# STUDIES OF ELECTRON TRANSPORT COMPLEXES

By

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This thesis is dedicated to my beloved wife, Deana. Her constant encouragement and loving companionship have made even the most difficult of times enjoyable. I would also like to dedicate this thesis to my parents, Robert and Roberta, my sister Stephanie, and my brothers Jeremy and Justin. The loving family support they have constantly given has been an inspiration to me throughout my lifetime and especially during the completion of this thesis.

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## NOMENCLATURE

| | |
|---|---|
| Azido-Q | 3-[$^3$H]azido-2-methy-5-methoxy-6-geranyl-1,4-benzoquinone |
| *cyt* | cytochrome |
| DCPIP | 2,6-dichlorophenolindophenol |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediaminetetraacetic acid |
| EPR | Electron paramagnetic resonance |
| g | Landé g factor |
| HPLC | High performance liquid chromatography |
| HQNO | 2-heptyl-4-hydroxyquinoline |
| kDa | Kilodaltons |
| NADH | Nicotinamide adenine dinucleotide (reduced) |
| NO | Nitric Oxide |
| NQR | NADH-Ubiquinoe reductase (Complex I) |
| $ONOO^-$ | Peroxonitrite |
| Q | Ubiquinone |
| $Q_0$ | 2,3-dimethoxy-5-methyl-1,4-benzoquinone |
| $Q_0C_1$ | 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone |
| $Q_0C_2$ | 2,3-dimethoxy-5-methyl-6-ethyl-1,4-benzoquinone |
| $Q_0C_3$ | 2,3-dimethoxy-5-methyl-6-propyl-1,4-benzoquinone |

| | |
|---|---|
| $Q_0C_4$ | 2,3-dimethoxy-5-methyl-6-butyl-1,4-benzoquinone |
| $Q_0C_5$ | 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone |
| $Q_0C_7$ | 2,3-dimethoxy-5-methyl-6-heptyl-1,4-benzoquinone |
| $Q_0C_9$ | 2,3-dimethoxy-5-methyl-6-nonyl-1,4-benzoquinone |
| $Q_0C_{10}$ | 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone |
| $Q_0C_{10}Br$ | 2,3-dimethoxy-3-methyl-6-(10-bromo-decyl)-1,4-benzoquinone |
| $Q_1$ | 2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone (ubiquinone-1) |
| $Q_1H_2$ | Ubiquinol-1 |
| QCR | Ubiquinol-cytochrome *c* reductase (Complex III) |
| SCR | Succinate cytochrome *c* reductase (Complexes II and III) |
| SDS | Sodium dodecyl sulfate |
| SDH | Succinate Dehydrogenase |
| SQR | Succinate ubiquinone reductase (Complex II) |

# CHAPTER I

## PREFACE

The enzymes involved in cellular bioenergetics comprise an intricate series of electron receptors which act together to produce the electrochemical energy necessary to drive proton translocation and ultimately, ATP synthesis. In mammalian cells, at least five complexes are operant with two mobile electron carriers (ubiquinone and cytochrome *c*). Each enzyme complex contains many subunits and each subunit can facilitate any of a host of functions ranging from maintaining structure to participation in the ligation of specific prosthetic groups directly involved in electron transport.

Because of the complexity of eukaryotic systems, a clearer understanding of protein function and architecture can often be elucidated when prokaryotic systems are examined. For example, while beef heart cytochrome *c* oxidase (complex IV) contains 13 subunits, cytochromes $bo_3$ and *bd* oxidase from *Escherichia coli*, which are both analogous to the mammalian complex IV, contain only 4 and 2 subunits respectively. Since the ultimate function of both systems is the same (i.e. energy production) it is easy to see how investigation of simpler systems leads to important insights about more

complicated systems. However, even though the simplicity of lower organisms sometimes makes them a better choice for study, a complete understanding of bioenergetics requires concurrent study of more complex cells.

There are several reasons which make prokaryotic organisms experimentally advantageous over eukaryotic equivalents. Foremost is the relative biological simplicity of prokaryotes compared to eukaryotes. As stated above, many bacterial enzymes accomplish the same reactions as their eukaryotic counterparts with a much simpler design. While many eukaryotic enzyme complexes contain supernumerary subunits with unknown functions, prokaryotic complexes typically only contain subunits which are involved in substrate binding or contain catalytic prosthetic groups. Many times, the catalytic subunits of prokaryotic enzyme complexes correspond to the mitochondrial encoded subunits of the eukaryotic systems, indicating they may share an early evolutionary history. This makes the prokaryotes ideal for structural and functional studies aimed at the mechanism of the catalytic core. Furthermore, many prokaryotic enzymes are easily expressed in recombinant cells. Thus, site-directed mutagenesis has become particularly valuable in determining information about the structure of these prokaryotic enzyme complexes.

Of considerable interest in the investigation of these enzymes are substrate and inhibitor binding sites. Although a compound may have been known to act as a natural substrate for years, many times even the general location of binding is unknown. The

same is true for inhibitors. Both the site and mechanism of binding are important for drug therapy, and by studying these sites, and the interaction of the substrate or inhibitor with the enzyme complex, a better understanding of the mechanism of electron transport can be acquired. Furthermore, studies of substrate and inhibitor binding can give important clues as to the three dimensional structure or subunit relationships of the complexes.

In this thesis, enzymes from both prokaryotic (*E. coli*) and eukaryotic (bovine heart mitochondria) sources which are well characterized electron transport complexes are investigated. In Chapter II, "Identification of the Ubiquinol-Binding Site in the Cytochrome $bo_3$ -Ubiquinol Oxidase of *Escherichia coli*," we use a substrate analog of one of the terminal oxidases of *E. coli*, cytochrome $bo_3$, to identify the most likely subunit involved in substrate binding. Similar approaches have been used to identify the binding sites of the other *E. coli* oxidase, cytochrome *bd*, as well as some eukaryotic enzymes.

In Chapter III, "The Effects of Nitric Oxide on Electron Transport Complexes," a general approach to investigate how nitric oxide inhibits electron transport complexes was taken by monitoring activity loss due to nitric oxide treatment, and changes in EPR spectrum. Because the EPR spectra of the bovine complexes II and III are well characterized, beef heart mitochondria was the major source of enzyme for the study. However, we were also interested in comparing the effects of a eukaryotic system with those of a prokaryotic system, so complex II was also purified from *E. coli* and the effects of nitric oxide were described for this enzyme as well.

In order to describe the significance of these studies, and to help understand why they were initiated, a brief review of the mammalian complexes of electron transport (I-IV) and their prokaryotic equivalents is provided. Complex V is the ATP synthase and is not directly involved in electron transport. Its function is to couple electron transport to ATP synthesis via the proton motive force generated by the previous four complexes.

Mitochondrial complex I (NADH-ubiquinone oxidoreductase), consisting of 41 or more polypeptides (1), is the most intricate complex, and probably the least understood. An intrinsic protein of the mitochondrial inner membrane, it serves as the entry point of NADH into the electron transport chain by catalyzing the reversible oxidation of NADH to $NAD^+$ coupled to the reduction of ubiquinone to ubiquinol. This reaction results in the translocation of 4 protons across the inner membrane per electron pair transferred from NADH to Q (2-4). The genetic origin of the subunits of complex I are both mitochondrial and nuclear (5-7). In respiratory bacteria, complex I contains a minimal amount of subunits. These include all subunits coded for by mitochondrial genes and homologues of subunits with prosthetic groups or substrate binding sites (5-7).

Complex II (succinate-Q reductase) not only functions as an enzyme of electron transport but is also involved in the tricarboxylic acid cycle, where it is responsible for the oxidation of succinate to fumarate. Complex II from mitochondria (8) contains both a water soluble succinate dehydrogenase and a membrane anchoring protein which aids in its

function. The first component, succinate dehydrogenase, is composed of two protein subunits. Fp is the larger of the two and contains a covalently linked FAD. The smaller one (designated Ip, or iron containing protein) contains three iron-sulfur clusters. The second component of complex II is the membrane anchoring protein fraction. Depending on the source of the complex, 2-3 subunits have been isolated. These proteins are generally referred to as QPs, cytochrome $b_{560}$, or hydrophobic protein fractions (9).

Complex II has been isolated from numerous eukaryotic and prokaryotic sources, and for the most part appears to be highly conserved (10, 11). This is especially true of both the Fp and Ip amino acid sequences. For example, in comparing Ip sequences from *Escherichia coli* and beef heart mitochondria, approximately 50% of the sequences are identical (12). However, in *Bacillus subtilis*, the function of Fp and Ip is carried out by a single transmembrane protein (13-15).

Complex III, also known as the $bc_1$ complex, is a member of a larger superfamily of enzymes found in mitochondria, some Gram-positive bacteria, and chloroplasts (where they are know as $b_6f$ complex) (16-21). Complex III is an integral membrane protein which spans either the mitochondrial inner membrane, bacterial plasma membrane, or the chloroplast thylakoid membrane. It catalyzes the two-electron oxidation of ubiquinol (or plastoquinol) with the concurrent one-electron reduction of cytochrome *c* (or plastoquinol or $c_2$). This reaction is coupled to generation of a proton gradient and membrane potential across the membrane for ATP synthesis. Three redox centers, a Rieske high potential

[2Fe-2S] cluster (22) and cytochromes *b* and $c_1$, are essential for activity (23), but no additional redox prosthetic groups are known to exist in any of the $bc_1$ complexes. Mitochondrial $bc_1$ contains several supernumerary subunits which lack prosthetic groups (24, 25).

Complex IV is known to catalyze reduction of $O_2$ to water and couples this reaction to proton-pumping. Four protons are translocated by cytochrome oxidase across the membrane per four electrons transferred from cytochrome *c* to $O_2$ in intact mitochondria (matrix side to cytoplasmic side) (26, 27) and in artificial phospholipid vesicles (28). Beef heart Complex IV is composed of 13 subunits (29) and all have been sequenced. Mitochondrial genes code for the three largest subunits (subunits I-III), but the rest are coded for by nuclear genes (30). Crystals of the complex have been made, and a three dimensional structure of the entire 13 subunit complex has been elucidated with a 2.8 Å resolution (31).

Complex IV is a member of the heme-copper oxidase superfamily (32-34) which includes the mitochondrial cytochrome *c* oxidases as well as most of the prokaryotic respiratory oxidases. This superfamily divides into two major branches: the cytochrome *c* oxidases and the quinol oxidases. The three mitochondrial encoded subunits of the eukaryotic oxidases are found in most of the bacterial oxidases. However, all members of the oxidase superfamily contain a subunit homologous to subunit I, the largest subunit of the mammalian cytochrome *c* oxidase. This subunit contains two hemes and one copper

($Cu_B$) prosthetic group. One of the hemes is six-coordinate, and the second is a component of the heme-copper binuclear center, which is the site where dioxygen binds and is reduced to water. Based on studies of mitochondrial complex IV and oxidases from *Rhodobacter sphaeroides* and *Paracoccus denitrificans* only subunits I and II are required for proton pumping coupled to electron transfer (35, 36).

The investigation of our first project involving cytochrome $bo_3$ from *E. coli* began in examining the structural relationship between this enzyme and beef heart cytochrome oxidase. It appears that the major structural differences of subunits I, II, and III of cytochrome $bo_3$ compared to cytochrome oxidase reside in subunit II. For example, subunit II of the cytochrome *c* oxidases, contains the immediate electron acceptor from cytochrome *c*, the $Cu_A$ redox center. Furthermore, the cytochrome *c* binding site appears to be on subunit II of these oxidases. In the quinol oxidases, $Cu_A$ is not present, and the amino acid residues implicated in either the binding of cytochrome *c* or ligating to $Cu_A$ are not conserved. Subunit II in *E. coli*, therefore, must have a function different than binding and reduction of cytochrome *c*. Since cytochrome $bo_3$ was a quinol oxidase (not cytochrome *c* oxidase), it seemed plausible that if subunit II had an analogous function in *E. coli* to the mitochondrial enzyme, subunit II would be the site of quinol binding. This in part warranted the investigation on our part to examine the quinol binding site of cytochrome $bo_3$ from *E. coli*. We therefore made determining the quinol binding site the major objective of the first part of this work. From this study, it appears that ubiquinol-8 specifically interacts with subunit II of cytochrome $bo_3$ (37).

As stated previously, inhibitors of electron transport can be important tools in investigating the mechanism of electron transport. This has been particularly true in the elucidation of the widely accepted Q cycle of complex III. The Q cycle is a complicated mechanism which describes the nonlinear transfer of electrons from ubiquinol to cytochrome *c* through the $bc_1$ complex (38-41). Briefly, the Q cycle can be broken down into two tightly coupled steps. In the first half, a sequence of electron transfer events transfers one electron from ubiquinol to cytochrome *c* and two protons are released to the outer side of the membrane. This generates one ubisemiquinone and one reduced cytochrome *c*. The ubisemiquinone generated is on the outer side of the membrane. It passes its electron to cytochromes $b_L$ and $b_H$ which then reduces a different ubiquinone on the inner side of the membrane to a ubisemiquinone. In the second half of the cycle, the same initial events reduce another cytochrome *c* and produce another ubisemiquinone on the outer side of the membrane. This time, however, the electrons from cytochrome $b_H$ along with two protons from the inner side of the membrane reduce a ubisemiquinone from the inner side of the membrane (previously reduced by the first half of the cycle) to ubiquinol. In this way, two cytochrome *c* molecules are reduced per one ubiquinol oxidized to ubiquinone while four protons are deposited on the outer side of the membrane and two protons consumed on the inner side of the membrane.

Antimycin A inhibits the Q cycle at the step where cytochrome *b* reduces ubiquinol and causes electrons to accumulate in the *b* cytochromes and in ubiquinol (42). This

specific inhibitor has been instrumental in the elucidation of the Q cycle. The oxidant-induced reduction of cytochrome *b* in slow respiring mitochondria is increased by addition of antimycin A (for a review see 43). However, the oxygen burst also causes increased oxidation of cytochromes *c* and $c_1$. These observations which cannot be explained by a linear electron transport pathway are consistent with the Q cycle.

