al, (1986); Colombani et al, (1985)]. Although calmodulin plays a critical role in T-cell activation, its capacity to bind both immuno- and non-immunosuppressive derivatives of CsA suggests that it is not in the crucial step of CsA’s mechanism of immunosuppression (see section 1.9.1).

CsA increases the sensitivity of multidrug resistant cells to chemotherapeutic agents. It binds to the 170kDa membrane-P glycoprotein that functions as an energy-dependent drug efflux pump. Both immunosuppressive and non-immunosuppressive derivatives of Cyclosporin A and $Ca^{2+}$ channel blockers (verapamil, dilatiazem) share binding sites on the protein [Foxwell et al, (1989)].

Using a crosslinking derivative of cyclosporin A a family of transporter proteins have been identified in both liver (bile transporter) and kidney (Na$^+$/ D-glucose transporter) cells that bind CsA. Although the transporters demonstrate no PPIase activity, they bind CsA. This might alter their activities. By affecting the transport capacity of the proteins, it is possible that the CsA interactions play a potential role in hepato- and nephrotoxicity [Ziegler and Frimmer, (1986); Ziegler et al, (1990)].

Cacalano and LeGrue report the existence of a specific CsA receptor located on the surface of lymphocytes. However the target protein is as yet unidentified and its relation to cyclophilin undetermined [Cacalano et al, (1992); LeGrue et al, (1993)]
Since cyclophilins and FKBPs are involved in immunosuppression they are collectively called immunophilins. With the findings that cyclosporin A inhibited the PPIase activity of cyclophilins, it was proposed that complexes of cyclophilins and FKBPs with CsA and FK506 respectively were directly responsible for preventing T-cell activation. However certain findings were inconsistent with this hypothesis;

Certain analogues of cyclosporin and FK506 readily inhibit PPIase activity of cyclophilins but are completely ineffective in immunosuppression [Bierer et al, (1990a); Dumont et al, (1990)]. The reverse is also true, certain analogues that fail to inhibit PPIase activity are potently immunosuppressive [Sigal et al, (1991)].

The immunosuppressive mechanism is further complicated by the finding that rapamycin (50-100 molar excess of FK506) antagonises FK506-dependent T-cell [Bierer et al, (1990a); Dumont et al, (1990); Koltin et al, (1991)] and B-cell activation [Wicker et al, (1990); Bierer et al, (1990b)]. Since both immunophilin ligands (FK506 and rapamycin) inhibit the same PPIase activity it was suggested that the drug/ immunophilin complexes (i.e. FK506/ FKBP and CsA/ cyclophilin) might interact with molecules involved in different signal transduction pathways [Bierer et al, (1990a); Bierer et al, (1990b); Lui et al, (1991); Schreiber, (1991); Fruman et al, (1992)].

Unless a specific pool of PPIase is involved in T-cell activation, the concentrations at which CsA and FK506 are effective at depressing T cell activation are very low with respect to the amounts that are needed to saturate the intracellular receptor [Schreiber and Crabtree, (1992)].

Studies in yeast Saccharomyces cerevisae and Neurospora crassa have clearly established a role for a drug/ cyclophilin complex in cell growth. The drug/ cyclophilin complex plays a critical role in signal transduction involving cell growth since mutants lacking the CsA or FK506 receptor (cyclophilin, FKBP) are rendered insensitive to the growth inhibitory effects of the drug [Tropschug et al, (1988); Heitman et al, (1991); Foor et al,
This suggests that the drug/immunophilin complex plays a critical role in signal transduction pathways involved in cell growth.

Clearly the drug/immunophilin complex is necessary for immunosuppression but not sufficient to activate T-cells.

[1.9.1] **Calcineurin.**

Using FKBP or cyclophilin affinity matrices Friedman and Weissman showed that a complex of immunosuppressant, calcineurin and calmodulin was retained by the affinity columns [Freidman and Weissman, (1991); Lui et al, (1991)]. Calcineurin otherwise known as Ca\(^{2+}\)/calmodulin-dependent serine/threonine phosphatase or Phosphatase 2B, is a heterodimer consisting of a 59kDa calmodulin binding catalytic unit A and a smaller 19kDa Ca\(^{2+}\)-binding subunit B [Guerini and Klee, (1989)]. CsA/cyclophilin, CsA/cyclophilin C and FK506/FKBP12 complexes were found to interact with the B unit of calcineurin [Ryffel et al, (1992); Li and Handschumacher, (1993); Husi et al, (1994); Woerly et al, (1994)] and inhibit the phosphatase activity of the enzyme [Swanson et al, (1992); O’Keefe et al, (1992); Bram et al, (1993); Etzkorn et al, (1994)]. Immunosuppressive activity was dependent upon the ability of CsA/cyclophilin or FK506/FKBP complexes to inhibit phosphatase activity of calcineurin. Although certain analogues of the drugs, e.g. [MeAla\(^6\)]-cyclosporin, FK506BD etc bind tightly to immunophilins their immunosuppressive value is rather poor since their complexes fail to inhibit the phosphatase activity of calcineurin. However other derivatives such as MeBm\(\_\)t-cyclosporin bind cyclophilins weakly but have a high affinity for calcineurin and thus exhibit potent immunosuppressive activity [Lui et al, (1991); Dumont, (1992); Lui et al, (1992); Nelson et al, (1993)]. Clearly the drug-immunophilin complex must expose both an immunophilin and a calcineurin binding domain. This may explain how FK506 and rapamycin can bind to the same target protein but interfere with completely different signalling pathways. Rapamycin once complexed with FKBP may expose epitopes that do not interact with calcineurin.
[1.9.2] Involvement of calcineurin in T-cell activation.

T-cell activation requires the transcription of lymphokine genes, e.g. interleukins 2 and 4 etc. Antigen-mediated T-cell activation via the T-cell receptor (TCR) induces the production of $2^\text{nd}$ messengers IP$_3$ and diacylglycerol. IP$_3$ dependent Ca$^{2+}$ release from both external and internal stores activates many cellular processes. Calmodulin after binding 4 Ca$^{2+}$ ions mediates a wide range of cellular processes that include gene and channel regulation as well as activation of calcineurin phosphatase activity. Inhibition of phosphatase activity is a prerequisite for immunosuppression. A strong correlation between calcineurin inhibition and immunosuppression activity was established using synthetic cyclosporin analogues [Dumont et al, (1992); Lui et al, (1992); Nelson et al, (1992)].

Recent work has established that the Nuclear Factor of Activated T-cells (NF-AT)c is a substrate for calcineurin phosphatase [Jain et al, (1993)]. This factor plays a key role in interleukin-2 (IL-2) gene transcription. The translocation of the factor from the cytoplasm to the nucleus is blocked by CsA and FK506 [Flanagan et al, (1991); Emmel et al, (1989)]. Two subunits of NF-AT appear to exist in T-cells. One subunit is localised to nucleus whilst the other is in the cytoplasm. Transcription of IL-2 requires the correct functional assembly of the 2 subunits. Inhibition of calcineurin phosphatase activity with CsA or FK506 treatment prevents the translocation of the cytoplasmic NF-AT subunit to the nucleus [Flanagan et al, (1991)]. Dephosphorylation of the cytosolic resident NF-AT subunit is a prerequisite for translocation into the nucleus [McCaffry et al, (1993)].

[1.10] Localisation and function of cyclophilins.

[1.10.1] Cyclophilins A, B, C, and D.

Different isoforms of cyclophilins and FKBPs have been identified in prokaroytic and eukaryotic cells. Besides immunosuppression they are also involved in other cellular functions e.g. regulating protein folding, trafficking and assembly of protein complexes etc. Cyclophilin A (CyPA) is a ubiquitous protein found in the cytoplasm of cells [Koletsky et al, (1986)]. Most cyclophilins have molecular weights close to 18kDa. They have been identified in
different species of bacteria for example E. coli [Lui and Walsh, (1990); Hayano et al, (1991); Rahfeld et al, (1994)], Legionella pneumophilia [Cianciotto et al, (1990)], Bacillus subtilis [Hayano et al, (1991)] as well as yeast and diverse mammalian cells. Cyclophilins have been demonstrated in Neurospora crassa in which a single cyclophilin gene transcribes two mRNAs with different 5' terminals. One exists in the cytosol whilst the other is targeted to the mitochondria [Tropschug et al, (1988)].

Cyclophilin B (CyPB) contains a discrete N-terminal sequence that localises the protein to the endoplasmic reticulum [Bose and Freedman, (1994); Bose et al, (1994)]. Arbers' group suggest that CyPB is closely associated with the Ca\(^{2+}\) binding protein, calreticulin, found in the endoplasmic reticulum. CyPB is also associated with microsomal ATPases in the myogenic cell line L6 and the Ca\(^{2+}\) binding protein of skeletal muscle, calsequestrin [Hasel et al, (1991); Arber et al, (1992)]. This indicated a possible role in Ca\(^{2+}\) mobilisation. However, contradictory to these findings, recent studies show that CyPB might be secreted into the extracellular media since the protein was isolated from blood and milk [Price et al, (1994); Mariller et al, (1996); Allain et al, (1996)]. A glycosylated cyclophilin, believed to be post-translationally modified CyPB, has been isolated from microsomal membranes [Thalhammer et al, (1992)]. The role of glycosylation of CyPB is unknown but it may serve a possible role in subcellular compartmentalisation and involvement in secretory pathways [Thalhammer et al, (1992)].

Cyclophilin C (CyPC) was isolated from a cDNA library prepared from murine bone-marrow derived cell line AC-6. It shared extensive sequence homology with previously reported cyclophilins [Friedman and Weissman, (1991)]. CyPC messenger RNA was produced at relatively high concentration in kidney cells [Friedman and Weissman, (1991); Bram et al, (1993); Ke et al, (1993)]. Based on this finding it was assumed that CyPC was the relevant target for CsA-mediated nephrotoxicity [Walsh et al, (1992)]. However, distribution studies by Schneider [Schneider et al, (1994)] showed CyPC to be present in other tissues that were unaffected by CsA therapy, namely skeletal muscle, heart, liver and pancreas.

Cyclophilin D (CyPD) contains a N-terminal hydrophobic signal sequence that translocates it to mitochondria [Bergsma et al, (1991)]. An 18.6kDa PPIase activity isolated
from mitochondria showed sequence similarity to CyPD [Connern and Halestrap, (1992)]. Interestingly, another cyclophilin isolated from mitochondria displayed 96% sequence similarity with CyPD and 66% homology with human CyPB [Inoue et al, (1993); Tanveer et al, (1996)]

A 150kDa tumour recognition factor (NK-TR) has been found on the surface of natural killer cells. The N-terminal domain of the receptor is homologous to cyclophilin domain. The receptor displayed a weak CsA binding constant ($K_d$ 770nM) and inhibited PPIase activity of the protein [Anderson et al, (1993); Rinfret et al, (1994)]. The PPIase activity was postulated to be involved in correct assembly of the receptor.

Cyclophilins have also been detected in the nucleus and nucleolus. Their functional role in these organelles has yet to be established [Jin and Burakoff, (1993); Shan et al, (1994)].

[1.10.2] **The role of cyclophilins.**

Besides their role in immunosuppression, cyclophilins are intimately involved in protein folding. They catalyse the cis trans isomerisation of Xaa-proline bonds in proteins such as transferrin, calcitonin, and collagen to their correct conformations [Lodish and Kong, (1991); Schonbrunner et al, (1991); Steinmann et al (1991); Kern et al,(1993)]

Probably the most intriguing and informative member of the cyclophilin family is the $ninaA$ gene product found in the retinal cells of Drosophilia [Shieh et al, (1989); Stamnes et al, (1991); Ondek et al, (1992)]. The gene is expressed tissue-specifically only in retinal cell. Mutation in the gene produces a 10-15 fold reduction in the levels of rhodopsin in the cells that leads to impaired visual function [Larivee et al, (1981)]. The gene product of $ninaA$ is a glycosylated cyclophilin that, according to its amino acid sequence, is bound to the ER membrane. Since alterations to the gene lead to depressed levels of active rhodopsin in the cell, it was assumed that $ninaA$ gene product played a critical role in the correct folding and transport of rhodopsin from the ER to the cytoplasm. The folding activity of $ninaA$ is
selective since other isoforms of rhodopsin are expressed normally in ninaA mutant flies. Homologues of the protein may also exist in bovine retinal tissue [Stamnes et al, (1991)].

Cyclophilins interact with other proteins. They play an important role in the heat shock response in which proteins are protected from the detrimental effects of widespread aggregation [Gething and Sambrook, (1992); Sykes et al, (1993)]. The physiological resemblance between heat shock protein 70 (hsp70) and proline isomerases is striking, both families of proteins are highly conserved and ubiquitously expressed. They are found almost in all compartments of cells and whilst some members are constitutively expressed others are heat-inducible. Many hsp70s function as chaperones in protein folding and assembly. This characteristic is displayed by CyPA which increases the rate of protein folding and prevents the aggregation of incompletely folded carbonic anhydrase [Freskgard et al, (1992)]. Cyclophilin was shown to interact with the 90kDa heat shock protein of the steroid receptor indicating a role in steroid-dependent gene regulation. Interestingly, this interaction was unaffected by CsA which suggested that hsp90 did not bind to cyclophilin via the CsA binding site [Nadeau et al, (1993)]. FKBP52 not only associates with hsp90/ glucocorticoid complex but itself functions as a heat shock protein [Sanchez, (1990); Renoir et al, (1990)]. This supported the chaperoning nature of cyclophilins and FKBP52.

Cyclophilins might be involved in T-cell Ca^{2+} signalling. Stimulation of the TCR (T-cell receptor) activates calcium and PKC dependent signal transduction pathways. Activation of these pathways leads to the translocation of the NF-AT factor to the nucleus [Emmel et al, (1989); Flanagan et al, (1991)] (see section 1.9.2). Cyclosporin A prevents the Ca^{2+} - dependent interaction between cyclophilin and calcineurin (see section 1.9.1). As mentioned earlier (section 1.9) the concentrations at which CsA is effective as an immunosuppressant is much lower than the amount needed to saturate the intracellular receptors (cyclophilin). This suggests that only a limited number of CsA/ cyclophilin complexes are capable of inhibiting the phosphatase activity of calcineurin to cause immunosuppression. So what is the biological partner of cyclophilin when it is not complexed to CsA? A cellular homologue of Cyclosporin has been identified in Jurkat T cells that binds CyPB and potentiates IL-2 transcription. The
33kDa CyPB receptor protein has been termed CAML (Calcium-signal modulating cyclophilin ligand). Addition of CsA or FK506 to Jurkat T cells overexpressing CAML completely abolished CAML-mediated NF-AT activity. This suggested that CAML did not directly interact with NF-AT but rather upstream of calcineurin. CAML does not directly activate calcineurin but causes an influx of extracellular Ca\(^{2+}\) which activates the Ca\(^{2+}\) dependent pathway of signal transduction, (i.e. activation of protein tyrosine kinase, phospholipase C). The removal (by EGTA) of extracellular Ca\(^{2+}\) prevented NF-AT activation [Bram and Crabtree, (1994)]. In association with cyclophilin CAML plays a critical role in regulating the Ca\(^{2+}\) influx signal from the T cell surface. A close correlation between the need for increased intracellular [Ca\(^{2+}\)] and CsA /FK506 -dependent inhibition of T-cell activation had earlier been recognised. Matilla et al [Matilla et al, (1990)] had reported that CsA and FK506 were only effective at inhibiting transcriptional activation of T-cells if intracellular [Ca\(^{2+}\)] was raised using a calcium ionophore (ionomycin). This suggested that the immunosuppressive agents were inhibiting a particular Ca\(^{2+}\) signalling pathway responsible for interleukin-2 transcription.

PPIase activity has been show to play a regulatory role in the release of Ca\(^{2+}\) in muscle cells. FKBP12 was shown to be necessary for proper function of the ryanodine receptor in skeletal muscle. The PPIase specifically regulates the activity of the Ca\(^{2+}\) channel by stabilising the close conformation of the complex [Timerman et al, (1993); Brillantes et al, (1994)].

Using GST-CyPC affinity chromatography and AC-6 cell lysate Friedman and Weissman (1991) demonstrated the selective binding of a 77kDa component to CyPC in the absence of CsA. In the presence of CsA CyPC affinity column retained a 55kDa protein. The binding of the 55kDa partner was also observed using a FKBP-FK506 affinity matrix [Friedman and Weissman, (1991)]. The lower molecular weight component was identified as calcineurin [Lui et al, (1991)] whilst the identity of the 77kDa protein is unrecognised.

In vivo studies have shown cyclophilin to display chemotactic behaviour in human peripheral neutrophils and monocytes [Sherry et al, (1992); Xu et al, (1992)]. Chemotactic behaviour was prevented by Cyclosporin A indicating that other than the inactivation of NF-AT factor in T cells, this characteristic might be an additional mechanism by which immunosuppressive drugs function to prevent graft rejection. The blockage of chemotactic
behaviour by CsA prevents and delays the migration of lymphocytes to the inflammation sites [Sherry et al, (1992)]. This might explain the anti-inflammatory effects of CsA in psoriasis disease where CsA depletes both activated and non-activated T-cells from the epidermis and dermis of psoriatic patients [Gupta et al, (1989); Bang et al, (1993)]. Interestingly, interleukin-8, a potent chemotactic peptide, has been shown to bind CsA. Although the lymphokine does not exhibit PPIase activity it does share a similar tertiary structure to cyclophilin [Bang et al, (1993)]. Again, this may represent another mechanism active in immunosuppression. Other cyclophilin-like proteins might play a role in immunoregulation. A 24kDa glycosylated protein secreted by fibroblast cells that displayed similar sequence identity to CyPA was postulated to be involved in immunoregulation during wound healing [Davis et al, (1991)]

Cyclophilins might be involved in targeting CsA to T-cells. CyPB/ CsA complexes have been shown to bind to T cell surface and undergo endocytosis. CyPB/ CsA binding sites were recycled back to the surface and the drug accumulated within the cell. These findings suggest that extracellular CyPB might interact indirectly with the immunosuppressive mechanism through the specific targeting of the drug to sensitive cells [Allain et al, (1994); Allain et al, (1996); Mariller et al, (1996)].

Cyclophilins are involved in parasite invasion. PPIase activity might be required in biochemical pathways essential for the intracellular survival of prokaryote and eukaryote parasites. The Legionella MIP (macrophage infectivity potentiator) component is an outer membrane virulence protein that displays PPIase activity and a high affinity for FK506. Whether the PPIase functions as a chaperone (modulating the folding of multimeric proteins involved in invasion) or interacts with the Ca$^{2+}$ dependent signalling mechanism of the host organism has yet to be clarified [Cianciotto et al, (1989); Cianciotto et al, (1990)].

Cyclophilins might be involved in AIDS. Recent in vitro studies on the HIV virus have shown CyPA to interact with the viral gag polyprotein complex (consisting of one cyclophilin molecule to 10 gag proteins). The cyclophilin is incorporated into HIV-1 virions through contact with the gag polyprotein complex. The HIV-gag interaction is necessary for proper HIV-1 replication. A single proline mutation in the gag protein not only disrupts gag-CyPA
interactions, but also prevents viral replication and CyPA integration into virions. Cyclosporin A and two of its non-immunosuppressive derivatives block all these activities whilst FK506 has no effect [Franke et al, (1994); Thali et al, (1994)]. The role of CyPA in the HIV-1 life cycle remains unclear. Nevertheless the use of CsA and its non-immunosuppressive derivatives in the treatment of AIDS is being investigated. Unfortunately the levels of CsA required to prevent gag-CyPA interactions are in the order 10 to 100 fold higher than are necessary for immunosuppression. Since these levels are equivalent to the concentration of cyclophilins in the cytoplasm, long term inhibition of cyclophilins will lead to severe cellular toxicity effects as well as possible Cyclosporin A resistance by virus [Franke et al, (1994); Thali et al, (1994); Luban et al, (1993)]

[1.10.3] Cyclophilin and the Ca\(^{2+}\)-activated membrane pore.

Cyclophilin has been identified in both heart and liver mitochondria [Griffiths and Halestrap, (1991); Inoue et al, (1993); Connem and Halestrap, (1994); Andreeva et al, (1995); Tanveer et al, (1996)]. Since PPIase activity has been demonstrated to modulate Ca\(^{2+}\) channel activity in skeletal muscle cells [Timerman et al, (1993)] it was conceivable that such an activity might also play a regulatory role in the Ca\(^{2+}\) activated pore of mitochondria [McGuinness et al, (1990); Halestrap and Davidson, (1990); Connem and Halestrap, (1994); Andreeva et al, (1995); Tanveer et al, (1996)]. A case has been made for the involvement of ADP translocase in pore formation on the basis that effectors of the translocase such as carboxyatracylactone, bongkrekic acid, ADP, and pyridoxal-5-phosphate all modulated pore opening [LeQuoc and LeQuoc, (1988); Halestrap and Davidson, (1990)]. Direct interaction between the translocase and CsA does not occur. The amount of immunosuppressant needed to block the pore was considerably lower (5pmoles of binding sites/ mg of mitochondrial protein) than the amount of translocase (roughly 150-350pmoles/ mg of liver mitochondrial protein) present in mitochondria [McGuinness et al, (1990)] unless a small, specific, population of translocases were responsible for pore formation. As mentioned earlier in section 1.7.7 two CsA-binding sites were demonstrated in liver mitochondria. The high affinity CsA binding site was attributed to mitochondrial cyclophilin (molecular mass 18kDa)
[McGuinness et al, (1990)]. The high affinity CsA binding site demonstrated Ca\(^{2+}\) sensitivity, (i.e. CsA binding was depressed by approximately 50% in the presence of [Ca\(^{2+}\)] sufficient to cause mitochondrial permeabilisation) [McGuinness et al, (1990)] and ADP sensitivity, (i.e. increased CsA binding in the presence of ADP) [Andreeva and Crompton, (1994)]. Since the membrane pore was sensitive to the same effectors and the same effectors modulated CsA binding to the high affinity CsA binding sites, it was postulated that cyclophilin played a critical role in pore function. It was envisaged that matrix cyclophilin might bind to the membrane-bound translocase in presence of elevated free [Ca\(^{2+}\)] and cause it to behave as a non-specific pore [Halestrap and Davidson, (1990); Griffiths and Halestrap, (1991); Halestrap, (1994)]. There is evidence to suggest that under defined conditions specific carrier proteins of mitochondria can function as non-specific channels. The mitochondrial aspartate/glutamate transporter and the ADP translocase can behave as non-specific pores [Dierks et al, (1990); Brustovetsky and Klingenberg, (1996)]. Interestingly, the proposed structure of the translocase placed conserved proline residues at the end of the three transmembrane alpha helices as they emerge onto the matrix surface of the membrane [Aquila et al, (1987)]. These were postulated to function as potential cyclophilin binding sites. If the ADP translocase played a critical role in pore formation/ pore opening then it was proposed that CsA inhibition of cyclophilin would prevent cyclophilin/ translocase interaction and as result block pore formation/ pore opening.

[1.11] Other models of the Ca\(^{2+}\) activated pore.

[1.11.1] Membrane impairment hypothesis.

Mitochondrial permeabilisation has been attributed to the damage of the inner membrane lipid bilayer. Origins of this hypothesis can be traced back to the association of fatty acid production (the ‘U factor’) with uncoupling and swelling of mitochondria [Wojtczak and Lehninger, (1961)]. A further model, suggesting the involvement of phospholipase A\(_2\) and lysophospholipid acyl CoA transferase, has been proposed to explain Ca\(^{2+}\) induced mitochondrial permeabilisation [Pfeiffer et al, (1979); Beatrice et al, (1980); Beatrice et al, (1982); Beatrice et al, (1984); Broekemeier et, (1985)]. It was proposed that deranged
phospholipid cycling (as a result of the activities of the two enzymes) was responsible for Ca\(^{2+}\) dependent mitochondrial depolarisation and swelling. Phospholipase A\(_2\) is found on both the inner and outer membranes of mitochondria [Levrat and Louisot, (1992)]. The enzyme activity is Ca\(^{2+}\) dependent, inhibited by Mg\(^{2+}\), and activated by fatty acids [Waite and Sisson, (1971)]. It was proposed that mitochondrial membrane defects (mitochondrial permeabilisation) would occur upon the accumulation of polyunsaturated fatty acids and lysophospholipids if the activity of lysophospholipid acyl CoA transferase was inhibited and the activity of the phospholipase was activated [Pfeiffer et al, (1979); Beatrice et al, (1980)]. Under permeabilisation conditions (high [Ca\(^{2+}\)], oxidative stress) phospholipase A\(_2\) is activated. The accumulation of fatty acids and lysophospholipids (phospholipase activaters) under conditions leading to permeabilisation have been reported [Pfeiffer et al, (1979); Beatrice et al, (1980)]. Inhibitors of phospholipase activity such as nupercaine and dibucaine [Palmer and Pfeiffer, (1981); Broekemeimer et al, (1985); Rizzuto et al, (1987)] have been shown to protect against mitochondrial permeabilisation and swelling by preventing the accumulation of phospholipid degradation product. Although an interesting hypothesis, the membrane defective mechanism was unable to accommodate certain observations;

- Cyclosporin A prevents Ca\(^{2+}\) induced permeabilisation by inhibiting the pore. It does not appear to effect phospholipase A\(_2\) activity [Broekemeier et al, (1985)].

- The pore mechanism displays all -or -nothing behaviour [Hunter et al, (1976); Hunter and Haworth, (1979a)] and a gradual change in the membrane permeability is insufficient to accommodate this characteristic.

- Deacylation/ reacylation reactions of phospholipids require co-factors i.e. ATP, Mg\(^{2+}\) [Al-Nasser and Crompton, (1986a/b); Crompton and Costi, (1988)]. These would be lost on permeabilisation from the matrix or exist at very low concentration insufficient to sustain reacylation of hydrolysed phospholipids.

- Spermine and Mg\(^{2+}\) prevent mitochondrial permeabilisation yet they have been demonstrated to stimulate phospholipase A\(_2\) activity [Saris, (1994)].
lysophospholipid acyl CoA transferase activity was not found in mitochondria. Rather the transferase activity was due to microsomal contamination of mitochondrial preparations [Broekemeier et al, (1991)].

[1.11.2] The mitochondrial megachannel (MMC).

Refinement of patch clamp techniques to investigate mitochondrial membrane conductivity revealed the existence of large channels that displayed variable conductances [Sorgato et al, (1987); Martinac et al, (1987); Zoratti and Petronilli, (1988); Moran et al, (1990); Szabo and Zoratti, (1992); Bernardi et al, (1994)]. These were called MMCs (Mitochondrial MegaChannel). The mitochondrial megachannel is a voltage dependent, high conductance channel recorded from mitoplast membranes. Observations using giant mitochondria have shown the channel to exhibit conductance changes from 30-1300pS in media containing 150mM KCl [Kinally et al, (1989); Petronilli et al, (1989); Szabo and Zoratti, (1992); Zorov et al, (1992)]. The conductance channel is believed to represent the Ca\(^{2+}\) activated membrane pore since both structures share similar properties for example;

-the different substates of conductances observed are believed to be due to the channel functioning both independently as a single channel and co-operatively when aggregated. The Ca\(^{2+}\) sensitive membrane pore also displays co-operative ADP and Ca\(^{2+}\) binding sites [Haworth and Hunter, (1980); Novgorodov et al, (1992)].

-both structures are voltage dependent. Collapse of membrane potential leads to pore opening [Petronilli et al, (1994); Bernardi et al, (1992); Bernardi et al, (1993)] whilst the MMC is fully open at zero voltage [Szabo and Zoratti, (1993)].

-both are activated by Ca\(^{2+}\) in the \(\mu\)molar range from the matrix side and inhibited by Mg\(^{2+}\), Mn\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) [Szabo and Zoratti, (1992); Zoratti and Szabo, (1994); Hunter and Haworth, (1979a,b)].

- both are inhibited by submicromolar levels of ADP on the matrix side [Szabo and Zoratti, (1992); Hunter and Haworth, (1979a); Hunter and Haworth, (1980)].

-both are inhibited by amiodorone in the \(\mu\)molar range [Antonenko et al, (1991)].

- both are inhibited by Cyclosporin A [Szabo and Zoratti, (1991); Bernardi et al, (1994)].
Mitochondrial benzodiazepine receptor (mBzR).

Porin (30-35kDa) mediates the high ionic permeability of the mitochondrial outer membrane [De Pinto and Palmer, (1992); Manella et al, (1992). The protein exhibits ion selectivity, preferring Cl⁻ over K⁺, when reconstituted into a black phospholipid bilayer. The mBzR is a voltage-dependent channel, closing as the membrane potential increases and opening as the membrane potential decreases. Based on these characteristics porin is also called VDAC (voltage dependent anion-selective channel) [Benz et al, (1988); Colombini, (1989)]. VDAC forms large channels in the outer membrane at contact sites [Konstantinova et al, (1995)] and regulates the ionic homeostasis of mitochondria [Moran et al, (1990)]. Besides functioning as a pore, porin is also an intracellular receptor for peripheral kinases such as hexokinase and glycerol kinase [Fiek et al, (1982)]. Thus it appears to be intimately involved in the glycolytic pathway. The conductance of VDAC is almost half that of the fully open MMC complex. Since patch clamp studies postulate that the MMC channel may aggregate as a dimer, it can be suggested that the fully open MMC complex might be due to two VDAC monomers co-operating together to form a high conductance channel [Szabo et al, (1992); DePinto and Palmieri, (1992); Manella et al, (1992); Szabo and Zoratti, (1993); Colombini, (1994)].

Purification of the mitochondrial benzodiazepine receptor (mBzR) revealed the copurification of VDAC, adenine nucleotide translocase and a 18kDa benzodiazepine binding protein [McEnery et al, (1992); McEnery et al, (1993); Kinally et al, (1993)]. Since VDAC and the 18kDa protein were localised to the outer membrane and the adenine nucleotide translocase to the inner, it was postulated that the mBzR formed at regions of mitochondria where the inner and outer membranes come together (‘contact sites’). Isoquinol carboxamides (RO5-4864, PK11195, clonazepam) are classical non-competitive inhibitors of the benzodiazepine receptor found on the plasma membrane. These ligands bind to the 18kDa subunit of the mBzR [McEnery et al, (1992); McEnery et al, (1993); Kinally et al, (1993)]. Interestingly ligands of the mBzR also influence the activity of the MMC channel at concentrations consistent with their affinities (in the nanomolar range) to the mBzR [Verma and Synder, (1988); Hirsch et al, (1989); Kinally et al, (1993)].
Since adenine nucleotide translocase can function as a dimer and a tetramer [Klingenberg, (1993)], it was suggested that two translocases co-operated with two VDAC molecules and presumably two 18kDa benzodiazepine binding proteins to form a high conductance channel with characteristics identical to the MMC. This would probably account for co-operative binding sites for Ca\(^{2+}\) [Haworth and Hunter, (1979); Novogordov et al, (1992)] and ADP [Haworth and Hunter, (1980)] on the translocase in mitochondria.

If the Ca\(^{2+}\) activated membrane pore is the mitochondrial benzodiazepine receptor, then how are the effects of CsA observed in conductance and permeabilisation studies explained by the receptor?

The effect of CsA can be explained if it is assumed that the mitochondrial cyclophilin associates with the translocase component of the receptor. Cyclophilin (5pmol/ mg of mitochondrial protein) was calculated to be 50-70% in excess of the pore (estimated to be at approx. 2pmol/ mg of mitochondrial protein). Interestingly the density of the pore as predicted by McGuinness et al, [McGuinness et al, (1990)] correlated well with the amount of benzodiazepine receptor in mitochondria (3pmol/ mg of mitochondrial protein) [Awad and Gavish, (1987)]. The cyclophilin, if it interacts with the ADP translocase, catalyses a reaction that favours pore opening. CsA, by preventing cyclophilin interaction with the mBzR, favours pore closure.

[1.11.4] Ca\(^{2+}\) release without the induction of the membrane pore (i.e. prooxidant - induced Ca\(^{2+}\) release).

Oxidation of matrix reduced pyridine nucleotides (NADH, NADPH) by enzymic activity, (i.e. reduction of oxidised glutathione by glutathione reductase -see earlier, section 1.6.4) and non-enzymic activity (direct oxidation of nucleotides by alloxan) has been observed to induce mitochondrial Ca\(^{2+}\) efflux of Ca\(^{2+}\) loaded mitochondria. The efflux was considered to be through an electroneutral Ca\(^{2+}\) / H\(^+\) exchange [Moore et al, (1983); Vercesi, (1984); Traber et al, (1992); Schlegel et al, (1992)]. This efflux occurred without swelling, sucrose entry into mitochondria and K\(^+\) efflux (providing that Ruthenium Red or EGTA was present to
inhibit Ca$^{2+}$ reaccumulation by mitochondria). These findings do not agree with established characteristics that reflect pore opening, but rather suggest an alternative mechanism of Ca$^{2+}$ efflux.

