This is to certify that the

dissertation entitled

Stopped-Flow Studies of the Reduction of Cytochrome c Oxidase and Magnetic Susceptibility Studies of Cytochrome c Oxidase and Some of Its Derivatives

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Chemistry

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Date 7/21/86

STOPPED-FLOW STUDIES OF THE REDUCTION OF CYTOCHROME *c* OXIDASE AND MAGNETIC SUSCEPTIBILITY STUDIES OF CYTOCHROME *c* OXIDASE AND SOME OF ITS DERIVATIVES

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1986

ABSTRACT

STOPPED-FLOW STUDIES OF THE REDUCTION OF CYTOCHROME *c* OXIDASE AND MAGNETIC SUSCEPTIBILITY STUDIES OF CYTOCHROME *c* OXIDASE AND SOME OF ITS DERIVATIVES

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Stopped-flow spectrophotometry was used to study the anaerobic reduction of cytochrome *c* oxidase by 5, 10-dihydro 5-methylphenazine (MPH). Cu_A reduction was complete after 400 ms. The 830 nm absorbance decay lagged the 605 nm absorbance growth. Analyses of data collected at various MPH concentrations showed that Cu_A was reduced via cytochrome *a*, not directly by MPH. The same reaction was followed at 444 nm in the presence of Tween-20 and lauryl maltoside. Both reactions had a second-order phase followed by two first-order phases. The rate constants were not affected by the detergent used. Aerobic reduction of oxygenated enzyme by MPH was followed at 444 nm. There was a bimolecular phase, a steady-state phase, and two phases that were first order in enzyme. Of the last two, one contributed only slightly, indicating almost complete enzyme homogeneity. The reaction of MPH and cytochrome *a* was rate-limiting; the rate constant of the intramolecular reaction was greater than 4.9 s^{-1}.

The temperature dependence of the magnetic

susceptibility was measured for resting, cyanide-bound and formate-bound cytochrome c oxidase. The a_3 center of the resting and formate-bound enzyme showed antiferromagnetic coupling. The cyanide-bound enzyme was magnetically heterogeneous with 20% of the enzyme having a -J value of 30 cm^{-1} and 80% having a J value of magnitude 1-2 cm^{-1}. The type of coupling in the latter is unknown. The magnetic behavior of the resting and cyanide-bound enzyme was unaffected by the enzyme isolation technique. Use of Tween-20 instead of lauryl maltoside did not affect the susceptibility of the resting enzyme or that of the major component of the cyanide-bound enzyme. The coupling factor of the minor component of the cyanide-bound enzyme was doubled in lauryl maltoside. Use of glucose oxidase and glucose to remove oxygen affected the susceptibilities of resting and formate-bound enzyme, presumably by causing peroxide binding. The spin state of cytochrome a_3 was found to be 3/2 in peroxide-bound cytochrome oxidase.

ACKNOWLEDGMENTS

I would like to thank Dr. James L. Dye for suggesting this project, for the many helpful discussions, and for encouraging me when I needed it.

I would also like to thank Dr. Gerald T. Babcock for his help with this project and for the financial support. I also wish to thank the Department of Chemistry, the National Science Foundation, and the National Institute of Health for financial support.

I would like to thank Dr. Thomas V. Atkinson, Martin Rabb, and Manfred Langer for the technical assistance always cheerfully given. "Without them there is no science."

I would also like to thank the many friends at Michigan State and at Purdue, as well as elsewhere, and my family, for encouraging me as I did experiments and wrote this manuscript.

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CHAPTER I

INTRODUCTION

A. Cytochrome c Oxidase

Cytochrome c oxidase (ferrocytochrome c:O_2 oxidoreductase:E.C. 1.9.3.1) catalyzes the transfer of electrons from cytochrome c to oxygen:

$$4 \text{ cytochrome } c^{2+} + O_2 + 4H^+ \longrightarrow 4 \text{ cytochrome } c^{3+} + 2H_2O.$$

More than 90% of the oxygen consumption by living organisms on earth occurs through this reaction (Wikstrom et al, 1981). The free energy change is about -192 kJ per four electrons transferred. This energy is stored as an electrochemical proton gradient and is used by the cell in subsequent ATP synthesis.

A.1 Structure

Cytochrome oxidase is a Y-shaped protein which spans the inner mitochondrial membrane. Twelve or thirteen subunits copurify in stoichiometric amounts with the metal centers (Kadenbach and Merle, 1981), but only eight of these subunits are required for electron transport activity

and for generation of a transmembrane proton gradient (Azzi, 1980; Downer, et al, 1976).

The arrangement of the subunits has been investigated by using cross-linking and chemical binding (Briggs and Capaldi, 1977; Fuller, et al, 1981). A model consistent with the data from these studies is shown in Figure I.1 (Capaldi et.al., 1983).

A.2 Metal Centers

There are two iron and two copper ions in each cytochrome oxidase monomer. Although both iron species are isolated from the enzyme as heme *a* (Figure I.2), they have different structural and functional properties in the enzyme. When there, they are called cytochrome *a* and cytochrome a_3. The copper complexes also differ from each other. Because of an assumed structural and functional association with the cytochromes, the copper species are known as Cu_A and Cu_B.

The traditional distinction between the cytochrome moieties is that cytochrome a_3 will bind ligands such as CO, HCN, and NO while cytochrome *a* will not. In addition, cytochrome a_3 exhibits no epr signal in the oxidized enzyme while cytochrome *a* has signals of a low-spin heme at g=3.0, 2.2, 1.5. Evidence suggests that cytochrome a_3 has one histidine ligand (Chan et.al., 1982) and that cytochrome *a* has two histidine ligands (Stevens et.al., 1982)

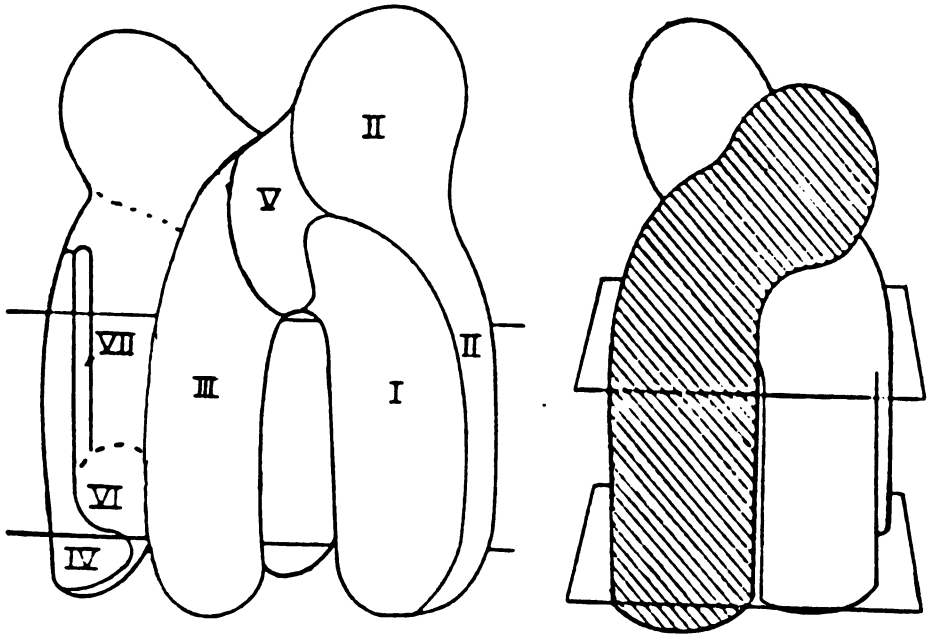
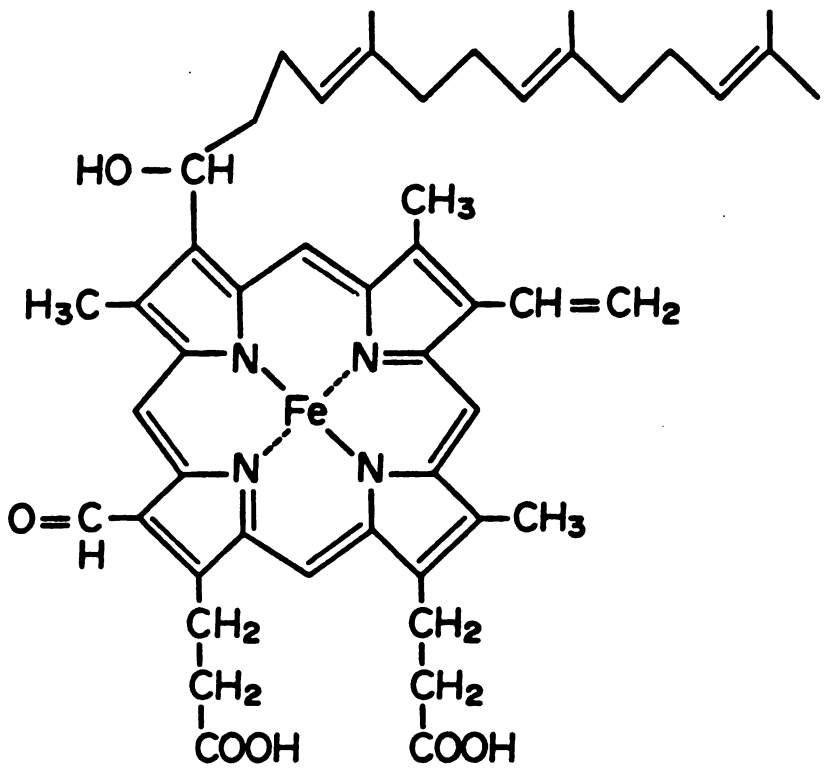


Figure I.1 The Arrangement of the Subunits in Cytochrome Oxidase in the Membrane (from Capaldi et.al., 1983)



heme a

Figure I.2 The Structure of Heme a.

Different epr and optical properties show that the environments of the two copper ions differ. No epr signal is exhibited by Cu_B while Cu_A has a g=2 signal. Isotopic substitution studies (Capaldi et.al., 1983) indicate two histidine ligands for Cu_B. ENDOR and epr studies (Stevens et.al.,1982) on yeast cytochrome oxidase identify one cysteine and one histidine as ligands of Cu_A. Because there are no epr signals from Cu_B or from cytochrome *a*3 in the resting or oxidized cyanide-bound forms of the enzyme, it is believed that these two metals are magnetically coupled in those enzyme forms. The pair may then be refered to as the *a*3 site.

A.3 Optical Spectra

The optical spectra of the fully oxidized and fully reduced enzyme are shown in Figure I.3. The bands in the near-UV ("Soret") and visible ("α") regions are due to the porphyrin ring of the hemes. A discussion of the origin of porphyrin spectra is given by Gouterman (1959). The two cytochromes contribute about equally to the absorbance at 444 nm. Cytochrome *a* contributes about 80% of the absorbance at 605 nm, with most of the remainder due to cytochrome *a*3. The broad band at 830 nm is at least 85% due to Cu_A (Wharton and Tzagoloff, 1964; Boelens and Wever,1980; Beinert et.al., 1980).

