A MITOCHONDRIAL CYCLOPHILIN

A thesis submitted for the degree of

Doctor of Philosophy

by

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Cheers guys.

For Mohammed Abbas and Zahra.
ABSTRACT

Cyclophilins (CyPs) are a family of proteins which catalyse the *cis/trans* isomerisation of prolyl-peptide bonds in proteins. The activity is blocked by the immunosuppressant drug Cyclosporin A. Cellular CyPs have been implicated in the refolding and trafficking of proteins and in Ca$^{2+}$ signalling pathways but their precise roles have yet to be elucidated. Mitochondrial CyP is of potential clinical significance as it may control the activity of a mitochondrial inner membrane pore relevant to certain forms of cell injury brought about by Ca$^{2+}$ and oxidant stress. In the present study, purification of PPIase from the matrix and intermembrane space fractions of rat liver mitochondria yielded a 21kDa CyP (CyP21) and an 18kDa CyP respectively on SDS-PAGE. Their locations were confirmed by mitochondrial marker enzyme analyses. The peptide sequence of CyP21 was determined and used to design degenerate primers; the aim was to generate a probe by PCR to screen a cDNA library for the full length cDNA of CyP21. However, degenerate primers were unsuccessful. Accordingly, the published human mitochondrial CyP base sequence was used to design non-degenerate primers. A fragment of DNA was obtained, inserted into the plasmid vector pCR-Script, and cloned in *E.coli*. Dideoxy sequencing confirmed it was from the correct protein and so it was radiolabelled and used to screen a rat liver cDNA library in bacteriophage λ. A positive phage clone was obtained, inserted into the plasmid vector pBluescript, cloned into *E.coli* and sequenced to confirm its identity. The cloned cDNA was then used to construct a CyP-GST fusion protein using a pGEX plasmid vector. The fusion protein was expressed and used to prepare a CyP affinity matrix. The binding of mitochondrial constituents under various conditions relevant to activation of the mitochondrial pore was investigated. A mitochondrial inner membrane protein of approximately 32kDa molecular weight was found to bind to the CyPD part of the fusion protein in a manner insensitive to traditional pore effectors. The possible nature of this binding protein is discussed.
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<th>Definition</th>
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<tr>
<td>PPIase</td>
<td>peptidyl prolyl <em>cis-trans</em> isomerase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methyl sulfonyl fluoride</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl thiogalactoside</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiothreitol</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N’, N’-tetramethylethylene-diamine</td>
</tr>
<tr>
<td>CyP</td>
<td>cyclophilin</td>
</tr>
<tr>
<td>MMC</td>
<td>mitochondrial megachannel</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>AdNT</td>
<td>adenine nucleotide translocase</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>PT</td>
<td>permeability transition</td>
</tr>
<tr>
<td>VDAC</td>
<td>voltage dependent anion channel</td>
</tr>
<tr>
<td>DHase</td>
<td>dehydrogenase</td>
</tr>
<tr>
<td>SM</td>
<td>sarcolemmal membrane</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>IIME</td>
<td>inner mitochondrial membrane extract</td>
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CHAPTER 1: INTRODUCTION

[1.1] Background

In oxidative phosphorylation, the transfer of electrons from a substrate to oxygen pumps protons out across the mitochondrial inner membrane. On their return, they drive the synthesis of ATP. This study relates to a pore structure in the inner membrane. Under normal physiological conditions it is closed, allowing oxidative phosphorylation to proceed. There is evidence however, that under certain conditions, in particular high intracellular calcium and oxidant stress, that the pore is open. Under these conditions ATP would be hydrolysed rather than synthesised. These conditions are characteristic of myocardial reperfusion injury, which can lead to cell death. The pore structure has not yet been isolated. The inhibition of pore activation by the immunosuppressant cyclosporin A (CsA) led to the proposal of a CsA binding protein, probably a member of the cyclophilin family with a mitochondrial origin, controlling pore activity. The following chapter discusses the history of the permeability transition pore, provides an outline of its characteristics and effectors, and gives a brief discussion of how abnormal cellular calcium fluxes affect mitochondrial calcium levels which in turn affect pore activity. The resulting consequences are outlined. Finally, evidence is presented for the involvement of cyclophilin in pore activity.
[1.2] **History of the Permeability Transition**

In the 1960s, groups including Lehninger and co-workers (Lehninger *et al* [1967]) showed that Ca\(^{2+}\) and inorganic phosphate (Pi) induce permanent uncoupling of respiration due to interference of the permeability properties of the inner mitochondrial membrane (IMM). Adenine nucleotides were shown to have a protective effect. Later work demonstrated how the redox state of pyridine nucleotides affected Ca\(^{2+}\) passage across the inner membrane (Lehninger *et al* [1978]). Agents that promoted the oxidised states (e.g. oxaloacetate) induce the release of Ca\(^{2+}\) accumulated in the matrix of Ca\(^{2+}\)-overloaded mitochondria and promoted mitochondrial uncoupling; on the other hand, reducing agents (e.g. β-hydroxybutyrate) allow mitochondria to accumulate large amounts of Ca\(^{2+}\) without uncoupling energy transduction.

The hypothesis that permeability properties of the IMM were affected due to the presence of a transmembrane channel or pore that was activated by Ca\(^{2+}\) was proposed in the late 1970s by Haworth & Hunter [1979]. They demonstrated that molecules of less than 1000 molecular weight became able to pass through the membrane in the presence of high Ca\(^{2+}\), irrespective of their charge. They later identified NADH as an inhibitor of pore activity in a manner similar to ADP (Haworth & Hunter [1980]).

The permeability features of the IMM were further studied by Crompton and co-workers with a novel \(^{14}\)C-sucrose entrapment technique in the mid-1980s (Al-Nasser & Crompton [1986]). Their findings supported the earlier hypothesis of the presence of a Ca\(^{2+}\)-induced inner membrane pore affecting permeability of the IMM. This group went on to study the kinetics of pore activity by developing a
rapid pulse flow solute entrapment technique (Crompton & Costi [1988]). Relative permeabilities of the compounds investigated indicated permeation via a pore of around 2nm diameter (Crompton & Costi [1990]).

In 1987, Fournier et al [1987] reported that cyclosporin A (CsA), a potent immunosuppressant extensively used in transplant surgery, inhibited Ca\(^{2+}\)-induced uncoupling of mitochondrial respiration and promoted Ca\(^{2+}\) retention. They attributed this to CsA blocking Ca\(^{2+}\) efflux and so inducing mitochondria to accumulate large amounts of calcium. Crompton et al [1988] demonstrated that these effects reflected the ability of CsA to block the Ca\(^{2+}\)-activated inner membrane pore in nanomolar concentrations.

[1.3] **The Permeability Transition Pore**

Ca\(^{2+}\) alone is sufficient to induce the activation of the permeability transition (PT) pore in at least liver, heart, kidney and adrenal cortex mitochondria (e.g. Haworth & Hunter [1979], Pfeiffer et al [1976], Chavez & Jay [1987]) although the presence of inducers markedly accelerates activation. Matrix Ca\(^{2+}\) has been established as the relevant parameter using the Ca\(^{2+}\) uniporter inhibitor Ruthenium Red (RR). If the inhibitor is added before Ca\(^{2+}\) loading, pore activation is blocked. Some authors have reported that mitochondrial populations show heterogeneous characteristics with respect to the permeabilisation of the inner membrane associated with pore activation. The lag time before onset, as well as the rate and final extent of pore opening is dependant on the amount of Ca\(^{2+}\) taken up (Vercesi et al [1988]). This apparent heterogeneity of mitochondria, with respect to pore activity, has been attributed to variations in their content of pore effectors.
However, since all pores open simultaneously in any single mitochondrion (permeabilization) and mitochondria ‘permeabilise’ according to a single, first order process, it is clear that there is no heterogeneity, and that the phenomenon represents the fact that the rate of permeabilization is determined simply by the fraction of unpermeabilized mitochondria at any point in time (Crompton & Costi [1988]).

[1.3.1] Inducers

Inorganic Phosphate (Pi)

Inorganic phosphate potentiates the effect of Ca$^{2+}$ on pore activity (Al Nasser & Crompton [1986]). Pi$^{-}$ enters the mitochondria by symport with H$^{+}$; this coentry of Pi$^{-}$-H$^{+}$ with Ca$^{2+}$ prevents the alkalinization of the matrix that otherwise occurs on Ca$^{2+}$ loading of mitochondria, allowing large amounts of Ca$^{2+}$ to enter the matrix (e.g. Vasington & Murphy [1962]). However, this simple explanation does not account for Pi as a PT promoter as lipid-soluble acids (e.g. acetate, Petronilli et al [1993]) are also taken up with Ca$^{2+}$ and increase Ca$^{2+}$ uptake, but they are not as effective inducers.

Another explanation offered is that Pi reduces the matrix ADP concentration, thereby inducing the PT. An increase in Pi would increase the ATP/ADP ratio (Kunz et al [1981]), resulting in an ease of pore opening (ADP>ATP as pore inhibitor, see later). However, this hypothesis is confused by observations with arsenate (As$^{3+}$). It is as good an inducer as Pi (e.g. Hunter & Ford [1955]), and yet it cannot be expected to effect adenine nucleotide (AdN) ratios. Recent evidence has been presented that a high external Pi concentration depletes matrix AdNs via
the Pi/ATP-Mg\(^{2+}\) or Pi/HADP\(^{2-}\) antiporter (Joyal & Aprille [1992], Aprille [1993]).

Other inducers (this list is by no means exhaustive)

**Oxidising agents** - Oxidisers of the pyridine nucleotides (PyNus) (e.g. oxaloacetate, acetoacetate) induce the PT (e.g. Palmer & Pfeiffer [1981]). Hydroperoxides oxidise reduced glutathione (GSH) and PyNus via enzymatic processes involving GSH peroxidase and reductase (Belloma [1984]). Thus, pore activation by hydroperoxides might reflect decreased matrix NADH and consequent deinhibition (see 1.2). Alternatively, free radicals may be involved in PT induction by hydroperoxides and thiol reagents as demonstrated by the protective effect of BHT (Novgoradov et al [1987], Carbonera & Azzone [1988]) and catalase (Valle et al [1993]), and by spin-trapping experiments (Kennedy et al [1986]).

**Thiol reagents** - These include heavy and transition metals and their complexes such as Hg\(^{2+}\) and mercurials (e.g. Hunter et al [1956]), probably via formation of coordination complexes with two -SH groups. Cross-linkers and disulphide bridge formers (e.g. diamide) have also been reported to have an inductive effect (e.g. Palmer & Pfeiffer [1981], Novgorodov et al [1987]).

**Fatty acids** - Fatty acids uncouple mitochondria by diffusing into the matrix as the protonated electroneutral form, and exiting as anions via the action of the adenine nucleotide translocase (AdNT) (Skulachev [1991]). The inducing effect may be due to the resulting decrease in transmembrane potential, although evidence (Petronilli et al [1993]) points to direct interaction with the pore as addition of fatty acids causes an upward shift of the curve relating permeabilization rate to transmembrane potential.
[1.3.2] *Inhibitors* (this list is by no means exhaustive)

Many inhibitors of the permeability transition act by preventing or counteracting an inductive effect. Since $\text{Ca}^{2+}$ is the primary activator of the pore, any intervention that prevents $\text{Ca}^{2+}$ accumulation also prevents pore activation, e.g. respiratory inhibitors, anaerobiosis, uncouplers.

The inhibitory effects of adenine nucleotides on the PT are well documented, with ADP being the principle, some say only, relevant nucleotide. It appears the affinity of $\text{Ca}^{2+}$ for PT activating sites is decreased by ADP (Novgorodov *et al* [1992]). Nanomolar concentrations of ADP increase the rate of EGTA-induced rescaling of permeabilised mitochondria (Crompton & Costi [1988]) and it has been suggested that ADP may facilitate, in some way, the interconversion of pores between the open and closed states (Crompton & Costi [1990]).

**Cyclosporin A (CsA)** - Following the finding that cyclosporin A (CsA), a cyclic undecapeptide, promoted mitochondrial $\text{Ca}^{2+}$ retention (Fournier *et al* [1987]) a number of groups (Crompton *et al* [1988], Broekemeier & Pfeiffer [1989], Broekemeier *et al* [1989], Novgorodov *et al* [1990]) demonstrated that CsA inhibited the PT at sub-$\mu$M concentrations. Subsequent patch clamp studies (Szabo *et al* [1992]) revealed that CsA inhibits the pore in a $\text{Ca}^{2+}$-competitive manner, i.e. increasing the $\text{Ca}^{2+}$ concentration overcomes the CsA inhibition. This correlates with earlier binding studies showing competition between $\text{Ca}^{2+}$ and $^3\text{H}$-CsA in mitochondria (McGuinness *et al* [1990]). AdNs have a synergic inhibitory effect with CsA.
The target molecule of CsA might be a protein(s) belonging to the ‘cyclophilin’ family. These ubiquitous cis-trans peptidyl-prolyl isomerases (PPIases) are present in all eukaryotic cells and most bacteria (Ryffel [1980], Handschumacher et al [1984]), and also in the mitochondria of Neurospora crassa (Tropschug [1988]), yeast (McLaughlin et al [1992], Davis [1992]), and liver and heart (e.g. Bergsma et al [1991]). It seems logical to consider that the inhibitory effect of CsA on the PT might be mediated via a mitochondrial cyclophilin. This concept is supported by observations that the relative potencies of CsA derivatives as inhibitors of mitochondrial PPIase are paralleled by their potencies as inhibitors of the pore (Petronilli et al [1994], Griffiths & Halestrap [1991]).

McGuinness et al [1990] showed CsA binds to two mitochondrial components, one (component I) with relatively low capacity and high affinity and the other (component II) with relatively high capacity and low affinity. Pore inhibition correlated with CsA-binding to component I which was identified as a CsA-sensitive PPIase (and, presumably, a cyclophilin). In addition, Ca\(^{2+}\) decreased CsA binding to component I, further indicating an involvement of the PPIase. The same study showed that CsA not only prevented pore opening, but also closed pre-opened pores. This suggests that CsA blockade of the pore cannot simply be due to PPIase inhibition. Rather, it suggests that cyclophilin binds to a pore-forming protein resulting in induction or stabilisation of the open pore. On binding CsA, this activity of cyclophilin is inhibited, causing the pore state equilibrium to shift towards closed.
Today the general consensus is that the permeability transition of mitochondria is due to the activity of a proteinaceous pore. However, for many years previously the preferred explanation was that damage to the lipid bilayer of the mitochondrial inner membrane led to the influx of ions and metabolites and so to swelling. This hypothesis began with the association of fatty acid production with uncoupling and swelling (Wojtczak & Lehninger [1961]), and the identification of the (opposing) activities of two enzymes in mitochondria: phospholipase A2 (PA2) (Bjornstad 1966) and lysophospholipid-acyl-CoA-transferase (lysophospholipid acylase) (Webster & Alpern [1964]). Pfeiffer and co-workers developed a working model for the hypothesis whereby the mitochondrial inner membrane would accumulate lysophospholipids resulting in permeability defects (Beatrice et al [1980]). The permeability transition would result from above normal activation of PA2 and/or inhibition of lysophospholipid acylase. Many experimental observations supported this model including Ca\(^{2+}\) (Waite & Sisson [1971]) and fatty acids (Bjornstad [1966]) have similar effects on the PT and on PA2 activity. However, many observations could not be explained by the membrane damage hypothesis:

- the PT displays an all-or-nothing characteristic (Hunter et al [1976]) which is contradictory to the gradual onset of membrane defects via PA2
- certain inhibitors of the PT do not inhibit PA2 (Epps et al [1982], Broekemeier et al [1985])
- under the minimal conditions required to open the pore, there is no detectable increases in free fatty acid concentration (McGuinness et al [1990])
pore closure on Ca$^{2+}$ removal occurs rapidly (<1msec) in the absence of ATP and CoA (which would be required for phospholipid reacylation) and is unaffected by addition of ATP (Crompton & Costi [1988]).

probably the clearest indication that, at the very least, the PA2-hypothesis is not the only one in operation is that CsA inhibits the PT (Crompton et al [1988], Broekemeier et al [1989], Fournier et al [1987]) but does not affect PA2 activity (Broekemeier et al [1989]).

electrophysiological experiments have recently been carried out on mitochondrial membranes and have led to the identification of a ‘mitochondrial megachannel’ (MMC), which by comparison of its properties to those of the PT pore have been reconciled as one and the same (Bernardi et al [1992], Szabo et al [1992], Szabo & Zoratti [1991], Szabo & Zoratti [1992]).

The identity of the pore-forming protein(s) is yet to be determined. The inhibitory effect of CsA on pore activation indicates a cyclophilin or other CsA-binding protein would be an obvious candidate; this forms the basis of the research presented here and is discussed. However, the nature of the PT pore has prompted other researchers to suggest two well-known proteins as being involved: the adenine nucleotide translocase (AdNT) and the outer membrane protein porin.

[1.5] Candidates for Pore-Forming Protein(s)

[1.5.1] Adenine Nucleotide Translocase (AdNT)

The AdNT has been implicated in the PT process due to the observation that many of its ligands have been shown to affect the PT. Le Quoc and Le Quoc (Le Quoc & Le Quoc [1988], Le Quoc & Le Quoc [1989]), from investigations of the
effects of these inhibitors of the AdNT, have concluded that the conformation of the AdNT determines whether the PT occurs in Ca\(^{2+}\)-loaded mitochondria. Ligands that stabilise the ‘M’ conformation (e.g. bongkrekate) act as PT inhibitors. Ligands that stabilise the ‘C’ conformation i.e. nucleotide binding site facing the cytoplasm (e.g. ATR, CATR, palmitoyl CoA, pyridoxal-5-phosphate; e.g. Klingenburg [1993]) have the opposite effect.

Results of Fournier et al [1987] indicate that CsA does not significantly affect the normal functions of the AdNT, but as the PT pore is hypothesised to correspond to an altered form of the AdNT, this observation does not necessarily oppose its involvement. This altered form might involve an association with porin (see below).

[1.5.2] Porin (or VDAC)

The PT phenomenon has not been observed in mitochondria stripped of their outer membrane (sub-mitochondrial particles) and this has prompted researchers to hypothesise that the PT pore forms at contact sites between the outer and inner membrane (Crompton & Costi [1988]). The outer membrane protein porin which forms voltage-dependant anion selective channels (VDAC) has been suggested as a possible candidate for recruitment into the pore structure at contact sites. This hypothesis has gained credence by the observation that the conductance of VDAC is approximately half that obtained from the MMC (Petronilli et al [1989], Szabo et al [1992], Szabo & Zoratti [1993], Manella et al [1992]). This has led to the proposition that the MMC is formed from two porin monomers. In 1992 it was suggested (McEnery [1992]) that the mitochondrial benzodiazepine receptor (mBzR) consisted of VDAC, the AdNT and an 18kDa peptide. This instantly
became a candidate for the PT pore, although the involvement of CsA remains unaccounted for.

[1.6] **Physiological functions of the permeability transition pore**

Evidence for the activation of the pore under normal conditions is limited, however, experimental observations have led to a number of suggestions for *in vivo* roles of the PT.

**Cell Death**

The collapse of the mitochondrial transmembrane potential has been demonstrated as an early event in the apoptotic process in a variety of cell types, irrespective of the apoptosis-inducing stimulus (e.g. the induction of apoptosis by tumour necrosis factor in myelomonocytic cells (Zamzami *et al* [1995]b). Inhibitors of the PT pore (e.g. CsA) suppress this apoptotic collapse of the transmembrane potential, indicating the mechanism involves the PT. Overexpression of the proto-oncogene Bcl-2 inhibits apoptosis induction. In mouse L929 fibrosarroid cells it enhances the steady state mitochondrial inner membrane potential (Hennet *et al* [1993]). Bcl-2 is expressed mainly in the mitochondrial outer membrane, although its distribution does appear to correlate with contact sites between the outer and inner membrane (Akao *et al* [1994]; de Jong *et al* [1994]). Bcl-2 overexpression inhibits the PT induced by certain PT inducers (e.g. atractyloside) but not all (calcium or diamide) (Zamzami *et al* [1996]).

**Inter-mitochondrial signalling**

The formation of gap junction type connections between mitochondria has been hypothesised, with the PT pore forming a component (Tanveer *et al* [1996]). Evidence for long-distance power transmission along mitochondrial membranes
has been provided in animal cell cultures; an analogous cell-cell transmission of membrane potential has been reported for filamentous bacteria. This suggests power transmission may be a fundamental function of extended membrane systems such as mitochondria (Skulachev [1990] and refs. within).

[1.7] *The involvement of the permeability transition pore in ischaemia- and reperfusion-induced injury*

There is correlation between the factors required for PT activation in isolated mitochondria and those implicated in ischaemia/reperfusion injury (Crompton & Costi [1988], Crompton *et al* [1988]). In this type of injury, cells that have withstood a period of ischaemia are irreversibly damaged on reperfusion. This indicates changes take place during the ischaemic period which damage the cells on re-supply of blood. Relevant changes are thought to include mitochondria accumulating large amounts of Ca\(^{2+}\) and other ions, a drop in ATP levels and the development of oxidant stress. It seems relevant at this stage to outline the role of calcium in cells generally and mitochondria specifically, and briefly discuss the effects of abnormal calcium fluxes on the inner membrane pore.

In the majority of mammalian cells, most of the energy requirements are met by the generation of ATP by mitochondrial oxidative phosphorylation (the remainder made up by glycolysis). Ca\(^{2+}\) has emerged as an important second messenger to integrate the utilisation of ATP extramitochondrially with its synthesis in the mitochondrial matrix. The Ca\(^{2+}\) transport systems of the inner mitochondrial membrane are believed to extend this second messenger function to the mitochondrial matrix. Key enzymes involved in the regulation of intramitochondrial oxidative metabolism are regulated by Ca\(^{2+}\).
Ca\(^{2+}\) cycles continuously across the inner membrane. The Ca\(^{2+}\) uniporter allows the passive influx of Ca\(^{2+}\) down its electrochemical gradient. Charge compensation occurs by the extrusion of 2H\(^+\) by the respiratory chain pumps (Crompton & Heid [1978]). Ca\(^{2+}\) efflux occurs via a Ca\(^{2+}/Na^+\) exchanger. This requires energy as Ca\(^{2+}\) travels against its electrochemical gradient. The Na\(^+\) entering by exchange with Ca\(^{2+}\) is then removed by exchange with H\(^+\), which is pumped out by the respiratory chain (Crompton et al [1976]).

Free Ca\(^{2+}\) levels in the matrix established by Ca\(^{2+}\) cycling are appropriate to regulate activity of key dehydrogenase enzymes involved in the rate control of intramitochondrial oxidative metabolism (Denton & McCormack [1980], Hansford [1987]), thus implicating this rate control as the natural role of the mitochondrial Ca\(^{2+}\) transport cycle. These key enzymes are the dehydrogenases (DHases): pyruvate DHase, oxoglutarate (DHase) and NAD-linked isocitrate DHase. These three enzymes catalyse oxidative decarboxylations at key control points in mitochondrial oxidative metabolism.

Although the adenine and pyridine nucleotides have long been considered as the principle regulators of metabolism, Ca\(^{2+}\) has emerged as the key regulator of oxidative metabolism in those tissues in which oxidative metabolism predominates. In conditions where the more conventional metabolic regulators offer no control, e.g. cardiac muscle, Ca\(^{2+}\) may play a critical role. The levels of Ca\(^{2+}\) in the matrix have been shown to be increased in mitochondria where cells have been subjected to conditions requiring greater than normal energy consumption, e.g. adrenaline-perfused hearts (Crompton et al [1983]) with corresponding increases in certain intramitochondrial dehydrogenases (McCormack et al [1984]).
There is a large Ca\(^{2+}\) gradient across the sarcolemmal membrane (SM) of heart cells.

On depolarisation of the SM, a voltage dependant Ca\(^{2+}\) channel lets Ca\(^{2+}\) into the cell. This Ca\(^{2+}\) induces Ca\(^{2+}\) release from stores in the sarcoplasmic reticulum (SR), and the resulting Ca\(^{2+}\) levels stimulate contraction of the cell. This rise in cytosolic free Ca\(^{2+}\) levels is rapidly reduced by ATP-dependant Ca\(^{2+}\) pumps in the SR membrane and the SM and the Na\(^+\)/Ca\(^{2+}\) exchange of the SM. This removal of Ca\(^{2+}\) from the cytosol enables the relaxation of contractile protein.

The only recognised way in which Ca\(^{2+}\) levels are controlled in mitochondria is by the Ca\(^{2+}\) levels in the cytosol. Ca\(^{2+}\) cycling by mitochondria is a slow process. The level of Ca\(^{2+}\) in mitochondria is determined by the height and frequency of the cytosolic transients. Under physiological conditions, matrix free Ca\(^{2+}\) is maintained within the range required for the regulation of the dehydrogenase enzymes (0.2-2\(\mu\)M). Under pathological conditions such as anoxia and ischaemia, resting cytosolic free Ca\(^{2+}\) levels rise (Allshire et al [1987]). If this is maintained, the inevitable result would be an increase in matrix free Ca\(^{2+}\) levels.

Mitochondrial Ca\(^{2+}\) overload is the inevitable outcome of ischaemia, and it results in respiration becoming uncoupled from energy-dependant reactions such as ATP synthesis.

ATP depletion during ischaemia results in the increase of free Ca\(^{2+}\) levels in the cytosol as the active Ca\(^{2+}\) extrusion via the ATP-dependant Ca\(^{2+}\) pumps in the SR membrane and the SM is diminished. Ca\(^{2+}\) efflux is also dependant on the Na\(^+\) gradient maintained by the ATP-dependant Na\(^+\)/K\(^+\) exchanger in the SM. Cytosolic free Na\(^+\) levels are increased, and as the Na\(^+\)/K\(^+\) exchanger is inhibited due to ATP depletion, Ca\(^{2+}\) levels rise.
Cell damage that occurs during periods of ischaemia can only be reversed by reperfusion if it occurs before the cytosolic Ca\(^{2+}\) levels reach a critical point of around 2\(\mu\)M (Allshire et al [1987]). Reperfusion after this point will not salvage the cell, and cell death occurs at an accelerated rate than if reperfusion had not taken place at all. This is reperfusion or reoxygenation injury. It is around this critical point that mitochondrial Ca\(^{2+}\) overload takes place and this is therefore considered to be significant in the onset of this type of injury (for review, see Crompton [1990]).