A mechanism for inducing Ca$^{2+}$ efflux was suggested by Richter and co-workers. They proposed that oxidation of mitochondrial reduced pyridine nucleotides and hydrolysis of the oxidised nucleotide, followed by ADP-ribosylation at one or more membrane sites, mediated prooxidant induced Ca$^{2+}$ release [Frei and Richter, (1988)]. NAD$^+$/NADP$^+$ glycohydrolase and ADP-ribosyl transferase activities were presumed to be responsible for ADP-ribosylation of a 30kDa protein postulated to mediate Ca$^{2+}$ efflux [Frei and Richter, (1988)]. Inhibition of NAD$^+$ hydrolysis by ATP [Hofstetter et al, (1981)], Cyclosporin A [Schlegel et al, (1991); Weis et al, (1992); Richter et al, (1990)] and ADP-ribosylation by the competitive inhibitor MIBG (m-iodobenzylguanidine) [Richter, (1990); Weis et al, (1992)] prevented prooxidant induced Ca$^{2+}$ efflux.

Besides sharing a general requirement for elevated free [Ca$^{2+}$] and cyclosporin A, both the membrane pore and the prooxidant-induced Ca$^{2+}$ efflux mechanism also display other similar characteristics that suggest that a common mechanism is involved [Novogordov and Gudz, (1996)].


A large amount of evidence is emerging that implicates the Ca$^{2+}$ activated membrane pore in tissue damage during ischaemia/reperfusion. The consequence of ischaemia depends on the length of ischaemia. Reoxygenation after a certain length of ischaemia (15 minutes) returns the cell/tissue back to almost normal function (reversible injury). However, reoxygenation after a longer duration of ischaemia (40-60 minutes) causes irreversible cell/tissue injury [Jennings and Reimer, (1983)]. Concentrations of ions remain normal in heart during 15 minutes ischaemia followed by reperfusion. However total tissue levels of Na$^+$, H$^+$, phosphate and other ions and molecules are severely deranged after prolonged ischaemia followed by reperfusion. Exhausted high energy creatine phosphate and ATP levels lead to loss of ATP-
dependent ionic gradients (see earlier, section 1.4). As a result myocardial cells besides accumulating Na\(^+\) and H\(^+\) also amass huge amounts of Ca\(^{2+}\). Since the raised levels of non-transient cytosolic free [Ca\(^{2+}\)] are transported to the mitochondria [Crompton, (1990)-Calcium and the heart, Gunter and Pfeiffer, (1990)] mitochondrial Ca\(^{2+}\) overload is inevitable [Allshire et al, (1987); Steenbergen et al, (1990); Bowers et al, (1992); Ferrari et al, (1993); Allen et al, (1993)]. Reperfusion of the cell/tissue at this stage, rather than restore ionic gradients to normal, accelerates cell injury. These findings appear to implicate mitochondrial Ca\(^{2+}\) in irreversible cell injury. It is hypothesised that activation of the membrane pore/MMC leads to mitochondrial uncoupling. ATPsynthase works in reverse hydrolysing any remaining ATP to maintain the fallen H\(^+\) gradient. Oxidative stress during reperfusion exasperates the process. Under these conditions, once uncoupled, mitochondria will never be able to develop a inner membrane potential that will sustain oxidative phosphorylation unless the pore is closed and the Ca\(^{2+}\) removed [Crompton et al, (1988); Crompton and Costi, (1990)]. Closure of the pore would prevent membrane depolarisation. CsA has been demonstrated to protect against the deleterious effects of Ca\(^{2+}\) induced cell/tissue damage by inhibiting the non-specific pore [Nazareth et al, (1991); Kass et al, (1992); Griffiths and Halestrap, (1993); Imberti et al, (1993); Duchen et al, (1993); Pastorino et al, (1993); Nieminen et al, (1995); Crompton et al, (1995)]


[1.13.1] Cell Death:

Cell death is associated with prolonged elevation of cytosolic [Ca\(^{2+}\)]. Under this condition, activation of the Ca\(^{2+}\) sensitive mitochondrial pore would be expected. This would result in the uncoupling of mitochondria. The collapse of the membrane potential may be a step in the cause of irreversible injury (cell death) since mitochondria, once uncoupled, will drive the cell into a vicious cycle of energy deprivation. The cells are unable to generate ATP to repair damage (i.e. ionic imbalances, protease and nuclease activity, oxidised proteins and phospholipids) caused by elevated levels of Ca\(^{2+}\) and oxidative stress. Any available ATP would be hydrolysed by mitochondria in a futile attempt to sustain the fallen proton gradient.

47
Since increased cytosolic free $[\text{Ca}^{2+}]$ and severely depressed ATP levels (90% loss) lead to membrane depolarisation via pore activation, components that prevent dissipation of membrane potential will protect cells against the effects of cell death. Cyclosporin A was demonstrated to prevent hepatocytes from dying [Broekemeier et al, (1992); Kass et al, (1992); Imberti et al, (1993); Pastorino et al, (1993)]. The cyclic peptide appeared to prevent uncoupling of mitochondrial oxidative phosphorylation which suggested that pore opening caused dissipation of membrane potential. Interestingly the ADP translocase inhibitor, bongkrekic acid, also inhibits apoptotic dissipation of membrane potential [Zamzam et al, (1996)].

Preservation of cell death by Cyclosporin A has also been explored in T-cells [Mercep et al, (1989); Shi et al, (1990)].


The $\text{Ca}^{2+}$ activated membrane pore functions as a non-specific transport system. It might serve as a mechanism for eliminating unwanted components from mitochondria (e.g. excess $\text{Ca}^{2+}$, degraded matrix proteins from protein turnover etc.) that do not possess a specific transport system [Igbavboa et al, (1989); Gunter and Pfeiffer, (1990)].


The role of the membrane pore in heat production has also been proposed. Like thermogenesis in brown fat tissue, it was postulated to direct substrate energy for ATP synthesis to heat generation [Himm-Hagen, (1976)]. The occurrence of the pore has been linked to the $\text{Ca}^{2+}$ dependent activation of respiration of liver mitochondria from hibernating squirrels [Brustovelsky et al, (1993)].
Mitochondrial signalling.

Under conditions of energy deficiency such as hypoxia, mitochondria in cells aggregate. This might be an attempt by the cell to unite functionally impaired mitochondria, that uncoordinated are unable to maintain necessary energy production, to form a large continuum capable of energy production and transmission [Skulachev, (1990)]. Mitochondria have been demonstrated to form tightly associated, long, thread-like structures at sites believed to be gap junctions. Since porin is localised at these sites it was postulated that it may function to co-ordinate electrical signals between mitochondria that are necessary for regulating energy production [Amchenkova et al, (1988); Skulachev, (1990); Bakeeva et al, (1993); Konstantinova et al, (1995)].

Aims of the study.

The long term aim of this work is to resolve the protein components of the membrane pore. Functional characteristics of the pore have been thoroughly investigated however the molecular components of the structure have not been conclusively identified. Photolabelling studies on intact mitochondria using a photoactive derivative of CsA ($^3$H[PA-CS] ) will be conducted to detect CsA-binding proteins. Since Ca$^{2+}$ and ADP are recognised pore ligands that open and close the pore respectively, their effect on $^3$H[PA-CS] binding to mitochondrial components will be analysed.

Once the relevant target protein is detected in heart and liver mitochondria it will be purified and its mitochondrial distribution investigated.

Activity studies and protein sequencing of the relevant target component should reveal whether the protein displays characteristic behaviour associated with CsA binding protein (PPIase activity).

Time allowing, studies will be conducted to isolate any proteins that interact with the CsA receptor.
Chapter 2:

[2.1] Preparation of rat heart and liver mitochondria.

The heart has some advantages over other mammalian tissues as a source of mitochondria. Mitochondria derived from heart are fairly stable, i.e. can be stored for long periods of time and are more resistant to oxidation damage. A standard method (described below) was used to isolate mitochondria derived from rat hearts and livers.

Typically, two Sprague-Dawley rats (250-300g body weights) were killed by cervical dislocation. The hearts were quickly removed and placed on tissue paper. They were cut, blotted and then transferred to ice-cold 210mM Mannitol/ 70mM Sucrose/ 10mM Tris-HCl/ 1mM EGTA/ BSA (1mg/ml)/ pH 7.2 (MSTEB). The tissue was then finely chopped and allowed to settle. After 1 min the upper layer containing blood was discarded and the tissue washed in MSTEB a further 3 times. The chopped tissue was transferred to 30ml centrifuge tubes (2 hearts or 1 liver per tube) and homogenised for 12 secs using a Polytron homogeniser with the rheostat set at position 4. The polytron probe was pre-cooled to 4°C before use. The tissue homogenate was centrifuged (Sorvall RC5B Refrigerated Centrifuge) at 1800g for 4.5 mins at 5°C to sediment cell debris, nuclei, and unbroken cells. Supernatants were decanted and centrifuged at 8000g for 8 mins at 5°C to sediment mitochondria. The mitochondrial pellets were resuspended in ice-cold 210mM Mannitol/ 70mM Sucrose/ 10mM Tris-HCl/ pH 7.2 (MST) and sedimented again. This was repeated a further 3 times. The mitochondria were suspended finally in MST (15-20mg of protein/ml) and kept on ice.

Rat liver mitochondria were prepared similarly but with the following modification: After sedimenting unbroken cells, cell debris, nuclei etc., the supernatants were centrifuged at 8700g for 8 mins at 5°C. The mitochondrial pellets were resuspended and MST added to 30 ml. The mitochondria were washed 4 times more by centrifuging at sequentially lower speeds i.e. 8700, 8200, 7500, and 7000g, 8 mins at 5°C. This procedure removes lysosomes [Bustamante et al, (1977)], adsorbed endoplasmic reticulum membranes [Katz et al, (1983)], and microsomes from the mitochondrial pellet. Lysosomal enzymes damage mitochondrial
function whilst non mitochondrial protein in general reduces the yield of intact mitochondria.

All centrifugation steps were conducted at 5°C whilst all other procedures were carried out on ice.

[2.2] Preparation of Sheep heart mitochondria.

To obtain more mitochondrial protein, sheep hearts were also used. These were readily available from the University’s Medical school. The sheep was killed by a lethal injection and 15 mins after death the chest cavity was cut open by sawing through the rib cage. The heart was removed and transported back to the laboratory covered in ice. Visible fat was removed and the heart diced into small pieces (1-2cm²) in ice-cold MSTE. The diced tissue was quickly cut into smaller pieces and allowed to settle. After 1 min. the supernatant, containing blood, was discarded. The chopped tissue was washed a further 3 times in MSTE to remove as much blood as possible. The tissue was homogenised for 12-15 secs until no visible pieces of heart were left. The thick layer of fatty material resting on the surface of the homogenate was “spooned off” and the homogenate filtered through 3 layers of cheese cloth to remove any unhomogenised material. The filtered homogenate was centrifuged in 6 x 250 ml plastic centrifuge tubes at 1700g for 4.5 mins at 5°C. The supernatants were retained and the mitochondria isolated as described for rat heart mitochondria (section 2.1).

Mitochondria used for protein purification were stored at -70°C for up to 3 weeks whilst those needed in photolabelling studies (section 2.4.3) were used immediately on the day of preparation.


The mitochondrial protein content was routinely estimated by a modified Biuret method [Kroger and Klingenberg, (1966)] in which BSA was used as standard. Briefly, 50µl of the mitochondrial suspension was mixed with 200µl of sodium cholate (4%), 2 ml NaOH
(10%), and 450µl of H₂O in a plastic cuvette. 300µl of CuSO₄ (1%) was added and the suspension mixed thoroughly. After 15 mins at room temperature, the absorbance at 540nm was determined using a Cecil CE1020 spectrophotometer. The blank cuvette contained H₂O in the place of sample.


[2.4.1] Introduction

When the therapeutic potential of Cyclosporin A (CsA) (fig. 2) was recognised there was an obvious need for derivatives that were not provided by nature or could not be obtained by chemical modification of naturally occurring compounds. The biosynthesis of Cyclosporins does not occur on ribosomes like proteins but rather on a multifunctional enzyme complex. The biosynthesis of Cyclosporins by this enzyme is comparable to the biosynthesis of fatty acids (in fatty acid biosynthesis the product of one enzyme is a substrate for another and no intermediate is released but rather passed from one enzyme to the next until the fatty acid is complete). An enzyme preparation capable of Cyclosporin A synthesis was isolated by Lawen and Zocher [Lawen and Zocher, (1990)] from Tolypocladium. The various derivatives of Cyclosporin (designated CsA, CsB, CsC etc) were synthesised by the addition of the required amino acids to the fermentation media, for example, the addition of DL-2 aminobutyric to the media results in the exclusive production of CsA.

Studies of structure-function relationships have revealed that the amino acids 1, 2, 3, 10, and 11 (fig. 2) of CsA are intimately involved in the immunosuppressive activity and that they are clustered on the surface of the molecule [Durette et al. (1988)]. Modification at these positions results in derivatives with reduced immunosuppressive activities. In the derivative described below and employed in this study, the amino acid at position 8 was modified.

[2.4.2] Photolabel.

A tritiated, photoreactive derivative of Cyclosporin A (³H[PA-CS]) (Sandoz 212-122) was supplied by Sandoz (fig. 4a) to identify CsA binding components. This has been used
previously to label CsA binding proteins of human lymphoid and fibroblast cells [Foxwell et al, (1992)] as well as the multidrug resistance glycoprotein of ovary cells [Foxwell et al, (1989)], and the Na⁺-D - glucose co-transporter in renal brush boarder membranes [Ziegler et al, (1988); Ziegler et al, (1990)]. By introducing different functional groups into the molecule (by adding different amino acids to the fermentation reaction) it was possible to obtain derivatives with different properties. These were used to distinguish specific groups involved in inhibiting the PPIase activity of cyclophilins [Bierer et al, (1990); Dumont et al, (1992)] as well those responsible for conferring immunosuppressive activity [Sigal et al, (1991)]. Recent structural studies on the CsA-cyclophilin complex [Pflugel et al, (1993)] showed that position 8 is not intimately involved in CsA-cyclophilin interaction. Therefore on this basis it was possible to introduce an amino acid with a reactive side chain at this position of the molecule without interfering with its cyclophilin interactions. Cyclosporin A was modified at this position with an aminobutanoyl spacer arm attached to a photoreactive (diazirine) group (fig. 4a). Upon activation (illumination) diazirine groups covalently bind to the nearest group in the vicinity. Diazirines offer a number of advantages over nitrenes as photoactive groups. The trifluoromethyl derivative of the diazirine group was chosen because it was found to be exceptionally stable. The activation of the photoactive diazirine group generates a carbene electrophile that is far more reactive than the nitrene (N₃) group. This makes it a much more short-lived species (which is favoured) so that only components in the very near vicinity (to be almost specifically interacting) are covalently linked. Nitrene groups suffer from an extended length of activation which means that the activated photoreactive group has sufficient time to covalently bind different components (i.e. crosslink more unspecifically). Another unfavourable feature of prolonged lengths of activation is that some of the nitrene intermediates generated will only react with specific functional groups in polypeptides. If such functional groups do not exist on the component that is in close vicinity, then the component will not be detected. Another important advantage that diazirines have over nitrene groups is that they are activated at higher wavelengths (340-360nm) that are non-damaging to proteins [Church and Weiss, (1970)]. Ultraviolet light with λ <300nm is needed to activate nitrene groups. This is harmful to polypeptides.
To allow crosslinked products to be analysed on gel filtration chromatography and gel electrophoresis the photoactive derivative was usually tritiated. The double bond on carbon 9 of the amino acid (4R)-4-(E)-2-butenyl)-4-methyl-L-threonine (MetBmt) at position 1 of the molecule (see fig. 4a) was reduced to allow the incorporation of $^3$H.

[2.4.3] Protocol for photolabelling.

The standard method adopted for photolabelling was as follows:

Mitochondria (20mg of protein/ml) were suspended in 6 ml of MST pH 7.2 containing a cocktail of protease inhibitors (PI). The protease inhibitors are largely small peptides that inhibit thiol, serine/threonine and acid proteases. Leupeptin (thiol protease inhibitor), antipain (thiol protease inhibitor) and pepstatin (acid protease inhibitor) were added at 1 μg/ml mitochondrial suspension whilst aprotinin (serine protease inhibitor) was added at 5 μg/ml. An ethanolic solution of phenylmethylsulphonyl fluoride (PMSF) (serine protease inhibitor) was added at 0.4mM. (Also, PMSF at this concentration almost completely inhibits the carboxylesterase activity observed in a sonicated suspension of mitochondria) (fig. 17). The mitochondrial suspension was split into 3 x 2ml aliquots in 50ml beakers that were cooled to 0°C.

It has been established that Ca$^{2+}$ and ADP affect the activity of CsA on membrane permeabilisation in an opposite and reverse manner (Ca$^{2+}$ depresses CsA- induced pore closures whilst ADP stimulates it) [Novogordov et al, (1992); Andreeva and Crompton, (1994)]. On this basis, photolabelling was conducted under the following conditions; in the presence of Ca$^{2+}$, in the absence of Ca$^{2+}$, and in the presence of ADP. Accordingly, 100μM of CaCl$_2$ was added to the first beaker to induce Ca$^{2+}$ repletion of mitochondria whilst 1mM of EGTA was added to the other beakers to ensure that mitochondria were Ca$^{2+}$ depleted. The beakers were then incubated on ice for 30 mins after which 0.5μM ethanolic $^3$H[PA-CS] was added to all the beakers in the dark under a red safety light. When added, 2mM ADP was introduced together with $^3$H[PA-CS] into the third beaker. After 7 mins continuous mixing in the dark the suspensions were exposed to long U.V light (Mineralight lamp, model UVGL-58)
at a distance of 10cm. The preincubation with $^3$H[PA-CS] in the dark was carried out to maximise interaction between the $^3$H[PA-CS] and its protein target(s).

In the majority of protein/ligand interactions the protein component of the reaction changes conformation to bind the ligand. However in this case it is the ligand ($^3$H[PA-CS]) that must adopt a different configuration to allow protein-ligand interaction since it displays a different structural surface in polar and apolar (i.e. ethanol) environments. In a typically apolar environment the hydrophobic side groups of CsA are folded away from the backbone structure whilst in an aqueous environment they are largely folded over from the ring [Lautz et al, (1992)].

After 20 mins irradiation at 0°C with continuous stirring the mitochondrial samples were transferred to eppendorf tubes and sedimented (2-3 mins at full speed in Eppendorf Bench Centrifuge) and then treated as described below.

[2.4.4] Preparation of photolabelled submitochondrial particles (SMPs)

Photolabelled mitochondria (20mg of protein/ ml) were suspended in ice-cold 10mM Tris-HCl/ 0.1mM EDTA/ protease inhibitors (PI)/ pH 8.1 and sonicated. Sonication (12-14A, MSE Soniprep F150 sonifier) of the samples was conducted on ice for a total of 2-3 mins at 15 sec intervals until the sonicate was translucent. The 15 sec intervals between sonication was to prevent the probe from heating up and consequently warming the sample. Sonication was routinely used to “break open” mitochondria and release the matrix content. Sonication also inverts the inner membrane so that inside-out vesicles are formed with the matrix side of the inner membrane facing the outside (SMPs). To isolate SMP’s (pellet) from the matrix fraction (supernatant) the sonicate was centrifuged 120,000g for 90 mins at 5°C (Beckman Ultracentrifuge, rotor 70.1Ti). The sediment and the supernatants were then stored at -70°C.

[2.5.1] Use of CHAPS as detergent.

Photolabelled SMP's were extracted in CHAPS (3-cholamidopropyl dimethylammonio-1-propane sulphonate). CHAPS is a mild detergent used routinely in protein purification procedures. It is a bile salt derivative containing a sulphonic acid and a tertiary amine group. With no net charge, it may be used with ion exchange resins. In addition CHAPS has a number of other favourable properties that make it suitable for use in protein purification methods. Chromatographic separations of proteins are routinely monitored at 280nm or higher wavelengths. Some detergents interfere with U.V measurements e.g. Triton X-100 absorbs strongly at this wavelength. CHAPS however has negligible absorption at 280nm and is thus unlikely to affect absorption measurements. Also, it has a high critical micelle concentration (CMC) of 6.5mM. This is the concentration of detergent beyond which it aggregates to form micelle vesicles. This means that it can be used at relatively high concentrations during solubilisation procedures. A high CMC combined with a low aggregation number (ten molecules of detergent per micelle) allows CHAPS to be easily removed from protein samples by ultrafiltration (centriconing) and also be used in gel filtration procedures.

CHAPS was routinely used at a concentration of 0.5% (mass/ vol.) throughout FPLC column chromatography to ensure that the extracted components remained soluble throughout the separation process and to prevent the blockage of the columns. A concentration of 6% (mass/ vol.) was used to extract protein components from the membranes.

[2.5.2] The determination of $^3\text{H}\text{[PA-CS]}$ binding to mitochondrial phospholipids.

Mitochondrial phospholipids were extracted in CHCl₃/ CH₃OH (1:5:1) and evaporated to dryness. After suspension in 100mM KCl/ 5mM Hepes/ pH 7.4 the sample was sonicated (30-40mins) under nitrogen until it was clear. This was then photolabelled in the same way as mitochondria except that the particles were sedimented using a Beckman Airfuge (100,000g...
for 30mins). The sedimented vesicles were extracted using CHAPS as detergent and analysed on Superose 12 gel filtration column.

[2.6] Calibration of gel permeation columns.

Gel filtration columns were calibrated using Bovine Serum Albumin (66kDa), Ovalbumin (45kDa), Carbonic Anhydrase (29kDa), Chymotrypsinogen (24kDa), Ribonuclease A (13.7kDa), Cytochrome C (13kDa). A plot of $K_{AV}$ (partition coefficient) versus Mw is demonstrated in figs 8a, b, c, and d.

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o}$$

$V_e$ = elution volume of standard or unknown;
$V_o$ = void volume of column. Void volumes of both Superose 12 and Superdex 75 were 6 ml each whilst the two columns in series had a void volume of 12 ml;
$V_t$ = the total column volume. Column volume of Superose 12 and Superdex 75 are 24 ml each.


Submitochondrial particles (SMP's) that were stored at -70°C were thawed at 5-10°C and extracted in CHAPS buffer. The following procedure describes the extraction and gel filtration steps employed:

The SMP pellet was extracted in 6% (w/v) CHAPS/ 150mM NaCl/ 20mM Hepes/ 0.5mM EGTA/ PI/ pH 7.2 at 0°C (10-15mg of protein/ ml of solubilisation buffer). After 30 mins. agitation on ice, insoluble components were sedimented at 120,000g, 45 mins at 5°C (Beckman Ultracentrifuge, 70.1Ti rotor). The soluble material (supernatant), usually 1 ml in
volume, was concentrated by ultrafiltration using a centricon 10 concentrating cell (Amicon ultrafiltration cell, 10,000 Mr cut off). Ultrafiltration was conducted at 6000g, at 5°C (using a SS34 rotor type) until the volume of the extract was 400μl or less. Reduction in volume lower then 400μl was difficult since there was tendency for the extract to become quite viscous around this mark. This impeded further ultrafiltration. Protein resolution is enhanced on gel filtration chromatography when small volumes of sample are applied.

Extract samples were clarified by centrifugation (10,000g for 8 mins at 5°C ) or filtration (0.22μm , Millipore filter) before applying to gel filtration columns.

The photolabelled extract was fractionated on a Superose 12 gel filtration column (24ml, Pharmacia) equilibrated in 0.5% CHAPS/ 150mM NaCl/ 20mM Hepes/ 0.5mM EGTA/ PI (omitting PMSF)/ pH 7.2. Superose 12 is an agarose-based matrix that separates biomolecular components between 1-300kDa in molecular mass. The matrix can interact hydrophobically with some components which means that they are sometimes eluted later than predicted by their molecular masses.

After fractionation 5-10% of each fraction was mixed with 4ml of Ecoscint H and counted for ^H[PA-CS] activity. Fractions exhibiting ADP/ Ca^{2+} sensitive photolabelling (see Results, figs11a and 12a) were pooled and centriconed to 200-300μl volumes. Even though the centicon membranes are supposed to be inert to almost all biological molecules, small amounts of fractionated proteins were found adsorbed to them. Bound proteins were routinely removed by treating the membrane with a small volume of a high salt buffer (10μl of 500mM NaCl) pH 7.2. After 10-15 mins incubation with ice-cold high salt buffer the desorbed material was pooled with the previously concentrated sample.

In some cases, concentrated fractions displaying ADP/ Ca^{2+} sensitive photolabelling were further analysed using a narrower range gel filtration column, Superdex 75 (HR 10/50, 24 ml, Pharmacia, 3-70kDa). In other cases, in an effort to improve resolution and reduce fractionation times, samples were resolved on a Superose 12 in series with a Superdex 75. Throughout all the fractionation procedures fractions were kept at 0°C to minimise proteolytic damage.
Inhibition of liver mitochondria carboxylesterase activity using substituted trifluoromethylketones.

More then 90% of the covalently bound label becomes deconjugated from photolabelled SMP's when they are left overnight at 0°C. The cause of this was unknown. $^3H[PA-CS]$ contains a butanoyl spacer between the photoreactive diazirine group and the CsA ring (see fig. 4a). This ester bond was considered to be a potential target for enzymatic hydrolysis. Carboxylesterases catalyse the hydrolysis of a wide range of aliphatic and aromatic esters, as well as amides and thioesters [Ashour and Hammock, (1987)]. Mitochondria contain a matrix resident carboxylesterase which might be responsible for $^3H[PA-CS]$ hydrolysis. However the source might also be a contaminatory enzyme of non-mitochondrial origin. Para-nitrophenylacetate (p-NAc) is rapidly hydrolysed by carboxylesterases and this was used to monitor the activity and its inhibition by trifluoromethylketones [Allen and Abeles, (1989)].

Carboxylesterase assay.

0.1ml of rat liver mitochondria (5mg of protein/ml) was diluted 30 fold with 100mM Tris-HCl pH 7.0. The suspension was sonicated for 15-20 secs on ice (MSE Soniprep F150 sonifier, 12-14A). The sonicate was transferred to a cuvette and equilibrated to 37°C (approx. 5 mins.). The carboxylesterase reaction was started with the addition of 0.25mM ethanolic p-nitrophenylacetate. The production of the chromophore nitrophenol was measured at 400nm. Substituted trifluoromethylketones were added at various concentration from an ethanolic stock solution (100mM). The rate of uncatalysed p-NAc hydrolysis (control) was obtained with the addition of 0.1ml H$_2$O in place of mitochondrial sonicate.

Measurement of Peptidylprolyl cis trans isomerase (PPIase) activity.

Rationale of assay.

PPIase activity was routinely assayed by the procedure of Fischer [Fischer et al, (1989)] using the test peptide N-succinyl-ala-ala-pro-phe-nitroanilide. The assay is based on
the conformational specificity of α-chymotrypsin that hydrolyses the test peptide only when
the ala-pro bond is in the \textit{trans} configuration (fig. 3). The yellow chromophore released (4-
nitroanilide) was measured at 390 minus 480nm.

In an aqueous solution, 90% of the commercially available peptide is in the \textit{trans}
conformation whilst the remaining 10% is in the \textit{cis}. In the presence of high concentration of
α-chymotrypsin, 90% of the hydrolysis reaction occurs within the dead time of manual mixing
(3-5secs). Hydrolysis of the remaining 10% is slow, limited in rate by \textit{cis} to \textit{trans}
isomerisation of the ala-pro bond of the peptide. This slow rate of hydrolysis is accelerated in
the presence of PPIase. The reaction can be illustrated schematically as follows;

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {N-succ-ala-ala-pro-phe-4-nitroanilide (\textit{trans})};
\node (B) at (3,0) {N-succ-ala-ala-pro};
\node (C) at (0,-2) {N-succ-ala-ala-pro-phe-4-nitroanilide (\textit{cis})};
\draw [->, thick] (A) -- (B);\node (D) at (1.5,0) {\textit{FAST} +};
\draw [->, thick] (C) -- (B);
\end{tikzpicture}
\end{center}

\textbf{Determination of PPIase activity and }k_{\text{cat}}/K_m\textbf{.}

Cyclophilins have a relatively low affinity for the peptide N-succ-ala-ala-pro-phe-4-
nitroanilide. For example, the $K_m$ value for cyclophilin A (bovine) for this peptide is about
1mM [Kofo et al, (1991)]. The maximal concentration of test peptide used in the PPIase
assays in the study was 14\mu M ; of this only about 10\% is in the \textit{cis} configuration. Thus
[test peptide] << $K_m$ value for test peptide. Under these conditions, the reaction will follow a first order reaction. The use of first order reaction is explained as follows;

With excess chymotrypsin to remove the product (trans peptide) the isomerization will proceed unidirectionally,

\[
\begin{align*}
E + cis & \xrightarrow[k_1]{k_2} E . cis \xrightarrow{k_{Cat}} E + trans \\
Velocity & = k_3 [E . cis], \quad \text{where} \quad [E . cis] = \frac{[E] . [cis]}{K_m}
\end{align*}
\]

Therefore Velocity = \( \frac{k_3 [E . cis]}{K_m} \)

Since [cis] << $K_m$, then [E . cis] is very small and [E] is the same as [E_t] where E_t refers to total enzyme.

Therefore Velocity = \( \frac{k_3 [E_t] [cis]}{K_m} \)

\[
= \frac{k_{Cat} [E_t] [cis]}{K_m}
\]

or, Velocity = $k$ [cis] \[1\]

where $k$, a pseudo first order rate constant, is given as

\[
k = \frac{k_{Cat}}{K_m} [E_t] \]

\[2\]

From [1]

\[
dcis / dt = -kcis
\]

where cis is the concentration of the cis isomer at a given time t and k is the rate constant for the reaction.
\[ \text{cis}_t = \text{cis}_0 e^{kt} \quad (3) \]

where \( \text{cis}_t \) is the concentration of the \( \text{cis} \) isomer at time \( t \) and \( \text{cis}_0 \) is the initial concentration of the \( \text{cis} \) isomer.

Now,

\[ \text{cis}_0 = \text{cis}_t + \text{trans}_t \]

Therefore:

\[ \text{cis}_t = \text{cis}_0 - \text{trans}_t \]

where \( \text{trans}_t \) is the concentration of the \( \text{trans} \) isomer formed at time \( t \). Substituting this into equation (3),

\[ (\text{cis}_0 - \text{trans}_t) / \text{cis}_0 = e^{-kt} \]

Now;

\[ \text{cis}_0 = \text{trans} \]

where \( \text{trans} \) is the concentration of the \( \text{trans} \) isomer on the complete isomerisation of the \( \text{cis} \) isomer. Therefore;

\[ \text{trans} - \text{trans}_t / \text{trans} = e^{kt} \]

in practice

\( \text{trans}_t \) is proportional to \( A \)

where \( A \) is absorbance change which has occurred at any time \( t \) due to the hydrolysis of the \( \text{trans} \) isomer.

So;

\[ A_a - A_t / A_a = e^{kt} \]

or

\[ \ln (A_a - A_t / A_a) = -kt \]

or simply
\[ \ln \left( \frac{A_t}{A_a} \right) = -kt \]

where \( A_t \) is the absorbance change that has occurred during time \( t \), and \( A_a \) is the total absorbance change which has occurred.

Plots of \( \ln \left( \frac{A_t}{A_a} \right) \) versus \( t \) were linear and used to estimate \( k \) (-slope, s\(^{-1}\)).

From [2], plots of \( k \) versus \( E_t \) were linear and used to estimate \( k_{cat} / K_m \) (μM\(^{-1}\) s\(^{-1}\))

[2.9.2] PPIase activity.

A 2mM stock solution of the 4-nitroanilide test peptide was made up in 50% ethanol. Such a solution contains an equilibrium mixture of approximately 10% cis and 90% trans conformers [Kofran et al, (1991)]. The solution was clarified with a brief pulse of sonication (10-15 sec, 12-14A) using MSE Soniprep F150 sonifier or in a sonication bath. The standard method for the assay is explained as follows:

60mM KCl/ 20mM Hepes/ 10mM Tris-HCl pH 8.1 assay buffer (0.8ml) was added to a glass cuvette equilibrated to 12°C. Protein sample (5-50μl) and 14μM N-succ-ala-ala-pro-phe-4-nitroanilide were added, and the mixture incubated for 2 mins at the same temperature. Under these conditions the equilibrium distribution remains constant at (approx.) 10% cis/90% trans. The reaction was started with the addition and rapid mixing (2-3 secs) of 15μM \( \alpha \)-chymotrypsin. The amount of chymotrypsin is sufficient to hydrolyse pre-existing trans conformer within the mixing time. The cis/ trans distribution is now no longer at equilibrium and there follows a net conversion of the cis to trans. This reaction occurs in the absence of PPIase (thermal cis-trans isomerization) giving a ‘basal’ rate of isomerization. The increase in the ‘basal’ rate in the presence of extract is due to PPIase activity.

When the inhibition of PPIase activity by CsA was measured, CsA was incubated for 7 mins with the protein sample before the start of the reaction. Since CsA adsorbs to glassware the cuvette was routinely washed in ethanol after use.

Covalently labelled proteins were rapidly deconjugated (section 2.8). Therefore there was a need to detect CsA binding proteins and to measure their $[^3]H$CsA binding capacities by some means other than photolabelling. Sephadex LH-20 minicolumns were routinely used to detect CsA binding proteins (CSBP). Sephadex LH-20 were first employed by Koletsky and colleagues [Koletsky et al, (1986)] to identify CSBPs in thymus cells and since then they have been widely used to look at CSBPs in other tissues. LH-20 minicolumns were therefore employed in this study as well.