Recently nitric oxide has been shown to be an inhibitor of electron transport. Specifically, three mitochondrial enzymes have been shown to be inhibited by macrophage derived nitric oxide (44-46). They are mitochondrial aconitase, complex I, and complex II. Although the interaction appears to be less specific than that of other inhibitors such as Antimycin A, a precise mechanism of inhibition may be operant. Presumably, nitric oxide reacts with the iron sulfur clusters of the mitochondrial complexes. Each of the enzymes or complexes mentioned above are known to contain iron sulfur clusters of the [4Fe - 4S] type.

Moreover, unlike Antimycin A, and most other inhibitors of electron transport, nitric oxide occurs naturally in the cell. In mammalian cells, nitric oxide is synthesized from L-arginine by an endogenous biosynthetic pathway (47-51) catalyzed by nitric oxide synthase. Several types of cells contain nitric oxide synthase, including endothelial cells (52, 53), macrophages (54, 55), neutrophils (56), and cerebellar neurons (57). It is in the macrophages and neutrophils that the action of nitric oxide is cytotoxic. These cells release nitric oxide on surrounding cells targeted for destruction by the immune response.

Cell death is thought to occur by the inhibition of electron transport. However, the extreme reactivity of nitric oxide makes the precise mode of action difficult to investigate as nitric oxide also reacts with numerous other cellular components as well. But these effects are not clearly defined *in vivo*.

If the interaction of nitric oxide with either aconitase, complex I, or complex II could be more completely understood, nitric oxide could become important in their further characterization. A specific inconsistency concerning the effects of nitric oxide on these enzymes that requires attention has to due with nitric oxide's effect on the iron sulfur clusters of these enzymes. If nitric oxide was specific for iron sulfur clusters, why were the effects of nitric oxide on complex III not significant? As described above, complex III contains an iron sulfur cluster of the Rieske type which is essential to activity of the complex. However, when the effects of mitochondrial enzymes were monitored after treatment with macrophage induced nitric oxide, only aconitase and complexes I and II were markedly inhibited. This observation brought about the question of how nitric oxide reacted with the iron sulfur clusters. Do different types of clusters react to nitric oxide differently? And are other iron sulfur clusters nitric oxide resistant?

Since inhibitors have been so effective in elucidating complicated mechanisms of electron transport, especially in complex III, investigation of the effects of nitric oxide on this complex and others appeared to be merited. Of specific interest was comparing the effects of nitric oxide on complex II to any effects on complex III. Furthermore, if nitric

oxide effected different iron sulfur clusters in different manners, or if the effects of nitric oxide were specific for only certain types of clusters, enzymes from different sources but with the same types of clusters should be effected by nitric oxide in similar manners. These questions formed the basis for the objectives set forth in the second half of this work. Therefore, to test these hypotheses, the effects of nitric oxide on complexes I and II from beef heart mitochondria and complex II from *Escherichia coli* were monitored.

The results reported in this thesis show that complex III can be inhibited by nitric oxide, but only when purified as a single complex. When copurified with complex II, complex III is not effectively inhibited by nitric oxide. It is not certain that this selective inhibition is due to a difference in iron sulfur cluster arrangement. Perhaps a more likely conclusion is that complex II protects complex III form the effects of nitric oxide when the two are copurified as a super complex (58).

Our studies on electron transport complexes which began as broad questions about the basic designs of these eukaryotic and prokaryotic systems have now been focused to specific investigations about substrate binding and inhibitor interactions, which have been completed. In the case of *E. coli* cytochrome $bo_3$, the major objective was to identify the site of quinol binding. This was accomplished by determining a suitable substrate photoaffinity analog, establishing a clear correlation between the rates of photoinactivation and covalent attachment of the substrate to the enzyme, and showing that alternative substrates or competitive inhibitors reduce the rate of photoinactivation.

In studying the effects of nitric oxide on electron transport complexes, the primary objective was to define the effects of nitric oxide on SQR and QCR given their relationship in electron transport. This was accomplished by monitoring the effects of nitric oxide on SQR and QCR either as single enzyme complexes or copurified as SCR. The results of the effects of NO on mitochondrial SQR were compared with *E. coli* SQR. The results were determined by monitoring activity loss and changes in EPR spectra.

# CHAPTER II

# IDENTIFICATION OF THE UBIQUINOL-BINDING SITE IN THE CYTOCHROME *bo*$_3$-UBIQUINOL OXIDASE OF *ESCHERICHIA COLI*

## SUMMARY

The cytochrome *bo*$_3$-ubiquinol oxidase, one of two ubiquinol oxidases in *Escherichia coli*, is a member of the heme-copper oxidase superfamily. The enzyme contains four protein subunits (I-IV) with apparent molecular masses of 58, 33, 22, and 17 kDa, respectively. Cytochrome *bo*$_3$ catalyzes the 2-electron oxidation of ubiquinol and the reduction of molecular oxygen to water. Although the primary structures of all four subunits have been determined, the ubiquinol-binding site has not been investigated. The photoreactive radiolabeled azido-ubiquinone derivative 3-[$^3$H]azido-2-methy-5-methoxy-6-geranyl-1,4-benzoquinone (azido-Q), which has been widely used in locating the ubiquinone-binding sites of other enzymes, was used to identify the subunit(s) involved in the binding of quinol to cytochrome *bo*$_3$. When reduced by dithioerythritol, the azido-Q derivative functioned as a substrate with partial effectiveness, suggesting that azido-Q interacts with a legitimate quinol-binding site. When cytochrome *bo*$_3$ was

incubated with an 8-fold molar excess of azido-Q, illumination by UV light for 10 min resulted in a 50% loss of activity. The uptake of radiolabeled azido-Q by the oxidase complex upon illumination correlated with the photoinactivation. In the presence of the competitive inhibitor 2-heptyl-4-hydroxyquinoline (HQNO) or ubiquinol, the rate of azido-Q uptake and loss of enzyme activity upon illumination decreased. Analysis of the distribution of radioactivity among the subunits after separation by SDS-polyacrylamide gel electrophoresis showed that subunit II was heavily labeled by azido-Q, but that the other subunits were not. This suggests that the ubiquinol-binding site of the cytochrome $bo_3$ complex is located at least partially on subunit II.

# INTRODUCTION

The cytochrome $bo_3$-ubiquinol oxidase is one of two terminal ubiquinol oxidases in the aerobic respiratory system of *Escherichia coli* (59, 60). This relatively simple respiratory chain branches at the level of ubiquinol, which can be oxidized by either the cytochrome $bo_3$ or cytochrome *bd* oxidase (59). While the cytochrome $bo_3$-ubiquinol oxidase has been shown to predominate under highly aerobic growth conditions, cytochrome *bd* predominates when oxygen is limited (61). Cytochrome $bo_3$ catalyzes the 2-electron oxidation of ubiquinol-8 within the cytoplasmic membrane and the 4-electron reduction of molecular oxygen to water (62, 63). In the cell, this reaction generates a transmembrane charge separation and is coupled to proton pumping, thus generating a proton-motive force (64). The formation of a proton electrochemical gradient has been demonstrated by reconstitution of the oxidase in artificial phospholipid vesicles (65-67).

SDS-polyacrylamide gel electrophoresis analysis of the purified oxidase reveals four or five subunits, depending on the purification protocol employed (68, 69). The four-subunit preparation of the enzyme, utilized in this work, contains subunits with apparent molecular masses of about 58, 33, 22, and 17 kDA for subunits I-IV respectively. All the of the subunits are encoded by the cyoABCDE operon. The genes encode subunits II (cyoA), I (cyoB), III (cyoC), and IV (cyoD). The fifth gene, cyo E,

has been shown to be a farnesyl transferase, required for the biosynthesis of heme O prosthetic group in the oxidase.

Cytochrome $bo_3$ is a member of the heme-copper oxidase superfamily (32-34). This superfamily includes most prokaryotic respiratory oxidases as well as the mitochondrial cytochrome *c* oxidases. All members of this oxidase superfamily contain a subunit homologous to the largest subunit of the mammalian cytochrome *c* oxidase (subunit I). This subunit contains two hemes and one copper ($Cu_B$) prosthetic group. One of the hemes is six-coordinate, and the second is a component of the heme-copper binuclear center, which is the site where dioxygen binds and is reduced to water. In most cases, the bacterial oxidases contain homologues of the three mitochondrial encoded subunits of the eukaryotic oxidases (subunits I-III). For example, subunits I-III of cytochrome $bo_3$ from *E. coli* are homologous to the corresponding subunits of the mitochondrial cytochrome *c* oxidases. However, subunit IV of the *E. coli* oxidase appears to be unrelated to any eukaryotic protein.

There are two major branches of this superfamily: the cytochrome *c* oxidases and the quinol oxidases. The major structural differences appear to reside in subunit II. In the cytochrome *c* oxidases, for example, subunit II contains the $Cu_A$ redox center, which is the immediate electron acceptor from cytochrome *c*. Subunit II also appears to be the site where cytochrome *c* binds to these oxidases. In the quinol oxidases, $Cu_A$ is not present,

and the amino acid residues implicated in either the binding of cytochrome *c* or ligating to $Cu_A$ are not conserved.

Little information concerning the quinol-binding site is available. A labeled substrate analogue that could be covalently attached to the binding site was synthesized to locate the subunit(s) that presumably compose the quinol-binding site. This analogue is a photoreactive radiolabeled azido-ubiquinone derivative, specifically 3-[$^3$H]azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone (azido-Q) (70). The success of azido-Q in the identification of quinone-binding subunits has been demonstrated in cytochrome *bd* oxidase from *E. coli* and ubiquinol-cytochrome *c* reductases from various sources (70-72). A similar technique has also been successfully employed to identify the quinone-binding subunit in NADH-ubiquinone reductase (73, 74). The results reported herein indicate that the quinol-binding site of cytochrome $bo_3$-ubiquinol oxidase is on subunit II. When 2,3-dimethoxy-5-methyl-6-ethyl-1,4-benzoquinone ($Q_0C_2$) was incubated along with azido-Q, the rates of activity loss and azido-Q uptake were decreased. The presence of 2-heptyl-4-hydroxyquinoline (HQNO), an inhibitor of cytochrome $bo_3$-ubiquinol oxidase, in the incubation system also resulted in a decrease in azido-Q uptake upon illumination. These results suggest that there is some competition for the binding site between the substrate analogues and azido-Q and between HQNO and azido-Q.

# EXPERIMENTAL PROCEDURES

Materials-Cytochrome *bo*$_3$-ubiquinol oxidase was prepared by a reported procedure (69) and stored in 50 mM potassium phosphate buffer, pH 8.1, with either Triton X-100 or Sarkosyl detergents. Neither of these detergents was suitable for azido-Q labeling experimentation. Since Triton X-100 and Sarkosyl interfered with the labeling study, these detergents were exchanged for 1% cholate by the following procedures. The enzyme was precipitated by 50% ammonium sulfate and then dissolved in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 1% sodium cholate and 10% glycerol. Alternately, the oxidase was treated with BIO-BEAD and then solubilized in buffer containing 1% sodium cholate. The cholate-solubilized quinol oxidase was stored at -20 °C until use.

The concentrations of the oxidase (69), ubiquinol-1, and azido-Q were determined spectrophotometrically. Quinone concentrations were measured in 95% ethanol at a $\lambda_{max}$ of 276 nm for ubiquinol-l and 291 nm for azido-Q using an extinction coefficient of 12.25 x $10^3$ $cm^{-1}$ $mol^{-1}$ liter for both.

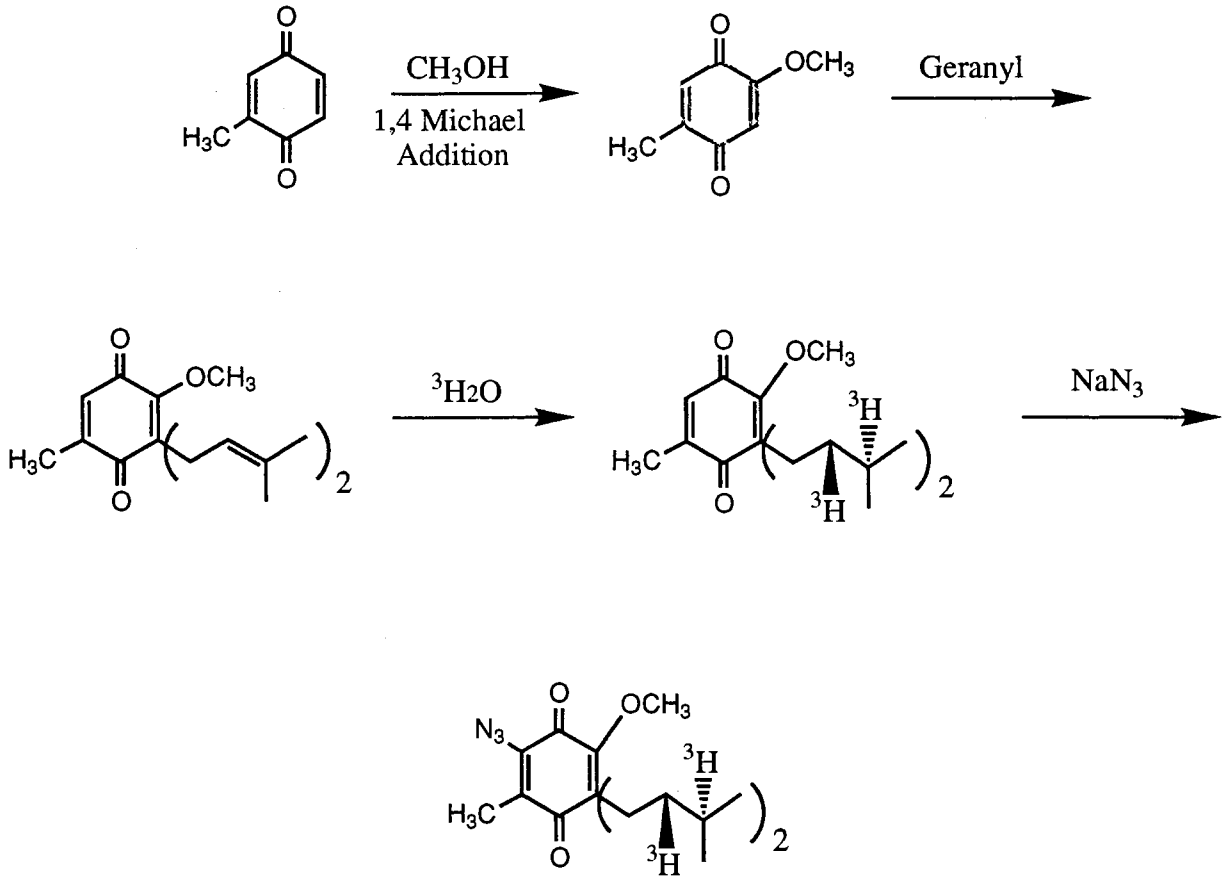
Azido-Q (10,000 cpm $^3$H/nmol), 2,3-dimethoxy-5-methyl-1,4-benzoquinone ($Q_0$), 2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone ($Q_1$), 2,3-dimethoxy-5-methyl-6-

geranyl-1,4-benzoquinone, 2,3-dimethoxy-5,6-dimethyl-1,4-benzoquinone ($Q_0C_1$), 2,3-dimethoxy-5-methyl-6-ethyl-1,4-benzoquinone ($Q_0C_2$), 2,3-dimethoxy-5-methyl-6-butyl-1,4-benzoquinone ($Q_0C_4$), 2,3-dimethoxy-5-methyl-6-pentyl-1,4-benzoquinone ($Q_0C_5$), 2,3-dimethoxy-5-methyl-6-heptyl-1,4-benzoquinone ($Q_0C_7$), and 2,3 dimethoxy-5-methyl-6-nonyl-1,4-benzoquinone ($Q_0C_9$) were synthesized by reported methods (75, 76). The synthesis of azido-Q is outlined in Scheme I-(a) and quinone structures are shown in Scheme I-(b). Dithioerythritol and cholic acid were purchased from Sigma; Tween 20 and electrophoresis reagents were purchased from Bio-Rad. All other chemicals and reagents were of the highest purity available.

