UBIQUNONE REACTS WITH NITRIC OXIDE TO PREVENT THE ACTIVITY OF PURIFIED COMPLEX I

Thesis submitted in fulfilment of the requirements of the University of London for the degree of Doctor of Philosophy in the Faculty of Medicine

Enrique Eduardo TERAN TORRES

May 2001
Abstract.

Mitochondrial complex I is a protein of 800 kDa consisting of 41 subunits. It contains flavin mononucleotide (FMN) and between 22-24 iron-sulphur (Fe-S) centres. Complex I is responsible for the oxidation of NADH to NAD\(^+\) in the presence of ubiquinone (CoQ) as electron acceptor. In this process, CoQ is reduced to ubiquinol (CoQH\(_2\)) which is re-oxidised to CoQ by complex III. Recently, it was shown in intact cells that prolonged exposure to nitric oxide (NO) results in a persistent inhibition of complex I activity, which is preceded by a decrease in the concentration of intracellular glutathione. The objective of this study was to investigate the possible mechanism(s) of the inhibition of complex I by NO using purified complex I from bovine heart mitochondria. The oxidation rate of NADH in the presence of complex I and CoQ was measured as an indicator of complex I activity, and the effect of different NO donors and incubation times on complex I activity was evaluated. Incubation of purified complex I with NO donors revealed no differences in the oxidation rate of NADH as compared to untreated controls. These results suggest that NO does not affect the activity of purified complex I. However, it was found that NADH added serially could be repeatedly oxidised by purified complex I and CoQ. In these conditions, exposure of CoQ to NO resulted in a reduction of the initial NADH oxidation and prevented almost completely the subsequent oxidation. Interestingly, the inhibitory effect of NO on CoQ was temporary and was completely reversible with time, suggesting the formation of a labile NO-CoQ adduct. Further biochemical and pharmacological analyses provided evidence of a chemical reaction between NO and CoQ.
Acknowledgements

• I am grateful to the School of Medicine at the Central University of Ecuador for their support and to the “Fundación para la Ciencia y Tecnología – FUNDACYT- for financial assistance.

• My thanks to Professor Salvador Moncada, who has always been available for discussion and for allowing me to work in his laboratory.

• I would like to thank to Mr. Richard O. Campbell, “Dick”, as his friendship, patience and helpful comments have made it possible for me to understand some of the principles and secrets of liquid chromatography. Another special friend I would like to thank is Mr. Neale Foxwell, whose support and knowledge were essential during my time as a “cell culture man”.

• There are not enough words to thank and express how important Doctor Edgar Lobos has been during my time in London, not only as a good friend, but also as a most valued critic of my work. I also owe thanks to Doctor Hannah Missfelder Lobos, a very good co-worker and a lovely friend.

• To Mrs. Suzanne McKnight, the friendliest lady I have met in London, thank you so much.

• I owe many thanks also to almost everybody at what was formerly the “Cruciform Project”, but especially I would like to thank Dr. Selim
Cellek, Mr. Dana Davis, Dr. Martin Feelisch, Ms. Gill Henderson and Ms. Annie Higgs.

- I am in a debt to my parents, Eduardo and Mabel, for their continuing support during all my studies. Also, thanks to my brother Cesar.

- To those guys, Julio, Santiago and Andres, who had no idea what I was doing here. They only knew Daddy was away. I know you missed me very much my boys! ... I want to say I love you my sons, and I missed you all very much also.

- Last but not least, this thesis is dedicated to my best friend, my best partner, the best idea someone can have, the most fresh and wonderful air I can breath and all the sense of my life. In other words, to the only woman I have loved and do love... my wife Isabel. I never ever will forget your patience and love, although you have suffered very much while I have been away. Now I can tell you “I will go back home my love”.

iii
# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>List of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>viii</td>
</tr>
<tr>
<td>List of Tables and Figures</td>
<td>xii</td>
</tr>
</tbody>
</table>

## Section I. General Introduction

1.1. Mitochondria. 2

1.1.1. Oxidative phosphorylation. 5

1.1.2. The respiratory chain. 6

1.1.2.a. From NADH to NADH dehydrogenase. 8

1.1.2.b. From NADH dehydrogenase to ubiquinone. 8

1.1.2.c. From ubiquinol to cytochrome bc₁ complex. 9

1.1.2.d. From cytochrome bc₁ complex via cytochrome c to cytochrome oxidase. 10

1.1.2.e. From cytochrome oxidase to oxygen. 10

1.2. NADH:ubiquinone oxidoreductase or complex I. 11

III.2.a. Measurement of complex I activity. 32

III.2.b. Determination of the amount of NO generated from NONOates. 33

III.3. Results. 35

Section IV. Effect of nitric oxide and peroxynitrite on complex I activity after removal of dithiothreitol

IV.1. Introduction. 39


IV.2.a. Removal of dithiothreitol from complex I preparation by size exclusion chromatography. 41

IV.2.b. Quantification of the total concentration of thiols. 41

IV.3. Results 42

Section V. Alternative mechanism(s) of action of nitric oxide related to complex I activity

V.1. Introduction. 46


V.2.a. Oxidation of NADH by purified complex I after pre-incubation of ubiquinone with an NO-donor. 47
V.3. Results.

Section VI. Interactions between ubiquinone and nitric oxide: a pharmacological and biochemical approach

VI. Introduction. 58


VI.2. Vascular reactivity experiments.

VI.2.a. Methods 60

VI.2.b. Results 61

VI.3. Electrochemical detection of NO.

VI.3.a. Methods 68

VI.3.b. Results 68

VI.4. Chromatographic determination of ubiquinone.

VI.4.a. Methods 71

VI.4.b. Results 72


VI.5.a. Methods 76

VI.5.b. Results 77
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.u.</td>
<td>Absorbance units</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CN-</td>
<td>Cyanide</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q or ubiquinone</td>
</tr>
<tr>
<td>CoQH⁺</td>
<td>Ubisemiquinone</td>
</tr>
<tr>
<td>CoQH₂</td>
<td>Ubiquinol</td>
</tr>
<tr>
<td>Cu⁺</td>
<td>Cuprous ion</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>Cupric ion</td>
</tr>
<tr>
<td>DEA/NO</td>
<td>2-(N,N-diethylnimo)-diazenolate-2-oxide</td>
</tr>
<tr>
<td>DETA/NO</td>
<td>(Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EC</td>
<td>Effective concentration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Reduced flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Fe-S</td>
<td>Iron-sulphur clusters</td>
</tr>
<tr>
<td>Fe$^{2+}$</td>
<td>Ferrous ion</td>
</tr>
<tr>
<td>Fe$^{3+}$</td>
<td>Ferric ion</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>FMNH$_2$</td>
<td>Reduced flavin mononucleotide</td>
</tr>
<tr>
<td>FP</td>
<td>Flavoprotein fragment</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
</tr>
<tr>
<td>H$^+$</td>
<td>Hydrogen ion</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>Water</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HP</td>
<td>Hydrophobic protein fraction</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IP</td>
<td>Iron protein fragment</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>M$^+$</td>
<td>Molecular ion</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>N$_2$</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>N$_2$O</td>
<td>Nitrous oxide</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$\text{N}_2\text{O}_3$</td>
<td>Dinitrogen trioxide</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>Oxidised nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>$\text{NH}_2\text{OH}$</td>
<td>Hydroxylamine</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO$^+$</td>
<td>Nitrosonium ion</td>
</tr>
<tr>
<td>NO$^-$</td>
<td>Nitrooxyl</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NONOates</td>
<td>Diazeniumdiolates</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NO$_x$</td>
<td>Nitric oxide-derived products</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>pA</td>
<td>Picoampers</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>pK</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>R-SH</td>
<td>Sulphydryl groups</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIN-1</td>
<td>3-morpholinosydnonimine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SNAP</td>
<td>S-nitroso-N-acetylpenicillamine</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Sper/NO</td>
<td>(Z)-1-{N-[3-aminopropyl]-N-[4-(3-aminopropylammonio) butyl]-amino}-diazen-1-ium-1,2-diolate</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet light</td>
</tr>
</tbody>
</table>
List of Tables and Figures

Table 1. Effect of purified complex I incubation by different times at 37°C before addition of NADH and CoQ-1 on the oxidation rate of NADH. 27

Table 2. Effect of the addition of increasing concentrations of CoQ-1 on the oxidation rate of NADH by purified complex I resting at room temperature. 27

Table 3. Addition of DEA/NO immediately after completely oxidation of NADH by purified complex I in a solution containing CoQ-1 results in a significant reduction of the oxidation rate of a second amount of NADH. 52

Figure 1. The mitochondria. 4

Figure 2. Scheme of the respiratory chain. 7

Figure 3. Structures of ubiquinone, semiubiquinone and ubiquinol. 10

Figure 4. Structural model of complex I. 15

Figure 5. Actions of nitric oxide and peroxynitrite on the mitochondrial respiratory chain. 19

Figure 6. Oxidation of NADH by purified complex I pre-incubated with DETA/NO for different periods. 36

Figure 7. Oxidation of NADH by purified complex I pre-incubated with SIN-1 for different periods. 37

Figure 8. Oxidation of NADH by purified complex I after removal of dithiothreitol. The new preparation was pre-incubated at 37°C for 20 min either with DETA/NO or SIN-1. 44

Figure 9. Repeated oxidation of NADH by purified complex I in a buffer containing CoQ-1. After completed oxidation of initial amount of NADH and OD remains constant, a second amount of NADH was added to the original amount of purified complex I and CoQ-1, restoring the OD to its initial value. 49
Figure 10. Repeated oxidation of NADH by purified complex I in a solution containing CoQ-1 is inhibited by addition of DEA/NO.

Figure 11. Inhibition of the repeated oxidation of NADH by purified complex I following pre-incubation of CoQ-1 with DEA/NO.

Figure 12. Repeated oxidation of NADH by purified complex I following pre-incubation of CoQ-1 with different amounts of DEA/NO.

Figure 13. Inhibition of the repeated oxidation of NADH by purified complex I following pre-incubation of CoQ-1 with DEA/NO disappears once NO is no longer generated by the NO-donor.

Figure 14. Addition of ubiquinone-1 results in further contraction of endothelium-intact rat aortic rings pre-contracted with phenylephrine (PE, EC₁₀).

Figure 15. Ubiquinone-1 produces a concentration-dependent inhibition of ACh-induced vascular relaxation in endothelium-intact rat aortic rings pre-contracted with phenylephrine.

Figure 16. Effect of ubiquinone-1, hydroquinone and duroquinone on ACh-induced vascular relaxation in rat aortic rings in the absence or presence of superoxide dismutase.

Figure 17. Ubiquinone-1 produces a concentration-dependent inhibition of S-nitrosoglutathione-induced vascular relaxation in endothelium-denuded rat aortic rings.

Figure 18. Effect of the addition of cumulative amounts of ubiquinone-1 on the NO signal (pA) recorded by an NO-selective electrode. NO was generated into a buffer containing Spermine/NO.

Figure 19. Addition of cumulative amounts of ubiquinone-1 decreased the NO signal recorded by an NO-selective electrode. NO was generated in a buffer containing Spermine/NO.

Figure 20. Effect of cumulative concentrations of S-nitroso-N-acetylpenicillamine on the height of the peak corresponding to ubiquinone-10. Ubiquinone-10 was identified by high performance liquid chromatography and the NO-donor was co-incubated for 30 min before the injection into the system.
Figure 21. Effect of co-incubation of S-nitroso-N-acetylpenicillamine with ubiquinone-10 produces a time reversible decrease in the height of the peak corresponding to ubiquinone-10.

Figure 22. Mass spectrometric analysis of ubiquinone-10. Peak corresponding to ubiquinone-10 showed a M+ of 885.

Figure 23. Mass spectrometric analysis of ubiquinone-10 after pre-incubation with S-nitroso-N-acetylpenicillamine (SNAP). A new molecule with a M+ of 1750 was found. SNAP was used in a 10 times molar excess and pre-incubated with CoQ-10 for 60 min before injection in the system.

Figure 24. Suggested chemical structure of a new compound resulting from the reaction between NO and CoQ-10.
Section I

General Introduction
Living organisms can be divided into two major groups. The most complex group comprises the eukaryotes, whose distinguishing feature is the presence of a true nucleus containing chromosomes and bounded by a nuclear membrane. The other group of organisms comprises the prokaryotes, which do not possess these features but characteristically have a nucleotide consisting of tightly packed DNA which lacks a limiting nuclear membrane [Fawcett, 1981]. Apart from differences in the organisation of the genetic material there are a number of other structural and biochemical features that differ between the two groups. One of the most significant differences is the possession by eukaryotes of mitochondria [Whittaker and Danks, 1978].