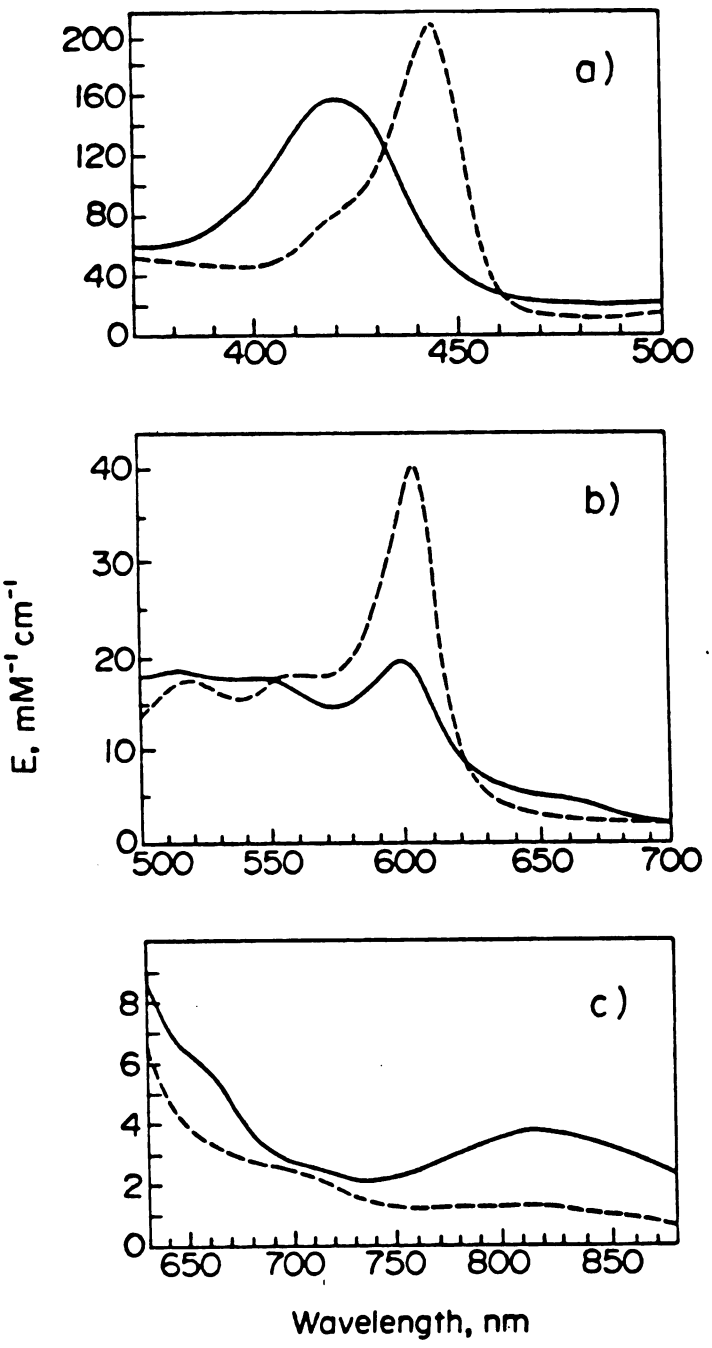


Figure I.3 The Optical Spectra of Oxidized and Reduced Cytochrome Oxidase: ___ , oxidized, and – – – , fully reduced. Molar absorptivities are expressed per unit containing two hemes and two copper ions. From Halaka (1981)

The optical spectrum of the oxidized cyanide-bound enzyme is shown in Figure I.4. (Nicholls and Chance, 1974). It is similar to that of the oxidized resting enzyme, with the most important difference being the lack of a shoulder at 650 nm. The absence of this shoulder is used as a check for complete cyanide binding of the enzyme.

A.4 Forms of Cytochrome Oxidase

Cytochrome oxidase as isolated is said to be in its "resting" state. When reduced and reoxidized it is converted to a form called "oxygenated" (Okunuki et.al., 1959). The oxygenated form, so called because it was originally believed to be an oxygen adduct, is more active than the resting enzyme (Antonini et.al., 1977; Brunori et.al., 1979; Peterson and Cox, 1980). Because its greater activity may mean it is the form present during catalytic cycles, the oxygenated enzyme is of great interest.

The traditional method used to make the oxygenated form involves reduction of the enzyme with an excess of sodium dithionite and subsequent reoxidation with oxygen. This procedure yields the more active form with a near-UV absorpton at 428 nm (vs 418-424 nm for the resting form) and a slightly enhanced absorption in the visible region (Lemberg and Stanbury, 1967). This enzyme will decay in a two-step process to a form with an activity and spectrum very similar to those of the resting oxidase. The shift in

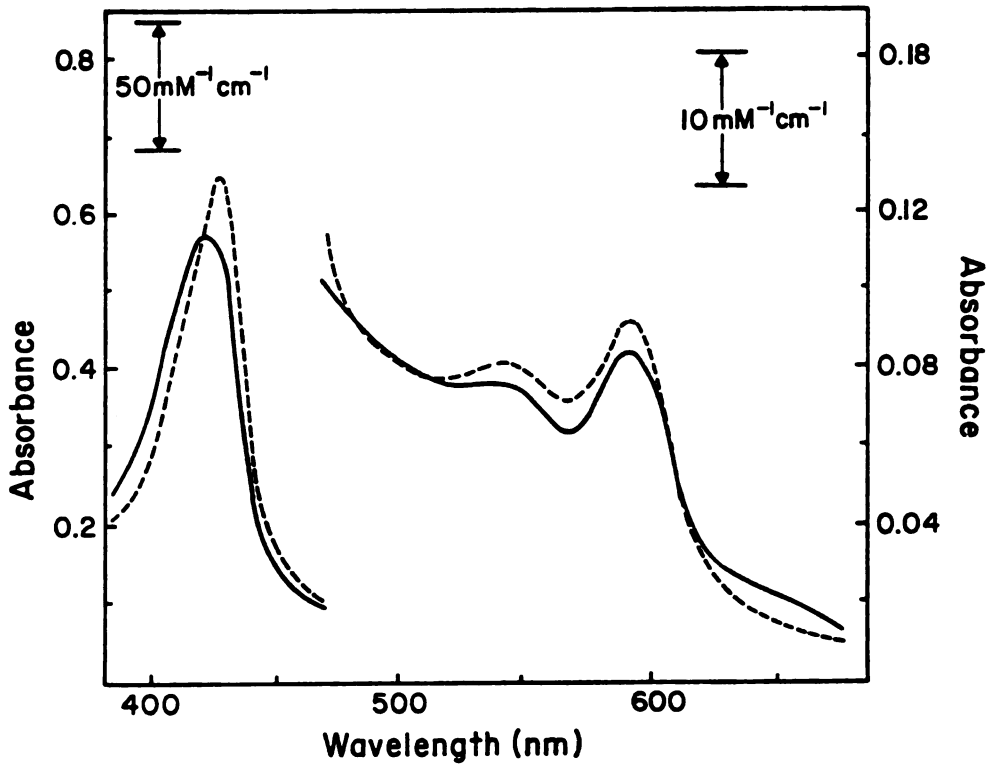


Figure I.4 The Optical Spectra of Oxidized and Cyanide-Bound Cytochrome Oxidase.
— oxidized, --- cyanide-bound

Soret maximum from 428 nm to shorter wavelengths has been correlated with the loss of enhanced activity (Brunori et.al., 1981) and the decay in the visible region has been shown to follow the disappearances of a g=5, 1.78, 1.69 epr resonance (Armstrong et.al., 1983).

Kumar et.al. (1984) have recently shown that the more active form of the enzyme can also be produced with its near-UV maximum at 420 nm. This is accomplished by using catalase to prevent formation of peroxide (due to the presence of dithionite) during enzyme reoxidation with O_2. Addition of peroxide to this 420 nm form of the more active enzyme produces the 428 nm form of the oxygenated enzyme. This indicates that the latter is a peroxide derivative or reaction product.

Oxidized cytochrome oxidase is believed to exist in more than one conformation because the resting oxidase is heterogeneous in its behavior with several ligands. Work done at Michigan State University by F. Halaka showed that the resting enzyme isolated by the method of Hartzell and Beinert (1975) is heterogeneous in its reduction by sodium dithionite. From 15 to 30 percent of the cytochrome $\underline{a_3}$ is reduced directly in an initial fast phase. The remaining enzyme is reduced at the cytochrome $\underline{a}$ site, with its cytochrome $\underline{a_3}$ reduced through intramolecular electron transfer. Using enzyme isolated by the Yonetani method, Jones et.al. (1983) found that all of the enzyme is reduced

by dithionite via cytochrome *a*. Brunori et.al. (1985) found no difference in the dithionite reduction of cytochrome oxidase isolated by using either the Yonetani or the Hartzell-Beinert techniques. These results may indicate a difference in the properties of the enzyme isolated in different laboratories or a preparation-to-preparation variation with the same isolation technique.

Bickar et.al. (1982) reported that the enzyme they isolated by the Yonetani method always bound at least some peroxide, but that the extinction coefficient varied. This indicates that the proportion of enzyme that bound the peroxide changed from one isolation to the next, even though the same procedure was used each time.

Brudvig et.al. (1981) report that three conformations may be present in the enzyme as isolated. Addition of NO induces a high-spin cytochrome epr signal in one fraction of the enzyme, presumably by uncoupling the cytochrome a_3 and Cu_B. Another fraction exhibits a g'=12 epr signal and is further distinguished from the first fraction by the slowness with which it binds cyanide. A third fraction does exhibit the high-spin cytochrome signal in the presence of NO alone, but does when both NO and F^- are present.

Brudvig et.al. (1981) also looked at the oxygenated enzyme. They found that the presence of NO by itself does

not induce a high-spin cytchrome epr signal, but addition of F^- or of F^- and NO induces the high-spin cytochrome epr signal. The oxygenated form does not show the g'=12 signal. Its behavior thus corresponds to that of the third fraction of the resting enzyme. A g'=12 signal appears as the 428 nm band shifts to 420 nm when the oxygenated form decays. The heterogeneity seen by Brudvig et.al. was confirmed by Wilson et.al. (1982), who found that the differences disappeared during turnover.

The kinetics of cyanide binding has been used as a probe of the a_3 site in both the resting and the oxygenated enzyme. The number of phases and their rate constants have been reported for the oxygenated enzyme and for resting cytochrome oxidase isolated by several different procedures.

Van Buuren et.al. (1972) reported that the binding of cyanide to the resting enzyme consists of more than two components with the initial second-order phase having a rate constant of 1.8 M^{-1} s^{-1}. Brittain and Greenwood (1976) prepared oxygenated enzyme and found that cyanide binds in one phase with a rate constant of 22 M^{-1} s^{-1}.

Naqui et.al. (1984) found that the resting enzyme bound cyanide multiphasically. The phases present and the percent contribution of each phase vary not only with the method of enzyme isolation, but from preparation to preparation when the same isolation procedure is used. For

all the enzyme except one of the two Yonetani preparations, the initial phase was second-order. The average rate constant was 2.5 M^{-1} s^{-1}. Other phases were first-order in enzyme and had rate constants of either 1.1 s^{-1} or 0.06 s^{-1}. Naqui et.al. suggest that the first-order phases are due to conversion of enzyme forms incapable of binding cyanide to a form that is capable. The fast phase would then be due to the binding of cyanide by the capable form initially present.

Like Brittain and Greenwood, Naqui et.al. found monophasic binding of cyanide to the oxygenated enzyme, but with an average second-order rate constant of 2.3 M^{-1} s^{-1}. All preparations of oxygenated enzyme showed the same behavior, regardless of the method used to isolate them from the membrane. Naqui et.al. noted the similarity of the second-order rate constants in the resting and oxygenated cases and suggested that the portion of the resting enzyme capable of binding cyanide may actually be in the oxygenated form.

While the rate constants for cyanide binding to the oxygenated enzyme vary considerably from one investigation to another, it is plain that the oxygenated enzyme does not show the heterogeneity that the resting enzyme presents.

A.5 Kinetics

A.5.1 Effects of Detergents on Activity Since cytochrome oxidase molecules span the inner mitochondrial membrane, at least some of their *in vivo* environment must be hydrophobic. When removed from the lipid environment of the membrane, the enzyme requires phospholipids or nondenaturing detergents to form a solution and to maintain electron transport activity (Awasthi et.al., 1971; Brierly et.al., 1962).

The steady-state activity of the soluble enzyme is affected by the dispersing detergent used. A higher activity results from the use of nonionic detergents such as Triton X-100 or the Tween series than from the use of ionic bile salts such as cholate. Robinson and Capaldi (1977) report that the higher activity is due to the greater fluidity of the fatty acid chain of the nonionic detergents. The identity of the detergent headgroup also has an effect since cytochrome oxidase has a higher steady-state activity in solutions of lauryl maltoside than in solutions of Tween-20, though these two detergents have identical fatty acid chains (Rosevear et.al., 1980). Robinson et.al. (1985), using several types of detergents, found that the rates of electron transport were dependent upon the structure of the head group and the length of the hydrocarbon tail.

The variation of activity with the nonionic detergent used may be related to the aggregation state of the enzyme. In lauryl maltoside the enzyme is monodispersed and is 2-10 times more active than in Tween-20, where it is oligomeric and polydispersed (Rosevear et.al., 1980).

A.5.2 Anaerobic Reduction by Cytochrome c Because reduced cytochrome c is the natural substrate of cytochrome oxidase, the reaction between them has been studied in several laboratories. Two of the difficulties associated with using cytochrome c as a reductant in transient-state kinetic studies should be noted: (1) the reaction is very fast (the second-order rate constant is about 8×10^7 M^{-1} s^{-1}) and much of the reaction is over within the deadtime of many of the stopped-flow systems used, and, (2) under anaerobic conditions, only two electrons are transferred from cytochrome c to an oxidase molecule, so complete four-electron, anaerobic reduction cannot be studied.