PT pore opening has been detected in vivo during myocardial reperfusion injury associated with tissue Ca\(^{2+}\) overload and oxidative stress (Griffiths & Halestrap [1995]) and oxidant stress- (hydroperoxide) induced injury in hepatocytes (Nieminen et al [1995]). CsA provides protection against reperfusion and hydroperoxide-induced injury in heart and liver cells (Pastorino et al [1993], Imberti et al [1992], Nazareth et al [1991], Griffiths & Halestrap [1993]) possibly by blockade of the PT pore.

[1.8] **Cyclosporin A (CsA)**

CsA is currently the most important immunosuppressive agent in use. Since its approval for clinical use in 1983, it has revolutionised solid organ and bone marrow transplantation through its widespread use in the prevention of graft rejection.

CsA is a naturally occurring secondary metabolite which was isolated from the Norwegian soil fungus *Tolypocladium inflatum*. It is a lipid-soluble, cyclic undecapeptide with a molecular mass of 1203. While most immunosuppressive
drugs act preferentially on dividing cells and are therefore generally toxic at immunosuppressive doses, CsA appears to have greater specificity for T lymphocytes. Certain Ca^{2+}-dependant cellular processes are inhibited by the drug, among which is the transcriptional activation of a number of cytokine genes. The inhibition of one of these genes, that of interleukin-2 (IL-2), is the major contributory factor in the prevention of T lymphocyte growth and functional ability. The major side effect of CsA use is its nephrotoxicity.

The majority of the CsA molecule appears to be involved with its immunosuppressive activity as modifications at most residues cause a significant loss in potency (Quesniaux et al [1987], Sigal et al [1991]).

Two other naturally occurring secondary metabolites under evaluation as therapeutic agents are the structurally related lipid-soluble macrocyclic lactones FK506 and rapamycin (with molecular masses of 822 and 915 respectively). Although their biological effects appear to be similar to those of CsA, they are chemically quite different. FK506 is produced by Streptomyces tsukubaensis, a bacterium discovered in a soil sample from Tsukuba, Japan. Rapamycin is produced by Streptomyces hygroscopicus, a bacterium isolated from a soil sample in Rapa-Nui, Easter Island.

Although better known for their immunosuppressive activity, CsA, FK506 and rapamycin are valuable research tools to probe signal transduction pathways. CsA and FK506 have identical effects in a number of immunosuppression models. They inhibit signal transduction pathways characterised by an initial rise in intracellular Ca^{2+} levels.
The CsA “receptor” has been shown to be a family of proteins known as cyclophilins (CyPs) (Fischer et al [1989], Takahashi et al [1989]). CyPs are one of a number of protein families that are peptidyl-prolyl cis/trans isomerases (PPIases) [Fig. 1.9A]. They catalyse the interconversion of cis and trans isomers of bonds preceding proline in peptides and proteins. Other currently known PPIase families are FK506-binding proteins (FKBPs) (Siekierka et al [1989], Harding et al [1989]) parvulins (Rahfeld et al [1994]) and trigger factors (Stoller et al [1995]) (the latter may make up a subfamily of FKBPs).

Proteins are a hugely heterogeneous class of macromolecules which differ greatly in their biological and physio-chemical properties. They can fulfil their vital functions only because of their very specific but still flexible conformation. Learning about the characteristics of protein folding is therefore both important and interesting.

In slow protein conformational processes prolyl cis/trans isomerisations are often rate-limiting (Davis et al [1989], Lang et al [1987]). Slow steps can cause problems as intermediates accumulate prior to them and these have a tendency to aggregate in competition with correct folding. PPIases catalyse slow prolyl isomerisations and are unique in that they catalyse interconversion by accelerating rotation of a single chemical bond. PPIases are ubiquitous in tissues and organisms and act in an ATP-independent manner.
[Fig. 1] *Cis/trans* isomerisation about a peptidyl-prolyl bond accelerated by a PPIase (from Galat [1993]).

\[ \text{cis} \quad \text{trans} \]
[1.9.1] Cyclophilins

The first PPIase was identified through research into protein folding in pig kidney cortex. A protein molecular mass less than 20kDa was found to catalyse prolyl isomerisation of a proline-containing oligopeptide 4-nitroanilide. It was not until 1989 that this enzyme was found to be identical to the 18kDa cytosolic receptor cyclophilin (CyPA) already known (Fischer et al [1989], Takahashi et al [1989]). A number of other cyclophilin isoforms have been discovered. All exhibit PPIase activity, with close homology at the PPIase domains. The isoforms differ in their subcellular localisations and CsA binding affinity. CyPB (Kₐ=9nM) is a 21kDa protein containing a signal sequence thought to target its translocation to the endoplasmic reticulum (ER) (Price et al [1991], Hasel et al [1991]). An avian homologue of CyPB co-localises with the calcium-storage protein calreticulin, suggesting CyPB resides within the calciosomes of the ER lumen (Arber et al [1992]).

CyPC, a 23kDa cyclophilin isoform, also contains an ER signalling sequence. Compared to the other isoforms CyPC has restricted tissue distribution, with highest expression in the kidney (Friedman et al [1994]). This may be relevant when considering the major drawback of the therapeutic use of CsA is its nephrotoxicity (Otsuka et al [1994]).

A fourth mammalian CyP isoform, CyPD, contains a N-terminal presequence (Bergsma et al [1991]) subsequently shown to be a mitochondrial targeting sequence.

Other CsA-binding proteins include a 150kDa protein specific to natural killer cells (NK-TR) (Rinfret et al [1994]). It is believed to be part of the tumour recognition complex. The N-terminal end of this protein has been found to possess
considerable homology to CyPs, and does possess PPIase activity. CsA inhibition of this activity was considerably lower than for other CyPs. This is thought to be due to the absence of a specific tryptophan residue in the NK-TR CyP domain. This residue is also absent in E.coli CyP which shares this lower affinity for CsA.

1.9.2 Interactions of cyclophilins with other proteins

CyPC has been reported to associate with a 77kDa glycoprotein in vitro in the absence of CsA. The protein contains a cysteine-rich domain found in a variety of cell-surface molecules (Friedman et al [1993]). This suggests a possible role of CyPC as a mediator or regulator of an unknown signal or cellular process initiated via this 77kDa protein.

A CyPB-binding protein has been cloned from a lymphocyte cDNA library that, when overexpressed, induces IL-2 transcription by activation of the T-cell specific transcription factor NF-AT. This protein has been termed calcium-signal modulating CyP ligand (CAML) (Bram & Crabtree [1994]). Activation of NF-AT requires stimulation of protein kinase C by phorbol ester, unlike TCR-mediated activation which activates both the calcium and PKC signal transduction pathways. This indicates CAML produces its effect downstream of the TCR. The specific calcineurin inhibitors CsA and FK506 completely abolish CAML-mediated activation, indicating the protein does not act directly on NF-AT, but upstream of calcineurin. There was an increase in the levels of intracellular Ca^{2+} in CAML-overexpressing cells and removal of extracellular Ca^{2+} inhibited CAML activation of NF-AT but had little effect on constitutive calcineurin activation. This shows CAML activates calcineurin by causing Ca^{2+} influx rather than directly binding to
and activating calcineurin. What significance the binding of CAML to CyPB has with respect to calcium homeostasis has yet to be discerned.

[1.9.3] Functions of Cyclophilins

The physiological significance of the various CyP isoforms have yet to be determined but a number of their cellular functions and relevant associations have been demonstrated.

Genetic analysis of the fruit fly Drosophila melanogaster has supplied important insights into CyP function. The Drosophila ninaA gene encodes a retinal photoreceptor specific CyP homologue with PPIase activity (Stamnes et al [1991]). It has been implicated in the post-translational regulation of specific isoforms of rhodopsin. Mutations resulting in loss of function of ninaA result in an increase in the levels of certain rhodopsin isoforms within the ER and a significant decrease outside the ER, causing deficient visual signal transduction. It has yet to be established however whether this is due to incorrect protein folding or a necessity for the ninaA gene product to assist the exit of rhodopsin out of the ER and into the Golgi apparatus (protein ‘trafficking’). Bovine retinal CyPs analogous to the ninaA protein of Drosophila have been identified (Ferreira et al [1995]) which are preferentially expressed by photoreceptors. This suggests these proteins facilitate proper folding and intracellular transport of mammalian opsins.

CyPs have been implicated in having molecular chaperone activity with evidence of complex formation with heat shock proteins. A 40kDa CyP (CyP40) has been characterised (Kieffer et al [1993]) which associates with the heat shock component, hsp 90, of the inactive steroid hormone receptor. This association is not affected by CsA and it does not affect the PPIase activity of the protein.
(Hoffman & Handschumacher [1995]). CyP40 shares homology at its COOH-terminal domain with another subunit of the steroid receptor which has recently been found to contain a FKBP-like PPIase domain (p59 or FKBP52, see later).

Retroviruses require host factors for their life cycle and replication. The retroviral Gag polyprotein of human immunodeficiency virus type I (HIV-1) and related retroviruses directs virion particle formation and release from the host cell membrane. This is an essential part of the early stage of the infection of the target cell. It has been demonstrated that the Gag polyprotein contains a central proline-rich region to which CyPs A and B can bind (Luban et al [1993]). CsA prevents the interaction between CyPA and Gag (Pflugl et al [1993]), and addition of CsA to HIV-1 infected cells causes a reduction of HIV-1 expression and retroviral infectivity (Karpas et al [1992]. The infectivity of HIV-1, but not HIV-2 or simian immunodeficiency virus (SIV), depends on the presence of CyPA in the virion (Franke et al [1994], Thali et al [1994]). Disruption of a single Gag proline in the capsid domain blocks the Gag-CyP interaction in vitro, preventing CyPA incorporation into (and inhibiting replication of) HIV-1 virions. Interestingly, CyPs are not required for the replication of HIV-2 or SIV, and their Gag protein can bind CyPB but not CyPA. Treatment of HIV-1 with CsA was attempted but unfortunately the doses of CsA required to disrupt virion formation in vitro are 10-100 fold higher than humans can tolerate in vivo. Research is ongoing.

Apoptosis is an important physiological process that occurs under numerous conditions where the selective deletion of cells from tissues and organs is required, without the production of an inflammatory response. One of the cellular responses on receiving an apoptotic stimulus is DNA cleavage by a calcium-dependant nuclease. CyPs A, B and C have been reported to contain sequence homology
with the apoptotic nuclease NUC18 from rat thymocytes with which they also share calcium/magnesium-dependant nuclease activity with similar biochemical and pharmacological properties (Montague et al [1994]). This nuclease activity appears to be distinct from the PPIase activity of the CyPs (Montague et al [1997]). These results indicate a possible involvement of CyP in degradation of the genome during apoptosis.

CyPs have been shown to be involved in the folding of newly imported proteins in yeast mitochondria. The import of the proteins was not affected by CsA, but it displayed an inhibitory effect on refolding (Rassow et al [1995]). In mutant yeast mitochondria that did not contain CyP, the same reduction in rate of refolding was observed, and this was not affected further by CsA (Matouschek et al [1995]).

CyPs may be produced in the course of inflammation and act as leukocyte chemotactic factors. CyPA has been identified in the synovial fluids of patients with rheumatoid arthritis (Billich et al [1997]), and its presence correlates with disease. This suggests CyPs may contribute to the pathogenesis of inflammatory diseases, possibly by acting as cytokines, and may explain the effectiveness of CsA in the treatment of rheumatoid arthritis.

CyPs may be stress-related proteins. Higher than normal CyP mRNA levels have been detected in bean and maize plants after a variety of stress-inducing conditions (Marivet et al [1994]), e.g. heat shock and salt stress; however, their accumulation profiles differed.

CsA has been reported to inhibit the growth of malarial parasites both in vitro and in vivo, and apart from the subsequent assumption that CyPs are involved, little is known about the mechanism of action. There is no correlation between anti-
malarial and anti-PPIase activities of CsA or the analogues investigated (Bell et al [1994]). FK506 and rapamycin also have this anti-malarial activity.

[1.9.4] **FK-506 binding proteins (FKBPs)**

The next family of PPIases to be discovered was the FK506-binding proteins (FKBPs), which were completely unrelated to CyPs (Siekierka et al [1989], Harding et al [1989]). However, FKBPs share many characteristics with CyPs. In addition to their PPIase activity, there are many FKBP isoforms in mammalian cells with distinct subcellular localisations, and these isoforms are conserved throughout evolution with greatest homology at the PPIase active site. Harrison & Stein [1990] showed CyPA and FKBP12 to exhibit different substrate selectivity towards proline containing peptides *in vitro*, suggesting the polypeptide substrates of FKBPs and CyPs *in vivo* may well be different.

The term FKBP refers to any protein which binds to both FK506 and rapamycin. So far, FKBPs have been named according to their molecular mass.

FKBP12, a 12kDa protein, is the predominant isoform in most cells, but there appears to be significant variation in expression in different cell types (Hultsch et al [1991], Kaye et al [1992]). FKBP12 binds both FK506 and rapamycin with high affinity (Harrison & Stein [1990], Bierer et al [1990a]). The cytoplasmic FKBP12 has been shown to associate with a Ca$^{2+}$ channel in the sarcoplasmic reticulum of skeletal muscle, the ryanodine receptor. This channel provides the pathway for Ca$^{2+}$ efflux required for excitation-contraction coupling in skeletal muscle. It consists of four identical subunits and it appears four FKBP12 molecules bind to each channel (Jayaraman et al [1992]).
FKBP13 is localised in the ER lumen (Nigam et al [1993]), and binds FK506 with a lower affinity than does FKBP12 (Nielsen et al [1992]). FKBP25 differs from other FKBP isoforms by its low affinity for FK506 but high affinity for rapamycin (Galat et al 1992).

Subcellular fractionation shows FKBP25 is found predominantly in the nucleus; in vitro it forms complexes with the predominantly nuclear kinase casein kinase II and the major nuclear protein nucleolin which is involved in ribosome biogenesis (Jin & Burakoff [1993]). Rapamycin does not disrupt the complexes suggesting the association is not mediated via the PPIase active site of FKBP25.

A fourth FKBP isoform is FKBP52, which contains three domains homologous to FKBP12, one of which is the PPIase active site. In vitro binding of FKBP52 to calmodulin has been reported (Massol et al [1990]). FKBP52 has been demonstrated to be a component of the inactive steroid hormone receptor complex, as has CyP40, apparently via binding to the hsp90 component (Lebeau et al [1992]). FK506 and rapamycin do not disrupt this complex.

[1.9.5] Other PPIase proteins

In addition to CyP-like PPIases, E.coli has more recently been discovered to have two other proteins with PPIase activity. The first of these to be discovered has been named ‘parvulin’. This protein has a low molecular weight, 10.1kDa, and its functional role is unclear. However, there are clues from homology analyses (Rahfeld et al [1994]). Several parvulin-like proteins (e.g. PrtM protein of Lactococcus lactis and the PrsA lipoprotein of Bacillus subtilis) are known to be involved in the protein maturation machinery and protein export in bacteria.
The second PPIase is specifically associated with the 50S subunit of the *E.coli* ribosome, and is identical to the *E.coli* trigger factor (Stoller *et al* [1995]). Its activity towards oligopeptide substrates and its subsite specificities resembles FKBPs, although it is insensitive to FK506. *In vitro*, the trigger factor has been found to catalyse the proline-limited refolding of a RNase T1 variant with more efficiency than the other PPIases studied.

[1.9.6] *Inhibition of PPIase activity* hypothesis of the immunosuppression pathway

The immunosuppressive compound CsA (a cyclic undecapeptide) and FK506 and rapamycin (pипелосic acid-derived peptidomacrolides) bind tightly to cyclophilins and FKBPs respectively. Enzymatic activity of the PPIases is inhibited in a reversible and competitive manner. Inhibition kinetics are difficult to investigate as members within each family vary greatly in affinity to the immunosuppressive compounds. It was originally hypothesised that since these compounds inhibited the PPIase activity of their respective cytosolic binding proteins, that PPIase activity may be involved in signal transduction and the inhibition had to be a significant step in the immunosuppressive pathway. This hypothesis has now largely been discarded due to a number of subsequent observation which include:

- Other PPIase inhibitors are not necessarily immunosuppressive agents (*Bierer et al* [1990], *Dumont et al* [1990]).

- Other immunosuppressive agents are not necessarily PPIase inhibitors. This was demonstrated aptly by *Sigal et al* [1991] with two analogues of CsA which were modified at opposite ends of the molecule. A methyl group was added to a
side chain, and this caused the analogue to retain a significant amount of its immunosuppressive activity but lose most of its cyclophilin inhibitory potential. A second analogue, which had a propyl group removed, remained a potent inhibitor of cyclophilin but was almost non-immunosuppressive.

- The low concentrations of the drugs required to produce an immunosuppressive response do not correlate with the high concentration of PPIase molecules present in the cell (Schreiber [1991], Schreiber & Crabtree [1992]).

- Since the drugs do not cross-inhibit each other’s binding proteins’ PPIase activity, cells treated with one drug would still have functional PPIase present.

[1.10] **Calcineurin**

Observations from chemical and genetic experiments led to the ‘toxic complex’ hypothesis: that it was the drug/binding protein complex which was required for CsA and FK506 to mediate their immunosuppressive activities. Many analogues of FK506 and CsA which contained the immunophilin binding domains, but other regions had been disrupted, were capable of inhibiting PPIase activity of the corresponding binding proteins but had no significant immunosuppressive effects (Bierer et al [1990], Sigal et al [1991], Dumont et al [1992]). Experiments with the yeast *Saccharomyces cerevisiae* where CYP1, one of its three cyclophilin genes, was inactivated, demonstrated resistance to CsA (Tropschug et al [1989]) in contrast to CsA toxicity in the wild type. These experiments separated the ability to inhibit PPIase activity from immunosuppression and implicate the CsA-CyP complex in toxicity.
Thus it has widely been accepted that the immunosuppressive entity is the immunosuppressant/immunophilin complex. This stimulated the search for cellular components which interact with the complex and thereby may interfere with signal transduction pathways leading to immunosuppression. The first of these targets to be identified was calcineurin, an intracellular Ca\(^{2+}\)- and calmodulin-dependant phosphatase (also known as serine/threonine phosphatase 2B); thus, the complexes CsA/CyPA, CsA/CyPC and FK506/FKBP12 bound three proteins on affinity matrices in a Ca\(^{2+}\)-dependant manner; i.e. the A and B subunits of calcineurin, and calmodulin (Friedman & Weissman [1991], Liu et al [1991]). The heterodimeric complex of calcineurin is composed of a catalytic A subunit of 59kDa and a regulatory B subunit of 19kDa. The complexes were shown to bind to the B subunit, but the A subunit needed to be present for the binding to occur. The phosphatase activity of calcineurin was inhibited by the complexes but not by the drugs or immunophilins alone. Treatment of cells by CsA or FK506 resulted in inhibition of cellular calcineurin (Liu et al [1992]). The activities of other cellular serine/threonine phosphatases were unaffected by the drugs in vivo, or the complexes in vitro.

No binding of calcineurin was observed with rapamycin or the rapamycin/FKBP12 complex, indicating a separate target for this toxic complex. Suggestions include other intracellular signalling molecules such as p70 S6 kinase. Rapamycin induces cell cycle G1 arrest in yeast and mammalian cells, and inhibits IL-2 receptor-induced S phase entry and subsequent T cell proliferation in mammals, resulting in immunosuppression. Kuo et al [1992] demonstrated that IL-2 selectively stimulates the phosphorylation and activation of p70 S6 kinase, and rapamycin inhibits the IL-2-induced activity at concentrations comparable to those blocking S
phase entry of T cells (0.05-0.2nM). FK506 completely inhibits rapamycin, indicating these effects are mediated via a FKBP. Rapamycin and FK506 bind to the same intracellular receptor, FKBP12. A mammalian FKBP-rapamycin-associated protein (FRAP) has been isolated (Brown et al [1994] whose binding to rapamycin analogues complexed to FKBP12 correlates with the ability of these ligands to inhibit cell-cycle progression.

Kunz et al [1992] have shown that mutations in the TOR2 gene in yeast, which encodes a phosphatidylinositol-3-kinase homologue, confer resistance to rapamycin. This indicates an involvement in cell signalling pathways.

[1.10.1] The role of calcineurin in signal transduction pathways

T cell activation can be divided into three stages: antigen binding to the membrane-spanning T cell receptor (TCR), the cytoplasmic signal transduction cascade that transmits information to the nucleus, and gene activation in the nucleus.

After the initial transmembrane signal, a membrane phospholipid is hydrolysed to produce intracellular messengers: inositol triphosphate (IP$_3$) and diacylglycerol (DAG). IP$_3$ causes a rapid increase in intracellular Ca$^{2+}$ levels via the opening of calcium channels in the plasma membrane or in the intracellular membranes of Ca$^{2+}$ stores within the cells (in most cells, the endoplasmic reticulum). The elevated Ca$^{2+}$ levels trigger many processes within cells. Calmodulin acts as a calcium detector in most eukaryotic cells. Each calmodulin molecule binds four Ca$^{2+}$ ions and the binding of each Ca$^{2+}$ ion facilitates further binding (in a sigmoidal fashion). This results in small cytosolic Ca$^{2+}$ level increases causing large
active calmodulin level increases. Binding of Ca\(^{2+}\) ions to calmodulin stimulates it to activate a variety of enzymes, one of which is calcineurin.

The involvement of calcineurin in IL-2 gene transcription activation has been demonstrated by over-expressing calcineurin in cells. Ca\(^{2+}\)-dependant transcription from the IL-2 promoter was less sensitive to CsA and FK506 (O'Keefe et al [1992], Clipstone & Crabtree [1992]).

Indications on how calcineurin exerts its effect on gene activation come from observations that certain enhancer elements attached to the IL-2 promoter region are essential for the successful transcription of the IL-2 gene; specifically the lymphoid-specific protein NF-AT (nuclear factor of activated T cells) which is one of many transcription factors required for antigen-induced gene expression (Emmel et al [1989], Graneeli-Piperno et al [1990], Schreiber [1992]). This suggests that the correct assembly and attachment of this transcription factor is the step which is interfered with by the complexes, causing their immunosuppressant activity. NF-AT has at least two subunits, one of which is T cell cytoplasm specific and the other is ubiquitous and mainly nuclear (Flanagan et al [1990]). Both subunits need to be assembled together for it to be functionally active. Evidence indicates that the translocation of the cytoplasmic subunit (NF-ATc) into the nucleus is calcium-dependant, and when calcineurin is deactivated on binding of the 'toxic complex', its induction of NF-ATc nuclear translocation is prevented without affecting synthesis of the nuclear subunit. This suggests NF-ATc is a direct or indirect substrate for calcineurin. One example of dephosphorylation controlling nuclear translocation is of yeast transcription factor SW15. It is involved in the activation of the HO endonuclease gene that controls mating-type switch. Phosphorylation of three serine residues by CDC28-dependant kinase
activity is crucial in keeping the protein cytoplasmic. On dephosphorylation, the transcription factor enters the nucleus and activates the HO endonuclease gene (Moll et al [1991]). It is possible that activation of the IL-2 gene occurs via a similar mechanism.

Calcineurin may not be the only target for the immunosuppressive complexes. The second intracellular messenger formed from the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate, is DAG. DAG activates protein kinase C (PKC), a key event in gene expression regulation and subsequent protein translation. PKC has many isoforms. It has been shown that CsA has no effect on the expression and activity of PKC-α. However, PKC-β is completely inactivated by the drug. The same study (Szamel et al [1993]) showed CsA inhibits activation of lysophosphatid acyltransferase-catalysed incorporation of cis-polyunsaturated fatty acids into plasma membrane phospholipids. It has therefore been suggested that the interference of CsA with plasma membrane fatty acid metabolism could selectively inhibit a signal transduction pathway involving PKC-β. Neutralisation of PKC-β by anti-PKC-β antibodies prevent IL-2 synthesis suggesting an inhibition of activation and translocation of PKC-β by CsA may result in inhibition of IL-2 gene expression, leading to immunosuppression.

[A.11] Aims of the study

CsA blockade of the pore and identification of a cyclophilin in mitochondria should enable the isolation of the pore component(s). The aim of this study was to isolate mitochondrial cyclophilin in rat liver and verify its locality. It was then anticipated that its full length cDNA would be obtained and used to construct a glutathione-S-transferase (GST) / CyP fusion gene. After expression of the fusion
protein and preparation of an affinity matrix, binding proteins from mitochondrial extracts could be isolated. Relevant proteins would be expected to display sensitivity to Ca$^{2+}$, ADP, and CsA.
CHAPTER 2: METHODS AND MATERIALS

[2.1] Preparation of rat liver mitochondria

Mitochondria were isolated from the livers of male Sprague-Dawley rats (200-300g body weight) using the following protocol, a modification of the method described by Pederson et al [1979]. The rats were killed by cervical dislocation and their livers removed and immediately placed into an ice-cold solution of 210mM mannitol, 70mM sucrose, 10mM Tris-HCl, 1mM EGTA and 0.5mg/ml BSA (bovine serum albumin) (MSTEB) at pH 7.2. The liver was chopped into small pieces with a pair of scissors and transferred to 30ml centrifuge tubes (2 tubes for each liver) and homogenised for approximately 10 seconds using a Polytron homogeniser on setting 5. The polytron probe was precooled to 4°C before use. The samples were then spun at 2200rpm for 5 minutes in a refrigerated superspeed centrifuge, Sorvall RC5B (rotor: SS34) at 4°C. The supernatants were decanted into new 30ml centrifuge tubes and spun at 9000rpm for 8 minutes at 4°C to sediment the mitochondria. The pellets were washed by resuspending in a small volume of ice-cold 210mM mannitol, 70mM sucrose, 10mM Tris-HCl (MST) at pH 7.2 using a glass test-tube filled with crushed ice. The volumes were then made up to 30ml with ice-cold MST and spun again at 8700rpm for 8 minutes at 4°C. Pellets were washed again as before. Three further spins were carried out under the same conditions but at 8000, 7500 and 7200 rpm, with the pellets being washed between each spin. After the final spin the mitochondrial pellets were resuspended in MST at around 10mg/ml and kept on ice until ready for use.
[2.2] Quantitation of mitochondrial protein

Protein was estimated by a modified biuret method [Kroger and Klingenberg, (1966)]. BSA was used as a standard. 0.5ml of sample and 0.5ml water were mixed with 0.2ml 4% sodium cholate, 2ml 10% sodium hydroxide and 0.3ml 1% copper sulphate. The reactions were mixed well and left to stand for 10 minutes, after which the absorbance of the sample was read against the blank at 540nm.