1.1. Mitochondria.

Mitochondria occupy a substantial portion of the cytoplasmic volume of eukaryotic cells, and they have been essential for the evolution of complex animals. The major function of mitochondria (although by no means the only one) is the synthesis of ATP from ADP and inorganic phosphate by the process of oxidative phosphorylation [Alberts et al., 1994]. Without mitochondria present-day animal cells would be dependent on anaerobic glycolysis for all of their ATP. However, when glucose is converted to pyruvate by glycolysis, only a very small fraction of the total free energy potentially available from glucose is released. In mitochondria
the metabolism of sugar is completed: pyruvate is imported into the mitochondrion and oxidised by molecular oxygen (O$_2$) to CO$_2$ and H$_2$O. The energy release is harnessed efficiently, with about 30 molecules of ATP produced for each molecule of glucose oxidised. By contrast, only 2 molecules of ATP are produced by glycolysis alone [Marks et al., 1996a; Marks et al., 1996b; Mayes, 1996].

Two highly specialised membranes that play a crucial part in its activities bound each mitochondrion. Together they create two separate mitochondrial compartments: the internal matrix space, and a much narrower intermembrane space (Figure 1). The outer membrane contains many copies of a transport protein called porin, which forms large aqueous channels through the lipid bilayer. The major working part of the mitochondrion is the matrix space and the inner membrane that surrounds it. The inner membrane is highly specialised, and is virtually impermeable to polar and ionic substances. The matrix enzymes include those that metabolise pyruvate and fatty acids to produce acetyl CoA, and those that oxidise acetyl CoA in the citric acid cycle. The principal end products of this oxidation are CO$_2$, which is released from the cell as waste, and NADH, which is the main source of electrons for transport along the respiratory chain. The enzymes of the respiratory chain are embedded in the inner mitochondrial membrane, and they are essential to the process of oxidative phosphorylation, which generates most of the mammalian
cell's ATP [Horton et al., 1993; Marks et al., 1996a; Marks et al., 1996b; Mayes, 1996; Hames et al., 1997].

**Figure 1.** The mitochondrion. The outer mitochondrial membrane is freely permeable to substances of molecular weights up to 10,000 due to the presence of the channel-forming protein porin. The highly folded inner membrane, which is virtually impermeable to polar and ionic substances, contains the protein complexes that carry out oxidative phosphorylation. (Reproduced from [Horton et al., 1993]).
1.1.1. Oxidative phosphorylation.

The energy for the synthesis of the high-energy phosphate bond of ATP is provided by the oxidation of NADH and FADH$_2$ by the electron transport chain (Figure 2). In this process electrons are transferred, in a stepwise fashion, from NADH and FADH$_2$ that arise from the citric acid cycle (located in the mitochondrial matrix), glycolysis (located in the cytoplasm) and fatty acid oxidation (located in the mitochondrial matrix), to O$_2$ [Horton et al., 1993; Marks et al., 1996a; Marks et al., 1996b; Mayes, 1996; Hames et al., 1997].

The oxidation of a molecule involves the loss of electrons whereas the reduction of a molecule involves the gain of electrons. Since electrons are not created or destroyed in a chemical reaction, if one molecule is oxidised, another must be reduced (i.e. oxidation-reduction or redox reaction). Thus, by definition, oxidation-reduction reactions involve the transfer of electrons. In the redox reaction:

\[
\text{NADH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \rightarrow \text{NAD}^+ + \text{H}_2\text{O}
\]

where NADH is oxidised to NAD$^+$, it loses electrons. When the molecular oxygen is reduced to water, it gains electrons [Alberts et al., 1994]. However, electrons are not transferred from NADH to oxygen directly. Rather, the electrons are transferred from NADH to oxygen along a chain of electron carriers collectively known as the electron transport chain (also
called the respiratory chain) [Horton et al., 1993; Marks et al., 1996a; Marks et al., 1996b; Mayes, 1996; Hames et al., 1997].

### 1.1.2. The respiratory chain.

The individual respiratory chain components are indicated in figure 2. Each complex also contains phospholipid. The complexes, numbered I to IV, catalyse the following reactions:

- **Complex I**  NADH oxidation - ubiquinone reduction
- **Complex II**  Succinate oxidation - ubiquinone reduction
- **Complex III**  Reduced ubiquinone oxidation - cytochrome c reduction
- **Complex IV**  Cytochrome c (Fe^{2+}) oxidation - oxygen reduction.

It has been shown that ubiquinone can transfer reducing equivalents between complexes I and III and between II and III and that cytochrome c can transfer reducing equivalents between complexes III and IV. This represents the structural basis for the previously established functional organisation.
Figure 2. Scheme of the respiratory chain redox components. Iron-sulphur (Fe-S) clusters in the NADH-ubiquinone (complex I) and succinate-ubiquinone (complex II) oxidoreductase segments are distinguished with suffixes Nx and Sx, respectively. Q_N, Q_S, and Q_o and Q_i denote specific ubiquinone binding sites in complex I, complex II and ubiquinol-cytochrome c oxidoreductase (Complex III) segment, respectively. (Modified from [Ohnishi, 1998]).
1.1.2.a. From NADH to NADH dehydrogenase.

The transfer of electrons from NADH to O\textsubscript{2} starts with the donation of 2 electrons from NADH to the first protein complex in the chain, NADH dehydrogenase (also called NADH-ubiquinone oxidoreductase or complex I). This enormous complex (800 kDa) consists of 41 subunits, containing FMN and between 22-24 iron-sulphur (Fe-S) centres. FMN accepts electrons from NADH to form FMNH\textsubscript{2}. Each electron is accepted together with a hydrogen ion, H\textsuperscript{+}, such that two electrons and two protons are accepted in total. The electrons are then transferred, within the NADH dehydrogenase complex, to Fe-S clusters (also called nonhaem iron proteins). Within an Fe-S cluster, an electron is carried by the iron atom which changes from the Fe\textsuperscript{3+} (ferric) state to the Fe\textsuperscript{2+} (ferrous) state. As the electron is passed to another electron carrier, the iron atom of the Fe-S cluster changes back to the Fe\textsuperscript{3+} state. NAD\textsuperscript{+} returns to the tricarboxylic acid cycle or another metabolic pathway to accept further electrons.

1.1.2.b. From NADH dehydrogenase to ubiquinone.

The next acceptor of electrons is ubiquinone (2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone), frequently called coenzyme Q (CoQ). Ubiquinone is the only component of the electron transport chains that is not protein bound. The hydrophobic side chain varies between 7 and 10 isoprenyl
residues, conferring lipid solubility to the quinone moiety and allowing ubiquinone to diffuse through the lipids of the inner mitochondrial membrane. Electrons from the Fe-S clusters of NADH dehydrogenase are passed on to ubiquinone. This molecule can act as an electron carrier by accepting up to two electrons and two H\(^+\) ions. In so doing, ubiquinone is converted to ubiquinol (CoQH\(_2\)).

**I.1.2.c. From ubiquinol to cytochrome bc\(_1\) complex.**

When ubiquinol donates its two electrons to the next carrier in the chain, the cytochrome bc\(_1\) complex (also called cytochrome reductase or complex III), the H\(^+\) ions are released once more. The cytochrome bc\(_1\) complex contains two types of cytochromes, cytochrome b and cytochrome c\(_1\), as well as an Fe-S protein. As the electron is accepted, the iron atom of the haem group changes from the Fe\(^{3+}\) state to the Fe\(^{2+}\) state. Since ubiquinol is a two-electron carrier whereas the cytochromes are one-electron carriers, the pathway of electron transfer within the cytochrome bc\(_1\) complex is more complex and involves ubiquinol releasing first one electron and an H\(^+\) ion to become ubisemiquinone (CoQH\(^+\)) and then the second electron and H\(^+\) ion to become ubiquinone (Figure 3).
Figure 3. Structures of (A) ubiquinone - CoQ; (B) ubisemiquinone - CoQH$^+$; and (C) ubiquinol - CoQH$_2$. In the side chain, $n$ ranges from 6 to 10.

1.1.2.d. From cytochrome bc$_1$ complex via cytochrome c to cytochrome oxidase.

Cytochrome c is a peripheral membrane haem protein that is loosely bound to the outer surface of the inner mitochondrial membrane. It binds to the cytochrome bc$_1$ complex and accepts an electron via an Fe$^{3+}$ to Fe$^{2+}$ transition. Then it binds to cytochrome oxidase and donates the electron, with the iron atom of the haem of cytochrome c then reverting to the Fe$^{3+}$ state.
I.1.2.e. From cytochrome oxidase to oxygen.

Cytochrome oxidase (also called complex IV) contains two cytochromes (cytochrome a and a₃). Cytochrome a is paired with a copper atom, Cuₐ, and cytochrome a₃ is paired with a different copper atom, Cuₐ₃. During electron transfer, the iron atoms of the cytochromes cycle between the Fe³⁺ and Fe²⁺ states whilst the copper atoms cycle between Cu²⁺ and Cu⁺. The cytochrome oxidase reaction is complex; it transfers four electrons from four cytochrome c molecules and four H⁺ ions to molecular oxygen to form two molecules of water. The cytochrome oxidase complex is the site of cyanide inhibition. Cyanide (CN⁻) is highly toxic for mammalian organisms. It binds to the Fe³⁺ in the haem of the cytochrome oxidase complex and prevents transfer of the electrons to O₂. Cytochrome oxidase is also the site of carbon monoxide (CO) poisoning.

I.2. NADH:ubiquinone oxidoreductase or complex I.

Mitochondrial complex I was first isolated from bovine heart mitochondria by Hatefi and co-workers [Hatefi et al., 1962]. The physiological reaction of the enzyme, namely, oxidation of NADH by long side-chain ubiquinones, can be conveniently assayed in membranes or in the isolated enzyme by replacing the natural acceptor with short-chain
homologues, e.g. CoQ-1, CoQ-2 or the CoQ-alkyl analogues [Hatefi et al., 1962; Lenaz et al., 1978; Ragan, 1978]. The electron transfer mechanism is carried out by a large number of redox groups whose sequence of operation and function is largely unknown. In the case of bovine complex I, it has been purified by two independent routes: (a) the classical method, by treatment of mitochondria with detergents and ammonium sulphate fractionation [Hatefi, 1978]; and (b) a procedure that involves extraction of mitochondrial membranes with lauryl maltoside followed by chromatographic steps [Finel et al., 1992]. Complex I, either in isolation or in the membrane, can be degraded to smaller fragments in several ways. These include incubation with ethanol at acidic pH and high temperature, incubation with proteolytic enzymes, phospholipase A, or NADH, and treatment with urea and other chaotropic agents [Hatefi, 1985; Ragan, 1987; Singer and Ramsay, 1992]. The most widely studied of these is to treat the isolated enzyme with the chaotropic anion, perchlorate. This produces soluble material and a precipitate, which is known as the hydrophobic protein (HP) fraction. There is no evidence that the HP retains any structural integrity. From the soluble fraction a flavoprotein (FP) fragment and an iron-protein (IP) fragment can be isolated. The FP fragment is a low-molecular mass flavoprotein which catalyses NADH oxidation by a wide variety of electron acceptors, including cytochrome c and ubiquinone analogues [Galante and Hatefi, 1979]. The FP fragment accounts for all the FMN and six of the iron atoms of complex I [Galante
and Hatefi, 1979]. The fragment consist of three polypeptides (51, 24, and 10 kDa) in a 1:1:1 molar ratio. The IP fragment is also water soluble, but is neither homogeneous nor monodisperse. The iron content (48 nmol/mg of protein) accounts for 9 to 10 of the iron atoms in complex I, i.e. at least three Fe-S clusters [Ragan et al., 1982]. Complex I can also be disrupted in other ways. For example, the bovine enzyme can be split by detergent treatment into two subcomplexes, named Iα and Iβ, which represent largely extrinsic and intrinsic membrane domains of the enzyme, respectively [Finel et al., 1992]. Subcomplex Iα can transfer electrons from NADH to ferricyanide or CoQ-1, and contains all of the Fe-S clusters that have been detected by electron paramagnetic resonance (EPR) spectroscopy in intact complex I.