The most comprehensive anaerobic study was done by Antalis and Palmer (1982), who varied the ionic strength and cytochrome c:oxidase ratio. Both changes affect the number of phases observed. Low ionic strength or reductant:oxidase ratios of 1:1 or 2:1 lead to monophasic kinetics. High ionic strength or high cytochrome c:oxidase ratios result in a biphasic reaction. In additon, the ionic strength affects the amplitudes of the two phases.

This explained the disagreements in the literature about the number of electrons transferred in the fast initial phase (Andreasson et.al., 1975; Wilson et.al., 1975; Van Buuren et.al., 1974) and in the slower second phase (Gibson et.al., 1965). Antalis and Palmer also found that the total number of electrons transferred did not depend on the ionic strength, but varied form 0.8 to 2.0 as the reductant:enzyme ration went from 1 to 8. This explained the differing reports of the number of electrons transferred (Andreasson et.al., 1975; Wilson et.al., 1975).

A.5.3 Reduction of Oxygenated Oxidase Reduction of oxygenated cytochrome oxidase by cytochrome c has been studied in several laboratories. Comparisons with the reduction of the resting enzyme indicate a difference in the kinetic behavior of the two forms.

Antonini et.al. (1977) found that the reduction of oxygenated cytochrome oxidase by cytochrome c had a steady state velocity 4-5 times greater than that of the resting oxidase. This difference disappeared at very low oxidase:cytochrome c ratios. The difference in reaction rates observed was due to faster reduction of cytochrome a_3 during a phase that was first-order in enzyme, rather than the faster reaction between cytochrome c and the enzyme. Peterson and Cox (1980) extended these studies by using both cytochrome c and reduced methyl and benzyl viologens

as reductants. They reported a rate constant of 7.5 s^{-1} for cytochrome a_3 reduction in the oxygenated enzyme. Gibson et.al. (1965) reported a rate constant of 0.5 s^{-1} for this reduction in the resting enzyme.

Thus the kinetic difference between resting and oxygenated enzyme is due to enhanced intramolecular electron transfer in the latter form. The disappearance of this kinetic difference at low oxidase:reductant ratios was confirmed by Antonini et.al. (1985) and is believed to be due to conversion of the resting form to the oxygenated form by multiple turnovers.

A.5.4 Reduction by 5-Methyl Phenazinium Methylsulfate

Anaerobic stopped-flow studies of the reduction of cytochrome oxidase by 5-methyl phenazinium methylsulfate (MPH) were done by F. Halaka (1981). Comparison of initial and final spectra with those of the oxidized and reduced MPH and the oxidized and reduced enzyme showed that the reaction has the overall stoichiometry:

$$2MPH + \text{cytochrome oxidase (oxidized)} \longrightarrow 2MP^{+} + \text{cytochrome oxidase (reduced)}.$$

The changes of absorbance in the difference spectra showed that the species that absorbed at 430 nm (cytochrome a) was reduced more quickly than the species that absorbed at 410 nm (cytochrome a_3).

Nonlinear least-squares fitting of the data showed that the reaction had a fast phase that was first-order in each reactant and was followed by two slow phases that were both first-order in enzyme concentration.

The absorbance change at 830 nm, due mainly to the reduction of Cu_A, lagged the change at 605 nm at the one set of concentrations used. The model proposed to account for this was

$$MPH + [\text{cyt } \underline{a}^{3+} \quad Cu_A^{2+}] \longrightarrow$$

$$MPH^{\cdot+}[\text{cyt } \underline{a}^{2+} \quad Cu_A^{2+}] \longrightarrow$$

$$MP^{+} + [\text{cyt } \underline{a}^{2+} \quad Cu_A^{+}]$$

Electron transfer to cytochrome a was proposed to be fast relative to electron transfer from cytochrome a to Cu_A; thus the cytochrome would always appear reduced.

The two phases that were first-order in enzyme must represent intramolecular electron transfer. The presence of two first-order phases may be due to heterogeneity of the enzyme. Both of these phases are too slow to be significant in the catalytic cycles of the enzyme.

A.6 Magnetic State of the Cytochrome a_3-Cu_B Center

Because neither the iron ion nor the copper ion of the a_3 center exhibits an epr signal in the resting or oxidized cyanide-bound enzyme, it has been proposed that the two metals are magnetically coupled (Van Gelder and Beinert,

1969) in these forms of the enzyme. It is generally believed that the coupling is a superexchange interaction operating through the orbitals of a bridging ligand.

The magnetic states of the a_3 center of the cyanide-bound and resting forms of the enzyme have been studied by using Mössbauer and magnetic-circular dichroism (MCD) spectroscopies and magnetic susceptibility measurements. The conclusions drawn from these studies are conflicting, with some studies indicating ferromagnetic coupling and some indicating antiferromagnetic coupling.

A.6.1 Mossbauer Because of the low (2%) natural abundance of ^{57}Fe, Mossbauer studies of cytochrome oxidase were done with bacterial enzyme isolated from Thermus thermophilis (Kent et.al., 1982). Comparative studies were later done with bovine cytochrome oxidase (Kent et.al., 1983).

For the a_3 center of the oxidized cyanide-bound bacterial enzyme, Kent et.al. report a zero-field quadrupole splitting and an isomer shift typical of a low spin ferric heme. Application of a magnetic field broadened the spectrum, indicating a ground state of integer spin, $S \geq 1$. These data were explained by ferromagnetic coupling of an S=1/2 iron ion with an S=1/2 copper ion.

The bovine cyanide-bound cytochrome oxidase had zero-field parameters similar to those of the bacterial

enzyme. The presence of a quadrupole doublet evinced a zero or integer spin ground state. Application of a magnetic field broadened the spectrum, indicating an integer spin. The similarity of parameters and the effect of the field suggest ferromagnetic coupling to yield an S=1 ground state in the bovine enzyme as in the bacterial enzyme. It must be noted, however, that the data for the bovine enzyme are extremely noisy and these conclusions are based mostly on comparison with the bacterial data.

The zero-field parameters of the a_3 center in the resting oxidized bacterial enzyme were typical of a high-spin ferric heme. The lack of zero-field hyperfine structure indicated that the ground state spin was zero or an integer. Surprisingly, application of a magnetic field gave results that were preparation dependent. The lack of change in the spectrum of one preparation indicated an S=0 ground spin state. The spectrum of a second preparation broadened when the field was applied, indicating an $S \geq 1$ spin ground state. Kent et.al. were unable to provide an explanation for this heterogeneity, or a mechanism by which a high-spin iron ion could couple with an S=1/2 copper ion to yield a diamagnetic state.

At zero field the a_3 center of the resting oxidized bovine cytochrome oxidase exhibited a quadrupole doublet with parameters similar to those of the bacterial enzyme. Application of a 60 mT field did not broaden the spectrum

within the limits of their signal-to-noise ratio. This ratio was poor enough that Kent et.al. do not believe the lack of broadening conclusively shows an S=0 ground state. If they are correct, then a zero or integer spin ground state is indicated with clear-cut conclusions about the spin and the nature of the coupling being impossible.

A.6.2 Magnetic Circular Dichroism Thomson et.al. (1981), used mcd spectroscopy to study the magnetic properties of cytochrome a_3 of the oxidized cyanide-bound bovine enzyme. Their enzyme was isolated using the Yonetani technique. The magnetization curve of the a_3 site of the cyanide-bound enzyme was typical of a ground state electronic doublet. The sigmoidal nature of the curve indicated a low-lying excited state. These curves and the lack of an epr signal were explained by Thomson in terms of an S=1 ground state with an axial distortion such that the $M_s = \pm 1$ components were at least 10 cm^{-1} lower in energy than the $M_s=0$ component. An epr signal would not be expected because $M_s = \pm 2$ transitions are forbidden. This model implies ferromagnetic coupling between two S=1/2 centers. The coupling constant was greater than 10 cm^{-1}.

A.6.3 Magnetic Susceptibility Tweedle et.al. (1978) measured the magnetic susceptibilities of the oxidized resting and oxidized cyanide-bound forms of bovine

cytochrome oxidase isolated with the Hartzell-Beinert technique. Their temperature range was 7 K to 200 K. Moss et.al. (1978) extended the temperature range to 1.4 K for the oxidized resting form.

Tweedle et.al. found that, except at the lowest temperatures, the magnetic susceptibility of the resting oxidized bovine enzyme conformed to the Curie Law.

$$\chi = \frac{(\tilde{g})^2 \, N \, \beta^2 \, S(S+1)}{3kT} = \frac{2 \, N \, \beta^2 \, \mu_{eff}^2}{3kT}$$

The slope of the χ vs T^{-1} plot corresponds to a $\mu_{eff}^2 = 31.5$. The contribution to this by cytochrome a^{3+} and Cu_A^{2+} was calculated from their epr parameters and found to be 7.2. Thus the a_3 site contribution was 24.3. The most plausible coupling scheme that could produce this value for μ_{eff}^2 is antiferromagnetic coupling of an S=5/2 cytochrome a_3^{3+} and the S=1/2 Cu_B. The linearity of the χ vs T^{-1} plots at temperatures as high as 200 K led to the conclusion that $-2J > 200$ cm^{-1}. The nonlinearity and the decrease in slope at low temperatures obtained by Moss et.al. were accounted for by reasonable values for the zero-field splitting and the rhombic zero-field parameter (D=9 cm^{-1} and E=0.1 to 1 cm^{-1}).

The plot of susceptibility vs reciprocal temperature for the oxidized cyanide-bound enzyme was curved. The limiting slope at low and high temperatures corresponded to a μ_{eff}^2 of 7.8 and 15.1 respectively. These values were expected to be 7.2 and 15.2 if an antiferromagnetically coupled center composed of two S=1/2 ions was present and if the absolute value of 2J was about kT. The curvature of the plot would arise as the excited state became more extensively populated at higher temperatures.

The equation for the classical model of such a two-spin interaction is

$$\chi = \frac{2 g^2 N \beta^2}{3kT} \left[1 + \frac{1}{3} \exp (-2J/kT) \right]^{-1}$$

where 2J is the difference in energy between the singlet and triplet levels. The values of the susceptibility observed by Tweedle et.al. fit this equation when a term was added for the temperature-independent paramagnetism. They obtained a nonlinear least-squares value of $-J = 38.5$ cm^{-1}.

A.6.4 Model Compound Gunter et.al. (1984) have synthesized a model for the cyanide-bound form of oxidized cytochrome oxidase. It is a cyanide-bridged Fe(III)-Cu(II)

heterobinuclear complex of the ligand α,α,α, α -tetrakis (o-nicotinamidophenyl) porphyrin [Fe(P)CNCu(N_4)]($ClO_4.3H_2O$). They interpret the bulk magnetic susceptibility, epr, and Mossbauer data in terms of weak ferromagnetic coupling between low-spin Fe(III) and Cu(II) ions. The magnetic behavior in solution seems to vary with solvent, however. In 10% CH_3OH-$CHCl_3$ the model shows properties similar to those of the solid state, but in CH_3CN and $(CH_3)_2SO$ little or no exchange coupling is seen. In DMF an epr silent species is formed.

B. Magnetic Studies of Enzymes

When a substance is placed in a magnetic field, the magnetic lines of flux change. This effect is normally measured via the magnetic susceptibility, χ , of the substance:

$$\chi = \frac{M}{H}$$

where M is the intensity of magnetization and H is the strength of the magnetic field.

B.1 Types of Magnetic Behavior

There are several types of magnetic behavior to be considered when dealing with enzyme systems: paramagnetism, diamagnetism, ferromagnetism and antiferromagnetism.

Paramagnetism is temperature dependent and arises from the interaction of the orbital and/or spin angular momenta of unpaired electrons with a magnetic field. The value of the susceptibility of a paramagnetic substance is positive. Paramagnetism in enzymes is usually due to the presence of metal ions, and it is often diagnostic of their magnetic state.

Diamagnetism is temperature independent and arises from the motion of paired electrons in a magnetic field. The susceptibility of a diamagnetic substance is negative. All molecules have a diamagnetic contribution to their magnetism. Diamagnetism is not diagnostic of the types of atoms that are in an enzyme or of the magnetic states of those atoms, so it is itself of little interest. However, because diamagnetism contributes to the susceptibility, its contribution must be subtracted in order to measure the absolute paramagnetism. A diamagnetic correction is unnecessary in magnetic studies when only the rate of change of the signal with temperature is of interest rather than the absolute signal.

In addition to the above types of magnetic behavior, there are two types that are due to interactions of spin moments on atoms which are close to each other. This interaction formally resembles direct dipole-dipole interactions, but is quantum-mechanical in nature. Antiferromagnetism arises from an antiparallel alignment of

spins from interactions, while ferromagnetism arises from a parallel alignment of spins due to dipole interactions. The contributions to the susceptibility from both of these effects are generally positive. Because the protein shields metal ions in one molecule from those in other molecules, spin alignment in enzymes is usually intramolecular rather than intermolecular.