Sephadex LH-20 is a crosslinked dextran in which the hydroxyl groups have been alkylated. This modification allows it to function as a weak hydrophobic matrix column (in which the elution of free hydrophobic CsA would be delayed), as well as a gel filtration matrix column, separating components according to molecular size.

Sephadex LH-20 (Sigma) minicolumns with 2ml bed volumes were constructed using glass pasteur pipettes. They were equilibrated in 20mM Tris-HCl/4mM 2-mercaptoethanol pH 7.4 [Koletsky et al, (1986)]. The binding of free $[^3]H$CsA was determined by mixing 90µl of 20mM Tris-HCl/4mM 2-mercaptoethanol pH 7.4 with 1µM $[^3]H$CsA (30nCi) and 3-4µl of (20mg/ml) of blue dextran. The mixture was applied to a column and eluted with the equilibrating buffer. The void volume (approx. 2 ml) of the column and the elution pattern of $[^3]H$CsA was determined (see fig. 7a).

The effect of CHAPS on the adsorption of free $[^3]H$CsA to the matrix was also investigated since it competes with $[^3]H$CsA for the non-polar groups as well as forming micelles with the CsA. This would reduce the retention of free $[^3]H$CsA so that it appears in the void volume of the column. Minicolumns were equilibrated in 150mM NaCl/10mM Hepes/0.5mM EGTA/ pH 7.2 and CHAPS (w/v) at varying concentrations (0, 0.125, 0.6, and 6%). 1µM $[^3]H$CsA (30nCi) was diluted in 150mM NaCl/10mM Hepes/0.5mM EGTA/ pH 7.2 containing 0.5 or 6% CHAPS (w/v) and applied to the columns (fig. 7a).

Elution of free $[^3]H$CsA containing 6% CHAPS on a minicolumn equilibrated in 6% CHAPS was considerably affected. Almost all of the CsA was eluted in the void volume (fig. 64).
7c). Only very little was retained. This suggests that at high detergent concentrations either a) CHAPS forms large mixed micelles (composing of CHAPS/[^3]H]CsA vesicles) that are of sufficient size to be excluded in the void volume or b) sites responsible for retaining free[^3]H]CsA are unavailable (since they are detergent associated) so that it appears in the void volume of the column.

The elution of[^3]H]CsA was unaffected by low concentrations of CHAPS (0.125, 0.5, and 0.6%) when measured on minicolumns equilibrated in 0.6% CHAPS (fig. 7a) however it was slightly affected when[^3]H]CsA in a 0.6% CHAPS buffer was fractionated on a minicolumn equilibrated in 6% CHAPS (see fig. 7b).

From these findings it can be concluded that analysis of[^3]H]CsA binding proteins in high [CHAPS] will give misleading results since the detergent affects the retention of unbound[^3]H]CsA. Thus[^3]H]CsA binding assays should be conducted in which the [CHAPS] of the sample is <<6% and the minicolumns are equilibrated in no greater then 0.5% [CHAPS].

LH-20 columns were routinely used to measure[^3]H]CsA binding of gel filtration and Mono-S column fractions. Between 5-10% of each fraction (approx. 100μl mixed with 1μM[^3]H]CsA (30nCi) and 3-4μl of 20mg/ml blue dextran and applied to a LH-20 column. Proteins binding[^3]H]CsA were eluted in the void volume (<1.5ml) whilst unbound[^3]H]CsA was retained and eluted later in the remaining 3ml.

Besides using LH-20 to detect[^3]H]CsA binding proteins they were also used to separate[^3]H][PA-CS] labelled proteins from non-labelled/unbound[^3]H][PA-CS]. This was necessary for purifying CsA binding proteins in mitochondria. Labelled protein was added to a much larger volume of extracted protein and analysed on gel chromatography. This allowed the purification procedure to be scaled up.

Since[^3]H]CsA adheres to glassware tubes containing LH-20, fractions were washed in ethanol, and the wash volumes added to the scintillation counting vials. After use LH-20 columns were cleaned in 100% ethanol to remove residual[^3]H]CsA. Columns not in use were stored in the dark (to prevent algal growth) with 20% ethanol to prevent microbial growth.
[2.11.] Preparation of mitochondrial subfractions

Patch clamp and permeability studies have shown the Ca\(^{2+}\) activated membrane pore to be blocked by CsA in an ADP/ Ca\(^{2+}\) sensitive manner. Since CsA binds cyclophilins it must be assumed that a mitochondrial cyclophilin is involved in pore opening and closing. Earlier work, based on CsA binding and PPIase activity analysis, proposed the involvement of cyclophilin in pore function [McGuinness et al, (1990); Halestrap and Davidson, (1990)]. Halestraps’ group [Halestrap, (1994)] have proposed a 18kDa PPIase (CyP18) to play a catalytic role in opening and closing of the pore. In contrast to this Cromptons’ group demonstrated the presence of a 10kDa CsA binding component that photolabelled (\(^3\)H[PA-CS]) in an ADP/ Ca\(^{2+}\) sensitive manner [Andreeva and Crompton, (1994)]. The same group then went on to isolate a 22kDa PPIase from the membrane fraction of mitochondria which also labelled in an identical manner [Andreeva et al, (1995)].

From the above findings mitochondria were fractionated into different compartments. PPIase activity in each fraction was then measured and resolved by gel filtration chromatography. This procedure would allow:

a) the localisation and distribution of PPIase activity to be determined in each of the different fractions

b) the isolation and subsequent identification of the relevant protein involved in pore activity.

Another reason for fractionating mitochondria into different subfractions was to remove a suspected protease activity. The ADP/ Ca\(^{2+}\) sensitive labelled 22kDa protein isolated from SMP’s was unstable. A protease thought to be responsible for this was suspected of being present in the SMP fraction. Removal of the proteolytic activity would improve the stability of the 22kDa PPIase.

The PPIase activity in mitochondrial subfractionations was determined. Mitochondria were fractionated into;

i) outer membrane plus intermembrane space (OM/ IMS)

ii) peripheral components binding to the outside of the inner membrane
iii) matrix fraction plus peripheral components binding to the inside (matrix facing) of the inner membrane

iv) components integral to the inner membrane (CHAPS extracted fraction)

The first step in mitochondrial subfractionation was the preparation of mitoplasts. Mitoplasts are mitochondria with their outer membrane and intermembrane space removed. They were prepared by a standard method [Pedersen et al, (1979)] using digitonin. The outer membrane is lysed by digitonin. Digitonin selectively binds cholesterol that is found abundantly in the OM [Pedersen et al, (1979)]. At low concentrations digitonin selectively lyses the outer membrane leaving the inner membrane intact whilst at higher concentrations the inner membrane is also lysed. In this study a fixed concentration of 0.12mg of digitonin/mg of mitochondrial protein was found to be optimal for mitoplast preparation. The following procedure used was as follows: A mitochondrial pellet was resuspended in MST (50mg of protein/ml) containing protease inhibitors. Digitonin (0.12mg digitonin/mg of mitochondrial protein) made in MST was gently warmed (to aid dispersion of detergent) and then cooled on ice. The mitochondrial suspension and the digitonin solution were gently mixed together and left on ice for 15 mins with occasional stirring. After centrifugation (8000g, 8 mins at 5°C) of the mixture the pellet (mitoplasts) and the supernatant (OM/IMS) were separated.

To separate components electrostatically associated with the outside of the inner membrane (cytoplasmic facing) the mitoplasts were treated with high salt. Mitoplasts were suspended in 0.5M KCl/20mM Hepes/0.5mM EGTA/PI/0.5mM dithiothreitol (DTT)/pH 8.1 and gently mixed for approx. 2 mins on ice. Dithiothreitol (DTT) is commonly used at concentrations between 0.5-1mM to prevent the oxidation of SH groups in proteins. The KCl treated mitoplasts were sedimented (8000g, 8 mins at 5°C) and the supernatant (KCl wash), containing components electrostatically associated with the outside of mitoplasts, decanted and stored at -70°C. KCl treated mitoplasts (pellet) was resuspended in 20mM Hepes/0.5mM EGTA/PI/0.5mM dithiothreitol (DTT)/pH 8.1 and sedimented to remove the salt.

To fractionate the matrix and components attached to the inside of the inner membranes the salt washed mitoplasts were sonicated in a high salt buffer. After removal of
the KCl wash the sedimented mitoplasts were resuspended in 0.5M NaCl/ 20mM Hepes/ 0.5mM EGTA/ PI/ 0.5mM dithiothreitol (DTT)/ pH 8.1 and sonicated on ice until translucent (12-14mA setting for a total length of 10 mins with 15 sec intervals). The inner membrane fraction was sedimented (120,000g, 70 mins at 5°C , rotor 70.1 Ti) and the supernatant (matrix and proteins electrostatically associated with the inside of the inner membrane) stored at -70°C.

Proteins located within or anchored to the inner membrane were solubilised using CHAPS as detergent. The inner membrane sediment was homogenised on ice in 6% CHAPS/ 150mM NaCl/ 20mM Hepes/ 0.5mM EGTA/ PI/ 0.5mM dithiothreitol (DTT)/ pH 8.1 for 30 mins after which the non-solubilised material was sedimented (120,000g, 70 mins at 5°C ) and the supernatant (solubilised fraction) stored at -70°C.

The PPIase was purified from each of the fraction isolated. The whole fractionation procedure is summarised in fig. 4b.

Labelled CyP22 from SMP’s was found to be unstable which meant that further studies on the pure protein were not possible. It was found that the stability and consequently the yields of the protein were considerably improved if the OM/ IMS were removed and the resulting mitoplasts treated with high salt buffer. Since the stability of the protein was now much improved it was believed that it might have been exposed to proteolytic hydrolysis that was either mitochondrial (OM/ IMS or activity attached to the outside of the inner membrane) or non-mitochondrial in origin (microsomal).

[2.11.1] Preparation of PPIase from mitochondrial subfractions.

PPIase from each of the subfractionations of mitochondria was purified to a single band on SDS-PAGE. The purification of each PPIase was carried out in almost identical manner but with minor modifications. The procedure for the purification of PPIase isolated from mitoplasts sonicated in high salt (0.5M NaCl) is described as follows:
a) Dialysis;

50ml of the supernatant (8mg of mitochondrial protein/ml) isolated from mitoplasts sonicated in high salt (matrix and proteins electrostatically associated with the inside of the inner membrane) was thawed at 5-10°C and dialysed. Dialysis tubing was boiled for 20 mins in 1mM EDTA. Heavy metals that may be found on the dialysis tubing might effect PPTase activity. Dialysis tubing was then extensively rinsed with water to remove all traces of chelating agent. After overnight dialysis of the sample at 0°C against 500ml 20mM Hepes/0.5mM EGTA/PI/0.5mM DTT/pH 7.8 the dialysate was diluted 1:2 to yield a final [NaCl] <25mM.

b) Cation-exchange (S-Sepharose);

The first step in PPTase purification involved the use a cation exchange step. An S-Sepharose cation exchange column (25ml) was constructed at 4°C. The amount of protein that could be applied to the column in a single loading was between 0.5-1g. The diluted extract ([NaCl] <25mM) was clarified (10,000g, 10 mins at 5°C, Sorvall RC5B) and applied at 1ml/min (LKB Bromma, Varoperpex II 2120) to the column equilibrated in 20mM Hepes/0.5mM EGTA/PI (omitting PMSF)/0.5mM DTT/pH 7.8. At this pH the majority of cyclophilins (PPIases) express a net positive charge on their surface. As a consequence all the PPIase activity was retained on the column. Retained proteins were eluted with step gradients (100, 200, and 500 [NaCl]) and fractions exhibiting isomerase activity were pooled and dialysed overnight (as previously) against 500ml 20mM Hepes/0.5mM EGTA/PI/0.5mM DTT/pH 7.7.

c) FPLC chromatography (Mono-S).

The following day the dialysed sample was diluted ([NaCl] < 10mM) and applied to an equilibrated Mono-S cation exchange column (8ml, Pharmacia). All samples applied to FPLC (Fast Protein Liquid Chromatography) columns were clarified by centrifugation (10,000g, 5°C, 8-10 mins) or filtration (0.22μm Millipore filter) whilst all buffers were filtered (0.22μm
Millipore filter) and air equilibrated at room temperature overnight before use. Proteins were eluted by means of a [NaCl] gradient (0-500mM) and fractions (maintained on ice) exhibiting PPIase activity were pooled and diluted 10 fold using 10mM MOPS (morpholino propane sulphonate, Cl- salt)/ 0.5mM EGTA/ PI/ 0.5mM DTT/ pH 6.7. The diluted sample was resolved on an equilibrated Mono-S cation exchange column (1ml, Pharmacia) and eluted using a single 0.5M [NaCl] step. All the isomerase activity was accumulated in a 1-1.5ml volume (2-3 fractions).

d) FPLC chromatography (Gel filtration).

Each fraction (0.5ml) from the small Mono-S column was fractionated on a Superdex 75 (24ml, Pharmacia) gel filtration column equilibrated in 100mM NaCl/ 20mM Hepes/ 0.5mM EGTA/ PI (omitting PMSF)/ 0.5mM DTT/ pH 7.7. Fractions containing isomerase activity from each run were pooled and diluted 10 fold with 10mM MOPS/ 0.5mM EGTA/ PI (omitting PMSF)/ 0.5mM DTT/ pH 6.7 and applied to Mono-S (1ml). On this occasion, using a single gradient step (300mM [NaCl]), a single sharp protein peak was eluted (0.5ml volume) which displayed all the PPIase activity measured. This was fractionated for the final time on Superdex 75 equilibrated in pH 7.7 buffer to obtain the pure protein. A single peak eluting at fractions 14, 15, and 16, peaking at 15, displayed maximal isomerase activity. The activity was pooled (1.5ml), diluted 2 fold with glycerol and stored at -70°C in 2 aliquots (1.5ml each).

Glycerol at this concentration (50%) protects against the damaging effects of freeze thawing (see results section 3.3.1).

Protein samples for SDS-PAGE analysis were freeze dryed to reduce their volumes and enable minigels to be run.


This procedure separates proteins primarily on the basis of their molecular weights [Schagger and von Jagow, (1987)]. SDS (1.4mg of SDS/ mg of protein) binds along the length of the polypeptide chain (but not to regions of glycosylation). SDS (anionic detergent)
disrupts almost all non-covalent interaction in native proteins. A complex of anionic SDS/ denatured protein has a net negative charge that is directly proportional to the mass of the protein. Upon electrophoresis the net negative charge acquired on binding SDS moves the protein towards the direction of the anode. Migration is proportional to the molecular weight of the protein.

Purified proteins were analysed by SDS-PAGE. Two systems of gel electrophoresis were used. One is the continuous buffer system in which the same buffer ions are present throughout the sample, gel, and electrode vessel reservoirs (albeit at different concentration) at a constant pH. In this system the protein sample is loaded directly onto the separating gel.

In contrast, the second type of system, discontinuous (multiphasic) buffer, employs different buffer ions in the gels compared to those in the electrode reservoirs. Most of these have discontinuities in buffer composition and pH. The major advantages of the discontinuous buffer over the continuous buffer systems are that i) a relatively dilute protein sample can be applied to the gels and ii) high protein resolution can be obtained, the reason being that proteins are concentrated into narrow zones (stacks) during migration through the stacking gel. They are partially resolved during migration through the spacer gel and are completely separated during migration through the resolving gel.

Electrophoresis was conducted using two kinds of apparatus. Initially, large slab gels (18cm x 20cm) were cast and run. These required extensive cooling during the lengthy runs (4-5hrs) and large sample volumes (60-80μl). Routinely, mini-gels (Mighty Small II, model SE250, Hoeffer) were used as they offered considerably reduced running times (1-2hrs) and needed small protein samples (10-20μl). The procedure for the preparation of mini-gels used with a discontinuous buffer system is as follows:

Glass plates for the mini-gels were washed extensively in water after storage in concentrated H₂SO₄. After drying with ethanol and acetone, they were clamped to the gel electrophoresis apparatus as described in the users manual. A 16% plug was cast (0.5cm from bottom of plates). This was followed by a 16% resolving gel overlaid with a 9.7% spacer gel. A layer of 50% butanol was applied to the top of the spacer gel to provide a sharp, straight edge to the top of the gel. The gel was then left to polymerise at room temperature (30-60
mins). After the spacer gel had extensively been washed with water (any butanol remaining interferes with the polymerisation reaction) a 1.7% stacking gel was overlaid on it. A 0.5mm thick plastic comb (10 wells) was inserted into the spacer gel at a slight angle to provide a way for the bubbles to escape. The comb was aligned into position whilst ensuring no air bubbles became trapped under the teeth. After polymerisation (30-40 mins) of the stacking gel the wells were thoroughly rinsed with cathode buffer to remove any unpolymerised gel. Unpolymerised gel can interfere with the migration of the sample into the gel.

Before use the gel was left to stand at 4°C overnight to allow complete polymerisation and equilibration. During storage the upper (cathode) and lower (anode) buffer reservoirs were filled, and the gel apparatus was covered in plastic wrap to prevent dehydration of the gel. (Wrapping was essential since the gels were routinely stored in a cold room in which very cold air was continuously circulated).

The unpolymerised components of the gel were made as follows;

A stock solution of acrylamide and bisacrylamide was made. 48g of acrylamide and 1.5g bisacrylamide were mixed vigorously with water to give a final volume of 100ml after which it was filtered (Whatman No.1) in the dark (to minimise light induced polymerisation reaction). The filtered solution was stored in the fridge (4°C) wrapped in aluminium to minimise light-induced polymerisation. The solution is stable for approx. 1 month at 4°C. Before use the solution required gentle warming (to dissolve the precipitated acrylamide) and degassing (trapped oxygen slows down the polymerisation reaction).

The unpolymerised components of the 16% plug were mixed in a small beaker and placed in a bowl of warm water (this speeds up the polymerisation process). To initiate the polymerisation reaction between acrylamide and bisacrylamide a small amount (10-20mg) of ammonium persulphate was added. Once the ‘crackling’ sound of the ammonium persulphate had stopped the polymerisation reaction was accelerated by the addition of 5-10μl TEMED (tetramethylethyldiamine). Once TEMED was quickly mixed (1-2 secs), the gel mixture was immediately poured as polymerisation was now underway.
The resolving and the spacer gels are poured one after the other so that a smooth, intermixed boundary between the two is formed.

Gel buffer.
The gel buffer used was 3M Tris-HCl, 0.3% SDS. The pH was adjusted to 8.45 with concentrated HCl.

16% plug and resolving gel.
10.0 ml 48% (w/v) acrylamide stock
10.0 ml gel buffer
3.2 ml glycerol
6.8 ml H$_2$O

9.7% spacer gel.
6.1 ml 48% (w/v) acrylamide stock
10.0 ml gel buffer
13.9 ml H$_2$O

1.7% stacking gel.
1.0 ml 48% (w/v) acrylamide stock
3.1 ml gel buffer
8.4 ml H$_2$O
Sample Loading Buffer (SLB).

4.0 ml 20% (w/v) SDS
2.4 ml glycerol
0.4 ml 2-Mercaptoethanol
2.0 ml Brilliant Blue G (marker dye, 50mg/100ml H_{2}O)
1.0 ml 1M Tris-HCl, pH 6.8

Preparation of protein samples for loading.

Equal volumes of Sample Loading buffer (SLB) were added to eppendorf tubes containing the protein samples. Samples were denatured in boiling water for 5 mins to allow SDS to bind, cooled at room temperature and applied to the SDS gel.

Cathode buffer.

0.1M Tris-HCl,
1.92M Tricine,
0.1% (w/v) SDS,
The pH of this solution should be 8.2.

Anode buffer.

A 5x stock solution was prepared at 1M Tris-HCl. The pH was adjusted to 8.9 using concentrated HCl.
Electrophoresis.

Electrophoresis was conducted at 4°C, 20mA (constant current) for 1 hr during which time the protein sample had migrated into the resolving gel. The current was increased to 40mA for a further 1-2 hrs until the dye front was approximately 1-1.5cm from the bottom of the gel. Electrophoresis generates heating currents that slow down the migration of proteins. To minimise band distortion and decrease run lengths the gels were cooled. When conducting electrophoresis using large slab gels the glass plates were placed at 4°C in front of an electric fan. This dissipated heat away from the polymerised gel. Water was circulated through the electrophoresis chamber to cool minigels.

Staining of gels.

Coomassie Blue stain:

Staining with Coomassie Blue can detect as little as 0.1μg of protein. The method was as follows:

On completion of electrophoresis the gel was transferred to a 50% (v/v) methanol/10% (v/v) glacial acetic acid solution for fixing. After 1 hr of gentle agitation at room temperature, the gel was transferred to a filtered (Whatman No.1) solution of Coomassie Blue R250 (10% glacial acetic acid/ Coomassie R250 at 1mg/ml) and stained overnight. On the following day the gel was destained in 10% (v/v) glacial acetic acid (2-3 changes) until a clear background was observed.

The Coomassie Blue stain can be stored for a month at room temperature and be used several times. However since it is easily prepared it was made fresh each time.

Silver stain:

This procedure of staining can detect as little as 2ng of protein in a single band. Prior to silver staining, the proteins are fixed. The fixing step has two roles. First, it immobilises the
proteins in the gel, or at least greatly retards their diffusion. Second, it removes substances
that may interfere with the staining procedures such as detergents, reducing agents or reactive
buffer components like glycine. A key factor for success in silver staining after SDS-PAGE is
the removal of SDS from the gel. This was carried out by several washes with methanol/
glacial acetic acid. The basic reaction in silver staining is the reduction of silver nitrate to
metallic silver at a protein band, leading to the deposition of silver grains. The solution for the
stain are as follows:

Solution A) 0.8g AgNO₃ in 4 ml H₂O.
Solution B) 21 ml of 0.36% (w/v) NaOH/ 1.4 ml of 14.8M (30%) NH₄OH

The silver stain (solution C) was prepared by adding solution A dropwise to a vigorously
stirring solution B and allowing the brown precipitate to clear each time. This was made up to
100ml and used within 15-20 mins of preparation

Solution D) 0.5ml 1% (w/v) Citric acid was mixed with 50μl of 38% (v/v) Formaldehyde and
made up to 100ml with water. This was freshly prepared before use.

The procedure for silver staining was as follows:

On completion of electrophoresis the gel was soaked in 50% (v/v) methanol/ 10%
(v/v) glacial acetic acid for 1-2 hrs with 2-3 changes of the solution. The gel was transferred
to the silver stain (solution C) in a glass container. The gel was stained for 15 mins in solution
C with gentle agitation. The gel was then developed using solution D. Once a pale yellow
background began to appear the reaction was terminated with 1% (v/v) glacial acetic acid.
Proteins bands are stained brown/black. Stained gels were rinsed with 2-3 changes of water
for 1-2 hrs before being stored in H₂O.
Blue toning of silver stained gels:

Blue toning is a procedure which amplifies the silver staining of polyacrylamide gels by a factor of 3 to 7 fold [Berson, (1983)]. It was used to enhance light bands obtained using silver stain. The procedure was as follows:

Silver stained gels were thoroughly washed in tap water for 30 mins. 100 ml of toning bath was prepared just before further treatment of the gel by mixing 10 ml 5% (w/v) ferric chloride/ 10 ml 3% (w/v) oxalic acid/ 10 ml 3.5% (w/v) potassium hexacyanoferrate III/ 70 ml H$_2$O. Stock solutions were stable for a few months but if the toning bath appeared blue-green instead of brown it was discarded. The gel was soaked in the toning bath between 30 - 120 secs. Proteins band appeared blue and when the desired degree of staining was obtained the reaction was terminated by washing the gel in tap water for 10 mins. Stained bands were immobilised by soaking the gel in 20% (v/v) methanol/ 5% (v/v) glacial acetic acid for 10 mins.

Gel slicing and $^3$H elution.

Direct autoradiography of $^3$H-labelled proteins in gels cannot be conducted since the low energy of $\beta$ particles fail to penetrate through the gel matrix and expose the X-ray film. To determine the position of the photolabelled protein on SDS-PAGE the gel was sliced and the slices (1mm wide) dissolved. The gel slicer consisted of razor blades (spaced out at 1mm distances) separated by plastic spacers. The blades and the plastic spacers were tightly bonded together to give a rigid slicing tool. The gel was placed on a wax sheet for slicing. After slicing the gel pieces were transferred to plastic tubes, macerated, and dissolved in 30% H$_2$O$_2$ by treating at 55°C overnight.
CyP22 - Affinity Chromatography.

PPIases and their interactions.

Cyclophilins, through their PPIase activity, are capable of changing the conformation of other proteins. They have been demonstrated to bind and interact with a number of proteins e.g. HIV-1 gag [Luban et al, (1994)], a 55kDa Ca$^{2+}$/ Calmodulin-dependant serine/threonine phosphatase [Friedman and Weissman, (1990)], Heat Shock Protein (HSP-90) [Walsh et al, (1993)] via their enzymic activity.

Affinity chromatography is a technique used to explore protein-protein interactions. Used appropriately it can detect weak interaction ($K_d \ 10^{-5}$) between components [Phizicky and Fields, (1995)]. Attempts at using affinity chromatography to investigate cyclophilin binding protein(s) were made.

Attempts to isolate CyP22 binding proteins using affinity chromatography.

Modification of CyPD using Bolton and Hunter Reagent.

To investigate CyP22 binding proteins using affinity chromatography it became necessary to consider the effect of protein coupling to a support matrix and the protein's catalytic activity. This was done using the Bolton and Hunter reagent (3-(4-hydroxyphenyl) propionic acid hydroxysuccinimide ester (fig. 5). Since it is readily iodinated, the crosslinker was originally used to introduce $^{125}$I into proteins lacking a tyrosine residue i.e. secretin, porcine parathyroid hormone. A covalent amide bond between the $\varepsilon$-amino group of lysine of the protein and the ester group of the crosslinker (fig. 5) would occur. The succinimide ester was used to investigate whether, a) CyP22 could be covalently attached to a rigid support through an amide bond and b) whether modifications by Bolton and Hunter reagent (BHR) at a lysine residue effected the cis trans isomerase activity of the enzyme.
A modified method of Bolton and Hunter [Bolton and Hunter, (1972)] was used to modify CyP22. This was as follows: A stock solution of BHR (0.2mg of reagent/ ml of dimethylformamide) was prepared. The solvent was evaporated from a 25µl aliquot (5µg of BHR) at 18°C, 1hr under a vacuum using a DNA dryer (DNA Concentrator). Eppendorf tubes containing BHR were tightly sealed with nescofilm to prevent moisture-dependant hydrolysis and stored at -20°C. CyP22 was purified as described in section 3.2 from rat liver mitoplasts. Dithiothreitol (DTT) and protease inhibitors were omitted from the final gel permeation procedures (Superdex 75) since DTT can interfere with coupling chemistry and modifications of inhibitor peptides (which contain a high percentage of basic amino groups) were unwanted. The pH of CyP22 (10µg) in 1.4 ml of 50% Glycerol/ 10mM Hepes/ 0.25mM EGTA/ 0.4mM PMSF/ pH 7.7 was adjusted to 8.5 on ice using 50% Glycerol/ 100mM Bicine/ 0.5mM EGTA/ pH 9.4. The protein sample was concentrated to 50µl (centricon 10, 6000g, 5°C, 48 hrs). Protein adsorbed to the centricon membrane was and pooled with the 50µl after 20 mins incubation with 10µl of 500mM NaCl/ 50% Glycerol/ 100mM Bicine/ 0.5mM EGTA/ pH 8.5 at room temperature. To covalently link BHR to CyP22, 2-2.5µg of protein was added to an eppendorf tube containing 5µg of succinimide ester. The reaction was incubated at 4°C for 1hr with gentle agitation. A parallel reaction was carried out containing no BHR which served as control (unmodified CyP22). Unchanged succinimide ester was reacted with 250mM Glycine/ 50% Glycerol/ 100mM Bicine/ 0.5mM EGTA/ pH 8.5 for 20 mins at 4°C. This procedure terminates and neutralises any unreacted ester groups that may exist. Modified CyP22 was diluted 2 fold using sample loading buffer (see later) and centrifuged at full speed (Eppendorf Bench Centrifuge, 4°C, 3-4 mins) to pellet aggregated protein which causes bands to streaking on isoelectrofocusing. Samples were electrofocused to determine the extent of modification.


Isoelectrofocusing involves electrophoresis in a pH gradient. Proteins are separated on the basis of their pI values. Proteins migrate until they reach a pH equal to their pI, at which point they carry no net charge and concentrate. Isoelectrofocusing (IEF) is therefore an
equilibrium technique in which the effects of diffusion are overcome. It is a sensitive technique where components differing in 0.001 of a pH unit can be resolved.

[2.14.1] **Carrier Ampholytes.**

Carrier ampholytes are a mixture of synthetic amphoteric buffers that form a smooth pH gradient in polyacrylamide gels. Usually, they are mixed polymers (0.2-1kDa) of aliphatic amino and carboxylic acids (polyamino-polycarboxylic acids). They are generally used at 2% concentration (v/v); concentrations greater then 3% are difficult to remove from the gel after focusing and have a tendency to be stained; concentrations lower then 1% form an unstable gradient. Carrier ampholytes (Pharmalyte, Pharmacia) with a range 3.5-10 were employed in this study.

[2.14.2] **Procedure for Isoelectrofocusing (IEF).**

IEF was conducted using Mighty Small II mini gel electrophoresis apparatus (SE250, Hoeffer). Acid treated plates were thoroughly washed, acetone-dried, and assembled according to manufacture's instruction manual.

The unpolymerised components of IEF are as follows:

Solution A) 30% (w/v) acrylamide, 1% (w/v) bisacrylamide (prepared as described in section 2.13).

**Preparation of IEF gel (5% T, 3.3% C).**

- 5.4 ml H₂O
- 2.0 ml solution A
- 0.32 ml ampholyte solution pH 3.5-10
- 6.0 g ultrapure urea
- 10-20 mg ammonium persulphate
- 10-15μl TEMED
Sample loading buffer [Robertson et al. (1987)]

0.48 g ultrapure urea

100μl 2-mercaptoethanol

40μl 1% (w/v) bromophenol blue

0.34 ml H₂O

45μl ampholytes pH 3.5-10

Anolyte.

10mM phosphoric acid

Catholyte.

20mM NaOH

The procedure for preparation of IEF gels was as follows;

Solution A, urea, water and ampholytes were mixed together in a warm water bath to dissolve the urea. Ammonium persulphate (10-20mg) was added to polymerise the gel and this reaction was accelerated by 10ul of TEMED which generates free-radicals from persulphate which in turn catalyses polymerisation. The gel mixture was quickly poured into the top of the gel cassettes whilst ensuring no air bubbles became trapped in the gel mould. Immediately afterwards a plastic comb was inserted at an angle to ensure no air remained trapped under the wells. Trapped air bubbles give uneven displacement of polypeptide bands since they interfere with protein migration. After polymerisation (1hr, room temperature), the comb was removed and the wells thoroughly rinsed with water to remove any unpolymerised material.
Unpolymerised acrylamide solution has a tendency to polymerise during focusing [Giulian et al, (1984)] and interfere with the results.

**Focusing conditions.**

Isoelectrofocusing was carried out at 150V (constant voltage) for 30 mins followed by 2.5 hrs at 200V (constant voltage). A 10mA current was observed at the beginning of the run which then steadily declined during focusing. Focusing was conducted at room temperature and the gels were cooled by circulating water through the gel apparatus.

**Fixing IEF gels.**

The gel was transferred to a 10% trichloroacetic acid solution and agitated for 10 mins after which it was left to soak in a 1% trichloroacetic acid overnight. This step ensures the complete removal of ampholytes from the gel which may interfere with the subsequent staining procedure.

Staining and destaining of the gel is identical to that described in section [2.13.1]

**Determination of pH gradient.**

The pH gradient of the gel was measured as follows:

A small strip of the gel was removed and cut into 5mm slices. These were placed in glass tubes containing 2 ml KCl and broken into small pieces and. After 1 hrs mixing at room temperature the pH of each tube was measured using 10mM KCl as control.

[2.15.] **Attempt at immobilising CyPD to activated Sepharose column.**

After investigating the effects of lysine modification on PPIase activity (which showed it to be largely unaffected) using the Bolton and Hunter reagent, it was necessary to covalently attach the protein to a support matrix. Activated matrices are commercially available that
allow proteins to be coupled through amide bonds under appropriate conditions. A commonly used linking reagent is N-hydroxysuccinimide ester (NHS esters). Sepharose 4B was bought containing 6-aminohexanoic acid N-hydroxysuccinimide bound to the matrix via the 6 amino group as shown in fig. 6. CypD was attached to the sepharose column matrix via the 6-aminohexanoic acid N-hydroxysuccinimide ester. The ester reacts with free amino groups, notably ε-amino groups of lysine or terminal α-amino groups of proteins or peptides to form amides [Anderson et al, (1964)]. The hydroxysuccinimide ester reacts optimally with ε-amino group of lysine at high pH (> 8.5). This is consistent with the need for an unprotonated form of the amino group that is observed at the higher pH (fig. 5). However hydrolysis of the active succinimide ester is considerably augmented at the elevated pH [Cuatrecasas and Parikh, (1972)]. Therefore a compromise between the stability of the reagent at the higher pH and the degree unprotonation of amino groups needs to made.