Because of the lipophilic nature of ubiquinone, the site at which it becomes reduced has been assumed to be within the membrane domain of the enzyme. A variety of inhibitors such as piericidin, rotenoids, barbiturates, pethidine [Filser and Werner, 1988], capsaicin [Shimomura et al., 1989] and myxalamids [Gerth et al., 1983] inhibit electron flow to ubiquinone by binding close to ubiquinone. The inhibition is non-competitive with ubiquinone, and is considered to arise from steric hindrance or from a conformational change preventing passage of electrons to ubiquinone [Singer and Ramsay, 1992]. A CoQ-10 binding protein has been isolated from bovine complex I by a mild dissociation procedure that results in an alternative IP fragment, in which ubiquinone
remains bound to the protein. It was suggested from its apparent molecular weight that this ubiquinone binding protein is a 15 kDa protein normally found in the IP fragment of the bovine enzyme [Suzuki and Ozawa, 1986].

The electron microscopic reconstruction of the membrane and peripheral arms of *Neurospora crassa* complex I have been combined into a model of the L-shaped membrane-bound enzyme in which the extrinsic peripheral arm protrudes into the mitochondrial matrix [Hofhaus et al., 1991]. Given that the bovine enzyme appears to have a similar subunit composition, this model can also be taken to approximate to bovine complex I. Therefore, the bovine enzyme can be depicted as being made largely of the subcomplex Iα (Figure 4). An important feature of this model is that it places all of the known redox centres of complex I outside the lipid bilayer.
**Figure 4.** Structural model of complex I. Arrangement of some subunits in bovine complex I based upon the polypeptide compositions of subcomplexes \(I\alpha\) and \(I\beta\) of the FP and IP fragments. All the known redox centres are in subcomplex \(I\alpha\). The dotted part of subcomplex \(I\beta\) indicates that it is not known whether the two complexes contact each other directly, or whether other subunits of complex I that appear not to be in either subcomplex form this contact area (Reproduced from [Walker, 1992]).

\[
\text{NADH} + \text{H}^+ \rightarrow \text{NAD}^+ + 2\text{H}^+
\]

- \(\text{FMN, N-1b, N-3, 24, 31, L-200, 75}\)
- \(\text{Hox} \, \gamma \, \text{dimer}\)
- \(\text{FP}\)
- \(\text{Lx} \rightarrow \text{10}\)
- \(\text{IP}\)
- \(\text{ND2}\)
- \(\text{Cytoplasm}\)
- \(\text{Ca. 11 other proteins}\)

\(\text{ND4, ND5}\)
1.3. Ubiquinone or Coenzyme Q.

Ubiquinone is an extremely versatile molecule which functions in a number of essential cellular processes. It was discovered as an obligatory component of the succinate oxidase portion of the mitochondrial electron transfer chain [Crane et al., 1957], and later was shown to be involved in the NADH oxidase portion [Szarkowska, 1966]. The essential role of CoQ in the function of the electron transport chain was confirmed with experiments involving reincorporation of the quinone into CoQ-depleted submitochondrial particles [Norling et al., 1974]. Oxidation-reduction cycling of CoQ during electron transport has been observed directly [Kroger and Klingenberg, 1973]. CoQ thus allows reversible interactions between the NADH dehydrogenase, succinate dehydrogenase, and cytochrome bc₁ portions of the electron transport chain [Emster, 1962; Chance and Hollunger, 1961; Low and Vallin, 1963].

Mitochondrial electron transfer chain activity is associated with free radical formation, the rate of superoxide formation being directly proportional to the rate of mitochondrial oxygen consumption [Boveris and Chance, 1973]. Studies with electron transport chain inhibitors [Loschen et al., 1971; Trumpower and Simmons, 1979] indicated that the locus of oxyradical formation was in the span between succinate dehydrogenase and cytochrome b. The authors postulated a mechanism implicating the semiquinone form of ubiquinone in superoxide radical formation. After
formation of the semiquinone form of ubiquinone during submitochondrial electron transport was demonstrated at the level of complex I [Backstrom et al., 1970; Burbaev et al., 1989]. CoQ binding proteins or CoQ-apoproteins have been isolated from each of complexes I, II, and III of the mitochondrial electron transfer chain and have been shown to differ structurally and interact specifically with the complex from which they were derived [Yu et al., 1986]. Takeshige and Minakami [1979] have localised the site of free radical formation with NADH or NADPH as substrate within the NADH dehydrogenase complex between a mercurial-sensitive and a rotenone-sensitive component, most likely a nonhaem Fe-S function.

1.4. Nitric Oxide.

Nitric oxide (NO, nitrogen monoxide) is a widespread intra and intercellular messenger with a broad spectrum of actions in the central and peripheral nervous systems, the cardiovascular and the immune systems [Moncada and Higgs, 1993]. The biosynthesis of NO is accomplished by oxidation of a terminal guanidine nitrogen of L-arginine, yielding citrulline as a coproduct [Palmer et al., 1988], and is catalysed by members of the NO synthase (NOS) family [Griffith and Stuehr, 1995]. One of the key targets through which NO exert its effects on cells appears to be the soluble guanylate cyclase. NO bound to the haem moiety of this
protein leads to an increase in enzyme activity and a consequent increase in the intracellular concentration of cyclic GMP [Moro et al., 1996].

1.4.1. Nitric oxide and mitochondrial function.
Before NO was discovered to be the agent responsible for many cyclic GMP-mediated effects, it was known that activated macrophages produced an agent that was cytotoxic to other cells by irreversibly inhibiting their mitochondrial respiration [Hibbs et al., 1987]. It has been shown that very low levels of NO (nM) cause a reversible inhibition of mitochondrial respiration at the level of complex IV in competition with oxygen [Brown and Cooper, 1994; Cleeter et al., 1994; Richter et al., 1994]. In contrast, addition of peroxynitrite (ONOO') to mitochondria causes irreversible inhibition of respiration at a number of sites (complexes I, II, III, aconitase, creatinine kinase and ATP synthase; figure 5) [Radi et al., 1994; Cassina and Radi, 1996; Bolaños et al., 1995].
**Figure 5.** Actions of nitric oxide (NO) and peroxynitrite (ONOO⁻) on the mitochondrial respiratory chain (Modified from [Brown, 1999]).

\[ \text{NAD}^{+} \rightarrow \text{NADH} \rightarrow \text{Complex I} \rightarrow \text{Complex II} \rightarrow \text{Complex III} \rightarrow \text{Complex IV} \rightarrow \text{ATP synthesis} \]

\[ \text{O}_2 \rightarrow \text{ONOO}^{-} \rightarrow \text{NO} \rightarrow \text{ONOO}^{-} \rightarrow \text{O}_2 \rightarrow \text{H}_2\text{O} \rightarrow \text{ADP} \rightarrow \text{ATP} \]

1.4.2. Nitrosylation and nitrosation of proteins by NO.

Nitrosylation means the covalent attachment of the NO group to sulphhydryl residues in proteins, whereas nitrosation means general NO attachment to nucleophilic centres [Simon et al., 1996]. NO reacts in biological systems with oxygen (O₂), superoxide (O₂⁻), and transition metals to form a variety of nitrogen oxides: NOₓ, ONOO⁻, and metal-NO adducts, respectively [Stamler et al., 1992]. These species can effect nitrosative reactions at responsive nucleophilic centres [Stamler, 1994]. Thiol groups are preferentially modified by nitrosylation [Stamler et al., 1992]. Although the mechanisms by which glutathione (GSH) modulates
cellular responses to NO$_x$ are not clear, it has been suggested that GSH, present in relatively high concentrations in cells, acts as a sink, scavenging NO$_x$ to form S-nitrosoglutathione (GSNO) and thereby preventing NO$_x$ from reacting with protein thiols [Stamler et al., 1992; Padgett and Whorton, 1995]. However, many protein thiols have lower pK$_a$ values than GSH and are more chemically reactive toward NO$_x$. Thus, besides reacting with NO$_x$ directly, GSH may be important in regulating protein thiol status by reversing S-nitrosoprotein formation and restoring basal protein function [Padgett and Whorton, 1995; 1997]. Recently, in our laboratory it has been found that prolonged exposure of cells to NO results in a gradual and persistent inhibition of complex I which is preceded by a reduction in the intracellular concentration of GSH [Clementi et al., 1998]. Moreover, this effect was completely reversed by the reducing agent dithiothreitol (DTT), high intensity light or removal of NO donor from the medium. These findings together suggested that inhibition of complex I activity by NO might be due to S-nitrosylation of critical thiols in the protein.
1.5. Aims of the study.

This study was aimed at understanding the mechanism(s) involved in the inhibition of mitochondrial complex I activity by NO. Specific objectives include: (1) investigation of the probable location and extent of S-nitrosylation of thiol groups in complex I; and (2) identification of alternative mechanisms by which NO might produce inhibition of complex I activity.
Section II

Activity of complex I in basal conditions
II.1. Introduction.

It has been reported that the composition of bovine complex I purified by solubilization with the detergent n-dodecyl-β-D-maltoside (lauryl maltoside), ammonium sulphate fractionation, and chromatography on MonoQ is very similar to that of purified by conventional methods [Finel et al., 1992]. However, three basic differences in the polypeptide composition were demonstrated by SDS-PAGE analysis. The first difference is the absence of transhydrogenase usually present in conventional preparations as a faint band near the top of the gel. Secondly, the relative amount of the 42-kDa subunit is significantly diminished in the new purification procedure. Thirdly, traces of other respiratory complexes are almost entirely absent from the complex I purified with the new method [Finel et al., 1992]. The enzymatic activity of complex I prepared by the new procedure is slightly lower than the activity of the complex I isolated by the conventional method, however these differences fall within the previously reported range of values [Ragan et al., 1987]. We have used complex I prepared by the method of Finel et al [1992]. It is important to mention that in beef heart mitochondria or submitochondrial particles (SMP), CoQ-1 exhibited high rotenone sensitivity (>90%) [Degli Esposti et al, 1996]; however, even in beef heart SMP and in purified complex I from bovine heart [Ragan, 1978], a high rotenone-insensitive rate with CoQ-1 was found in absence of added
phospholipids, but significant only at high quinone concentrations. This is in agreement with the observation of Finel [1992].

The polypeptide composition of the purified complex I was analysed by SDS-PAGE, and its enzymatic activity monitored by its ability to oxidise NADH in presence of CoQ-1.


II.2.a. Mitochondrial complex I purified from bovine heart.

Purified NADH:ubiquinone oxidoreductase (Complex I, EC 1.6.5.3) was kindly provided by Doctors Leonid Sazanov and John E. Walker from the MRC Laboratory of Molecular Biology (Cambridge, UK). Briefly, complex I protein was purified from bovine heart mitochondria by solubilization with lauryl maltoside, followed by ammonium sulphate fractionation, and chromatography on Mono Q in the presence of the detergent as previously described [Finel et al., 1992]. The purified complex I (5 mg/ml) was stored at -80°C in a buffer containing Tris HCl 20 mM, pH 7.4, EDTA 2 mM, glycerol 20%, and 0.1% of dodecyl maltoside.

II.2.b. Identification of the components of purified complex I.

Identification of the components of purified complex I was carried out by loading 20 µg samples of the protein on a 15% SDS-PAGE using a Protean II xi electrophoresis cell equipped with a PowerPac 300 system.
(Bio-Rad, Hertfordshire, UK). Gels were stained with Coomassie brilliant blue R-250. Briefly, polyacrylamide gels are chemically inert and particularly mechanically stable. By chemical co-polymerisation of acrylamide monomers with a cross-linking reagent, usually N,N'-methylenebisacrylamide, a clear transparent gel which exhibits very little electroendosmosis is obtained [Westermeier, 1997]. During electrophoresis in polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) the separation is exclusively according to molecular weight [Westermeier, 1997]. The molecular weight of the proteins can be estimated with a calibration curve using marker proteins.

**II.2.c. Measurement of complex I activity.**

Complex I activity was evaluated by its ability to oxidise NADH to NAD$^+$ in the presence of CoQ-1 as electron acceptor. This enzymatic reaction was started by addition of 0.1 mM of NADH and 0.1 mM of CoQ-1 to 20 μg of complex I protein resuspended in a buffer containing 20 mM Tris, pH 7.5, 2 mM EDTA and 0.1% dodecyl-maltoside (identical composition as the storage buffer, except for the absence of glycerol). Oxidation of NADH, either at room temperature or at 37°C, was followed spectrophotometrically at 340 nm and changes in optical density were recorded every minute.