[2.3] Separation of mitochondria into intermembrane space and mitoplast fractions

Low digitonin concentrations selectively lyse the mitochondrial outer membrane leaving the inner membrane intact. After protein quantitation, mitochondrial suspensions were treated with 0.19mg digitonin/mg mitochondrial protein (unless otherwise stated) and incubated on ice for 15 minutes. Mitoplasts were then spun down as mitochondria, leaving the intermembrane space components in the supernatant. EGTA and dithiothreitol were added to 0.5mM. 1mM PMSF and protease inhibitors (1µg/ml leupeptin, antipain and pepstatin) were also added.

[2.4] Purification of cyclophilin(s) by column chromatography

[2.4.1] Preparation of samples

[2.4.1.1] Intermembrane space fraction: The intermembrane space fraction was prepared [2.3] and then clarified by high speed centrifugation at 40,000rpm in Beckman ultracentrifuge (rotor: 70Ti) before loading.
[2.4.1.2] **Matrix fraction**: Mitoplasts [2.3] were sonicated to rupture the inner membrane in sonication buffer comprising 100mM NaCl, 10mM HEPES, 0.5mM EGTA, 0.5mM dithiotreitol, 1mM PMSF and 1µg/ml protease inhibitors at pH 8.0. This was clarified by high speed centrifugation at 40,000rpm in Beckman ultracentrifuge (rotor: 70Ti) before loading.

[2.4.1.3] **Inner membrane fraction**: The pellet from [2.4.1.2] was regarded as the inner membrane fraction.

[2.4.1.4] **Dialysis of samples in high salt buffers**: Samples in high salt buffers were dialysed against 10-50 times sample volume of 10mM HEPES, 0.5mM EGTA, 0.5mM dithiothreitol, 1mM PMSF and 1µg/ml protease inhibitors (low salt buffer) at pH 7.7 for 12 hours at 4°C.

[2.4.2] **Column chromatography of cyclophilin**

[2.4.2.1] **S-Sepharose cation exchanger**: To avoid overloading/blocking the FPLC columns [2.4.2.2] an initial fractionation was made using a standard cation exchange resin. A 25ml column was prepared using S-Sepharose fast flow cation exchanger (Sigma S-1264). The active group is sulphonic acid (-CH₃-SO₃⁻) with a wet bead size of 45-165µm and a capacity of 180-250µeq/ml gel. The approximate exclusion limit is around 4x10⁶ molecular weight. The column was equilibrated with 3 volumes of low salt buffer [2.4.1.3] at pH 8.0. 90% of mitochondrial protein did not bind to the column under these conditions, but 80% of PPIase activity was retained. This was eluted off with 0.5M NaCl in the low salt buffer. The remaining 20% was not recovered in the passthrough and was assumed to have denatured.
**[2.4.2.2] Mono-S cation exchange column** (Mono-S HR 5/5 Pharmacia). This column was used with the FPLC system and has a volume of 10ml. Its particle size is 10μm, the active group is sulphonic acid and the protein capacity is around 20-50mg/column. Substances with a molecular weight of <10^7 are separated successfully. The column was equilibrated with low salt buffer [2.4.1.3] at pH 7.7. Mitochondrial protein was eluted off the column using a salt gradient from 0-0.5M NaCl in the low salt buffer. Using a 1ml/min flow rate, 1ml fractions were collected. PPIase activity was detected in the fractions obtained at 0.2-0.3M NaCl.

**[2.4.2.3] Superdex 75 gel filtration column** (Superdex 75 HR 10/30 Pharmacia). This column is used with FPLC system and has a volume of 24ml. Its mean particle size is 13μm and for globular proteins the exclusion limit is 100,000 molecular weight. The column was equilibrated with 100mM NaCl in the low salt buffer [2.4.1.3] at pH 7.7. 0.5-1.0ml sample volumes were loaded. Using a 0.5ml/min flow rate, 0.5ml fractions were obtained. PPIase activity was detected in fractions 20-25.

**[2.5] Assay for peptidyl-prolyl cis-trans isomerase (PPIase) activity**

**[2.5.1] Rationale of PPIase assay**

PPIase activity was determined by the assay of Fischer et al (1989). The cis-trans isomerisation of the ala-pro peptide bond in the test peptide N-succinyl-ala-ala-pro-phe-4-anilide is measured in a coupled assay with chymotrypsin. Chymotrypsin cleaves the test peptide when the ala-pro is in the trans isomeric form. 85% of the substrate is already trans when commercially obtained. This pre-
existing \textit{trans} isomer is hydrolysed before monitoring is begun (within 5 seconds) to release the yellow chromophore nitroaniline. Hydrolysis of the remaining \textit{cis} isomer (15\%) is limited by the \textit{cis-trans} isomerisation of the ala-pro bond. This slower reaction is monitored by following the increase in absorbance due to the formation of the yellow chromophore.

\textbf{[2.5.2] Protocol for assay of PPIase activity}

A stock solution of the test peptide was made up at 1.2mM in 50\% ethanol and sonicated in a bath sonicator to aid solvation. A 20mg/ml solution of chymotrypsin was made up in PPIase buffer 120mM KCl, 10mM HEPES at pH 7.0. The assays were carried out at 14\(^\circ\)C in 3ml glass cuvettes containing buffer, an aliquot of the protein sample and 0.6\(\mu\)M CsA (when added). After a 5 minute preincubation, 50\(\mu\)M chymotrypsin was added. After 1 minute, the reaction was started by adding 15\(\mu\)M test peptide. Formation of the yellow chromophore was monitored by following the increase in absorbance on a Perkin Elmer dual beam spectrophotometer using the wavelength pair 390-480nm.

\textbf{[2.5.3] Derivation of the rate equation}

Rate of disappearance of the \textit{cis} isomer is represented by:

\[ \frac{dcis}{dt} = -kcis \]

where \(cis\) is the concentration of the \textit{cis} isomer at time \(t\), and \(k\) is the rate constant for the reaction.

\[ cis_t = cis_0 e^{kt} \]

where \(cis_t\) is the concentration of the \textit{cis} isomer at time \(t\), and \(cis_0\) is the initial concentration of the \textit{cis} isomer.
\[ \text{cis}_t = \text{cis}_i + \text{trans}_t \]

where \( \text{trans}_t \) is the concentration of the \( \text{trans} \) isomer which has been formed from the \( \text{cis} \) at time \( t \).

Therefore, \( \text{cis}_i = \text{cis}_T - \text{trans}_i \)

So, \( (\text{cis}_T - \text{trans}_i)/\text{cis}_T = e^{kt} \)

Now, \( \text{cis}_T = \text{trans}_T \)

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

Therefore, \( (\text{trans}_T - \text{trans}_i)/\text{trans}_T = e^{kt} \)

For experimental purposes:

\[ \text{trans}_i \propto A \]

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

So, \( (A_T - A_i)/A_T = e^{kt} \)

Or, \( \ln[(A_T - A_i)/A_T] = -kt \)

Therefore the rate constant of isomerisation \( k \) is given by the negative slope of the graph \( \ln(A_T/A_i) \) against time \( t \).

[2.6] Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

[2.6.1] Introduction to SDS-PAGE

The polyacrylamide gel electrophoresis was a modification of the discontinuous procedure of Schagger and von Jagow, (1987), using the SE 250-Mighty Small II Slab Gel Electrophoresis Unit (Hoeffer Scientific Instruments, San Francisco).
SDS binds to most proteins causing the dissociation of most into their monomer subunits and disrupting their secondary structure. Heating in the presence of disulphide reducing agents such as β-mercaptoethanol or dithiothreitol further dissociates proteins. Also, when proteins are saturated with the negatively charged SDS molecules (at 1.4g/g protein), migration during electrophoresis will depend solely on molecular weight.

The pore size in polyacrylamide gels is determined by the total acrylamide concentration and the amount of cross-linking agent (N,N'-Methylene-bis-acrylamide).

The resolving gel was 10-16% polyacrylamide depending on the molecular weight of the protein of interest.

Table [2.1] shows a general guide for %T needed to sieve proteins of differing sizes.

\[
% T = \frac{([\text{Acrylamide (g)} + \text{Bis-Acrylamide (g)}] \times 100)}{100\text{ml}}
\]

\[
% C = \frac{\text{Bis-Acrylamide (g)} \times 100}{\text{Acrylamide (g)} + \text{Bis-Acrylamide (g)}}
\]

<table>
<thead>
<tr>
<th>% T</th>
<th>molecular weight of proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>15%</td>
<td>12000-45000</td>
</tr>
<tr>
<td>10%</td>
<td>16000-70000</td>
</tr>
<tr>
<td>5%</td>
<td>60000-200000</td>
</tr>
</tbody>
</table>

Table [2.6A] General guide for acrylamide concentrations required to resolve proteins of varying molecular weights.
Ammonium persulphate and N, N, N’, N’-tetramethylethylene-diamine (TEMED) were added just before pouring each gel layer to catalyse polymerisation. Polymerisation is catalysed by free radicals generated by ammonium persulphate. The formation of free radicals from ammonium persulphate is catalysed by TEMED. Before addition of these final two components, the gel solutions were degassed. The plug gel was poured into the trough and taken up to about 1cm by capillary action. At room temperature this took 15-30 minutes to polymerise. The resolving gel was poured on top of the plug and overlaid with water saturated butanol. On polymerisation, this was poured off and the top of the gel was washed with water. A 4% polyacrylamide stacking gel was poured on top of the resolving gel and the comb (0.75mm thick, 10 tooth) inserted, ensuring there was a 0.5cm gap between the bottom of the comb and the top of the resolving gel.

[2.6.2] Reagent preparation

Acrylamide solution

Acrylamide 48g
N,N’-Methylene-bis-acrylamide 1.5g

Dissolve in water to a final volume of 100ml.

Gel Buffer

Trizma base 36.34g
Sodium dodecyl sulphate 0.30g

Dissolve in 60ml water. Adjust pH to 8.45 with concentrated HCl.

Anode buffer

Trizma base 121.1g
Dissolve in 1L water. Adjust to pH 8.9 with concentrated HCl. Make up to final volume of 5L with water.

**Cathode buffer**

- Trizma base: 12.11g
- Tricine: 17.92g
- Sodium dodecyl sulphate: 1.0g

Dissolve in 1L water. The pH of the solution should be approximately 8.2.

**Fixative solution**

- Methanol: 50ml
- Glacial acetic acid: 10ml

Make up to 100ml with water.

**Staining solution**

- Coomassie Brilliant Blue: 50mg

Dissolve in 200ml 10% glacial acetic acid. Stir for 30 minutes and filter before use.

**Destaining solution**

- Glacial acetic acid: 100ml

Make up to 1L with water.

**Ammonium Persulphate solution**

Dissolve 100mg in 1.0ml water. Prepare fresh daily.

*For each gel*: 30μl Ammonium Persulphate solution and 3μl TEMED were added.
<table>
<thead>
<tr>
<th></th>
<th>Stacking gel</th>
<th>Resolving gel</th>
<th>Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4% T 3% C</td>
<td>10% T 3% C</td>
<td>16% T 3% C</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>0.3ml</td>
<td>2.0ml</td>
<td>3.3ml</td>
</tr>
<tr>
<td>Gel Buffer</td>
<td>1.0ml</td>
<td>3.3ml</td>
<td>3.3ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>1.1ml</td>
<td>1.1ml</td>
</tr>
<tr>
<td>Water</td>
<td>2.7ml</td>
<td>3.6ml</td>
<td>2.3ml</td>
</tr>
</tbody>
</table>

Table [2.6B] Gel recipes for SDS-PAGE.

[2.6.3] Preparation of protein samples for loading

Samples were heated at 95°C for 5 minutes in an equal volume of 2 x sample buffer containing 0.125mM Tris.HCl at pH 6.8, 2mM EDTA, 6% SDS, 20% glycerol, 0.025% bromophenol blue. β-mercaptoethanol was added to 5% just before use.

[2.6.4] Electrophoresis

The gel was electrophoresed at 20mA (current limiting) until the dye front moved into the resolving gel. The current was then raised to 40mA until the dye front had reached the bottom of the gel.

[2.6.5] Fixing, Staining and Destaining

The gel was first fixed in the fixative solution for 30 minutes and then transferred to the staining solution for 2 hours. The gel was then destained in two successive washes of destaining solution over 4 hours, with gentle shaking.
[2.7] Marker Enzyme Assays

[2.7.1] Malate dehydrogenase – mitochondrial matrix marker

Malate dehydrogenase oxidises malate to form oxaloacetate.

\[
\text{malate} + \text{NAD}^+ \rightleftharpoons \text{oxaloacetate} + \text{NADH} + \text{H}^+
\]

The decrease in absorbance at 340nm as NADH is converted to NAD\(^+\) was measured spectrophotometrically. 50mM phosphate buffer at pH 7.3 was mixed with 5\(\mu\)g rotenone from a 1 mg/ml stock solution in ethanol and a small aliquot of the protein sample in a 4ml glass cuvette. The reaction was started by adding 0.1mM NADH followed by 0.5mM oxaloacetate.

[2.7.2] Monoamine oxidase – outer mitochondrial membrane marker

Monoamine oxidase oxidatively removes amino groups. The assay is based on the oxidation of benzylamine to benzaldehyde. The reaction is monitored by measuring the absorbance change at 250nm spectrophotometrically.

Cuvettes contained (3ml final volume) 50mM K\(_2\)PO\(_4\) buffer pH 7.2, and protein sample (test cuvette); reactions were started with 4mM benzylamine. The contents of both cuvettes were mixed and the test read against the reference at room temperature.

[2.7.3] Adenylate kinase – mitochondrial inter-membrane space marker

Adenylate kinase (AK) catalyses the interconversion of AMP, ADP and ATP.

\[
\text{AMP} + \text{ATP} \rightleftharpoons \text{ADP} + \text{ADP}
\]

The assay is based on the measurement of NADP reduction (an increase in absorbance at 340nm) on the formation of 6-phosphogluconate from glucose and
ATP in the presence of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH).

\[ 2\text{ADP} \rightleftharpoons \text{ATP} + \text{AMP} \]
\[ \text{ATP} + \text{glucose} \rightleftharpoons \text{glucose-6-phosphate} \]
\[ \text{glucose-6-phosphate} + \text{NADP} \rightleftharpoons 6\text{-phosphogluconate} + \text{NADPH} \]

Buffer containing 50mM KCl and 10mM KH$_2$PO$_4$ was added to a 4ml glass cuvette along with 1.5mM MgCl$_2$, 10mM glucose, 1mM NADP, 5 units HK and 20 units G6PDH. The reaction was started by adding 2mM ADP.

[2.7.4] *Succinate - cyt c reductase - inner mitochondrial membrane marker*

Succinate - cyt c reductase catalyses the oxidation of succinate to fumarate with concomitant reduction of cyt c.

\[
\text{complex II} \\
\text{succinate} + \text{cyt c}_\text{ox} \rightleftharpoons \text{fumarate} + \text{cyt c}_\text{red} \\
\text{complex III}
\]

The assay is based on the reduction of cytochrome c which can be monitored by the change in absorbance at 550nm. Phosphate buffer [2.7.2] at pH 7.0 was added to a 4ml glass cuvette along with 25μM cytochrome c, 5μg rotenone, 0.5mM KCN and an aliquot of the sample. The reaction was started by the addition of 10mM succinate.
2.8 Protein Sequencing

Proteins were separated by SDS-PAGE and stained lightly with Coomassie Blue. The protein band of interest was excised and sent to the Ludwig Institute where it was kindly sequenced by Dr. J.J. Hsuan and Dr. N. Totty. In brief, the band was digested by endoproteinase Asp-N (Boehringer). Peptides were recovered by means of sonication and applied to Aquapore AX-300 and OD-300 columns connected in series on a Hewlett-Packard 1090M HPLC system. The columns were developed with a acetonitrile gradient in 0.1% trifluoracetic acid, and peptide elution was monitored by means of diode-array detection (200-600nm). Fractions were collected and applied to a modified Applied Biosystems 477A pulsed-liquid automated sequencer (Totty et al [1992]).

2.9 Amplification of a cDNA library

A sample of rat liver cDNA library in λgt11 was kindly supplied by Dr. E.A. Shepherd (this Dept.). This was amplified before use.

2.9.1 Preparation of plating cells

5ml of sterile 2.5% nutrient broth (Oxoid No.2) containing 0.2% maltose and 5mM MgCl₂ was inoculated with an appropriate E.coli strain. After incubation overnight at 37°C in a rotary shaker, the cells were spun down by centrifugation in an Eppendorf centrifuge 5412, at full speed for 2 minutes, and then resuspended in SM buffer containing 0.1M NaCl, 8mM MgSO₄, 0.05M Tris.HCl at pH 7.5.
Plating cells were prepared fresh daily.

[2.9.2] Preparation of phage plate lysate

Preliminary experiments were carried out to determine phage titre. For the most efficient yield of phage DNA, the plates had to be prepared such that the outer edges of the plaques just touched each other. This was referred to as a 'standard' plate. This type of plate contained approximately 4000 plaques. A small aliquot of library (approximately 10μl) was mixed with 100μl plating cells in a sterile tube. To this was added 3.5ml molten top nutrient agar (0.7% agar, 2.5% nutrient broth) prewarmed to 50°C. After brief mixing this was immediately poured on to a 90mm plate containing 20ml hardened bottom nutrient agar (2% agar, 2.5% nutrient broth). Top nutrient agar was distributed evenly by gentle swirling of plate. This was left to set for 10 minutes at room temperature and then incubated overnight at 37°C.

The plate was cooled at 4°C for 1 hour and then 5ml of SM buffer was added to the surface. The plate was incubated at room temperature for 4 hours with gentle, intermittent shaking. The phage suspension was harvested by tilting the plate and aspirating and was placed in a sterile tube. 100μl chloroform was added and the suspension shaken before centrifugation at 4000g for 10 minutes at 4°C to pellet debris. The supernatant was transferred to a fresh tube and stored at 4°C with a drop of chloroform. It can be stored at -70°C after addition of dimethylsulfoxide (DMSO) to 7%.
**[2.9.3] Preparation of bacteriophage \( \lambda \) DNA**

The lysate from one plate was centrifuged at 4000g for 10 minutes at 4°C to remove debris. 1µl each of RNase A (1mg/ml) [2.16.3] and deoxyribonuclease I (1mg/ml) were added to the supernatant. This was incubated for 15 minutes in a 37°C water bath. An equal volume of a solution containing 20% polyethylene glycol 8000 and 2M NaCl was added and the suspension was mixed gently. This was incubated on ice for 1 hour. Precipitated phage were recovered by centrifugation at 10000g for 10 minutes at 4°C. The supernatant was poured away and the tube was left to stand inverted on a paper towel until all the fluid had drained away. Phage were then resuspended by vortexing in 0.5ml TE buffer containing 10mM Tris.HCl, 1mM EDTA, 20µg/ml RNase A [2.16.3] at pH 8.0. SDS was added to 0.1% and incubated for 5 minutes at 68°C. NaCl was added to 0.1M. The DNA was then extracted once with phenol/chloroform (see below) and once with chloroform alone. An equal volume of isopropanol was added to the aqueous layer, mixed and placed at -70°C for 15 minutes. DNA was recovered by centrifugation at full speed in an eppendorf centrifuge 5412 for 15 minutes at 4°C. The pellet was washed with 70% ethanol and air-dried for 5-10 minutes. DNA was dissolved in 50µl TE buffer.

**[2.10] The Polymerase Chain Reaction (PCR)**

**[2.10.1] PCR**

PCR enables amplification of an area of DNA that lies between two areas of known DNA sequence. Amplification is achieved by using oligonucleotide
primers. These are short, single-stranded DNA molecules complementary to the ends of the sequence on the DNA template which is to be amplified. The primers are extended by a DNA polymerase in the presence of deoxynucleoside triphosphates (dNTPs). Strand synthesis is repeated by heat denaturation of the double-stranded DNA, annealing of primers by cooling the reaction and extension of primers at a suitable temperature for the DNA polymerase.

The basic components for PCR are: DNA template, primers, enzyme, dNTPs, and buffer. These are then placed in a thermal cycler for amplification.

[2.10.2] **DNA template**

This was in the form of a rat liver cDNA library in bacteriophage λgt11. DNA from library amplification (see [2.9]) was diluted by a tenth before addition to the PCR reaction.

[2.10.3] **Primers**

Primers are usually designed to be exactly complementary to the template DNA. The PCR primers designed were between 20-30 nucleotides long to enable a reasonably high annealing temperature. Areas of unusual sequence or sequences with significant 2° structure were avoided. It was ensured there was no complementarity either inter or intra the individual primers.

To amplify an uncloned gene it is possible to back-translate from the protein sequence. The amplified DNA is then used as a probe to screen a suitable library for the whole gene.

Designing degenerate primers was complicated by the fact that most amino acids are encoded by more than one codon. When these primers were designed it was
done such that there was a bias for areas of the template where there were amino acids encoded by only one or two codons.

Primers were ordered from Pharmacia Biotech and arrived lyophilised. They were dissolved in water to a 10μM working solution.

An approximate annealing temperature for the primers was calculated by the formula:

\[(\text{number of A + T}) \times 2^\circ C + (\text{number of G + C}) \times 4^\circ C\]

where A, C, G and T are the four bases.

A value 5-10°C below the average value for the two primers was used as a starting point to determine the optimum annealing temperature.

[2.10.4] *Enzyme*

There are a number of commercially available thermostable DNA polymerases but throughout the course of this project, Pfu DNA polymerase was used for PCR. It has both 5' to 3' DNA polymerase activity and also 3' to 5' exonuclease proof-reading activity. This latter characteristic increases the accuracy of DNA synthesis as it excises incorrectly added, mis-matched 3'-terminal nucleotides from the primer/template and then adds the correct nucleotide. It is essential to add the enzyme last, as in the absence of dNTPs the exonuclease activity of the enzyme causes degradation of primer and template DNA.

[2.10.5] *Deoxynucleotide triphosphates (dNTPs)*

dNTPs (Utrapure dNTP Set, Pharmacia Biotech) were supplied as 4 individual 100mM stock solutions. Small aliquots were mixed together and diluted to 2mM before use in PCR.
[2.10.6] Buffer

Supplied as a 10 times concentrate with Pfu DNA polymerase. Mg\(^{2+}\) ions are an essential component as they form a soluble complex with dNTPs which is required for dNTP incorporation. The amount of Mg\(^{2+}\) ions may need to be varied to determine optimal concentration for maximum product yield.

[2.10.7] Typical PCR reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA template</td>
<td>2.5(\mu l) (0.1ng-1ng)</td>
</tr>
<tr>
<td>Primers 1 &amp; 2</td>
<td>2.5(\mu l) (25 pmols of each)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>1(\mu l) (2.5 units)</td>
</tr>
<tr>
<td>dNTPs</td>
<td>2.5(\mu l) (20(\mu )mols of each)</td>
</tr>
<tr>
<td>Buffer</td>
<td>5(\mu l) (10 times concentrate)</td>
</tr>
</tbody>
</table>

Volume made up to 50\(\mu l\) with water.

[2.10.8] PCR conditions

The most common set of PCR conditions used during the project was:

- Denaturation temperature: 94°C
- Annealing temperature: 60°C
- Extension temperature: 72°C

for the 30 cycles on a Biometra Personal Cycler with a heated lid.
DNA Agarose Gel Electrophoresis

DNA was analysed on 0.5-2% agarose gels prepared from molten agarose made up in electrophoresis buffer containing 100mM Tris base, 90mM Boric acid, 10mM Na$_2$EDTA at pH 8.3 (TBE buffer). The molten gel was poured into the horizontal gel electrophoresis apparatus (Horizon® 11.14 Gibco BRL) as according to manufacturers instructions, ensuring the correct well-forming comb for the volume of sample to be loaded was inserted. The agarose concentration of the gel was chosen depending on the size of the fragments of interest. Large DNA fragments require low percentage gels. Generally, for fragments between 0.5 and 10kbp, a 1% gel is adequate. DNA samples were prepared for electrophoresis by addition of 0.1 volume of 10 x sample loading buffer containing 50% glycerol, 100mM Na$_2$EDTA, 1% (w/v) SDS, 0.1% (w/v) bromophenol blue, and immediately loaded. Gels were electrophoresed at 90mA (current limiting). The DNA was visualised by staining for 30 minutes in 0.5µg/ml ethidium bromide in water and placing on a transilluminator and viewing on UV light. Gels were photographed on Ilford film for a permanent image. DNA was approximately quantified by comparison with known amounts of marker fragments generated from restriction digests by the enzyme Pst I of λDNA which range from 0.16-14.16 kbp.
[2.12] *Purifying DNA from agarose gels (the Geneclean II® kit, BIO 101 Inc.)*

The procedure is based on the elution of DNA by binding to a silica matrix termed GLASSMILK® by the manufacturers.

The DNA band of interest was excised from the ethidium bromide-stained gel with a scalpel, cut into 2mm cubes and put in a tube. 4.5 volumes of sodium iodide solution and 0.5 volumes of TBE modifier (for agarose gels made up with TBE buffer) where 1g equals 1ml. Both reagents are provided in the kit. The tube was placed in a water-bath at 45-55°C for approximately 5 minutes with occasional mixing until the gel had completely dissociated. 5µl of glassmilk was added (provided with kit) and incubated at room temperature for 15-30 minutes. The glassmilk was spun down by centrifugation for approximately 5 seconds in an eppendorf centrifuge. The supernatant was removed and the pellet washed 3 times with NEW (a wash solution provided as a concentrate with the kit). DNA was eluted from the glassmilk with 10-20µl water or TE buffer. The yield was usually assessed by analysing a small amount of product on an agarose gel.