Using the CyPD affinity column, attempts were made to investigate specifically interacting proteins from different subfractionations of mitochondria under different conditions i.e. high Ca²⁺, EGTA etc. After equilibrating the column with mitochondrial extract and minimising non-specifically bound components (low salt wash), fractions eluted from the columns (using salt gradient) were analysed using SDS-PAGE. The same was repeated for a control column. Results from the two columns were then compared.
Fig. 4a. A Schematic representation of $^3$H[PA-CS].
Mitochondria suspended in MST, pH 7.2

Add a solution 0.12mg Digitonin per mg of mitochondrial protein, pH 7.2.

Leave on ice for 15 minutes with occasional stirring.

Centrifuge at 8000g, 5°C, 8 minutes.

Mitoplasts.

Resuspend mitoplasts in 20mM Hepes, 0.5mM EGTA, PI, PMSF, DTT, pH 8.1.

Add [KCl] to a final concentration of 0.5M, pH 8.1.

Mix gently for 2 minutes.

Centrifuge at 8000g, 5°C, 8 minutes.

Salt treated mitoplasts.

Sonicate in 0.5M NaCl, 20mM Hepes, 0.5mM EGTA, PI, PMSF, DTT, pH 8.1. for 5-10 minutes until translucent.

Centrifuge at 40,000g, 95 minutes, 5°C.

SN store at 70°C (matrix/proteins associated with the inside of the inner membrane).

Pellet

Extract in 6% CHAPS, 150mM NaCl, 20mM Hepes, 0.5mM EGTA, PI, PMSF, DTT, pH 8.1.

Leave on ice for 15 minutes with occasional homogenisation.

Centrifuge at 40,000g, 95 minutes, 5°C.

SN store at 70°C (integral membrane proteins of the inner membrane).

Pellet (detergent unextracted components).

Fig. 4b. Procedure for the subfractionation of mitochondria.
Fig. 5. Modification of lysine residues by Bolton and Hunter reagent.
Fig. 6. Reagent (6-aminohexanoic acid N-hydroxysuccinimide ester) used to construct a CyP1D affinity column.
Fig. 7a. *Elution pattern of $[^3]H$CsA in different concentrations of CHAPS.* Sephadex LH-20 columns were equilibrated in 0, 0.125, and 0.6% CHAPS/150mM NaCl/10mM Hepes/0.5mM EGTA/pH 7.2. Ninety-five microlitres of each buffer at 0, 0.125, 0.6% CHAPS was mixed with 10nM $[^3]H$CsA (5nCi) and Blue dextran. After the dye had entered the matrix 200μl fractions were collected and their $[^3]H$CsA activity measured.
Fig. 7b. The elution of $^{3}H$CsA in 6% CHAPS/ pH 7.2 on a LH-20 minicolumn equilibrated in 0.6% CHAPS.

LH-20 minicolumn was equilibrated in 0.6% CHAPS. 95 μl of 6% CHAPS/150 mM NaCl/10 mM Hepes/0.5 mM EGTA/ pH 7.2 was mixed with 10 nM $^{3}H$CsA (5 nCi), Blue dextran and applied to the column. After the dye entered the matrix, 200 μl fractions were collected and their $^{3}H$CsA activity measured.

Fig. 7c. The elution of $^{3}H$CsA in 6% CHAPS/ pH 7.2 on a LH-20 minicolumn equilibrated in 6% CHAPS.

LH-20 minicolumn was equilibrated in 6% CHAPS. 95 μl of 6% CHAPS/150 mM NaCl/10 mM Hepes/0.5 mM EGTA/ pH 7.2 was mixed with 10 nM $^{3}H$CsA (5 nCi), Blue dextran and applied to the column. After the dye entered the matrix, 200 μl fractions were collected and their $^{3}H$CsA activity measured.
Fig. 8. *Calibration of Superose 12 and Superdex 75 (Pharmacia) gel permeation columns at pH 7.2.*

a) Superose 12, 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ pH 7.2.

b) Superdex 75, 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ pH 7.2.
Fig. 8. Calibration of Superose 12 and Superdex 75 (Pharmacia) gel permeation columns at pH 7.2.

c) Superose 12/ Superdex 75 in series, 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ pH 7.2.
d) Superose 12/ Superdex 75 in series, 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ pH 7.2
[3.1] **Identification of mitochondrial CsA binding proteins by photolabelling using**

$^3$H[PA-CS]

[3.1.1] **Introductory: Identification of CsA binding protein.**

CsA binding proteins have been thoroughly investigated in eukaryotes as well as yeast and bacterial lysates [Galat, A. (1993) and refs within]. They have been found in almost all compartments of cells. However, mitochondrial CsA binding proteins have not been pursued with the same intensity as those of the other cellular compartments (such as ER, nucleus, cytosol).

Most CsA binding proteins belong to a family of proteins called cyclophilins. These have been isolated and studied in a number of different ways. One of the earliest methods to detect CsA binding proteins used hydrophobic chromatography. By measuring $[^3$H]CsA binding capacities using sephadex LH-20 columns Handschumacher et al were able to recognise CsA binding proteins in cellular extracts [Handschumacher et al, (1984); Kolestsky et al, (1986)]. Crystallographic examination of CsA-cyclophilin complexes have offered other ways of isolating cyclophilins. Recent structural studies of CsA-cyclophilin complexes demonstrated that position 8 of the CsA molecule is not involved in the interaction [Pflugel et al, (1993)]. This provided a convenient site at which the immunosuppressant could be covalently attached to an inert matrix via a spacer arm to generate an affinity column in the isolation of cyclophilins from cellular extracts. Using a CsA derivative (8-orthino-CsA) this method has been successfully used to isolate a range of cyclophilins from different types of cells, both bacterial and mammalian [Thalhammer et al, (1992); High et al, (1994)].

A photoactive derivative of CsA, $^3$H[PA-CS], has also been employed to detect and isolate CsA binding proteins. In this case tritiated CsA is modified at position 8 with an aminobutanoyl diazirine group. This allows the molecule to crosslink to cyclophilins or
components that recognise a CsA-cyclophilin complex. The tritiated form of the photolabel has been used to detect intracellular targets of CsA or CsA-cyclophilin complexes in human lymphoid and fibroblast cells [Davis et al, (1991); Foxwell et al, (1992); Ryffel et al, (1993)]

In addition to binding Cyclosporin A, cyclophilins display peptidyl prolyl cis trans isomerase (PPIase) activity. The PPIase activity of protein extracts containing cyclophilins can be examined using the peptide N-succ-ala-ala-pro-phe-4-nitroanilide. The PPIase activity is determined from the rate at which the peptide (trans configuration) is cleaved by chymotrypsin [Fischer et al, (1989)] (see Material and Methods section 2.9).

[3.1.2] Introductory: Mitochondrial CsA binding proteins.

Since the opening of the Ca\(^{2+}\) activated membrane pore in mitochondria is blocked by Cyclosporin A at very low concentrations (30nM) [Fournier et al (1987); Crompton et al (1988)], it became obvious that CsA could be used as a high affinity ligand to isolate or detect the relevant CsA binding protein in mitochondria.

Mitochondria have been reported to contain a cyclophilin [Tropschug et al, (1988)]. Based on its amino acid sequence cyclophilin D (CyPD) was thought to be of mitochondrial origin. The N-terminal amino acid sequence of CyPD (predicted from its cDNA) showed similarities to signal sequences that target proteins to the mitochondria [Bergsma et al, (1991)]. Subsequent studies by a number of groups using \(^{3}H\)CsA binding, PPIase activity and photolabelling methods confirmed the presence of the enzyme in mitochondria [Tropschug et al, (1988); McGuinness et al, (1990); Halestrap and Davidson, (1990); Connern and Halestrap, (1992); Inoue et al, (1993); Andreeva and Crompton, (1994)].

Recent studies in mitochondria have provided evidence that cyclophilin is involved with the membrane pore. McGuinness et al showed that pore opening (\(^{14}\)C sucrose entry) was blocked by CsA (30nM) [McGuinness et al, (1990)]. Within the same study, Scatchard analysis of CsA binding to liver mitochondria revealed the presence of two CsA binding components. Component I, a high affinity, low capacity CsA binding site, was Ca\(^{2+}\) sensitive. Thus, in the presence of 0.5\(\mu\)M buffered [Ca\(^{2+}\)] (sufficient to cause pore opening) the apparent
CsA binding affinity of component 1 was reduced by almost 50%. Component II, a low affinity, high capacity site, failed to show any Ca\(^{2+}\) sensitive CsA binding. This suggested that component 1 might be the relevant component involved in mitochondrial permeabilisation (i.e. pore opening). The binding affinity of component 1 for CsA (8nM) was comparable to the K_i value for CsA inhibition of the PPIase activity of cyclophilin [McGuinness et al, (1990)], suggesting that of component I was cyclophilin. In agreement the amount of CsA binding to component I (5pmol of CsA/ mg of mitochondrial protein) correlated well with the amounts of CsA (6pmol/ mg of protein) needed to block the pore [McGuinness et al, (1990)]. On the other hand recent electrophysiological studies in excised mitoplast patches have shown that the so-called megachannel is activated by Ca\(^{2+}\) and blocked by CsA. Since cyclophilin is a H\(_2\)O soluble protein it would be unlikely to be associated with excised patches and the CsA sensitivity observed might implicate the involvement of some other CsA binding protein other than cyclophilin. However it is possible that cyclophilin might bind tightly to discrete areas of the membrane containing pore components [Szabo and Zoratti, (1991); Szabo et al, (1992); Szabo and Zoratti, (1992)].

**Photolabelling of heart mitochondria.**

In an attempt to identify the CsA binding protein photolabelling studies were carried out in heart mitochondria. The SMP (submitochondrial particles) fraction from photolabelled mitochondria was solubilised using CHAPS as detergent and resolved on Superose 12 gel filtration column (fig. 9). A wide range of components between 11-66kDa in size were photolabelled. From this it was difficult to decide which of the labelled component(s) was involved in the membrane pore.

The soluble fraction of photolabelled mitochondria was also investigated. Two main labelled components (I and II) eluting at 90kDa and 18kDa respectively were observed (fig. 10). Again, it was not possible to conclude which, if any, was involved in the pore.
Fig. 9. A wide range of membrane components (13-66kDa) are covalently labelled by \(^3\)H[PA-CS]. Freshly prepared rat heart mitochondria (10mg of protein/ml) were photolabelled using \(^3\)H[PA-CS] (0.5\(\mu\)M). After sonication membranes were pelleted at 4°C. Dissolved membranes were fractionated on a gel permeation column (Superose 12 equilibrated in 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ PI/ pH 7.2). Graph shows protein elution profile as measured at 280nm with \(^3\)H[PA-CS] content in each fraction. Volume of each fraction was 0.5ml. 
( - ● - ) radioactivity activity.
Fig. 10. The soluble fraction of mitochondria does not demonstrate ADP/ Ca\(^{2+}\) sensitive photolabelling.
Mitochondria were photolabelled in the presence of ADP (■) and Ca\(^{2+}\) (▲). The soluble fraction was separated into 0.5ml fractions using a Superdex 75 column (24ml, Pharmacia). The column was equilibrated in 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ PI/ pH 7.2. The radioactivity activity was determined of each fraction. Where the values in the presence of ADP and Ca\(^{2+}\) are identical only one symbol (○) is used.
3.1.3 Introductory: The effects of ADP and Ca\(^{2+}\) on pore function.

Since it was difficult to pinpoint the correct CsA binding protein from the labelled membranes (components eluting between 45-6kDa) (fig. 9), a criterion for selecting the appropriate one was needed. As described below, it appears that ADP and Ca\(^{2+}\) can modify the binding of CsA to the pore/ component 1. These characteristics were used then to identify the relevant CsA binding protein of the pore.

Patch clamp studies on excised membranes showed clearly the competition between Ca\(^{2+}\) and CsA to open and close the megachannel respectively [Szabo and Zoratti, (1991); Szabo et al, (1992); Szabo and Zoratti, (1992)]. Whereas Ca\(^{2+}\) activation of the megachannel was blocked by CsA, reactivation occurred at higher [Ca\(^{2+}\)] , which, in turn, was blocked by higher [CsA]. In addition, as mentioned above [3.1.2], [\(^3\)H]CsA binding to component 1 was decreased by Ca\(^{2+}\). This seemed to suggest that Ca\(^{2+}\) and CsA competed for binding to the megachannel.

The prevention of pore opening by CsA was potentiated by ADP. Earlier studies showed ADP to prevent Ca\(^{2+}\) induced dissipation of the membrane potential (pore opening) in a synergistic manner with CsA [Novgorodov et al, (1992)]; Andreeva and Crompton, (1994)]. If added before Ca\(^{2+}\) induced activation of the pore, 30nM of CsA could prevent dissipation of membrane potential. However, if CsA was added after Ca\(^{2+}\) induced activation of the pore, in addition to a higher concentration of the immunosuppressant (400nM) ADP (200\(\mu\)M) was also required to block the pore. CsA and ADP added alone to Ca\(^{2+}\) treated mitochondria were unable to close the pore [Andreeva and Crompton, (1994)].

A similar relationship between ADP and CsA was reported by Novgorodov et al [Novgorodov et al. (1992)]. A plausible reason for why ADP might be needed for reversal of pore opening is that endogenous nucleotides, needed for pore closure, are lost during pore opening [Novgorodov et al. (1990)]. Since pore opening allows solute permeation (1500Da in size) the loss of mitochondrial adenine nucleotides would be anticipated [Andreeva and Crompton, (1994)]. Pore opening with subsequent loss of adenine nucleotides would mean that CsA alone (unless exogenous ADP was added) would be insufficient to reverse and close
the pore. CsA (30-60nM) if added before pore opening can delay Ca\(^{2+}\) induced activation in the absence of ADP. This suggests that retained adenine nucleotides potentiate the capacity of CsA. The capacity of CsA to delay pore opening is probably a reflection on its ability to retain or prevent the loss of adenine nucleotides through the pore.

All the previous studies show that Ca\(^{2+}\) prevents the capacity of CsA to close the membrane pore. In contrast to the effects of Ca\(^{2+}\), ADP facilitates the effects of CsA to block the pore. These two characteristics of the CsA sensitive pore (i.e. activation by Ca\(^{2+}\) and deactivation by ADP) can be used to selectively identify the relevant CsA binding protein involved in the pore.

[3.1.4] Introductory: How might photolabelling of the relevant CsA binding component of the pore be affected by ADP and Ca\(^{2+}\) ?

Ca\(^{2+}\) induced pore opening in mitochondria can be blocked by CsA. To induce further pore activation a higher concentration of Ca\(^{2+}\) is needed and to block this again a higher concentration of CsA is required. Therefore, based on this characteristic, it seems conceivable that Ca\(^{2+}\) prevents the binding of CsA to a component that regulates the opening and closing of the pore. It is unlikely that Ca\(^{2+}\), CsA, and ADP effect the pore through a single site on a protein since there is no structural similarity whatsoever between them. It is possible that after binding Ca\(^{2+}\), a Ca\(^{2+}\) sensitive component of the pore stimulates the association of CsA binding protein with the pore complex. A Ca\(^{2+}\) sensitive component/ CsA binding protein/ pore complex might then favour pore activation. However a CsA-CsA binding protein interaction would make the CsA binding protein unavailable for inducing pore activation. ADP might facilitate CsA-CsA binding protein interaction (perhaps interacting with the Ca\(^{2+}\) sensitive component or a different component) so that it is less likely to associate with pore (see hypothetical model below).
Based on the experimental evidence showing the effects of Ca\(^{2+}\) and ADP on CsA-induced pore blockage [Novgorodov et al, (1992); Andreeva and Crompton, (1994)] and the hypothetical model proposed by Halestrap [Halestrap, 1994], it is possible to suggest that Ca\(^{2+}\) and ADP will exert opposite and reverse effects on the binding of CsA to the relevant protein of the pore. Since Ca\(^{2+}\) activates the pore it is likely to depress photolabelling (\(^{3}\)H[PA-Cs]) to the relevant component. ADP, since it behaves synergistically with CsA to induce pore closure, is likely to increase labelling to the relevant component. Ca\(^{2+}\) will limit the amount of CsA binding protein available for photolabelling whilst ADP will depress and reverse this.

[3.1.5] The photolabelling of mitochondria in the presence of Ca\(^{2+}\) and ADP.

As suggested in the previous section, ADP would be expected to increase photolabelling of the relevant CsA binding component in mitochondria whilst Ca\(^{2+}\) would decrease it. The effects of ADP and Ca\(^{2+}\) on mitochondrial photolabelling in heart and liver were investigated. Mitochondria from a single preparation were either photolabelled under high Ca\(^{2+}\) (100μM), EGTA (0.5mM) or ADP (2mM) concentrations. Mitochondria were then fractionated into soluble and membrane (SMP) fractions. SMPs dissolved with detergent were
resolved on a Superose 12 gel filtration column. The soluble fraction was also analysed for ADP and Ca\(^{2+}\) effects using Superdex 75 gel filtration column. Figure 10 shows how ADP and Ca\(^{2+}\) affected the labelling of the soluble fraction of rat heart mitochondria. The soluble fraction yielded two main photolabelled fractions (I and II). Neither ADP nor Ca\(^{2+}\) significantly affected the degree of labelling in the two fractions. Interestingly, CyP18 (CsA binding protein isolated from the soluble fraction of rat liver mitochondria) has been shown to elute at the same position as fraction II (fig 22). These findings suggested that the relevant CsA binding factor of the pore was likely to be absent from the soluble fraction. On the other hand photolabelling of protein extracted from SMPs was considerably affected by ADP and Ca\(^{2+}\). Figure 11a reports the labelling of pattern obtained from rat heart SMPs. Although the labelling pattern was similar to figure 9, a region between 11-22kDa (peaking at fraction 20) clearly displayed ADP/ Ca\(^{2+}\) sensitive photolabelling. Labelling of the 11-22kDa region was increased by a factor of 2 in the presence of ADP (increase of approx. 117% ± 23% in fraction 20) and decreased by a factor of 2 in the presence of Ca\(^{2+}\) (decrease of approx. 54% ± 8% in fraction 20). This was observed in 4 experiments (fig. 11b) (see also table below).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Photolabelling % (SMPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>EGTA</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>217 ± 23</td>
</tr>
</tbody>
</table>

Table 1. The effects of ADP and Ca\(^{2+}\) on photolabelling of rat heart mitochondrial membranes.

Rat heart mitochondria (15-20mg of mitochondrial protein/ ml) were photolabelled in the presence of Ca\(^{2+}\) (200\(\mu\)M), EGTA (1mM), and ADP/ EGTA (2mM/ 1mM). Solubilised SMP components were analysed on Superose 12 gel filtration column. Percentage labelling of fraction 20 (11-22kDa region) was calculated
After photolabelling, liver mitochondria also displayed a similar distribution of \(^3\)H activity in the soluble and the membrane fraction (fig. 12a). Photolabelling of the 11-22kDa (fraction 19) region was increased in the presence of ADP (approx. 80%) and decreased (approx. 50%) in the presence of Ca\(^{2+}\) (fig. 12a). This was repeated 3 times and on each occasion ADP increased labelling whilst Ca\(^{2+}\) decreased it (fig 12b) (see also table below).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Photolabelling % (SMPs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})</td>
<td>65 ± 9</td>
</tr>
<tr>
<td>EGTA</td>
<td>100</td>
</tr>
<tr>
<td>ADP</td>
<td>213 ± 30</td>
</tr>
</tbody>
</table>

Table 2. The effects of ADP and Ca\(^{2+}\) on photolabelling of rat liver mitochondrial membranes

Rat liver mitochondria (15-20mg of mitochondrial protein/ml) were photolabelled in the presence of Ca\(^{2+}\) (200\(\mu\)M), EGTA (1mM), and ADP/EGTA (2mM/1mM). Solubilised SMP components were analysed on Superose 12 gel filtration column. Percentage labelling of fraction 20 (11-22kDa region) was calculated

[3.1.6] Resolution of photolabelled SMP components on a Superose 12 gel filtration column connected in series with a Superdex 75 gel filtration column.

Two FPLC columns connected in series were used to fractionate photolabelled SMP components. The use of two columns in series was based on two findings; firstly, the component displaying ADP/ Ca\(^{2+}\) sensitive photolabelling was unstable. As a result further purification of it was difficult. Overnight concentration (freeze-drying), a step needed to reduce the volume of the sample to enable loading onto the FPLC column, resulted in substantial degradation of the labelled protein. Using two columns in series, the need to concentrate the sample for the next chromatographic step was eliminated. This minimised the
Membrane components with approximate mol. wts 13kDa are covalently labelled in an ADP- Ca\(^{2+}\) - sensitive manner.

Rat heart mitochondria were photolabelled in the presence of Ca\(^{2+}\) (200μM) (•), ADP/EGTA (2 and 1mM respectively) (○) and EGTA (1mM) (○). Solubilised SMPs extracts were separated into 0.5ml fractions on Superose 12 gel permeation column equilibrated in 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ PI/ pH 7.2. Positions of molecular weight markers (6-45kDa) are indicated. Where one symbol is given the others are superimposable.
Fig 11b. *The effect of ADP and Ca\(^{2+}\) on the photolabelling of heart mitochondrial membranes.*
Fig. 12a Gel filtration analysis of the effects of ADP, Ca\(^{2+}\) and EGTA on membrane labelling of liver mitochondria.

Rat liver mitochondria (20mg of protein/ml) were covalently labelled with \(^3\)H[PA-CS] in the presence of ADP/EGTA (○ - ○), EGTA (△ - △), and Ca\(^{2+}\) (■ - ■). Membranes extracted with 30mM CHAPS were fractionated on an equilibrated Superose 12 column at pH 7.2. The \(^3\)H activity of the fractions (0.5ml in size) was determined. Positions of molecular weight standards (6-66kDa) are indicated.
Fig 12b. The effect of ADP and Ca\(^{2+}\) on the photolabelling of liver mitochondrial membranes.

ADP n = 3
Ca\(^{2+}\) n = 4
+- SEM
extent of degradation and subsequently improved yields of the labelled protein. Secondly, free \[^3\text{H}]\text{CsA}\ (\text{fig. } 16)\ eluted very close to the ADP/ \text{Ca}^{2+} \text{ sensitive photolabelled components (fig. 16 broken lines). Since the labelled component (11-22kDa) was prone to deconjugation two columns in series improved the separation of the deconjugated \[^3\text{H}]\text{PA-CS}\] (taken to be equivalent to free \[^3\text{H}]\text{CsA})\ from the ADP/ \text{Ca}^{2+} \text{ sensitive labelled protein. In addition to allowing the amounts of specifically labelled components and deconjugated label to be measured, the two columns minimised contamination of the 11-22kDa components by the deconjugated label.}

As before, heart mitochondria were either photolabelled in the presence of high \text{Ca}^{2+}, \text{EGTA}, or ADP/\text{EGTA}. The elution pattern of \[^3\text{H}]\text{CsA}\ mixed with unphotolabelled SMP extract was also followed to determine the distribution of deconjugated label, if any. A region between 15.3-13kDa (fractions 21, 22, 23 and 24) exhibited ADP and \text{Ca}^{2+} \text{ sensitive photolabelling (fig 13). Free }[^3\text{H}]\text{CsA (equivalent to any deconjugated label) eluted later at approx. 29.5 ml (fraction 25). Fractions displaying ADP and \text{Ca}^{2+} \text{ sensitive labelling were further purified. Fractions 21, 22, 23, and 24 from each condition, (i.e. photolabelling in the presence ADP or \text{Ca}^{2+})\ were pooled, concentrated (using a centricon 10), and separated on a Superdex 75 gel filtration column. ADP and \text{Ca}^{2+} \text{ sensitive labelling was still prevalent. The }[^3\text{H}\text{ activity corresponding to both the ADP and the \text{Ca}^{2+} \text{ samples was observed to peak at fraction 11 and 12 (elution volume 11.5 ml) (fig. 14a). These peaks corresponded to a molecular mass between 29-24kDa. In order to clearly confirm the position of the ADP/ \text{Ca}^{2+}\ sensitive labelled components on a narrow range gel filtration column, the experiment was repeated. However on this occasion, since the label was continuously being deconjugated (which limited the amount of }[^3\text{H activity detectable on the column), fractions exhibiting maximal ADP and \text{Ca}^{2+} \text{ sensitive labelling (fractions 22 and 23 from both fractionations) were } all\ pooled together to increase the total amount of }[^3\text{H activity and separated on Superdex 75. This time the }[^3\text{H activity eluted a fraction later at fraction 12 and 13 (fig. 14b). Similarly fractions 24 from both fractionations (i.e. fraction 24 from components labelled in the presence of ADP and fraction 24 from components labelled in the presence of \text{Ca}^{2+})\ were also pooled together and analysed on Superdex 75 (fig 14b).}
Fig. 13. Fractionation of $^{3}H$[PA-CS] labelled rat heart mitochondria on Superose 12/ Superdex 75 series column demonstrates components (approximately 15kDa in size) that covalently label in an ADP/ Ca$^{2+}$-dependent manner.

Rat heart mitochondria (5mg of protein/ml) were photolabelled in the presence of 2mM ADP/1mM EGTA (- ● -), 1mM EGTA (- ▲ -), and 200μM Ca$^{2+}$ (- ■ -). Membrane extracts were separated on a Superose 12/ Superdex 75 column equilibrated in 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ PI/ pH 7.2. The elution profile of $^{3}H$[CsA] mixed with membrane extract is as also illustrated (- O -). The radioactivity of each fraction was measured. Where only one symbol is represented the others are superimposable. The positions of molecular weight standards (14-45kDa) are indicated.
Fig. 14a. Gel filtration analysis of mitochondrial membranes revealed a component between 26-29kDa that photolabelled in ADP/ Ca\(^{2+}\) -sensitive manner.

Rat heart mitochondria (5mg of protein/ml) were photolabelled in the presence of ADP (-○-) or Ca\(^{2+}\) (-■-). Membrane extracts were fractionated on Superose 12/ Superdex 75 series column equilibrated in 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ PI/ pH 7.2. Fractions demonstrating ADP and Ca\(^{2+}\) -sensitive labelling were pooled, concentrated and further chromatographed on Superdex 75. The \(^3\)H activity in these fractions (0.5ml in size) was determined. The positions of molecular weight standards (13-66kDa) are indicated.
Fig. 14b  Gel filtration analysis of rat heart mitochondrial membranes revealed a photolabelled component between 26-29kDa.
Rat heart mitochondria (5mg of protein/ml) were photolabelled in the presence of ADP. Membrane extracts were fractionated on Superose 12/Superdex 75 series column equilibrated in 0.5% CHAPS/150mM NaCl/10mM Hepes/0.5mM EGTA/PI/pH 7.2. Fractions known to demonstrate ADP sensitive labelling were pooled. Fractions 22 and 23 were pooled, concentrated and separated on a narrow range gel filtration column, Superdex 75 (Pharmacia) (- ■ -). Fraction 24 (- ○ -) from the series column was also concentrated and fractionated on Superdex 75.

$^3$H activity of the Superdex 75 fractions (0.5ml in size) was determined.
From these findings it was concluded that the ADP/\( \text{Ca}^{2+} \) sensitive labelled fractions interacted to a small degree with the Superose matrix. As a consequence they were marginally retained. Although this appeared to give a molecular mass between 11-22kDa, the molecular mass of the ADP/\( \text{Ca}^{2+} \) sensitive labelled component may have been higher (between 29-24kDa) (fig. 14a).

These results appeared to agree with the model predicted earlier which attempted to explain how ADP and \( \text{Ca}^{2+} \) might affect CsA blockage of the pore (section 3.1.4). \( \text{Ca}^{2+} \) depressed photolabelling of the CsA binding component. In its absence, (i.e. in the presence of EGTA) photolabelling was increased. ADP potentiated photolabelling of the CsA binding component. As well as agreeing with the predicted model these findings also agreed with permeabilisation studies in mitochondria [Crompton et al, (1988); Andreeva and Crompton, (1994)] and electrophysiological experiments [Szabo and Zoratti, (1992); Szabo et al, (1992)].

As discussed earlier, the components that mediate the effects of ADP and \( \text{Ca}^{2+} \) on CsA blockage of the pore are unknown. However, in light of these findings it may be postulated that the two pore ligands might affect CsA binding to the 11-22kDa fraction of SMPs through a common mechanism. This mechanism may involve two different proteins which either bind ADP or \( \text{Ca}^{2+} \) or a single protein that can bind both ADP and \( \text{Ca}^{2+} \).


Apart from the ADP/\( \text{Ca}^{2+} \) sensitive labelled peak observed at 11-22kDa (figs. 11a and 12a) other regions also displayed photolabelling. The radioactivity peak (11-22kDa) observed in heart and liver SMPs (figs. 11a and 12a) was frequently accompanied by decreased labelling of components eluting at approximately 13.5 ml (fraction 15, molecular weight 45-30kDa). Since the photoactive derivative (\(^3\text{H}[\text{PA-CS}]\)) is lipid soluble the photolabelling of mitochondrial phospholipids was expected. In order to clarify whether or not these were labelled phospholipids, purified mitochondrial phospholipids, in the form of sonicated vesicles treated with \(^3\text{H}[\text{PA-CS}], \) were dissolved in CHAPS and analysed on Superose 12 gel filtration.
column. A single $^{3}$H[PA-CS] peak was demonstrated at an elution volume of 13.0-13.5 ml (fractions 14 and 15) (fig. 15). This suggested that labelled phospholipids were responsible for the radioactivity peak observed at approx. 13.5 ml (figs. 11a and 12a). The labelled phospholipid peak observed at 13.5 ml (fig. 15) was likely to consist of mixed micelles, i.e. phospholipids/$^{3}$H[PA-CS]/ detergent that eluted at the higher molecular weight (30-45kDa).

Although the position of the photolabelled phospholipids was always consistent, changes in the extent of their labelling was quite variable in the presence of ADP or Ca$^{2+}$. Decreased photolabelling in the presence of ADP of fraction 15 (elution volume 13.5 ml) might reflect decreased $^{3}$H[PA-CS] solvated by lipid when binding to the 11-22kDa component was increased. The decrease in this region (elution volume 13.5 ml) in the presence of ADP was usually small (<20-25%) but on one occasion a reduction of 60% was observed.

[3.1.8] Inhibition of mitochondrial carboxylesterase activity by substituted trifluoromethylketones.

As already discussed the photoactive label (fig. 4) contains an ester link that is susceptible to carboxylesterase activity Many esterases contain an active serine residue in their active sites and can be inhibited by PMSF (phenylmethylsulphonylfluoride). Therefore PMSF (0.4mM) was added throughout all the procedures except when the labelled protein extracts were resolved on FPLC gel filtration columns. PMSF was not added to the buffers used in gel filtration chromatography because of its tendency to precipitate.

PMSF decreased esterase activity (as measured by p-nitrophenylacetate hydrolysis by sonicated rat heart mitochondria) by approx. 80-90%. The residual esterase activity (i.e. in the presence of PMSF) was inhibited by trifluoromethylketone derivatives (0.7mM). Of these, 4, 4, 4 trifluoro-1-phenyl 1, 3 butandione (TFPB) was most effective (fig. 17).

Photolabelling of heart mitochondria in the presence of 4, 4, 4 trifluoro-1-phenyl 1, 3 butandione (fig. 18) produced no change in the labelling profile on gel filtration. Nor did it
Mitochondrial phospholipids (1.4mg) were sonicated until clear under nitrogen. After photolabelling with $^3$H[PA-CS] the particles were sedimented. The particles were extracted using CHAPS as detergent and fractionated (0.5ml fractions) on a Superose 12 gel filtration column equilibrated in 0.5% CHAPS/ 150mM NaCl/ 10mM Hepes/ 0.5mM EGTA/ PI/ pH 7.2. The positions of molecular weight standards (6-66kDa) are indicated.

Fig. 15. The photolabelling of mitochondrial phospholipids.
Fig. 16 The elution pattern of free \[^{3}H\]CsA mixed with membrane extract on Superose 12 gel filtration column.

Mitochondrial membranes (10 mg of mitochondrial protein/ml) were extracted using 6% CHAPS/150 mM NaCl/10 mM HEPES/0.5 mM EGTA/Pi/pH 7.2. An aliquot (50 μl) was mixed with 0.6 μl \[^{3}H\]CsA (30 μCi/ml) containing 0.5% CHAPS and fractionated on a Superose 12. The \[^{3}H\]CsA activity in each fraction was measured (• -). Taken directly of figure 12a, the dotted lines show the elution profile of the ADP/\textsuperscript{2+} sensitive labelled component. The positions of molecular weight standards (6-66 kDa) are indicated.
Fig. 17. Inhibition of carboxylesterase activity by substituted trifluoroketones. Carboxylesterase activity in 0.4mg of rat heart mitochondria was measured as a percentage of p-NAc hydrolysis. 80-90% of the activity was inhibited by PMSF. The effect of different substituted trifluoroketones on the residual activity (PMSF uninhibited) was measured between 400-500nm using a dual beam spectrophotometer (Elmer-Perkin). 
1,1,1 Trifluoro-2,4-pentanedione (TFA). 
1,1,1 Trifluoro-2,4-pentanedione (TFA). 
1,4,4 Trifluoro-1-phenyl-1,3-butanedione (TFPB).
prevent the loss of covalently bound label on further analyses (Superdex 75). These findings further support the fact that the ADP/\(\text{Ca}^{2+}\) sensitive labelled component was prone to severe deconjugation or proteolysis that is unaffected by specific esterase inhibitors.