Complex I activity was expressed as (1) variations in the optical density at 340 nm, or (2) changes in the oxidation rate of NADH in μmol/mg
protein/min. To calculate the oxidation rate of NADH the following equation was used:

$$\text{NADH oxidation} = \frac{(\Delta \text{Abs}) \times 100}{\text{CE}} \times 50 \times \frac{\mu\text{mol/mg protein/min}}{1000}$$

Where: $\Delta \text{Abs} =$ Absorbance at time 0 – absorbance at time 1

$\text{CE} =$ Coefficient of extinction for NADH (6.22 mM$^{-1}$ cm$^{-1}$)

II.3. Results.

Complex I protein from bovine heart mitochondria, purified at the MRC Laboratory of Molecular Biology (Cambridge, UK) was identical to the previously published (not shown), and for a detailed picture showing the subunit composition of complex I, please refer to the original publication. Experiments performed using complex I preparation resting at room temperature oxidised NADH (0.1 mM) in the presence of 0.1 mM of CoQ-1 with a specific activity of 0.78±0.04 µmol/min/mg protein, which is in good agreement with previously published data [Finel et al., 1992]. Remarkably, the enzymatic activity of complex I, when the protein was incubated at 37°C by different times before the addition of NADH (0.1 mM) and CoQ-1 (0.1 mM), was significantly higher at all time-points in comparison to the activity values obtained at room temperature (Table 1).
Thus, at 37°C complex I oxidises 0.1 mM NADH in the presence of CoQ-1 (0.1 mM) faster than at room temperature.

Interestingly, the oxidation rate of 0.1 mM NADH by complex I resting at room temperature was also faster in the presence of increasing amounts of CoQ-1 (Table 2). This finding demonstrates that NADH oxidation is not only dependent on complex I activity, but also on the availability of electron acceptor.

**Table 1. Effect of purified complex I (20 μg) incubation by different times at 37°C before addition of 0.1 mM NADH and 0.1 mM CoQ-1 on the oxidation rate of NADH (μmol/min/mg protein).**

<table>
<thead>
<tr>
<th>Complex I incubation’s time</th>
<th>Room temp</th>
<th>5 min</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
<th>40 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.78±0.04</td>
<td>1.29±0.06</td>
<td>1.34±0.07</td>
<td>1.77±0.10</td>
<td>1.64±0.05</td>
<td>1.43±0.12</td>
<td></td>
</tr>
</tbody>
</table>

* p<0.001 vs. 0 min. Data represent mean ± SEM from 5 separate experiments.

**Table 2. Effect of the addition of increasing concentrations of CoQ-1 on the oxidation rate of 0.1 mM NADH by 20 μg of purified complex I resting at room temperature (μmol/min/mg protein).**

<table>
<thead>
<tr>
<th>CoQ-1 concentration</th>
<th>0.1 mM</th>
<th>0.3 mM</th>
<th>0.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH oxidation rate</td>
<td>0.78±0.04</td>
<td>1.19±0.06</td>
<td>3.15±0.08</td>
</tr>
</tbody>
</table>

* p<0.001 vs. 0.1 mM. Data represent mean ± SEM from 3 separate experiments.
Section III

Effect of nitric oxide and peroxynitrite on complex I activity
III.1. Introduction.

Nitric oxide reacts in biological systems with oxygen (O$_2$), superoxide (O$_2^-$) and transition metals to form a variety of nitrogen dioxides: NO$_x$, peroxynitrite (ONOO$^-$), and metal-NO adducts, respectively [Stamler et al., 1992]. These species can effect nitrosative reactions at responsive nucleophilic centers [Stamler, 1994]. Nitrosylation of sulphhydril centers has been shown to confer NO-like biological activities and/or to regulate protein functions in numerous systems, including low-molecular-weight thiols, such as cysteine [Ignarro et al., 1981]; plasma proteins, such as albumin (BSA) [Stamler et al., 1992], enzymes, including glyceraldehyde-3-phosphate dehydrogenase [Mohr et al., 1996] and cathespin B [Stamler et al., 1994].

There is a great deal of literature on NO-related mitochondrial inhibition or damage in cells and tissues in relation to pathology [Brown, 1999]. For example, in tumour cells co-cultured with activated macrophages, some mitochondrial iron-sulphur proteins have been shown to be inhibited by NO before others, with aconitase being inhibited first ($t_{1/2}$ 4h), followed by complex I ($t_{1/2}$ 8h), then complex II ($t_{1/2}$ 14h), while complex III was not inhibited [Drapier and Hibbs, 1986]. The inhibition of mitochondrial iron-sulphur proteins in activated macrophages and target cells is accompanied by the appearance of an electron paramagnetic resonance (EPR) signal at $g$=2.035 indicating the presence of a complex between
NO and iron, probably of the [Fe (NO)$_2$(SR)$_2$] type [Drapier et al., 1991; Lancaster and Hibbs, 1990]. Both the appearance of this signal and the loss of aconitase activity are associated with NO production in a range of different cells, leading to the suggestion that the EPR signal derives from iron released from mitochondrial iron-sulphur centres by NO [Drapier, 1997; Henry et al., 1993]. However, the EPR signal in some cells also may derive from ferritin or other cytosolic pools of iron [Drapier, 1997; Henry et al., 1993], and is not necessarily a marker for damage to mitochondrial iron-sulphur centres.

Using isolated heart mitochondria, Radi et al. [1994] demonstrated ONOO$^-$-mediated impairment of oxygen consumption by mitochondrial complexes I and II when succinate or NAD-linked substrates (pyruvate, maleate or glutamate) were used. NADH dehydrogenase is not as sensitive as succinate dehydrogenase, a component of complex II, to the actions of ONOO$^-$ [Radi et al., 1994]. Later studies using isolated heart mitochondria revealed that NO caused reversible inhibition of complex I-, II- and IV-dependent respiration [Cassina and Radi, 1996]. However, it is likely that this observation arises from the inhibition of complex IV by NO which, as a consequence leads to a secondary impairment of electron transport and hence to glutamate/maleate and succinate driven respiration [Heales et al., 1999]. Complex I in isolated brain mitochondria also appears to be relatively resistant to the direct actions of ONOO$^-$ [Bolaños et al., 1995]. However it has been reported that the sensitivity of
complex I to ONOO⁻ may be critically dependent on the concentration of mitochondria utilised in the incubations, and whether respiratory substrates are present [Brookes et al., 1998].

Induction of iNOS in astrocytes or brief exposure of cultured neurones to ONOO⁻ does not result in any loss of complex I activity [Bolaños et al., 1994]. In contrast, when neurones are exposed to an NO donor for 24 h, a marked loss of complex I activity occurs in conjunction with a loss of activity of complexes II-III and IV [Bolaños et al., 1996]. Under such conditions, there is also a marked loss of the intracellular antioxidant, GSH [Bolaños et al., 1996].

It has also been shown recently that prolonged exposure of intact murine macrophage cells to NO results in a gradual and persistent inhibition of complex I preceded by a decrease in the concentration of GSH [Clementi et al., 1998]. This inhibition could not be prevented by scavengers of ONOO⁻ or superoxide, and both the inhibition of respiration and of complex I could be completely reversed by either light or thiol reducing agents [Clementi et al., 1998]. In summary, this work suggested that inhibition of complex I activity after prolonged exposure of intact cells to NO was due to direct S-nitrosylation of crucial thiols in complex I. Therefore, we decided to investigate the effect of NO and ONOO⁻ on the activity of purified complex I.


The activity of complex I was evaluated by its ability to oxidise NADH to NAD$^+$ in the presence of CoQ-1 (see section II.2.c). Enzyme activity was measured every minute at 37°C, and the reactions were started by addition of 0.1 mM of NADH and 0.1 mM of CoQ-1 (to have a slower oxidation of NADH) to the assay buffer containing 20 µg of complex I protein alone or complex I pre-incubated for different periods (up to 60 min) with varying concentrations (ranging from 0.5 to 5 mM) of the slow-releasing NO donor, DETA/NO [Maragos et al., 1991] or with 1 mM of the ONOO$^-$ generator SIN-1.

III.2.b. Determination of the amount of NO generated from NONOates.

To calculate the concentration-time profiles of NO released from ideal-compounds, e.g. NONOates, used in our experimental conditions, a mathematical model [Schmidt et al., 1998] was used. This model is based on a system of two differential equations describing the first-order decomposition of the NO donor in association with the overall third-order reaction of NO with oxygen. Though, there is no close formula to express the solution of this equation system, the solution can be computed by any standard numerical-equation solver or simulation software, provided that the following input parameters are known:
1. Initial concentration of the donor;
2. Decomposition rate constant of the donor;
3. Stoichiometry of NO release;
4. $O_2$ concentration of the buffer; and
5. Rate constant of NO autoxidation.

For our experimental conditions, most of these parameters are published, making a determination of these values unnecessary [Maragos et al., 1991; Wink et al., 1993; Lewis and Deen, 1994; Hrabie et al., 1993]. Upon decomposition of donor compounds in aerobic solutions, the actual concentration of NO depends on the decomposition rate of the donor and the autoxidation rate of NO. The following system of differential equations describes the concentration of NO $[c_{NO}(t)]$ and the concentration of the donor $[c_D(t)]$ at time $t$.

$$\frac{d}{dt} c_D(t) = -k_1 c_D(t)$$

$$\frac{d}{dt} c_{NO}(t) = k_1 c_D(t) e_{NO} - k_2 o_2 [c_{NO}(t)]^2$$

$$c_D(0) = c_0, c_{NO}(0) = 0$$
Equation (1) describes the decomposition of the donor following first order kinetics with a rate constant of $k_1$. The first term in equation (2) models the production of NO, assuming that each mol of donor yields $\epsilon_{\text{NO}}$ mol of NO. The second term in equation (2) describes the autoxidation of NO as a third-order reaction, depending on $[\text{NO}(t)]^2$ and the oxygen concentration $O_2$. If the latter is not limiting, we may assume that $O_2$ remains constant. The rate constant for the autoxidation reaction is denoted by $k_2$. Equation (3) gives the initial state of the solution with $c_0$ denoting the initial concentration of the donor. As equation (1) is a differential system with independent variables, it should be integrated directly as follows:

(5) \[ \frac{dc_D(t)}{c_D(t)} = -k_1 dt \]

(6) \[ \ln c_D(t) = -k_1 t \]

(7) \[ \ln c_D(t) - \ln c_D(0) = -k_1 t - (-k_1 0) \]

(8) \[ \ln \frac{c_D(t)}{c_D(0)} = -k_1 t \]

(9) \[ c_D(t) = c_D(0) \exp(-k_1 t) \]

Therefore, equation (10) may be obtained by replacement of equation (9) into equation (2):
(10) \[ \frac{d}{dt} c_{NO}(t) = k_1c_D(0) \exp(-k_1t)e_{NO} - k_2o_2[c_{NO}(t)]^2 \]

Finally, equation (10) was solved using a second-order Runge-Kutta algorithm and with the help of a commercially available software package Polymath (Version 3.0.1., The Cache Corp., Austin, TX).

### III.3. Results.

Purified complex I incubated at 37°C was exposed to increasing concentrations of DETA/NO (from 0.5 to 5 mM), to reach a steady-state concentration of NO ranging from 45 to 150 µM. In all cases, NO release from the donor was demonstrated with an NO-electrode. Surprisingly, we found no differences in the oxidation rate of NADH by purified complex I incubated with these concentrations of DETA/NO compared to the control carried out in the absence of the NO donor (data not shown). Pre-incubation of purified complex I with DETA/NO (5mM) equivalent to 150 µM of NO for up to 1 hour also failed to show any differences in enzyme activity (Figure 6).

Moreover, pre-incubation (varying from 5 up to 60 min) of purified complex I with 1 mM of the peroxynitrite generator, SIN-1, did not produce any difference compared to the control (Figure 7).
**Figure 6.** Oxidation of NADH by purified complex I pre-incubated with DETA/NO (5 mM) equivalent to 150 μM of NO for different periods. The graph is representative of 5 separate experiments.
**Figure 7.** Oxidation of NADH by purified complex I pre-incubated with SIN-1 (1 mM) for different periods. The graph is representative of 5 separate experiments.
Section IV

Effect of nitric oxide and peroxynitrite on complex I activity after removal of dithiothreitol
IV.1. Introduction.