B.2 Diamagnetic and Background Corrections

There are two methods commonly used to measure the diamagnetic contribution of the protein. If the paramagnetic contribution is removed, then the diamagnetism can be measured directly. The paramagnetism may be removed by extracting the metal ions or by reducing them to a state where they have no unpaired electrons. The diamagnetism of the resulting protein is then used as the value of the diamagnetism of the actual enzyme. The second way to correct for diamagnetism utilizes the temperature independence of diamagnetism and the inverse temperature dependence of paramagnetism. Magnetic measurements are made on the enzyme system and the susceptibility values obtained as a function of temperature are extrapolated to infinite temperature. At this point the paramagnetic signal would be zero, so the residual susceptibility is due to diamagnetism. This value of diamagnetism is then used for the entire temperature range. Because the absolute

signal was not of interest in the present study, it was not necessary to correct for the diamagnetism of the protein.

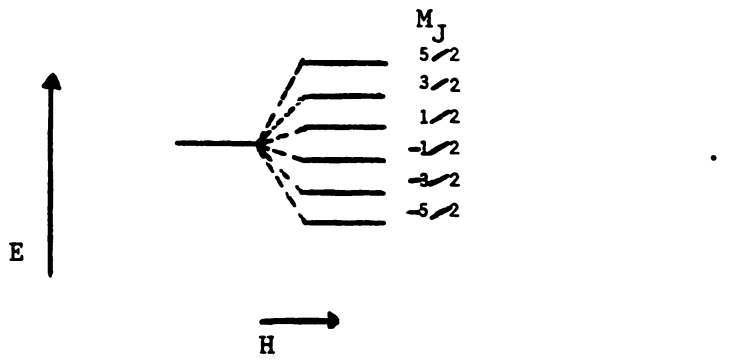
For enzyme solutions, an additional correction must be made for the magnetism of the buffer. In this study the susceptibility of the pure buffer was measured and used as a correction. Because the mass of buffer present in the enzyme sample was not known, the mass of the entire solution was used as the mass of buffer present. This method ignores the mass of the enzyme itself. An alternative would have been to calculate the mass of enzyme and buffer present by using the approximate molecular mass of the enzyme and the measured concentration of the enzyme. In this study these two methods give different absolute values for the susceptibility, but gave the same temperature dependence of the susceptibility. The susceptibility of the empty sample holder was measured separately and subtracted from the signal measured for the sample.

It must also be noted that isolation techniques for cytochrome oxidase are not developed well enough to ensure zero contamination from other metal ions (such as other cytochromes originally present in the membrane), but such contaminations are low. The concentration of unbound metal ions is kept low by use of EDTA in solutions during enzyme isolation and by avoiding any contact between the enzyme solutions and metal.

B.3 Curie Law

The magnetic properties of an ion depend on the spin angular momentum and the orbital motion of electrons in its occupied energy levels. The properties thus depend on the quantum numbers L and S (or J if Russell-Saunders coupling applys) of the occupied energy levels. The population distribution is governed by the Boltzmann distribution, so the electrons in the ground state and/or in levels within approximately kT of the ground state energy level are the ones that contribute most significantly.

An external magnetic field will resolve the degeneracies of an energy state, splitting the levels with different M_J quantum numbers. If the original (degenerate) energy level is taken as the zero of energy, then the energy of each sublevel is $E = M_J\, g\, \beta\, H$, where β is the Bohr magneton, g is the Lande constant, and H is the field. For example, a d^5 ion in an external magnetic field shows the following splitting:



The magnetic moment of an ion in level n is $\mu_n = \frac{\partial E}{\partial H}$. For an ion with quantum number J=L+S, the energy is $M_J\, g\, \beta\, H$, so $\mu_n = M_J g\beta$. The Boltzmann factor may be used to calculate the average magnetic moment of one ion:

$$\langle \mu_z \rangle = \frac{\sum_{-J}^{J} \mu_n \exp(-E_n/kT)}{\sum_{-J}^{J} \exp(-E_n/kT)} \qquad (I.1)$$

$$\langle \mu_z \rangle = \frac{\sum_{-J}^{J} (-M_J g\beta) \exp(-\eta M_J)}{\sum_{-J}^{J} \exp(-\eta M_J)}$$

where $\eta = g\beta H/kT$.

Now,

$$\sum_{-J}^{J} \exp(-\eta M_J) = [\sinh (J+\tfrac{1}{2})\eta \,/ \sinh(\eta/2)$$

and

$$\sum_{-J}^{J} (-M_J g\beta) \exp(-\eta M_J) = kT \frac{\partial}{\partial H_z} \left[\sum_{-J}^{J} \exp(-M_J \eta) \right]$$

Consequently,

$$\langle \mu_z \rangle = kT \frac{\partial}{\partial H_z} \left\{ \ln \left[\sum_{-J}^{J} \exp(-M_J \eta) \right] \right\}$$

Combining these equations gives

$$\langle \mu_z \rangle = g\beta \{ (J+\tfrac{1}{2}) \coth [(J+\tfrac{1}{2})\eta] - \tfrac{1}{2} \coth (\eta/2) \}$$

$$\langle \mu_z \rangle = g\beta J B_J \eta$$

where $B_J(\eta)$ is the Brillouin function.

The macroscopic magnetic moment for N noninteracting ions is then

$$M = gJ\beta\, B_J(\eta)$$

For large η (high fields or low temperatures), $B_J \simeq 1$ and

$$\chi = \frac{M}{H} = Ng\beta J/H$$

Note that this susceptibility does not depend on the temperature, but is a constant value known as the "saturation moment". This physically corresponds to all ions being in the ground magnetic state.

For small η (low fields and high temperatures),

$$B_J(\eta) \simeq (J+\tfrac{1}{2})\ \eta/3$$

and

$$\chi = Ng^2\ \beta^2\ J(J+1)/3kT = \frac{C}{T} \qquad \text{(I.2)}$$

where $C=Ng^2\beta^2J(J+1)/3k$. Equation I.2 is known as the Curie Law.

Another common expression of the Curie Law is called the spin-only formula for the susceptibility:

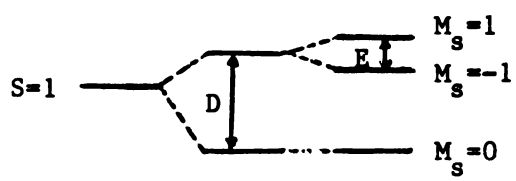
$$\chi = Ng^2\ \beta^2\ S(S+1)/3kT = N\ \beta^2\ \mu_{eff}^2/3kT$$

where $\mu_{eff}^2 = g^2\ S(S+1)$

This equation applys for many complexes of ions of the iron series, such as heme proteins, because their orbital angular momentum is quenched (L=0) by the ligand field.

B.4 Zero-Field Splitting

Spin-orbit interactions may partially lift the degeneracy of the ground state, leaving a set of spin states. This splitting is commonly called zero-field splitting because it occurs in the absence of an external magnetic field. It is characterized by the axial and rhombic zero-field splitting factors, D and E, respectively:



The effect of this splitting on the susceptibility becomes most apparent as kT approaches the energy difference between the split states.

For many heme proteins D≈10 cm^{-1} (Tasaki et.al., 1966; Uenoyama et.al., 1968; Behere et.al., 1979). Since the symmetry of a heme approaches axial, the rhombic splitting factor, E tends to be much smaller and the spin Hamiltonian $H = D S_z^2$ is sufficient to represent the fine structure of the electronic ground state of many heme proteins.

Although the average susceptibility is affected by the zero-field splitting, it is not the best parameter to use to determine the zero-field splitting. If crystals of the material in question are available, measurements of the magnetic anisotropy provide a better value.

Some heme proteins have been found to have magnetic behavior that deviates from the Curie Law at high temperatures. The magnetic susceptibility of cytochrome c peroxidase, for example, deviates form the Curie Law behavior above 170 K. Its μ^2_{eff} was found to vary from 5 to 35 (Izuka, 1968). These are the values expected from pure low- and high-spin behavior d^5 ions. The behavior of the system matches that expected if there is a thermal equilibrium between low and high spin states.

B.5 Van Vleck Equation

An equation for the susceptibility which includes field dependent effects on the energies can be derived by returning to Equation I.1. Assume that the energy can be expanded as a power series in the field:

$$E_n = E_n^{(0)} + H E_n^{(1)} + H E_n^{(2)} + \ldots$$

Then

$$\exp(-E_n/kT) \approx (1-E_n^{(1)}/kT) \exp(-E_n^{(0)})$$

and

$$\mu_n = -\partial E/\partial H = -E_n^{(1)} - 2HE_n^{(2)}$$

Substitution of these into Equation I.1, expansion of the exponentials, and elimination of terms involving powers of H larger than one (except for the exponential involving E_n) gives the Van Vleck equation:

$$\mu_n = \frac{N \sum_n (-E_n^{(1)} - 2HE_n^{(2)}) (1-HE_n^{(1)}/kT)}{\sum_n \exp(-E_n^{(0)} / kT) (1-HE_n^{(1)} / kT)}$$

where $E_n^{(0)}$ is the zero-field energy and $E_n^{(1)}$ and $E_n^{(2)}$ are the first and second order Zeeman terms, respectively.

B.5 Intramolecular Effects

While the size of enzyme molecules often prevents intermolecular interaction between metal ions, the ions within a molecule may be close enough for their spins to interact. One way that spin-spin coupling can occur is by direct contact of the orbitals on adjacent metal ions. The Pauli principle then requires that the spins be aligned antiparallel, so the coupling is antiferromagnetic. Another mechanism of spin-spin coupling is that the orbitals of the metal, which have unpaired electrons, overlap with filled orbitals of bridging atoms and the unpaired electrons are thus delocalized. This is referred to as superexchange, and the resultant coupling can be either ferromagnetic or antiferromagnetic.

The spin interaction between two metal ions with spins S_1 and S_2 can be described by the Hamiltonian $\hat{H} = -2J\,\vec{S}_1 \cdot \vec{S}_2$ where J in energy units is called the exchange coupling constant and is different than the quantum number J. With this Hamiltonian an antiferromagnetic interaction leads to a negative J and a ferromagnetic interaction leads to a positive J. Using the relationships

$$\vec{S}_1 \cdot \vec{S}_2 = \tfrac{1}{2}(S^2 - S_1^2 - S_2^2) \qquad \text{and} \qquad S^2 = S(S+1)$$

a system with $S_1 = S_2 = \tfrac{1}{2}$ and antiferromagnetic coupling has an energy of 3J/2 for the S=0 level and and energy of -J/2 for the S=1 level. The energy difference between the levels is thus 2J. Substituting these energies into the Van Vleck equation gives, upon rearrangement,

$$\chi = \frac{2Ng^2\beta^2}{3kT}\left[1 + \frac{1}{3}\exp\left(-2J/kT\right)\right]^{-1}$$

This equation does not consider the effects of any zero-field splitting which may be present.

CHAPTER 2

MATERIALS AND METHODS

A. Materials

The beef hearts used to isolate cytochrome oxidase were obtained fresh from Van Alstine's Packing House in Okemos, Michigan. Phenazine methosulfate (MPMS), β-nicotinamide adenine dinucleotide (NADH), cholic acid, N-2-hydroxyethyl piperazine N-2-ethane sulfonic acid (HEPES), polyoxyethylene sorbitan monolaurate (Tween-20) and Triton X-114 were obtained from Sigma Chemical Company.

Argon was purified by passing it through a 50 cm BASF R3-11 catalyst column at 100 °C. Cholic acid was purified by recrystallization from 95% ethanol. The crystals were mixed with equivalent amounts of potassium hydroxide to give a 20% (w/v) solution in cholate ion. Hydrochloric acid was used to adjust the pH to 8.0. Tween-20 and Triton X-114 were kept refrigerated as 20% (v/v) solutions. All other reagents were of analytical grade and were used without further purification.

B. Stopped-flow Experiments

B.1 Stopped-flow system

The stopped-flow system used a double-beam, vacuum-tight, rapid-scanning stopped-flow spectrophotometer (Papadakis et.al., 1975; Coolen et.al., 1975). Up to three solution bottles were attached to each side of the system at the top of burets. Liquids flowed from the burets into a reservoir containing a magnetic stir bar. Thus, the concentrations of the solutions mixed in the cell could be varied in the reservoir before their introduction into the push syringes. The scanning collection mode was used to follow absorption changes at several wavelengths during a single mix of solutions within the cell. When better time resolution was desired, data were collected in the fixed wavelength mode. Detailed descriptions of the stopped-flow system are given in the Ph.D. dissertations of N. Papadakis and R.B. Coolen. Details of the periodic checks run on the system are given in the Ph.D. dissertation of F. Halaka.