Assay Conditions-Quinol oxidase activity was assayed spectrophotometrically and polarographically. Ten microliters of pure cytochrome *bo*$_3$-ubiquinol oxidase (0.5 mg/ml) in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 0.1% sodium cholate was used. The assay mixture for the spectrophotometric assay contained 50 mM potassium phosphate, pH 7.4, 0.025% Tween 20, and 25 μM substrate. Oxidation of ubiquinol was followed by an increase in absorbance at 280 nm with an extinction coefficient of 12.25 x $10^3$ $cm^{-1}$ $mol^{-1}$ liter (70).

In some experiments, activity was measured by following $O_2$ uptake at 25 °C using a Yellow Springs Model 53 oxygen monitor. For these measurements, the assay mixture contained 50 mM potassium phosphate buffer, pH 7.4, 0.06% Tween 20, and 2 mM dithioerythritol. Quinol concentrations ranging from 3 to 16.7 μM were used as

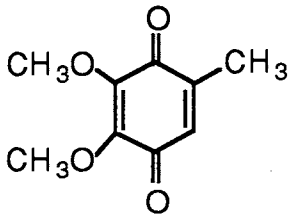
**Scheme I-(a): Synthesis of 3-[$^3$H]azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone (azido-Q)**



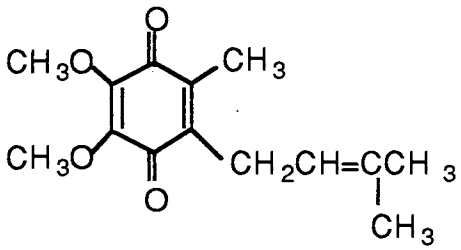

[$^3$H] 3-Azido-2-methyl-5-methoxy-6-geranyl-1,4-benzoquinone

**[$^3$H] 3-Azido Q**

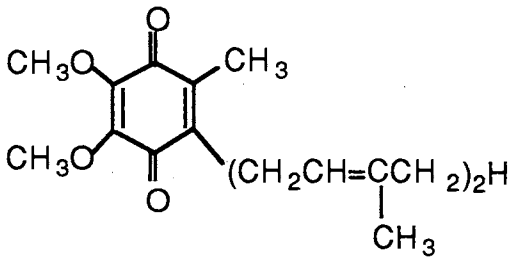
## Scheme I-(b): Important Quinone Structures



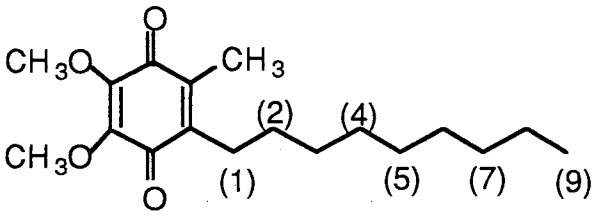
2,3-dimethoxy-5-methyl-
1,4-benzoquinone
($Q_0$)



2,3-dimethoxy-5-methyl-6-isoprenyl-
1,4-benzoquinone
($Q_1$)



2,3-dimethoxy-5-methyl-6-geranyl-
1,4-benzoquinone
($Q_2$)

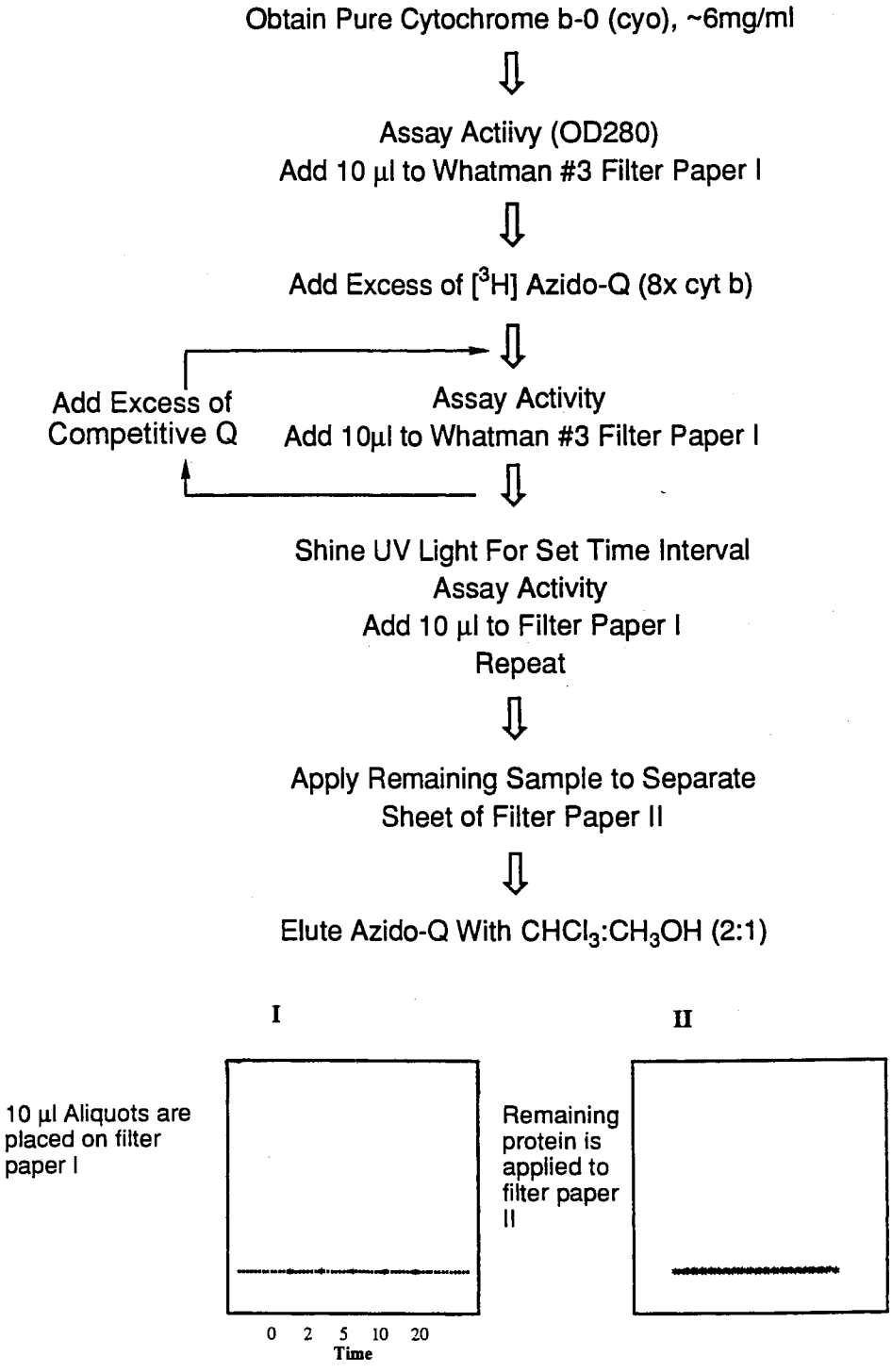


2,3-dimethoxy-5-methyl-6-R-
1,4-benzoquinone
($Q_0C_{1\text{-}9}$)

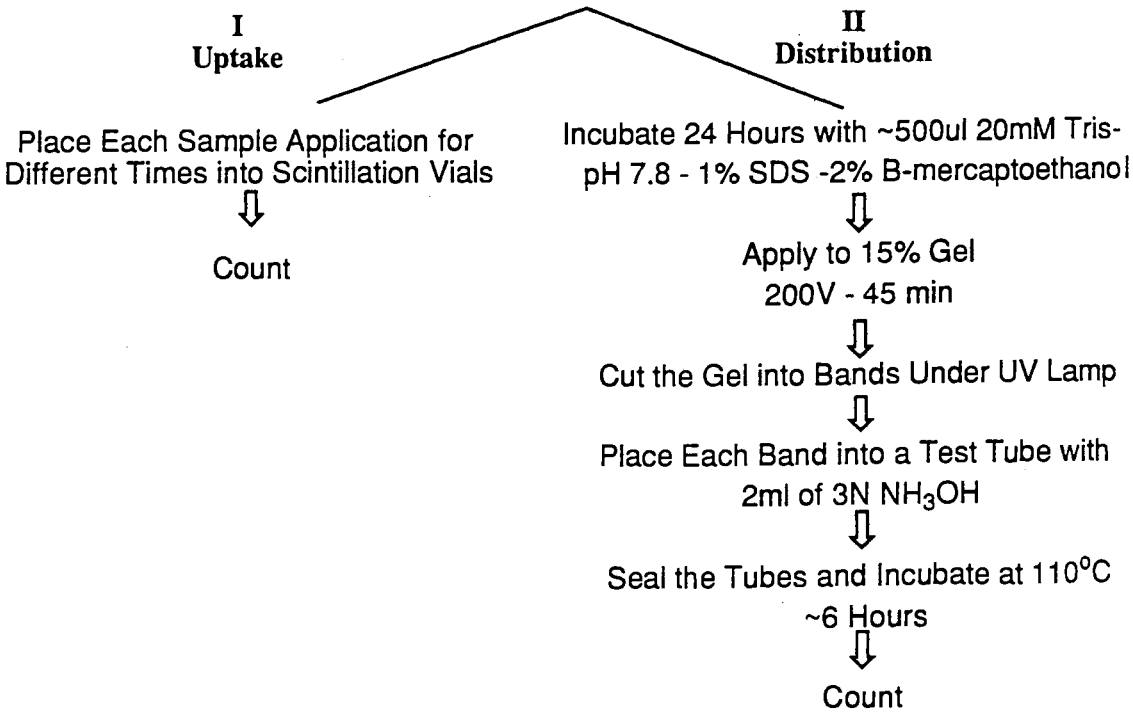
substrate.

Photoinactivation-Scheme II outlines the photoinactivation and subunit labeling procedure. The enzyme (40 μM in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 0.5% sodium cholate) was incubated with radiolabeled azido-Q (8-fold molar excess) and subjected to UV illumination (70). The stock solution of azido-Q was 5 mM in ethanol. In some experiments, an additional quinone derivative or inhibitor was included in the solution. The enzyme solution was placed into a 2-mm light path cuvette. This was incubated in ice water in a container with a quartz window. For the time course study of both inactivation and azido-Q uptake upon illumination, aliquots of enzyme sample were withdrawn from the illuminated cuvette at various time intervals. One portion was used to determine the enzyme activity, and the other was used to measure uptake of azido-Q by the protein. Ten microliters of the illuminated samples was withdrawn at various time intervals and spotted onto a piece of Whatman No. 3MM filter paper (15 x 15 cm) 0.5 inch from the bottom. The paper was kept in the dark. Once all the samples were spotted, the paper was then developed with a 2:1 chloroform/methanol solution and air-dried. The spots, which retained the protein at their original positions, were cut into small pieces. They were then placed into 7-ml scintillation vials with 6 ml of Packard Insta-Gel XF, and their radioactivity was determined. Radioactivity was measured in a Packard 1900CA liquid scintillation analyzer.

## Scheme II: Subunit Labeling Procedure Using Azido-Q



## Scheme II: Subunit Labeling Procedure Using Azido-Q (cont.)



Determination of the Distribution of $^{3}H$ Radioactivity Among the Subunits of the Enzyme Complex-After 10 min of UV illumination, 1-1.5 mg of protein was spotted onto filter paper, and unbound quinone was eluted as described above. The strip containing the protein was then cut into small pieces and incubated in 400 µl of 20 mM Tris-CI, pH 7.8, containing 1% SDS and l% 2-mercaptoethanol for ~40 hours. The subunits were separated by electrophoresis on a 15% polyacrylamide gel run at 200 V for 50 min. Before addition of the sample, glycerol was added to the digestion buffer to a final concentration of 12%, and fluorescamine was also added. After running, the gel (viewed under UV irradiation) was cut into strips, and the band pattern as seen by fluorescamine was recorded. For each experiment, one lane of standard and one lane of sample were reserved for staining with Coomassie Blue as previously described (77). The strips were placed into preweighed test tubes so that the weights of the gel strips could be determined. To each tube was added 2 ml of 3 N ammonium hydroxide, and the strips were broken into small pieces. The tubes were sealed and incubated at 110 °C for 6 hours. The contents of each tube were then placed into 20-ml scintillation vials with 18 ml of the mixture, and radioactivity was counted. Since the presence of gel causes a significant amount of quenching, a correction of this quenching in the counting system must be made. A given amount of radioactive azido-Q was mixed with a given amount of gel solution before polymerization and subjected to the same treatment as that of gel strips of the samples. The ratio of radioactivities determined in the presence of gel was used as a factor for the correction of the quenching.