The reactivity of NO with sulphydryl (R-SH) groups depends on the electron configuration of its $2p-\pi$ antibonding orbital [see Stamler et al., 1992]. The presence of one (radical) electron in this orbital does not ordinarily confer reactivity with R-SH groups, though it clearly allows reaction with thiol radical species. On the other hand, loss of this electron to form NO$^+$ confers strong electrophilicity and reactivity towards most biological R-SH species [Stamler et al., 1992; Arnelle and Stamler, 1995]. A second electron in the $2p-\pi$ orbital, forming NO$, may under certain circumstances confer reactivity with relatively electropositive R-SH species, particularly in the presence of ferrous ion or other transition metals [Stamler et al., 1992; Arnelle and Stamler, 1995]. Also, nitric oxide synthase (NOS) may produce nitrous oxide (N$_2$O) and hydroxylamine (NH$_2$OH) from NO$^-$ [Schmidt et al., 1996]. Further coproduction of NO and superoxide may form ONOO$^-$. This is a potent nitrosating species, especially in the presence of excess NO [Gaston, 1999].

The third-order reactivity of NO with oxygen under physiological conditions (rate constant in aqueous phase $\sim 6.6\times10^6$ M$^{-1}$ s$^{-1}$) [Wink et al., 1993] predicts relatively slow production of dinitrogen trioxide (N$_2$O$_3$) as a nitrosating agent acting via NO$^+$ and NO$_2^-$. However, this reaction is accelerated 300-fold in the hydrophilic core of biological membranes [Liu et al., 1998]. Reactions of NO with superoxide, on the other hand, are so
rapid as to be diffusion limited \((k= \sim 7 \times 10^9 \ M^{-1} \ s^{-1})\) [Haddad et al., 1994]. Both amines and sulphydryl groups are highly susceptible to reactions with \(N_2O_3\) and ONOO\(^-\) [Schmidt et al., 1996; Gow et al., 1997; Liu et al., 1998]. Thiol nitros(yl)ation is preferred under physiological conditions, both because of the tendency of amines to be more basic and because, unlike deamination of primary amines after NO\(^+\)-induced diazotization [Stamler et al., 1992], loss of the S-NO group thiolate does not generally occur rapidly. Reactivity of these nitrosating species toward carbon groups is less than toward amino groups and sulfhydryl groups. It is therefore not surprising that S-nitrosylation of proteins has been demonstrated to be preferred over N- and C-nitrosylation under physiological conditions [Simon et al., 1996] and that large, stable reservoirs of S-NO, but not N-NO or C-NO, species have been identified in tissues [Gaston, 1999].

Dithiothreitol (DTT) a strong reducing agent, is routinely added during the purification process of complex I, in order to prevent aggregation of the protein [Finel et al., 1992]. Thus, we decided to investigate the effect of removal of free thiol from the storage buffer of purified complex I on the actions of NO and ONOO\(^-\) on the rate of NADH oxidation.

IV.2.a. Removal of dithiothreitol from the complex I preparation by size exclusion chromatography.

Removal of dithiothreitol (DTT) from the complex I preparation was performed using a Sephadex G-25 column (Amersham Pharmacia Biotech, UK). Briefly, size exclusion chromatography, also called gel-filtration chromatography, separates molecules based on their size [Squire, 1964]. The gel medium consists of spherical beads containing pores of a specific size distribution. Separation occurs when molecules of different sizes are either included or excluded from the pores within the gel matrix. Molecules that are smaller than the pore size can enter the particles and therefore have a longer path and longer transit time than larger molecules that cannot enter the particles. To obtain the desired separation, the gel should have an exclusion limit smaller than the molecule of interest [Amersham Pharmacia Biotech, 1997].

IV.2.b. Quantification of the total concentration of thiols.

To determine the total concentration of thiols present in the new complex I preparation Ellman's reaction was used. Briefly, this reaction is performed by the addition of 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), to phosphate buffer (pH 8.0) containing the compound to be analysed. This mixture will develop colour rapidly (2 min) and changes in optical density are analysed.
at 412 nm [Ellman, 1959]. To calculate the concentration of thiols the following equation was used:

\[ C_0 = \frac{A}{\varepsilon D} \]

Where:  
- \( C_0 \) = original concentration  
- \( A \) = absorbance at 412 nm  
- \( \varepsilon \) = extinction coefficient (13,600 M\(^{-1}\) cm\(^{-1}\))  
- \( D \) = dilution factor

**IV.3. Results.**

First, the absence of reduced low molecular weight thiols in the new preparation of purified complex I was confirmed using the DTNB [Ellman, 1959].

Secondly, the oxidation of 0.1 mM NADH by complex I protein (20 ug) incubated by 20 min at 37°C and in the presence of 0.1 mM CoQ-1 did not show any difference between the new preparation (DTT-free) and the original one (data not shown). Also, there was no significant differences between the oxidation rate of 0.1 mM NADH by DTT-free complex I (20 ug) in the presence of 0.1 mM CoQ-1 alone or after incubation at 37°C for 20 min either with 5 mM of DETA/NO or 1 mM of SIN-1 (Figure 8).

Moreover, in another set of experiments the effect of other NO-donors on the oxidation of 0.1 mM NADH by complex I protein (20 ug) incubated by
20 min at 37°C and in the presence of 0.1 mM CoQ-1, was monitored in presence of either 1 mM S-nitrosoglutathione (GSNO) or 1 mM S-nitroso-N-acetylpenicillamine (SNAP). Unfortunately, because these are coloured compounds with a detectable wavelength close to the absorbance at which NADH oxidation is monitored (340 nm), it was impossible to reach any appropriate reading.

In summary, all together these results support the proposal that neither presence nor absence of free thiols into the preparation of purified complex I, had not any evident effect mediated by NO nor ONOO' on the enzymatic activity of complex I.
**Figure 8.** Oxidation of NADH by purified complex I after removal of dithiothreitol. The new preparation was pre-incubated at 37°C for 20 min either with DETA/NO (5 mM) equivalent to 150 μM of NO or SIN-1 (1 mM). Graph is representative of 5 separate experiments.
Section V

Alternative mechanism(s) of action of nitric oxide related to complex I activity
V.1. Introduction.

It is well known that the physiological electron donor to complex I is NADH while the physiological electron acceptor is the lipid-soluble redox-active quinone derivative coenzyme Q (CoQ-10 in most mammalian mitochondria) [Lass and Sohal, 1999]. It is also accepted that the assay to evaluate complex I activity requires the use of artificial electron acceptors, since physiological quinones, such as CoQ-10, are too insoluble in water to be added as substrates to the assay media. Thus, the most commonly used acceptors are short-chain CoQ homologues (starting from CoQ-0), analogues such as tetramethylbenzoquinone (duroquinone) or analogues having straight saturated chains, such as decylubiquinone [Ragan, 1976; Wan et al., 1975]. It is generally assumed that these ubiquinone homologues and analogues interact with the physiological site of endogenous CoQ, as derived from the sensitivity to rotenone. Estornell et al [1993] have shown that the water solubility of the quinones is the fundamental factor for assessing whether kinetic saturation is reached during the assay, a pre-requisite for enzymatic determinations. CoQ-1 has been found to be the best acceptor, yielding the highest rate of complex I activity, linear kinetics and relative independence of the phospholipid concentration [Estornell et al., 1993].

Our own experiments have shown that changes in the amounts of ubiquinone added to the assay cocktail resulted in different activities of complex I (see Table 2). Therefore, evaluation of complex I activity is not
only dependent on the presence of the enzyme (complex I) and the substrate (NADH), it is also dependent on the presence of the electron acceptor (ubiquinone).

Due to this close relationship between the evaluation of complex I activity and ubiquinone, we decided to investigate possible interactions between NO and ubiquinone, as an alternative mechanism for NO-induced inhibition of NADH oxidation by complex I.


V.2.a. Oxidation of NADH by purified complex I after pre-incubation of ubiquinone with an NO donor.

Complex I purified from bovine heart mitochondria was used in all experiments. Complex I activity was measured in a buffer containing 20 mM Tris, pH 7.5, 2 mM EDTA and 0.1% dodecyl-maltoside. The oxidation of NADH was followed spectrophotometrically at 340 nm. Enzyme activity was measured every 30 seconds at room temperature (as there were not significant differences in the oxidation rate between 37°C and room temperature). In order to test if equal amount of complex I protein (20 ug) in a solution containing 0.1 mM of CoQ-1 as previously used, is able to oxidise half amount of NADH in repetitive occasions, the reaction was started by addition of only 0.05 mM. After complete oxidation of NADH a second amount (0.05 mM) of NADH was added. Using this
protocol and after complete oxidation of the first amount of NADH, a fast-releasing NO donor, DEA/NO 1 mM was added. In another set of experiments, CoQ-1 was pre-incubated with DEA/NO 10 minutes before addition of NADH and complex I protein.

For statistical comparisons the student t test was used and a p value lower than 0.05 was considered significant.

V.3. Results.

When only 0.05 mM of NADH was added to complex I in a solution containing CoQ-1, the substrate was oxidised completely at a similar rate to when the original amount of NADH (0.1 mM) was added (0.76±0.08 vs. 0.78±0.04 μmol/min/mg protein, respectively; p NS). This process could be repeated at least two times with a similar rate of substrate oxidation between second and first additions (0.57±0.10 vs. 0.76±0.08 μmol/min/mg protein, respectively; p NS; figure 9).
Figure 9. Repeated oxidation of NADH by purified complex I (20 ug) in a buffer containing CoQ-1 (0.1 mM). After completed oxidation of initial amount of NADH (0.05 mM) and OD remains constant, a second amount of NADH (0.05 mM) was added to the original amount of purified complex I and CoQ-1, restoring the OD to its initial value. Depicted data are representative of 15 separate experiments.
As shown in figure 9, addition of 0.05 mM NADH reach an OD of 0.30 a.u. and once it was completely oxidised after 2 min the OD remains constant in 0.05 a.u.. Addition of second amount of NADH at 4 min restores the OD to 0.30 a.u. which is one time more completely oxidised after two minutes.

However, the addition of 1 mM of DEA/NO, equivalent to 67 μM of NO, immediately after complete oxidation of the first amount of NADH (0.05 mM) resulted in an increase in the OD, which in control experiments using DEA/NO and DTT alone without complex I protein confirmed the formation of nitrosothiols. This increase in OD remains constant after 2 min of DEA/NO addition. That is the reason why second addition of same amount of NADH (0.05 mM) results in a high OD than the initial one (Figure 10).

Finally, and the most important, the addition of 1 mM of DEA/NO, equivalent to 67 μM of NO, immediately after complete oxidation of the first amount of NADH (0.05 mM) resulted in a significant reduction in the oxidation rate of a subsequent addition of NADH (Figure 10 and table 3).
Figure 10. Repeated oxidation of 0.05 mM NADH by purified complex I (20 µg) in a solution containing CoQ-1 (0.1 mM) is inhibited by addition of DEA/NO (1 mM), equivalent to 67 µM of NO. An increase in the absorbance was observed which remained constant after 2 minutes of DEA/NO addition. Depicted data are representative of 15 separate experiments.
**Table 3.** Addition of 1 mM DEA/NO, equivalent to 67 μM of NO, immediately after complete oxidation of 0.05 mM NADH by purified complex I protein (20 μg) in a solution containing CoQ-1 (0.1 mM) results in a significant reduction of the oxidation rate of a second amount of NADH (umol/mg protein/min).