B.2 Data Handling

Data were collected with a PDP 8/I or PDP 8/E computer interfaced to the stopped-flow system, then transferred to the MSU Cyber 750 for analyses. Correction was made for finite scan times, channel numbers were converted to

wavelength, and voltages were changed to absorption values by using programs described in Apendix E of R. Cochran's Ph.D. dissertation. The resulting time-absorption-wavelength data were fit by using the nonlinear least-squares fitting program KINFIT4, a modified version of KINFIT (Dye and Nicely, 1971).

B.3 Deoxygenation

All solutions were deoxygenated by alternately evacuating their bottles and allowing purified argon to equilibrate with the solutions. This was repeated six to ten times and then 3 psig of argon was introduced. The sealed bottles of buffer, MPH, and water were attached to the stopped-flow apparatus with 5 mm Fischer-Porter solv-seal joints.

After the bottles of solutions (except the enzyme) were attached, the stopped-flow system was made anaerobic by evacuation to 100 microns pressure four times, with each evacuation followed by an equilibration with purified argon at 3 psig. The system was then evacuated to less than 1 micron pressure and water was allowed to enter the cell. Then 1-2 psig of argon was introduced to prevent foaming of the detergent-containing solutions when they were allowed to flow into the burets.

The anaerobicity of the stopped-flow and the buffers was checked by monitoring the MPH spectrum for several

minutes. Any oxygen present would have reacted with this species, oxidized it, and produced an increase in the absorbance at 388 nm. After anaerobicity checks and calibrations, the liquid-containing parts of the system were isolated with Kontes valves and the enzyme bottle was attached to the system. The part of the system not isolated was then evacuated and filled with argon several times. The enzyme bottle was not hung until this point of the experiment so it could be kept cold as long as possible.

B.4 MPH Preparation

MPH was prepared by anaerobically titrating MPMS with NADH by using the bottle shown in Figure 2.1. Because MPH showed an instability when kept in HEPES buffer overnight, no buffer was used in its solutions. NADH was added with a calibrated Hamilton gas-tight syringe. The cell on the bottle allowed the reduction of MPMS to be monitored with a Cary 17 spectrophotometer. NADH was added until only a shoulder remained at 388 nm; this shoulder indicated that a small amount of MPMS remained, ensuring that no excess NADH was present. A typical set of titration spectra is shown in Figure 2.2. The concentration of MPH was calculated by using $\Delta\varepsilon_{388} = 21.0\ mM^{-1}\ cm^{-1}$ (Halaka, unpublished). The MPH was allowed to stand overnight before the final spectrum was taken.

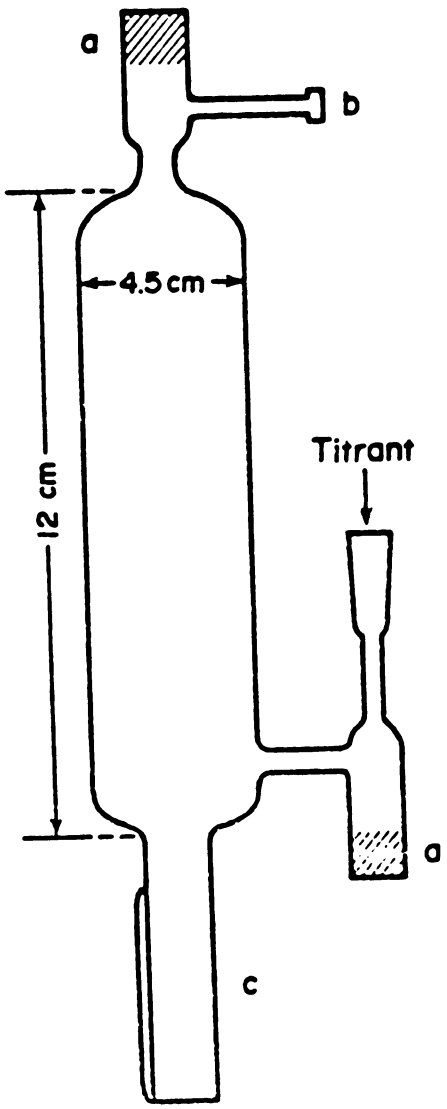


Figure II.1 A Schematic of the Bottle Used for the Anaerobic Titration of MPMS with NADH. (a) Kontes valve (b) Fischer-Porter solv-seal joint (c) one centimeter quartz cell (from Halaka, 1981)

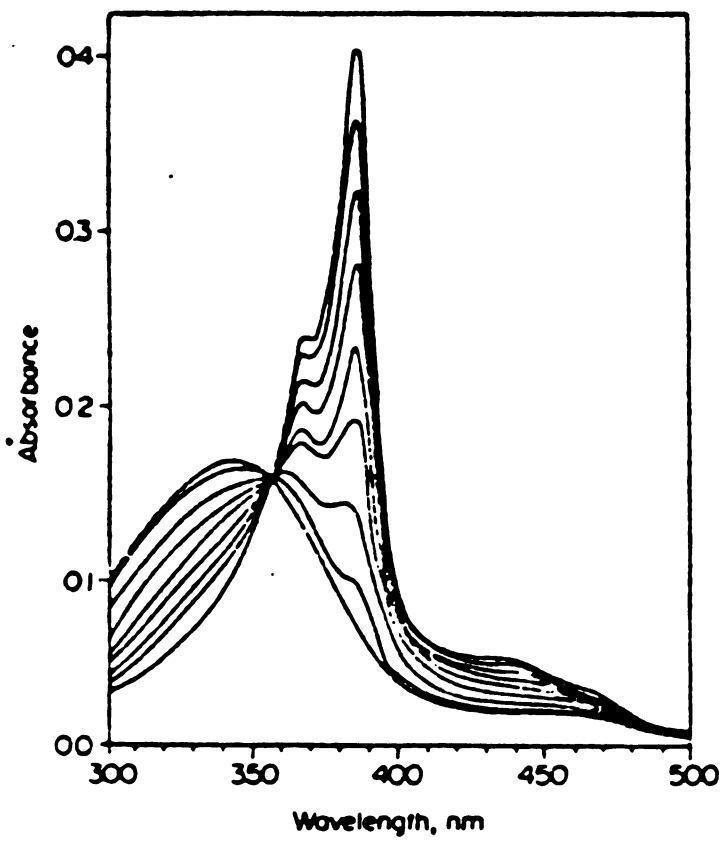


Figure II.2 The Spectra of an Anaerobic Titration of MPMS with NADH (from Halaka, 1981)

All bottles and burets used to handle MPH or MPMS were wrapped in aluminum foil due to the photosensitivity of these compounds. Room lights were turned off during the experiment. There are no windows in the room.

B.5 Enzyme Preparation

The cytochrome oxidase used in stopped-flow experiments was isolated from the membrane by the method of Hartzell and Beinert (1975). During all steps the enzyme was kept between 0 °C and 10 °C. The final pellet was solubilized in 1-2 mL of 50 mM HEPES buffer (pH=7.4) containing 0.5% Tween-20. The enzyme solution was stored in liquid nitrogen until the day of the experiment, when it was diluted with more of the same buffer. For the reduced enzyme, the ratio of the absorbance of the reduced enzyme at 444 nm to that at 420 nm was at least 2.4 (Gibson et.al., 1965).

C. Magnetic Susceptibility Experiments

Magnetic susceptibilities were measured with an S.H.E. Corporation Variable Temperature Susceptometer, which utilizes superconducting quantum interference devices (SQUID) as a basis for its measuring system. Data were taken at 7.00 kG over a temperature range of 2 to 200 K.

Sample holders were made from either an aluminum-silicon alloy obtained from S.H.E. Corporation or from

poly(monochlorotrifluoroethylene), whose trade name is Kel-F. The Kel-F sample holders were cleaned in boiling nitric acid before use.

The enzyme used was prepared either by the method of Hartzell and Beinert (1975) or Yonetani (1966). The latter was a gift from P. Moroney. Hartzell and Beinert enzyme was in 50 mM HEPES solution containing 0.5% Tween-20. Yonetani enzyme was in 100 mM phosphate buffer containing Tween-80 or in Tween-20.

Because oxygen is paramagnetic, the enzyme and buffer samples were made anaerobic before they were frozen. This was done by one of two methods: (1) a series of evacuations of the sample bottle, each followed by argon equilibration, or (2) addition of small amounts of glucose and glucose oxidase to the sample while it was in a helium-flushed glovebag. After the oxygen had been removed, the samples were frozen and stored in liquid nitrogen.

CHAPTER III

STOPPED-FLOW KINETICS OF CYTOCHROME OXIDASE

A. Concentration Dependence of the Cu_A Reaction

Several laboratories (Wilson et.al., 1975; Gibson et.al., 1965; Antalis and Palmer, 1982) have demonstrated that electrons are donated to at least two sites during the rapid initial phase of the reaction between oxidized cytochrome oxidase and ferrous cytochrome c. Wilson et.al. (1975) showed that a decay of the 830 nm band that is assigned to Cu_A occurs during the initial rapid phase of that reaction. The 605 and 444 nm bands also change during this phase, an indication of cytochrome a^{3+} reduction. The decay at 830 nm either lagged or was simultaneous with formation of the 605 nm band. It has been commonly postulated that electrons enter through the cytochrome a, which is then in rapid equilibrium with Cu_A. It has also been suggested several times (Wikstr m and Casey, 1985; Capaldi, 1983; Azzi, 1980) that Cu_A may be the site where some or all of the electrons enter the enzyme.

When the enzyme is reduced with MPH the decay at 830 nm lags the growth of absorbance at 605 nm; this led Halaka (1981) to propose that Cu_A receives its electrons via cytochrome a.

Halaka demonstrated this lag in the decay at 830 nm at only one MPH concentration, so the experiment described here was designed to do two things: (1) to see if this lag is reproducible at other MPH concentrations, and (2) to see whether the rate constant obtained from the absorbance change at 830 nm depends on the reductant concentration. The existence of such a dependence would indicate that the MPH was reacting at the Cu_A site rather than only at the cytochrome *a* site. The absorbance change at 605 nm was also followed.

The typical absorbance decrease at 830 nm during the first 300 ms is shown in Figure III.1. The lag in absorbance is evident at the two MPH concentrations used, showing that the presence of the lag does not depend on reductant concentration.

The scheme that Halaka proposed to account for the lag at 830 nm was

$$\underset{(A)}{MPH + [\text{cyt } a^{3+} \quad Cu_A]} \xrightarrow{k_1} \underset{(B)}{MP^{+}[\text{cyt } a^{2+} \quad Cu_A^{2+}]} \xrightarrow{k_2} \underset{(C)}{MP^{+} + [\text{cyt } a^{2+} \quad Cu_A^{+}]}$$

The concentration of B+C at time t is given by

$$([B]+[C]) = \frac{L_o\,(1-\exp(-kt))}{R-\exp(-kt)} \qquad \text{(III.1)}$$

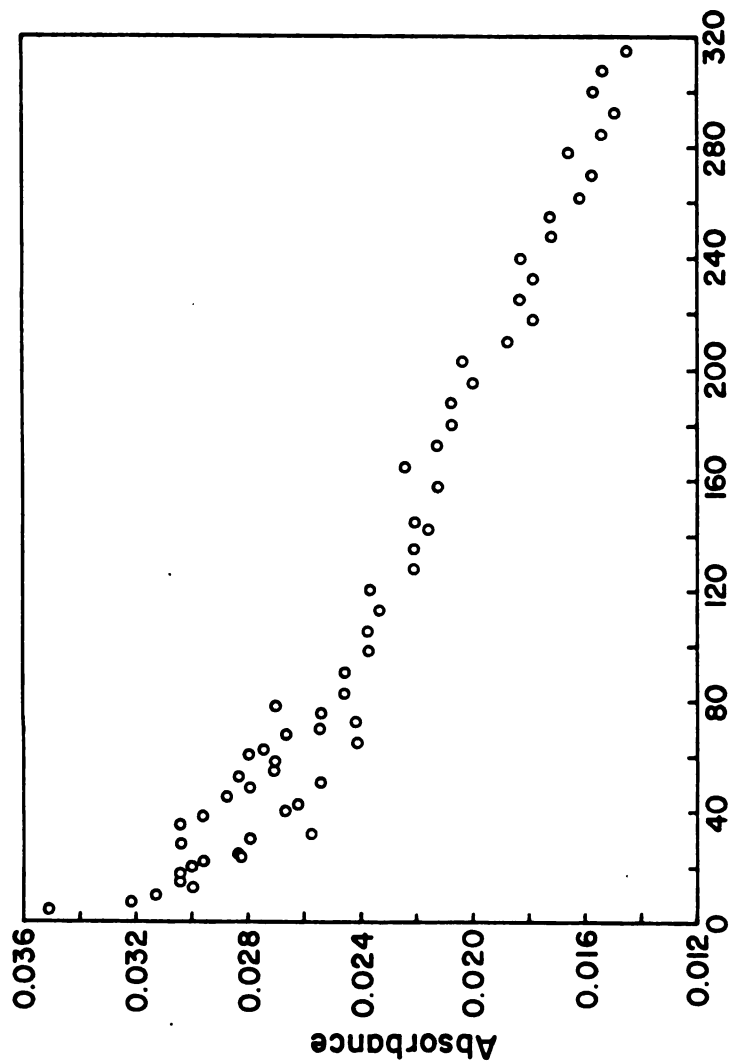


Figure III.1 The Absorption Change at 830 nm During the Reduction of Cytochrome Oxidase with MPH. $[aa_3]$=16.5 μM [MPH]= 55.0 μM

where R is $[L]_o/[M]_o$, $[L]_o$ and $[M]_o$ are the initial concentrations of MPH and cytochrome oxidase respectively, and k is $k_1([L]_o-[M]_o)$. The rate of change of absorbance at 830 nm is proportional to the production of C,

$$dC/dt = k_2(([B] + [C]) - [C])$$

The right side of Equation III.1 was substituted for ([B] + [C]) above and the resulting equation was solved numerically with the value of k_2 being adjusted.