# RESULTS

Effect of Varying the Quinone Side Chain on Cytochrome *bo*$_3$-Ubiquinol Oxidase Activity-The natural substrate of cytochrome *bo*$_3$-ubiquinol oxidase is ubiquinol-8. This quinone has a carbon side chain consisting of eight isoprenoid units or a total of 40 carbons. The low solubility of ubiquinol-8 in aqueous solution makes it less attractive to use as a substrate in routine assays. Lower quinol homologues are generally used in the determination of oxidase activity. Ubiquinol-1 ($Q_1H_2$), which contains only one isoprenoid unit, is the most commonly used substrate for cytochrome *bo*$_3$-ubiquinol oxidase. It is of interest to investigate the substrate specificity of other low homologues of synthetic ubiquinones. Fig. 1 summarizes the effects of different quinone derivatives on oxidase activity. The quinone derivatives examined contain unbranched saturated side chains that vary only in the length of their carbon side chains (denoted $Q_0C_n$). The activity of the oxidase was followed both spectrophotometrically and polarographically.

In polarographic assays, the quinones were maintained reduced by dithioerythritol, and oxygen uptake was measured directly with an oxygen electrode. Considering the ubiquinol-1 activity to be 100%, the activity with the other quinone derivatives is expressed as a percentage of ubiquinol-1 activity under the same experimental conditions (Fig. 1). $Q_0C_4$ is as equally effective a substrate as ubiquinol-1.

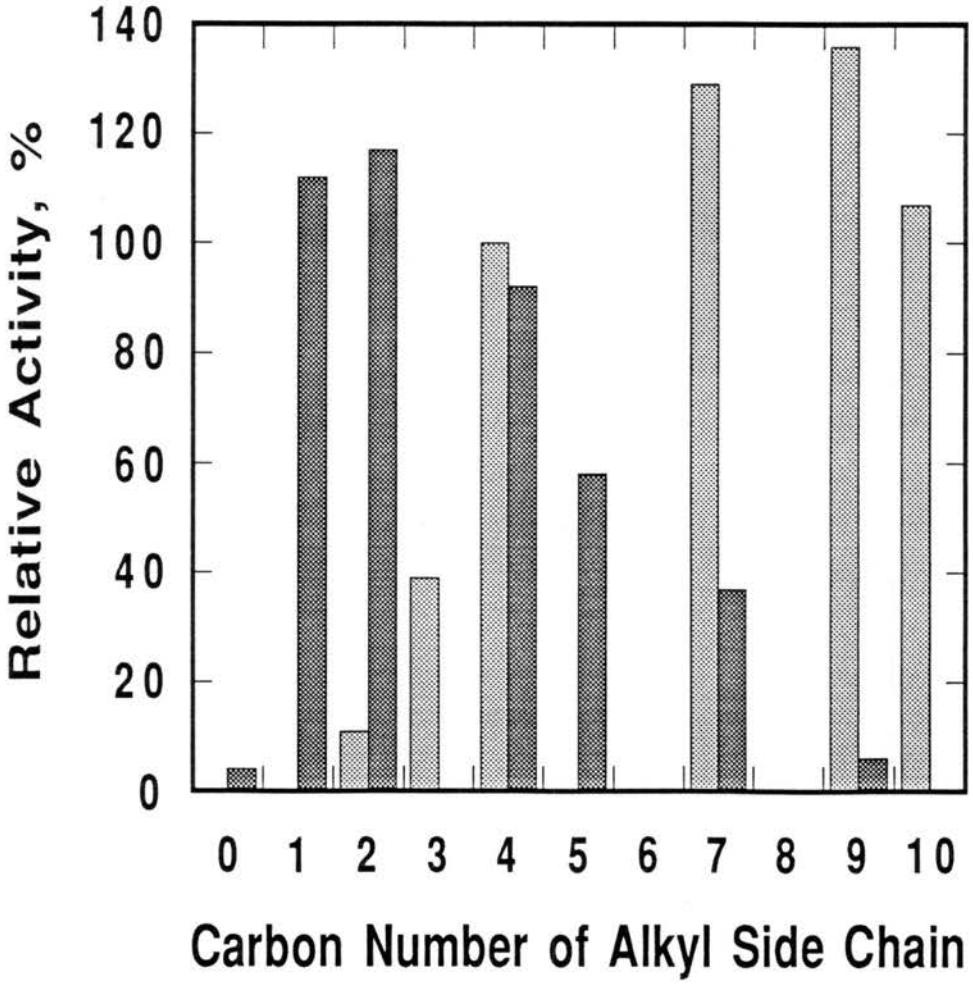



Fig. 1. Effectiveness of ubiquinone analogues as substrates of the cytochrome $bo_3$ complex. $Q_1H_2$ activity (29.1 μmol of $O_2$ $min^{-1}$ $mg^{-1}$) was taken to be 100%. The darker bars represent the oxygen uptake measurements, while the lighter bars show the spectrophotometric assay results. The graph shows an average of five values obtained for each substrate. The quinone derivatives used were $Q_0C_1$, $Q_0C_2$, $Q_0C_3$, $Q_0C_4$, $Q_0C_5$, $Q_0C_7$, $Q_0C_9$, and $Q_0C_{10}$.

$Q_0C_1$ and $Q_0C_2$ appear to be even better substrates than $Q_1H_2$, but $Q_0$ is inactive as a substrate for the cytochrome *bo*$_3$ complex.

The surprisingly low rate of activity observed polarographically with $Q_0C_9$ is due to the slow rereduction rate of $Q_0C_9$ by dithioerythritol under the assay conditions. This was experimentally verified by directly observing the rate of reduction spectrophotometrically. The slow rereduction rate of $Q_0C_9$ by dithioerythritol may be due to the more hydrophobic side chain of $Q_0C_9$, which could hinder its association with dithioerythritol. This was partially overcome by increasing the detergent concentration in the assay, resulting in an increase in oxidase activity specifically with $Q_0C_9$, but not with the other analogues, which are less hydrophobic and more water-soluble. In contrast, using the spectrophotometric assay, analogues with a side chain longer than that of ubiquinol-l were shown to have activity comparable to that of ubiquinol-1. In this assay, all the substrates were reduced prior to the start of the assay.

Oxidase Activity of Cytochrome *bo*$_3$ Using Reduced Azido-Q as a Substrate-The azido-Q synthesized and used in this experiment has a 10-carbon side chain. To determine the feasibility of utilizing this azido-Q derivative as a probe of the quinol-binding site, the substrate activity of azido-Q was compared with that of ubiquinol-l. Using the polarographic assay, the specific activity using azido-Q is 11% of that observed with ubiquinol-1, which is comparable to the results obtained with $Q_0C_9$. The specific activity of $Q_0C_9$ increases to 135% of that of ubiquinol-1 when the spectroscopic assay is

employed but the comparable experiment with azido-Q could not be performed due to the chemical reactivity of the azido group of azido-Q. It is reasonable to conclude, however, that azido-Q is as effective a substrate as $Q_0C_9$ and therefore a reasonable substrate analogue.

Correlation Between Inactivation of Oxidase Activity and Azido-Q Uptake Upon Illumination-As demonstrated in Fig. 2 the rate of ubiquinol-1 oxidation by the purified cytochrome *bo*$_3$ complex decreases rapidly during the first 10 min of illumination in the presence of azido-Q to 50% of the original activity. Beyond 10 min, very little inactivation is observed, similar to the behavior when the oxidase is subjected to UV irradiation in the absence of azido-Q (Fig. 2). The incomplete ( 50%) photoinactivation of the cytochrome *bo*$_3$ complex in the presence of azido-Q is comparable with results obtained previously with other membrane protein complexes using similar derivatives (70, 71). The incomplete inactivation may be due to the presence of some of the endogenous quinone substrate present in the enzyme preparations. Fig. 2 also shows the rate of incorporation of the azido-Q label into the protein as a function of the time of irradiation. The label is covalently attached to the protein over the first 10 min of irradiation and parallels the observed inactivation of the oxidase (Fig. 2). It is reasonable to conclude that the inactivation during the first 10 min of illumination is due mostly to specific covalent linkage of azido-Q at the substrate-binding site, while the incorporation

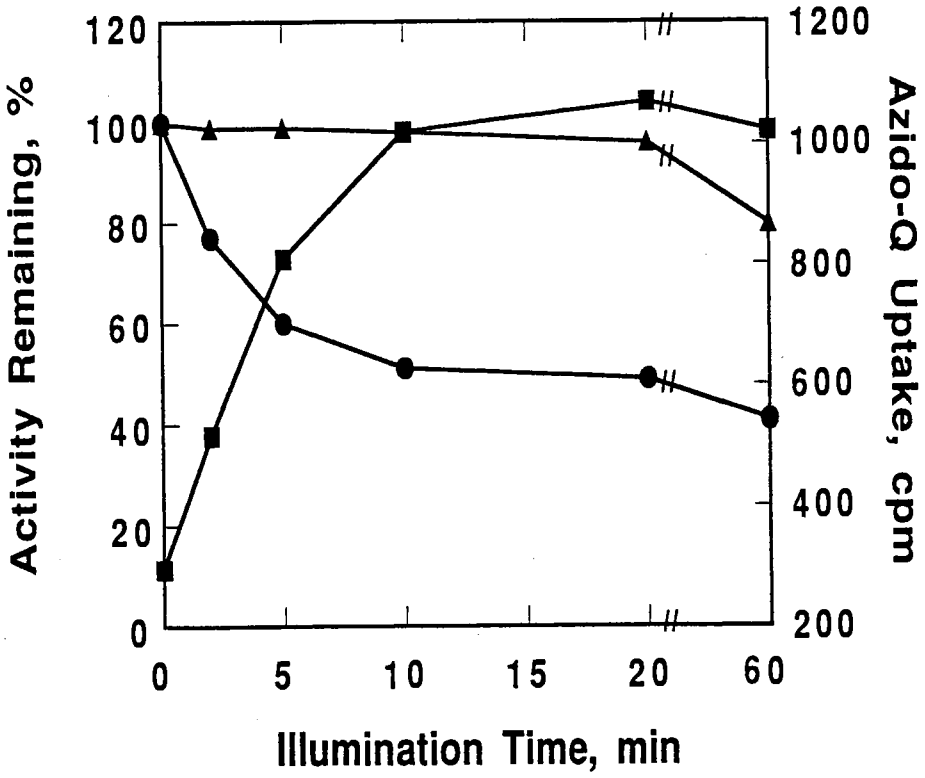



Fig. 2. Correlation of photoinactivation of and azido-Q uptake by pure cytochrome $bo_3$ oxidase treated with radiolabeled azido-Q. The enzyme (41 μM cytochrome *b* in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 0.5% sodium cholate) was incubated with 320 μM radiolabeled azido-Q (8-fold molar excess) and subjected to illumination at 0 °C. For the time course study of inactivation and azido-Q incorporation upon UV irradiation, a given amount of the sample was withdrawn from the illuminated cuvette at various time intervals. One portion was used for the activity assay, and the other portion was used for determining the uptake. Activity was assayed spectrophotometrically in 50 mM potassium phosphate buffer pH 7.4 containing 0.025% Tween 20. Oxidation of $Q_1H_2$ was followed at 280 nm. ●, activity remaining after photoinactivation; ■, uptake of azido-Q; ▲, control that contained no azido-Q.

of azido-Q after that is due to nonspecific interaction.

Distribution of Radioactivity Among the Subunits of Cytochrome *bo*$_3$-Fig. 3 shows an SDS-polyacrylamide gel electrophoresis analysis of the oxidase after the protein had been subjected to the treatment designed to remove noncovalently bound azido-Q. Subunits I-III are apparent, and their relative intensities are comparable to those observed prior to irradiation. Subunit IV is difficult to detect, as is often the case. Also apparent is a small amount of a high molecular mass aggregate that is not due to the irradiation or labeling procedure, but is present in the gel pattern of the protein prior to treatment. Fig. 3 demonstrates that the subunits are recovered after irradiation and the organic extraction treatment. Hence, the possibility that the more hydrophobic subunits might aggregate and not be recovered for this analysis can be excluded.

Fig. 4 shows the distribution of radioactivity among the subunits of the cytochrome complex after 10 min of illumination. After photoinactivation of the enzyme, unbound azido-Q was removed, and the remaining sample was used for SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedures." After viewing the band pattern as labeled by fluorescamine, the gel was cut into slices, which were used to evaluate the distribution of [$^3$H]azido-Q by scintillation counting. The results are shown in Fig. 4 and clearly indicate that subunit II is preferentially labeled with azido-Q. Some azido-Q is also covalently linked to subunit III, but very little label is observed on subunit I. Although a role for subunit III in quinone binding certainly

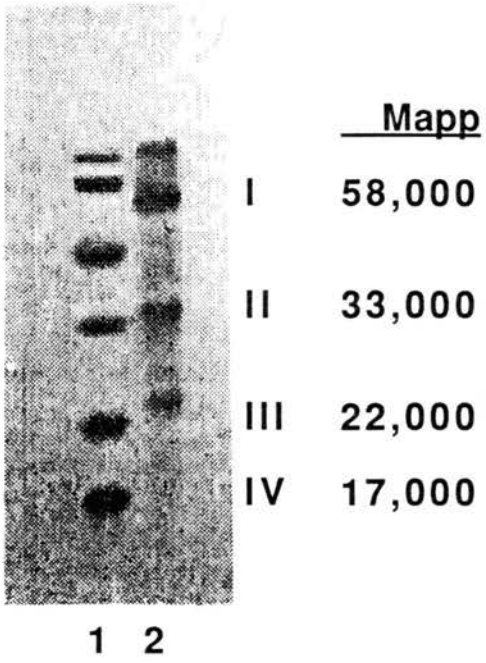



Fig. 3. SDS-polyacrylamide gel electrophoresis pattern of cytochrome $bo_3$-ubiquinol oxidase. After elution of unbound quinone, the sample was incubated in 20 mM Tris-Cl, pH 7.8, containing 1% SDS, 1% 2-mercaptoethanol, and 12% glycerol and then applied to a 15% polyacrylamide gel run at 200V for 50 min. Ten to fifteen micrograms of protein solution was loaded onto the sample lane. Lane 1, low molecular mass standards (in daltons); lane 2, cytochrome $bo_3$-ubiquinol oxidase.