<table>
<thead>
<tr>
<th></th>
<th>30 s</th>
<th>60 s</th>
<th>90 s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before DEA/NO</td>
<td>0.76±0.038</td>
<td>0.75±0.038</td>
<td>0.30±0.015</td>
</tr>
<tr>
<td>After DEA/NO</td>
<td>0.61±0.031</td>
<td>0.30±0.015</td>
<td>0.16±0.008</td>
</tr>
<tr>
<td>p value</td>
<td>0.0049</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

These results show a significant reduction in the oxidation rate of NADH by purified complex I in a solution containing CoQ-1, after the addition of NO in an activated protein. It means that to NO acts was necessary an initial amount of NADH to be oxidise by complex I otherwise no action has been observed. In order to clarify this observation CoQ-1 was pre-incubated with DEA/NO (1 mM), equivalent to 67 μM of NO, for 10 minutes before the addition of complex I protein (20 μg) and 0.05 mM NADH. In this situation, a significant decrease in the oxidation rate of NADH was observed upon addition of the initial amount of NADH. Moreover, subsequent additions of the same amount of NADH revealed an absence of further oxidation of NADH by complex I (Figure 11).
**Figure 11.** Inhibition of the repeated oxidation of 0.05 mM NADH by purified complex I (20 ug) following pre-incubation (10 min) of CoQ-1 (0.1 mM) with DEA/NO (1 mM), equivalent to 67 μM of NO. Graph is representative of 5 separate experiments.
More detailed studies showed that this inhibition of NADH oxidation by complex I was only present when CoQ-1 was pre-incubated by 10 min with DEA/NO at a concentration of 100 μM or more (n=3; figure 12), equivalent to 15 μM of NO or more. Interestingly, when CoQ-1 was pre-incubated with DEA/NO for a longer period than the half-life of the donor, i.e. 60 min, the above described effect seemed to be reduced (n=5; figure 13). In other words, the inhibition of purified complex I after pre-incubation of CoQ-1 with DEA/NO was reversible, so that only while NO was being continuously generated by the NO-donor did CoQ-1 lose its ability to accept electrons and therefore prevent NADH oxidation by complex I.
**Figure 12.** Repeated oxidation of 0.05 mM NADH by purified complex I (20 ug) following pre-incubation (10 min) of CoQ-1 (0.1 mM) with different amounts of DEA/NO (ranging from 1 to 100 μM), equivalent to 0.2 to 15 μM of NO. Graph is representative of 3 separate experiments.
**Figure 13.** Inhibition of the repeated oxidation of 0.05 mM NADH by purified complex I (20 ug) following pre-incubation (10 min) of CoQ-1 with DEA/NO (1 mM), equivalent to 67 uM of NO, disappears once NO is no longer generated by the NO-donor (after 60 min). Graph is representative of 5 separate experiments.
Section VI

Interactions between ubiquinone and nitric oxide:

A pharmacological and biochemical approach
VI. Introduction.

Quinones represent an important class of naturally occurring compounds that are found in plants, fungi, and bacteria, primarily as components of the electron-transport chain involved in cellular respiration and photosynthesis [Nohl et al., 1986; Monks et al., 1992]. Most quinones can also undergo one-electron reduction by the membrane-bound flavoprotein NADPH-cytochrome P450 reductase to form a semiquinone radical which can reduce oxygen to superoxide [O'Brien, 1991; Monks et al., 1992].

The most recognised physiological function of CoQ is the transfer of electron from complexes I and II to complex III and the translocation of protons in the mitochondrial respiratory chain [Crane and Navas, 1997; Ernster and Dallner, 1995]. Autoxidation of ubisemiquinone is believed to be the major source of mitochondrial superoxide anion radical ($O_2^-$)/$H_2O_2$ generation [Boveris and Chance, 1973; Boveris and Cadenas, 1975]. More recently, CoQ has been recognised as an important antioxidant in the inner membrane, where it scavenges radicals directly [Takayanagi et al., 1980; Kagan et al., 1990] and regenerates $\alpha$-tocopherol from the tocopheroxyl radical [Kagan et al., 1998; Lass and Sohal, 1998; Packer and Maguire, 1994].

Ubiquinone is not only present in the inner mitochondrial membrane, but in low density lipoproteins (LDL), plasma membranes, and all intracellular membranes [Dallner et al., 1992]. In membranes other than the inner
mitochondrial membrane CoQ is found largely in the reduced state [Dallner et al., 1992], an observation that raises the question of its recovery. There are currently two schools of thought about the distribution of CoQ in the mitochondrial membrane. One school subscribes to the view that CoQ exists as a freely diffusable homogenous pool, shuttling electrons between different complexes [Kroger and Klingenberg, 1973; Lenaz et al., 1997; Gupte et al., 1984]. The other view, based on the variations in the reducibility of CoQ by different substrates [Lass and Sohal, 1998; Jorgensen et al., 1985], is that CoQ is distributed heterogeneously, forming separate and distinct pools within the hydrophobic domain of the membranes.

Interestingly, there are few reports studying the interaction between quinones and NO. The majority of those that have been carried out have shown that quinones (e.g. hydroquinone and duroquinone) act as NO scavengers through the generation of $O_2^-$, an action that has been characterised either using relaxation of vascular smooth muscle or stimulation of nitrergic neurotransmission [Moncada et al., 1986; Lilley and Gibson, 1995; Paisley and Martin, 1996]. Studies have also been performed using quinones with well known anti-carcinogenic properties (e.g. adriamycin) as well as various quinones that are ubiquitous contaminants in urban air (e.g. phenanthraquinone and anthraquinone), which have shown that their mechanism of action might be through
inhibition of NO synthase [Kumagai et al., 1998; Vasquez-Vivar et al., 1997].

Taking this information together with our own previous results showing that inhibition of complex I activity might be mediated by a direct reaction between NO and ubiquinone, we decided to investigate the pharmacological and biochemical interactions, between ubiquinone and NO.

VI.2. Vascular reactivity experiments.

VI.2.a. Methods

Female Harlan-Sprague Dawley rats (220–240 g) were killed by stunning and cervical dislocation. The thoracic aorta was dissected and cleared of connective tissue and blood products. Intact rings, 2–3 mm in width, were mounted in 25-ml glass organ baths containing Krebs bicarbonate buffer (composition mM: NaCl, 118.1; KCl, 4.7; MgSO₄, 1.0; KH₂PO₄, 1.0; CaCl₂, 2.5; NaHCO₃, 25.0; glucose, 11.1) which was maintained at 37°C. Tissues were gassed continuously with 95% O₂, 5% CO₂. A resting tension of 1 g was applied to each tissue and changes in tension recorded with a Grass FT03 force displacement transducer attached to a Rikadenki (R-64) chart recorder. Where indicated, the endothelium was removed from the tissues by gently rubbing the luminal surface with forceps. The rings were allowed to equilibrate for 1 h prior to experimentation. Vessels that generated an active tone of less than 1 g in response to a submaximal concentration of
phenylephrine (PE; 1 μM) were discarded. To test the functional integrity of the endothelium, PE-precontracted vessels showing >50% relaxation to 1 μM acetylcholine (ACh) were deemed endothelium-intact, those exhibiting <15% relaxation were regarded as endothelium-denuded, and vessels not satisfying either criterion were discarded.

After washout, the rings were re-contracted with PE to the EC_{10}, and cumulative concentrations of CoQ-1 (1 – 20 μM) were added to either endothelium-intact or -denuded rings.

Using another set of vessels, after washout, the rings were re-contracted with PE to the EC_{90}, and relaxation was induced by 1 μM ACh in presence of cumulative concentrations of CoQ-1 (1 – 20 μM). The effects of hydroquinone (2.5 μM) and duroquinone (2.5 μM) were compared with that of CoQ-1 (2.5 μM). Some of the experiments were carried out in the presence of superoxide dismutase (SOD; 30-120 U ml⁻¹) added two minutes before the addition of the different quinones.

In another set of experiments, rings pre-contracted with PE to the EC_{90} were used to study the effect of cumulative concentrations of CoQ-1 on the relaxation induced by exogenous NO generated by S-nitroso-glutathione (GSNO; 100 nM).

**VI.2.b. Results**

CoQ-1 (1 - 20 μM) induced a concentration-dependent contraction (EC_{50} = 9.3±0.7 μM) in endothelium-intact isolated rat aortic rings pre-contracted
with an EC$_{10}$ of PE (n=6; figure 14). No contractile action of CoQ-1 was observed in endothelium-denuded aortic rings (n=6; data not shown). ACh (1 μM)-induced relaxation of rat isolated endothelium-intact aortic rings pre-contracted with PE (EC$_{90}$) was in average 73±3.75%. Cumulative amounts of CoQ-1 produce a concentration-dependent inhibition of ACh-induced relaxation with a EC$_{50}$ of 1.27±0.34 μM (Figure 15).

Inhibition of ACh-induced relaxation was also observed in the presence of hydroquinone (2.5 μM) and duroquinone (2.5 μM), but this was much less than seen with the same amount of CoQ-1 (Figure 16). Pre-treatment of the tissues with SOD (30-120 U/ml) before the addition of either hydroquinone or duroquinone resulted in a significant (p<0.05) abrogation of the inhibitory effect previously seen. Interestingly, however pre-treatment of the tissues with SOD has no effect on the inhibitory action of CoQ-1 (2.5 μM) on the ACh-induced relaxation (n=5; Figure 16), considered together, these data suggest by which CoQ-1 inhibits Ach-induced vascular relaxation in different from that of hydroquinone and duroquinone.
Figure 14. Addition of ubiquinone-1 (CoQ-1) results in further contraction of endothelium-intact rat aortic rings pre-contracted with phenylephrine (PE, EC\textsubscript{10}). Trace is representative of 6 separate experiments.
Figure 15. Ubiquinone-1 (CoQ-1) produces a concentration-dependent inhibition of ACh (1 μM)-induced vascular relaxation in endothelium-intact rat aortic rings pre-contracted with phenylephrine (EC$_{90}$). Data are expressed as mean ± SEM of 5 experiments.
Figure 16. Effect of ubiquinone-1 (2.5 μM), hydroquinone (2.5 μM) and duroquinone (2.5 μM) on ACh (1 μM)-induced vascular relaxation in rat aortic rings in the absence (-) or presence (+) of superoxide dismutase (SOD; 30 U/ml). Control value for relaxation induced by ACh alone was 73±3.75%. Data are expressed as mean ± SEM of 5 experiments.
Finally, the addition of cumulative concentrations of CoQ-1 resulted in a dose-dependent inhibition ($EC_{50} = 32.8\pm0.02 \ \mu M$) of the relaxation induced by GSNO (100 nM) in endothelium-denuded rat aortic rings ($n=5$; Figure 17).
**Figure 17.** Ubiquinone-1 (CoQ-1) produces a concentration-dependent inhibition of S-nitrosoglutathione (100 nM)-induced vascular relaxation in endothelium-denuded rat aortic rings. Data are expressed as mean ± SEM of 5 experiments.
VI.3. Electrochemical detection of NO.

VI.3.a. Methods

For the electrochemical detection of NO, an isolated NO meter and sensor was used (Iso-NO, World Precision Instruments Inc., Hertfordshire, UK) enclosed in an iron shield (Faraday cage) to protect against stray electric field. The probe tip was immersed in an incubation glass chamber with a total volume of 2.5 ml, sealed by a plunger assembly (Rank Brothers Ltd, Cambridge, UK) and connected to a Model 300 Stirrer Control Box (Rank Brothers Ltd, Cambridge, UK). Data were collected and analysed by computer using the Microsoft® Windows® -based Duo.18 software (World Precision Instruments Inc., Hertfordshire, UK). NO was generated in the incubation chamber by 1 mM of the NO donor Sper/NO which has a half-life of 230 min at 22°C [Keefer et al., 1996]. Once the NO signal recorded by the NO-selective electrode stabilised, cumulative amounts of CoQ-1 (ranging from 100 μM to 1 mM) were added.

VI.3.b. Results

Using an NO-sensitive electrode, addition of CoQ-1 to the chamber containing 1 mM of Sper/NO resulted in a concentration-dependent decrease in the NO signal coming from the NO donor (n=3; figure 18). The calculated NO/CoQ-1 ratio from these experiments was 1:487 M. Interestingly, as NO is released continuously from this NO-donor, i.e. NONOates, once all CoQ-1 was apparently consumed by NO, the NO-
selective electrode began to detect a signal again [Feelisch, M., personal communication] (Figure 19).

**Figure 18.** Effect of the addition of cumulative amounts of ubiquinone-1 (CoQ-1) on the NO signal (pA), equivalent to 410 nM, recorded by an NO-selective electrode. NO was generated into a buffer containing 1 mM of Sper/NO.
Figure 19. Addition of cumulative amounts of ubiquinone-1 (CoQ-1) decreased the NO signal recorded by an NO-selective electrode. NO was generated in a buffer containing Spermine/NO (1 mM). The NO/CoQ-1 ratio in this experiment was 487:1. Graphic is representative of 3 separate experiments.
VI.4. Chromatographic determination of ubiquinone.