[2.13] *Cloning DNA into vectors*

Plasmids are small, circular DNA molecules that can exist within bacterial cells.

Bacteriophages (or phages) are viruses that specifically infect bacteria. They consist of a molecule of DNA (or occasionally RNA) surrounded by a protein coat.
Both plasmids and bacteriophages share essential characteristics of a cloning vehicle: they are small and are able to survive and replicate independently of their host.

Recently, plasmids and phages have been combined to form phagemid vectors. They contain a plasmid and a filamentous phage replication origin. They can be handled as plasmids but can be induced to package and release themselves into culture by infection with a helper phage.

[2.13.1] **Cloning into a phagemid vector by ligation of ‘sticky-ended’ DNA fragments**

This is an efficient procedure as compatible sticky-ends can base pair by hydrogen bonding with each other forming a stable complex for the ligase enzyme to work on.

The vector used during this project for this type of cloning was the pBluescript™ II SK+ phagemid.

[2.13.2] **Cloning into a phagemid vector by ligation of ‘blunt-ended’ DNA fragments**

PCR reactions usually produce blunt-ended DNA fragments which are not as efficiently ligated into vectors as sticky-ended fragments as they have to wait for chance associations to bring the ends together.

pCR-Script (provided in the pCR-Script™ Amp SK(+) Cloning kit from Stratagene), derived from the pBluescript phagemid, was the vector used for blunt-ended cloning. A restriction enzyme included in the ligation mix, *Srf I*
(provided with kit), acting on a very rare restriction site, maintained a high-steady-state concentration of digested vector DNA.

[2.13.3] Ligation

0.2μg of vector DNA was digested with the appropriate restriction enzyme to generate ends compatible to the DNA fragment to be inserted. After purification from the digestion mix by precipitation [2.14], vector was mixed with insert in a 2:1 ratio of insert:vector, measured in available picomole ends.

This was calculated by the formula:

\[
\text{picomole ends} / \mu g \text{ of DNA} = \frac{2 \times 10^6}{(\text{number of base pairs} \times 660)}
\]

The volume was reduced to 5μl by precipitation [2.14] and resuspension in water.

The ligation reaction consisted of the following: (*provided with kit)

- 5μl digested vector + insert
- 1μl ligase buffer*
- 0.5μl rATP (10mM)*
- 1μl T4 DNA ligase (4units/μl)*
- Water up to 10μl

This was placed at room temperature for 2 hours.

[2.13.4] Transformation

2μl of the ligation mix from [2.13.2] was used to transform Epicurian Coli® XL1-Blue MRF’ Kan supercompetant cells (provided with kit). A 40μl aliquot of the cells in Falcon 2054 tubes was thawed on ice and β-mercaptoethanol was added to 25mM. Cells were left on ice for a further 10 minutes. 2μl of ligation mix
was added and cells were left on ice for a further 30 minutes. This was followed by a heat pulse in a 42°C water bath for 45 seconds and then the cells were placed on ice for 2 minutes. 0.45ml of nutrient broth at 42°C was added and the mixture incubated at 37°C for 1 hour in a rotary shaker.

[2.13.5] **Plating**

Approximately 30 minutes before plating of transformed cells, 20μl 0.2M IPTG (isopropyl-thiogalactoside) and 20μl 10% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) had to be spread on nutrient agar plates containing 20μg/ml ampicillin. 50μl, 100μl, 150μl and 190μl of the transformation mixture was spread onto the previously prepared plates, and incubated overnight at 37°C.

[2.13.6] **Screening**

[2.13.6.1] **Transformation** - Plasmid vectors usually carry a selectable marker to determine cells that have been transformed. pBluescript carries the gene for ampicillin resistance. Selection is achieved simply by plating cells on to nutrient agar containing ampicillin.

[2.13.6.2] **Recombinants** - Recombinants are usually selected for by the insertional inactivation of a further selectable marker. The new DNA fragment destroys one of the genes on the plasmid. pBluescript carries the gene lacZ' which codes for part of the enzyme β-galactosidase. Some *E.coli* strains lack this part of the β-galactosidase gene and can synthesise the enzyme only when containing plasmid. However, if the plasmid has had its lacZ' gene disrupted by insertion of DNA within the gene, the enzyme cannot be synthesised. Screening
for this enzyme involves the lactose analogue X-gal which is broken down by
the enzyme to a blue product. An inducer of the enzyme such as IPTG should
also be present. Non-recombinant cell colonies are blue and recombinants are
white.

Screening for recombinant clones without insertional inactivation of a selectable
marker can be achieved by a variety of methods:

1. Rapid plasmid DNA preparation for identification of recombinants on basis of
   size.

   Potential recombinant colonies were resuspended in 40μl cell disruption buffer
   buffer (10mM Tris/HCl, 100mM NaCl, 10mM EDTA, pH 8.0) in a 1.5ml
   eppendorf tube. 50μl phenol/chloroform (1:1) was added and the tube vortexed
   for 1 minute. Sample was microcentrifuged for 5 minutes. 1μl of a 1mg/ml RNase
   A solution was added to the supernatant and incubated for 10 minutes at room
temperature. Sample was run on a 1% agarose gel (see [2.11]). Plasmids
   containing insert were identified by comparing mobility alongside similarly
   prepared non-recombinant plasmid DNA.

2. PCR.

   If primers for the ends of the insert are available, a standard PCR reaction can be
   used to identify recombinants. A tiny amount of a potential recombinant colony
   was added to a tube containing the rest of a typical PCR reaction reagents (minus
   template, see [2.10.7]). This was run under common PCR conditions (see
   [2.10.8]) with an initial 5 minute incubation at 94°C to effect cell lysis. PCR
   products were analysed by electrophoresis. Recombinants were identified by
   amplification of the insert.
3. Restriction analysis.

DNA from potential recombinant colonies was prepared (see [2.16.1]). A pair of restriction sites within the insert or at each side of the insert in the plasmid were digested. The resulting products(s) were analysed to determine whether colonies were recombinant.

[2.14] Precipitation / Concentration of DNA

Sodium acetate pH 6.0 was added to a final concentration of 0.4M, followed by 2 volumes of ethanol. This was vortexed and placed at -20°C for 15 minutes. The precipitated DNA was spun in a centrifuge for 10 minutes at full speed. The supernatant was removed and the pellet washed with ethanol. This was spun for a further 2 minutes and after removal of the ethanol, was allowed to air-dry for a few minutes.

[2.15] DNA sequencing

Plasmid DNA sequencing was carried out to validate fragments generated by PCR and clones isolated from the cDNA library. Sequencing reactions were based on the dideoxy method of Sanger et al [1977] which is dependant on base-specific termination of enzyme-catalysed primer-extensions. Four reactions are performed, each containing one of four chain-terminating dideoxynucleotides in addition to the four deoxynucleotides. Dideoxynucleotides are incorporated just as efficiently as normal nucleotides but as they lack a hydroxyl group at the 3' position of the sugar component, further nucleotides cannot be added. DNA chains of a mixture
of lengths are generated, each terminated by the dideoxynucleotide present in the reaction. The position of chain termination represents the occurrence of the corresponding deoxynucleotide. The products of the four reactions are electrophoresed side-by-side so the sequence can be deduced. Fragments increase in length by one nucleotide successively. They are visualised by introduction of a label into the fragments by including a radioactive deoxynucleotide \((^{35}S\text{-dATP})\) into the reaction mixture at the beginning of the experiment.

The electrophoresis gels are very thin for better resolution. They contain urea to denature DNA, ensuring newly synthesised strands dissociate from the template. Gels are electrophoresed at high voltages so that high temperatures are generated (50-60°C) also ensuring strands do not reassociate.

DNA sequencing was carried out using the T7 Sequencing™ Kit (Pharmacia Biotech) *indicates reagents provided with the kit [2.15.1-2.15.3].

[2.15.1] 

Annealing of primer to template

Primers are complementary to a site on the plasmid adjacent to the cloned insert.

Two sets of reactions were set up for each template using the T3 20-mer Primer (Stratagene) to sequence 3'→5' on the positive strand, and the KS 17-mer Primer to sequence 3'→5' on the negative strand.

40μl containing 1.5-2μg template DNA was precipitated in the usual way [2.14] and resuspended in 10μl water. Primer and annealing buffer were added to the resuspended template as follows:
Template DNA  10μl  (1.5-2μg)
Primer                  2μl  (5-10pmol)
Annealing buffer*  2μl

Tubes were mixed, centrifuged, and incubated at 65°C for 5 minutes then quickly transferred to a 37°C water bath for a 10 minute incubation. This was followed by placing at room temperature for 5 minutes and centrifugation.

The composition of the annealing buffer was 1M TrisHCl pH 7.6, 100mM MgCl₂ and 160mM DTT.

[2.15.2] **Labelling reaction**

The primer undergoes enzyme-catalysed extension in the presence of limiting concentrations of the four deoxynucleotides, one of which (dATP) is labelled with $^{35}$S.

To the sample obtained from [2.15.1], the following were added:

Annealed template/buffer [2.15.1]  14μl
Labelling mix*                  3μl
Labelled $^{35}$S-dATP        1μl  (12.5μCi)
T7 DNA polymerase               2μl  (3.2 units)

This was mixed and incubated at room temperature for 5 minutes.

The composition of the labelling mix was 1.375μM each of dCTP, dGTP and dTTP and 333.5mM NaCl. The polymerase was dissolved in buffered glycerol solution.
[2.15.3] Termination reaction

The primer which has now been labelled and extended is terminated in four separate reactions, each containing a specific dideoxynucleotide in addition to non-limiting concentrations of all four deoxynucleotides.

4.5μl of the sample obtained in [2.15.2] was transferred to 2.5μl of each of the four pre-warmed (to 37°C) sequencing mixes*. This was mixed and incubated at 37°C for 5 minutes. 5μl of Stop Solution* was added to each of the four samples and mixed.

The compositions of the sequencing mixes were as follows:

'\text{A}' Mix: 840μM each dCTP, dGTP and dTTP; 93.5μM dATP; 14μM ddATP; 40mM TrisHCl pH 7.6 and 50mM NaCl. '\text{C}' Mix: 840μM each dATP, dGTP and dTTP; 93.5μM dCTP; 17μM ddCTP; 40mM TrisHCl pH 7.6 and 50mM NaCl.

'\text{G}' Mix: 840μM each dATP, dCTP and dTTP; 93.5μM dGTP; 14μM ddGTP; 40mM TrisHCl pH 7.6 and 50mM NaCl. '\text{T}' Mix: 840μM each dATP, dCTP and dGTP; 93.5μM dTTP; 14μM ddTTP; 40mM TrisHCl pH 7.6 and 50mM NaCl.

The composition of the Stop Solution was 0.3% each Bromophenol Blue and Xylene Cyanol FF, 10mM EDTA pH 7.5 and 97.5% deionized formamide.

[2.15.4] Electrophoresis (using the sequencing gel electrophoresis apparatus, Model S2, Gibco BRL)

Preparation of gel

Two glass plates (one long, one short) were cleaned with soap, rinsed with water and dried. This was followed by rinsing with ethanol and drying. The small glass plate was then swabbed with Silane (water-repellent) and allowed to dry. The
A glass plate sandwich was assembled with the silane-treated side facing inwards and the plate separated by 0.4mm thick spacers. The plates were taped together, and the following gel solution prepared:

- 20ml 40% acrylamide (acrylamide : N,N'-methylenebisacrylamide, 19:1)
- 10ml 10x TBE buffer
- 50ml 46.7% urea solution
- 20ml water

NB. The urea solution was prepared in advance by dissolving 467g of urea in distilled water to a final volume of 1 litre. 20-30g Amberlite MB-3 was added and stirred for 30 minutes. This was filtered and the solution at room temperature until use.

After degassing, gel formation was initiated by addition of 0.5ml 10% ammonium persulphate solution and 100μl TEMED. The solution was immediately poured between the 2 plates and a surface former (reverse side of a 0.4mm shark's tooth comb) was inserted at the top. The gel was left at a 45° angle for 1 hour to polymerise. After removal of the surface former, the surface was immediately rinsed with distilled water to remove any unpolymerised acrylamide. The gel was then placed in the electrophoresis apparatus and TBE buffer added, ensuring the sample wells were filled. The gel was pre-run for 45-60 minutes at 40W constant power. The power was switched off and the shark's tooth comb inserted until the points just touched the surface of the gel. 1.5-6μl of the samples obtained from the termination reactions [2.15.3] were heated for 2 minutes at 75-80°C and immediately loaded into appropriate wells of the gel in sets of four. The gel was reconnected to the power supply and electrophoresed at 40W constant power. If additional samples from the same sequencing reactions were loaded, this was done...
when the xylene cyanol in the last sample loaded was 4-5cm from the bottom. The power supply was switched off and the adjacent group of four empty wells were washed out before loading. The final period of electrophoresis was stopped when the bromophenol blue in the samples loaded last reached the bottom of the gel.

[2.15.5] Autoradiography and Analysis

After switching off the power supply, the gel/plate assembly was removed from the apparatus and laid horizontal. The plates were carefully separated so that the gel remained attached to one of them. The gel/plate was placed in a tray containing 10% glacial acetic acid / 10% methanol in distilled water for 30 minutes and the solution was then removed by aspiration. After rinsing with water the gel was transferred to a supporting sheet of filter paper (Whatman, 3mm). This was covered with Saran wrap and the gel dried using a vacuum gel dryer. This took approximately 2 hours, after which the Saran wrap was removed and the gel on its filter paper support was transferred to a film cassette. A sheet of X-ray film was laid over the gel in a darkroom, and exposed overnight.

[2.16] Preparation of plasmid DNA from transformed cells


5ml of nutrient broth was inoculated with a loop-full (one or two colonies) of transformed E.coli cells. This was incubated overnight (>7 hours) at 37°C in a rotary shaker. 1.5ml was pipetted into an eppendorf and microcentrifuged for 2 minutes. The supernatant was removed and the pellet was resuspended in 100μl resuspension buffer (100μg/ml RNase A, 50mM Tris/HCl, 10mM EDTA pH 8.0)
by vortexing and then placed at room temperature for 5 minutes. 200μl lysis buffer (200mM sodium hydroxide, 1% SDS) was added and the tube inverted repeatedly to mix. 150μl neutralisation buffer (3M sodium acetate pH 5.5) was added and the tube vortexed briefly and placed on ice for 5 minutes. This was followed by a 5 minute spin and the supernatant transferred to a fresh tube. An equal volume of phenol/chloroform (1:1) was added, vortexed to mix and spun for 2 minutes to separate the phases. The upper layer was transferred to a fresh tube, 1ml ethanol was added and the tube mixed thoroughly and placed on ice for 5 minutes. This was followed by a 10 minute spin. The supernatant was discarded and the pellet washed with ethanol and air-dried. The DNA was redissolved in either water or TE buffer (10mM Tris/HCl, 1mM EDTA, 100μg/ml RNase A pH8.0)

[2.16.2] QIAprep method using the QIAprep Spin Plasmid Kit (*indicates reagents supplied with kit).

1-5ml of an overnight culture of transformed *E.coli* cells in nutrient broth was microcentrifuged for 2 minutes. The pelleted bacterial cells were suspended in 250μl of Buffer P1*. 250μl Buffer P2* was added and the tube inverted to mix. 350μl of Buffer N3* was added and the tube inverted to mix, and then spun for 10 minutes. The supernatant was applied to a QIAprep column* and this was spun for 30-60 seconds. The flow-through was discarded. The column was washed by adding 0.5ml Buffer PB* and spinning for 30-60 seconds, discarding the flow-through. A further wash was carried out by adding 0.75ml Buffer PE* and spun for 30-60 seconds. The flow-through was discarded and the column spun for an additional 60 seconds to remove residual wash buffer. The column was placed in a
fresh 1.5ml eppendorf and the DNA eluted with either water or TE buffer [2.16.1] by adding 50-100μl to the column, letting it stand for 60 seconds and then spinning for 60 seconds, collecting the flow-through.

The compositions of the buffers provided with this kit were not given.

[2.16.3] **Preparation of DNase free RNase solution**

A 20mg/ml stock solution of pancreatic RNase A (Sigma) was prepared using sterile distilled water and heated to 100°C for 10 minutes. This inactivates any contaminating DNases. The stock solution was stored at -20°C until use.

[2.16.4] **Preparation of buffer-saturated phenol for phenol/chloroform**

To a 500g bottle of phenol (Analar), the following were added: 50ml 1M TrisHCl pH 7.5, 20ml disodium EDTA pH 8.0, and 200ml distilled water. The mixture was shaken to dissolve the phenol crystals and mix the buffer thoroughly. An aliquot of the (lower) phenol layer was added to an equal volume of chloroform just before use. The remaining buffer-saturated phenol was stored at -20°C.

[2.17] **Labelling DNA for use as a probe to screen a cDNA library** using the RadPrime DNA Labelling System, GibCO BRL (* indicates reagents provided with kit)

25ng of DNA was dissolved in 20μl TE buffer and denatured by heating for 5 minutes in a boiling water bath, then immediately cooled on ice. The following additions were performed on ice:
1μl 10mM dATP
1μl 10mM dGTP
1μl dTTP
20μl 2.5 x Random Primer Solution
5μl (approximately 50μCi) α-32P dCTP
made up to 49μl with water

After brief mixing, 1μl Klenow fragment was added. The sample was mixed again, microcentrifuged briefly and incubated at 37°C for 10 minutes. This was heated for 5 minutes in a boiling water bath before adding to 50ml pre-hybridisation solution (6 x SSC solution, 5 x Denhardts solution, 0.5% SDS and 100μg/ml denatured DNA).

20 x SSC solution
21.5g NaCl was added to 63g citric acid and made up to 250ml with water. pH was brought to 7 with NaOH.

50 x Denhardt's solution
5g Ficoll (Type 400, Pharmacia) was added to 5g polyvinyl pyrrolidone and 5g BSA. This was made up to 500ml with water and stored in 50ml aliquots at -20°C until use.

Denatured DNA
10mg/ml salmon sperm DNA solution was heated for 10 minutes in a boiling water bath and stored at -20°C. Before use, solution was heated again for 5 minutes in the same way and chilled on ice.
[2.18] Screening of a cDNA library in bacteriophage

The cDNA library used in this project was in λgt11. Phage plaques on nutrient agar plates were prepared as described earlier ([2.9.2]). There were 50-4000 plaques/plate and 2-20 plates depending which round of screening was being attempted. Pre-titration was necessary to obtain the desired number of plaques/plate. Plates were cooled for 1 hour at 4°C to harden top agar.

Nitocellulose filters were placed on to the surface for 15 minutes, during which filter orientation was marked uniquely and non-symmetrically. 3 stacks of 3 sheets of 3mm Whatman filter paper was arranged. The first stack was saturated with solution 1 (5g NaOH and 21.9g NaCl in 250ml water), the second stack was saturated with solution 2 (30.3g Tris/HCl and 21.9g NaCl in 250ml, pH 7.5) and the third stack with solution 3 (2 x SSC, see[2.17]). After the 15 minutes adsorption was completed, filters were laid plaque side up on the first stack for 10 minutes, the second stack for 10 minutes and the third stack for 5 minutes. Filters were dried by blotting on clean filter paper and cross-linked in a UV oven at 70,000 joules.

Up to 8 nitrocellulose filters, separated by nylon sheets, were placed in a screw-top glass tube. The pre-hybridisation solution containing the probe (from [2.17]) was added and the filters incubated at 60°C in a rotary oven overnight. The filters were placed side-by-side between 2 sheets of Saran wrap in a film cassette. A piece of X-ray film was laid over the Saran wrap and left to expose overnight at room temperature.

Positive plaques were identified and removed from the plates by ‘plugging’ with the end of a glass pasteur pipette and placing into 0.5ml SM buffer [2.9.1] with
10μl chloroform and left for 2 hours at 4°C to allow phage to soak out. Phage were amplified [2.9] and the DNA analysed by sequencing [2.15].

[2.19] Expression of a GST fusion protein using the pGEX system

pGEX plasmid expression vectors contain the glutathione S-transferase (GST) gene with a series of downstream restriction sites (e.g. Bam H1, Eco R1) allowing expression of fusion proteins with GST at the N terminus and the C terminus derived from the cloned insert. Ampicillin resistance is the selectable marker. Fusion protein expression is controlled by the inducible tac promoter. The plasmid contains the lacP gene which confers relatively tight control of the tac promoter via the lac repressor.

Vector and insert DNA were prepared for ligation by digestion with appropriate restriction enzymes to generate compatible sticky ends.

Ligation [2.13.3], transformation [2.13.4] and plating [2.13.5] were carried out. Recombinants were identified by restriction analysis [2.13.6].

Amplification was achieved by inoculation of 5ml nutrient broth with the recombinant colony and incubating overnight at 37°C with shaking. 1ml was then added to 9ml fresh nutrient broth containing 100μg/ml ampicillin. Cells were left to grow at 37°C to mid-log phase (A600~0.6). Expression was induced by adding IPTG at this stage to a final concentration of 0.1mM and allowing cells to grow for a further 3-5 hours.
Purification of fusion protein from bacterial cell lysate

An immobilised glutathione matrix (Sigma G-4510) was obtained as lyophilised powder and swelled in water (200ml/g) for 2 hours. 80mg gave 1ml of matrix, which bound 17μmol glutathione.

Matrix was loaded into narrow glass columns plugged at one end with 40μm polyethylene pore discs. Columns were equilibrated with 10 volumes of equilibration buffer, EB (10mM HEPES / 100mM NaCl / 0.5mM EDTA, pH 7.5). Cells were sedimented by centrifugation, resuspended in EB and sonicated for 30-60 seconds. Cell debris was microcentrifuged into a pellet and the supernatant loaded onto the column. After bacterial cell lysate had passed through, the column was washed with a further 2 volumes of EB and the fusion protein eluted with 20mM reduced glutathione in EB.

Cleavage of the fusion protein

The pGEX-3X plasmid contains a Factor Xa protease recognition site (see Fig. 5.1A for restriction map of pGEX-3X). This enables the enzymatic cleavage of the fusion protein in a simple digestion step. The eluate from the affinity column [2.20] was incubated with Factor Xa for 6 hours at room temperature. The digestion contained 1:5 w/w Factor Xa to the fusion protein, and 1mM CaCl₂.
CHAPTER 3: PURIFICATION & LOCALISATION ANALYSES OF RAT LIVER MITOCHONDRIAL CYCLOPHILINS.

[3.1] Background

Cyclosporin A (CsA) had been found to block the inner mitochondrial permeability transition pore [1.3.2]. Previous work in the laboratory had demonstrated that this blockade by CsA was potentiated by ADP, whereas on the other hand, increasing intramitochondrial Ca\(^{2+}\) caused a decrease in CsA blockade of the pore and CsA binding to mitochondria (Andreeva & Crompton [1994]). These observations provided two potential means of identifying the CsA-binding constituent of the pore (Crompton & Andreeva [1994]). Both criteria, (i.e. Ca\(^{2+}\) and ADP sensitivities) were used to isolate the CsA binding component by utilising a tritiated photoactive CsA derivative to covalently label the CsA binding component. The majority of the photolabelling which occurred in an ADP/Ca\(^{2+}\) sensitive manner was analysed by SDS-PAGE and found to migrate at approximately 22kDa. PPIase activity purified from the same fraction was also found to behave in the same way on SDS-PAGE (Andreeva et al [1995]).

The first stage of this project was to identify the location of this protein in rat liver mitochondria. This information would then be used to purify the protein and characterise it further. As a subsidiary aim, the relation of an 18kDa cyclophilin reported in mitochondrial preparations (Crompton et al [1992], Connern & Halestrap [1992]) was investigated.
[3.2] Fractionation of rat liver mitochondria

The use of digitonin is a standard established means of fractionating mitochondria. Digitonin is built from one digitogenin molecule, two galactose molecules and one xylose molecule. At low concentrations, digitonin forms an insoluble complex with cholesterol, a component not present in the inner membrane. This causes the outer membrane to break-up and come away from the inner membrane, releasing the components of the intermembrane space. However, at higher concentrations, digitonin acts as a general detergent and solubilises both the outer and inner membranes. The variation in composition means relatively low concentrations of digitonin can be used to selectively lyse the mitochondrial outer membrane (releasing the intermembrane space components) and leave the inner membrane (and therefore the matrix) largely intact. At higher concentrations, both membranes are lysed and the contents of the mitochondrial matrix are released (Pedersen et al [1979]).

Membrane markers were required to quantify protein release from the intermembrane space and matrix fractions to monitor the effects of digitonin. Adenylate kinase was chosen as the intermembrane space marker and malate dehydrogenase was chosen as the matrix marker. This was due to the high specific activity of both enzymes and the ease of their measurement.

Fig. 3.2A reports the effects of varying digitonin concentration on the distribution of adenylate kinase, malate dehydrogenase and PPIase in mitochondrial fractions. The results reflect the nature of digitonin to selectively lyse the outer membrane at relatively low concentrations. At 0.2mg digitonin/mg mitochondrial protein, >90% of the mitochondrial adenylate kinase was detectable in the supernatant, suggesting the intermembrane space components have been released by lysis of the
[Fig. 3.2A] Separation of mitochondrial fractions by means of incubation with varying concentrations of digitonin.

Mitochondria in MST were treated with digitonin and centrifuged. The activity of adenylate kinase ○, malate dehydrogenase △ and PPIase ● in the supernatant were expressed as a percentage of their activities in whole mitochondria treated with 30mM CHAPS.
outer membrane. It was not until the digitonin concentration reached 0.4mg digitonin/mg mitochondrial protein that >90% of the malate dehydrogenase activity could be detected in the supernatant indicating release of the matrix components by lysis of the inner membrane. The distribution of PPIase at increasing concentrations of digitonin differed from the release of either marker, prompting the theory that cyclophilin(s) may be present in both compartments. >95% of the PPIase activity detected in the mitochondria was inhibited by CsA.

From Fig. 3.2A it is apparent that the best separation between inner membrane plus matrix (mitoplast) and intermembrane space plus outer membrane was obtained using 0.19mg digitonin/mg mitochondrial protein. Accordingly, this concentration of digitonin was used to investigate the localisation of the cyclophilin(s) in these fractions.