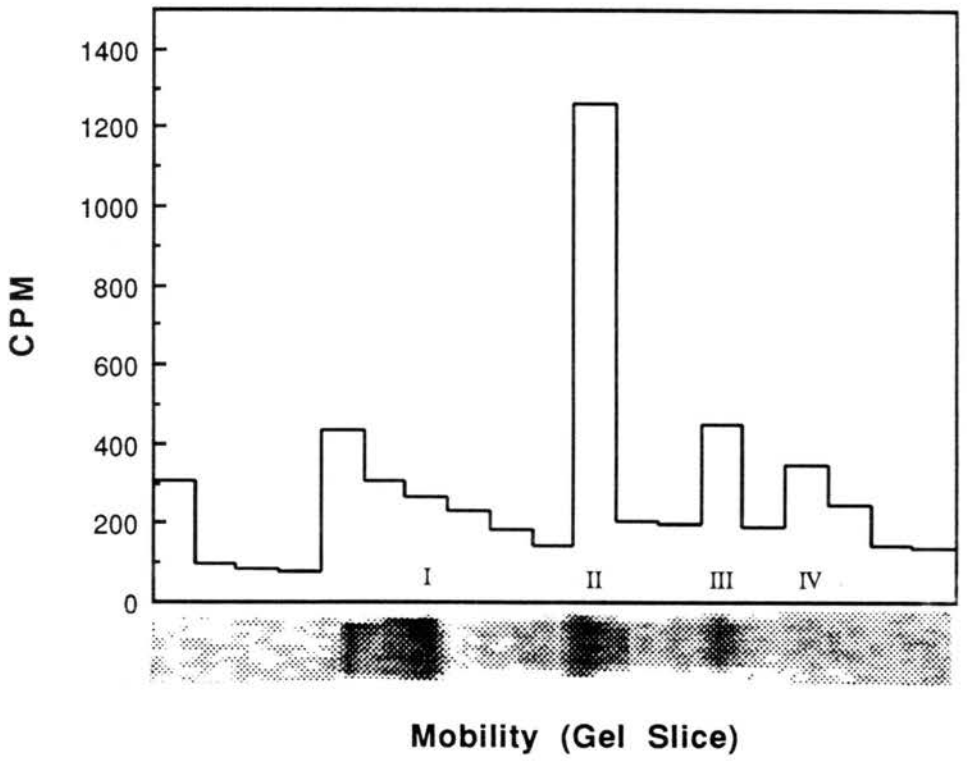



Fig. 4. Distribution of radioactivity among the subunits of the cytochrome $bo_3$ complex. Unbound azido-Q was removed from the treated cytochrome complex after 10 min of illumination. The remaining protein was then digested and subjected to SDS-polyacrylamide gel electrophoresis analysis. After electrophoresis, the gel pattern was recorded. A portion of the gel was saved for staining, while the rest of the gel was cut into slices for determination of radioactive incorporation. The positions of the four subunits of the cytochrome complex are indicated.

cannot be excluded, it is worth noting that the high hydrophobicity of subunit III favors nonspecific interactions. The relative hydrophobicities of the first three subunits calculated from their amino acid compositions are 54, 46, and 66% for subunits I-III, respectively. (Relative hydrophobicity was calculated by considering Pro, Ala, Val Met, Ile, Leu, and Phe as hydrophobic amino acids).

A quantitative analysis indicates that 33% of the counts applied to the SDS gel are recovered. This loss is presumably due to the quenching effects of the gel. Of these counts, about half are associated with the polypeptides (shown in Fig. 4), whereas the remainder are residual label bound to phospholipids or in noncovalently bound form. The amount of azido-Q bound to total protein after electrophoresis was calculated to be 0.30 mol of quinone/mol of oxidase.

Competitive Effects of HQNO and $Q_0C_2$-To further strengthen the argument that azido-Q labeled an authentic quinol binding site on the enzyme, the effects of both HQNO and $Q_0C_2$ were investigated. HQNO is an effective inhibitor of the quinol oxidase and is thought to be a competitive inhibitor of quinol. $Q_0C_2$, as shown in Fig. 1, is an effective substrate of the oxidase. Fig. 5 shows the effects of varying concentrations of HQNO on the ubiquinol-1 oxidase activity of cytochrome $bo_3$. Under the conditions of the assay, the maximal inhibition is 82%, and no additional effect is observed beyond ~25 μM. Fig. 6 shows the effect of the presence of HQNO and $Q_0C_2$ on the rate of photoinactivation of the oxidase by azido-Q. Both compounds cause a reduced rate of inactivation of the oxidase. As observed in Fig. 6, the inactivation due to HQNO and that due to azido-Q

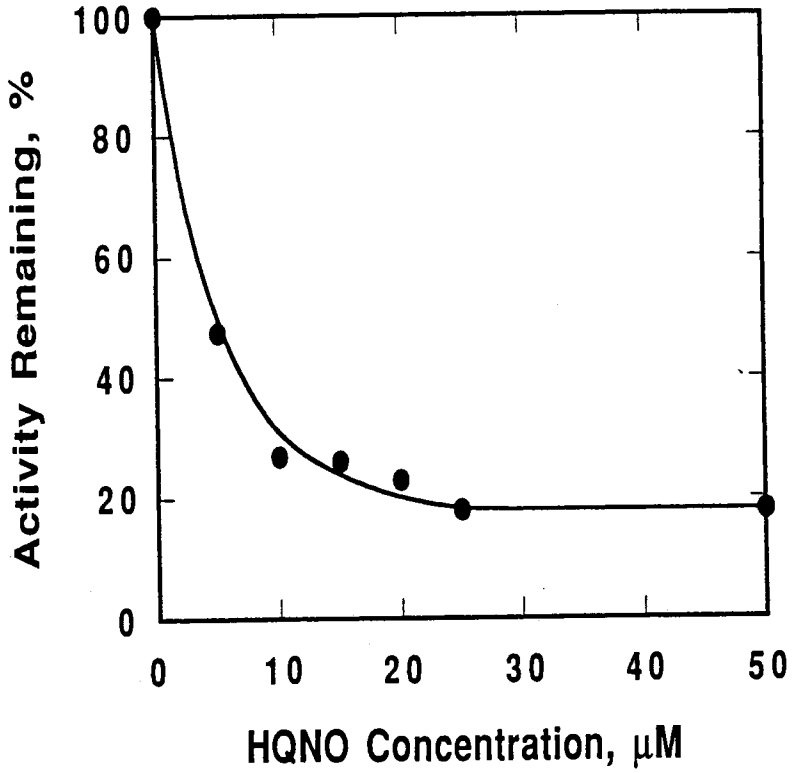



Fig 5. Inhibition of the cytochrome *bo*$_3$ complex by HQNO. Pure cytochrome *bo*$_3$-ubiquinol oxidase (0.6 mg/ml in 50 mM $K_2HPO_4$ containing 0.1% Triton X-100, pH 8.1) was used for assay in 5-μl amounts. The assay mixture was 1 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 0.025% Tween 20, and 50 nmol of ubiquinol-l was used as substrate. Oxidation of $Q_1H_2$ was followed by absorption at 280 nm. HQNO was 10 mM in ethanol, and the amount in the assay mixture was varied from 5 to 50 nmol of total HQNO present.

appear to be additive. The extent of inactivation at 20 min is clearly not affected by the presence of $Q_0C_2$. This is probably due to the fact that the binding of $Q_0C_2$ is reversible, whereas the photoinactivation is not reversible. On the other hand, HQNO does affect the extent of inactivation at all time observed. In these experiments, the amount of HQNO used was adjusted to that sufficient to result in 50% inhibition in the presence of azido-Q. As noted in Fig. 6, inhibition due to HQNO in the presence of azido-Q without illumination is also observed, suggesting that HQNO binding to the oxidase is stronger than that of azido-Q. The inhibitory effect is thus not eliminated by either azido-Q or by other substrates under the given conditions. Fig. 7 shows the effects of both HQNO and $Q_0C_2$ on the incorporation of azido-Q into the oxidase. These data mimic those in Fig. 6 in that both compounds slow the rate of incorporation into the oxidase. The presence of HQNO significantly decreases the noncovalent uptake of azido-Q by oxidase. Since inactivation by azido-Q is irreversible, whereas HQNO is a reversible inhibitor, complete blocking of the incorporation of azido-Q into the oxidase by HQNO is not expected.

These data are consistent with the conclusion that azido-Q is binding to a specific quinol-binding site in cytochrome $bo_3$ and that the observed results are not due to nonspecific binding to the oxidase. As expected, both the competitive inhibitor, HQNO, and the alternative substrate, $Q_0C_2$, reduce the rate of photoinactivation by azido-Q. If azido-Q showed no preference for the binding site, one would expect the effects of HQNO or $Q_0C_2$ to be minimized. However, since results reported in this work show HQNO as a competitive inhibitor and $Q_0C_2$ a suitable substrate, they must both interact with the quinol

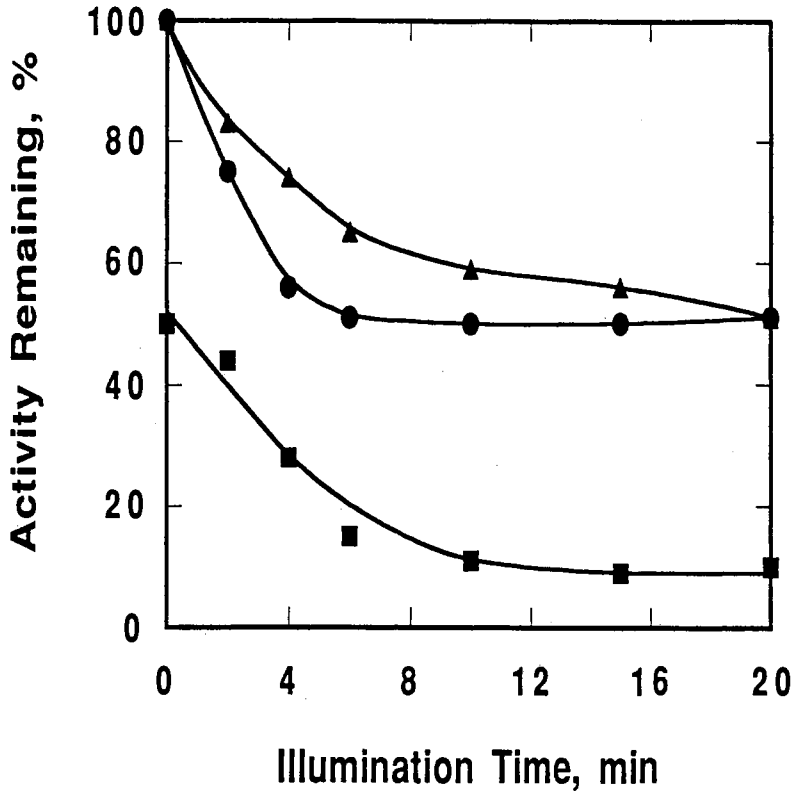



Fig. 6. Effect of HQNO and $Q_0C_2$ on the photoinactivation of cytochrome *bo*$_3$-ubiquinol oxidase. Cytochrome *bo*$_3$-ubiquinol oxidase (40 μM cytochrome *b* in 50 mM sodium/potassium phosphate buffer, pH 7.8, containing 0.5% sodium cholate) was subjected to UV illumination at 0 °C as described under "Experimental Procedures." The sample was incubated with azido-Q (●), azido-Q and $Q_0C_2$ (▲), or azido-Q and HQNO (■). All substrates were added to a final concentration of 320 μM total quinone present.

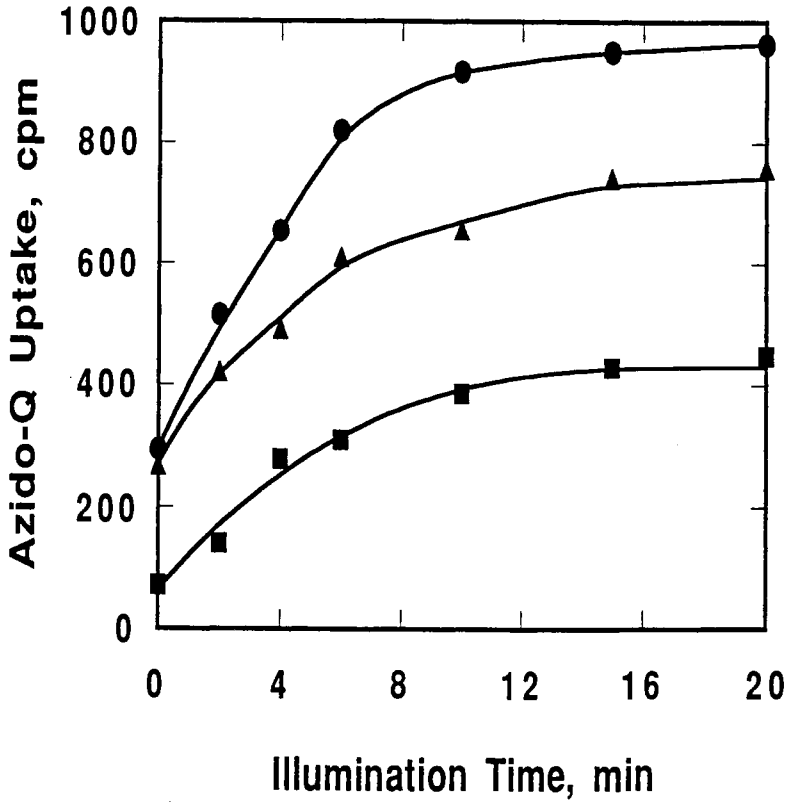



Fig 7. Effect of HQNO and $Q_0C_2$ on azido-Q uptake by the cytochrome complex. The same sample as in Fig. 6 was treated in the same manner as described, except that radioactivity was measured. Plotted is the sample when incubated with azido-Q (●), azido-Q and $Q_0C_2$ (▲), or azido-Q and HQNO (■). Again, all substrates were added to a final concentration of 320 μM total quinone present

binding site. They therefore must interfere with azido-Q binding by preventing its ability to enter the binding site as well. The result is a decrease in the rate of photoinactivation by azido-Q.