VI.4.a. Methods

To characterise the novel reaction that we have observed between ubiquinone and NO, we used high performance liquid chromatography (HPLC). As we have been working with the synthetic CoQ-1, a second aim was to investigate if this reaction might occur with the physiological ubiquinone variety, i.e. CoQ-10. The HPLC system consisted of an Applied Biosystems 1783A gradient pump system controller with an 1480A injector/mixer, a Hibar RT 125-4 pre-packed RP-18 column (5 μm; LiChrosorb, Merck Co., UK) with a 4-4 RP-18 pre-column (5 μm; LiChroCART, Merck Co., UK), an Applied Biosystems 1783A absorbance detector, and an Antec digital electrochemical amperometric detector with glassy carbon electrode. The detectors were set-up in line, with the eluent passing first through the absorbance detector. Samples were eluted isocratically with a running buffer containing 70% ethanol and 30% methanol at 1 ml/min. Each run lasted 40 min. The UV detector was set at 275 nm for detection of ubiquinone and the electrochemical detector was in the oxidising mode, potential 0.6V, full recorder scale at 50 nA [Podda et al., 1996; Mohr and Stocker, 1996; Yamashita and Yamamoto, 1997; Rousseau and Varin, 1998]. Data were collected with an Applied Biosystems pen recorder. CoQ-10 was a kind gift of Eisai Pharma-Chem Europe Ltd. (London, UK) and standards were prepared in absolute ethanol after an initial dilution in a non-polar solvent (chloroform).
Ubiquinol-10 standards were synthesised by reduction of ubiquinone with a saturated solution of sodium dithionite. To study the interaction between CoQ-10 and NO, some experiments were performed in the presence of different concentrations (ranging from 1 μM to 10 mM) of S-nitroso-N-acetylpenicillamine (SNAP), an alcohol-soluble NO-donor. In these experiments, ubiquinone-10 and SNAP were first injected separately, and then a mixture of the two compounds was injected after different co-incubation periods (ranging from 5 to 240 min).

**VI.4.b. Results**

Our experiments confirmed that the HPLC with electrochemical detection is a useful tool to evaluate the ratio of ubiquinone/ubiquinol, since, using the UV detector at 275 nm the peak corresponding to ubiquinone-10 appeared around 16 min, whereas the signal of ubiquinol was recorded by the electrochemical detector at −0.6 mV with a delay of around 40 seconds. It is important to note that under normal conditions ubiquinol is not detected by HPLC with electrochemical detection, normally it is necessary to create an oxygen-free atmosphere or to use a special reducing column. To test our system, addition of a saturated solution of sodium dithionite, a strong reducing agent, to a sample of ubiquinone-10 resulted in disappearance of the peak corresponding to ubiquinone-10, whereas the electrochemical signal of ubiquinol-10 remained present. Thus, was possible to establish a good correlation between the decrease in the height of the peak corresponding to ubiquinone-10 and the increase
in the height of the peak corresponding to ubiquinol-10 (data not shown). Experiments carried out to assess the interaction between NO and ubiquinone demonstrated a dose-dependent decrease in the height of the ubiquinone-10 peak after co-incubation with 1 mM of SNAP (n=5; figure 20). Interestingly, despite the decrease in the height of the peak corresponding to ubiquinone-10, we were unable to detect at the same time any increase in the peak corresponding to ubiquinol-10. Thus, this result strongly suggested that NO was reacting with ubiquinone-10, but not by a direct conversion of ubiquinone to its reduce form, ubiquinol. Moreover, after 6 hours co-incubation of CoQ-10 with SNAP, the chromatographic peak corresponding to CoQ-10 showed almost the same height as in the absence of the NO-donor indicating that the interaction of CoQ-10 with NO was reversible with time (n=5; figure 21).
Figure 20. Effect of cumulative concentrations of S-nitroso-N-acetylpenicillamine (SNAP) on the height of the peak corresponding to ubiquinone-10. Ubiquinone-10 was identified by high performance liquid chromatography and the NO-donor was co-incubated for 30 min before the injection into the system.
Figure 21. Effect of co-incubation of S-nitroso-N-acetyl-penicillamine (SNAP) with ubiquinone-10 (CoQ-10) produces a time reversible decrease in the height of the peak of ubiquinone-10.

VI.5.a. Methods.

To test the formation of a new compound after the interaction between ubiquinone and NO, mass spectrometric analysis was performed. The system consisted of an HP1100 Series Liquid Chromatography/Mass Spectrometry Detector (Hewlett Packard, Cheshire, UK). The source temperature was 100°C, with N2 drying gas flow of 813 l/h. The capillary voltage was set at 3.5 kV and cone voltage set at 40 V. The spectrum was recorded continuously from 200 to 2000 m/z with a scan time of 3 s for the entire duration of the HPLC elution. Chromatographic steps were performed as previously described with a Hibar RT 125-4 pre-packed RP-18 column (5 μm; LiChrosorb, Merck Co., UK) and a 4-4 RP-18 pre-column (5 μm; LiChroCART, Merck Co., UK), while mass spectrometry was performed using an atmospheric pressure ionisation (API)-electrospray accessory (Hewlett Packard, Cheshire, UK). In both cases, samples were eluted isocratically with a running buffer containing 70% ethanol and 30% methanol at one ml/min. After recording the mass spectrum of ubiquinone-10 and SNAP separately, their mixture was analysed. Data were collected and analysed in a computer using the Microsoft® Windows® -based HP3DLC ChemStation software (Hewlett Packard, Cheshire, UK). Briefly, in mass spectrometry, a substance is bombarded with an electron beam having sufficient energy to fragment the molecule. The positive fragments which are produced (cations and
radical cations) are accelerated in a vacuum through a magnetic field and are sorted on the basis of mass-to-charge ratio. Since the bulk of the ions produced in the mass spectrometer carry a unit positive charge, the value m/e is equivalent to the molecular weight of the fragment. The output of the mass spectrometer shows a plot of relative intensity vs. the mass-to-charge ratio (m/z). The most intense peak in the spectrum is termed the base peak and all others are reported relative to its intensity. The peaks themselves are typically very sharp, and are often simply represented as vertical lines. The process of fragmentation follows simple and predictable chemical pathways and the ions that are formed will reflect the most stable cations and radical cations which that molecule can form. The highest molecular weight peak observed in a spectrum will typically represent the parent molecule, minus an electron, and is termed the molecular ion (M+). Generally, small peaks are also observed above the calculated molecular weight due to the natural isotopic abundance of $^{13}$C, $^2$H, etc. Fragments can be identified by their mass-to-charge ratio, but it is often more informative to identify them by the mass which has been lost. That is, loss of a methyl group will generate a peak at m-15; loss of an ethyl, m-29, etc.

**VI.5.b. Results**

Mass spectrometric analysis revealed ubiquinone-10 as a parent molecule (M+) of 885 m/z, with the presence of multiple fragments ranging between 270 and 650 m/z (Figure 22). However, according to its chemical
structure, ubiquinone-10 should be a parent compound of 863 m/z, therefore it seems like a sodium atom is associated, due to contamination of the equipment. After co-incubation of ubiquinone-10 with a 10-times molar excess of SNAP, the mass spectrometric analysis again revealed the presence of the parent molecule of ubiquinone-10 (M, 885), but this time a peak with a M+ of 1750 was also observed, suggesting the formation of a new compound (Figure 23). The high molecular mass suggests perhaps the existence of two molecules of ubiquinone bound through a molecule of NO [Feelisch, M., personal communication] (Figure 24).
Figure 22. Mass spectrometric analysis of ubiquinone-10. Peak corresponding to ubiquinone-10 showed a M+ of 885.
Figure 23. Mass spectrometric analysis of ubiquinone-10 after pre-incubation with S-nitroso-N-acetylpenicillamine (SNAP). A new molecule with a M+ of 1750 was found. SNAP was used in a 10 times molar excess and pre-incubated with CoQ-10 for 60 min before injection in the system.
Figure 24. Suggested chemical structure of a new compound resulting from the reaction between NO and ubiquinone-10. It might be possible to bound two molecules of CoQ-10 through an NO molecule.
Section VII

General discussion
Our biochemical analysis of complex I purified from bovine heart mitochondria used in this study revealed the same polypeptide composition as previously reported [Finel et al., 1992]. The enzymatic analysis of the complex I activity evaluated by the rate of oxidation of NADH was in agreement with the values reported by other authors [Finel et al., 1992]. We found that oxidation of NADH by purified complex I was increased after pre-incubation of the protein at 37°C. This is not surprising because the rate of an enzyme-catalysed reaction usually increases with increasing temperature up to an optimum point, then decreases because enzymes are thermolabile. [Davidson and Sittman, 1994]. Also, we demonstrated that the activity of the purified complex I was not only dependent on the concentration of the enzyme (complex I) and the substrate (NADH), but also on the concentration of the electron acceptor (CoQ-1). Therefore, our results indicate that the oxidation of NADH by purified complex I does not follow the classical Michaelis-Menten equation, in which the reaction velocity depends on substrate and enzyme alone [Davidson and Sittman, 1994]. Thus, changes in the availability of CoQ are crucial factors in the overall capacity of purified complex I to oxidise NADH. Although our results were obtained under well-controlled conditions, we suggest that the concentration of CoQ might be relevant in the evaluation of complex I activity. Indeed, using isolated mitochondria, Lenaz et al. demonstrated that complex I has a lower affinity for CoQ than complex II. The high $K_m$ for CoQ exhibited by
complex I during NADH oxidation suggests that changes in the mitochondrial CoQ concentration, occurring in pathological situations, might play an important role in the function of the mitochondrial respiratory chain [Lenaz et al., 1997]. However, in many tissues the mitochondrial CoQ concentration lies within the range of the $K_m$ values determined experimentally and the rate of electron transfer is expected to be maximal [Battino et al., 1990].

Clementi et al. [1998] reported that long term exposure to NO produces inactivation of complex I in intact murine macrophage J774 cells and this effect has been confirmed in other cell lines, i.e. lymphocytes (Jurkat cells) and fibroblasts (L929) [Beltran et al., 2000]. Surprisingly however, using the purified protein and under the experimental conditions applied, no inhibition of the oxidation of NADH by complex I was seen after pre-incubation with DETA/NO, an NO-donor.

Our purified complex I preparation is insensitive to rotenone, a classical inhibitor of complex I activity, and this was attributed to the purification process, which results in removal of the membrane components, e.g. phospholipids [Finel et al., 1992]. For this reason, our finding that pre-incubation of purified complex I with NO did not inhibit the oxidation of NADH could be explained by the removal of membrane components which might interact with NO to inhibit complex I activity. Another possible interpretation for the absence of effect of NO on the purified complex I is
that in intact cells NO might be transformed and/or modified before acting on complex I.

There is currently a general consensus that complex IV is the main mitochondrial target for endogenous and exogenous NO [Cleeter et al., 1994; Richter et al., 1994; Brown and Cooper, 1994; Bolaños et al., 1994; Brown et al., 1998]. Also, it is well known that $O_2^-$ is produced in large quantities in mitochondria, especially if complex IV is inhibited [Turrens, 1997]. Thus, in the presence of NO, ONOO$^-$ might be formed. Once ONOO$^-$ is present then other components of the mitochondrial respiratory chain, i.e. complexes I to III, become more susceptible to inactivation [Radi et al., 1991; Radi et al., 1994; Bolaños et al., 1995; Cassina and Radi, 1996; Gadelha et al., 1997; Brookes et al., 1998].

Therefore, it is logical to assume that the inhibition of complex I observed in intact cells could be due to the presence of ONOO$^-$. In fact, it has been demonstrated, using isolated mitochondria and submitochondrial particles, that ONOO$^-$ does inhibit complex I activity [Bolaños et al., 1995; Cassina and Radi, 1996; Brookes et al., 1998]. However, when we evaluated the activity of purified complex I previously exposed to SIN-1, a generator of ONOO$^-$, the oxidation of NADH was not affected. Thus, in contrast to the NO-induced inhibition of NADH oxidation observed when working with whole cell homogenates, no inhibition by either NO or ONOO$^-$ was observed if purified complex I was used.
From our experimental approach it was not possible to guarantee that NO and ONOO' did not produce changes in the protein of complex I, i.e. nitrosylation or nitration, but, assuming that that was the case, apparently those changes were not crucial to produce inhibition of the oxidation of NADH by complex I. Nevertheless, a missing point in our experimental design was the use of authentic NO and ONOO', rather than their respective donors. This might be especially important in the case of SIN-1, because it has recently been shown that, in the presence of an electron acceptor, SIN-1 acts preferentially as an NO-donor [Singh et al., 1999].