In the experiment described in [3.3], PPIase was isolated from the matrix fraction of rat liver mitochondria after sub-fractionating the mitochondria with 0.19mg digitonin/mg mitochondrial protein as described. In the experiment described in [3.4], PPIase activity was isolated from the intermembrane space fraction of rat liver mitochondria using the same digitonin concentration as described.

[3.3] Purification of cyclophilin from rat liver mitochondrial matrix fraction

A purification procedure encompassing a variety of chromatography columns was employed to isolate any cyclophilin(s) in the matrix fraction of eight rat livers. Mitochondria from the rat livers were prepared [2.1]. After determining the amount of protein [2.2], the mitochondria were separated into matrix and intermembrane space fractions using the digitonin concentration that gave the best separation as determined in Fig. 3.2A [2.3]. The matrix fraction was prepared for
column chromatography as described [2.4.1.2]. This was the starting point for 
matrix cyclophilin isolation (* see Table 3.3A). PPIase activity was measured as a 
marker of the protein of interest. Table 3.3A summarises the purification, 
describing the use of ion exchange and gel filtration columns. These results are 
representative of four such experiments.

Cation exchange resins retain proteins with an overall positive charge. Most 
PPIases have been shown to have an isoelectric point between 4 and 10 (Galat 
[1993]), with the majority being towards 10. In step 2 of the purification 
procedure, S-Sepharose cation exchange chromatography was carried out. This 
was because 90% of mitochondrial protein was found not to bind to this matrix 
(under the conditions described [2.4.2.1]) but all the PPIase activity did. This 
relieved a huge amount of pressure from the sensitive FPLC columns used further 
on in the purification. All the bound protein was eluted off in one step with 0.5M 
NaCl in the running buffer (trace not shown). PPIase activity eluted over 10ml. 
This was dialysed to remove the salt as described [2.4.1.4].

Step 3 employed a mono-S cation exchange column, with the pH of the running 
buffer at 6.7. The column was run as described [2.4.2.2]. PPIase activity eluted 
over 7mls (fractions 21 to 27), between 0.2-0.3M NaCl. Activity peaked in 
fraction 24. The trace from this run is shown in Fig. 3.3A.

Steps 4 and 5 in the purification procedure employed the Superdex-75 gel 
filtration column. This fractionates proteins on the basis of their size. Fig. 3.3B 
shows the traces from Steps 4 and 5. The column was run as described [2.4.2.3]. 
The 7mls containing PPIase activity from Step 3 were pooled, dialysed, freeze-
dried and resuspended in 1ml of the running buffer. This was loaded onto the 
Superdex-75 column (Step 4). PPIase activity eluted over 3mls (fractions 13 to
18). These 3mls were concentrated to 1ml by freeze-drying and loaded onto the Superdex-75 column (Step 5). PPIase activity eluted over 2mls (fractions 14 to 17). PPIase activity peaked in fraction 15 in both cases. This corresponds to a molecular weight of approximately 21kDa. The positions of previously determined molecular markers at 13 and 29kDa are shown.

The final step in this purification (step 6, Table 3.3A) is shown in Fig. 3.3C. Here the trace from the final mono-S column run is displayed. There are two distinct peaks, or sets of peaks. PPIase activity was only detected in the fractions associated with the proteins that eluted at the lower salt concentration (~0.08M). The table accompanying the trace gives the PPIase activities of these fractions. 20% of the fraction containing the majority of the activity (fraction 17) was concentrated by freeze-drying and run on a SDS-PAGE gel. Fig. 3.3D is a photograph of the gel. The protein was loaded onto lane A.

This purification demonstrated the presence of a 21kDa cyclophilin, designated CyP21, in the mitochondrial matrix; the smaller 18kDa cyclophilin that had been reported (see [3.1]) was not detected.

The recovery of the protein at the end of the purification procedure was only 6%. The stability of CyP21 was investigated further. The remaining 80% of the protein from the purification was stored for 14 days at -20°C. This was then thawed on ice and re-run on the mono-S column. The trace from this run is shown in Fig. 3.3E. There was hardly any CyP21 left in the sample. However, the peak that had eluted at ~0.19M salt in the mono-S run of two weeks previous had increased in size. 20% of fraction 31 was concentrated and run alongside the original purified CyP21 (lane B, Fig. 3.3D). A variety of components can be seen, with the principle one running at approximately 11kDa on SDS-PAGE.
<table>
<thead>
<tr>
<th>Step</th>
<th>Treatment</th>
<th>Total protein (mg)</th>
<th>Total PPIase activity (s⁻¹)</th>
<th>Specific activity (s⁻¹mg⁻¹)</th>
<th>Purification fold</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
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<td>685.810</td>
<td>161.3</td>
<td>0.24</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2.</td>
<td>S-Sepharose pH 8.0</td>
<td>101.350</td>
<td>131.3</td>
<td>1.29</td>
<td>5</td>
<td>81</td>
</tr>
<tr>
<td>3.</td>
<td>Mono-S pH 7.7</td>
<td>15.160</td>
<td>78.1</td>
<td>5.15</td>
<td>21</td>
<td>49</td>
</tr>
<tr>
<td>4.</td>
<td>Superdex-75</td>
<td>0.360</td>
<td>57.0</td>
<td>158.33</td>
<td>660</td>
<td>35</td>
</tr>
<tr>
<td>5.</td>
<td>Superdex-75</td>
<td>0.075</td>
<td>30.6</td>
<td>408.00</td>
<td>1700</td>
<td>19</td>
</tr>
<tr>
<td>6.</td>
<td>Mono-S pH 6.7</td>
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<td>10.2</td>
<td>377.78</td>
<td>1574</td>
<td>6</td>
</tr>
</tbody>
</table>
Step 3 of purification of cyclophilin from rat liver mitochondrial matrix fraction as summarised in Table 3.3A.

Protein eluted from the S-Sepharose column (Step 2) was dialysed overnight and run on a mono S column at pH 7.7 as described in [2.4.2.1]. All fractions showing protein at $\text{OD}_{280}$ were assayed for PPIase activity. Activity was detected in fractions 21-27, eluted between 0.2-0.3M NaCl. These values are reported in Table 3.3B.
[Fig. 3.3B] **Steps 4 and 5 of purification of cyclophilin from rat liver mitochondrial matrix as summarised in Table 3.3A.**

Fractions eluted from the mono S column (Step 3) containing PPIase activity were pooled, dialysed, concentrated by freeze-drying and resuspended in 1ml of Superdex 75 gel filtration column running buffer as described [2.4.2.3]. This was loaded onto the column (Step 4). All fractions showing protein at OD$_{280}$ were assayed for PPIase activity. Activity was detected in fractions 13-18. These were concentrated and reloaded (Step 5). PPIase activity was detected in fractions 14-17. Positions of molecular weight markers at 13K and 29K are shown. PPIase values are reported in Table 3.3B.
Step 6 (final step) of purification of cyclophilin from mitochondrial matrix fraction as summarised in Table 3.3A. The fractions containing PPIase activity from the Superdex 75 column (Step 5) were pooled, diluted 10-fold and loaded onto the mono S column at pH 6.7 as described [2.4.2.3]. All fractions showing protein at OD$_{280}$ were assayed for PPIase activity. These values are reported below.

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>PPIase activity (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>1.1</td>
</tr>
<tr>
<td>17</td>
<td>10.2</td>
</tr>
<tr>
<td>18</td>
<td>3.0</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>31</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
</tr>
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</table>
[Table 3.3B] PPIase activity of fractions from steps 3, 4 and 5 of purification procedure summarised in Table 3.3A

Step 3 from Table 3.3A

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>PPIase activity ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>0.5</td>
</tr>
<tr>
<td>22</td>
<td>1.5</td>
</tr>
<tr>
<td>23</td>
<td>10.2</td>
</tr>
<tr>
<td>24</td>
<td>38.3</td>
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<tr>
<td>25</td>
<td>15.4</td>
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<tr>
<td>26</td>
<td>9.0</td>
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<tr>
<td>27</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Step 4 from Table 3.3A

<table>
<thead>
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<th>Fraction number</th>
<th>PPIase activity ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>2.9</td>
</tr>
<tr>
<td>14</td>
<td>8.6</td>
</tr>
<tr>
<td>15</td>
<td>26.0</td>
</tr>
<tr>
<td>16</td>
<td>10.2</td>
</tr>
<tr>
<td>17</td>
<td>5.8</td>
</tr>
<tr>
<td>18</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Step 5 from Table 3.3A

<table>
<thead>
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<th>Fraction number</th>
<th>PPIase activity ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>5.6</td>
</tr>
<tr>
<td>15</td>
<td>16.1</td>
</tr>
<tr>
<td>16</td>
<td>5.0</td>
</tr>
<tr>
<td>17</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Purification of cyclophilin from rat liver mitochondrial intermembrane space fraction

Another purification procedure encompassing a variety of chromatography columns was employed to isolate any cyclophilin(s) in the intermembrane space. After preparation of mitochondria from eight rat livers [2.1] and determining amount of protein [2.2], mitochondria were separated into intermembrane space and matrix fraction using the appropriate digitonin concentration as determined in Fig. 3.2A [2.3]. The intermembrane space fraction was prepared for column chromatography as described [2.4.1.1]. This was the starting point for the isolation of cyclophilin from intermembrane space fraction (* see Table 3.4A). PPIase activity was measured as a marker of the protein of interest. Table 3.4A summarises the purification, describing the use of ion exchange and gel filtration columns. These results are representative of three such experiments.

The total protein in the intermembrane space fraction was passed through a S-Sepharose column as in [3.3] (trace not shown). As observed in the earlier experiment, most of the protein did not bind, but the PPIase activity did. No activity was detected in the passthrough. The activity was eluted in one step with 0.5M NaCl in the running buffer, and eluted it over 8mls. This was dialysed to remove salt.

Step 3 utilised a mono-S column to further purify PPIase from the sample. The trace is shown in Fig. 3.4A. PPIase activity eluted over 6mls (fractions 23 to 28), peaking at fraction 25. This was between 0.2 and 0.35M NaCl. The 6mls were pooled, dialysed, freeze-dried and resuspended in 1ml
[Fig. 3.3D] **SDS-PAGE analysis of purified CyP D and its breakdown products.**

**A.** CyP D - Fraction 17 of Fig. 3.3C (Step 6, Table 3.3A) concentrated by freeze-drying and 20% loaded onto the gel.

**B.** Breakdown products - Fraction 31 of Fig. 3.3E concentrated by freeze-drying and 20% loaded onto the gel.

![Protein markers](image)
[Fig. 3.3E] Analysis of CyPD stability by separation of stored protein on Mono S column.

80% of fraction 17 (CyP D) from Fig. 3.3C (Step 6, Table 3.3A) was stored for 14 days at -20°C. This was then treated and re-run on the Mono S column in an identical manner to Fig. 3.3C.
The 1ml sample from Step 3 was loaded onto the Superdex-75 column (Step 4, Run 1). The traces for step 4, Runs 1-3 are shown in Fig. 3.4B. The fractions containing PPIase activity were pooled, concentrated by freeze-drying and re-run. PPIase activity peaked over fractions 15 and 16 in all three cases. This corresponds to a molecular weight of approximately 18kDa. The positions of previously determined molecular markers at 13 and 29kDa are shown. The fractions from the final run (step 4, Run 3) were concentrated individually and run on an SDS-PAGE gel, a photograph of which is shown in Fig. 3.4C. The protein profile corresponds to the PPIase activity profile, ensuring a single PPIase protein was purified which ran at ~18kDa.

These results indicate that only the smaller 18kDa cyclophilin, designated CyP18, could be detected in the intermembrane space fraction; the larger CyP21 was not visible on SDS-PAGE analysis. Recovery of CyP18 was equally low as with CyP21 (5%) but the degradation products were not as easily identifiable.

[3.5] Marker Enzyme Analyses

To verify CyP21 as a mitochondrial matrix protein, the location of CyP21 in mitoplasts was investigated further. Mitoplasts were obtained in the usual way, i.e. treatment of mitochondria with 0.19mg digitonin/mg mitochondrial protein followed by centrifugation. The supernatant (intermembrane space fraction) was retained. Purification of PPIase yielded an 18kDa protein [3.4]. The pellet (mitoplasts) was suspended in 500mM KCl, 10mM Hepes, 0.5mM EGTA, 1μg/ml protease inhibitors pH 8.0 and centrifuged. The supernatant (intermembrane-side salt wash) was retained. The salt-washed mitoplasts were suspended in the same
**Table 3.4A** Summary of purification of cyclophilin from rat liver mitochondrial intermembrane space fraction.

<table>
<thead>
<tr>
<th>Step</th>
<th>Treatment</th>
<th>Total protein (mg)</th>
<th>Total PPIase activity (s⁻¹)</th>
<th>Specific activity (s⁻¹mg⁻¹)</th>
<th>Purification fold</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
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<td>804.000</td>
<td>36.6</td>
<td>0.05</td>
<td>1</td>
<td>100</td>
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<tr>
<td>2.</td>
<td>S-Sepharose</td>
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<td>25.6</td>
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<td>70</td>
</tr>
<tr>
<td></td>
<td>pH8.0</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Mono-S</td>
<td>22.000</td>
<td>17.1</td>
<td>0.78</td>
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<td>47</td>
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<td></td>
<td>pH7.7</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4i.</td>
<td>Superdex-75</td>
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<td>10.1</td>
<td>20.2</td>
<td>404</td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>ii.</td>
<td>Superdex-75</td>
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<td>2.7</td>
<td>90.0</td>
<td>1800</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>iii.</td>
<td>Superdex-75</td>
<td>0.006</td>
<td>1.8</td>
<td>300.0</td>
<td>6000</td>
<td>5</td>
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</table>
Step 3 of purification of cyclophilin from mitochondrial intermembrane space fraction as summarised in Table 3.4A.

Protein eluted from S-Sepharose column (Step 2) was dialysed and run on a Mono S column at pH 7.7 as described [2.4.2.2]. All fractions showing protein at OD\textsubscript{280} were assayed for PPIase activity. Activity was detected in fractions 23-28, eluted at 0.2-0.35M NaCl. These values are reported in Table 3.4B.
[Table 3.4B] PPIase activity of fractions from steps 3 and 4 from purification procedure summarised in Table 3.4A

Step 3 from Table 3.4A

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>PPIase activity (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>0.9</td>
</tr>
<tr>
<td>24</td>
<td>3.2</td>
</tr>
<tr>
<td>25</td>
<td>8.4</td>
</tr>
<tr>
<td>26</td>
<td>2.4</td>
</tr>
<tr>
<td>27</td>
<td>1.8</td>
</tr>
<tr>
<td>28</td>
<td>0.4</td>
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</table>

Step 4, Run 1 from Table 3.4A

<table>
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<th>Fraction number</th>
<th>PPIase activity (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>1.9</td>
</tr>
<tr>
<td>15</td>
<td>3.4</td>
</tr>
<tr>
<td>16</td>
<td>3.3</td>
</tr>
<tr>
<td>17</td>
<td>0.7</td>
</tr>
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<td>18</td>
<td>0.3</td>
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Step 4, Run 2 from Table 3.4A

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</thead>
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<tr>
<td>14</td>
<td>0.3</td>
</tr>
<tr>
<td>15</td>
<td>0.9</td>
</tr>
<tr>
<td>16</td>
<td>0.9</td>
</tr>
<tr>
<td>17</td>
<td>0.4</td>
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<tr>
<td>18</td>
<td>0.1</td>
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</table>

Step 4, Run 3 from Table 3.4A

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>PPIase activity (s⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>13</td>
<td>0.1</td>
</tr>
<tr>
<td>14</td>
<td>0.2</td>
</tr>
<tr>
<td>15</td>
<td>0.5</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>17</td>
<td>0.3</td>
</tr>
<tr>
<td>18</td>
<td>0.2</td>
</tr>
</tbody>
</table>
[Fig. 3.4B] Step 4 (final step) of purification of cyclophilin from mitochondrial intermembrane space fraction as summarised in Table 3.4A.

Three successive gel filtration runs on the Superdex 75 were carried out as described [2.4.2.3]. All fractions showing protein at OD$_{280}$ were assayed for PPIase activity. Activity was only detected in fractions 13-17, peaking (equally) in 15 and 16 in all three cases. PPIase activities are reported in Table 3.4B. Positions of molecular weight markers at 29K and 13K are shown.

**Run 1.** Fractions containing PPIase activity from Mono S column (Step 3, Table 3.4A) were pooled, dialysed, freeze-dried, resuspended in 1ml and loaded.

**Run 2.** Fractions 15 and 16 from Run 1 were pooled and loaded.

**Run 3.** Fractions 15 and 16 from Run 2 were pooled and loaded.
Run 1

Run 2 Fractions 15 and 16 from Run 1

Run 3 Fractions 15 and 16 from Run 2
[Fig. 3.4C] SDS-PAGE analysis of cyclophilin purified from the mitochondrial intermembrane space fraction on elution from the Superdex 75 gel filtration column.

10% of fractions 13-17 obtained from Run 3, Step 4 (Fig. 3.4B) were assayed for PPIase activity. The remaining 90% were concentrated by freeze-drying and loaded onto the gel. Two bands of equal density can be seen in lanes 15 and 16 (corresponding to fractions 15 and 16 respectively) at approximately 18kDa.
buffer, sonicated and separated into supernatant (matrix) and pellet (inner membrane). Purification of PPIase from the intermembrane-side salt wash, matrix and inner membrane fractions produced 21kDa bands on a SDS-PAGE gel [Fig. 3.5A]

The detection of CyP21 outside of the matrix initially seemed to contradict our earlier theory of it being a matrix enzyme. However, the following experiment [Fig. 3.5B] showed that some matrix marker enzyme malate dehydrogenase was also present in the intermembrane space fraction (as discussed later). The CyP21 on the outside of the inner membrane was only recovered after a salt wash of the mitoplasts. This suggested CyP21 bound electrostatically to the outside of the inner membrane in mitoplasts, indicating a more basic nature than the smaller 18kDa protein of the intermembrane space. The separation of the two activities in this way was observed in four experiments, and allowed the amounts of CyP18 and CyP21 in the intermembrane space to be estimated from initial PPIase activities of the intermembrane space fractions and intermembrane-side salt wash fractions respectively. In addition, it allowed the estimation of how much total mitochondrial PPIase activity was attributable to each cyclophilin. It was estimated that CyP18 accounted for 22 ± 6% of the total mitochondrial PPIase activity with the remainder being due to CyP21.

This more basic nature of CyP21 may be due to a net positive charge in the additional residues contained in CyP21 but not the smaller protein. Connern & Halestrap [1992] report two mitochondrial PPIases of 18.6kDa and 17.5kDa. Presuming these correspond to CyP18 and CyP21 respectively from this study, the peptide sequence data they published was analysed. The larger isoform contained an extra ten residues. Of these ten, seven are usually uncharged at neutral pH.
Fig. 3.5A] **SDS-PAGE analysis of CyP 21 locality in mitochondria.**

Mitochondria were treated with 0.19mg digitonin / mg and centrifuged. This yielded the intermembrane space fraction (supernatant) and mitoplasts (pellet). Mitoplasts were resuspended in 500mM KCl, 10mM Hepes, 0.5mM EGTA, 1µg/ml protease inhibitors pH 8.0 and centrifuged. The supernatant (intermembrane space-side salt wash) was retained. The salt-washed mitoplasts were resuspended in the same buffer, sonicated and separated by centrifugation into the matrix (supernatant) and inner membrane (pellet) fractions. Purification of PPIase activity from intermembrane space-side salt wash (A), matrix (B) and inner membrane (C) fractions yielded 21kDa bands on SDS-PAGE.
Two of the residues were arginine molecules, which are usually positively charged at neutral pH. One was aspartic acid, which is usually negatively charged at neutral pH. This would result in a net positive charge of the larger isoform over the smaller one. This is in line with the theory of the larger PPIase having a more basic nature than the smaller one.

To verify the location of CyP21, its distribution was compared to those of a variety of mitochondrial marker enzymes chosen for their high specific activity and ease of measurement:

- monoamine oxidase - outer membrane
- succinate cyt.c reductase - inner membrane
- malate dehydrogenase - matrix
- adenylate kinase - intermembrane space

The results are shown in Fig. 3.5B. The distributions of the four enzymes in three mitochondrial fractions are compared to that of cyclophilin in the three fractions. An outer membrane containing fraction is not specified in Fig. 3.5B as monoamine oxidase partitioned almost equally between the intermembrane space and inner membrane fractions. This was not entirely unexpected. The results of Ono & Tuboi [1987] suggest that along with the outer membrane protein porin, monoamine oxidase may be integrated in the outer membrane at points where it meets the inner membrane ('contact sites'), although unlike porin these may not be the only points in the outer membrane which contain the enzyme. Thus on fractionation of mitochondria a significant amount of the enzyme may sediment along with the inner membrane. In addition to this, fragments of the outer membrane anchored by these contact sites would be expected to be present in the
The intramitochondrial location of CyP21 and four mitochondrial marker enzymes.

Mitochondria were treated with 0.19 mg digitonin / mg and centrifuged. The supernatant was retained. The mitoplast fraction was fractionated as in Fig. 3.5C into intermembrane space-side salt wash, matrix and inner membrane. The following fractions were assayed for CyP21 (cyp), malate dehydrogenase (md), adenylate kinase (ak), monoamine oxidase (mao) and succinate cyt. c reductase (sd): intermembrane space (intermembrane space-side salt wash + intermembrane space), matrix and inner membrane.
inner membrane fraction, thereby increasing the amount of outer membrane proteins in the inner membrane sample.

The flow chart shown in Fig. 3.5C summarises the following preparation. As was determined by results shown in Fig. 3.2A, the digitonin concentration that provided the best separation of intermembrane space and matrix fractions was 0.19mg digitonin/mg mitochondrial protein. Thus, mitochondria were treated with this concentration of digitonin and centrifuged. The supernatant (i.e. intermembrane space fraction, 1a, Fig. 3.5C) was retained. The mitoplasts were suspended in salt wash buffer comprising 500mM KCl, 10mM HEPES pH 8.0, 0.5mM EGTA, protease inhibitors and centrifuged. The supernatant (i.e. intermembrane space-side salt wash, 1b, Fig. 3.5C) was retained. The salt-washed mitoplasts were suspended in the same medium, sonicated and separated into supernatant (matrix, 2, Fig. 3.5C) and salt-washed inner membrane (3, Fig. 3.5C). Three fractions were assayed for cyclophillin (cyp), malate dehydrogenase (md), adenylate kinase (ak), monoamine oxidase (mao) and succinate cyt.c reductase (sd): intermembrane space (intermembrane space fraction [1a]+ intermembrane space-side salt wash [1b]), matrix [2] and inner membrane [3].

The distribution pattern of cyclophilin in the three fractions was almost identical to that of the mitochondrial matrix marker enzyme malate dehydrogenase, and distinctly unlike the distribution patterns of the other marker enzymes. It may be that the CyP21 detected on the outside of the mitoplasts is an artefact and was released along the same route as the malate dehydrogenase detected outside the matrix fraction.

These results confirm that CyP21 is located in the mitochondrial matrix.
[Fig. 3.5C] Flow chart summarising the preparation of the mitochondrial fractions.

1a. mitochondria

0.19 mg digitonin/mg mitochondrial protein

Centrifugation

Supernatant: (intermembrane space fraction)

Pellet: (mitoplasts)

Salt wash buffer

1b. Supernatant: (intermembrane-side salt wash)

Salt wash buffer

Centrifugation

Pellet: (salt washed mitoplasts)

Sonication

2. Supernatant: (matrix)

Pellet: (salt washed inner membrane)

3
[3.6] SDS-PAGE of the two mitochondrial cyclophilins

From this series of experiments, the unusual distribution of PPIase on increasing digitonin concentration shown in Fig. 3.2A can now be explained. The distribution of PPIase did not follow that of the intermembrane space enzyme adenylate kinase or that of the matrix enzyme malate dehydrogenase as it appears a PPIase is present in both of these mitochondrial compartments. This gives total PPIase a distribution pattern somewhere in the middle of the two marker enzymes. Fig. 3.6A shows a photograph of an SDS-PAGE gel of the two cyclophilins from the two mitochondrial compartments which were fractionated using a digitonin concentration of 0.19mg digitonin/mg mitochondrial protein and without washing the outside of the mitoplasts before sonication.

Cyclophilins in mitochondria have been reported previously. As was mentioned earlier, Connern & Halestrap [1992] detected two cyclophilins in the matrix of rat liver, the major 18.6kDa protein and a minor 17.5kDa protein. These are likely to correspond to the two cyclophilins discovered in this investigation, although these were found in separate mitochondrial compartments. N-terminal sequencing of the two proteins demonstrated to the authors the presence of a 10 residue presequence on the 18.6kDa isoform. Although the remaining sequence shows much similarity to other cyclophilins, parallel purification and N-terminal sequencing of rat cytosolic PPIase with the mitochondrial form showed the proteins to contain significant differences suggesting the proteins were encoded by different genes. Tropschug et al [1988] reported a cyclophilin in the mitochondrial matrix of the yeast Neurospora crassa. This cyclophilin was encoded by a gene which also encoded a cytosolic cyclophilin that was the precursor of the
mitochondrial protein. The mature protein was formed by a two-step cleavage of
the precursor after its translocation from the cytosol into the matrix.
[Fig. 3.6A] **SDS-PAGE analysis of the two cyclophilins isolated from rat liver mitochondria.**

Mitochondria were fractionated using 0.19mg digitonin/mg and without washing the mitoplasts before sonication. **A.** 18kDa cyclophilin isolated from the intermembrane space fraction as described [3.4]. **B.** 21kDa cyclophilin isolated from the matrix fraction as described [3.3].

Protein markers

- 97K
- 66K
- 45K
- 29K
- 21K
- 14K
On obtaining a purified sample of CyP21, the next step was to obtain peptide sequence data of the protein. This would serve to confirm its cyclophilin classing. In addition to this, learning more about the structure of this likely inner membrane pore component might broaden the knowledge of the pore structure and so aid in the possible elucidation of its native function.