A typical fit of the equation to the data is given in Figure III.2. The residuals from this fit are given in Figure III.3. Their randomness indicates that the equation fits the data and that the above mechanism, where Cu_A receives its electrons via cytochrome a, is sufficient to explain the data of this experiment.

The values found for k_2 are given in Table III.1 Examination of these values shows that the rate constants exhibit no dependence on the concentration of MPH, again indicating that Cu_A does not receive its electrons directly from the reductant.

The initial part of the reaction was also followed through the absorbance change at 605 nm. The first step in the above reaction scheme was used to develop the following second-order rate equation that related absorbances and the initial concentrations of reactants:

$$A_t = (A_\infty-A_o)\ \{[1-\exp(-k't)]/[1-\exp(-k't)/R]\} + A_o$$

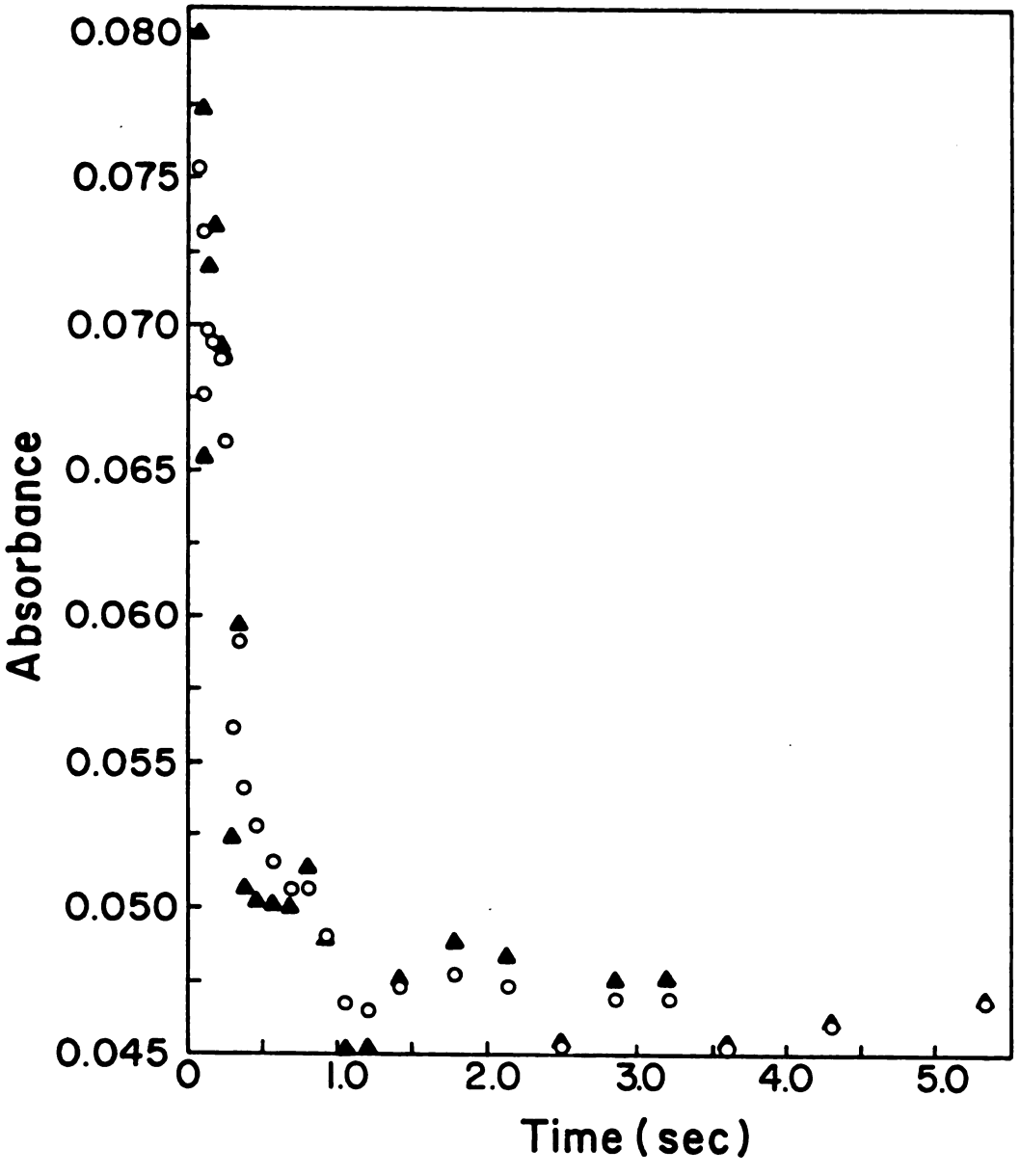


Figure III.2 The Calculated and Experimental Absorbances at 830 nm for the Reduction of Cytochrome Oxidase with MPH. o calculated, ▲ experimental, $[aa_3]$=16.5 μM, [MPH]= 80.2 μM

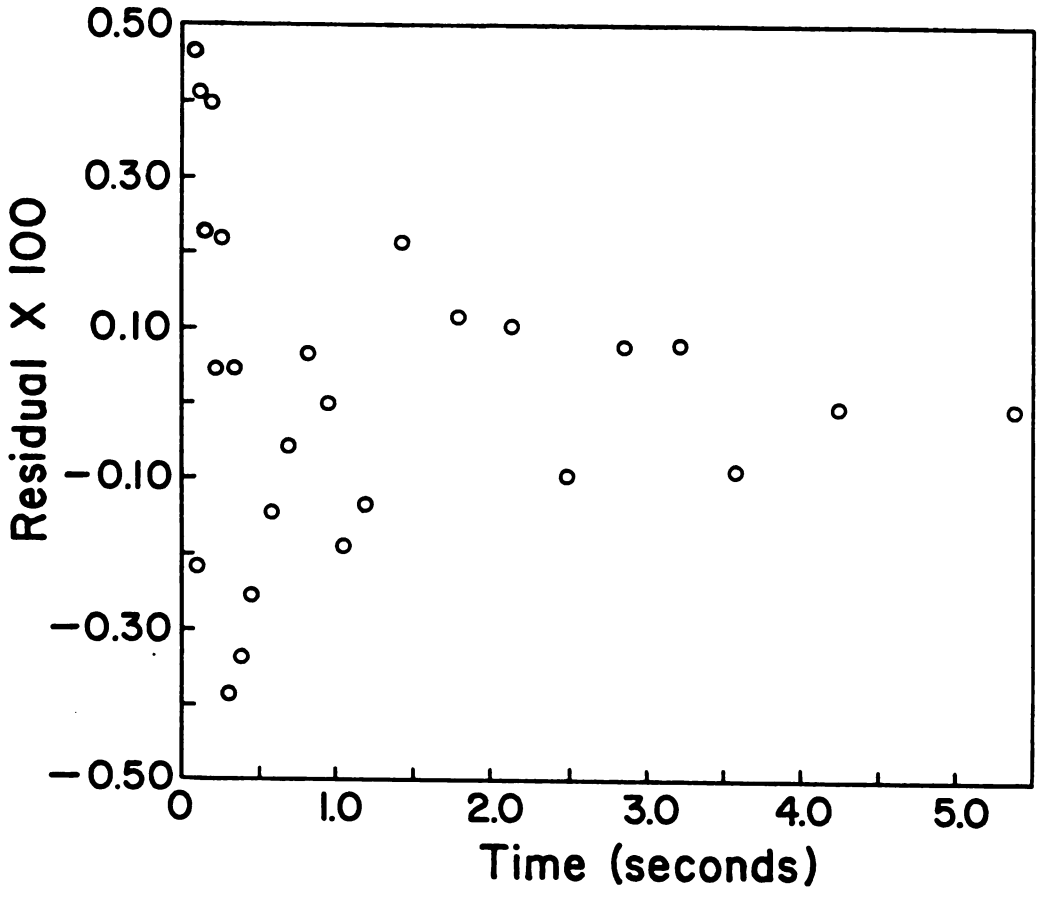


Figure III.3 The Residuals of the Calculated Absorptions at 830 nm for the Reduction of Cytochrome Oxidase with MPH. $[aa_3]$=16.5 μM, [MPH]=80.2 μM

Table III.1 Rate Constants For the Initial Phase of the Reaction of MPH and Cytochrome Oxidase at Varying MPH Concentrations

[MPH], μM	[aa_3], μM	$k_1 \times 10^5$ M^{-1} s^{-1} *	k_2 s^{-1}**
27.8	8.2	1.10±0.04	
40.1	8.2	1.07±0.02	
40.1	8.2	1.10±0.05	
11.2	30.0	2.8±0.3***	
40.1	8.2		11.8±0.5
40.1	8.2		13.4±2.3
40.1	8.2		13.4±2.5
27.8	8.2		17.0±4.9
27.8	8.2		12.2±0.4
10.8	27		17.8±0.5***

* from data at 605 nm

** from data at 830 nm

*** from Halaka (1981)

where A_t, A_0, and A_∞ are the absorbances at time t, zero, and at the end of the phase, respectively, k' is $k_1(L_0-M_0)$ and R is L_0/M_0. Since this second-order equation fit the initial absorbance changes, the MPH is reacting at cytochrome a. The fit of this equation to the data is given in Figure III.4 , and the residuals of this fit are given in Figure III.5. The rate constants found were about one-third of those found by Halaka, apparently indicating a difference in the enzyme preparations.

B. Detergent Effects on Kinetics

The steady-state activity of soluble cytochrome oxidase varies with the type of nonionic detergent present. The identity of the detergent head group affects the activity. Rosevear et.al.(1980) found that cytochrome oxidase was two to ten times more active in lauryl maltoside than in Tween-20, though the two detergents have identical fatty acid chains. Robinson et.al. (1985) used a variety of types of detergents and found that the electron transport activity of the enzyme depended on both the head group and the fatty acid chain of the detergent present.

Cytochrome oxidase as isolated is heterogeneous. None of its soluble forms are as active as the membrane-bound enzyme. The more active forms of the solubilized enzyme may be structurally more similar to the enzyme in the membrane.