In view of these observations, the possibility was considered that the correctly photolabelled protein was being deconjugated in an ADP/\(\text{Ca}^{2+}\) sensitive manner and that the deconjugate, still tritiated, was then "mopped up" by some other CsA binding component i.e. 11-22kDa in size. This was examined in the one of the following two ways:-

Firstly, free \(^{3}\text{H}\)CsA mixed with dissolved SMPs was resolved on Superose 12, free \(^{3}\text{H}\)CsA (equivalent to the deconjugate) was eluted at 16 ml (fig. 16 - continuous line). This was different to the elution profile of the ADP/\(\text{Ca}^{2+}\) sensitive labelled region (22-11kDa) (fig. 16 - dotted lines). This procedure also showed that the free \(^{3}\text{H}\)CsA, if deconjugated from the relevant protein, would not bind to any significant amount with the resolved proteins (fig. 16).

Secondly, on analysis of the ADP/\(\text{Ca}^{2+}\) sensitive labelled region (11-22kDa) on SDS-PAGE, the possibility that an 'incorrectly' labelled protein was being studied became very unlikely. Although deconjugation was widespread, a small yet sufficient amount of residual radioactivity was preserved on the components. This was enough to identify the radiolabelled component, as well as confirm that it was the correct one being studied. A component of approx. 22kDa was shown to be photolabelled in an ADP/\(\text{Ca}^{2+}\) sensitive manner (fig. 19). A small amount of \(^{3}\text{H}\) activity was also observed between 10-12kDa. This was thought to be a breakdown product of the 22kDa component.

Based on these findings, the 22kDa component is more than likely to be the relevant protein that photolabels in an ADP/\(\text{Ca}^{2+}\) sensitive manner in intact mitochondria.

[3.1.9] Detection of \(^{3}\text{H}\)CsA binding proteins using Sephadex LH-20 minicolumns.

Since the photolabel was prone to deconjugation, another method for detecting and identifying CsA binding proteins was also employed. Sephadex LH-20 minicolumns have been used to investigate \(^{3}\text{H}\)CsA binding proteins. LH-20 is a crosslinked dextran support in which the hydroxyl groups have been alkylated. This allows the LH-20 support to function as a
0.7 mM TFPB was added to rat heart mitochondria before photolabelling in the presence of Ca\(^{2+}\) (- ■ -), EGTA (- ▲ -) and ADP (- ○ -). SMPs were prepared and extracted proteins analysed on Superose 12 gel permeation column. 10% of each fraction was counted for \(^3\text{H}[\text{PA-CS}]\) activity. Fraction size is 0.5 ml. The positions of molecular weight standards (6-66 kDa) are indicated.
Fig. 19. SDS-PAGE analysis of the Ca^{2+}-sensitive and ADP-sensitive photolabelling of heart mitochondria.

Rat heart mitochondria from a single preparation were photolabelled in the presence of ADP/EGTA (-△-), EGTA (-○-), or Ca^{2+} (-●-). Membrane extracts were fractionated on Superose 12 to remove labelled phospholipids. Fractions eluting between 11-22kDa region of the column were analysed by SDS-PAGE. The positions of molecular markers (8-29kDa) are indicated.
hydrophobic affinity column in which the elution of free \(^{3}H\)CsA is considerably delayed. However, in the presence of detergent (CHAPS), the columns are more likely to separate components on the basis of molecular size since the detergent limits the hydrophobic characteristics. LH-20 can separate molecular weights between 100-4000Da in size (components >4000Da appear in the void volume). Before measuring the CsA binding capacities of the resolved protein fractions, the columns were tested to determine at which detergent concentration they ceased to separate free \(^{3}H\)CsA from mixed micelles of \(^{3}H\)CsA / detergent. Mixed micelles, due to their size, permeated freely into the void volume of the column. This gave the false impression that a CsA specific binding protein was present (fig. 7b). Besides being able to separate \(^{3}H\)CsA/ protein complex from unbound \(^{3}H\)CsA, another useful property of the gel is that it is compatible with both organic and non-organic buffers. Thus, CsA adsorbed to conventional gel matrixes can be extremely difficult to remove. Flushing LH-20 with ethanol removed all traces of bound CsA which might have affected subsequent \(^{3}H\)CsA binding measurements.

To examine whether CsA binding proteins were detectable using Sephadex LH-20, solubilised SMP extract with 0.6% CHAPS was mixed with free \(^{3}H\)CsA (1\(\mu\)M, 30nCi) and separated on LH-20 (fig. 20). \(^{3}H\)CsA binding proteins were eluted in the first 1.5 ml (void volume) whilst almost no \(^{3}H\)CsA was retained on the column. In the absence of solubilised SMP proteins with 0.6% CHAPS, \(^{3}H\)CsA was retained (fig. 20). From these findings it was evident that Sephadex LH-20 minicolumns could be used to determine which protein fractions would bind \(^{3}H\)CsA.

[3.1 10] LH-20 and PPIase activity measurements also demonstrated the existence of a cyclophilin between 11-22kDa in size.

With few exceptions CsA binding proteins belong to a family of proteins called the cyclophilins, all of which display peptidylprolyl cis trans isomerase activity. This key feature was used in conjunction with LH-20 measurements to provide alternative methods to detect CsA binding proteins. As before solubilised proteins from SMPs were fractionated on a Superose 12 gel filtration column. The \(^{3}H\)CsA binding capacity and PPIase activity were
Mitochondrial binding proteins are excluded in the void volume.

SMP proteins from rat heart mitochondria (5mg of protein/ml) extracted in 6% CHAPS/150mM NaCl/10mM Hepes/0.5mM EGTA/2mM ADP/pH 7.2 were concentrated (Centricon 10, Amicon). Protein sample was diluted 10 fold ([CHAPS] 0.6%) and re-centrifuged. [³H]CsA binding capacity of concentrated protein (■) was measured using LH-20 column equilibrated at 0.6% CHAPS/150mM NaCl/10mM Hepes/0.5mM EGTA/2mM ADP/pH 7.2. The [³H]CsA binding capacity of 0.6% CHAPS was also measured on the same column.
measured in the fractions. Components between 11-22kDa exhibited[^3]H]CsA binding and PPIase activity (fig. 21). Besides being almost superimposable the two peaks correlated extremely well with ADP/ Ca^{2+} sensitive labelled region (11-22kDa) of rat heart and liver mitochondrial membranes (figs. 11a and 12a).

The PPIase that was detected may essentially provide all the high affinity CsA binding sites expressed in the ADP/ Ca^{2+} sensitive labelled region (11-22kDa). The two parameters,[^3]H]CsA binding and PPIase activity, were virtually superimposable (fig. 21).[^3]H]CsA binding analysis showed a single peak corresponding to the photolabelled peak (11-22kDa). PPIase activity also corresponded to this peak. Therefore it was assumed that the photolabelled component (11-22kDa) was a cyclophilin.

[3.1.11] Comparison of the ADP/ Ca^{2+} labelled PPIase with CyP18.

A cyclophilin (CyP18) has been isolated from the soluble fraction of mitochondria [McGuinness et al, (1990); Connem and Halestrap, (1992); Inoue et al, (1993)]. A hypothetical model, based on mitochondrial permeabilisation and CsA binding studies, was proposed by Halestraps’ groups in which CyP18 was postulated to be intimately involved in the opening and closing of the Ca^{2+} activated pore [Halestrap and Davidson, (1990)]. A similar model involving cyclophilin in membrane pore regulation was also proposed by Cromptons’ group [McGuinness et al, (1990)]. However, based on some of the evidence presented here, CyP18 was unlikely to play a role in the membrane pore. One of the main reasons why CyP18 was unlikely to be involved in pore function was that photolabelling of the protein was completely unaffected by ADP or Ca^{2+}. As reported in figure 10 photolabelling of the soluble fraction of mitochondria was shown to be insensitive to high concentrations of ADP and Ca^{2+}. No ADP or Ca^{2+} sensitive photolabelling was observed at the higher (fraction 3 and 4) or the lower molecular weight fraction (fraction 13 and 14). This differed considerably from the ADP/ Ca^{2+} sensitive labelling pattern observed of the PPIase detected from SMPs (figs. 11a and 12a).
Fig. 21 Membrane component expressing ADP-Ca⁺-sensitive photolabelling also displays peptidylprolyl cis-trans isomerase activity and [³H]C₅₆ binding capacity. Solubilised membrane extract from rat heart mitochondria (10mg of protein/ml) was fractionated on Superose 12 gel filtration column (equilibrated in 0.5% CHAPS/2mM ADP/150mM NaCl/10mM Hepes/0.5mM EGTA/PI/PH 7.2). The [³H]C₅₆ binding capacity (I.H-20) and the PPIase activity of the region known to exhibit ADP/Ca⁺-sensitive photolabelling is shown. The positions of molecular weight standards (6-66kDa) are indicated.

(- - -) [³H]C₅₆ binding, (■ - ■) PPIase activity
The ADP/ Ca\(^{2+}\) sensitive labelled 22kDa protein and CyP18 were analysed on gel filtration chromatography. With respect to the 22kDa protein, CyP18 ran a fraction later on Superdex 75. Separation of the two proteins was examined on Superdex 75 gel filtration column. A small amount (10μg) of CyP18 that had been purified by O. McGuinness and stored at -20°C was photolabelled and separated from non-specifically labelled \(^{3}\)H[PA-CS] using LH-20. The labelled PPIase was resolved on Superdex 75 (fig. 22 - continuous line). An absorbance and \(^{3}\)H activity peak corresponding to 18kDa was observed (elution volume 12.5 - 13 ml). An unknown absorbance peak was also detected at fraction 21 - 22. This peak might have reflected breakdown products of CyP18 that did not bind the photolabel, or a low molecular weight contaminant. The protein purified from the ADP/ Ca\(^{2+}\) sensitive labelled region of Superose 12 (11-22kDa) was also analysed on the same column. It ran a fraction earlier than CyP18 (fig. 22, dotted line) indicating it to be of a slightly higher molecular weight than CyP18. The actual size of the of the ADP/ Ca\(^{2+}\) sensitive labelled protein (22kDa) was determined using SDS-PAGE (fig. 19).

Although the two proteins were similar in molecular size, it was just possible to separate them on gel filtration chromatography.

[3.1.12] Examination of PPIase binding to Ca\(^{2+}\) and ADP

As clearly established, photolabelling of intact mitochondria resulted in the selective labelling of a membrane localised PPIase in an ADP/ Ca\(^{2+}\) dependant manner. It was unknown whether this protein had ADP and/ or Ca\(^{2+}\) binding sites and if it did whether they were responsible for modulating CsA binding to it. Therefore it was vital to determine whether the PPIase bound ADP and/ or Ca\(^{2+}\) (ligands that affect CsA binding to it). The effects of ADP and Ca\(^{2+}\) on CsA binding (LH-20) were examined.

As before SMPs from rat heart mitochondria were solubilised using CHAPS and fractionated on Superose 12/ Superdex 75 series column. Fractions exhibiting \(^{[3]}\)H CsA binding and PPIase activity (26.0 - 28.1 ml) (fig. 23) agreed well with fractions that displayed ADP/ Ca\(^{2+}\) sensitive photolabelling (fig. 13).
Fig. 22. Elution profile of CyP18 and CyP22 on a Superdex 75 (Pharmacia) gel permeation column.

Pure CyP18 (10 μg) was photolabelled (³H[PA-CS]) (5 mins incubation/ 15 mins irradiation with long U.V) in 0.5% CHAPS/ 10mM Hepes/ 0.5mM EGTA/ pH 7.2 buffer. Labelled Cyp18 was separated (LH-20 column) and fractionated on Superdex 75 equilibrated at 0.5% CHAPS/ pH 7.2. Radioactivity (- - ) in the fractions (0.5ml in size) was measured. The discontinous line represents the elution profile of CyP22. The positions of molecular weight standards (13-66kDa) are indicated.
Fig. 23. Fractions exhibiting $^3$H/CsA binding and PPIase activity correlate well with ADP/Ca$^{2+}$ sensitive photolabelling on series column.

SMPs from rat heart mitochondria (13 mg mitochondrial protein/ml) were dissolved (30 mM CHAPS/150 mM NaCl/10 mM Hepes/0.5 mM EGTA/PI/2 mM ADP(K' salt)/pH 7.2) and fractionated on Superose 12/ Superdex 75 series column. $[^3]$H/CsA binding capacity (- O -) was determined in 0.5% CHAPS/2 mM ADP/pH 7.2 buffer. PPIase activity (- ○ -) was determined in each fraction at 14°C. The positions of molecular weight standards (14-45kDa) are indicated.
Fractions eluting between 26.0 and 28.8ml demonstrated ADP/ Ca$^{2+}$ sensitive photolabelling in intact mitochondria (fig. 13). The effect of ADP and Ca$^{2+}$ on the CsA binding capacity of these fractions was examined. Using LH-20, [$^3$H]CsA binding to these fractions (26.0 to 28.8ml) was measured in the presence of ADP/ EGTA (2mM/ 50μM), EGTA (50μM), or Ca$^{2+}$ (150μM). [$^3$H]CsA binding to the semi-pure protein was almost completely unaffected by ADP or Ca$^{2+}$ (fig. 24).

These experiments suggested that the protein, although photolabelling in an ADP/ Ca$^{2+}$, did not itself contain sites from which ADP or Ca$^{2+}$ could modulate [$^3$H]CsA binding.

Although the PPIase was photolabelled in an ADP/ Ca$^{2+}$ sensitive manner in intact mitochondria, the protein itself appeared to be ADP and Ca$^{2+}$ insensitive. ADP and Ca$^{2+}$ failed to affect [$^3$H]CsA binding to CyP22. This reinforced the earlier interpretation that the ability of ADP and Ca$^{2+}$ to modify CsA interaction with CyP22 in situ (i.e. photolabelling) was mediated by another protein that bound ADP, Ca$^{2+}$ and CyP22.

After binding ADP or Ca$^{2+}$ this protein may then modify the photolabelling of the membrane localised PPIase of intact mitochondria. ADP binding may enhance photolabelling and [$^3$H]CsA binding of the PPIase whilst Ca$^{2+}$, binding to the same component, might depress it. If this was so then a single protein capable of binding either ADP or Ca$^{2+}$ would be responsible for modulating CsA binding to the PPIase. On the other hand, the effects of ADP and Ca$^{2+}$ might be mediated through 2 discrete proteins; a Ca$^{2+}$ binding PPIase target protein and an ADP binding PPIase target protein.

These assumptions appear to support the model proposed in section 3.1.4. Ca$^{2+}$ by binding to its target protein, would decrease the binding of CsA to the cyclophilin thus favouring pore activation. ADP, by binding to its target protein, would increase the binding of CsA to the cyclophilin to inhibit pore activation. Whether one or more components was responsible for this property is as yet unknown.
Fig. 24. $[^3]$H]CsA binding capacity of 22kDa PPlase from rat heart mitochondria is unaffected by pore ligands Ca$^{2+}$ and ADP.

Detergent extracted proteins from rat heart SMPs (10mg of mitochondrial protein/ml) were fractionated on Superose 12/ Superdex 75 column. The binding capacity of the region known to exhibit ADP/ Ca$^{2+}$ sensitive photolabelling was measured. To determine the effects of ADP/ EGTA, EGTA, and Ca$^{2+}$ on the $[^3]$H]CsA binding capacity, relevant fraction (20, 21, 22, 23 and 24) of the region were dialysed (against 0.2% CHAPS/150mM NaCl/10mM Hepes/50uM EGTA/PI/0.4mM PMSF/pH 7.2) overnight and concentrated (Centricon). The effects of ADP/ EGTA, EGTA, and Ca$^{2+}$ on the $[^3]$H]CsA binding capacities (LH-20) of the concentrated fractions was investigated.
1) The photolabelling of heart and liver mitochondria was ADP/ Ca\(^{2+}\) sensitive. Photolabelling of a region between 11-22kDa from solubilised SMPs was increased in the presence of ADP and decreased in the presence of Ca\(^{2+}\). This region also displayed \(^{3}\)H]CsA binding and PPIase activity. This suggested that the relevant CsA binding component involved in the pore was membrane located.

2) The photolabelling of the soluble fraction of mitochondria was unaffected by ADP and Ca\(^{2+}\). This appeared to suggest that soluble CyP18 (believed to be located in the matrix) was unlikely to be involved in pore activity.

3) PPIase activity, separate to that of CyP18, was found at the membranes. This protein (22kDa in size) was likely to be the relevant component involved in pore function since photolabelling of it by \(^{3}\)H[PA-CS] was affected by ADP and Ca\(^{2+}\) (ligands that affect the permeability and conductivity of the membrane pore).

4) Under the conditions used the \(^{3}\)H]CsA binding capacity of the semi-pure protein was unaffected by high concentrations of ADP and Ca\(^{2+}\). Similarly the PPIase activity of the protein (fig. 53) was also unaffected by ADP and Ca\(^{2+}\). Usually a 22kDa protein was was photolabeled in an ADP/ Ca\(^{2+}\) sensitive (figs. 11a, 12a, 19) but occasionally the ADP/ Ca\(^{2+}\) sensitive labelled protein existed at higher molecular weights (approx. 29kDa) (fig. 14a). The effects of ADP and Ca\(^{2+}\) on \(^{3}\)H]CsA binding and inhibition of PPIase activity by CsA of the protein, and the fact that the protein displayed ADP/ Ca\(^{2+}\) sensitive photolabelling at a higher molecular weight, suggested that some other protein(s) (that presumably was ADP and/or Ca\(^{2+}\) sensitive) was responsible for ADP/ Ca\(^{2+}\) sensitive photolabelling of the 22kDa protein.
[3.2] **Purification of the ADP/ Ca$^{2+}$ sensitive labelled PPIase from mitochondria.**

[3.2.1] **Analysis of solubilised SMPs from photolabelled mitochondria on ion exchange chromatography.**

In the previous section mitochondria were photolabelled in the presence of ADP and Ca$^{2+}$. This identified the position of the relevant CsA binding protein on Superose 12 gel filtration column and showed it to correspond to a PPIase. This PPIase was likely to be the relevant CsA binding protein as $^3$H[PA-CS] labelling to it was significantly affected by the pore ligands ADP and Ca$^{2+}$. ADP increased and Ca$^{2+}$ decreased labelling of the protein.

At this stage it was not possible to be certain if the ADP/ Ca$^{2+}$ sensitive labelled PPIase was involved in pore function. In order to investigate its possible role in membrane pore activity and to determine its identity the protein needed to be purified. Information from the previous section (behaviour on gel filtration columns, PPIase activity etc.) was used to isolate and purify the relevant CsA binding protein. Once purified the protein would allow experiments examining the interaction of the PPIase with other pore components to be performed. The involvement of CyP22 binding components seemed likely since preliminary studies on the semi-pure protein (fig. 24) indicated that $[^3$H]CsA binding capacity was unaffected by high concentrations of ADP/ Ca$^{2+}$. This suggested that some other protein (that presumably was ADP/ Ca$^{2+}$ sensitive) interacted with the PPIase to modulate its CsA binding capacity.

As discussed briefly in the photolabelling section [3.1.6], the ADP/ Ca$^{2+}$ sensitive labelled PPIase (11-22kDa) was prone to deconjugation which meant that further purification of the enzyme (by gel filtration chromatography) was almost impossible. In an attempt to determine the correct molecular weight of the relevant protein the ADP/ Ca$^{2+}$ sensitive labelled fractions (11-22kDa, Superose 12) were pooled, freeze dried overnight and analysed on SDS-PAGE. It was hoped that sufficient $^3$H activity would remain bound to the relevant component for it to be detectable on the gel. Although on some occasions the label was just detectable at approx. 22kDa (fig. 19), virtually every SDS-PAGE analysis of this region
resulted in the accumulation of radioactivity at the bottom of the polyacrylamide gel. As the protein of interest was deconjugating very quickly, the only way to increase its yield was to increase the amount of starting protein. However, since gel filtration chromatography is limited by the amount of protein that can be fractionated by each run, this was not possible and therefore another fractionating procedure was introduced.

In ion exchange chromatography proteins are separated on the basis of their overall charge in the separating media. Besides this feature, it is a high capacity separating technique that allows relatively large amounts of proteins to be quickly and easily fractionated. The first step was to identify the position of the relevant CsA binding protein on ion exchange column. SMPs from heart mitochondria (20mg of protein/ ml) photolabelled in the presence of 2mM ADP (to potentiate labelling) were solubilised with 6% CHAPS and fractionated on a small Mono S column (1ml, Pharmacia). Mono S is a cation exchange column that selectively retains proteins that display an overall positive charge in the separating media. This column was chosen since most PPIases display pi (isoelectric point) values between 4 and 10 with the majority towards pH 10 [Galat, (1993)]. Therefore at a pH of 6.7 (pH of fractionation media) most PPIases, exhibiting a net positive charge, would remain stuck to the column until eluted by salt. Almost all $^3$H activity (approx. 95%) was measured in the pass through (not shown). A single radioactive peak eluting at 75-85mM [NaCl] was observed at pH 6.7 (fig. 25). This was assumed to be the protein of interest. Once the elution profile of the protein on the Mono-S column was determined, the procedure was scaled up. [3.2.2.

[3.2.2] A photolabelled component with a high molecular weight was also fractionated on Mono S cation exchange.

As discussed above (fig. 25) a single radioactivity peak eluting at 75-85mM [NaCl] was observed. In an attempt to increase the yield of this CsA binding protein, extracted SMPs from a small amount of photolabelled mitochondria (20-25mg of protein/ ml) were added to a much larger amount (15 ml) of dissolved SMPs (40-50mg of mitochondrial protein/ ml) and fractionated on Mono-S (8ml, Pharmacia). On some occasions two radioactive peaks were observed. A major peak (approx. 50% of total $^3$H activity) was resolved between 100-120mM
Fig. 25. Fractionation of photolabelled membranes reveals a single $^3$H peak between 75-100mM [NaCl] on a Mono-S cation exchange column.

Rat heart mitochondria (10mg of protein/ml) were photolabelled by standard procedure. Membranes extracted in 6% CHAPS/150mM NaCl/10mM NaHPO$_4$/0.5mM EGTA/pH 6.7 were diluted 10 fold with 0 salt/pH 6.7 buffer and fractionated on a cation exchange column (Mono-S, 1ml, Pharmacia). Protein absorbance was measured at 280nm using a [NaCl] gradient between 0-500mM. The volume of each fraction was 1ml

(- ● -) $^3$H counts
Fig. 26. Two radioactivity peaks are revealed on cation exchange chromatography. Soluble SMP proteins from rat heart mitochondria (20mg of protein/ml) were photolabelled in the presence of 2mM ADP/1mM EGTA. After separation of unbound photolabel (LH-20) the labelled protein sample was added to a much larger volume of dissolved membranes (40-50mg mitochondrial protein/ml). The mixture was developed [0-500mM NaCl gradient] on Mono-S column (8ml, Pharmacia) equilibrated at pH 6.7. The $^3$H activity (- ● -) of the fractions (1ml in size) was measured.
Fig. 27. The tighter binding component (figure 26 (II)) is probably the high molecular weight contaminant observed in the soluble fraction of mitochondria. Fractions (32 to 35) containing the tighter binding component from figure 26 were pooled, concentrated and analysed on a Superdex 75 gel filtration column. $^3$H activity (- - -) in all fractions was determined. The positions molecular weight markers (12-66kDa) are indicated.
[NaCl] (I) whilst a minor peak (approx. 15% of total activity), which bound tightly to the column, eluted at 275-300mM [NaCl]) (II) (fig. 26). To investigate the nature of the minor peak, fractions containing the minor peak were pooled, concentrated and analysed on Superdex 75 gel filtration column. A high molecular weight contaminant (>90kDa) was observed (fig. 27). Since this was observed sometimes but not others it was presumably identical to the higher molecular weight peak observed when the photolabelled soluble fraction of mitochondria was resolved on Superdex 75 (fig 10).

From these findings it was concluded that photolabelled SMPs might still be contaminated with the soluble fraction of mitochondria by a component that binds tightly to the cation exchange column.

[3.2.3] The major peak observed on Mono S column eluted at the same position as CyP18 on Superdex 75 gel filtration column

Since the minor peak that binds tightly to the cation exchange column was shown to be a high molecular weight contaminant, only the purification of the major peak (containing the relevant CsA binding protein) was pursued.

Since the position of the relevant CsA binding protein had been identified on a cation exchange column (elution at 75-85mM [NaCl] on Mono S), further purification of the protein using gel filtration chromatography was carried out. The relevant fractions from Mono S column were pooled, concentrated and photolabelled in the presence of ADP (to potentiate $^3$H[PA-CS] binding). Specifically labelled protein was separated (LH-20) and resolved on a Superdex 75 (fig. 28). Although fractions from the cation exchange column were still largely contaminated (absorbance profile of fig. 28), a single radioactivity peak migrating between 12.5 - 13 ml (fractions 13-15) was observed (fig. 28). Further purification of this peak on the same column failed to display any $^3$H activity in any of the fractions (fig. 29). Based on the absorbance measurements approx. 2.5μg of protein (eluted between 12.5 - 13 ml) was obtained from a total of 250-300mg of mitochondrial protein. Although no $^3$H activity was preserved in any of the fractions the protein was still heavily contaminated with high and low...
Fig. 28. A single radioactivity peak corresponding to a molecular weight of 18kDa is resolved on narrow range gel filtration column.

Fraction exhibiting maximal $^3$H[PA-CS] binding (fractions 26 - 29) of figure 26 were pooled and concentrated (Centricon 10). The concentrated sample was fractionated on Superdex 75 column equilibrated at pH 7.2. 10% of the total fraction volume was measured for $^3$H[PA-CS] activity (- - -). Graph shows protein profile (280nm) against $^3$H activity. Fraction size was 0.5ml. The positions of molecular weight markers (13-66kDa) are indicated.
Fig. 29. A single protein peak corresponding to 18kDa is observed with contamination at high and low molecular weights. Fractions 13 and 14 from figure 28 were pooled, concentrated, and analysed on Superdex 75. No $^3$H[PA-CS] counts were detected in any of the fractions. Graph shows absorbance profile (280nm). Fraction size was 0.5ml. The positions of molecular weight markers (13-29kDa) are indicated.
molecular weight components (fig. 29). These findings consolidated the known phenomena of
deconjugation established in photolabelling studies (see section 3.1.6).

Based on the elution profile of $^3$H activity observed in fig. 28, it was concluded that
the photolabelled component resolved on ion exchange chromatography shared a similar
molecular weight (based on molecular weight markers) with CyP18 (fig. 22).

[3.2.4] The ADP/ Ca$^{2+}$ sensitive photolabelled PPIase displays a molecular weight
between 22-23kDa.

To improve the purification protocol, a slightly modified method to the one above was
used. Extracted SMP proteins (12-15mg of mitochondrial protein/ ml) were fractionated on a
Superose 12 gel filtration column. The fractions known to exhibit ADP/ Ca$^{2+}$ sensitive
photolabelling (11-22kDa region, elution volumes 14 - 15.5 ml) (figs 11a and 12b) were
pooled, concentrated and photolabelled (5 mins. incubation, 3 mins. irradiation). The semi-
pure PPIase (now labelled) was added to a much larger amount of dissolved SMPs and
fractionated on the Mono S column (8ml, Pharmacia). On this occasion (fig. 30) no $^3$H
activity was observed between 275 - 300mM [NaCl] which seemed to suggest, as found
earlier, that the tighter binding protein was a higher molecular weight contaminant (probably
from the soluble fraction of mitochondria) that bound some $^3$H[PA-CS] (approx. 15%).
Separation of the dissolved SMPs on Superose 12 gel filtration column clearly removed the
high molecular weight component from the relevant PPIase. The single radioactivity peak that
eluted at 100mM (fig. 30) corresponded well with the major peak observed in figure 25. This
indicated that the $^3$H activity peak observed in figure 25 was identical to that resolved from
the ADP/ Ca$^{2+}$ sensitive photolabelled region (11-22kDa) of Superose 12 (fig. 30). This
indicated that the correct protein was being studied.

Further fractionation of the $^3$H activity of figure 30 on Superdex 75 showed a $^3$H peak
at fraction 14 (elution volume 13.0ml) (fig. 31). As discussed earlier in figure 22 this protein
appeared to elute at the same position as CyP18. Approx. 3μg of protein (fraction 14) was
purified on this occasion (fig. 31). Also, with regards to the absorbance profile, the
Fig. 30. A single $^3$H[PA-CS] is observed at 100mM [NaCl].
Soluble extract from rat heart SMPs (15-20mg of mitochondrial protein/ml) was fractionated on Superose 12 column at pH 7.2. Fractions known to exhibit ADP/ Ca$^{2+}$-sensitive photolabelling (i.e. fractions 16, 17, 18) were concentrated and labelled ($^3$H[PA-CS]) in the presence of 2mM ADP. Specifically labelled components were separated (LH-20) and mixed with a larger SMP extract (100-120mg of mitochondrial protein). The mixture was developed on Mono-S (8ml, Pharmacia). 1ml fractions were collected of which 10% was measured for $^3$H activity (- ● -).
Fig. 31. $^3$H[PA-CS] activity peaks at a molecular weight around 18kDa. Fractions 25-29 of figure 30 were pooled, concentrated and fractionated on Superdex 75 column. Fractions (13 and 14) from the Superdex 75 gel filtration column were pooled, concentrated, and refractionated on the same column. 0.5ml fractions were collected and 5% of each fraction was measured for $^3$H activity (- - -). The positions of molecular weight markers (13-29kDa) are indicated.
protein at fractions 13 and 14 of figure 31 appeared much less contaminated than observed previously in figure 29.

SDS-PAGE analysis (using large slab gel, see Materials and Methods section 2.12) of figure 31 (fractions 13, 14, and 15) showed a major band migrating at approx. 22 - 23kDa (fig. 32). Although these fractions appear to be relatively ‘clean’ based on the absorbance profile (fig. 31), analysis on SDS-PAGE revealed minor contaminatory components at 66, 14, and 11kDa. In a similar experiment in which the photolabelled protein was analysed on mini-gel SDS-PAGE and sliced to determine the position of the label, a 22- 23kDa band was clearly shown to be specifically labelled (fig. 33). The minor components displayed no $^3$H[PA-CS] labelling.

[3.2.5] The single radioactivity peak observed on Mono S is identical to the CsA binding protein that displays ADP/ Ca$^{2+}$ sensitive photolabelling.

Fractions known to exhibit ADP/ Ca$^{2+}$ sensitive photolabelling on Superose 12 gel filtration column (11-22kDa region) were photolabelled and analysed on Mono S column. Figure 30 demonstrates a single radioactivity peak at a salt concentration of approx. 100mM. The finding that this protein was the relevant CsA binding component was further supported by PPIase and $[{}^3$H]CsA binding measurements. Proteins from dissolved SMPs fractionated on the Mono S column showed the component eluting at 100mM [NaCl] to clearly display $[{}^3$H]CsA binding (fig. 34) and PPIase activity (fig. 35).

Taken together these studies imply that the 22- 23kDa membrane localised PPIase (CyP22) is the relevant CsA target that is photolabelled in an ADP/ Ca$^{2+}$ sensitive manner in intact heart and liver mitochondria.

Although CyP18 and CyP22 can be differentiated on Superdex 75 gel filtration column (fig. 22), they elute close to each other. However, upon SDS-PAGE analysis, the difference in size is much clearer (fig. 36).
Fig. 32. The labelled protein from rat heart mitochondria demonstrates a molecular size of 22-23kDa.

SDS-PAGE analysis of fractions 13, 14, and 15 of figure 36. Large SDS-PAGE gel was run under standard conditions and stained with Coomassie Blue. The positions of molecular weight markers (14 - 97kDa) are indicated.
Fig. 33. SDS-PAGE reveals a single peak corresponding to a molecular weight of 22kDa. The photolabelled 22kDa was purified from rat liver mitochondria using gel filtration chromatography (Superose 12 and Superdex 75). The purified protein was examined on mini-gel SDS-PAGE. Gel slices (2mm) were cut and $^3$H[PA-CS] activity counted. The positions of molecular weight markers (14-45kDa) are indicated.
Fig. 34. The elution profile of photolabelled SMP's on Mono-S chromatography correlates well with $[^{1}H]CsA$ binding profile of extracted proteins on the cation exchange column.