# DISCUSSION

The results of the experiments reported in this work clearly point to subunit II as being intimately involved in the quinol-binding site of the cytochrome $bo_3$-ubiquinol oxidase from *E. coli*. This conclusion is supported by 1) the fact that the photoreactive label is itself a substrate for the enzyme; 2) the correlation between the rates of photoinactivation and of covalent attachment to the oxidase; and 3) the influence of an alternative substrate ($Q_0C_2$) and a competitive inhibitor (HQNO) on reducing the rate of photoinactivation of the oxidase by azido-Q.

Furthermore, the selective labeling of subunit II rules out nonspecific hydrophobic interactions as the major contributor to the labeling pattern since subunit II is the least hydrophobic of the subunits. This subunit appears to contain two transmembrane helical spans and a large hydrophilic carboxyl-terminal domain (78). It is the hydrophilic domain in the homologous subunit of the cytochrome *c* oxidases that contains the cytochrome *c*-binding site (34) and that contains the $Cu_A$ redox center (79). Hence, the results presented in this report indicate that the function of subunit II in both the cytochrome *c* oxidases and the quinol oxidases may be similar. In both cases, the subunit appears to be directly involved in binding to the substrate to be oxidized, either cytochrome *c* or ubiquinol.

It must be pointed out that these data by no means exclude other subunits from being directly involved in forming the quinol-binding site. Furthermore, the recent suggestion (80) that there could be two different quinol-binding sites within cytochrome $bo_3$ needs to be investigated, and the use of the azido-Q label might prove useful to define such sites, if this proves to be the case. Clearly, the identification of the specific site labeled within subunit II under the present conditions will be the first priority, and such experiments are currently in progress.

# CHAPTER III

## THE EFFECTS OF NITRIC OXIDE ON ELECTRON TRANSPORT COMPLEXES

## SUMMARY

The effect of nitric oxide on mitochondrial electron transfer complexes was studied by comparing the activities of nitric oxide treated and untreated, deoxygenated samples of purified beef heart succinate-cytochrome *c* reductase, succinate-ubiquinone reductase, and ubiquinol-cytochrome *c* reductase. More than 90% of succinate-cytochrome *c* reductase activity is lost during nitric oxide treatment. The activity of the succinate-ubiquinone reductase component of succinate-cytochrome *c* reductase decreases 95%, while the ubiquinol-cytochrome *c* reductase component is unaffected by nitric oxide. This inactivation is due primarily to the destruction of iron sulfur clusters from succinate-ubiquinone reductase. When purified beef heart succinate-ubiquinone reductase was treated with nitric oxide, virtually all activity was irreversibly lost. The EPR spectra of the treated complex showed typical iron-nitric oxide complex signals, confirming that inactivation is due to destruction of the iron-sulfur clusters. Similar results were obtained with purified *E. coli* succinate-ubiquinone reductase. Pure beef heart ubiquinol-cytochrome *c* reductase treated with nitric oxide loses 40% of its initial

activity, but regains most of it (90-100%) after 24 hours of incubation at 0 °C in the absence of nitric oxide. This suggests that ubiquinol-cytochrome *c* reductase is protected from nitric oxide when complexed with succinate-ubquinone reductase or that when split from succinate-ubiquinone reductase, ubiquinol-cytochrome *c* reductase undergoes a conformational change which allows access of nitric oxide to the Rieske iron-sulfur center. Such access is not possible when ubiquinol-cytochrome *c* reductase is complexed with succinate-ubiquinone reductase. The loss of ubiquinol-cytochrome *c* reductase activity correlates with a decrease in the Rieske protein EPR signal intensity without formation of any new EPR signal. The Rieske iron-sulfur cluster signal is recovered after 24 hours incubation in the absence of nitric oxide.

# INTRODUCTION

Nitric Oxide (NO) is a small diatomic free radical, and a gas at room temperature. It is slightly soluble in water (2mM at 1 atm, 20 °C) and highly reactive. It reacts with molecular oxygen to yield nitrogen dioxide, a brown gas (81-83). NO also reacts with the superoxide anion $O_2^-$ to form peroxonitrite ($ONOO^-$) (83). Other properties of NO include a remarkable ability to coordinate transition metal ions such as iron ($Fe^{2+}$ and $Fe^{3+}$), manganese, and copper. Many of these complexes have been well characterized, and several, including complexes formed when NO coordinates iron sulfur centers, can be detected by EPR spectroscopy (82).

In mammalian cells, NO is synthesized from L-arginine by an endogenous biosynthetic pathway (47-51) catalyzed by NO synthase. Biosynthesis of NO occurs in many types of cells including endothelial cells (52, 53), macrophages (54, 55), neutrophils (56), and cerebellar neurons (57). The high reactivity of NO accounts for its involvement in diverse physiological functions. Three mitochondrial enzymes have been shown to be inhibited by macrophage derived NO (44-46). They are mitochondrial aconitase, NADH-ubiquinone (Q) reductase (complex I) and succinate ubiquinone reductase (complex II or SQR). Each of these enzymes or complexes are known to contain iron sulfur clusters of the [4Fe - 4S] type. Complexes I and II in murine tumor cells are inhibited by direct

exposure of the cells to NO gas (53, 84). This inhibition appears to result from NO interaction with the iron sulfur clusters of these enzymes.

Succinate-Q reductase (8) has two major components: a water soluble succinate dehydrogenase and a membrane anchoring protein. Succinate dehydrogenase is composed of two protein subunits. The larger one (Fp) houses a covalently linked FAD and the smaller one (Ip) binds three iron-sulfur clusters. The membrane anchoring proteins have 2-3 subunits depending on the source of the complex. These proteins are generally referred to as QPs, cytochrome $b_{560}$, or hydrophobic protein fractions (9).

Ubiquinol-cytochrome *c* reductase (complex III or QCR) generates a proton gradient and membrane potential across the inner mitochondrial membrane for ATP synthesis. Its three redox centers, a Rieske high potential [2Fe-2S] cluster (22) and cytochromes *b* and $c_1$, are essential for QCR activity (23) . Succinate-Q reductase and QCR can be purified individually or co-purified as succinate cytochrome *c* reductase (SCR). There are two schools of thought regarding the physical state of succinate-Q reductase (SQR) and QCR in the mitochondrial inner membrane. Some evidence suggests that these reductases exist separately (85), while other evidence supports the existence of a SCR super-complex (86).

In an effort to more clearly define the physical interactions between SQR and QCR and the action of NO on the electron transport pathway, we treated purified SCR, SQR,

and QCR with NO gas, individually and in combination. The results reported herein show that the effect of NO on SQR ranges from complete inhibition when SQR alone is treated, to a substantial activity loss (up to 95%) when SQR, as a component of SCR, is treated. EPR spectra confirm that these inhibitions are due to the destruction of iron sulfur clusters. Ubiquinol-cytochrome *c* reductase alone is partially susceptible to NO treatment but is not inactivated when it is in SCR. The protection of QCR from NO by SQR suggests that these two complexes may exist as a super-complex (86).

# MATERIALS AND METHODS

Materials- Purified SCR, SQR, and QCR were prepared by published procedures (87, 88). Succinate cytochrome *c* reductase was stored in 50 mM sodium/potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. Succinate-Q reductase was stored in 0.2 M potassium phosphate buffer, pH 8.0, with 20 mM succinate, 10% glycerol, and 0.2% sodium cholate. Ubiquinol-cytochrome *c* reductase was stored in 50 mM Tris-Cl, pH 8.0, with 0.67 M sucrose. Succinate-Q reductase from *E. coli* was prepared by a previously reported method and stored in 20 mM Tris-Cl, pH 7.5, containing 2 mM phenylmethylsulfonyl fluoride, 100 mM NaCl and 0.2% Lubrol PX (52). For EPR measurements, glycerol was added to all samples to a final concentration of 10%. Although each enzyme was stored and used in different buffers, optimal for their preservation, the type of storage buffer does not affect experimental results. To change buffers, the enzymes were precipitated with ammonium sulfate and redissolved in the desired buffer.

Protein assays were according to Lowry (90), with crystalline bovine serum albumin as standard. Ubiquinone was measured spectrophotometrically in 95% ethanol using an extinction coefficient of 12.25 $cm^{-1}$ $mmol^{-1}$ L at a $\lambda_{max}$ of 278 for 2,3-

dimethoxy-3-methyl-6-(10-bromo-decyl)-1,4-benzoquinone ($Q_0C_{10}Br$). Spectrophotometric measurements were done on a Shimadzu UV-2101PC spectrophotometer at room temperature.

Nitric oxide gas was obtained from Matheson Gas Products, Inc., and passed through sodium hydroxide pellets before use. Argon gas was from Sooner Airgas Inc.EPR spectra were obtained with a Bruker ER 200D spectrometer at 77 K. The details of the instrument settings are given in the figure legends.

Assay Conditions- All enzyme activities were determined spectrophotometrically. Five µl each of 1 mg/ml SCR, SQR, or 5 µl of 0.05 mg/ml QCR was used in a 1 ml assay mixture. Succinate-cytochrome *c* reductase was assayed in 100 mM sodium/potassium phosphate buffer, pH 7.4, containing 100 µM cytochrome *c*, 300 µM EDTA, and 20 mM succinate. The assay mixture for QCR contained the same phosphate buffer, at pH 7.0, without succinate, but with 25 nmol of reduced $Q_0C_{10}Br$ as substrate. For SCR and QCR, cytochrome *c* reduction was assayed at 550 nm using a millimolar extinction coefficient of 18.5 $cm^{-1}$ $mmol^{-1}$ L. Succinate-Q reductase assay mixture was 50 mM sodium/potassium phosphate buffer, pH 7.0, containing 50 µM 2,6-dichlorophenolindophenol (DCPIP), 100 µM EDTA, and 20 mM succinate. 25 nmol $Q_0C_{10}Br$ was added as substrate and activities were measured by following the absorbance decrease at 600 nm as DCPIP was reduced, using a millimolar extinction coefficient of 21.0 $cm^{-1}$ $mmol^{-1}$ L.

Nitric Oxide Treatment- Figure 8 diagrams the apparatus for treatment with NO and Scheme III outlines the procedure. All connections are either made of tygon or glass. Diluted (for activity assay) or undiluted (EPR spectra measurement) enzyme in a Thunberg tube at room temperature was stirred and flushed with argon gas for 1 min. The tube was then evacuated until the solution stopped bubbling. The argon and vacuum steps were repeated and argon was applied a third time for 2 min to remove oxygen. Vacuum was applied for a few seconds to empty the chamber, and discontinued as soon as bubbles appeared. The sample was then flushed with NO for 1 min with stirring and shaking to assure saturation. A 2 mM concentration of NO in the system was confirmed by titration of a buffer solution subjected to the same treatment. At the end of the reaction period, vacuum was applied briefly prior to flushing with argon to remove residual NO. It is important to note that although this procedure appears to be overly rigorous, these steps were necessary to assure that the enzyme did not denature due to pH changes caused by the reaction of NO with oxygen. Specific activities of control samples, treated in the same manner, but without the NO step, remained unchanged. The concentration of protein increases 5 to 6% during this treatment due to repeated evacuation and flushing with argon.

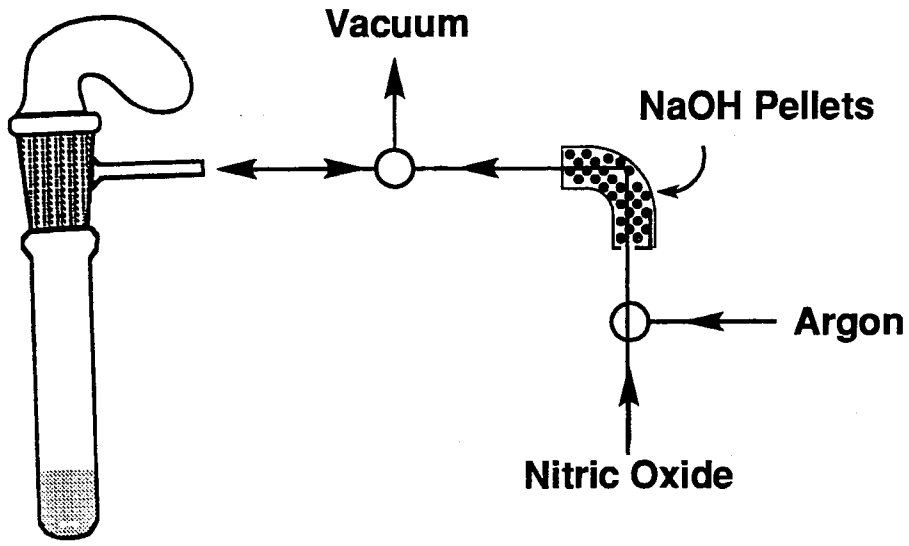



Fig. 8 Nitric oxide reaction apparatus.

## Scheme III: Outline of Procedure for Treatment of Enzymes With Nitric Oxide

**Enzyme Solution Placed Into Thunberg Tube at Room Temperature**

↓

**Stir and Flush with Argon (1 min)**

↓

**Apply Vacuum**

↓

**Stir and Flush with Argon (1 min)**

↓

**Apply Vacuum**

↓

**Flush with Argon (2 min)**

↓

**Carefully Evacuate Chamber Without Disturbing the Solution**

↓

**Flush with Nitric Oxide**

↓

**Stir and Shake**

↓

**Carefully Evacuate Chamber Without Disturbing the Solution**

↓

**Flood Chamber with Argon**

# RESULTS

Effects of Nitric Oxide on Succinate Cytochrome *c* Reductase- The SCR preparation had about 4 nmol cytochrome *b*/mg protein and an activity of approximately 1.5 μmol cytochrome *c* reduced $min^{-1}$ $mg^{-1}$ protein. The corresponding SQR and QCR activities were about 4 μmol DCPIP reduced $min^{-1}$ $mg^{-1}$ protein and 8 μmol cytochrome *c* reduced $min^{-1}$ $nmol^{-1}$ cytochrome *b*, respectively, as components of SCR. After treatment of purified SCR with NO, the SCR, QCR, and SQR activities were assayed. Results are presented as percent of initial activity (Figure 9).