Once determined, the peptide sequence could then be utilised to obtain the base sequence of the gene encoding the protein. Degenerate PCR primers could be designed that would specifically amplify from a DNA library a piece of DNA from CyP21.

Two types of gene libraries in use today are genomic and cDNA libraries. The preparation of a genomic library involves the purification of total cell DNA, which is then digested by a restriction enzyme. This results in DNA fragments that can be inserted into a suitable vector. The principle problems of this approach to isolating a gene are:

1. the desired piece of DNA may have been cut by the restriction enzyme used and is therefore not obtainable from a single fragment;
2. if the gene is contained within a piece of the genome that has relatively few of the relevant restriction sites, the resulting fragment may be too large to have been ligated into a vector, and would not be present in the library;
3. the presence of introns decreases the specificity of this type of library.

The alternative to a genomic library is a cDNA library. cDNA libraries originate from the mRNA in a particular cell type. The mRNA in a cell will consist only of those genes that are expressed in that cell type. The mRNA is converted into
cDNA and ligated into a suitable cloning vector. For this investigation a rat liver
cDNA library in λgt11 was used to isolate the gene for CyP21.

After PCR pulled out a fragment of the CyP21 gene from the cDNA library, it
could be radiolabelled and used as a probe to pull out the full length cDNA. If the
protein were then expressed, the tight association of CsA to cyclophilin could be
utilised to isolate other pore components.

[4.1] CyP21 peptide sequencing - identification of CyP21 as CyPD

A sample of CyP21 was obtained and prepared for peptide sequencing as
described [2.8]. Purified CyP21 was digested with endoproteinase Asp-N. The
enzyme cleaved at some glutamic residues in addition to aspartic residues. There
were eleven resulting peptides. The amino acid sequences of the CyP21 peptides
were obtained (as outlined in [2.8]) and are displayed in Fig. 4.1A. With the
exception of one of the peptides, a search of data bases revealed a 96% sequence
homology of the peptides to human CyP3 (Bergsma et al [1991]), which is also
more commonly designated CyPD (Galat [1993]). The second part (B) of Fig.
4.1A shows the CyP21 peptides (with the exception of one) aligned beneath the
full length sequence of human CyP3 (CyPD).

The presence of a NH₂-terminal hydrophobic extension on CyPD suggested that
this was a possible signal sequence of the protein, serving to transport the
polypeptide across the membrane of a sub-cellular organelle (Bergsma et al
[1991]. Subsequently, Connem & Halestrap [1992] sequenced, by means of
Edman degradation, the N-terminus of an 18.5kDa cyclophilin from rat liver
mitochondria. Except for one residue, this sequence is identical to that of the one
peptide of CyP21 that could not be aligned with human CyPD. This can be seen in
[Fig. 4.1A] Amino acid sequences of the eleven peptides obtained by digestion of CyP21.

A

1  ARDGGARGANSSQNPLVYLDVGADGQPL
   DGGARGANSSFQN

B

1  MLALRCGSRWLGLLSVPRSVPRLPAARACSKGSGDPSSSSSSGNPLVYL
   *  *  *
51  DVDANGKPLGRVVLELKADVPPKTAENFRALCTGEKFGYKGSTFHRVIX
     DGQPLGRVV     ENFRALXTEKFGYKGSTFHRVIX
     DVVKPITA     EKFGYKSTFHRVIX
   *
101  SFMCQAGDFTNHNTGGKSIYGSRFPDENFTLKVPGVLSMANAGPNNTN
     AFXDENFTLKVPGVLSMANAGPNNTN
     AFMXOA DGTGG
   *
151  GSQFFICTIKTDWLDGKHVFHVKEGMDVKKIESFGSKSRTSKKIVI
     DGKHVFHVKEGMDVKKIESFGSKSRTSKKIVI
     DVKKIESFGSKSRTSKKIVI
     DGKHVFHVKEGMD

201  TDCGQLS

Amino acid residues that differ from previously identified sequences are marked with an asterisk, unidentified residues are designated X.

(A) The amino acid sequence of one peptide (underlined) was found to be nearly identical to the previously defined N-terminal sequence of rat liver mitochondrial cyclophilin (Connem & Halestrap [1992]).

(B) The amino acid sequences of the remaining ten peptides (underlined) are aligned with the full length sequence of human CyPD (Bergsma et al [1991]).
the first part (A) of Fig. 4.1A. This suggests this peptide is located in the N-terminal region of the protein.

The peptide sequence data from CyP21 was aligned with other CyP isoforms from a data base search and this yielded the following best sequence homologies: 79% to rat CyPA (Danielson et al [1988]), 66% to human CyPB (Bergsma et al [1991]) and 60% to murine CyPC (Friedman & Weissman [1991]).

From the available sequence information it appears that the human and rat CyPD proteins differ considerably at the N-terminus. Little sequence similarity has been noted among mitochondrial targeting sequences and the divergent N-terminal regions may form a part of such a sequence.

A smaller cyclophilin (17.8kDa) was isolated from rat liver mitochondria (Connern & Halestrap [1992]). This presumably corresponds to CyP18 from this study. In this protein the different N-terminal region (10 residues) was not present, suggesting the protein may have arisen by cleavage of the larger isoform. Whether any such cleavage occurs physiologically or artefactually requires study.

[4.2] Generation of a probe by PCR using degenerate primers

The next stage of the investigation was to obtain the full length cDNA of rat liver CyPD. To do this, a rat liver cDNA library had to be screened. First, a radiolabelled probe had to be generated which would then hybridise and thereby identify the correct cDNA. To generate a probe, primers had to be designed which could then be used in a PCR reaction to amplify a piece of cDNA from the library. In addition to the usual considerations (see [2.10]), the primers had to be selective for CyPD, i.e. at least one of the two primers had to be homologous for a region of CyPD that contained sequence differences from the other cyclophilin isoforms
**Fig. 4.2A** Sequence comparison of peptides from rat CyPD with equivalent regions of other rat cyclophilin isoforms. ER sequence was obtained from Iwai & Inagami [1990]. Cytosolic sequence from Danielson et al. [1988]. The two regions of sequence indicated relate to the positions of the degenerate primers that were designed in order to amplify CyPD from a cDNA library.

| Mitochondrial | DGQLGRV | DVPKTAENFRLXTGKGSFTFRVIXAFMX |
| ER           | I-GRTCTSDWLWDKDCSVDAGNKNKHMKD |
| Cytosolic    | EE-CFELFAK-SGSSISGGENDFTN |

| Mitochondrial | QAXDFTNHNGTGGKSIYGSR DEFTLKHVGPGVLSMAGPNTN |
| ER           | GGRGDP-K-Y-WV-K-GSQF |
| Cytosolic    | GG-R-EKFE-I-T-I-N-GSQF |

| Mitochondrial | DGKHVVFGHVKEGNDVKKIESFGSKSGKTKSKKIVI |
| ER           | FITVKTSDL-R-V-NTKTD-RDKPLD-I |
| Cytosolic    | FICTAKEWL-R-MSI-EAM-R-N-ITS |
Degenerate primers P1 and P2.

The position of the degenerate primers P1 and P2 designed to amplify a piece of the CyPD gene from a rat liver cDNA library is shown here against the peptide sequence of CyP21 (underlined). CyP21 is aligned underneath the full length human CyPD peptide sequence (Bergsma et al [1991]).
in rat liver. This would ensure the cDNA being amplified was from the correct cyclophilin isoform. The sequences of rat cytosolic and ER cyclophilins were available (Danielson et al [1988] and Iwai & Inagami [1990] respectively). These were aligned against rat liver CyPD as shown in Fig. 4.2A. Taking the sequence data into account, a number of suitable regions became available. However, when the other degenerate primer design factors were considered [2.10.3], there were few regions of sequence left that were suitable. A primer was eventually designed that was homologous to a region of CyPD that showed a high degree of conservation through the cyclophilin isoforms (EKGFGYK). The second primer designed was homologous to a region of CyPD that was more specific to CyPD (NDVVKIE). Fig 4.2B shows the position of the primers against the peptide sequence of rat liver CyPD. The PCR reaction was expected to amplify a 303bp fragment of CyPD DNA.

Variations in a number of PCR conditions can vary the yield of the product in a reaction (for details see [2.10]). A series of PCR reactions under varying concentrations of Mg\(^{2+}\) in the buffer, annealing temperature (T\(_M\)) of the primers and amount of template in the reaction, were carried out. These conditions are specified in Table 4.2A. 10% of the products were run on a 2% agarose gel which was stained with ethidium bromide. Fig. 4.2C is a photograph of the gel.

From the results it can be seen that the correct sized fragment (i.e. a fragment of \(~0.3\text{kbp}\)) is only visible in tracks 3, 7, 8 and 9. The PCR conditions of these reactions are highlighted in Table 4.2A. The best yield of the desired product was obtained using standard conditions (see [2.10]) with the Mg\(^{2+}\) concentration in the buffer at 1.5mM, an annealing temperature of 40°C, and 1μl of template (underlined in Table 4.2A). Further PCR reactions were carried out under these
<table>
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<th>Well no.</th>
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$T_m = 40^\circ C$  

$T_m = 45^\circ C$
Analysis of PCR products generated using the degenerate primers P1 and P2.

A series of 12 different reactions were carried out which are specified in Table 4.2A. 10% of the products were run on a 2% agarose gel [2.11]. The fragment of interest can be seen in lanes 3, 7, 8 and 9 which correspond to the same well numbers in Table 4.2A.
[Fig. 4.2D] Peptide sequence of the PCR product amplified using the degenerate primers P1 and P2.

The 303bp fragment from lane 3, Fig. 4.2C was purified from the gel and cloned into pCR-Script [2.12-13]. This was used to transform E.coli. DNA was prepared and sequencing carried out [2.15-16]. The peptide sequence shown was derived from the base sequence obtained, and is completely unrelated to cyclophilin. Possible sites of primer recognition are highlighted.

Across the N-terminal:

+ strand
1  GGSARKFGYRGRMNQKFEAVNAIDRNWTDCA
2  GDPPEKGLDGAG*IKNLQKLTQLTGQIQIAL
3  GIRPKRQWIQGQDESI*KSS*RY*QELDLRL

- strand
1  PSGGSFPKSVPAHPHILFKFCNVSNVPIPCIAS
2  PDARFNPYLPYLIF*FNSATLAISLFLQVSQA
3  PIRGFLTQICPCSSDFIQLL*R*QCSNSLNR

Across the C-terminal

+ strand
1  SPSFLQQ*LNTNIDGGFSASQFLPPVDQSPASRSFYPL
2  AHLFYNVD*LTL*MAVLRNNFFRQLIKVQPVRFTHC
3  PIFFTTVTHK*HRWRF*RVATFSSAS*SKSSQSFVLPIV

- strand
1  GMKKVTVLC*CLHRN*RTAVKEEALQDFDLWDNNTKGMT
2  AWRK*LLS*VSVYIATKARRLKKKRWNILTGWGTRKQW
3  GDKKCCHSFVLMSPKLDACSRRGGTS*LGALEN*GN
optimal conditions for production of the 303bp fragment until there was enough DNA to conduct a sequencing reaction. The 303bp fragment was purified from the agarose gels [2.12] and cloned into the phagemid vector pCR-Script which was then used to transform *E.coli* [2.13]. DNA was prepared [2.16] and sequencing carried out [2.15].

When sequence data of the 303bp fragment was obtained, it was discovered that the fragment was completely unrelated to any known cyclophilin. Fig. 4.2D shows the peptide sequences derived from the base sequence data obtained. All six possible peptide sequences are given, i.e. reading in all three frames on both the positive and negative strands. It was concluded that due to the inherent low specificity of degenerate primers, unrelated sequences had been amplified. Highlighted on Fig. 4.2D are possible regions to which the primers may have hybridised and begun amplifying.

In all, DNA prepared from twelve recombinant colonies was sequenced in this way. All twelve had the same or similar sequences to that shown in Fig. 4.2D, i.e. unrelated to cyclophilin.

[4.3] **Generation of a probe by PCR using non-degenerate primers**

The low specificity of degenerate primers had led to the amplification of a cyclophilin-unrelated piece of cDNA. The solution to this problem was to increase the specificity of the primers. The most efficient way to do this was to switch to non-degenerate primers (i.e. primers based on base sequence information).

Since the base sequence of rat liver CyPD was not available at the time, the 96% homology of the CyP21 peptide sequence to human CyPD was taken as sufficient justification to use the base sequence of human CyPD (Bergsma *et al* [1991]) to
[Fig. 4.3A] **Non-degenerate primers P3 and P4.**
The peptide sequences of the non-degenerate primers P3 and P4 are shown against the full length peptide sequence of human CyPD (Bergsma et al [1991]). The base sequence of the human protein was used to design these primers.

1  MLALRCGSRWLGLLSVPRSVPLRLPAARACSKGSGDPSNSSSSSSGPNLVY
    \[=\]  

51  DVDANGKPLGRVLELKADVPKTAENFRALCTGEKGFGYKGFSTFHRVIP
    \[="\]

101  SFMCQAGDFTNHGTGGISKIYGSRFPDENFTLKVPGVLSMANAGPN

151  GSQFFICTIKTDWLGDGHVFHVKEGMDVVKKIESFGSKGRTSK
    \[="\]

201  TDCGQLS
design non-degenerate primers. As in the previous experiment [4.2], in addition to the usual considerations (see [2.10]), one or both primers had to be selective for CyPD above the other cyclophilin isoforms. Using Fig. 4.2A, two regions of sequence were chosen. The positions of the non-degenerate primers that were designed are shown in Fig. 4.3A aligned against the peptide sequence of human CyPD. The primers were termed P3 and P4. The peptide sequence data of rat CyPD had been determined for the region of primer P4, and was identical to the human peptide sequence (5' KKIESFGSK 3'). However, the region of primer P3 had not yet been determined in rat (5' NPLVYLDV 3'). The choice of primer turned out to be a good decision as the human and rat sequences were later discovered to be identical in this region [Fig. 4.4B]. However, at the time this was not known and there was the possibility that the human and rat sequences may have been drastically different in this region. It was decided the primer combination of the first series of PCR reactions would be varied to include the degenerate primers from the previous experiment. A series of PCR reactions under varying concentrations of Mg$^{2+}$ in the buffer, annealing temperature ($T_M$) of the primers and primer combination (P1 to P4), were carried out. These conditions are specified in Table 4.3A. 10% of the products were run on a 2% agarose gel which was stained with ethidium bromide. Fig. 4.3B is a photograph of the gel.

The PCR product of primers P3 and P4 was expected to be 438bp; the PCR product of primers P1 and P4 was expected to be 318bp; the PCR product of P2 and P3 was expected to be 423bp.

The results show that the PCR using only the pair of non-degenerate primers worked. From Fig. 4.3B it can be seen that the correct sized fragment (438 bp) is only visible in lanes 11 and 14. The PCR conditions of these reactions are
Table 4.3A] PCR reaction conditions using a combination of degenerate and non-degenerate primers.

<table>
<thead>
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<th>Well no.</th>
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[Fig. 4.3B] Analysis of PCR products generated using a combination of the degenerate primers P1 and P2 and the non-degenerate primers P3 and P4.

A series of 15 different reactions were carried out which are specified in Table 4.3A. 10% of the products were run on a 2% agarose gel [2,11]. The fragment of interest (438bp) can be seen in lanes 11 and 14 which correspond to the same well numbers in Table 4.3A. Both the successful reactions used the non-degenerate primers P3 and P4, with well no. 11 having a slightly higher [Mg] and yield of desired product.
[Fig. 4.3C] Amplification of the product obtained using the non-degenerate primers P3 and P4.

The optimal conditions used to obtain the 438bp fragment of interest (Well no.11, Table 4.3A) were used in 10 PCR reactions. 10% of the products were run on a 2% agarose gel [2.11]. One of the reactions (*) obviously contained some contaminatory DNA which caused non-specific amplification.
highlighted in Table 4.3A. Standard PCR conditions were used with an annealing temperature of 60°C using the pair of non-degenerate primers P3 and P4. The difference between the two reactions (11 and 14) was the Mg$^{2+}$ concentration in the buffer (1.5mM and 1.0mM respectively), the former (underlined in Table 4.3A) providing a better yield. These optimal conditions were used to amplify more of the 0.45kbp fragment until there was enough DNA to conduct a sequencing experiment. DNA was prepared for sequencing as in [4.2]. A photograph of the gel of the PCR products from the optimal reactions is shown in Fig. 4.3C.

Sequencing across the ends of the fragment confirmed the PCR reaction had indeed amplified a piece of CyPD. Fig. 4.3D shows the peptide sequence of the fragment, deduced from the base sequence obtained, aligned against the previously determined peptide sequence of human CyPD. Incorporated also into Fig. 4.3D is the peptide sequence of CyPD as determined earlier (Fig. 4.1A). Three discrepancies were observed between the two sets of peptide sequence data. The first at residue 60, the second at residue 84 and the third at residue 178. Base sequence data later resolved these residues as G, G and M respectively [Fig. 4.4B].

Fig. 4.3E shows the peptide sequence from the CyPD fragment obtained by PCR aligned against the corresponding regions of two other rat cyclophilin isoforms (cytosolic - Danielson et al [1988], ER - Iwai & Inagami [1990]). The regions of homology confirm the fragment is indeed of a gene encoding a protein from the cyclophilin family. The differences between the sequences confirm the fragment obtained is from the correct cyclophilin isoform.
[Fig. 4.3D] Peptide sequence of the PCR product amplified using the non-degenerate primers P3 and P4.
The 438bp fragment was purified from the gel and cloned into pCR-Script [2.12-13]. This was used to transform E.coli. DNA was prepared and sequencing was carried out across the ends of the fragment [2.15-16]. The peptide sequence shown (underlined) was derived from the base sequence obtained. The derived peptide sequence is aligned underneath the full length human CyPD sequence (Bergsma et al [1991]). Underneath in bold type is the previously determined rat CyP21 peptide sequence [Fig. 4.1A].

```
1  MLALRCGSRWGLLLSVPRS
21 PLRLPAARACSKGSGDPS
41 SSSSNLPLVYLDVANGKPLG
     NPLVLVDVGADGQPLA
61 RVVLELKADVVPKTAFRA
     DVPKTAFRA
81 LCTGEKFGKYKGSFHRV IP
     LCTGE
81 LXTSEKFGKYKGSFHRVIX
101 SFMCQADFTNHTGGKSI
     AFMDFTNHTGGKSI
121 YGSRFPDENFTLKHVGPGV
     YGSRDENFTLKHVGPGV
141 SMANAGPNTNGSFFICTIK
SMANAGPNTNGSFFICTIK
161 TDWLDGKHVFVGHVKEMDV
     TDWLDGKHVFVGHVKEMDV
     TDWLDGKHVFVGHVKEMDV
181 VKKIESFGSKSGRTSKKIL
     VKKIESFGSK
     VKKIESFGSKSGRTSKKIL
201 TDCGQLS
```
[Fig. 4.3E] Peptide sequence of PCR product amplified using the non-degenerate primers aligned against other rat cyclophilin isoforms. The region of the CyPD protein encoded by the PCR product is shown aligned against the corresponding regions of other rat cyclophilin isoforms. The peptide sequence of the cytosolic isoform was obtained from Danielson et al [1988]. The peptide sequence of the ER isoform was obtained from Iwai & Inagami [1990].

**mitochondria**

```
N P L V Y L D V G A D G Q P L A R V V L E L K A D V V P K T
```

**cytosol**

```
- - T - F F - I T - - - E - - G - - C F - - F - - K - - - -
```

**ER**

```
I - G R T C - T S D W T L W K D C R - -
```

**mitochondria**

```
A E N F R A L C T G
```

**cytosol**

```
- - - - - - S - -
```

**ER**

```
V D - - V - - A - -
```

**mitochondria**

```
E F F I C T I K T D W L D G K H V V F G H V K E G M D V V K
```

**cytosol**

```
- - - - - - A - - E - - - - - - - - - K - - - - - S I - E
```

**ER**

```
- - - - T - V - - S - - - - - - - - - K - L - - - - - R
```

**mitochondria**

```
K I E S F G S K
```

**cytosol**

```
A M - R - - - R
```

**ER**

```
V - N T K T D
```
[4.4] Identification and analysis of a positive bacteriophage clone from a cDNA library

The next step was to radiolabel the 0.438kbp PCR product and use it as a probe to identify the full length cDNA of CyPD. The rat liver cDNA library that was used was in λgt11, a bacteriophage λ expression vector. In the case of phage vectors, plaque formation is the selectable marker for the presence of the vector. λgt11 carries the E.coli lacZ gene which contains a unique EcoR1 site located within the β-galactosidase coding region. The recombinant library is constructed by insertion of eukaryotic cDNA into the EcoR1 sites. This insertionally inactivates the β-galactosidase. The vector can accept up to 8.3kbp of insert DNA and a complete cDNA library containing a large number of independent recombinants can be easily constructed. Screening for the desired insert is carried out on plaques of the recombinant bacteriophage. The library is plated out with a suitable E.coli host strain (see [2.19]). The resulting plaques are adsorbed onto nitrocellulose membranes which are then processed for screening by the radiolabelled probe. The treatment ensures the DNA molecules are denatured so that the hydrogen bonds keeping the double strands of the helix together, are broken. The single strands are bound tightly to the membrane by UV irradiation. The molecules are attached by their sugar-phosphate backbones, leaving the bases free to hybridise with complementary nucleic acid molecules. On identification of a positive signal, the positive plaque is removed from the agar plate and a recombinant bacteriophage has therefore been isolated.

In the present study, positive bacteriophage clones were isolated after two rounds of screening. The first round consisted of ten agar plates containing 4000-5000
[Fig. 4.4A] Analysis of positive bacteriophage clones obtained from a rat liver cDNA library.

The 438bp CyPD gene fragment was radiolabelled for use as a probe to screen a rat liver cDNA library in λgt11 for full length CyPD [2.17-18]. Details are given in [4.4]. Seven bacteriophage plaques gave positive signals after two rounds of screening. These seven clones were amplified and DNA prepared [2.9]. Restriction digests with EcoRI revealed the insert size of each clone. 10% of the digests were run on a 1% agarose gel [2.11]. Clone 2 (indicated) shows the longest insert.

DNA markers

- 2.84kbp
- 0.80kbp

Clone 2 (indicated)
[Fig. 4.4B] Base and peptide sequence of rat CyPD from Woodfield et al. [1997]. Clone 2, Fig. 4.4A was amplified as described [4.4] and sequencing was carried out across the ends [2.15]. Base sequence data was obtained for the region underlined.
plaques. These were each adsorbed onto duplicate nitrocellulose filters (i.e. 20 filters in total). The filters were incubated with the radiolabelled probe. Positive signals were obtained from four of the ten plates. Plaque density was too high to match the signals to single plaques, so areas of agar ~5mm diameter were excised and placed in 0.5ml SM buffer [2.9.1] with 10μl chloroform (to kill the bacterial cells). These soak-outs were used to prepare four more agar plates containing 50-100 plaques each and the procedure was repeated. Between 5-10 positive signals were obtained from each plate. The plaques on the second round plates were sufficiently well spaced out that signals could be traced to single plaques that should not contain phage from any surrounding plaques. The strongest signals were identified on the plates and the plaques excised. These amounted to seven plaques. The phage clones were amplified and bacteriophage DNA prepared [2.9]. Restriction digests with EcoRI revealed the insert size of each clone. Fig. 4.4A shows 10% of the DNA preparation digested with EcoRI on a 1% agarose gel. The inserts are visible at ~1kbp. Clone 2 shows the longest insert. Obtaining enough DNA of a quality sufficient for sequencing proved to be a problem. Initial sequencing reactions on the remaining 90% of phage of clone 2 did not work. This was presumed to be due to contaminants (agar, etc.) in the DNA preparation.

An additional step was introduced into the DNA isolation procedure. After phage were precipitated with PEG 8000, but before the addition of SDS, 3.77g CsCl was added for each 5ml of phage suspension and dissolved. This was centrifuged at 38,000rpm at 17°C overnight. If the phage layer was visible it was removed with a hypodermic needle and syringe. If the phage layer was not visible, the whole supernatant was taken. CsCl was removed by dialysis and the phage re-precipitated with PEG 8000 and the standard procedure continued. This
[Fig. 4.4C] **Base sequence of the CyPD clone from the cDNA library.**

Sequencing was carried out as described [2.15]. The 5' end of the sequence obtained begins at base number 68 of the published sequence of Woodfield et al [1997] and ends at 203. The 3' end of the sequence begins at base number 1288 and ends at 1020. Underlined bases are those which are not present in the published sequence. Bases substituted with * are those not detected. Above them are the bases from the published sequence. The base in bold type is different that published.

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<tr>
<td></td>
<td>GCGCCTCCTCCTCCTCTCCACGACCGTGACCGACG*</td>
</tr>
<tr>
<td></td>
<td>c c c c</td>
</tr>
<tr>
<td></td>
<td><em>GGAG</em>CCGAG**GCGAATCTTT*CTCCCCAAACCCGCTGCTGTAGTGGACGCTGCGG</td>
</tr>
<tr>
<td></td>
<td>GCCGACGGACAGCGCT 203</td>
</tr>
</tbody>
</table>

| 1020 | CAAAGCTACTACACTAACTGCACTTCTATGTTGACCTGAGTGG*CAAAGCCAGACAG |
|      | ACCCCACACCAACCACCCACACACCACACCACACCACCTCCC**CCTGCGCTTCTGAAACATAA |
|      | CTCAGGGCTCTTTGTGAAATTCCACCACATCAATGCGCTCTCTCTCTCTTGCTGACCTGTAATTCAG |
|      | CT**GACCTGTGAGCGCCGCTGCTGCTC 1288                    |
[Fig. 4.4D] **Segments of the sequencing gel that the base sequence of the CyPD clone was obtained from.**

(i) Base sequence was obtained from the 5' end using the T3 primer (see Fig. 4.4E). X shows the EcoR1 site where the gene was inserted into the vector. Y shows a region of ambiguity which made accurate reading of the gel difficult. (ii) Base sequence was obtained from the 3' end using the KS primer (see Fig. 4.4E). Z shows another region of ambiguity.