SMPs extracted (6% CHAPS) from rat heart mitochondria (200 - 250mg of mitochondrial protein) were fractionated on Mono-S (8ml, Pharmacia) column pH 6.7. Proteins were developed by salt gradient (0-500mM [NaCl]). Fractions (1ml in size) eluted between 75 - 150mM [NaCl] were measured for $[^{1}H]CsA$ binding capacity (- ● -).
Fig. 35. A single PPIase in rat heart mitochondria is developed between 100-125mM [NaCl] on Mono-S.
SMPS from rat heart mitochondria (15mg of protein/ml) extracted in CHAPS were analysed on Mono-S (Pharmacia) cation exchange column equilibrated in 0.5% CHAPS/10mM Mops/0.5mM EGTA/0.5mM DTT/PI/pH 6.7. Protein fractions (1ml in size) eluting between 100-125mM [NaCl] were measured for PPIase activity. Graph shows protein absorbance monitored at 280nm with PPIase activity (- - - -).
Fig. 36. 

CyP18 and CyP22 are observed as two different proteins on SDS-PAGE analysis. CyP22 purified from rat liver mitochondria was run against CyP18 purified by Orla McGuinness. 2.5μg of CyP22 (lane 1) and approximately 4μg of CyP18 (lane 2) were fractionated on a large gel SDS-PAGE electrophoresis. The positions of molecular markers (14-97kDa) are indicated.
[3.2.6] Measurement of $k_{cat}/K_m$ of CyP22 and its $K_d$ for CsA.

The peptidylprolyl *cis trans* isomerase activity of purified CyP22 and its inhibition by CsA was examined in detail using the test peptide N-succinyl-ala-ala-pro-phe-4-nitroanilide. Measurements of PPIase activity values differ depending upon the pH and temperature of the assay buffer. PPIase activity was measured at 13-14°C at pH 7.2. Briefly, PPIase activity was determined by measuring the rate at which the test-peptide (in *cis* configuration) was cleaved by α-chymotrypsin (when in the *trans* configuration) to release the α chromophore (nitroaniline). The faster the reaction comes to termination (i.e. when all the test-peptide was cleaved) the more PPIase activity is present. CsA slows down the rate of absorbance change by inhibiting PPIase activity.

Figure 37a shows absorbance change ($\lambda_{390-480}$nm) against time of CyP22 PPIase activity in the presence of different [CsA]. Data from figure 37a was then plotted according to the relation $\ln(A_t/A_a) = -kt$ where $A_t$ is the absorbance change that occurred subsequent to any time $t$, and $A_a$ is the maximal absorbance change recorded in curve 1 (control). From figure 37b a $k_{cat}/K_m$ value of $5.8 \mu M^{-1} s^{-1}$ was calculated. The PPIase activity was blocked by CsA ($K_d$ approx. 4nM) (fig. 37c). These values are similar to other cyclophilins acting on the same test-peptide ($k_{cat}/K_m$ 1-10µM$^{-1}$ s$^{-1}$ and $K_d$ for CsA between 1-100nM) [Lui et al, (1990)]; Connem and Halestrap, (1992)]

[3.2.7] The location of CyP22 in SMPs.

The way in which CyP22 associated with SMPs was investigated by exposing the membrane to different salt concentrations (0 and 500mM) with and without sonication. Most of the PPIase activity (>90%) remained membrane bound in low salt buffer whilst almost all of it became largely dissociated when SMPs were exposed to 500mM NaCl media (fig. 38). Resonication of SMPs in high salt media did not change the extent of PPIase dissociation. These findings suggested the PPIase activity was electrostatically bound to the outside of SMPs. Since the inner membrane becomes inverted during SMP preparation (the outside
Fig. 37a. Peptidylprolyl cis trans Isomerase activity of the heart 22kDa protein.

Trace shows the absorbance change (390-440nm) against time after addition (arrow) of test peptide to the pure protein. The absorbance change due to hydrolysis of pre-existing trans-peptide occurred within the mixing time (1s) and is not shown. PPIase activity was measured in 60mM KCl/ 10mm Hepes/ pH 7.2 at 14°C.

(1) no addition of pure protein,
(2) 0.4µg of 22kDa protein plus 5nM CsA
(3) 0.4µg of 22kDa protein plus 2nM CsA.
(4) 0.4µg of 22kDa protein.
0.4µg of 22kDa protein plus 10nM CsA (not shown)

Fig. 37b. Data from figure 37a plotted according to the relationship \( \ln \left( \frac{A_t}{A_0} \right) = -kt \) where \( A_t \) is the absorbance change that occurred subsequent to any time \( t \) and \( A_0 \) is the maximal absorbance change recorded.

Fig. 37c. The ratio \( k_{cat}/K_m \) (from figure 37b) was determined at the different CsA concentrations.
Fig. 38. Cyp22 is dissociated from membranes in high salt media.
Rat heart SMPs were suspended in 20mM Hepes/ 0.5mM EGTA/ PI (1μg/ ml)/ pH 7.4. The following additions were made: (I) nothing (untreated), (II) 500mM NaCl, and (III) 500mM NaCl plus sonication. SMPs were sedimented at high speed and PPIase activity in pellet (P) and supernatant (S) measured. Activities were measured as a percentage of that measured in SMPs suspended in buffer containing 30mM CHAPS.
corresponds to the matrix facing side of the membrane) it was assumed that CyP22 was bound facing the matrix compartment.

Up to this stage all attempts at purifying CyP22 were conducted in media containing CHAPS as detergent to ensure the membrane associated protein remained soluble. As almost all membrane bound CyP22 was found to be salt-dissociable (fig. 38), the need for CHAPS in future purification procedures was unwarranted.

[3.2.8] The salt-dissociable PPIase and the CHAPS extracted PPIase are the same proteins.

So far it has been established that the ADP/ Ca^{2+} sensitive labelled protein purified from SMPs has a molecular weight around 22-23kDa (CyP22). Figure 38 shows that CHAPS was probably not needed in the purification procedures since almost all PPIase activity associated with SMPs was dissociated by high salt. However at this stage there was no evidence to suggest that the salt dissociable PPIase was identical to the detergent extracted PPIase. In an attempt to clarify this ambiguity both PPIases (salt dissociable and CHAPS extracted) were purified to a single absorbance peak from sheep heart mitochondria. ADP/ Ca^{2+} sensitive labelled PPIases isolated from rat heart and liver mitochondria were prone to proteolytic activity and deconjugation and therefore the only alternative at this stage, to increase the yield of the purified protein, was to increase the amount of starting mitochondrial protein. Since sheep tissue was readily available at the time, it was decided that this was a good source of mitochondria.

Both the salt-dissociable and the CHAPS extracted PPIases were purified to a single absorbance peak on Superdex 75 gel filtration column. The relevant fractions (i.e. 13, 14 and 15) were pooled, freeze dried overnight and suspended in sample loading buffer (see Materials and Methods section 2.13). Upon SDS-PAGE analysis (minigels) the CHAPS extracted PPIase migrated at a higher molecular weight (approx. 25-27kDa) (lane 2) then the salt dissociable protein (lane 1) (fig. 39). It is known that high levels of salt (>2M) and detergent in protein samples prepared for SDS-PAGE analysis tend to interfere with the migration of proteins. In order to alleviate this problem equal amounts of the pure proteins
Fig. 39. *The detergent extracted PPIase displays a molecular weight around 26-27kDa on SDS-PAGE.*

The salt and detergent extracted PPIases from Sheep heart mitochondria were purified to single absorbance peaks on gel filtration chromatography (Superdex 75). Equal amounts of the protein (3μg) were analysed by SDS-PAGE using mini-gels (12% resolving gel). The positions of the molecular weight markers (14-97kDa) are shown.

Lane 1 = Salt dissociable
Lane 2 = Detergent extracted
(approx. 3µg) were ultrafiltered (Centricon 10) to remove salt and detergent and freeze dried overnight. Samples were diluted 2 fold with sample loading buffer and examined on SDS-PAGE (minigels) (fig. 40). On this occasion it was found that, even though equal amounts (3µg) of both proteins were examined, only the detergent extracted protein was visibly stained (fig. 40, lane 2). The salt-dissociable protein (lane 1) could not be seen. It was assumed that the salt dissociable PPIase was degraded as a result of proteolytic activity that probably co-dissociated with the protein. Cleavage of the protein was suspected to occur during the ultrafiltration step. To minimise exposure to proteolytic activity and prevent salt and detergent dependant interference on protein migration, equal amounts of salt and detergent extractable PPIases (approx. 3µg) were diluted 7 and 3.5 fold with equal volumes of sample loading buffer and water respectively. Large slab gel electrophoresis of the two samples was then conducted. Blue tone analysis of the large gel run revealed 2 bands (although faint) at approx. 22kDa in size (fig. 41). This was much more clearly demonstrated in figure 42 in which salt dissociated (lane 1), detergent extracted (lane 2, in which the detergent concentration was reduced) and detergent extracted (lane 3, in which the detergent had been removed) PPIases from sheep heart SMPs were analysed on large SDS-PAGE gel with specially constructed wells. These wells were narrower (almost half the standard width) and deeper (almost twice the standard length) than the gel in figure 42. On this occasion the effect of high [CHAPS] was clearly seen to influence the migration of the 22kDa PPIase (lane 3). Removal of the detergent (lane 2) showed a protein that migrated identically to the position of the salt dissociable PPIase (lane 1).

On the basis of these findings it was concluded that the 22kDa PPIase which photolabelled in an ADP/ Ca²⁺ sensitive manner (detergent extracted) was identical to the PPIase that was electrostatically associated with SMPs.

It seems that the purified salt dissociable CyP22 may be subjected to proteolytic activity that co-dissociates with it from the membranes. The proteolysis of the protein (fig. 40, lane 1) was likely to occur during the ultrafiltration procedure.

The migration of CyP22 is considerably affected by high [CHAPS]. In the presence of the detergent the protein is unlikely to be completely unfolded because SDS and CHAPS
Fig. 40. Salt dissociable PPIase is sensitive to proteolysis.
Approximately 3 - 4μg of salt dissociable and CHAPS extracted PPIase was purified from sheep heart SMPs. Salt and CHAPS concentrations were minimised by ultrafiltration (Centricon 10). Proteins were analysed on minigel SDS-PAGE (12% resolving gels). The position of the molecular weight markers (14-97kDa) are shown
Lane 1 = Salt dissociable PPIase ([NaCl] at 0.225M).
Lane 2 = CHAPS extracted PPIase ([NaCl] at 0.2M, [CHAPS] at 0.85%).
Fig. 4.1. Silver staining followed by Blue toning of salt dissociable and CHAPS extracted PPIases.

The salt dissociable and CHAPS extracted proteins of Sheep heart SMPs were purified to single absorbance peaks on Superdex 75. 3μg of salt dissociable PPIase was diluted 7 fold using equal volumes of SLB and water and 3μg of CHAPS extracted PPIase was diluted 3.5 in the same way. Samples were analysed on large slab SDS-PAGE gel. Gel was silver stained and then Blue toned to enhance protein bands. The position of the molecular weight markers (14-97kDa) are shown.

Lane 1 = Salt dissociable PPIase
Lane 2 = CHAPS extracted PPIase
Fig. 42. **CHAPS significantly affects the migration of the PPIase.**

Salt dissociable and CHAPS extracted PPIases from Sheep heart mitochondria were purified to single absorbance peaks on Superdex 75. Approximately 3μg of each protein was separated on large slab SDS-PAGE (12% resolving gel) with narrower and deeper wells. The positions of molecular weight markers (14-97kDa) are shown.

Lane 1 = Salt dissociable PPIase ([NaCl] at 3.3M).

Lane 2 = CHAPS extracted PPIase in which the [CHAPS] has been significantly reduced.

Lane 3 = CHAPS extracted PPIase ([NaCl] at 1.67M, [CHAPS] at 8.35%).
compete with one another for the same binding sites on the protein. Since CHAPS does not
denature proteins the PPIase will not be completely unfolded by SDS and, as a result will
migrate a little slower than expected. Removal of CHAPS allows the complete denaturation of
the protein by SDS (fig. 42, lane 2).

In order to study further the PPIase and its possible role in pore function, it was
necessary to decide the optimum purification procedure for the best yield of protein. Tables
43a, b, c and d show the purification tables of the salt dissociable and detergent extractable
PPIases from rat and sheep heart SMPs. Comparison of the purification data relating to the
detergent extractable and the salt dissociable PPIases showed that these proteins were about
equally stable during the purification procedure i.e. the proteins were about equally prone to
denaturation during purification. The lower purification with the salt dissociable protein was
due to the removal of the membrane proteins by the initial salt wash.

The salt dissociable protein was identical to the detergent extractable PPIase isolated
from the SMPs of rat and sheep mitochondria. The detergent extractable PPIase was
photolabelled in an ADP/ Ca^{2+} sensitive manner and therefore, based on these findings, it was
postulated that the ADP/ Ca^{2+} sensitive labelled PPIase was electrostatically associated with
the Ca^{2+} sensitive membrane pore.

[3.2.9] Investigation in to the stability and localisation of CyP22 in mitochondria.

The general distribution and stability of CyP22 was investigated by fractionation of rat
liver mitochondria. In addition to determining the correct size of the protein, the amount of
enzyme purified from each fraction would also indicate the most stable source of protein. To
achieve this, PPIase activity from each mitochondrial fraction was purified to a single band on
SDS-PAGE. The fractionation of mitochondria would:
### Table 43. Purification of salt dissociable and CHAPS extractable 22kDa PPIase from sheep and rat heart mitochondria.

(a) Detergent extracted PPIase from rat heart mitochondria.
(b) Salt dissociable " " " " "
(c) Detergent extracted PPIase from sheep heart mitochondria
(d) Salt dissociable " " " " "

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i) improve the final yield of CyP22 so that further studies looking at the role of CyP22 in membrane pore activity could be conducted. The salt dissociable PPIase associated with the membrane appeared to be unstable because it was susceptible to membrane bound protease activity. The proteolytic activity was believed to persist alongside the PPIase during the entire purification procedure. Proteolysis was believed to be the main cause of poor protein yields and therefore removal (separating CyP22 from the protease) or inhibition of proteolysis would ameliorate the amount of CyP22 isolated.

ii) remove any contaminatory components e.g. E.R, microsomes etc. (that also contain PPIases) from the mitochondrial fraction thus ensuring that the PPIase purified was of mitochondrial origin only.

Rat liver mitochondria were separated into different fractions. The outermembrane/intermembrane space (OM/IMS) fraction was isolated by the addition of low concentrations of digitonin (0.12-0.19 mg/mg of mitochondrial protein). At this concentration the outermembrane is selectively lysed whilst the inner membrane remains intact. The resulting mitoplasts were briefly (2 mins.) treated with 500mM [KCl] to remove any membrane associated protease activity that might be responsible for causing protein degradation as well as any other electrostatically bound components on the external face of the mitoplasts (cytoplasmic facing side). This step considerably increased the yield of CyP22 from the resulting mitoplasts. An increase of almost 10 fold was calculated suggesting the PPIase from these mitoplasts was considerably more stable than from the SMPs. Purification of CyP22 from rat heart SMPs gave yield of approx. 0.46 pmoles/mg of mitochondrial protein. Purification of CyP22 after salt treatment (500mM KCl) yielded approx. 4.7 pmoles/mg of mitochondrial protein.

2.3μg of salt dissociable PPIase and 1.4μg of PPIase isolated from sonicated mitoplasts after treatment with 0.5M KCl was analysed on SDS-PAGE. The salt dissociable protein stained comparatively less than the PPIase isolated from the sonicated mitoplasts, even though more (2.3μg) had been applied (fig. 44). The salt dissociable protein had degraded as a result of proteolysis before it could be analysed on SDS-PAGE. Purification of CyP22 from
Fig. 44. The purification of PPlases from different fractions of rat liver mitochondria. PPlase activity from each compartment (i.e. outer membrane/ intermembrane space, salt treated mitoplasts, sonicated mitoplasts and CHAPS extractable) of rat liver mitochondria was purified to homogeneity. Purified proteins were analysed on large slab SDS-PAGE gel. The position of molecular weight markers (14-97kDa) are shown
Lane 1 = PPlase from OM/ IMS.
Lane 2 = Salt dissociable PPlase.
Lane 3 = PPlase from sonicated mitoplasts after treatment with high salt.
Lane 4 = CHAPS extracted PPlase from sonicated mitoplasts
mitoplasts that had been "washed" with high [salt] was far more stable. This was indicated by considerably improved yields and intensified staining pattern.

The general distribution of PPIase activity between the different fractions of mitochondria was also investigated. The PPIase activity measured in each of the fractions was expressed as a percentage of the total activity determined. Figure 45 shows the percentage of PPIase activity measured in the different fractionations. Almost half of the activity was localised to the matrix and the matrix facing side of the inner membrane whilst approximately 38% was designated to the OM/ IMS fraction of mitochondria. 6-7% of the activity was CHAPS extractable only, whilst approx. 11% was displaced by 500mM KCl treatment of mitoplasts. These percentages were not confirmed by calculating the distribution of mitochondrial marker enzymes. In addition to this the OM/ IMS fraction was presumed to contain protease activity that was responsible for cleavage of PPIase. This activity would have consequently reduced the amount of PPIase activity measured in the OM/ IMS fraction. However, although incomplete, an overall picture of the distribution of PPIase activity in mitochondria can be obtained.

Other work in the laboratory [Tanveer et al, (1996)] compared the distribution of mitochondrial marker enzymes with that of PPIase activity. Malate dehydrogenase (matrix marker), adenylate kinase (IMS marker), succinate dehydrogenase (inner membrane marker) and monoamine oxidase (outer membrane marker) were measured. The results from these studies plus the work presented here demonstrated CyP22 to be a matrix enzyme that associated electrostatically with the inner membrane (matrix facing side).

[3.2.10] Purification of each PPIase activity from the fractionated mitochondria.

The PPIase protein from each of the mitochondrial fractions was purified to a single band by SDS-PAGE to determine the molecular weight. All PPIases (i.e., salt dissociable, matrix resident and detergent extractable) isolated from liver mitoplasts exhibited a molecular weight of approx. 22kDa (CyP22) (fig. 44). The OM/ IMS fraction (isolated using low
Fig. 45. Distribution of PPlase activity in rat liver mitochondria.
Graph shows the distribution of PPlase activity in the different compartments of rat liver mitochondria as a percentage of total PPlase activity measured.
concentrations of digitonin) showed the existence of a 18kDa (CyP 18) protein [Tanveer et al, (1996)]. Measurement of enzyme markers in the laboratory confirmed that CyP18 was localised to the OM/IMS fraction of mitochondria and CyP22 to the inner membrane and matrix fraction.

Based upon the above findings some interesting issues concerning the identity of the 2 proteins can be addressed. At this stage it is unknown whether CyP18 actually exists in vivo, i.e. if it is a native protein distinct from CyP22. Since the OM/IMS fraction of mitochondria is believed to contain protease activity, it is possible that some CyP22 might have been cleaved to a lower molecular weight PPIase, i.e. CyP18.

The amino acid sequence (fig 46B, italic) of CyP22 showed almost 96% sequence similarity with human CyP3 (fig. 46B non-italic). Human CyP3 is also designated CyPD [Bergsma et al, (1991); Inoue et al, (1993)]. Cyclophilin D was thought to be a mitochondrially located enzyme based on the N-terminal sequence (predicted from cDNA) which shared similar structural features to mitochondrial targeting sequences of other proteins. Mitochondrial targeting sequences of proteins are quite different from one another. They can be between 0.5 and 10kDa in size. Although formed from different amino acids (i.e., usually made of a high content of hydrophobic amino acids separated by positively charged amino acids) the overall charge and property of mitochondrial targeting sequences are similar. The N terminus presequence of CyP18 (fig. 46A, non-italsics) might be related to a targeting sequence based on the amino acid forming the structure (i.e. high content of alanine, serine and arginine). Interestingly, one of the peptides of CyP22 (fig. 46A italics) that could not be aligned with CyPD showed identical amino acid sequence with the N-terminal of 18.6kDa cyclophilin (fig. 46A, non-italic) isolated from rat liver mitochondria by Connem and Halestrap [Connem and Halestrap, (1992)]. This peptide probably corresponds to the N terminus of CyP22 and is probably involved in targeting the protein to mitochondria. In reality, since CyP18 showed similar sequence identity with human CyP3 (CyPD) [Inoue et al, (1993)] and CyPD showed 96% sequence similarity with CyP22, the 3 proteins were in all probability identical. Some proteins of mitochondria, after processing and transportation to the matrix, are processed again and transported to other compartments of mitochondria, i.e.
Fig. 46. Amino acid sequencing of CyP22
Amino acid sequence was determined by treating purified CyP22 with endoproteinase Asp-N. This enzyme cleaves at some glutamic acid residues in addition to aspartic residues. (A) One of the peptides (shown in italics) was found to be identical to the N-terminus of rat liver CyP18 identified by Connern and Halestrap, (1992). (B) The remaining ten sequences (shown in italics) were found to be aligned with full length sequence of human CyPD [Bergsma et al, (1991)].

Unidentified residues are designated X whilst those that differ from the previously identified sequences are marked with an asterisk.

A

1  | ARDG|ARGANSSQNPLL|YLDVGADGQPL
   |    |DGGARGANSSFQN

B

1  | MLAI|RCGSRW|GLL|SVP|SVF|LRLPAARAC|SK|GDPSSSSSS|GPNPLL

   |      |DVDANGKPLG|R|VLE|LKADVVPKTAENFRALCTG|KE|GFGYKG|ST|FHRVIP
   |      |DGQPLGRVV  |ENFR|LXTSEKGFG|YK|ST|FHRV|IX
   |      |DV|PK|TA    |EK|GFG|Y|ST|FHRV|IX

101 | SFMC|QAGDFTN|NH|GGKSI|YGSRFDEN|T|LKHVGP|VLSM|NAGPNTN

   | AFM DFT|NH|GGKSI|YGSRDEN|T|LKHVGP|VLSM|NAGPNT
   | AFMXQA DGT|GG

151 | GSQF|ICTIKTD|LDG|KH|VVF|HVKEGMDV|K|IESFS|G|K|RT|KKI|V

   | DG|KH|VV|F|HVKEGMDV|K|IESFS|G|K|RT|KKI|V

201 | TDCGQLS

T
OM, IMS etc. CyP18 might represent a processed product of CyP22 that is transported to the OM/IMS fraction of mitochondria. However, with respect to localisation and distribution studies presented earlier, it was unlikely that CyP22 was an intermediate for CyP18 since most of the mitochondrial CyP was present in this form. The only way the presence of CyP18 could be explained was to assume that only some of the CyP22 was processed to the smaller isoform. However, until complete sequence information of both isoforms is known, no conclusion about whether the small isoform is a processed product of the large isoform can be drawn.

As reported in figure 44 and demonstrated in figures 38 and 45, most of the PPIase activity of CyP22 (>80%) can be salt displaced from the inner membrane. This suggested the protein was electrostatically associated with the membrane. Other workers have only purified the 18kDa CyP from the "matrix" compartment of mitochondria [Connern and Halestrap, (1992); Inoue et al, (1993)] and suggested that it was the relevant CsA binding protein involved in the Ca^{2+} activated pore. These workers, although demonstrated PPIase activity and CsA inhibition within the correct ranges for cyclophilins, failed to detect CyP22. Under their conditions it was possible that CyP22 was either completely associated with the inner membrane or completely cleaved (by the proteolytic activity in the OM/IMS fraction) to the smaller protein.

Returning to the membrane pore, it was proposed by Halestrap and colleagues [Halestrap, (1994)] that the 18.6kDa CyP of mitochondria (postulated to be matrix localised) binds to the ADP translocase and then modulates its pore forming activity in the presence of Ca^{2+}. This model could be considered correct (on the presumption that the translocase is involved in the pore) if the smaller CyP isoform (18.6kDa) was replaced by CyP22 (CyPD). Since CyP22 was shown to exhibit ADP/ Ca^{2+} sensitive CsA binding, the involvement of CyP22 in pore function, rather than CyP18, seems more acceptable.
Separation of CyP22 and CyP18 on the basis of their overall charge.

The two cyclophilins could not be easily separated on the basis of molecular sieving by gel filtration chromatography. They elute close to one another on Superdex 75 column (fig. 22). The difference in behaviour of the two PPIases (as predicted at different pH) was used to fractionate the proteins on ion exchange chromatography.

The smaller CyP (CyP18) and CyP22 are both basic proteins at pH 7.8 and can be retained on a cation exchange column (Mono S) at this pH. CyP22 was the more basic of the two proteins as indicated by its greater retention on the ion exchange column (elution at approx. 100mM [NaCl]) whilst CyP18 eluted earlier (65-75mM [NaCl]) (fig. 47). On the basis of these characteristics the two PPIases could be separated on cation exchange chromatography.
Fig. 47. The partial fractionation of CyP18 and CyP22 using cation exchange chromatography.

Rat liver mitochondria (5.3 mg of protein/ml) were treated with 200mM KCl/digitonin (0.19 mg of detergent/mg of mitochondrial protein) and sedimented at high speed. PPIase activity (- - -) of the supernatant was chromatographed at pH 7.7 on Mono-S (8ml, Pharmacia) using a salt gradient [0-500mM].
Overall summary.

From this section it can been shown that;

i) removal of the digitonin treated fraction (OM/ IMS fraction) and the subsequent treatment of the mitoplasts with high salt significantly increased the stability and the yield of CyP22.

ii) CyP18 was likely to be localised to the OM/ IMS fraction of mitochondria.

iii) CyP22 was found almost exclusively on the inner membrane (matrix facing) and matrix fractions of mitochondria.
Analysis of CyPD (CyP22) binding partner(s) that are responsible for ADP/ Ca\(^{2+}\) sensitive photolabelling of the PPlase in intact mitochondria.

[3.3.1] Preservation of PPlase activity under different conditions.

CyP22 was photolabelled in an ADP/ Ca\(^{2+}\) sensitive manner in intact mitochondria (figs. 11a and 12a). In the presence of ADP, photolabelling of the protein was significantly increased (fig. 11b) whilst in the presence of Ca\(^{2+}\) it was depressed (fig. 11b). The \(^{3}H\)CsA binding capacity of the semi-pure protein was completely unaffected by high concentrations of ADP or Ca\(^{2+}\) (fig. 24). This suggested that the protein did not have sites from which ADP or Ca\(^{2+}\) could influence its CsA binding property. Therefore it was concluded that this characteristic (ADP/ Ca\(^{2+}\) sensitivity) was assigned to a component, other than the PPlase, which was ADP/ Ca\(^{2+}\) sensitive.

CyP affinity chromatography has been successfully used to detect and isolate cyclophilin-interacting proteins from cellular extracts [Friedman and Weissman, (1991)]. This technique could also be used to investigate CyPD binding proteins e.g. proteins either responsible for ADP/ Ca\(^{2+}\) sensitive photolabelling to the PPlase in situ or other protein components of the pore. In order to construct a CyPD affinity column a reasonable amount of protein had to be immobilised on the column. The purification of CyPD was difficult; the highest yield of pure PPlase was obtainable from the mitochondria that had the OM/ IMS fraction removed by low concentrations of digitonin. CyP22 from the mitoplast fraction of mitochondria was also considerably more stable than from the SMP fraction. Bearing these factors in mind large amounts of CyPD was purified and used to construct the protein affinity column.

However, before any attempts to crosslink CyPD to an activated matrix could be considered, it was important at this stage to ensure that;

a) the immobilised PPlase was absolutely pure so that any interacting protein(s) detected was binding to CyPD rather than to a contaminant present in the preparation.
b) the PPlase was in its native state in order to maximise the possibility of it interacting with the target protein. This condition was assessed from the preservation of PPlase activity.

Experiments were conducted investigating conditions that preserved maximal PPlase activity (i.e. protein stability). The PPlase was stored under these conditions of optimal stability. A CyPD affinity column was prepared and run under similar conditions to ensure protein stability. Salt dissociable PPlase activity from rat heart mitoplasts was left at 0°C for 72 hrs at different pH with and without PMSF and protease inhibitors. In addition to PMSF and protease inhibitors, figure 48 reports the need for a high pH to preserve PPlase activity. A more detailed investigation examining the effects of different conditions on PPlase activity was carried out. CyPD purified from rat liver mitoplasts (after high salt treatment) was subjected to different storage conditions over a 2 week period. Figure 49 shows the effects of these conditions on the PPlase activity of the protein. Three rounds a of freeze-thaw cycle of the protein stored at -70°C resulted in the loss of approximately 42% of total activity (fig. 49 - table below). Overnight freeze drying of the protein and resuspension in 50% glycerol resulted in a 55% loss of total PPlase activity.

All conditions investigated (i.e storage in ammonium sulphate, ammonium sulphate/50% glycerol, 50% glycerol at 0°C and -70°C) displayed equal amounts of activity at day 0 (fig. 49- graph). Maximum PPlase activity was preserved when the protein was stored at 0°C in 50% glycerol. A single round of freeze-thaw of the protein (that had been stored at -70°C in 50% glycerol) resulted in a loss of approx. 10% of total activity. A second round of freeze-thaw caused a further loss of 10% whilst a third round of freeze-thaw resulted in a loss of about 47%. The protein could sustain 2 cycles of freeze-thaw but any further cycles caused a considerable loss of activity. Ammonium sulphate protects most proteins from denaturation. Purified proteins are routinely stored in ammonium sulphate.
Fig. 48. *High pH, PMSF and protease inhibitors are needed to preserve PPlase activity of* Cyp22.

Rat heart mitochondria (23mg of protein/ml) were sonicated in 10mM Tris-HCl/0.5mM EDTA/0.5mM DTT/pH 8.3 containing protease inhibitors (leupeptin, antipain, pepstatin A at 1μg/ml) and aprotinin at 5μg/ml) plus PMSF (1mM), PMSF, or nothing. SMPs were suspended in 500mM NaCl/10mM Tris-HCl/0.5mM EDTA/0.5mM DTT/pH 8.3 and sedimented. The pH of supernatants was adjusted to 6.7 and 7.2 using 500mM Mops/0.5mM EDTA/pH 6.5 containing either PI and PMSF, PMSF, or nothing and placed at 0°C for 72hrs. PPlase activity of the supernatants was measured in 60mM KCl/50mM Tris-HCl/50mM Hepes/pH 7.2 at 14°C.
### Conditions

<table>
<thead>
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<th>Procedure</th>
<th>Initial activity (arbitrary units)</th>
<th>Final activity (arbitrary units)</th>
<th>% loss</th>
</tr>
</thead>
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<td>3 rounds of freeze-thaw</td>
<td>50</td>
<td>5.8</td>
<td>42</td>
</tr>
<tr>
<td>overnight freeze-drying</td>
<td>117</td>
<td>52</td>
<td>58</td>
</tr>
</tbody>
</table>

Fig. 49 *The PPlase activity of CyP22 is stable in 50% Glycerol.*

Approximately 2.5μg of CyP22 protein from rat liver mitochondria was stored for 2 weeks at 0°C under different conditions. The effects of freeze-thaw and desiccation (freeze-dry) were also investigated. Peptidylprolyl cis trans isomerase activity was measured at day 0, 7 and 14 in 60mM KCl/10mM Heps/ pH 7.2 at 14°C.
On this occasion a 3.2M solution (sufficient to protect against protein denaturation) was unable to preserve PPlase activity of CyPD at 0°C over a 7 day period (fig. 49).

From the results obtained, storage of CyPD at 0°C in 50% glycerol maintains the maximum amount of PPlase activity. Like most proteins the PPlase was particularly sensitive to changes caused by freezing and thawing at -70°C. These changes were minimised when the protein was stored in 50% glycerol. The protein is also sensitive to desiccating procedures (i.e. freeze drying) which caused >50% loss of total activity.

3.3.2 The use of iodinated CyPD to investigate potential interacting partners.

Introduction:

Protein affinity chromatography can be used to investigate components that, in situ, interact specifically with the immobilised protein. Similarly, crosslinking studies can also be used to isolate proteins that are in close vicinity of each other, enough to be almost specifically interacting. Both methods involve chemical modification of amino acid groups on the test-protein. Reagents used to crosslink and immobilise proteins should modify the protein with minimal change in protein activity and protein structure. Crosslinking studies, in addition to covalently modifying the protein, need to ‘tag’ the protein (e.g. using a radio- or fluorescent label etc.). Tagging allows the detection of crosslinked components on polyacrylamide gels and chromatographic columns.

Since CyPD dissociates from SMPs in high salt media (fig. 38), reassociation of the protein with the membranes in a low salt media could be used to investigate components that interact with it. To allow the detection and the measurement of CyPD association with membrane components, it was decided that the PPlase should be iodinated. The reassociation of iodinated CyPD with CyPD-depleted SMPs could then be analysed in the presence of agents known to modulate pore activity (i.e. Pi, ADP, Ca^{2+}, CsA). Once conditions for ^{125}I-CyPD reassociation with membranes were established, studies to determine whether the protein associated with a specific membrane component could be examined. The association between ^{125}I-CyPD and SMP components could be made permanent using a homobifunctional
crosslinker e.g. DSP. As before, crosslinked products could then be analysed on standard SDS-PAGE. Alternatively, CyPD could be pre-reacted with an iodinatable photoactive heterobifunctional crosslinking reagent before reassociation with CyPD-depleted SMPs. Again, crosslinked products would be analysed on SDS-PAGE. To investigate soluble components that interact with the protein, SMPs with labelled (iodinated photoactive heterobifunctional crosslinker) CyPD associated, could be exposed to soluble mitochondrial extract. Activation of the photoactive group would allow specific interactions with soluble proteins to be observed on SDS-PAGE.