As expected, the SQR component of SCR is very susceptible to NO. Nitric oxide treatment inhibits SQR activity up to a maximum of 95%, and the destruction of activity is irreversible. Conversely, QCR activity was only slightly affected by NO. The net effect of NO treatment on SCR was a 90% inhibition. NO inhibited all the complexes in a concentration dependent manner. The amount of SCR activity remaining correlated with the amount of SQR activity remaining. However, SQR was always more susceptible to NO than was SCR. This suggests that the site of action of NO on succinate cytochrome *c* reductase is in the SQR component, with QCR unaffected.

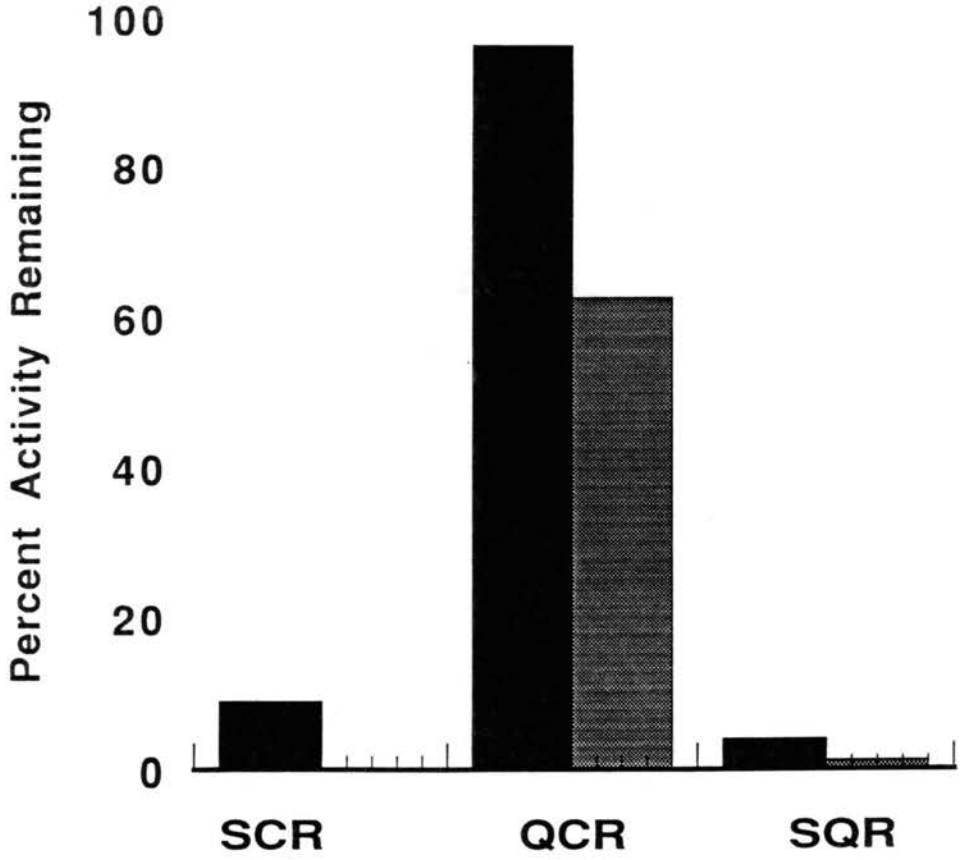



Fig 9. Effect of NO on the activities of succinate-cytochrome *c* reductase, ubiquinol-cytochrome *c* reductase or succinate ubiquinone reductase. After purified succinate cytochrome *c* reductase, SCR (1 mg/ml), QCR (1 mg/ml), or SQR (1 mg/ml) were treated with NO, the activities of the enzymes were measured and the results presented as percent of initial activity. The darker bars represent succinate cytochrome *c* reductase treated with NO. The lighter bars represent purified QCR or SQR treated with NO.

Effects of Nitric Oxide on Purified Succinate-Ubiquinone Reductase and Ubiquinol-cytochrome *c* Reductase- The effects of NO on SQR and on QCR were also examined. The specific SQR activity averaged 12 μmol DCPIP reduced $min^{-1}$ $mg^{-1}$ protein and QCR activity 8-10 μmol cytochrome *c* reduced $min^{-1}$ $nmol^{-1}$ cyt *b*. When SQR was treated with NO gas for 1 min, almost all (>99%) activity was lost, compared to a 95% loss in the case of SCR. Succinate-Q reductase activity is completely abolished during prolonged incubation with NO.

The effect of NO on QCR differs from its effect on QCR as a component of succinate cytochrome *c* reductase. Purified QCR is susceptible to NO treatment, losing 40% of its activity during a 1 min incubation period. Longer incubation times result in little further activity loss. Most (96%) of the lost QCR activity is recovered, after NO removal, during a 24 hour incubation under air at 0 °C. In contrast, no SQR activity is restored under the same conditions. The greater susceptibility of isolated QCR to NO, compared to QCR in SCR, suggests some physical association between SQR and QCR. Perhaps SCR is a true physical entity, not simply a mixture of SQR and QCR.

Activity Restoration of Nitric Oxide Treated Ubiquinol-Cytochrome *c* Reductase- Figure 10 shows the 24 hour time course of activity restoration of NO treated QCR. Since the protocol for NO treatment calls for argon gas as a final step to displace residual NO, air was passed over the sample to remove the argon and to facilitate NO removal. The small jump in activity observed (Fig 10) between the first and second points is the result of this additional step. Although most activity is regained during the first 12 hours

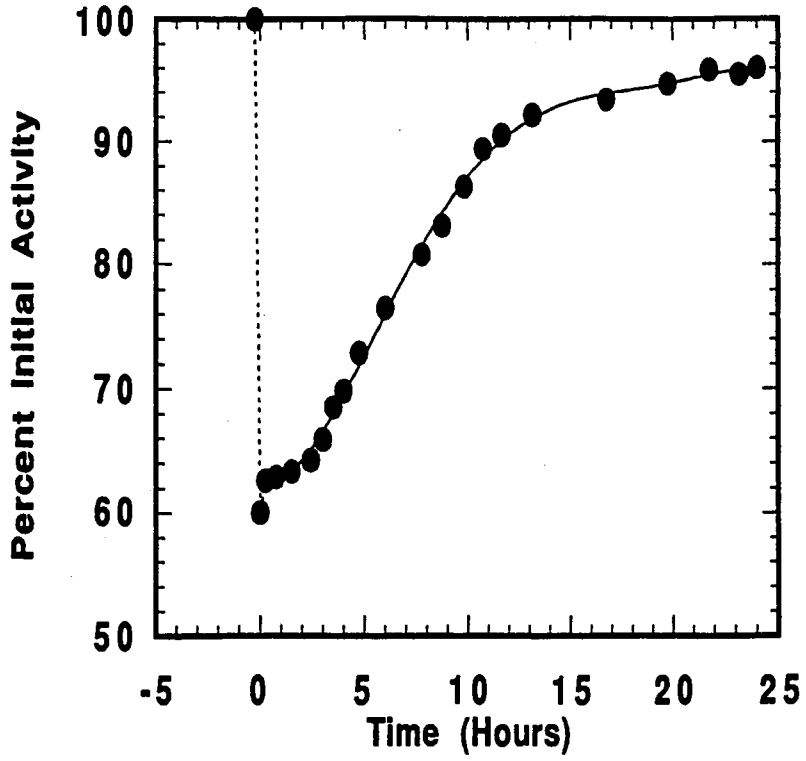



Fig 10. Time course of activity restoration of NO-treated ubiquinol-cytochrome *c* reductase. Purified QCR (0.07 mg/ml, 600 μM cyt *b*, in 50 mM Tris-Cl buffer, pH 8.0, containing 0.66 M sucrose) was treated with NO as described. After the initial activity loss was measured, air was passed over the sample for a period of 30 sec to remove argon gas from the Thunberg tube. Time zero represents the first measurement, made immediately after the argon was replaced by air.

of incubation, a recovery equal to 96% of initial activity is observed after 24 hours.

EPR Spectra of Succinate-Ubiquinone Reductase Before and After Nitric Oxide Treatment- Nitric oxide does not give an EPR signal at room temperature, or in the frozen state, for microwave frequencies from 9-35 GHz (82), but NO-iron complexes give very intense signals. Therefore, in an effort to characterize the effects of NO on the purified SQR and QCR complexes, EPR spectra of the complexes were obtained and compared.

Concentrated samples of SQR (10mg/mL) and QCR (14 mg/mL) were treated with NO gas and then reduced with sodium dithionite. EPR spectra were taken before and after treatment with NO. The spectra obtained from NO treated SQR corresponded to those previously reported (91).

The EPR spectra of *E. coli* SQR (12 mg/mL) treated with NO, were obtained for comparison with the mitochondrial SQR spectra. Although the *E. coli* SQR spectra are somewhat different from those from mitochondrial SQR, due to inherently different properties of the two enzymes, the NO treated enzymes showed identical EPR spectra. Both the mitochondrial SQR and *E. coli* SQR spectra (Figure 11) have large shoulders/signals at g=2.03 and g=1.93. However, the mitochondrial SQR spectrum has additional signals, missing in the *E. coli* SQR spectrum. The spectra of NO treated enzymes (Figure 12) are identical, with a very intense signal at g=2.04 and a lesser one at

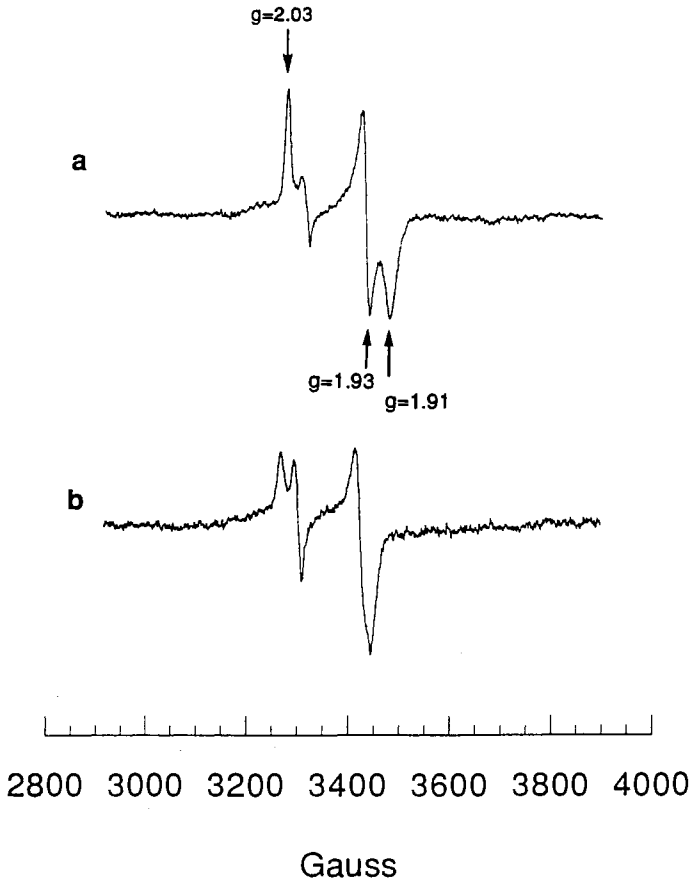



Fig. 11 EPR spectra of purified mitochondrial and *E. coli* succinate-Q reductases. Purified mitochondrial SQR (10 mg/ml) (a) and *E. coli* SQR (12 mg/ml) (b) were reduced with dithionite and their EPR spectra measured. The instrument settings were as follows: microwave frequency, 9.29 GHz; microwave power, 20 mW; modulation amplitude, 8 G; time constant, 0.5 s; temperature 77K and Gain $3.2 \times 10^5$.

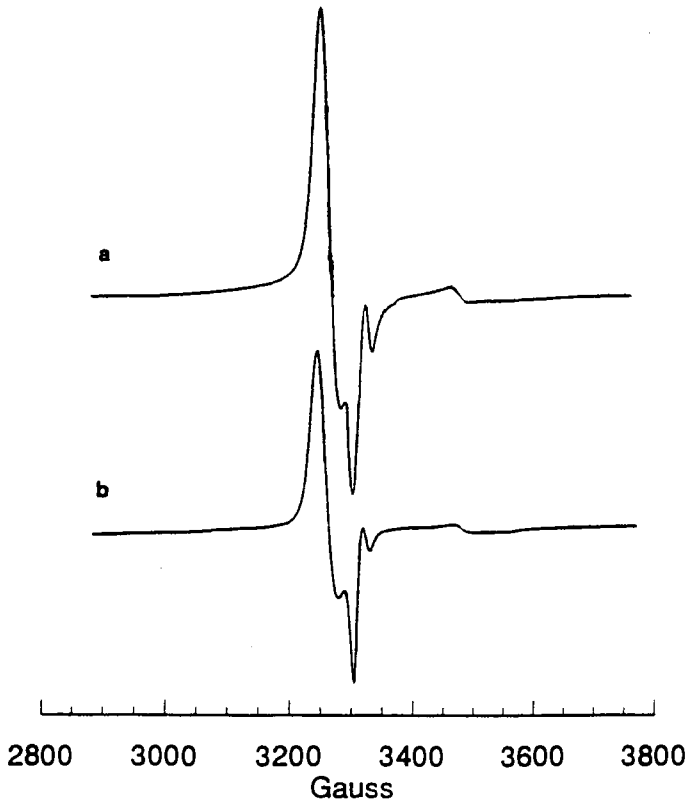



Fig. 12 EPR spectra of purified mitochondrial and *E. coli* succinate-Q reductases. Purified SQR (10 mg/ml) (a) and *E. coli* SQR (12 mg/ml) (b) were treated with NO, reduced with dithionite, and their EPR spectra measured. The instrument settings were as follows: microwave frequency, 9.29 GHz; microwave power, 20 mW; modulation amplitude, 8 G; time constant, 0.5 s; temperature 77K and Gain $3.2 \times 10^4$.

g=1.93. These are the well characterized NO-iron complex signals that appear when NO titrates iron in an iron-sulfur cluster (91).