Obviously, the specific conditions applied in every experimental model may be responsible for differences in the results obtained. As an example, Sharpe and Cooper [1998] using purified complex IV recently confirmed its reversible inhibition by NO, consistent with previous reports using intact cells and isolated mitochondria [Cleeter et al., 1994; Brown and Cooper, 1994; Richter et al., 1994]. Interestingly, and in contrast to some reports showing that ONOO' does not affect complex IV [Cassina and Radi, 1996; Poderoso et al., 1996a], when the purified complex IV was exposed to ONOO' an irreversible inhibition of its activity by as much as 50% was observed [Sharpe and Cooper, 1998].

In view of these findings, we decided it was necessary to look for other mechanism(s) by which NO can affect the activity of purified complex I. First, the possibility was considered that the high concentration of free thiol present in the storage buffer of the purified complex I might be
responsible for the lack of inhibition of the protein activity after exposure to either NO or ONOO$. It is well known that thiols can interact with NO and ONOO$, and also that this interaction could occur either by a direct chemical reaction to produce nitrosothiols or by transnitrosylation [Stamler, 1994]. Thus, in the presence of an excessive amount of free thiols in the buffer, it is possible that NO or ONOO$ might react preferentially with those rather than with the protein of complex I. However, comparative measurement of the oxidation rate of NADH before and after removal of thiols clearly revealed no difference. Our experiments demonstrated that in the absence of free thiols, pre-incubation of the complex I protein either with an NO-donor or a generator of ONOO$ produced no inhibitory effect on complex I activity.

Due to the close association between complex I and ubiquinone and based on our earlier results showing that complex I activity is extremely dependent on the availability of CoQ, we tested the effect of NO on the CoQ. For this purpose, after successful modification of the assay to evaluate the activity of complex I, we found that NO added during complex I activity produced a decrease in the oxidation of subsequent additions of NADH. Interestingly, if the CoQ was pre-incubated with the NO-donor, not only was the initial oxidation rate of NADH reduced, but the oxidation of subsequent additions of NADH were also almost completely inhibited.
Such a reaction has not been described previously, and ours is therefore the first report that pre-incubation of ubiquinone with NO results in prevention of the oxidation of NADH by purified complex I. In summary, although we found no inhibitory effect of NO or ONOO⁻ when incubated directly with the protein of complex I, our results demonstrated a novel, alternative mechanism by which NO can inhibit the activity of mitochondrial complex I.

It has recently been reported that complex I activity is inhibited following loss of GSH [Sriram et al., 1998; Balijepalli et al., 1999a]. Glutathione is present in virtually all cells at concentrations ranging from 0.5 to 10 mM, making it the most prevalent intra-cellular thiol [Wang and Ballatori, 1998]. Using isolated brain mitochondria, Balijepalli et al. [1999b] demonstrated that, following a decrease in GSH, the inhibition of complex I activity was not due to the loss of surface thiol groups, and suggested that the vital thiol groups necessary for complex I activity are not accessible to the thiol modifiers. Such modification of surface thiols might, however lead to secondary structural alteration resulting in the reversible oxidation of the vital thiol groups in the protein [Balijepalli et al., 1999b].

Treatment of cell types from different species with NO gas or NO-donors has been reported to result in a decrease [Clementi et al., 1998; Beltran et al., 2000; Bolaños et al., 1996; Wakulich and Tepperman, 1997; Kim et al., 1997; Clancy et al., 1994], in no change [Byrne and Hanson, 1998], or even in an increase [Moellering et al., 1998; White et al., 1995] in
intracellular GSH concentration. Similar variability was found when studying the effect of inducible NO synthase activity on cellular GSH levels [Hothersall et al., 1997; Kuo and Abe, 1996]. Despite the discordance in these results, it is well accepted that NO reactive species might produce nitrosative stress, the equivalent of oxidative stress that is produced by oxygen reactive species [Padgett and Whorton, 1995]. Also, it has been demonstrated recently that exposure to short- and long-term nitrosative stress in several cell types resulted in a differential ability to withstand a decrease in GSH [Berendji et al., 1999]. Therefore, the mechanism of NO-mediated intracellular GSH decrease is likely to involve reaction of NO with O$_2$ yielding NOx (N$_2$O$_3$, NO$_2$, N$_2$O$_4$, etc.), which subsequently nitrosylates the GSH thiol group [Wink et al., 1994; Kharitonov et al., 1995]. It is likely, then, that once the intracellular level of GSH is reduced, following either oxidative or nitrosative stress, complex I is inactivated.

At the time of our initial findings, the only relevant literature reference was an abstract suggesting an interaction between NO and ubiquinol, the reduced form of ubiquinone [Poderoso et al., 1996b]. According to these authors, interaction between NO and ubiquinol might in part explain the inhibitory effect of NO on complex III activity, together with the intramitochondrial formation of O$_2^-$ [Poderoso et al., 1996b]. Thus, having found a novel reaction between CoQ and NO, we set out to study such reaction in detail. Using a classical pharmacological assay, we
found that CoQ inhibited the vascular relaxation induced by ACh in rat aortic rings with an intact endothelium. Also, after contraction of the tissues with small amounts of PE, addition of CoQ produced further contraction, an effect that was not present in endothelium-denuded rings. Interestingly, when vascular relaxation was produced by exogenously supplied NO, the addition of CoQ also resulted in inhibition of the relaxation. Obviously, the EC\textsubscript{50} in each case was different, requiring higher amounts when exogenous NO was used. However, a similar effect has been demonstrated with other quinones, and was thought to be mediated by the generation of O\textsubscript{2}^{-} [Moncada et al., 1986; Lilley and Gibson, 1995; Paisley and Martin, 1996]. In our experiments, pre-treatment of the tissues with varying concentrations of SOD failed to influence the inhibitory effect of CoQ-1, therefore, it is possible to conclude that this reaction in contrast to that of other quinones was not mediated by generation of superoxide radical. Thus, CoQ-1 appeared to inhibit NO-induced vascular relaxation via a direct reaction with NO.

We then have obtained a second piece of evidence supporting the reaction between NO and CoQ-1. Using an NO-selective electrode we demonstrated that CoQ-1 can act as a scavenger of the NO signal. This effect was concentration-dependent and interestingly, it looks like once NO consumed all the CoQ-1, the electrode began to detect a signal again. If NO were able to reduce directly ubiquinone to ubiquinol, our findings of the inhibition of complex I activity by pre-incubation of ubiquinone with an
NO-donor might be completely explained [Rich, P., personal communication]. However, after chemical analysis of the reaction products, we confirmed that NO interacts with ubiquinone, and also demonstrated chromatographically that such a reaction did not involve reduction of ubiquinone to ubiquinol by NO. Moreover, using a separate approach, our results indicated that the product of the reaction between NO and CoQ is present only while the NO-donor was active. Again, these findings reinforce our initial results showing that inhibition of purified complex I activity after pre-incubation of ubiquinone with DEA/NO was temporary, and dependent on the continuing generation of NO by the donor. We tested other NO-donors, i.e. NONOates, using the HPLC method, and found a similar effect with Sper/NO, while no changes were found with DETA/NO (data not shown). Sper/NO produces high concentrations of NO at the beginning of its decomposition, and these high fluxes of NO may in turn produce significant levels of $N_2O_3$ after reaction with $O_2$ [Wink et al., 1994; Kharitonov et al., 1995]. By contrast, DETA/NO decomposes slowly and should give rise essentially to NO$^\cdot$. S-nitrosothiols can also generate NO$^\cdot$ by homolytic decomposition [Singh et al., 1996], the rate of decomposition depending greatly on the nature of the buffer, the presence of redox active species and contaminants such as cooper ions [Butler and Rhodes, 1997].

Despite the reversibility of the reaction between NO and CoQ, using mass spectrometric analysis we were able to detect the potential "new
molecule resulting from this reaction. Its relatively high molecular mass suggests that while NO is present, two molecules of CoQ might be able to bind to each other using a molecule of NO as bridge [Feelisch, M., personal communication].

Finally, Poderoso et al. [1999 and 2000] recently confirmed their initial observation that NO reacts with ubiquinol, the reduced form of ubiquinone [Poderoso et al., 1996b]. Interestingly, the experimental approach used by these authors to characterise the interaction between NO and ubiquinol is very similar to the approach used in one part of this thesis, i.e. electrochemical detection plus spectrophotometric measurements. Also, their main conclusion was that the reaction between NO and ubiquinol increases the intra-mitochondrial steady-state concentration of semiubiquinone, finally producing $O_2^-$ by auto-oxidation [Poderoso et al., 1999a and 1999b; Schopfer et al., 2000].

These data, together with our own results, suggest that ubiquinone is a very reactive species, which can interact with NO in its reduced form, in its familiar antioxidant role. However, it also can react with NO in its oxidised form, a novel and hitherto undescribed reaction.

Clementi et al. [1998] showed that prolonged exposure of intact cells to NO resulted in an inhibition of complex I activity, and suggested that S-nitrosylation of complex I might be responsible for that inhibition. However, we have found (i) an extreme dependence of complex I activity on the availability of ubiquinone, and (ii) that inhibition of the activity of
purified complex I occurs only after a direct reaction between ubiquinone and NO. Thus, it is possible that the previously reported inhibition of complex I activity in intact cells might in fact be due to the interaction between ubiquinone and NO. Nevertheless, although we found a novel interaction between NO and ubiquinone, the question remains whether NO can interact in intact cells with ubiquinone to inhibit the oxidation of NADH. One possibility is that NO, depending on the concentration and/or the time of exposure, might react first with complex IV, and after that interact with complex III. Once a complex is formed between NO and the cytochromes, a reaction between NO and ubiquinone might be possible [Cadenas, E., personal communication].
Section VIII

References


Chance, B. and Hollunger, G. (1961) The interaction of energy and
electron transfer reactions in mitochondria. I. General properties and
nature of the products of succinate-linked reduction of pyridine nucleotide.
Journal of Biological Chemistry. 236, 1534-1543.

Clancy, RM., Levartovsky, D., Leszczynska-Piziak, J., Yegudin, J., and
Abramson, SB. (1994) Nitric oxide reacts with intracellular glutathione and
activates the hexose monophosphate shunt in human neutrophils:
evidence for S-nitrosoglutathione as a bioactive intermediary.
Proceedings of the National Academy of Sciences USA. 91, 3680-3684.

Cleeter, MW., Cooper, JM., Darley-Usmar, V., Moncada, S., and
Schapira, AH. (1994) Reversible inhibition of cytochrome c oxidase, the
terminal enzyme of the mitochondrial respiratory chain, by nitric oxide.
Implications for neurodegenerative diseases. FEBS Letters. 345, 50-54.

Persistent inhibition of cell respiration by nitric oxide: Crucial role of S-
nitrosylation of mitochondrial complex I and protective action of
 glutathione. Proceedings of the National Academy of Sciences USA. 95,
7631-7636.


Drapier, JC. and Hibbs Jr, JB. (1986) Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-
sulfur prosthetic group and is reversible. *Journal of Clinical Investigation.* 78, 790-797.


Lass, A. and Sohal, RS. (1998) Electron transport-linked ubiquinone-dependent recycling of \( \alpha \)-tocopherol inhibits autooxidation of


determination of tissue tocopherols, tocotrienols, ubiquinols, and

Poderoso, JJ., Carreras, MC., Lisdero, C., Riobo, N., Schopfer, F., and
Boveris, A. (1996a) Nitric oxide inhibits electron transfer and increases
superoxide radical production in rat heart mitochondria and

Poderoso, J., Carreras, M., Schopfer, F., et al. (1996b) Ubiquinol-2 reacts
with nitric oxide. In: *Abstracts of the Second International Conference on
the Biochemistry and Molecular Biology of Nitric Oxide.* Anonymous Los
Angeles, CA.: p. 94

Poderoso, JJ., Carreras, MC., Schopfer, F., Lisdero, C., Riobo, N., Giulivi,
C., Boveris, AD., Boveris, A., and Cadenas, E. (1999a) The reaction of
nitric oxide with ubiquinol: kinetic properties and biological significance.
*Free Radicals in Biology and Medicine.* **26,** 925-935.

Poderoso, JJ., Lisdero, C., Schopfer, F., Riobo, N., Carreras, MC,
Cadenas E., Boveris, A. (1999b) The regulation of mitochondrial oxygen
uptake by redox reactions involving nitric oxide and ubiquinol. *Journal of
Biological Chemistry.* **274,** 37709-37716.


Wink, DA., Nims, RW., Darbyshire, JF., Christodoulou, D., Hanbauer, I., Cox, GW., Laval, F., Laval, JM., Cook, JA., Krishna, MC., DeGraff, WG.,