One of the main disadvantages of using crosslinking procedures to detect protein-protein interactions is that only neighbouring proteins that are in close vicinity to the labelled protein to be almost specifically interacting are detected. This means that non-specific protein-protein interactions (e.g. transient protein-protein interactions etc.) are also detected. However, crosslinking procedures can "cement" together very weak protein-protein interactions that would otherwise pass undetected, as well as identify relevant transient interactions.

Experimental method.

Attempts to iodinate CyPD were difficult since CyPD was extremely sensitive to iodination procedures. A direct iodination procedure using a modified Chloramine T method [Hunter and Greenwood, (1962)] resulted in almost complete loss (>90%) of PPlase activity. It has been reported that some proteins undergo substantial denaturation and loss of activity as a result of oxidation damage during this procedure. Another method using lodogen to iodinate CyPD [Salacinski et al, (1987)] was examined. This is similar to the Chloramine T method except that direct exposure of the protein to the oxidant is minimised. This was equally detrimental as the Chloramine T procedure; a PPlase activity loss greater then 80% was observed.

CyPB has been successfully iodinated using Iodogen to characterise CyPB binding sites on human T-cells [Allain et al, (1994)]. However, from the results obtained CyPD was susceptible to damage (probably oxidation) during the iodination procedure. Since $^{125}$I is exchanged on tyrosine residues, of which there are only two in the amino acid structure (fig. 170).
iodination at these particular residues probably rendered the protein inactive. On the other hand it was possible that the two tyrosine residues (tyr91, tyr122) were not available for iodination (i.e. not located on the surface of the protein). The result was no iodination of the protein and interference by the iodinating procedure to the PPIase activity of the protein.

[3.3.3] The use of Bolton and Hunter reagent (BHR) to modify CyPD.

As demonstrated in the previous section, the iodination of CyPD using conventional methods was difficult. PPIase activity of the protein was almost completely destroyed by the processes. Therefore, some other means of labelling CyPD was required. The Bolton and Hunter reagent (3- (4- hydroxyphenyl)propionic acid N- hydroxysuccinimide ester) (fig. 5) has been used to introduce $^{125}$I into proteins lacking accessible tyrosine residues [Bolton and Hunter, (1973)]. The reagent can be easily iodinated and then covalently linked to the protein. As briefly explained in fig. 5 the reagent specifically modifies basic residues, preferring lysine, at high pH. Reaction with Bolton and Hunter reagent (BHR) increases the net +ve charge on proteins. The change in +ve charge can be used to separate modified proteins from those that are unmodified. The change in +ve charge can also be used to separate different species of proteins that have been modified to different extents.

The effect of lysine-specific modification on the PPIase activity of CyPD was investigated. As reported in Figures 50 and 51 modification of the CyPD by Bolton and Hunter reagent resulted in relatively small losses (approx. 25%) of PPIase activity. This suggested that most of the protein was still active after incorporation of the reagent. However, it was unknown to what extent the PPIase had been modified. This was examined by isoelectrofocusing of the modified protein.

[3.3.4] Isoelectrofocusing of modified CyPD.

As reported in figures 50 and 51 PPIase activity was not severely affected by covalent modification of lysine residues. Since 6 lysine residues (see amino acid sequence, fig. 46b-italic) were potentially available for modification, it was anticipated that different species of
Fig. 50. Total PPlase activity before and after treatment with Bolton and Hunter reagent.
Fig. 51. Peptidylprolyl cis trans isomerase activity of CyP22 was preserved after treatment with Bolton and Hunter reagent. 10μg of CyP22 in 24% glycerol/10mM NaCl/ pH 8.5 was concentrated 70 fold (Centricon 10). After reaction with 0.1mM 3-(4-hydroxyphenyl)propionic acid N-hydroxysuccinimide ester (Bolton and Hunter reagent) and neutralisation with 0.4M glycine, the protein was fractionated on a Mono-S cation exchange column. PPIase activity was determined at each stage of the procedure. Data from the absorbance traces was plotted as $\ln (A_t / A_\infty)$ against t (secs) where $A_t$ is the absorbance change that occurred subsequent to any time t and $A_\infty$ is the maximal absorbance change recorded.
modified CyPD would arise. It was also anticipated that, in addition to displaying differences in net +ve charge, the different species of CyPD would also demonstrate different PPIase activities.

To separate the different species of modified CyPD (based on differences in net +ve charge) three types of electrophoretic techniques were examined. The gel stains used in these techniques can detect µg to ng amounts of proteins. Such sensitivity was important since very small quantities of proteins needed to be detected. Native gel electrophoresis of modified CyPD was abandoned. Conditions produced during native gel electrophoresis caused band distortion. The heat produced during electrophoresis was the likely cause. The use of native and denaturing isoelectrofocusing (IEF) was also examined. Denaturing IEF was most effective at separating modified CyPD from unmodified CyPD. Denaturing IEF can fractionate proteins that have a slight difference in charge between them. Proteins migrate along the pH gradient until they arrive at a point where they carry no net charge. This is the isoelectric point (pI) of the protein.

Figure 52 reports the focusing of modified and unmodified CyPD. An IEF gel with a pH gradient between 3-10 was used. Three, possibly four, bands of modified CyPD were observed with 2 major bands migrating between pH 7.9-8.3 (lane 1). Minor bands were detected between pH 8.6-9.4. Unmodified CyPD migrated as two bands with pIs near pH 9.9 (lane 2). The two bands detected in unmodified CyPD might casually be attributed to microheterogeneity within the pure protein. Slight damage to the protein during purification might give rise to the formation of artefacts (e.g. N-terminal blocking, acetylation etc). Such artefacts may pass unrecognised on SDS-PAGE or gel filtration chromatography whereas, on IEF, they were detected.

Since no protein band was detected at pH 9.8, it was presumed that all of CyPD was modified to the four different species using the Bolton and Hunter reagent (fig. 52). Although chemical modification did not adversely effect the PPIase activity of the protein (fig. 51), it remained unknown which specie of the protein displayed PPIase activity. Since 75% of PPIase activity was preserved after treatment with Bolton and Hunter reagent, it was assumed that
most, if not all of the PPIase activity, was attributed to the 2 major bands at pH 7.9-8.3 (fig. 52).

Knowing that CyPD remains active after modification at lysine residues, it was thought it may be possible to investigate CyPD interacting proteins using crosslinking procedures. Under conditions known to influence CsA binding to the PPIase (e.g. ADP, Ca$^{2+}$), an iodinated lysine-specific heterobifunctional crosslinker could be used to crosslink CyPD to either its ADP/ Ca$^{2+}$ sensitive binding partner (in SMPs or the soluble fraction of mitochondria) or to components of the pore.

Rather than employing crosslinking techniques, CyPD- affinity chromatography was used to investigate CyPD interacting components. Simply by attaching the active protein to an inert matrix (via a lysine residue) and passing mitochondrial extracts through the column, it was thought it may be possible to detect and isolate CyPD interacting proteins. Again, as with crosslinking studies, the effects of ADP and Ca$^{2+}$ (or other pore ligands) on CyPD interactions with mitochondrial proteins could be examined.

[3.3.5] The effects of ADP and Ca$^{2+}$ on the salt dissociable PPIase activity of rat liver mitoplasts.

Before any attempt to construct a CyPD- affinity column was considered, it was important to examine which fraction of mitochondria (i.e. OM/ IMS, inner membrane, matrix) was likely to contain CyPD binding proteins. Photolabelling studies demonstrated that $^3$H[PA-CS] binding to the soluble fraction of intact mitochondria was unaffected by ADP or Ca$^{2+}$ (fig. 10). This suggested that the component responsible for selectively labelling CyPD in an ADP/ Ca$^{2+}$ sensitive manner in SMPs was absent from the soluble fraction. Other evidence pointed to the existence of an ADP and Ca$^{2+}$ sensitive factor on the matrix facing side of the inner membrane. Single channel electrophysiological recordings of patch clamped mitoplast membranes showed an ADP and CsA inhibited Ca$^{2+}$ activated pore (i.e. megachannel) [Szabo and Zoratti, (1992); Szabo et al, (1992)]. Since these patches (free of the soluble fraction of mitochondria) were activated by Ca$^{2+}$ and inhibited by ADP and CsA, it was concluded that
Fig. 52. Isoelectrofocusing of modified CyP22 revealed 2 major bands between pH 7.9-8.3 on a denaturing gel.
the ADP/ Ca\(^{2+}\) sensitive component was membrane located. In addition to these findings, mitochondria were also shown to possess sites (cytosol facing) on the inner membrane that modulated the activity of the Ca\(^{2+}\) sensitive pore [Bernardi et al, (1993)]. Taking these findings into consideration and the fact that CyPD is photolabelled in an ADP/ Ca\(^{2+}\) sensitive manner at the membranes in intact mitochondria, it was suspected that the salt treated fraction of mitoplasts (after digitonin treatment of mitochondria), as well as containing PPIase activity, might also contain the ADP/ Ca\(^{2+}\) sensitive component responsible for ADP/ Ca\(^{2+}\) sensitive labelling of the PPIase.

The effects of ADP and Ca\(^{2+}\) on the PPIase activity of the salt dissociable fraction of rat liver mitoplasts was examined. Figure 53 reports the inhibition of the PPIase from the salt treated fraction of mitoplasts. Inhibition by CsA (14.8nM) was completely unaffected by high concentrations of ADP (1.9mM) or Ca\(^{2+}\) (1.45mM). This suggested the absence of any ADP/ Ca\(^{2+}\) sensitive component electrostatically attached to the outside of the inner membrane.

Present studies have shown the membrane attached CyPD to bind \(^3\)H[PA-CS] in an ADP/ Ca\(^{2+}\) sensitive manner in whole mitochondria. The component responsible for ADP/ Ca\(^{2+}\) sensitive CyPD labelling was unlikely to be located in the matrix (fig. 10) or on the outside of the inner membrane (fig. 53). The ADP/ Ca\(^{2+}\) sensitive component was more likely to be associated with the matrix facing side of the inner membrane and so this fraction of mitochondria was examined for CyPD interacting protein(s).


Protein affinity chromatography can be used to detect protein- protein interactions. Under appropriate conditions the technique can detect the weakest of binding constants (\(K_d 10^{-5}\)). It examines all the proteins of the test-extract equally for interactions with the immobilised protein. Any interacting protein detected by affinity chromatography has therefore successfully competed with all the proteins of the extract for the immobilised ligand. The technique can also be used to explore the effects of domain changes (i.e. using a mutant test-protein) on the interaction with immobilised protein.
Fig. 53. ADP/ Ca^{2+} -sensitive binding partner of CyP22 is not found attached to the outside of the mitochondrial inner membrane.
Salt washed fraction of rat liver mitoplasts (113mg of mitochondrial protein) was assayed for PPlase activity in 60mM KCl/ 20mM Hepes/ 20mM Tris-HCl/ pH 8.0 at 14°O. The effect of CsA, ADP, and Ca^{2+} is reported. Graph shows In (A_t/A_o) against t (secs) where A_t is the absorbance change that occurred subsequent to any time t and A_o is the maximal absorbance change recorded.
CyPD was immobilised onto a sepharose-4B matrix via 6-aminohexanoic acid-N-hydroxysuccinimide ester (fig. 6). The ester provided a 6 carbon spacer between the matrix and the protein. This allowed some flexibility between the matrix and CyPD so that different surface groups on the immobilised protein were available for interaction with proteins of the extract.

By preparing a control column (one that had no CyPD coupled to it), conditions under which minimal non-specific interactions occurred with the matrix were investigated. After slowly (3 - 4 hrs) passing mitoplast membranes extracted in 10% glycerol/ 6% CHAPS/ 1mM CaCl₂/ 20mM Hapes/ PI/ 0.5mM DTT/ pH 7.7 through the column, and analysing the eluted products on SDS-PAGE (fig 54A), it was found that non-specific interactions were minimised by increasing the pH of both the membrane extract and the elution buffers (fig. 54B). Although the same proteins adhered to the control column, their interactions with the matrix were minimised at the higher pH. The control column was replaced with the CyPD affinity column and the experiment repeated.

To examine CyPD interacting proteins, 15μg of CyPD (free of protease inhibitors and DTT) was coupled to the activated matrix. Inhibitor peptides were removed from CyPD because they contain a high % of basic residues (e.g. arginine etc.) which serve as sites for coupling reaction. Any inhibitor peptides immobilised to the column would be a source of undesirable protein interaction. DTT, by reacting with the succinimide ester of the activated matrix, would limit the amount of CyPD coupled to the column. Inner membranes from mitoplasts were prepared by sonicating salt treated mitoplasts in zero [salt]/ pH 8.1 media. The pellet was solubilised using CHAPS as detergent (discussed above). This ensured that components stuck to the inner membrane (matrix facing) were also solubilised and tested for interaction with CyPD. After circulating solubilised extract through CyPD-affinity column any interacting proteins were resolved using ammonium acetate (20mM, 100mM, 500mM and 500mM/ 0.5mM EGTA). Ammonium acetate was used to elute proteins from the column because, being a volatile buffer, it was completely removed upon freeze drying. Therefore, unlike high [NaCl], it did not interfere with protein migration on SDS-PAGE. Figure 55 compares the elution profiles of control and CyPD affinity columns on SDS-PAGE. No
Fig. 54. Non-specific interactions of inner membrane proteins with Sepharose-4B matrix activated column are minimised at high pH.

Sepharose-4B matrix columns activated with 6-aminohexanoic acid N-hydroxysuccinimide ester were equilibrated at 0.6% CHAPS/10% glycerol/20mM Hepes/0.5mM DTT/PI/1mM CaCl₂/pH 7.4 (A) or 8.1 (B). Salt treated mitoplasts from rat liver mitochondria (115mg of mitochondrial protein) were sonicated in zero salt buffer (pH 8.1) and sedimented at high speed. The pellets (inner membranes) were extracted in 6% CHAPS/100mM NaCl/10% glycerol/20mM Hepes/0.5mM DTT/PI/PMSF (0.4mM)/1mM CaCl₂ at pH 7.4 or 8.1. The solubilised extracts were diluted (1:10) and circulated through the columns (3-4hrs). The columns were developed using a 0-500mM NaCl gradient at pH 7.4 (A) or 8.1 (B). Eluted fractions were analysed on standard SDS-PAGE minigels and stained with Coomassie Blue.
Fig. 55. Cyp22- affinity column is unable to retain any specifically interacting component from mitochondrial inner membranes.

Cyp22 (15μg) was coupled to Sepharose-4B activated matrix at pH 8.5. Salt treated mitoplasts from rat liver mitochondria (113mg of mitochondrial protein) were sonicated in zero salt buffer (pH 8.1) and sedimented (120,000g 90mins. 5°C). The pellet (inner membranes) was extracted in 6% CHAPS/ 100mM NaCl/ 10% glycerol/ 20mM Hepes/ 0.5mM DTT/ PIPES (0.4mM)/ 1mM CaCl2/ pH 8.1, diluted (1:10), and circulated through affinity column (1ml/hr). Proteins fractions eluted using ammonium acetate (100 (i), 500 (ii), and 500mM/ 0.5mM EGTA gradients (iii)) were desiccated (freeze-dry) and analysed on standard SDS-PAGE mini-gels (A). Column with no Cyp22 attached was treated identically (B). Molecular weight standards (14-97kDa) are indicated.
significant difference was observed between the types of proteins retained by the two columns.

Since the binding affinity of CyPD for CsA is in the nanomolar range [McGuinness et al, (1990)] and Ca\(^{2+}\) competes with CsA (i.e. depresses and inhibits the effects of CsA in labelling, binding and electrophysiological studies), it was assumed that the affinity of the Ca\(^{2+}\) sensitive component for CyPD was just as high. Therefore, to potentiate the interactions of immobilised CyPD with the Ca\(^{2+}\) sensitive factor of the test-extract, affinity chromatography was performed in the presence of high [Ca\(^{2+}\)]. However affinity chromatography failed to retain any specifically interacting components from the membrane extract. One of the ways to explain the lack of specific binding to the immobilised protein was to assume that interaction between the PPIase in the extract (free PPIase) and the Ca\(^{2+}\) sensitive binding partner was very tight [McGuinness et al, (1990)]. In this way the Ca\(^{2+}\) sensitive factor (if it was present at a much smaller amount than the extracted PPIase) would be completely associated with the free PPIase. As a result none of the Ca\(^{2+}\) sensitive binding partner would be available for interaction with the immobilised protein.
The dissociation of CyPD from the inner membrane was potentiated by Ca$^{2+}$ and unaffected by other pore effectors.

[3.4.1] Introduction.

As it stands, both electrophysiological experiments using excised membrane patches [Szabo and Zoratti, (1991); Szabo and Zoratti, (1992)] and mitochondrial permeabilisation experiments measuring swelling and membrane potentials have demonstrated blockage of the Ca$^{2+}$ activated membrane pore by CsA. Pore blockage by CsA was depressed by Ca$^{2+}$ and potentiated by ADP and Mg$^{2+}$ [Novgorodov et al, (1992); Andreeva and Crompton, (1994)]. Photolabelling ($^3$H[PA-CS]) of the inner membrane PPIase (CyPD) was also selectively increased in the presence of ADP and decreased in the presence of Ca$^{2+}$ (figs. 11a and 12a). Interestingly, based on the $K_d$ for CsA and the amount of protein present, the PPIase was considered to be identical to the high affinity CsA binding sites involved in mitochondrial permeabilisation. CsA binding to these sites (and presumably to the PPIase) was also decreased in the presence of Ca$^{2+}$ [McGuinness et al, (1990)]. Considering all this evidence, it seemed likely that CyPD played a pivotal role in pore activity. The model proposed to explain how CyPD modulated pore activity suggested that in the presence of Ca$^{2+}$, CyPD bound tightly to the membrane pore [McGuinness et al, (1990); Halestrap, (1994)]. After association with the pore the PPIase catalysed a reaction that predisposed the pore to form and stabilise an open structure. CsA prevented CyPD association by sequestering the cyclophilin. By binding to and inhibiting the PPIase activity of CyPD, CsA limited the amount of protein available for pore activation, (i.e. formation of CyPD/pore structures). Presumably, ADP increased the probability of the pore to form a closed structure by potentiating the binding of CsA to CyPD.

No ADP and/or Ca$^{2+}$ sensitive sites were found on CyPD since the [$^3$H]CsA binding capacity of the pure protein was ADP and Ca$^{2+}$ insensitive (fig. 24). Therefore some component, other than the PPIase, was responsible for ADP/Ca$^{2+}$ sensitive photolabelling of CyPD in situ. It was unknown whether the effects of ADP and Ca$^{2+}$ on CsA binding to CyPD

183
were mediated through a single ADP/ Ca\(^{2+}\) sensitive binding component, or two different components that exclusively bound ADP or Ca\(^{2+}\). This component(s) was unlikely to be found in the soluble fraction of mitochondria. Photolabelling studies in intact mitochondria showed ADP/ Ca\(^{2+}\) insensitive \(^{3}\)H[PA-CS] binding of the soluble fraction (fig. 10). The ADP/ Ca\(^{2+}\) sensitive component was also unlikely to associate with the outside of the inner membrane since PPIase activity of the salt treated fraction of mitoplasts was unaffected by ADP and Ca\(^{2+}\) (fig. 53). As discussed in the previous section the ADP and/ or Ca\(^{2+}\) sensitive component was likely to be located on the matrix facing side of the inner membrane or actually within the membrane.

**Experimental method.**

Considering the predicted model [Halestrap, (1994)] and the fact that CyPD associated electrostatically with the membrane, it was postulated that pore ligands (e.g. ADP, Ca\(^{2+}\), NAD\(^+\) etc.) modulated the association/dissociation of CyPD with the membrane pore. By binding to their respective partners, ADP and Ca\(^{2+}\) modified the association and dissociation of CyPD with pore components. Ca\(^{2+}\) potentiated the association of CyPD with the pore (i.e. pore activation) whilst ADP prevented and depressed association (i.e. pore inactivation). Other pore effectors e.g. Pi, Mg\(^{2+}\), redox potential etc might also influence pore function (either directly or indirectly) by modulating the association/ dissociation characteristic of the PPIase. This characteristic was investigated further. It was concluded earlier that ADP and/or Ca\(^{2+}\) sensitive target protein may be located on or in the inner membrane. Therefore, inner membrane fractions supposedly containing components of the pore were prepared from mitoplasts. The effects of different pore ligands on the association of CyPD with the membranes were investigated. However, before exploring this, two issues concerning the experimental conditions under which association/ dissociation were to be studied were addressed. Since CyPD was easily displaced from membranes by high [salt], it was important to use a salt concentration that only produced partial dissociation (so that effects of pore ligands on dissociation or reassociation of CyPD could be measured). Also, the addition of pore ligands to inner membrane preparations changed the ionic strength of the experimental
media. This was suspected to affect the interaction of CyPD with the inner membrane. By adding equivalent amounts of KCl (to compensate for the change in ionic strength on addition of pore ligands) the effects of changes in ionic strength on the association of CyPD with the membranes were eliminated.

Liver mitoplasts were treated with high salt to remove PPIase associated with the outside of the inner membrane. These were then suspended in low salt media and sonicated. The isolated inner membranes were resuspended in zero and [50mM] salt buffer. Figure 56 shows that almost all PPIase activity associated with the inner membrane was dissociated by 50mM [NaCl] at pH 7.3 whilst only 36% was soluble in zero salt media. As a result the effects of different pore ligands on dissociation or association of CyPD with the inner membranes were examined in zero salt media.

The ability of different effector ligands to displace PPIase activity from inner membrane was investigated. Figure 57 reports the effects of Ca^{2+} (1mM), NAD^+ (2mM), NADP^+ (2mM), NaHPO_4 (2mM) and t-BuOOH (50μM) on the dissociation of CyPD from the membranes. In all cases Ca^{2+} potentiated the displacement of CyPD from the membranes (average increase of 35-40% in PPIase activity). All other pore ligands, except for NAD^+, showed no change in the displacement profile of CyPD. NAD^+ appeared to decrease the amount of CyPD displaced from the membrane. Interestingly this was reversed in the presence of Ca^{2+} (fig. 57). Tertiary butylhydroperoxide (t-BuOOH) did not influence CyPD displacement. To a certain extent this was expected since t-BuOOH was unlikely to interact directly with the PPIase to induce pore activation. Tertiary-BuOOH modulated pore activity by depressing the redox potential of mitochondria (i.e. potentiating oxidative stress) (see introduction, section [1.6.4]). NADP^+ and phosphate might modulate CyPD displacement through allosteric interactions with regulatory proteins involved in pore activity. These proteins may have been lost on membrane preparation.

Figure 58 reports the effects of NADH, NAD^+, NADPH and NADP^+ on the dissociation of CyPD from the inner membrane fraction (pellet) into the soluble fraction (SN) of mitochondria. Identical experiments were conducted in which varying KCl concentrations (1, 3, 5 and 10mM), replaced the nicotinamides. This compensated for the effect of the
Fig. 56. Almost all PPIase activity associated with the inner membrane is displaced by [50mM] NaCl.
Salt treated mitoplasts (293 mg of mitochondrial protein) suspended in 20mM Hepes/0.2mM EGTA/PI/0.4mM PMSF/0.5mM DTT/pH 7.2 were sonicated (10 mins with 15 sec intervals) and sedimented (120,000g, 45mins, 5°C). The pellet (inner membrane) was suspended in pH 7.2 buffer containing zero salt. The suspension was split into 3 and NaCl added to 2 of the suspension to give 50 and 100mM [NaCl]. The suspensions were placed on ice for 15-20mins. After sedimentation at high speed PPIase activity in the SNs was measured.
Fig. 57. $Ca^{2+}$ potentiates the dissociation of CyP22 from rat liver inner membranes whilst $NAD^+$, $NADP^+$, $Na_2HPO_4$ and tertiary Butylhydroperoxide were ineffective. Salt treated mitoplasts (293 mg of mitochondrial protein) suspended in 20mM Hepes/0.2mM EGTA/Pl/0.4mM PMSF/0.5mM DTT/pH 7.2 were sonicated (10 mins with 15 sec intervals) and sedimented (120,000g, 45mins, 5°C). The pellet (inner membrane) was suspended in pH 7.2 buffer containing zero salt. The effects of $Ca^{2+}$ (1mM), $NAD^+$ (2mM), $NaHPO_4$ (2mM) and t-BuOOH (50μM) on the dissociation of CyP22 from the membranes is examined. KCl is added at appropriate concentration to compensate for the change in ionic strength.
<table>
<thead>
<tr>
<th>Condition</th>
<th>% of total PPIase activity in SN</th>
<th>% of total PPIase activity in Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>1mM NADH</td>
<td>38.6</td>
<td>61.4</td>
</tr>
<tr>
<td>3mM KCl</td>
<td>40.2</td>
<td>59.8</td>
</tr>
<tr>
<td>1mM NAD⁺</td>
<td>34.7</td>
<td>65.3</td>
</tr>
<tr>
<td>1mM KCl</td>
<td>27.4</td>
<td>72.6</td>
</tr>
<tr>
<td>1mM NAD⁺/ 2mM ADP</td>
<td>51.4</td>
<td>48.6</td>
</tr>
<tr>
<td>5mM KCl</td>
<td>50.4</td>
<td>49.6</td>
</tr>
<tr>
<td>1mM NADPH</td>
<td>55.8</td>
<td>44.2</td>
</tr>
<tr>
<td>10mM KCl</td>
<td>42.7</td>
<td>57.3</td>
</tr>
<tr>
<td>1mM NADP⁺</td>
<td>48.6</td>
<td>51.4</td>
</tr>
<tr>
<td>5mM KCl</td>
<td>50.4</td>
<td>49.6</td>
</tr>
<tr>
<td>0 KCl</td>
<td>29.3</td>
<td>70.7</td>
</tr>
<tr>
<td>400mM KCl</td>
<td>87.23</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Table 58. NAD⁺, NADH, NADP⁺, and NADPH do not affect the association/dissociation of CyP22 with the inner membrane.

Salt treated mitoplasts (120 mg of mitochondrial protein) suspended in 20mM Hepes/0.2mM EGTA/PI/0.4mM PMSF/0.5mM DTT/PH 7.2 were sonicated (10 mins with 15 sec intervals) and sedimented (120,000g, 45mins, 5°C). The pellet (inner membrane) was suspended in pH 7.2 buffer containing zero salt. The distribution of PPIase activity between the membrane fraction (associated) and soluble fraction (dissociated) was investigated in the presence of pyridine nucleotides. Table shows the percentage distribution of total PPIase activity between the two fractions. KCl is added at appropriate concentration to compensate for the change in ionic strength.
nicotinamides on the ionic strength of the experimental media. The distribution of CyPD between the membranes and the soluble fraction was unaffected by the different nicotinamides.

The question arises as to how this finding - Ca$^{2+}$ induced CyPD displacement- can be incorporated into the pore model proposed earlier. Ca$^{2+}$ potentiated the dissociation of CyPD from the inner membrane. This was in complete contradiction to the predicted pore model [McGuinness et al, (1990); Halestrap, (1994)] in which, in the presence high [Ca$^{2+}$], CyPD was postulated to bind tightly to the pore and predispose it to form an open structure.
Chapter 4.

[4.1] Photolabelling of mitochondrial membranes is ADP/ Ca\(^{2+}\) sensitive.

The involvement of ADP in pore function has been well documented. However, depending on the experimental methods and conditions used to investigate pore function, the effects of ADP on pore closure can be interpreted in different ways. For example, experiments in which dissipation of the membrane potential was measured to indicate pore opening, showed ADP to behave synergistically with CsA at preventing loss of potential [Andreeva and Crompton, (1994)]. These experiments showed ADP and CsA had to be added together to induce pore closure. CsA, even at concentration 10 fold greater than needed to block the pore, was found to be ineffective at blocking the pore unless supplemented with ADP (200\(\mu\)M).

Using other methods to study pore activity (i.e., \[^{14}\text{C}\]sucrose entry, light scattering), the synergistic effects of ADP and CsA on pore closure were found to be inconsistent [Davidson and Halestrap, (1988); McGuinness et al, (1990)]. These studies showed that ADP and CsA added alone were sufficient to induce pore closure. Nevertheless, whether ADP behaves synergistically with or independently of CsA, the nucleotide clearly plays an important role in stimulating the closure of the membrane pore.

Using methods similar to those above, Ca\(^{2+}\) was shown to behave in completely the opposite manner to ADP and CsA. Rather than blocking, Ca\(^{2+}\) stimulated the opening of the membrane pore as indicated by sucrose entrapment, dissipation of membrane potential and conductivity measurements of excised membrane patches. Presumably, by preventing CsA interactions, Ca\(^{2+}\) competed with CsA for relevant binding site on the pore complex. This feature of the pore was clearly demonstrated in patch clamp studies in which membrane conductivity of the mitochondrial megachannel (MMC) was measured. On adding Ca\(^{2+}\) to membrane patches (sufficient to induce pore opening), membrane conductivity of the MMC (i.e., flicker between open and closed states of the pore) was rapidly increased. This was quickly decreased on adding CsA to the patches. To reactivate the MMC (after CsA
inhibition) a higher concentration of $\text{Ca}^{2+}$ was needed [Szabo and Zoratti, (1991); Szabo and Zoratti, (1992); Szabo et al, (1992)]. Similar findings were also reported in which the dissipation of membrane potential (as a reflection of pore opening) rather than conductivity changes was measured [Andreeva and Crompton, (1994)]. Taken together these studies clearly suggest that $\text{Ca}^{2+}$ and CsA compete with one another for the relevant binding sites of the pore.

All previous investigations have shown pore blockage by CsA to be greatly affected by $\text{Ca}^{2+}$ and ADP. $\text{Ca}^{2+}$, by preventing CsA blockage, stimulated pore opening whilst ADP, by potentiating the effects of CsA, stimulated pore closure. Since $\text{Ca}^{2+}$ and ADP affect the association of CsA with the pore in an opposite and reverse manner, they were considered to be ideal candidates that could be used to selectively identify (and later purify) the relevant CsA binding protein of the pore.

In light of these findings mitochondria were photolabelled ($^3\text{H}[\text{PA-CS}])$ in the presence of high $[\text{Ca}^{2+}]$ and $[\text{ADP}]$ in an attempt to identify the relevant CsA binding target involved in the membrane pore. Although both the soluble and the membrane fractions of mitochondria showed widespread labelling, ADP/ $\text{Ca}^{2+}$ sensitive photolabelling was only observed in the membrane fraction. This suggested the relevant CsA binding component was membrane localised. ADP increased photolabelling of the membrane fraction by approximately two fold whilst $\text{Ca}^{2+}$ decreased it by approximately the same magnitude (figs 11b and 12b). These findings agreed well with earlier reports of ADP/ $\text{Ca}^{2+}$ sensitive CsA binding in which ADP was shown to facilitate the effects of CsA on pore closure (i.e., increase CsA binding) and $\text{Ca}^{2+}$ to depress CsA induced pore closure (i.e., decrease CsA binding) [Andreeva and Crompton, (1994)]. Since the soluble fraction of mitochondria failed to express any ADP/ $\text{Ca}^{2+}$ sensitive photolabelling, it was assumed that the relevant CsA binding protein was absent from this fraction.

The competition between CsA and $\text{Ca}^{2+}$ to influence conductivity of the MMC was quite clear. CsA depressed membrane conductivity by closing the pore whilst $\text{Ca}^{2+}$ stimulated conductivity by opening the pore. The $\text{Ca}^{2+}$ activated megachannel could be blocked by increasing the concentration of CsA added to the patches. Any further activation of the CsA
blocked channel required a higher Ca\(^{2+}\) concentration. The effects of ADP on membrane conductivity were not so clear. Ca\(^{2+}\) and ADP did not behave in a competitive manner to influence the activity of the megachannel. Although pore opening (i.e., flickering of the channel) was completely inhibited by ADP, it could not be readily reversed by adding increasing concentration of Ca\(^{2+}\) [Szabo et al, (1992)]. This suggested that ADP and Ca\(^{2+}\) affected pore activity through different binding sites.

[4.2] **ADP and Ca\(^{2+}\) binding sites in mitochondria.**

Based on a number of studies that used different techniques to investigate the effects of Ca\(^{2+}\), ADP and CsA on pore function, some interesting points concerning the sites from which these agents might mediate their action have been raised. Patch clamp experiments suggested that both ADP and Ca\(^{2+}\) modulated pore activity through one or more binding sites [Szabo and Zoratti, (1992)]. These sites might be common to the two effectors but, because the two ligands are completely different in structure, it was very unlikely that ADP competed directly with the Ca\(^{2+}\) binding site. Rather, by decreasing the affinity for Ca\(^{2+}\), ADP modified Ca\(^{2+}\) binding through a specific ADP binding site [Novgorodov et al, (1992); Szabo and Zoratti, (1992)]. In this way, by limiting the interaction of Ca\(^{2+}\) with its binding site, ADP potentiated CsA binding to the relevant component of the pore.