EPR Spectra of Ubiquinol-Cytochrome *c* Reductase Before and After Nitric Oxide Treatment- Figure 13 shows the EPR spectra of dithionite reduced native and NO treated QCR. The native enzyme shows a peak at g=2.01, the Q radical signal, and another at g=1.90, the Rieske iron sulfur cluster signal. Although no NO-iron complex spectra appear, the Rieske iron sulfur signal disappears during NO treatment. After the 24 hour incubation period in the absence of NO, the Rieske iron-sulfur cluster EPR signal reappears. These data suggest two things. First, NO does not form a traditional NO-iron complex while inhibiting QCR activity. Second, this inhibition is clearly reversible. NO inhibition of QCR, which differs from the inhibition of SQR, is probably due to a conformational change caused by NO. Since no iron-NO spectra were observed and the loss of iron-sulfur cluster signal was reversible, the cluster was probably not totally destroyed. When denatured QCR is treated with NO, an NO-iron complex can be seen by EPR (data not shown). This indicates that in the denatured enzyme the iron is titrated by NO, and supports the speculation that in fully active QCR, the loss of activity during NO treatment is not due to NO titration of iron.

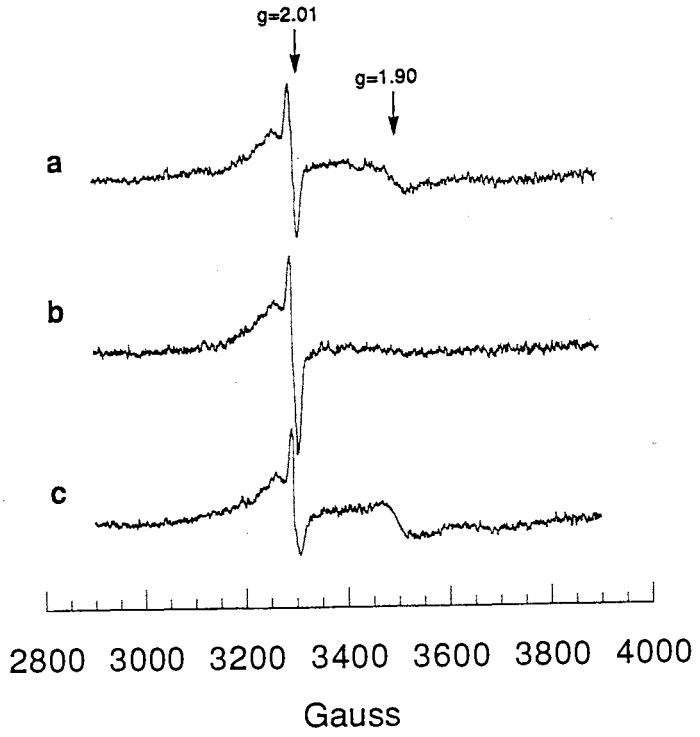



Fig. 13. EPR spectra of purified ubiquinol-cytochrome *c* reductase before and after treatment with NO and after a 24 hour incubation period of exposure to air. Purified QCR (14 mg/ml) was treated with NO as described above. The samples were divided into two portions. One (b) was immediately reduced with dithionite and frozen in liquid nitrogen. The other (c) was incubated at 4 °C, exposed to air for 24 hours, and then reduced with dithionite and frozen. Spectra (a) is an untreated control. The instrument settings were the same as those in Fig. 4.

# DISCUSSION

These experiments show that NO inhibits SQR (complex II) by destroying iron-sulfur clusters via formation of NO-iron complexes. More specifically, the target of action is succinate dehydrogenase (SDH), the subunit of SQR which contains iron-sulfur clusters. Although the precise configuration of the clusters in SDH is unknown, three clusters have been shown to exist. These are a [2Fe - 2S] (92), [3Fe - 3S or 4S] (8), and either [4Fe - 4S] or [2Fe - 2S] (93) type clusters. All or most of these clusters react with NO. The irreversible loss of activity in SQR is apparently due to the destruction of iron-sulfur clusters. Ubiquinol-cytochrome *c* reductase provides some protection against NO destruction of SQR.

The mode of action of NO on free QCR is less clear. Although no specific NO-iron complex is formed, NO inhibits QCR activity in a reversible manner. Since the activity loss in QCR is reversible, it is different from the irreversible loss of activity in SQR, in which the inhibition is entirely due to destruction of the iron-sulfur complexes.

Although the site of NO action in QCR is not known, the disappearance of the EPR signal of the Rieske iron-sulfur center indicates that a change in structure occurred on or around this center. Probably NO affects the iron-sulfur center, without removing

iron from it, since NO has a high affinity for free iron and no NO-iron complex EPR signal is observed. The diminished effect of NO on QCR in SCR may be explained in at least two ways. First, the presence of SQR may prevent access of NO to the target site in QCR. Alternatively, the formation of a super-complex between SQR and QCR may render the latter resistant to a conformational change, which would have occurred in the absence of the former. These explanations are consistent with the speculation that SQR and QCR exist as a super-complex, in isolated preparations or in the inner mitochondrial membrane.

Our results support earlier observations that NO affects different types of iron-sulfur complexes in different ways (45). Conflicting results have shown NO to be an ineffective inhibitor of aconitase activity under anaerobic conditions (94, 95) and suggest that NO is only an intermediate on the way to more oxidizing compounds, such as peroxonitrite, the true inhibitor. It is unclear whether this is true for all enzymes with [4Fe-4s] iron-sulfur complexes or only for aconitase. Under the strict anaerobic conditions we adhered to during NO-treatment of our enzymes, no oxidants such as peroxonitrite will form. This implicates NO as the effector. To date, destruction of iron-sulfur complexes by NO has been limited to enzymes which have the [4Fe-4S] type. Probably only certain types of clusters (e.g. ones where Fe is ligand to only one cysteinyl residue such as S2 and S3 of succinate dehydrogenase) are susceptible to NO titration, while others (such as the high potential Rieske [2Fe-2S] type, in which Fe is ligated to two cysteinyl residues) are not. This proposal remains to be confirmed experimentally.

# CHAPTER IV

## SUMMARY

Opening Remarks- The results reported in this thesis are the products of combined experiments designed to further elucidate the mechanisms of electron transport in cytochrome $bo_3$ of *E. coli* and complexes II and III of beef heart mitochondria. In this work, "Studies of Electron Transport Complexes," two different approaches are taken to further investigate both the prokaryotic enzymes cytochrome $bo_3$ and SQR from *E. coli* and SQR and QCR from beef heart mitochondria. Clear evidence for the site of ubiquinol binding being located on subunit II of cytochrome $bo_3$ was demonstrated in chapter II with evidence based on the photoaffinity labeling by a substrate analog. These results had implications for both the system investigated, but also for all cytochrome oxidases of the heme-copper terminal oxidase superfamily. In the same spirit, chapter III examines the effects of nitric oxide on mitochondrial and bacterial preparations of complex II and mitochondrial preparations of complex III. The results demonstrate that the effects of NO on complex II are similar for beef heart preparations and *E. coli* preparations. Thus the investigations reported herein not only provide specific evidence of discovery, but they also help to further the understanding of electron transport complexes as a whole.

Identification of the Ubiquinol-Binding Site in the Cytochrome $bo_3$-Ubiquinol Oxidase of *Escherichia coli*- In chapter II, a photoreactive radiolabeled azidoubiquinone derivative (azido-Q), was used to identify the subunit(s) involved in the binding of quinol to cytochrome $bo_3$. Although the primary structures of all four subunits of this enzyme have been determined, the ubiquinol-binding site had not been investigated. Cytochrome $bo_3$-ubiquinol oxidase is one of two ubiquinol oxidases in aerobic respiratory chain of *Escherichia coli*, and a member of the heme-copper oxidase superfamily. Our preparation showed four protein subunits (I-IV) with apparent molecular masses of 58, 33, 22, and 17 kDa, respectively. The reaction catalyzed by cytochrome $bo_3$ is the 2-electron oxidation of ubiquinol and the reduction of molecular oxygen to water.

It was demonstrated that when azido-Q was reduced by dithioerythritol, the derivative functioned as a substrate with partial effectiveness. This suggested that azido-Q was indeed interacting with a legitimate quinol-binding site. When cytochrome $bo_3$ was incubated with excess azido-Q, illumination by UV light for 10 min resulted in a 50% loss of activity. The uptake of radiolabeled azido-Q by the oxidase complex upon illumination correlated with the photoinactivation. In the presence of the competitive inhibitor HQNO or $Q_0C_2$, the rate of azido-Q uptake and loss of enzyme activity upon illumination decreased. Analysis of the distribution of radioactivity among the subunits after separation by SDS-poyacrylamide gel electrophoresis showed that subunit II was heavily labeled by azido-Q, but that the other subunits were not. This evidence suggested that the ubiquinol-binding site is located at least partially on subunit II.

Future Experiments on Cytochrome $bo_3$ of *Escherichia coli*- With molecular genetics in *E. coli* routine, and the bacterial oxidase operon cloned (96, 97) and expressed (98, 99), several labs are conducting studies on cytochrome $bo_3$, using it as the model system of the heme-copper respiratory oxidases. The aim is to use molecular genetic manipulation coupled with biophysical approaches to identify key residues which are involved in either electron transfer substrate binding, ligation of metal ions, or proton translocation. Since evidence now points to subunit II as the probable location of quinol binding, and subunit I is known to contain all the metal centers, most research will focus on these two subunits. Indeed it appears that subunits I and II, coded by *cyoA* and *B*, form a functional core for cytochrome $bo_3$, analogous to what has been observed in other heme-copper oxidases namely cytochrome *c* oxidase.

Concerning the quinol binding site on subunit II, there is much to be learned. Foremost is the determining the peptide sequence to which ubiquinol-8 has affinity. This can be best accomplished by techniques similar to those presented in this work. By using an azido-Q photoaffinity label and HPLC to separate digested protein fragments, labeled peptide fragments can be detected and their amino acid sequence determined. However, cytochrome $bo_3$ is a membrane bound protein and relatively hydrophobic making this procedure timely and difficult. Other investigations will focus on the tertiary structure of the quinone binding pocket, and how electrons are transferred to the first redox center in subunit I.

Simultaneous studies on complex I revolved around the molecular structure of the metal redox centers, and their three-dimensional arrangement. The use of many spectroscopic and biophysical approaches has revealed much about the arrangements of these centers and has led to the formulation of some structural models, but this runs beyond the scope of this paper (for review see 100). Of course all three-dimensional models will ultimately be tested against X-ray crystallographic studies and crystallization of the complex will be a priority until defractable crystals are obtained.

The Effects of Nitric Oxide on Electron Transport Complexes- In chapter III, the effects of nitric oxide on mitochondrial electron transfer complexes were studied by comparing the activities of nitric oxide treated and untreated, deoxygenated samples of purified beef heart succinate-cytochrome *c* reductase, succinate-ubiquinone reductase, and ubiquinol-cytochrome *c* reductase. Also investigated were the effects of nitric oxide on *E. coli* succinate-Q reductase. Both mitochondrial and *E. coli* succinate-Q reductase demonstrated the same reaction with nitric oxide, that is irreversible total activity loss due to the titration of their iron-sulfur clusters.

However when mitochondrial succinate cytochrome *c* reductase was treated with nitric oxide only 90% of succinate-Q reductase activity is lost and the ubiquinol-cytochrome *c* reductase component appeared to be unaffected. When purified mitochondrial ubiquinol-cytochrome *c* reductase was treated with nitric oxide, it lost 40%

of its initial activity, but recovered most of it (90-100%) after an incubation period in the absence of nitric oxide. The loss of ubiquinol-cytochrome *c* reductase activity correlated with a decrease in the Rieske protein EPR signal intensity without formation of any new EPR signal, but the original signal was recovered after the incubation period. The fact that ubiquinone-cytochrome *c* reductase was protected from nitric oxide in the presence of copurified succinate-Q reductase suggested a specific interaction between the two complexes. In addition, the possibility that nitric oxide effects different iron sulfur clusters in different ways was discussed based on the results which showed reversible activity loss for the Rieske center.

Future Experiments on the Effects of Nitric Oxide on Electron Transport Complexes- We proposed two explanations as to why the effects of NO on QCR in SCR are diminished compared to SQR in SCR. First was the possibility that access of NO to the target site in QCR may be prevented by SQR. The other explanation was that a super-complex of SQR and QCR could make QCR resistant to conformational changes which might occur in the absence or SQR, and in this way prevent NO inactivation. Either proposal supports the speculation that SQR and QCR naturally assume a super-complex formation in the mitochondria, or as SCR preparations. Further experimentation using biophysical techniques will be aimed at further defining the interactions of SQR and QCR in the presence and absence of nitric oxide.

The second major finding of our experiments centered around the manner in which NO effected the Rieske [2Fe-2S] iron sulfur cluster. Prior to this work, only enzymes of the [4Fe-4S] type clusters have shown NO inactivation. This raises the question as to whether or not NO has preference to different types of iron sulfur clusters. Are only certain types of clusters vulnerable to NO attack? This question has profound medical importance since many cells of the immune response including macrophages and neutrophils release NO as a destructive compound of attack. Curiously, some cells (such as microglia cells and other NO producing cells) are better at fending off macrophage derived NO than others (101). Some bacteria are also resistant. Perhaps some cells resistant to immune attack do not rely on [4Fe-4S] clusters for respiration or have other mechanisms of evasion. . Future experiments will therefore be aimed at the effects of NO on respiratory enzymes with non [4Fe-4S] clusters. Since the Rieske subunit of *Rhodobacter sphaeroides* can be expressed in *E. coli* in the absence of other subunits (102), this protein may prove of great interest in further defining these mechanisms.

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