The two sets of 15 bases shown underneath were deduced from the two segments of the gel beginning from the points indicated by the arrows.

(i) 5' (T3 primer)  
A C g T

(ii) 3' (KS primer)  
A C g T

(i) gggccgcagtaatt  
(ii) ccctgagttatgttt
modification drastically reduced yield but significantly improved the quality of the DNA obtained at the end of the isolation procedure. Sequencing was carried out across the ends of the DNA from clone 2 to confirm it was of CyPD origin.

At the same time as this data was obtained, Woodfield et al [1997] published the entire base sequence of rat liver CyPD. Fig. 4.4B shows this base sequence. The data obtained during this investigation is indicated by the regions underlined in Fig. 4.4B. As can be seen, it seems the cDNA clone obtained is shorter than the published data by 13 residues. The only explanation for this occurrence is that on the conversion of the mRNA to cDNA during the library construction, the entire mRNA script was not copied into cDNA. Conversion begins with the enzyme reverse transcriptase which synthesises a DNA strand complementary to an existing RNA strand. Once the cDNA strand is synthesised, the RNA strand is partially digested with ribonuclease H. The remaining RNA fragments then serve as primers for DNA polymerase I, which synthesises the opposite cDNA strand, to give double-stranded DNA for ligation into a vector, and cloning. Where the problem occurred is difficult to be certain, but it is likely to be at the stage of the reverse transcriptase activity. The clone sequenced was the longest obtained by the screening procedure. However, there is every chance that further screening could have produced the full length clone.

The actual base sequence obtained from across the ends of the positive clone is shown in detail in Fig. 4.4C. Indicated on this figure are the positions of bases not detected, additional bases detected, and bases which appeared different, all compared against Woodfield et al [1997]. The 5' end of the sequence starts at base number 68 of Fig. 4.4B and ends at base number 203. The 3' end of the sequence starts at base number 1288 and ends at base number 1020.
Circular map and polylinker sequence of the pBluescript II KS(+-) phagemid (Stratagene).
Fig. 4.4D shows segments of the sequencing gel that the base sequence data of the positive clone was obtained from. Fig. 4.4Di is from the 5' end of the sequence, using the T3 primer (see Fig. 4.4E). Reading from the bottom upwards, the EcoRI restriction site into which the cDNA inserts into the plasmid is indicated (X). Regions of sequence which initially showed base additions, deletions or differences against those of Woodfield et al [1997], see Fig. 4.4C, were later discovered to be at points of ambiguity in the gel. An example can be seen on Fig. 4.4Di, labelled as (Y). Fig. 4.4Dii is from the 3' end of the sequence using the KS primer (see Fig. 4.4E). Another region of ambiguity is indicated as (Z). Fifteen bases obtained from the lower end of both segments of the sequencing gel are shown at the bottom of Fig. 4.4E. The arrows beside the gel segments indicate the first of the bases in both series.

Fig. 4.4E shows a circular map of the phagemid into which the cDNA was inserted for sequencing, pBluescript II KS (+/-). Details of the sequencing primers are given underneath the map within the polylinker sequence.

In Connem & Halestrap [1992], the authors published N-terminal peptide data of a mitochondrial cyclophilin which matched one of the peptides obtained from the sequencing carried out in this investigation (see Fig. 4.1A). According to the base sequence data obtained here, the preceding two residues of the peptide would have been CS(DGG....). Connem & Halestrap [1992] originally reported these residues as AR(DGG....). However, in their more recent paper, where the base sequence of CyPD is published (Woodfield et al [1997]) these two residues are reported as CS(DGG....).
CHAPTER 5: CONSTRUCTION OF A GST-CyPD FUSION PROTEIN

[5.1] Construction of the GST-CyPD fusion gene

As the base sequence of CyPD was now available, a variety of options were presented to investigate the function of the protein with regards to pore activity. The principle objective was the identification of mitochondrial components (if any) which interact with CyPD under pore activating conditions. The simplest way to do this appeared to be the expression of the mature protein (i.e. without the targeting presequence) as a fusion protein. The use of carrier proteins that are easily purified on affinity matrices greatly simplifies purification procedures that are required to isolate soluble proteins.

One of the most popular fusion protein expression systems is the pGEX system (Smith & Johnston [1988]), since it allows easy affinity purification of the expressed protein. pGEX vectors contain the glutathione-S-transferase (GST) gene fused at its C-terminal to BamH1, EcoR1 and Sma1 restriction sites. Fusion protein expression is controlled by an inducible tac promoter, a stronger hybrid of the traditional lac and trp promoters. The plasmid contains the lacI$^q$ allele that over-produces the lac repressor allowing controlled expression in most of the commonly used E.coli host strains. The tac promoter is induced in culture by the addition of IPTG.

The advantages of the pGEX system relevant to this investigation were that GST fusion proteins are often soluble, and are easily purified from bacterial cell lysates by adsorbing to an immobilised glutathione matrix and then eluting with reduced glutathione. In addition to this, GST fusion proteins have already been established as a means by which the protein targets of other cyclophilin isoforms have been
[Fig. 5.1A] Restriction map of pGEX-3X.
identified (e.g. CyP40, Hoffmann et al. [1995]). On adsorption of the GST/CyPD fusion protein onto the matrix, binding proteins from rat liver mitochondrial fractions could be isolated. The relevant protein(s) might be expected to be sensitive to Ca$^{2+}$, ADP and CsA, enabling their identification.

The first step in the construction of a GST/CyPD fusion gene was the preparation of the mature CyPD gene so it could be inserted into the fusion vector. The specific vector available was pGEX-3X. A restriction map of the plasmid is shown in Fig. 5.1A. It was decided that the best method of preparing the CyPD gene was to amplify the mature sequence by PCR using primers that contained compatible restriction sites for cloning into the vector. Non-degenerate primers were designed based on the base sequence published by Woodfield et al. [1997] which added a restriction site onto either side of the DNA molecule. The N-terminal primer (P5) added a BamH1 site onto the N-terminal end of the CyPD gene, and the C-terminal primer (P6) added a EcoR1 site onto the C-terminal end of the CyPD gene.

The primer P5 consisted of a spacer region of four bases, followed by the six bases which made up the BamH1 restriction site. An additional two bases were added on here so that when the CyPD gene was inserted into the pGEX vector and fused to the GST gene, the CyPD gene would be in the correct reading frame for transcription. These two extra bases were followed by twenty bases homologous to the CyPD gene where the primer would hybridise to the template DNA. This resulted in a "32mer" (i.e. a primer 32 bases long).

The primer P6 consisted of twenty bases homologous to the end of the gene up to which the active protein would be transcribed, not including the stop codon. This
Fig. 5 IB] Primers P5 and P6 designed to generate compatible ends to the pGEX-3X vector.
The published base sequence of rat CyPD (Woodfield et al [1997]) was used to design the homologous regions of the two primers. Restriction sites were added onto the beginning of the 5' primer P5 (BamHI) and the end of the 3' primer P6 (EcoRI) so the PCR product could be directionally inserted into the vector.
[Fig. 5.1C] Analysis of the PCR products amplified using primers P5 and P6.

Three standard PCR reactions were carried out [2.10] with varying amount of template DNA: 0.2μl (1), 0.1μl (2) and 0.05μl (3) prepared as described [2.9.3]. The template DNA was the CyPD clone obtained by screening the cDNA library (‘Clone 2’). 10% of the products were run on a 1% agarose gel [2.11]. There appeared to be no difference in the yield of the fragment of interest (~0.54kbp).
was followed by six bases which made up the EcoRI restriction site and finally the spacer region of four bases.

A diagrammatic representation of primers P5 and P6 is shown in Fig. 5.1B. They are aligned against the peptide sequence of CyPD to indicate their positions relative to the entire CyPD protein (i.e. including the presequence).

The primers were used in three standard PCR reactions (see [2.10]). The only variable was the amount of template used. Tracks 1, 2 and 3 contained 0.2, 0.1 and 0.05µl of phage DNA respectively (prepared as [2.9.3]). The template DNA for all the reactions was the positive CyPD bacteriophage clone obtained from the λgt11 rat liver cDNA library [4.4]. 10% of the products from each of the reactions were loaded onto a 1% agarose gel. This was electrophoresed and stained with ethidium bromide. Fig. 5.1C is a photograph of the gel. The amount of DNA visible in tracks 1, 2 and 3 is the same. Varying the amount of template did not have much of an effect. The primers were expected to amplify a PCR product of 0.56kbp, and this was obtained with a satisfactory yield in all three reactions.

The 0.56kbp fragment was purified from the agarose gel. Before ligation of the vector and insert could be carried out, they both had to be digested with the appropriate restriction enzymes (BamHI and EcoRI) to generate ‘sticky ends’ [2.13.1]. After ligation, the vector (+ insert) was then used to transform E.coli cells. Transformed cells were selected for by the ampicillin resistance gene contained in the plasmid. In the first instance, it was attempted to identify recombinants by PCR screening [2.13.2] but the PCR reaction amplified a correct sized product even from the negative control (non-transformed E.coli cells). This indicated the E.coli genome must contain a similar sequence to which the primers were hybridising. Accordingly, the potential recombinants were then screened by
restriction analysis [2.13.2]. DNAs from these colonies were digested with EcoR1 and BamH1. Recombinant colonies yielded a fragment of ~0.56kbp.

[5.2] *Induction of fusion protein expression*

Two identical 10ml cultures of transformed *E.coli* [5.1] were prepared. Fusion protein expression was induced in one of the cultures by IPTG [2.19]. Both cultures were sedimented by centrifugation and resuspended in 1ml equilibration buffer [2.20]. The cells were sonicated to rupture their cell walls. The 1ml induced and 1ml non-induced samples were each divided into two 0.5ml samples. Fig. 5.2A is a summary of how each sample was treated. One 0.5ml sample from both the uninduced and induced cultures was regarded as the total protein (samples 1 and 2 respectively, Fig. 5.2A). The remaining 0.5ml from the induced culture was centrifuged. The supernatant was regarded as the induced soluble protein (sample 3, Fig. 5.2A) and the pellet as the induced insoluble protein (sample 4, Fig. 5.2A). Each 0.5ml sample was in equilibration buffer. 10µl each of samples 1-4 from Fig. 5.2A was added to an equal volume of 2 x sample loading buffer and run on a SDS-PAGE gel. Fig. 5.2B is a photograph of the gel.

The size of the fusion protein was expected to be ~44kDa as the GST protein is ~26kDa and mature CyPD ~18kDa. Unexpectedly, a small amount of a protein at approximately 44kDa is visible in track 1 (sample 1) of Fig. 5.2B. This sample had not been induced by IPTG. In addition to induction, the other major type of gene regulation is repression. Since the sample had not been induced, then repression of the gene must have been slightly inefficient. If sufficient amounts of repressor (produced by the *lacI* gene) was not available, then it would not bind to all the promoter regions and some
Fig. 5.2A] Preparation of induced and uninduced bacterial cultures transformed with the pGEX-3X plasmid containing the GST/CyPD gene.

1 ml induced sample

0.5 ml total induced sample

2

centrifuged

supernatant

0.5 ml induced soluble protein

3

resuspended in 0.5 ml

induced insoluble protein

1 ml uninduced sample

0.5 ml total uninduced sample

1

pellet

0.5 ml induced insoluble protein

4
Two bacterial cultures transformed with the pGEX-3X plasmid containing the GST/CyPD gene were prepared [2.19] One was induced for fusion protein expression with IPTG. Both cultures were spun down and resuspended in 1ml equilibration buffer [2.20]. Cell walls were ruptured by sonication. The two 1ml samples were treated as detailed in Fig. 5.2A, and aliquots of the four resulting samples were run on an SDS-PAGE gel.

1 Total protein in the uninduced culture.
2 Total protein in the induced culture.
3 Soluble protein in the induced culture.
4 Insoluble protein in the induced culture.
transcripts of the gene would be produced. This may have been the case with respect to sample 1.

The fusion protein is clearly visible at approximately 44kDa in tracks 2 and 3 (samples 2 and 3 respectively). Both samples had been induced by IPTG. IPTG binds and so inactivates repressor molecules, leaving promoter regions active to switch on transcription.

There is very little protein visible in track 4 (sample 4) and none around 44kDa, indicating the fusion protein is soluble.

[5.3] Purification of fusion protein from bacterial cell lysate

The soluble nature of the fusion protein meant that it was easily purifiable via affinity chromatography. The fusion protein was isolated from total bacterial cell lysate using an immobilised glutathione matrix, as described [2.20]. Two columns of 1ml volume were prepared, one for the uninduced culture and one for the induced culture. 10ml cultures were prepared and treated as in [5.2] to obtain the soluble protein. PPIase activity was measured before passing the 1ml samples through the columns. The first 3ml was collected and referred to as ‘passthrough’. The following 2ml were ‘wash’. At this point the buffer was switched to the elution buffer (20mM reduced glutathione in the equilibration buffer [2.20]). Every ml for the next 5 was collected separately.

Fig. 5.3A shows the PPIase activity recovered from isolation of fusion protein from an uninduced (i) and induced (ii) sample. There was approximately 2.5 times more PPIase activity in the induced than in the uninduced culture. This confirmed
PPIase activity recovered during purification of fusion protein from bacterial cell lysate.

Experimental details are given in [5.3]. PPIase activity was measured before passing the 1ml bacterial cell lysate samples through the glutathione agarose columns. The first 3mls were collected and referred to as 'passthrough'. The following 2mls were 'wash'. The buffer was switched to the elution buffer 'GSH' and every ml for the next 5 was collected separately.

![Graph showing PPIase activity recovery](image-url)
Table 5.3A] PPIase activity measurements to determine
stability of fusion protein.

<table>
<thead>
<tr>
<th></th>
<th>Stability</th>
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<tr>
<td></td>
<td>PPIase activity in total cell lysate (s⁻¹)</td>
<td>PPIase activity at t = 0 (s⁻¹)</td>
<td>PPIase activity at t = 90 (s⁻¹)</td>
</tr>
<tr>
<td>Uninduced</td>
<td>1) 6.8</td>
<td>3) 5.3</td>
<td>5) (+PMSF) 2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6) (-PMSF) 2.1</td>
</tr>
<tr>
<td>Induced</td>
<td>2) 17.1</td>
<td>4) 17.1</td>
<td>7) (+PMSF) 11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8) (-PMSF) 12.2</td>
</tr>
</tbody>
</table>

Numbers 1 - 8 (in bold type) correspond to the protein lanes in Fig. 5.3B.
[Fig. 5.3B] SDS-PAGE analysis of GST/CyPD fusion protein to determine stability.

Experimental details are given in [5.3]. Lane numbers 1 - 8 correspond to sample numbers 1 - 8 from Table 5.3A.

1. Non-induced total cell lysate
2. Induced total cell lysate
3. Non-induced purified fusion protein at t=0
4. Induced purified fusion protein at t=0
5. Non-induced purified fusion protein at t=90 minutes + 1mM PMSF
6. Non-induced purified fusion protein at t=90 minutes - 1mM PMSF
7. Induced purified fusion protein at t=90 minutes + 1mM PMSF
8. Induced purified fusion protein at t=90 minutes - 1mM PMSF
the functional integrity of the GST/CyPD fusion protein with respect to the PPIase activity of CyPD.

Approximately 40% of the PPIase activity of the uninduced sample loaded onto the column was recovered in the passthrough (Fig. 5.3Ai), and the rest came through in the next 7ml with a barely significant increase when the elution buffer was put through after the wash.

Approximately 25% of the PPIase activity of the induced sample was recovered in the passthrough. There was a significant increase in elution of PPIase activity in the 2nd ml after switching to elution buffer (~ 50% of the PPIase activity loaded was eluted in this ml, see Fig. 5.3Aii).

Table 5.3A refers to a repeat of the above experiment except that 30ml (not 10ml) of each culture was prepared. Therefore 3ml of each bacterial cell lysate was obtained. PPIase activity was measured and again 2-3 times more activity was observed in the induced sample compared to the uninduced. Each sample was passed through the column and the column was again washed for a further two column volumes before switching to the elution buffer. The first 3ml were collected.

To determine the stability of the fusion protein as a function of its PPIase activity, the activity was measured at t=0 (i.e. immediately on elution from the column) and at t=90 minutes. After taking the t=0 measurements, the two samples were divided into two and the protease inhibitor PMSF was added to one of the two to a final concentration of 1mM. The four samples were left at room temperature for 90 minutes.
[Fig. 5.3C] **Cation exchange chromatography of GST/CyPD fusion protein.**

2mls of bacterial cell lysate from an induced culture were prepared and fusion protein purified [2.19-20]. Protein was loaded onto a Mono S column and found to elute at ~100mM NaCl [2.4.2.2]. All fractions showing protein at OD$_{280}$ were assayed for PPIase activity. It was detected in fractions 11-13. These values are reported below.

![Chromatogram](https://via.placeholder.com/150)

<table>
<thead>
<tr>
<th>Fraction no.</th>
<th>PPIase activity / ml (s$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>0.07</td>
</tr>
<tr>
<td>12</td>
<td>0.63</td>
</tr>
<tr>
<td>13</td>
<td>0.27</td>
</tr>
<tr>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>
[Fig. 5.3D] SDS-PAGE analysis of GST/CyPD fusion protein after elution from Mono S cation exchange column.

Fractions 10-14, Fig. 5.3C, were concentrated by freeze-drying and 50% of each sample was loaded onto the gel. PPIase activity reported in Fig. 5.3C corresponded to the single protein band seen most prominently in fraction 12.

protein markers

97K→
66K→
45K→
29K→
21K→
14K→

fraction number
About 40% of the PPIase activity of the uninduced samples remained after 90 minutes; PMSF did not appear to have any effect. About 70% of the PPIase activity of the induced sample remained after 90 minutes; PMSF appeared to have a slightly detrimental effect.

The lack of beneficial effects on stability of PMSF and the general stable nature of the protein as determined by the PPIase activity indicates any proteolytic enzymes in the total bacterial cell lysate have been removed via the affinity matrix.

The above results are visualised in Fig. 5.3B. 10µl of the total bacterial cell lysate from both cultures, 10µl of both samples at t=0, and 10µl of the four samples at t=90 minutes were added to an equal volume of 2 x sample loading buffer and run on a SDS-PAGE gel. A photograph of the gel is shown in Fig. 5.3B. Tracks 1 and 2 show the protein content of total bacterial cell lysate in uninduced and induced cultures respectively. Again, as in Fig. 5.2B, the fusion protein is visible in both cultures with there being 2-3 times more in the induced culture. This difference remained consistent throughout the course of the experiment. Tracks 3 and 4 show the fusion protein immediately on elution from the GSH column (i.e. t=0) in the uninduced and induced cultures respectively. There is little difference visible between the amount of protein in track 3 and (after 90 minutes) in tracks 5 and 6 (with and without PMSF respectively). Likewise, there is little difference between the amount of protein visible in track 4 and (after 90 minutes) in tracks 7 and 8 (with and without PMSF respectively).

To further assess the purity of the fusion protein after its elution from the glutathione matrix, a cation exchange column was employed. 20ml induced bacterial culture was prepared in the same manner as in [5.2] except that the
equilibration buffer did not contain 100mM NaCl. The bacterial cell lysate was
passed through the 1ml column in the usual way. 10% of the eluate was passed
through a mono-S column equilibrated with equilibration buffer without 100mM
NaCl. The trace from the mono-S column is shown in Fig. 5.3Ci. Protein began
eluting at ~100mM NaCl. 1ml fractions were collected, and the PPIase activities
of fractions 10-14 are shown in Fig. 5.3Cii. There was no PPIase activity
associated with the passthrough. The protein content of fractions 10-14 is shown
in Fig. 5.3D, which is a photograph of a SDS-PAGE gel of the fractions. Each 1ml
fraction was concentrated by freeze-drying and 50% of each sample was loaded
onto the gel. PPIase activity values corresponded to the single protein band.

[5.4] Cleavage of the fusion protein into GST and CyPD

To enable comparison of the specific activities of the fusion protein and CyPD,
the Factor Xa protease recognition site contained between the GST and CyPD
was employed to cleave the GST from the CyPD. The procedure is outlined in
[2.21]. To separate the CyPD from the other proteins in the digestion mix (GST,
uncleaved fusion and protease enzyme), after the incubation period was over, the
digestion mix was passed through a gel filtration column (Superdex 75) so the
proteins would be separated on the basis of their molecular weight. As was
established earlier, CyPD eluted in fraction 15 (data not shown here). A 10%T
SDS-PAGE gel was carried out of an aliquot of the cleaved CyPD, a tiny amount
of CyPD that had previously been purified from rat liver mitochondrial matrix (as
described earlier, see Chapter 3) and had been stored at -20°C, and a sample of
CyPA that had been purified from rat liver cytosol. CyPA was purified in a series
of cation exchange and gel filtration steps (data not shown). A photograph of this
[Fig. 5.4A] SDS-PAGE analysis of CyPD cleaved from the fusion protein, native CyPD and native CyPA.

1. CyPD cleaved from the GST/CyPD fusion protein [2.21].
2. Native CyPD purified from rat liver mitochondrial matrix [3.3].
3. Native CyPA purified from rat liver cytosol in a series of cation exchange and gel filtration steps.

protein markers

\[34.5K \rightarrow 28.8K \rightarrow 20.5K \rightarrow 7.4K \rightarrow\]

1 2 3
gel is shown in Fig. 5.4A. The first track shows CyPD that was cleaved from the fusion protein and the second track shows CyPD purified from rat liver. Although not absolutely clear, due to overloading of the first track and rather a low %T (see [2.6], a %T of 15% would probably have demonstrated the molecular weights better), these two proteins were taken to be of a similar if not identical molecular weight of ~20kDa, as determined from the molecular weight markers. The third track shows CyPA purified from rat liver and this appears slightly smaller than its neighbours. The base and peptide sequences for CyPA of a number of species have actually been published and show CyPA to have a molecular weight between 17-18kDa (Harding et al [1986], Danielson et al [1988]).

[5.5] Calculation of the specific activities of the GST-CyPD fusion, the cleaved recombinant CyPD and the purified native CyPD

The PPIase activities of known amounts of the GST-CyPD protein, the cleaved CyPD protein and the native CyPD protein were calculated. The $k_{cat}/K_M$ value for the GST-CyPD protein was found to be an average of 23.7 $\mu M^{-1}s^{-1}$ (n=2).

The $k_{cat}/K_M$ value for the cleaved CyPD protein was found to be an average of 62.8 $\mu M^{-1}s^{-1}$ (n=3).

The $k_{cat}/K_M$ value for the native CyPD protein (purification of which is summarised in Table 3.3A) was found to be an average of 29.5 $\mu M^{-1}s^{-1}$ (n=3).

The three sets of data above are represented in Table 5.5A.
[Table 5.5A] $k_{cat}/K_M$ values for CyPD proteins

<table>
<thead>
<tr>
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<th>$k_{cat}/K_M$ (μM$^{-1}$s$^{-1}$)</th>
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<tbody>
<tr>
<td>GST/CyPD</td>
<td>27.2</td>
</tr>
<tr>
<td>CyPD (cleaved from fusion)</td>
<td>71.4</td>
</tr>
<tr>
<td>native CyPD</td>
<td>26.4</td>
</tr>
</tbody>
</table>

The figures displayed in Table 5.5A indicate the CyPD protein cleaved by Factor Xa from the fusion protein to be 2-3 fold more active than either the fusion protein or the native CyPD protein, which have similar turnover ($k_{cat}/K_M$) values. This indicates a possible partial activation of the CyPD protein by Factor Xa cleavage. Although CyPD does not contain the protease recognition site (IEGR↓), a structurally similar site may be present, which Factor Xa could be recognise, but is not immediately apparent. This is being investigated presently, and protease activation of proteins is discussed further in Chapter 6.

[5.6] The search for a mitochondrial binding protein to the GST-CyPD fusion protein

The results from the previous section indicate that the CyPD fused to GST has similar properties to that of the native protein, at least with regards to PPIase activity. To confirm this, the $K_i$ values of CsA inhibition of the PPIase activities of the fusion protein and of the native protein were obtained (data not shown). They were 5nM and 3nM respectively. These results together with the previous data
[Fig. 5.6A] **SDS-PAGE analysis of the mitochondrial binding protein to GST/CyPD.**

Experimental details are provided in [5.6].

Lanes 1 and 3: GST/CyPD incubated with soluble inner mitochondrial membrane extract (IMME).

Lanes 2 and 4: GST/CyPD incubated without IMME (control).

Lanes 5 and 6: GST incubated with IMME (control).
meant the use of the GST-CyPD protein to isolate mitochondrial binding proteins to CyPD could now be justified.

Mitochondria were prepared from ten rat hearts as described [2.1] and from these the matrix and inner membrane fractions were prepared [2.4.1]. The inner membrane was resuspended in 3ml 100mM NaCl, 10mM Hepes pH 7.2 (EB buffer) with 6% Chaps to solubilise the membrane. The EB buffer also contained 1mM PMSF and 1μg/ml of the following protease inhibitors: chymostatin, peptatin, leupeptin and antipain. This was hand homogenised thoroughly and left on ice for 30 minutes. The debris was spun down in an eppendorf centrifuge at 4°C for 10 minutes. The resulting supernatant was the source of mitochondrial binding protein(s) to GST-CyPD.

To prepare the GST-CyPD affinity matrix, 10ml induced bacterial cell lysate [2.19] were passed through 0.5ml of glutathione-agarose equilibrated with EB buffer [2.20]. This was washed through with a further 5 x 1ml of EB buffer. A 40μl aliquot of the affinity matrix-GST-CyPD gel slurry was incubated with 0.4ml of the solubilised inner membrane fraction in an eppendorf tube for 30 minutes on ice with intermittent gentle shaking. The gel beads were then spun down, the supernatant removed, and the beads resuspended in 0.4ml EB buffer containing 0.6% Chaps. The beads were spun down again and the supernatant discarded. This was repeated four times to ensure thorough washing of the beads of residual mitochondrial components.

The GST-CyPD protein and any binding proteins were then eluted from the affinity matrix by the addition of 40μl of 70mM reduced glutathione and gentle
mixing. After 5 minutes incubation on ice, the beads were spun down and the 40μl supernatant removed.