The location and the number of Ca\(^{2+}\) and ADP binding sites involved in pore function have also been investigated in some detail. These studies predicted that two Ca\(^{2+}\) binding sites, both localised to the inner membrane of mitochondria, were responsible for opening the membrane pore [Haworth and Hunter, (1979b); Haworth and Hunter, (1980); Bernardi et al, (1993)]. One of the Ca\(^{2+}\) binding sites faced the matrix whilst the other faced the cytoplasm. Based on the fact that intramitochondrial Ca\(^{2+}\) was responsible for activating the pore, it was assumed that the matrix facing Ca\(^{2+}\) site, rather than the cytoplasmic facing site, was involved in pore opening [Gunter and Pfeiffer, (1990); Szabo et al, (1992); Bernardi et al, (1993)]. Similarly, two ADP binding sites were predicted to exist on the matrix facing side of the inner membrane [Haworth and Hunter, (1980)]. One site was presumed to represent the ADP translocase whilst the identity of the other was unknown. The ADP translocase is a strong
candidate for involvement in the pore complex (see later) and is likely to play a critical role in pore function. However some doubt is cast upon its identity as the relevant ADP binding protein responsible for potentiating the inhibitory effects of CsA on pore opening [Novgorodov et al, (1992)]. Novgorodov et al demonstrated the presence of an ADP binding site capable of reversing pore opening that was distinct from the ADP binding site of the translocase [Novgorodov et al, (1992)].

[4.3] A 22kDa PPIase (CyP22), that is identical to CyPD, photolabels in an ADP/ Ca\(^{2+}\) dependent manner.

Using the selection criteria (i.e., photolabelling in the presence of high [ADP] and [Ca\(^{2+}\)]) a 22kDa protein that photlabelled in an ADP/ Ca\(^{2+}\) sensitive manner was identified and purified. The photolabelling profile of the protein agreed well with previous investigations that examined the effects of ADP and Ca\(^{2+}\) on CsA binding to sites in mitochondria (i.e., ADP increased CsA binding to mitochondrial sites whilst Ca\(^{2+}\) decreased CsA binding) [McGuinness et al, (1990); Andreeva et al, (1994)]. Interestingly, some of the features of the protein, (i.e., PPIase activity of k\(_{cat}\)/ K\(_{m}\) of 3-8\(\mu\)M\(^{-1}\)s\(^{-1}\) and a K\(_i\) between 5-8nM for CsA) correlated remarkably well with those of the high affinity (K\(_d\) 7-8nM for CsA) CsA binding sites studied by McGuinness et al [McGuinness et al, (1990)]. This indicated the two components were probably identical. Besides these features other properties of the high affinity CsA binding sites, (namely ADP/ Ca\(^{2+}\) sensitivity) also suggested that they were identical to the PPIase. CsA binding to the high affinity sites was reduced by Ca\(^{2+}\) concentrations similar to that encountered under permeabilisation conditions, (i.e. >5\(\mu\)M) [McGuinness et al, (1990)] and increased by ADP [Andreeva and Crompton, (1994)]. Therefore, considered alongside the photolabelling and the \[^{3}H\]CsA binding evidence presented earlier, the 22kDa PPIase isolated from the membrane fraction of mitochondria is, in all probability, the relevant CsA binding CsA target protein involved in the membrane pore.

Although the 22kDa PPIase (CyP22) is considered to be the relevant CsA binding target involved in pore function, other cyclophilins with molecular weights similar to the 22kDa protein (i.e., 17.6, 18 and 20kDa) were also considered as possible CsA binding
components of the pore. Recent studies looking at the involvement of cyclophilin in pore function suggested that CyP22 might not play a role in the pore because it was a contaminant of ER origin and therefore non-mitochondrial. [Nicolli et al, (1996)]. Nicolli et al, postulated that CyPM (20kDa in size) was likely to be the main and only relevant cyclophilin involved in pore function because in their investigations, interaction of the protein with SMPs (measured using western blotting) was disrupted by CsA and a low pH, (i.e., conditions known to prevent pore opening). However, because CyP22 was purified from mitoplasts (mitochondria that have had the OM/IMS and any membranous organelles associated with them e.g., ER, microsomes removed by digitonin) it was unlikely that it was of non-mitochondrial origin. This procedure not only stabilised the protein considerably (by removing protease activity found in OM/IMS fraction or in contaminatory organelles that was responsible for proteolytic cleavage of CyP22), but also demonstrated it to be of mitochondrial origin. Marker enzyme assays in conjunction with subfractionation of mitochondria, further supported the location of CyP22 as mitochondrial [Tanveer et al, (1996)]. Interestingly, photolabelling analysis of SMPs only revealed a 22kDa PPIase, no 20kDa PPIase was detected or isolated. A possible explanation for this is that in their purification procedure Nicolli et al [Nicolli et al, (1996)] did not include protease inhibitors in the isolation buffers. In which case CyPM (20kDa) might have been a cleaved product of CyP22. The inclusion of protease inhibitors in protein extracts and isolation buffers was shown to protect PPIase activity of CyP22 (fig. 48) from proteolytic damage. Another PPIase that has been postulated to play a critical role in pore opening is CyP18 (18.6kDa in size) [Halestrap and Davidson, (1990); Halestrap, (1994)]. A rather elegant model that attempted to explain pore function was proposed by Halestraps' group in which CyP18 was predicted to play a key role in pore activity (see Introduction, section 1.10.3).

Although the previous experiments show the possible involvement of different cyclophilins in pore function, based on their similar sequence data, in reality, CyP22, CyPM (20kDa) and CyP18 are likely to be identical proteins. The N-terminal sequence of CyPM was identical to the N terminus of CyP18 [Conner and Halestrap, (1992)]. Interestingly, except for one residue, the N-terminal sequence of CyP18 (fig. 46A non italic) was identical to a peptide sequence found in CyP22 (fig. 46A italics). This peptide sequence was likely to be
located on the N-terminal of CyP22. Besides a close homology with the N-terminal sequences of CyP18 and CyPM, CyP22 also revealed a close sequence homology with human CyP3 (CyPD) and other cyclophilins, i.e., rat CyPA (79%), human CyPB (66%), and rat CyPC (60%). This suggested that these cyclophilins have similar core sequences but differ in N-terminal sequence. Some N-terminal sequences target proteins to the ER whilst some target proteins to the mitochondria. As a result of either proteolytic activity during purification or protein processing, CyP22 was probably cleaved or processed to CyP20 and CyP18 so that, on SDS-PAGE analysis, different cyclophilins appeared to be present.

[4.4] CyPD (CyP22) contains no intrinsic ADP or Ca$^{2+}$ binding sites.

The photolabelling ($^3$H[PA-CS]) profile of CyP22 in situ was ADP/ Ca$^{2+}$ sensitive. Under the conditions used, [$^3$H]CsA binding (LH-20) to the semi-pure CyP22 demonstrated no sensitivity to ADP or Ca$^{2+}$ (fig. 24). It was considered that the concentration of [$^3$H]CsA used was too high. This meant that the effects ADP and Ca$^{2+}$, if any, on [$^3$H]CsA binding to the protein would not have been observed. However, further studies investigating the effects of high [ADP] and [Ca$^{2+}$] on the inhibition of PPIase activity by CsA of the protein did show it to be insensitive to the pore ligands (fig. 53). This suggested that the protein did not bind ADP or Ca$^{2+}$. This led to an interesting possibility where a component, other than CyPD that presumably binds ADP or Ca$^{2+}$ was responsible for ADP/ Ca$^{2+}$ sensitive photolabelling of the PPIase.

In some photolabelling studies CyP22 was found associated with a smaller molecular weight component. A 29kDa complex that consisted of CyP22 and a 7kDa factor continued to exhibit ADP/ Ca$^{2+}$ sensitive photolabelling (fig. 14a). Whether the associating factor (7kDa) is the relevant binding protein of CyP22 that displays ADP and/or Ca$^{2+}$ binding is unknown. It would be tempting to designate the ADP/ Ca$^{2+}$ sensitive characteristic of photolabelling to this factor. Since CyP22 was prone to proteolytic damage in photolabelling studies, the possibility that the 7kDa was part of a larger protein that had been partially degraded was also considered. With respect to the pore model proposed by Halestrap [Halestrap, (1994)] in which cyclophilin was postulated to interact with the translocase, and the fact that the
translocase was ADP and Ca\(^{2+}\) sensitive [Novgordov et al, (1992); Brustovetsky and Klingenberg, (1996)], the possibility that the low molecular weight component might be a proteolytic fragment of the translocase was considered. The 7kDa component might also represent proteolytic fragments of the matrix facing ADP and Ca\(^{2+}\) binding sites involved in pore activity that, during purification of labelled CyP22, remain attached to the PPIase.

The interaction of cyclophilins with other proteins is not an unknown feature of the proteins (see introduction, section 1.10.2). One of the more interesting roles of cyclophilins that may be relevant to this study is the involvement of PPIase activity in the modulation of Ca\(^{2+}\) release from the SR of muscle cells. FKBP12 is a small protein (12kDa in size) that binds tightly to each of the ryanodine receptors and controls the release of Ca\(^{2+}\) into the cytoplasm [Timerman et al, (1993)]. The protein displays PPIase activity which suggests that Ca\(^{2+}\) release was dependent upon a configuration change (\textit{cis} to \textit{trans} isomerisation) in the receptors. Other cases in which channel activity has been shown to involve PPIase activity have also been reported [Heleker et al, (1994), Bram and Crabtree, (1994)]. Therefore, on the assumption that it too is intimately involved in opening and closing of the membrane pore, a similar role for CyPD was also conceivable. By binding to the pore complex, the PPIase activity of the protein activates the opening of the pore. CsA, by blocking the activity, prevents interaction of the protein with the pore complex and as a result closes the pore. As mentioned earlier, by modulating the interaction of CsA with the PPIase, ADP and Ca\(^{2+}\) effect pore activity in opposite and reverse manners.


There is considerable evidence that implicates the involvement of the ADP translocase in pore activity [Le Quoc and Le Quoc, (1988); Halestrap and Davidson, (1990); Macedo et al, (1993)]. Some recent reports go so far as to say that the translocase is the channel forming protein of the pore structure [Brustovetsky and Klingenberg, (1996)]. A model that accounted for the involvement of the translocase in pore activity was proposed by Halestraps’ group
[Halestrap and Davidson, (1990); Halestrap, (1994)]. The hypothetical model suggested a direct interaction between the PPIase and the ADP translocase in the presence of high [Ca\(^{2+}\)] which occurred when the translocase was in the e-configuration. In this configuration specific proline residues that become exposed on the translocase were proposed to act as sites for PPIase interaction. The interaction of the PPIase with these sites was suggested to catalyse a conformational change that converted the translocase from a specific transporter into a non-specific pore-like structure. [Griffiths and Halestrap, (1991)]. The participation of the translocase in pore formation was further supported by findings that the Ca\(^{2+}\)-dependent decrease in translocase activity was alleviated and prevented by CsA. Since CsA can inhibit and reverse pore opening and its target protein is a cyclophilin, the model involving the interaction of the two proteins (i.e., cyclophilin with translocase) was quite favourable [Macedo et al, (1993)].

As proposed earlier the PPIase was likely to play a regulatory role in pore activity. It was not thought to be directly responsible for pore opening but (by catalysing a cis to trans isomerisation reaction in a protein) probably sensitised the pore to form an open configuration more easily. If this was correct, then by binding to its target protein, Ca\(^{2+}\) would minimise CyPD/ CsA interaction and allow CyPD dependent pore activation (i.e. more CyPD available to catalyse pore sensitization). In contrast, ADP (by binding to its target protein) would potentiate CyPD/ CsA interaction and minimise the amount of CyPD available for pore activation.

Whether ADP and Ca\(^{2+}\) modulate pore activity by modifying CyPD interaction with the translocase is unknown. However, with regard to its involvement in pore activity, recent studies clearly demonstrate the ability of the translocase to form large membrane channels. Reconstitution studies (involving patch-clamp techniques) of the translocase by Brustovetsky and Klingenberg [Brustovetsky and Klingenberg, (1996)] have shown the translocase to be reversibly converted into a large channel. Conductivity measurements of the channel have shown it to be Ca\(^{2+}\), pH, carboxyatractylate, bongkrekate and ADP sensitive. Interestingly the same effectors have also been shown to affect the activity of the pore (i.e. Ca\(^{2+}\) [Hunter and Haworth, (1979); Crompton et al, (1988)], low pH [Petronilli et al, (1993)], inhibition by
bongkrekic acid, ADP, and activation by carboxyatractylate [Hunter and Haworth, (1979); LeQuoc and LeQuoc, (1988)]. Considering these and their own findings, Brustovetsky and Klingenberg proposed that the key component forming the pore was the adenine nucleotide translocase (i.e. the translocase is the pore forming unit). These workers were unable to demonstrate any CsA sensitivity of the channel and suggested that this was expected, since in their reconstituted system, cyclophilin was absent. These studies provide strong evidence to support the suggestion that the translocase plays a crucial role in pore function.

Since CsA is clearly able to reverse and prevent pore opening in mitochondria, the role of cyclophilin in pore opening, (whether it involves the translocase or some other protein) becomes one that is vitally important.

Some studies rule out the involvement of the translocase in pore function. Work by Lohret et al [Lohret et al, (1996)] demonstrated the existence of a multiconductance channel from translocase-deficient yeast mutant in giant liposomes reconstituted with mitochondrial inner membrane. Although these findings are in conflict with those of Brustovetsky and Klingenberg [Brustovetsky and Klingenberg, (1996)] they indicate that proteins, besides the translocase, may be able to form channel-like structures.

[4.6] CyPD is found in the matrix and inner membrane fraction of mitochondria.

As suggested in the previous section the hypothetical model proposed by Halestrap suggests that CyP18 interacts with the translocase to cause pore activation. This model was based on the fact that CyP18 was isolated from the matrix fraction of mitochondria. However, work in our laboratory localised CyP18 to the IMS/OM fraction of mitochondria [Tanveer et al, (1996)]. CyPD (CyP22) was found in the matrix and in association with the inner membrane fraction of mitochondria. Using low concentrations of digitonin our group separated and purified the two cyclophilins and, based on the distribution of marker enzymes, assigned them to the different subfractions of mitochondria [Tanveer et al, (1996)]. However, since the two proteins (CyPD/CyP22 and CyP18) are almost certain to be of a single origin, the model proposed by Halestraps' group could be partially correct if CyP18 was replaced by
the larger protein, CyPD. As mentioned earlier Cyp18 might be a processed component of CyPD that somehow becomes localised to the IMS/OM fraction of mitochondria.

[4.7] **Proteins involved in pore activity.**

Although the involvement of CyPD (CyP22) in modifying the activity of the pore is undisputed, its interaction with the translocase is not so clear. According to the hypothetical model proposed by McGuinness et al, [McGuinness et al, (1990)], if the translocase was involved in pore activity then only 0.5 - 1.5% of the total protein was required to interact with CyPD and cause pore opening. This raises an awkward question. The ADP translocase is the most abundant protein of the inner membrane (150 - 300pmole/ mg of mitochondrial protein) [Klingenberg, (1976)]. If the calculations in the model were correct, then why would only a small and specific amount of translocase be capable of forming a channel or binding CyPD to cause pore opening? Interestingly, the model also proposed that between 30-50% of the total CyPD was required to interact with the pore complex to induce pore opening. Again, why would only some of the PPIase be capable of causing pore activation?

A possible answer to the previous questions might involve the participation of the mitochondrial benzodiazepine receptor (mBzR) [McEnery et al, (1992); Kinally et al, (1992)]. In terms of size and conductance properties the receptor complex displays characteristics similar to those exhibited by the megachannel and the membrane pore. The receptor is composed of two molecules of porin, two molecules of translocase and two molecules of an 18kDa protein that binds isoquinoline carboxamide ligands. Earlier studies had postulated the involvement of porin in the membrane pore. However, based on its distribution (in the OM), it was considered less likely to contribute to the activity of the pore [Haworth and Hunter, (1980); Le Quoc and Le Quoc, (1985); McGuinness et al, (1990)]. Nevertheless, considering recent work, the involvement of porin in pore activity seems much more likely [McEnery et al, (1992); Kinally et al, (1992)]. Since porin and the 18kDa protein are on the outer membrane and the adenine nucleotide translocase on the inner membrane, the whole receptor complex was considered to be associated with contact sites (i.e., regions where the outer and inner membranes come together) [Crompton and Andreeva, (1993)]. The 18kDa protein of benzodiazepine receptor is present at around 3pmol/ mg of mitochondrial protein [Awad and
Gavish, (1987)]. Considering this, a limited amount (0.5 - 1% of total) of translocase (in the c-conformation) and porin would be capable of binding to the receptor. This may explain why only a small and specific amount of translocase and CyPD are needed to participate in pore activity. Therefore, it is conceivable that only the translocase associated with the mBzR is capable of binding a single cyclophilin molecule and contributing to the formation of the pore complex. Since the amount of translocase associated with mBzR is limited, then only a limited amount (30-50 %) of CyPD could interact with the translocase. If indeed the mBzR complex is the pore complex, the porin molecules are likely to be the channel-forming components of the structure whilst the translocase molecules might function to regulate their activity. In turn, two molecules of CyPD binding to the translocase might modify the regulatory feature of the protein (cis to trans isomerisation of the translocase, possibly to the c-configuration, causing activation of the porin molecules).

Other proteins are also believed to participate in pore function in a regulatory capacity. The elucidation of components involved in forming the membrane pore was further complicated by the findings of Beutner et al, [Beutner et al, (1996)]. Besides involvement of the proteins forming the mBzR (i.e., porin, translocase, 18kDa protein) the interaction of hexokinase and creatine kinase was also implicated. Electrophysiological and transport studies of reconstituted mitochondrial hexokinase and creatine kinase complexes showed characteristics similar to the membrane pore. Complexes of hexokinase/ creatine kinase/ porin/ adenine nucleotide translocase added to artificial phospholipid membranes demonstrated conductance changes similar to those displayed by the MMC. The reconstitution of the hexokinase complex into asolectin/ cholesterol vesicles showed the transport of ATP or malate across the vesicular membrane. Transport of ATP and malate was affected by Ca^{2+} and N-methyl-Val-4-cyclosporin whereby Ca^{2+} potentiated their release and the CsA derivative inhibited it [Beutner et al, (1996)]. This study showed that the hexokinase and the creatine kinase complexes in mitochondria could form pore-like structures, probably at contact sites as the two enzymes are abundant here. From here they could regulate the conductance and transport of molecules across the two membranes. Even more interesting is that the hexokinase complex was sensitive to Ca^{2+}, CsA, atractyloside - ligands that have been shown to adversely affect the activity of the membrane pore. Evidence from this study suggests that
the Ca\textsuperscript{2+} activated membrane pore is likely to be composed of porin and adenine nucleotide translocase. The activity of this complex might be regulated by the two kinases found attached to it. Interestingly, because the reconstituted hexokinase/porin/translocase complex displayed N-methyl Val-4-cyclosporin-sensitivity, CyPD was tightly associated with the pore-forming complex. Again, this supports the fundamental role of the cyclophilin in pore activation.

These studies suggest the membrane pore is unlikely to be a simple structure responsible for inducing mitochondrial permeability changes. Rather, it is likely to be a complex system of different proteins that together form a pore-like structure that can be effected by different conditions (e.g. oxidative stress, high [Ca\textsuperscript{2+}], CsA, ADP etc.).

[4.8] **The association of CyPD with the membrane pore.**

As mentioned earlier subfractionation of mitochondria (using low concentrations of digitonin) showed CyPD to be found in the matrix compartment and associated with the inner membrane of mitochondria. It was assumed that the fraction electrostatically associated with the matrix facing side of the inner membrane was involved in pore activation [Tanveer et al., (1996)]. Similarly, electrophysiological studies of excised inner membrane patches also indicated the involvement of membrane associated protein with pore flickering (flickering indicated pore activation). Changes in membrane conductivity were stimulated by Ca\textsuperscript{2+} and depressed by CsA. This led to the hypotheses that only the membrane associated PPIase was capable of catalysing a step in pore structure that predisposed the pore to form an open structure. The unassociated PPIase was incapable of pore activation. Along with the effects of Ca\textsuperscript{2+} and CsA on mitochondrial permeabilisation, membrane conductivity (flickering of the patches) was also affected by other pore modulators (i.e., ADP, a low pH and Mg\textsuperscript{2+}) [Szabo and Zoratti, (1991); Szabo and Zoratti, (1992)]. Since CyPD recruitment to and interaction with the membrane pore appears to be a prerequisite for pore activation, the effects of these pore ligands suggested the involvement of other pore components that presumably modified the association/dissociation of CyPD with the pore. Ligands that potentiated CyPD association with the pore presumably caused pore opening whilst those that prevented association or stimulated CyPD dissociation caused pore closure.
The effect of pore ligands on the association and dissociation of CyPD with the membrane.

Virtually every model proposed to explain pore activation predicts that cyclophilin, on binding to the pore complex, catalyses a reaction that leads to pore opening [Halestrap and Davidson, (1990); McGuinness et al, (1990); Halestrap, (1994)]. As a result, the association of CyPD with the pore complex was an essential feature of pore activity. Photolabelling studies in intact mitochondria in the presence of ADP and Ca\(^{2+}\) supported this feature, i.e. in the presence of high [Ca\(^{2+}\)] all pore sites had CyPD bound to them which meant that less was available for interaction with the photolabel, this resulted in decreased photolabelling. In the presence of ADP, CyPD association with the pore sites was prevented which meant that more of the cyclophilin was available to interact with the photolabel, this resulted in increased photolabelling. These studies also indicated the involvement of component(s) (i.e., ADP and/or Ca\(^{2+}\) binding protein) that after binding CyPD, modulates its capacity to interact with the pore. Besides ADP and Ca\(^{2+}\), other pore effectors have also been shown to influence CyPD association with mitochondrial membranes [Connern and Halestrap, (1994); Connern and Halestrap, (1996); Nichollii et al, (1996)].

The association and dissociation of CyPD from the inner membranes of mitochondria was examined under different conditions (fig. 57). The effect of pyridine nucleotides and tBuOOH on the association/dissociation of CyPD with the inner membrane of mitochondria revealed something rather confusing. On each occasion when Ca\(^{2+}\) was present (irrespective of whether or not other pore effectors were present), the amount of PPIase activity measured in the supernatant (dissociated) was greater than in the control (where KCl replaced CaCl\(_2\) to maintain ionic balance). This suggested that, rather than potentiating, Ca\(^{2+}\) depressed the migration of CyPD to the membrane. Other pore effectors, (i.e., pyridine nucleotides, tBOOH, and phosphate) (fig. 58) failed to elicit any effects on the association or dissociation of the PPIase with the inner membrane. This suggested that rather then involving the direct migration of CyPD to the membranes, their mechanism of modifying pore activity involved some other pathway(s).
According to the pore model [Halestrap, (1994)], if association of the PPIase with the membrane pore was a prerequisite for pore activation, the pyridine nucleotides should have potentiated the dissociation of CyPD from the membrane. However, under the experimental conditions used, the pyridine nucleotides failed to demonstrate any change in the distribution of PPIase activity between the inner membrane and supernatant fractions of mitochondria (fig. 58). This suggested that the redox potential (i.e. \([\text{NADH}] / [\text{NAD}^+]\) did not play a direct role in influencing the association and dissociation of CyPD with the inner membrane. The redox potential maintains the pools of NADH and NADPH that are needed to preserve the levels of glutathione. Glutathione protects cells against the deleterious effects of oxidant stress by functioning as an antioxidant [Beatrice et al, (1984); Bellomo et al, (1984); Traber et al, (1992); Richter et al, (1992)]. Reduced pyridine nucleotides prevent the activation of the pore by preventing the formation of components that constitute oxidative stress. Oxidative stress (e.g. tBOOH) has been loosely shown to potentiate the migration of cyclophilin to the membrane [Connem and Halestrap, (1994)]. However the association/dissociation studies conducted in the presence of tBuOOH failed to elicit any change in the distribution of CyPD between the inner membrane and matrix fraction (fig. 57).

Other possible ways in which reduced pyridine nucleotides might prevent opening of the pore is by protecting against the formation of dysfunctional proteins. Loss or suppressed levels of reduced pyridine nucleotides (i.e. oxidative stress) lead to intra and intermolecular S-S crosslinkages of proteins. These modifications severely affect the activity and function of proteins. Such detrimental changes in some membrane proteins of mitochondria might lead to the formation of the non-specific pore.

Rather than directly measuring the effects of different pore ligands on the association/dissociation of CyPD with the inner membrane, Halestraps’ group used an immunological approach to investigate CyP binding [Connem and Halestrap, (1996)]. Employing western blot techniques (using N-terminal anti-peptide antibodies against mitochondrial CyP18) they showed that CyP18 binding to mitochondrial membranes was affected by two factors: chaotrophic agents (e.g. KSCN, guanidinium chloride) and an increased mitochondrial matrix volume (caused by decreasing osmolarity, 330 to 180mosM) caused an increase in the amount
of CyP bound to the membranes. Interestingly, the $\text{Ca}^{2+}$ sensitivity of the pore was also increased by these factors so that a lower $[\text{Ca}^{2+}]$ was needed to activate the pore. Chaotrophic agents stabilise hydrophobic groups in an aqueous environment. In the presence of chaotrophic agents it was suggested that hydrophobic groups (not usually accessible to cyclophilin) on the CyP target protein became exposed. These groups then allowed CyP to interact with its target protein (i.e. pore complex) and, as a result, CyP binding to the complex was enhanced [Connern and Halestrap, (1996)]. Other factors that modulate the $\text{Ca}^{2+}$ sensitivity of the pore (i.e., ADP, bongkrekic acid, membrane potential, carboxyatractylate) did not affect CyP binding to the membrane [Connern and Halestrap, (1994)]. Also, in contrast to the findings of Nicolli et al [Nicolli et al, (1996)], Halestrap’s group failed to detect any decrease in CyP binding to the membranes in the presence of ADP, bongkrekic acid, carboxyatractylate and a low pH, factors which all considerably affect pore activity. These findings indicated that regulatory mechanisms other than ones involving CyP binding to membranes, may be involved in modulating the $\text{Ca}^{2+}$ sensitivity of the pore [Connern and Halestrap, (1996)]. In respect to the pore model proposed by Halestrap, these agents, by binding to the translocase, might somehow bypass the need for ‘cyclophilin-dependent activation’ of the translocase. Halestrap’s group also proposed that intramitochondrial NADH might exert its inhibitory effect on pore opening by preventing CyP binding to the pore forming complex. However, in addition to work presented here, there is strong evidence that oxidation of matrix NADH/ NAD$^+$ (i.e., a decreased redox potential) rather than NADH directly can sensitise pore opening to $[\text{Ca}^{2+}]$ [Hunter and Haworth, (1979)].

[4.10] Cyclophilin and the membrane pore in SMPs

Similar experiments to those mentioned in the previous section were conducted by Cromptons’ group to study cyclophilin interaction with mitochondrial membranes [Crompton et al, (1992)]. Rather then using specific antibodies raised against CyP18 to detect association of the protein to the membranes, the effects of CyP18 on the permeability properties of SMPs (i.e., membrane potential, respiration) were examined. Interestingly, although CyP18 interacted with the respiratory chain in a $\text{Ca}^{2+}$ dependent manner (i.e., CyP18 increased the
rate of respiration in the presence of high [Ca\(^{2+}\)], it did not interact with the membrane pore. The fact that no pore activation (i.e., dissipation of membrane potential) was detected in the presence or absence of Ca\(^{2+}\) suggested that CyP18 interaction with SMPs was insufficient to activate the pore. This meant that other pore components, presumably lost on SMP preparation, were necessary for pore induction.

Similar experiments in which antibodies against CyPM were used to investigate pore activity were conducted with SMPs [Nicolli et al, (1996)]. These workers were unable to provide clear evidence that the membrane pore was functional in SMPs. Although liposomes reconstituted with SMP proteins displayed sucrose permeability, this was unaffected by Ca\(^{2+}\), CsA or EGTA. Similarly, membrane potential measurements of SMPs also failed to show operation of the Ca\(^{2+}\) sensitive pore. Although the membrane potential in SMPs was decreased (possible indication of pore activation) by Ca\(^{2+}\) and phenylarsine oxide (oxidant stress), the prevention of depolarisation was CsA insensitive [Nicolli et al, (1996)]. This agreed with the earlier finding of Crompton et al, (1992) in which CyP18 was found to be insufficient at inducing pore opening in SMPs.


\[^{3}\text{H}]\text{CsA} binding (LH-20) and photolabelling studies have indicated that CyPD interacts with other mitochondrial protein(s). Photolabelling studies demonstrated a complex of CyPD and some low molecular weight protein (fig 14a and 14b). LH-20 analysis suggested that another protein, after binding either ADP or Ca\(^{2+}\), modulated the \[^{3}\text{H}]\text{CsA} binding capacity of the PPIase. These techniques, although suggesting the involvement of other components, were not the most suitable to investigate and isolate CyPD binding components. Therefore, in an attempt to isolate CyPD binding components (either the ADP/ Ca\(^{2+}\) sensitive binding partner(s) or other components of the pore), the use of CyPD affinity chromatography was explored. Other workers [Friedman and Weissman, (1991)] have successfully used CyP affinity chromatography to isolate and purify cyclophilin binding proteins from cellular lysates. A CyPD affinity column was used on the basis that the CsA binding/ PPIase site was intimately involved in pore activity. The protein(s) responsible for ADP/ Ca\(^{2+}\) sensitive
photolabelling of CyPD (i.e., the ADP and/or Ca$^{2+}$ binding partners of the PPIase) would presumably influence pore activity by effecting the *cis-trans* isomerase activity of the PPIase in vivo. Therefore, by immobilising CyPD to an inert matrix and passing mitochondrial extracts over it, proteins that either bind CyPD via the catalytic site or influence the PPIase activity and CsA binding capacity feature of the cyclophilin, are retained.

CyPD was attached to a Sepharose matrix using a lysine specific crosslinker (fig. 6). From the protein sequence (fig. 46B *italics*) six lysine residues were potentially available for attachment. Modification at any of these residues might have resulted in the loss of PPIase activity and hence prevented CyPD/protein interaction. However, modification of CyPD at a lysine residue(s) showed a small loss in PPIase activity indicating that most of the active sites were intact and available for protein/protein interaction (figs 50 and 51). It was unknown whether lysine modification of CyPD would affect the binding capacity of ADP and/or Ca$^{2+}$ sensitive binding partners to the cyclophilin.

No detectable CyPD-binding proteins were retained by the affinity column (fig. 55). A number of possible causes might have obstructed CyPD/target protein interactions. Although modification of lysine residues did not considerably affect the PPIase activity of CyPD, modification of certain lysine residues on the surface of the protein (those involved in protein-protein interaction) may have prevented the PPIase from interacting with relevant binding proteins. Another reason to explain the lack of specifically interacting components retained by the affinity column might be that the Ca$^{2+}$ sensitive CyPD binding partner was completely sequestered. Since the Ca$^{2+}$ and/or ADP sensitive partner is likely to be a component of the membrane pore then, assuming that the pore is 70-50% less abundant than cyclophilin in mitochondria [McGuinness et al., (1990)], under the conditions chosen to extract membranes (i.e., high Ca$^{2+}$), all the relevant Ca$^{2+}$ sensitive binding protein may already be tightly associated with the extracted PPIase of the mitochondrial extract, so that none, or very little, would be available for interaction with immobilised CyPD.
Further studies.

Since CyPD can associate with the inner membrane under different conditions, this suggests that the protein interacts with some membrane component of the pore. Experiments could be conducted to resolve CyPD interacting proteins on the membrane. Radioiodinated CyPD ($^{125}$I-CyPD) could be returned to CyPD depleted SMPs under conditions that favour association with the pore. Once these conditions are clarified the effects of pore ligands (e.g. Ca$^{2+}$, ADP, phosphate etc.) on CyPD association/ dissociation with the pore can be investigated. Interacting components could then be isolated using a bifunctional crosslinking reagent that would covalently link CyPD to any component in the close vicinity (depending on the length of crosslinker). Crosslinked products would be analysed on SDS-PAGE gels. Alternatively, CyPD could be pre-reacted with a photoreactive heterobifunctional crosslinker and introduced to CyPD-free SMPs. Photoactivation of the light sensitive group of the molecule would crosslink CyPD with its relevant target protein.

An immunological approach could be used to identify CyPD interacting proteins. Polyclonal antibodies raised against CyPD would be purified on a CyPD affinity column. Mitochondria would be exposed to conditions known to affect pore activity. Proteins extracted under the same conditions would then be passed through a protein A-antibody affinity column. CyPD would be specifically retained by the column and components that interact with it (i.e. Ca$^{2+}$ and/or ADP sensitive target proteins and/or membrane target proteins that the PPIase binds through its CsA binding site) would also co-immunoprecipitate. Taken further, the effects of any co-immunoprecipitants on the CsA binding capacity of the PPIase could then be investigated.

Besides using CyPD antibodies to isolate target proteins, they could also be used to explore the role of the PPIase in mitochondrial energy transduction. Since CyP18 interacts with the respiratory chain in a Ca$^{2+}$ dependent manner (CyP18 increases the rate of respiration in the presence of Ca$^{2+}$) [Crompton et al., (1992)], and CyPD is probably identical to CyP18, it would be interesting to look at the effects of anti CyPD antibodies on mitochondrial respiration.


209


211


214


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219