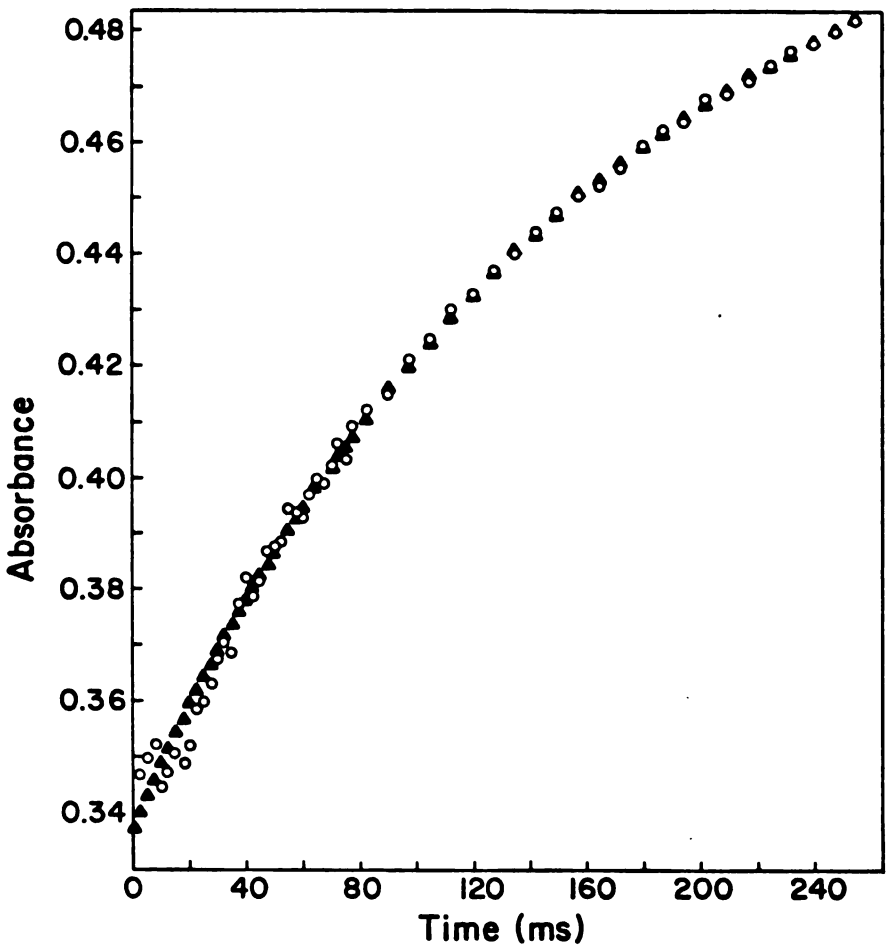


Figure III.4 The Calculated and Experimental Absorbances at 605 nm for the Reduction of Cytochrome Oxidase with MPH. $[aa_3]$=16.5 μM, [MPH]=55.0 μM

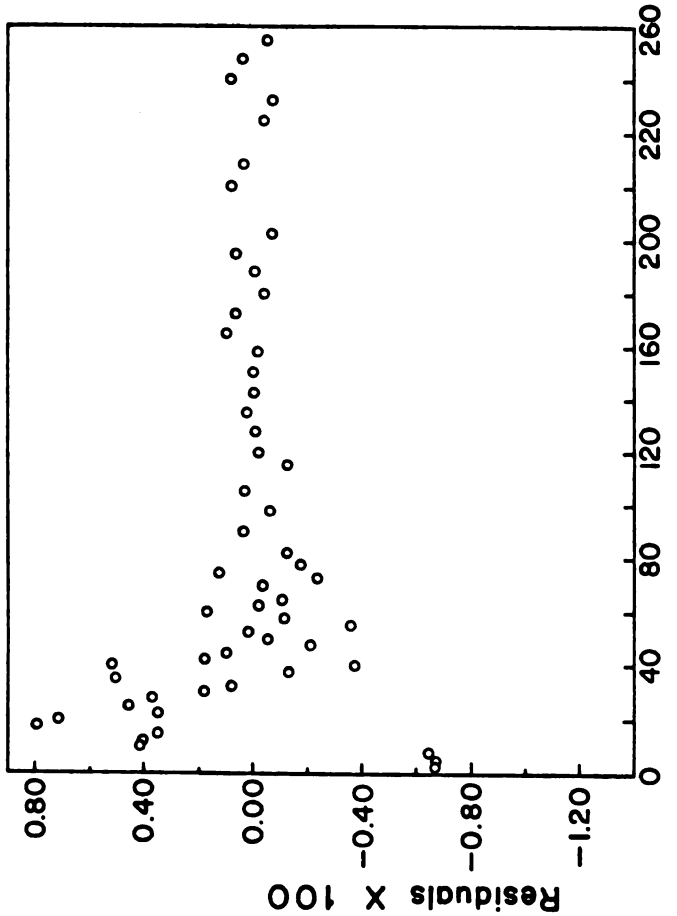


Figure III.5 Residuals of the Calculated Absorbances at 605 nm for the Reduction of Cytochrome Oxidase with MPH. $[aa_3]$=16.5 μM, [MPH]=55.0 μM

If the steady-state activity of an enzyme form is higher, then one or more steps in its turnover reaction must occur at a faster rate. Determination of which step is faster would give clues about the enzyme structure of the more active form.

The stopped-flow experiment described here was designed to see which step in the reaction is accelerated when the enzyme is reduced in the presence of lauryl maltoside. The enzyme solutions contained either 0.5% lauryl maltoside or 0.5% Tween-20. The kinetics of the anaerobic reduction of cytochrome oxidase by 5-methylphenazinium methylsulfate (MPH) was studied. MPH was used as the reductant because its oxidation is easily followed through changes in its optical spectrum and its reduction potential is low enough that it will completely reduce cytochrome oxidase under anaerobic conditions.

B.1 Spectral Changes and the Reaction

Spectral changes in the 340-500 nm region were followed while the enzyme was reduced by MPH. Oxidation of MPH led to an increase in absorbance at 388 nm, while reduction of cytochrome oxidase led to a decrease in absorbance at 420 nm and an increase in absorbance at 444 nm. The first and last spectra collected are shown in Figure III.6.

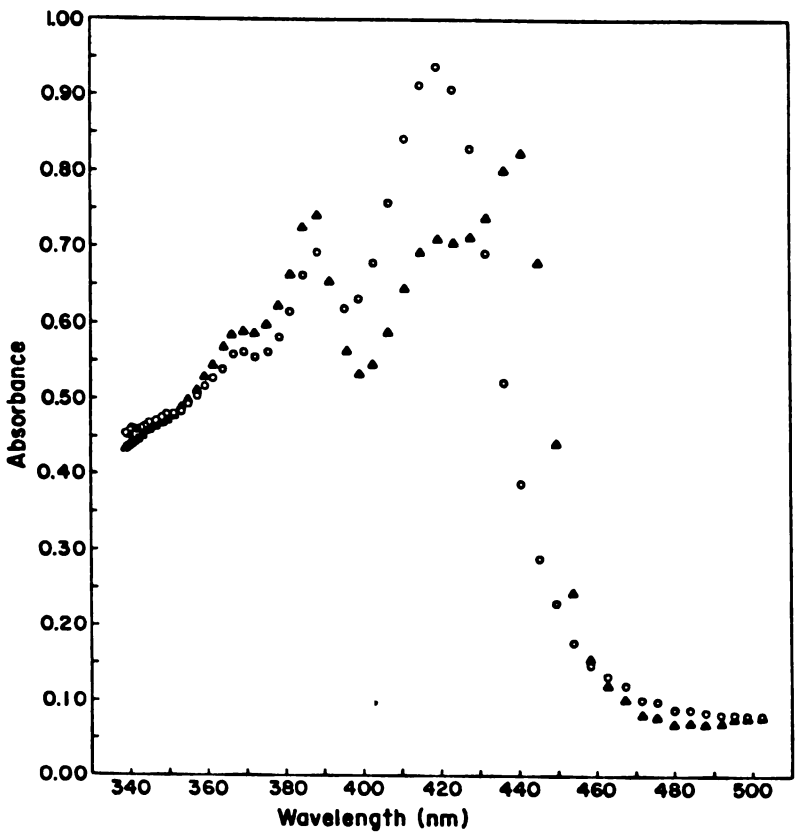


Figure III.6 The Spectra from the Partial Reduction of Cytochrome Oxidase with MPH in the Presence of Lauryl Maltoside. o first spectrum, ▲ last spectrum

It was shown by Halaka (1981) that the overall stoichiometry of the reaction of MPH and oxidized cytochrome oxidase, in the prescence of Tween-20, is

$$2\ MPH + \text{cytochrome oxidase (oxidized)} \longrightarrow 2\ MP^{+} + \text{cytochrome oxidase (reduced)}.$$

Halaka also found that the reaction was triphasic when carried out in Tween-20. The initial, fast phase was first order in both reactants. The two phases that followed were both slower, first order in enzyme, and zero order in MPH. The same triphasic behavior was observed when the enzyme was reduced by MPH in the prescence of lauryl maltoside instead of Tween-20.

B.2 Data Analysis

A second-order rate equation that related absorbances and the initial concentrations of reactants was used to do the nonlinear least-squares fitting. The equation was derived for the second-order reaction

$$L + M \xrightarrow{k} \text{products}$$

If x is the fraction of the reaction remaining, then the rate of the reaction may be expressed by

$$dx/dt = -kM_o(R-1+x)$$

where L_o and M_o are initial concentrations of L and M and $R=L_o/M_o$. Integration of this equation gives

$$\ln [x/(R-1+x)] = -k't + q$$

so

$$x = [(R-1)\exp(-k't+q)]/[1-\exp(-k't+q)] \qquad \text{(III.2)}$$

where $k' = k(R-1)M_0$ and q is the product of (R-1) and the integration constant. The fraction of reaction left, x, may also be expressed in terms of absorbances by

$$x = 1 - (A_t-A_0)/(A_\infty-A_0),$$

where A_t, A_0, and A_∞ are the absorbances at times t, 0, and at the end of the phase. Setting this equal to the right-hand side of Equation III.2 and solving for A_t gives

$$A_t = (A_\infty-A_0)\{[1-\exp(-k't)]/[1-\exp(-k't)/R]\} + A_0$$

(after the integration constant is evaluated by using $A_t=A_0$ at t=0).

Figure III.7 shows a typical fit of this second order equation to the data collected during the initial phase of the reduction. The randomness of the residuals indicates that the equation used fits the data. The second-order rate constant obtained was $5.74 \times 10^5\ M^{-1}\ s^{-1}$ (Table III.2).

The last two phases were fit by the equation representing two first order processes:

$$A_t = A_\infty - \Delta_1\exp(-k_1t) - \Delta_2\exp(-k_2t).$$

The fit of this equation to the data is given in Figure III.8. Again, the residuals are small and random, indicating the equation fits the data. The two rate constants obtained were $0.031\pm.002\ s^{-1}$ and $0.0021\pm.0003\ s^{-1}$ (Table III.2).

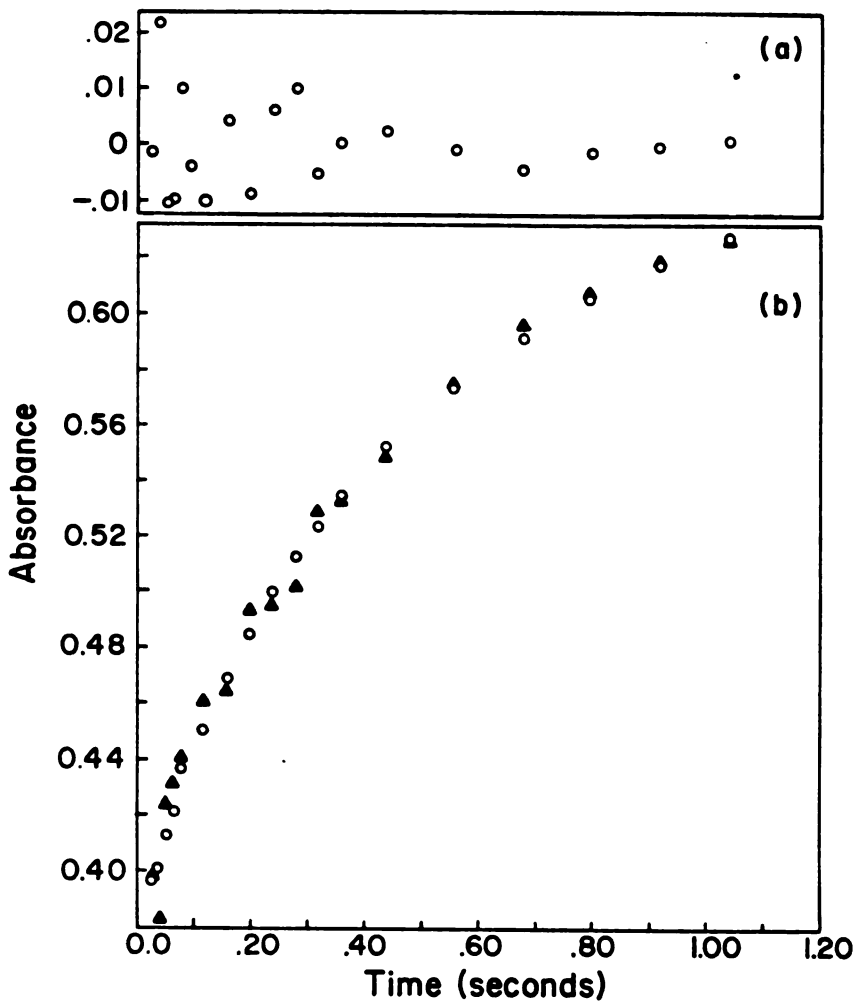


Figure III.7 The Calculated and Experimental Absorbances at 444 nm for the First Phase of Reduction of Cytochrome Oxidase with MPH in the Presence of Lauryl Maltoside. [aa_3]=3.0 μM [MPH]=2.7 μM

Table III.2 Rate Constants for the Anaerobic Reaction of MPH and Cytochrome Oxidase in the Presence of Lauryl Maltoside and Tween-20.