Two factors needed to be considered: whether any binding proteins observed were of mitochondrial origin or bacterial origin, and whether any binding proteins observed were interacting with the CyPD part of the protein or to the GST.

These were dealt with by running the following two controls alongside the test sample:

1. Bacteria were transformed with the pGEX plasmid containing only the GST gene and so on induction of these bacteria with IPTG only GST was expressed. 0.5ml of glutathione-agarose was prepared by loading with GST alone (i.e. no fusion protein and therefore no CyPD).

2. Affinity matrix loaded with fusion protein was incubated with 1ml EB buffer rather than soluble inner membrane fraction.

The 20μl of test sample plus the two 20μl control samples were run on a SDS-PAGE gel, a photograph of which is shown in Fig. 5.6A. As can be seen on track 1, a binding protein of approximately 32kDa is visible in the test sample, but not in either control(tracks 2 and 3). This means a 32kDa protein of mitochondrial origin binds to the CyPD part of the fusion protein.

The principle objective here was to further elucidate the role of CyPD in the action of the permeability transition pore. The pore has been demonstrated to be activated by Ca^{2+} and inhibited by ADP and CsA. To investigate the nature of the 32kDa binding protein interaction with CyPD with regards to pore activity, the above experiment was repeated three times with the following modifications, with
an additional modification to check for interactions in the mitochondrial matrix fraction with CyPD:

1. 1mM EGTA in the EB buffer;
2. 1mM CaCl₂ in the EB buffer;
3. CsA added to the affinity matrix-GST-CyPD 5 minutes before incubation with the soluble inner membrane fraction;
4. mitochondrial matrix fraction used as source of binding protein(s) instead of soluble inner membrane fraction.

It was expected that under conditions of pore activation CyPD-binding protein interaction would be potentiated, and that under conditions of pore inhibition interaction of CyPD with the binding protein would be inhibited. It was also expected that CyPD binding proteins would be discovered in the matrix as CyPD was originally isolated from the matrix fraction. However, neither of these was the case.

The first three experiments produced the same result as the original (i.e. a repeat of Fig. 5.6A). Pore inducing or inhibiting conditions did not seem to affect the interaction of CyPD with the 32kDa protein. The third experiment showed no binding proteins of CyPD to be present in the mitochondrial matrix fraction, 32kDa in size or otherwise.

These results were unexpected as it was assumed the interaction of CyPD to any binding protein would be sensitive to factors that influence the pore. However, this does not rule out the possibility of the 32kDa protein being a pore component as a series of proteins are likely to form the pore and a partially reconstituted entity may not be able exhibit pore-like characteristics.
Presently a sample of the 32kDa protein will be purified and sent for peptide sequencing. The possible identity of this protein is discussed further in Chapter 6.
CHAPTER 6: DISCUSSION

[6.1] Indications for cyclophilin involvement in the permeability transition pore

The cyclophilin family of proteins, characterised by their peptidyl-prolyl cis-trans isomerase activity which is inhibited by CsA, have been shown to be the principal binding proteins for CsA (see Chapter 1). The effect of CsA on the permeability transition indicated the likelihood of a mitochondrial cyclophilin playing a role in the process and possibly mediating the CsA effects. If cyclophilin(s) were identified in the locality of the pore, this would further enhance this hypothesis.

This investigation has discovered two cyclophilin isoforms within rat liver mitochondria. One was located in the matrix fraction and the other in the intermembrane space fraction. This further implicates cyclophilin in the activity of the mitochondrial inner membrane pore which is inhibited by CsA. Other observations support this.

Andreeva et al [1995] investigated the possible role of cyclophilins in the activity of the mitochondrial inner membrane pore. As CsA was well documented in the blockade of the pore, this group photolabelled rat heart mitochondria with a CsA derivative, and then subjected mitochondria to pore activating conditions. Their hypothesis was that the CsA binding protein would be a cyclophilin. Binding of CsA to this protein, induced by pore activating conditions, would label a cyclophilin involved in pore activity. The earlier work of this group had shown rises in intramitochondrial Ca$^{2+}$ levels to decrease the capacity of CsA to inhibit pore opening, and the opposite effect of rises in intramitochondrial ADP levels. These parameters were used to attempt the isolation of the relevant proteins by
the photolabelling studies. Mitochondria were photolabelled in the presence of either Ca\(^{2+}\), EGTA or EGTA and ADP. Membrane fractions were prepared by suspending mitochondria in a Tris/HCl buffer (pH 8.1) containing protease inhibitors, sonicating and then isolating membranes by centrifugation. Ca\(^{2+}\) was found to depress photolabelling of membrane fraction components eluting at 11-22kDa on a gel filtration column. ADP enhanced photolabelling in the same region. When they analysed this region by SDS-PAGE, the majority of the photolabel was in a 22kDa component, and a minor radiolabelled component was found at 11kDa. Purification of PPIase activity from the 11-22kDa region gave a protein which migrated at 22kDa on SDS-PAGE gel. The final preparation also showed minor protein bands at 21kDa and 11kDa.

The authors suggested that the 11kDa component may have arisen through proteolysis within the membrane fraction. Results displayed in Chapter 3 of this investigation support this. An 11kDa protein appears to be the principal degradation product of the larger of the cyclophilin isoforms (CyP21) isolated from the matrix fraction (Fig. 3.3D, lane B).

Nicolli et al [1996] isolated a mitochondrial cyclophilin from a rat liver subcellular fraction by constructing an affinity column with immobilised CsA. Other cyclophilin isoforms were eluted with CsA, but a further two proteins, of molecular weights 30 and 32kDa, could only be eluted on denaturation by urea. The latter was later determined as the outer mitochondrial membrane protein porin by Western blotting using a specific antibody against the rat liver protein. The former protein was not characterised. They found the mitochondrial cyclophilin (that they termed CyP-M) to be a matrix protein which interacted with the inner membrane under physiological conditions. This interaction was disrupted by CsA...
and mildly acidic pH values, conditions which inhibit the pore. They also observed that the potency of inhibition of the PPIase activity of CyP-M of various CsA derivatives was similar to that displayed at inhibition of pore opening.

Confirmation of the presence cyclophilin in the mitochondrial matrix lends further credence to the hypothesis of cyclophilin controlling the inner membrane pore. The use of cyclophilin as a tool in the isolation of other pore constituents is now justifiable.

[6.2] Cyclophilin isoforms in mitochondria

The discovery during this investigation of two cyclophilin proteins in distinct compartments within mitochondria raises the interesting question that is the larger isoform really the only cyclophilin of interest with regards to the inner membrane pore, or could it be possible that both isoforms are involved in its activity? Additionally, did these proteins enter the mitochondria separately, or was there one large cyclophilin precursor that was cleaved differentially into two smaller isoforms?

The presence of more than one cyclophilin isoform in rat liver mitochondria has been previously reported by Connern & Halestrap [1992]. Mitochondria were separated into mitoplasts and intermembrane space plus outer membrane fractions by incubation with 0.12mg digitonin / mg mitochondrial protein. Mitoplasts were sonicated and the inner membrane removed by centrifugation. This group carried out ammonium sulphate precipitation of the matrix fraction before passing extracts through ion exchange and gel filtration chromatography columns. Two PPIase activity peaks were eluted from the gel filtration column. On SDS-PAGE analysis, the larger of the peaks was found to contain a protein of 18.6kDa molecular
weight. The smaller peak contained this protein plus another protein of molecular weight 17.5kDa. Both proteins were N-terminally sequenced (see Chapter 3) and the results suggested to the authors that both proteins were encoded by the same gene and therefore initially both proteins were identical. After entry into mitochondria, the precursor protein was believed to have its mitochondrial targeting sequence differentially cleaved within the matrix to give the two forms.

Woodfield et al [1997] took the work of this group further by using a human CyPD cDNA clone to produce a radiolabelled probe. This was used to screen a rat skeletal muscle Uni-Zap XR library for the full length cDNA of CyPD. Positive clones were obtained and the complete base sequence was determined by a primer walking strategy. It contained an open reading frame of 207 amino acids. The starting point of the two cyclophilins reported earlier by Connern & Halestrap [1992], 18.6kDa and 17.5kDa, can be seen 29 and 39 amino acids into the full length peptide sequence. These two proteins are likely to correspond to the two cyclophilins isolated during this investigation.

A 3-4kDa variation in the estimates of the molecular weight of a protein from laboratory to laboratory is not uncommon. In the case of ornithine carbamoyltransferase, for example, when the base sequence was obtained for the gene encoding the rat protein, it put the molecular weight of the protein at 36,135 Da (Takiguchi et al [1984]). Before this, estimates varied from 35,300 - 39,600 Da. For example, Lusty et al [1979] and Hoogenraad et al [1980] both carried out sedimentation equilibrium analysis of the trimeric protein, and also SDS-PAGE analysis of the monomers. The earlier paper reported the molecular weight at 39,600 ± 1000Da, and the later paper estimated it at 35,300Da.
It is expected that the targeting sequence of a mitochondrial protein is cleaved on entry into the matrix. Mitochondrial proteins are synthesised as precursors in the cytosol and are imported post-translationally into the mitochondria (for review, see Neupert [1997]). These cytosolic precursors remain loosely-folded and translocation competent by interaction with chaperone molecules. They generally contain amino-terminal presequences containing the required information for targeting to mitochondria. These presequences are usually positively charged, rich in hydroxylated amino acids and form an amphipathic helix. Precursor proteins interact with surface receptors on the mitochondria, and insertion into the outer membrane is believed to be via a ‘general insertion protein’ (GIP) and ATP-dependant. Translocation into and across the inner membrane is probably at contact sites between the outer and inner membrane, through a proteinaceous channel or pore. Translocation is completed with the aid of mitochondrial molecular chaperones within the matrix such as hsp60. Presequences are cleaved during or after translocation by the matrix processing peptidase. Some proteins of the inner membrane and all proteins of the intermembrane space then undergo further treatment within the matrix in order to reach their target compartment, although these are less well understood.

The results had initially indicated that CyP21 did have such a sequence as on peptide sequencing, one peptide could not be aligned against the human CyPD peptide sequence (Fig. 4.1A, A). This one peptide was assumed to form part of a rat liver mitochondrial targeting sequence. This peptide fits in at residue 31 of the complete peptide sequence of Woodfield et al [1997]. This is all further complicated by the isolation of a cyclophilin with a molecular weight of 18kDa in the intermembrane space fraction of rat liver mitochondria. Its convenient
molecular weight of 18kDa does suggest its formation by cleavage of a targeting sequence from the 21kDa matrix protein. However this would mean that a targeting sequence first directed it to the matrix, then out into the intermembrane space, and was then cleaved off. It seems probable that targeting sequences contain two sets of information: the first specifies 'mitochondria', and the second 'intermembrane space'. There are two possible pathways. The protein may enter the matrix, lose a part of its presequence, and the remainder guides it to its target compartment. The alternative is the presequence contains some kind of stop signal which halts the path of the protein before it reaches the matrix. There is every reason to consider both pathways are in existence and which one is undertaken depends on the precursor being imported. Obtaining sequence data of CyP18 will further explain its origin.

One point that may be relevant is that CyPs 21 and 18 were isolated from rat liver, and base sequence data was obtained via a rat liver cDNA library. Connem & Halestrap [1992] used a rat skeletal muscle cDNA library to isolate full length cDNA clones of CyPD. However there is no evidence to suggest that the CyPDs of rat liver and skeletal muscle should not be identical. There has been no evidence presented of targeting sequences to various cell organelles differing between cell types. However, one published report suggests the efficiency of import may vary between cell types. Cote and Boulet [1985] compared the ability of mitochondria from four tissues to import and process the precursor to β-subunit of the F$_1$-ATPase, a protein common to all mitochondria, and the precursor to ornithine carbamolytransferase (OC), a tissue-specific protein found in the matrix of liver mitochondria. The precursors were generated in a reticulocyte lysate and labelled
with 35S-Methionine. The precursor of the F1-ATPase β-subunit was observed to import with more efficiency into spleen and liver mitochondria than heart or kidney mitochondria. The opposite was observed with the import of the OC precursor. The authors took this to indicate that mitochondria do not discriminate against tissue-specific mitochondrial proteins. In addition to this, variations in import efficiency of different tissue mitochondria suggests variations in the processing procedure.

Mitochondrial targeting sequences have been extensively studied in order to define any sequence motifs between them. Hendrick et al [1989] conducted a study comparing the peptide sequences of 50 precursors of mitochondrial proteins where the mature amino terminus had been determined by peptide sequence analysis.

Two possible models of processing of mitochondrial precursors into mature proteins emerged as a result. The first was a one-step cleavage of the leader sequence from the mature protein, and the second was a two-step cleavage. The one-step model was characteristic of precursors containing an arginine at position -2 relative to the cleavage site. The two-step model contained a second cleavage site eight residues further on from the cleavage site.

Multiple cleavage products as a result of a two-step cleavage are a possible explanation for detecting more than one CyPD isoform in mitochondria both in this investigation (CyP18 of the intermembrane space and CyP21 of the matrix) and Connern & Halestrap [1992] (the 18.5kDa and 17.6kDa proteins mentioned earlier). However, on examination of the CyPD precursor peptide sequence, the protein does not appear to strictly contain the two-step cleavage sequence motif. Two peptide bonds to the carboxyl end of the arginine at position 28 of the CyPD
[Fig. 6.1] The first 40 residues of rat CyPD (Woodfield et al [1997])

MLALRCGPRLGLLSGPRSAPLLLLSTTRTC

1. Possible second cleavage site if protein follows two-step model.
2. N-terminal of the smaller cyclophilin protein reported by Connern & Halestrap [1992].

[Fig. 6.2] The first 60 residues of the cyclophilin from Neurospora crassa (Tropschug et al [1988]).

MFGPRHFSVLTGSLVSTFSSSLKPTAT

1. Possible first cleavage site if protein follows two-step model.
2. Beginning of mature protein.
precursor is believed to be the site of cleavage into the mature protein. To fit the two-step model, another possible cleavage site would have to be present eight peptide bonds to the carboxyl side of the first site. Connern & Halestrap [1992] obtained N-terminal sequence data of their two cyclophilin proteins. There were ten residues between them. It may be that this a loose fit of the two-step model [Fig. 6.1].

A cyclophilin has been identified which contains the two-step sequence motif and has been demonstrated to be cleaved in two steps. The cyclophilin of Neurospora crassa, reported by Tropschug et al [1988], exists in two forms encoded by a single gene. One form resides in the cytosol, and the other in the mitochondria. The precursor of the mitochondrial form, a 24kDa polypeptide, was generated using reticulocyte lysate and labelled with 35S-Methionine. This was incubated with mitochondria in the presence or absence of a mitochondrial membrane potential. Only in the presence of a membrane potential was the 20kDa mature cyclophilin detected in the mitochondria. When the matrix-located processing activity was partially inhibited by a metal chelator, but a membrane potential was present, a 21kDa intermediate cyclophilin form, not the mature form, was detected. This indicated the mature protein was processed in two steps. The first cleavage site is not known, but if the two-step model is followed, an arginine should be present at position -2 relative to the cleavage site. This would indicate the first cleavage takes place after the alanine at position 36 of the precursor [Fig. 6.2]. The mature protein begins from the serine at position 45 of the precursor. It is worth noting that both the precursor of the mitochondrial cyclophilin, and the mature protein, are larger in the fungus than their mammalian counterparts.
One of the principal aims of this investigation was to attempt the elucidation of permeability transition pore components. Now that cyclophilin was identified and localised to the mitochondrial matrix, proteins that interacted with it could be investigated. If these proteins could be resolved, they could well prove to be those responsible for the mitochondrial malfunction that leads to cell damage during ischaemia and reperfusion injury and may even provide targets for drugs that protect against this form of injury.

The construction of a fusion protein consisting of a cyclophilin and glutathione-S-transferase (GST) (Chapter 5) has been carried out previously.

Friedman & Weissman [1991], Friedman et al [1993], isolated a cyclophilin from a cDNA library prepared from a bone marrow-derived stromal cell line. The cDNA was isolated from a subtracted sublibrary which contained genes induced by treatment of the cell line with interleukin-1. The CyPC mRNA levels were induced 2-3 fold by this treatment. CyPC was used as an affinity probe for cytoplasmic proteins involved in its functions via construction of a fusion protein with GST by cloning of the protein into one of the pGEX bacterial expression vector series.

CyPC-GST affinity purification of cell-line extracts revealed a number of proteins which bound specifically to CyPC-GST and were eluted by CsA. They appeared to have molecular weights of 77kDa, 60kDa, 37kDa and 25kDa on SDS-PAGE gels. Protein sequence data suggested that all four proteins had a common precursor, possibly the 77kDa protein. The protein sequences were used to design primers to screen the cDNA library by PCR. A partial clone was obtained, and this was used as a probe to isolate the full length cDNA. The longest clone obtained
encoded a protein of 574 amino acids. This cDNA was cloned into another vector and transfected into COS cells. This directed the production of a 77kDa glycoprotein which bound CyPC in the absence, but not the presence, of CsA. The protein was found to contain a cysteine-rich domain found in a variety of cell-surface molecules. This prompted the authors to suggest that CyPC may serve as a mediator or regulator of an unknown signal or cellular process.

This approach was used by Hoffmann & Handschumacher [1995] to identify cellular components of the ubiquitous cyclophilin 40 (CyP 40). After expressing it as a fusion with GST and coupling to a GSH affinity matrix, a heat-shock protein (hsp 90) was found to be the predominant associated protein in all the tissue extracts examined. This same protein was treated in a similar manner by Ratajczak et al [1995]. They incubated the fusion of GST and CyP 40 (which they termed GST-ERBC) with calmodulin-agarose. In the presence of Ca\(^{2+}\) the protein was completely retained on the resin. When EGTA replaced Ca\(^{2+}\) however, the majority of the protein remained unabsorbed.

As the GST-affinity column method for the purification of the fusion protein and identification of binding proteins had so successfully been demonstrated as in the above reports, this approach was undertaken with CyP D. Although the binding protein observed [Fig. 5.6A] has yet to be characterised, the observations of Nicoll et al [1996] suggest that it may be one of the two proteins they obtained (30 and 32kDa in size) with a CsA affinity column. Although the molecular weight of the binding protein appears to be 32kDa and therefore indicating porin, the outer mitochondrial membrane location of porin suggests this is unlikely, unless of course the cyclophilin in the intermembrane space interacts with porin. The second (30kDa) protein observed by Nicoll et al [1996] remained
uncharacterised and this seems more likely to correspond to the binding protein observed during this investigation.

As mentioned briefly previously [1.5], the involvement of porin in the permeability transition has been suggested as long ago as 1988 (Crompton & Costi [1998]). Observations such as the stripping of the outer membrane from mitochondria preventing the resulting sub-mitochondrial particles (mitoplasts) exhibiting permeability transition characteristics (Le Quoc & Le Quoc [1985]) suggest that components within the contact sites between the outer and inner mitochondrial membranes may be involved in pore formation and/or activation. Le Quoc and Le Quoc prepared mitoplasts by digitonin incubation of rat liver mitochondria. After swelling in an isotonic salt solution (100mM ammonium acetate) of N-butylmaleimide-treated mitochondria and mitoplasts, PEG 6000 was added. In intact mitochondria this caused rapid, though transient, shrinkage. This effect was not observed in mitoplasts. This suggests that pore activation in the inner membrane, allowing the influx of polyethylene glycol molecules, can only occur in intact mitochondria.

The tight association of the outer mitochondrial membrane protein porin to the inner membrane has been demonstrated (Ono & Tuboi [1987]). Porin synthesised in vitro in a cell-free translation system with rat liver RNA was only found to bind to the outside of the outer mitochondrial membranes of intact mitochondria, when incubated at 0°C for 5 minutes. No binding was observed with isolated outer membranes. When the incubation time was extended, porin was found in the inner membrane fraction. No porin was detected when incubations were carried out with isolated inner membranes.
Reports that the conductance of the voltage-dependent anion selective channel formed by porin was half that of the permeability transition (see [1.5]) led to the proposition that two porin monomers may be involved in its structure. The adenine nucleotide translocase (AdNT) was incorporated into the model on purification of the mitochondrial receptor for benzodiazepines. After solubilisation of rat kidney mitochondrial membranes using a non-ionic detergent, the fraction was chromatographed on a hydroxyapatite column. The peak of binding activity was analysed by SDS-PAGE and three proteins were observed. Their molecular weights were 18, 30 and 32kDa. On a gel filtration (sizing) column, these proteins migrated as a single peak of 50-70kDa, indicating an intact complex. Western blot analyses identified the 32kDa protein as porin, and the 30kDa protein as the AdNT. Could the 18kDa peptide be the smaller of the two cyclophilins discovered during this investigation, CyP18? The possibility that the mitochondrial benzodiazepine receptor may coincide with the inner membrane pore is a real one, but the evidence presented is not at all conclusive. One point is that a significant amount of contact sites between the mitochondrial membranes appear to remain intact on digitonin incubation, according to the results obtained in this investigation using the outer mitochondrial membrane marker monoamine oxidase. Additionally, the presence of porin in inner membrane extracts has been demonstrated, presumably originating from contact sites (Ono & Tuboi [1987]). If this is the case, then this does not explain the lack of activation of the pore in mitoplasts. However, the apparent failure of CsA to influence activity of the mitochondrial benzodiazepine receptor still remains the principle argument against this model, and also suggests the 18kDa peptide is highly unlikely to be from the cyclophilin family.
As further information becomes available on the 32kDa cyclophilin binding protein discovered here, some light may be shed on the workings of the inner membrane pore and its constituent parts; this in turn could lead to fresh ideas for the management of ischaemia/reperfusion injury and related disorders.

[6.4] *Implications of the specific activity variations of CyPD depending on its fused, native or recombinant form*

The values obtained for the specific activities of the GST-CyPD fusion protein and the cleaved CyPD protein (k_{cat}/K_{M} values of ~20.2μM^{-1}s^{-1} and ~62.8μM^{-1}s^{-1} respectively, see [section 5.5]) initially appeared higher than one would expect. Connem and Halestrap calculated a value of ~0.9μM^{-1}s^{-1} for the larger of the mitochondrial CyP isoforms they reported (Connem & Halestrap [1992]), which probably corresponds to CyPD from this investigation. However, the dependence of the rate of the PPIase activity of CyPs on reaction conditions is well known. The most apparent difference between the two sets of reaction conditions is with regards to the temperature. During this investigation, all PPIase measurements were taken at 15°C. In Connem & Halestrap’s paper, the reaction temperature was quoted as 10°C. Although it seems unlikely this 5°C difference would account for a 60 fold difference in the k_{cat}/K_{M} value, it may play a significant part and in conjunction with other factors may not seem like such a drastic difference.

The other obvious difference is that of course the specific activities here were calculated for recombinant proteins. The 0.9μM^{-1}s^{-1} was derived for a native protein. Post-translational modifications and post mitochondrial import reactions may have any number of untold effects on the PPIase activity of the protein, either positive or negative.
A GST fusion protein of the bovine estrogen receptor binding cyclophilin (ERBC), which is a cyclophilin component of the unactivated estrogen receptor, has been expressed in *E. coli* (Ratajczak et al [1995]). They found the $k_{cat}/K_M$ values of the fusion protein and the cleaved recombinant ERBC protein to be similar (0.5$\mu$M$^{-1}$s$^{-1}$ at 5°C). However, the native bovine ERBC protein has also been characterised. They found the $k_{cat}/K_M$ value to be almost 4 fold higher, at 1.9$\mu$M$^{-1}$s$^{-1}$, and these activities were measured at 10°C. This would even further increase the rate of PPIase activity by an unknown amount.

The 3 fold difference in specific activities of the GST-CyPD fusion protein and CyPD cannot be explained in any definite manner. However, the conformational ramifications of adding a 26kDa protein onto a 20kDa protein are bound to be significant, and may well account for the decrease in PPIase activity of the fusion protein.

The similar $k_{cat}/K_M$ values for the native CyPD protein and that of the fusion protein against the 3 fold greater value of that of the recombinant protein [5.5] suggests the possible enhancement of PPIase activity by proteolytic cleavage of the CyPD protein. This activation of an enzymatic process by proteolysis is not an unheard of phenomenon:

The natural protein regulator of the cardiac sarcoplasmic reticulum calcium pump, phospholamban, is phosphorylated via the enzymatic activity of a protein kinase, and this leads to increased calcium pump activity and so removal of Ca$^{2+}$ from the cytoplasm during cardiac muscle relaxation. Unphosphorylated phospholamban inhibits the pump. In addition to phosphorylation, removal of inhibition has been demonstrated *in vitro* by the proteolytic cleavage of the cytoplasmic domain of phospholamban (Kirchberger et al [1986]). Trypsin incubation of cardiac
microsomes stimulated calcium uptake over a wide range of trypsin concentrations. The optimal concentration produced a 3-fold increase. Other proteases also activated microsomal calcium uptake but higher concentrations were required. The maximal activation achieved by trypsin was found to be approximately the same as that obtained by protein kinase; the effects were not additive. The authors concluded a common pathway for the action of trypsin and protein kinase on the regulatory function of phospholamban on calcium uptake.

The 3-fold greater specific rate of PPIase activity of the cleaved cyclophilin protein against the native protein and the fusion protein may indicate a similar mechanism of activation as the trypsin cleavage of phospholamban. If CyPD does indeed contain such a proteolytic site, this adds an interesting angle on to the effects, and regulation of these effects, of CyPD on the inner membrane pore. These findings are currently under further investigation.
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Membrane and Regulation of the Permeability Transition Pore, a Cyclosporin A-sensitive Channel.


APPENDIX

Protein markers (1)

97K  phosphorylase b
66K  bovine serum albumin
45K  ovalbumin
29K  carbonic anhydrase
21K  lysozyme
14K  lactalbumin

Protein markers (2)

202.0K myosin
109.9K β-galactosidase
78.8K bovine serum albumin
46.7K ovalbumin
34.5K carbonic anhydrase
28.8K soybean trypsin inhibitor
20.5K lysozyme
7.4K aprotinin

Superdex 75 markers

150K alcohol dehydrogenase
66K bovine serum albumin
29K carbonic anhydrase
13K cytochrome c

DNA markers

Pst I restriction endonuclease digest of bacteriophage λ DNA.