Second Order Phase	First Order Phases	
$k \times 10^5$ M^{-1} s^{-1}	k_1 s^{-1}	k_2 s^{-1}
5.7±0.5 *	0.036±0.002 *	0.0028±0.0003*
7.3±0.8 *	0.031±0.002 *	0.0021±0.0003*
5.5±0.5 **	0.031±0.004 **	0.0041±0.0007**
7.±2. **	0.05±0.01 **	0.0014±0.0008**
4.9±0.5 **	0.030±0.002 **	0.0028±0.0004**

* in lauryl maltoside

** in Tween-20

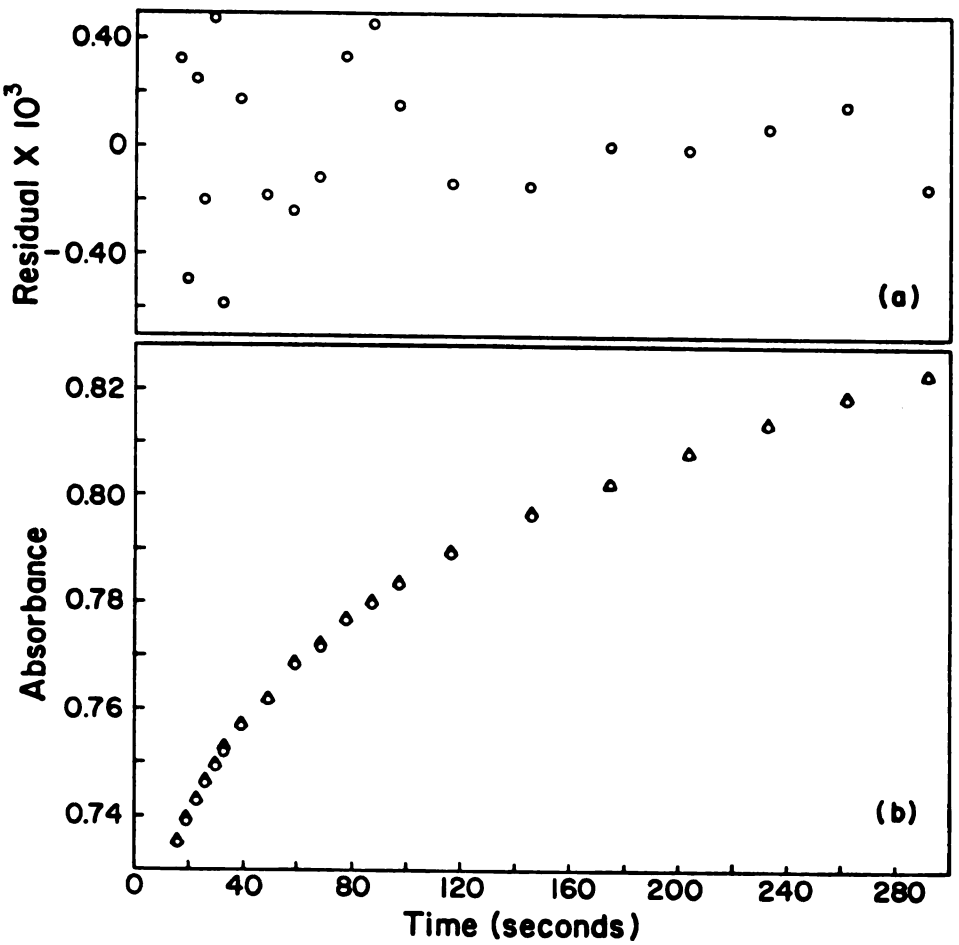


Figure III.8 The Calculated and Experimental Absorbances at 444 nm for the Final Phases of the Reduction of Cytochrome Oxidase with MPH in the Presence of Lauryl Maltoside. $[aa_3]$=2.8 μM, [MPH]=3.2 μM

The corresponding rate constants found for the three phases of the reaction between this preparation of the enzyme and MPH, carried out in Tween-20, are also given in Table III.2.

B.3 Discussion

There is no significant difference in the rate constants obtained when the enzyme is in Tween-20 and when the enzyme is in lauryl maltoside. This indicates that either (1) the step that is faster during turnover in lauryl maltoside is not one of the steps that can be observed at 444 or 605 nm, or (2) the enzyme converts to the oxygenated form during turnover and it is this form which has a higher activity in lauryl maltoside.

C. The Reduction of Oxygenated Cytochrome Oxidase

C.1 Procedure

Anaerobic solutions of 104 μM MPH and 23.1 μM oxidized cytochrome oxidase were mixed in a 2:1 volume ratio in the reservoir on one side of the stopped-flow instrument. Aerobic buffer was prepared in the reservoir on the other side of the instrument by mixing anaerobic and oxygen-saturated 50 mM HEPES buffer. All solutions contained 0.5% Tween-20. The enzyme solution was 150 mM in HEPES and the MPH solution had no buffer. 0.5 units of catalase were present per milliliter of enzyme (after

mixing) to prevent formation of peroxide. The proportion of anaerobic and oxygen-saturated buffers was varied during the experiment, resulting in different oxygen concentrations. Approximately ten minutes after the MPH and enzyme were mixed, the resulting solution of reduced enzyme and excess MPH was mixed with the aerobic buffer and the reaction was observed.

C.2 Reaction and Spectral Changes

A typical absorption-time curve at 444 nm is shown in Figure III.9. At the beginning of observation, about 75% of the enzyme was reoxidized, which produced the oxygenated enzyme. An increase in absorption at 444 nm is due to the reduction of cytochromes *a* and a_3. After observation was begun, the absorption initially increased, a plateau was reached, and then the absorption increase continued.

Before the plateau was established, electrons were entering the enzyme faster than they were leaving it. The absorbance increase after the plateau is due to continued reduction of the enzyme upon deletion of the oxygen. For the plateau to exist, the change in the sum of the concentrations of reduced cytochromes *a* and a_3 must be small. This implies that, during the plateau period, electrons entered the cytochrome *a* site at approximately the same rate as that at which they left the enzyme. The steps that occur after cytochrome *a* reduction are electron

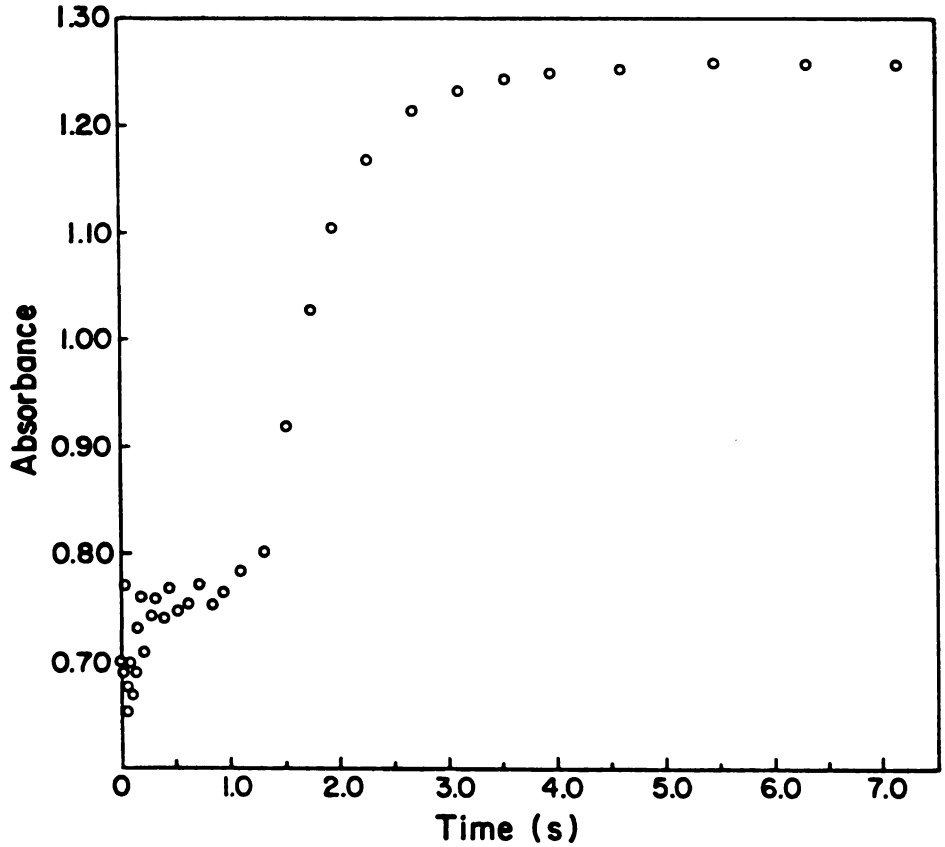


Figure III.9 The Change in Absorbance at 444 nm During the Reduction of Oxygenated Cytochrome Oxidase. $[aa_3]$=3.8 μM, [MPH]=13.0 μM

transfer through the enzyme, in one or several steps, and the transfer of electrons to oxygen. During the plateau period, one of these steps was occurring at the same rate at which electrons were entering the enzyme, resulting in the establishment of an approximate steady state.

If the oxygen reaction had been slower than the intramolecular electron transfer steps, then the level of oxidation of the enzyme during the steady state would have depended on the oxygen concentration. No such dependence was observed, so an intramolecular electron transfer reaction must be slower than the oxygen reaction.

If that step directly involved cytochrome $\underline{a}^{2+}$ or a species that it transfers electrons to quickly, then

$$d(\text{Absorbance})/dt = d(\underline{a}^{2+})/dt = k_1\,[\text{MPH}][\underline{a}^{3+}] - k_2[\underline{a}^{2+}] \approx 0.$$

Then $k_2 \approx k_1\,[\text{MPH}][\underline{a}^{3+}]/[\underline{a}^{2+}]$. Because there was oxygen present during the plateau period, the concentration of reduced cytochrome $\underline{a}_3$ was small. Since the absorbance level indicates the enzyme was very close to 50% reduced, most of the cytochrome $\underline{a}$ must have been reduced. Thus $[\underline{a}^{3+}]/[\underline{a}^{2+}]$ would have been small and k_2 would be small compared to the pseudo order rate constant for the MPH reaction.

C.3 Data Analyses

The initial data analyses were done in an empirical manner. Although most of the data were collected in scanning mode, fixed wavelength files were used to analyze

the increase in absorbance before the plateau. This was due to the increased time resolution of the fixed-wavelength data and the short time before the plateau. The following one exponential equation was found to fit this initial part of the absorbance increase:

$$A_t = A_0 + (A_\infty - A_0)\ [1 - \exp(-kt)]$$

A_t, A_0, and A_∞ are the absorbances at time t, time zero, and at the end of the phase. The calculated and observed absorbances and the residuals are shown in Figure III.10. The rate constants found, from two data files, were 12±2 s^{-1} and 14±2 s^{-1}. This region would include the changes due to a second-order reaction between MPH and cytochrome a. The rate constants are thus pseudo first-order constants. They correspond to the bimolecular rate constants $4.4\pm0.6 \times 10^5\ M^{-1}\ s^{-1}$ and $5.1\pm0.7 \times 10^5\ M^{-1}\ s^{-1}$, which agree well with the value $3.2\pm0.5 \times 10^5\ M^{-1}\ s^{-1}$ found for this phase of the anaerobic reduction of resting cytochrome oxidase by MPH (Halaka, 1981). It should be noted that the data used do not include those points just prior to the plateau since the oxygen reaction would be expected to be increasingly important as the steady-state phase is approached. Also, the initial absorbance found by fitting the equation indicates that about 20% of the enzyme was not reoxidized within the mixing time. This may be due to the competition between the oxygen and MPH reactions.

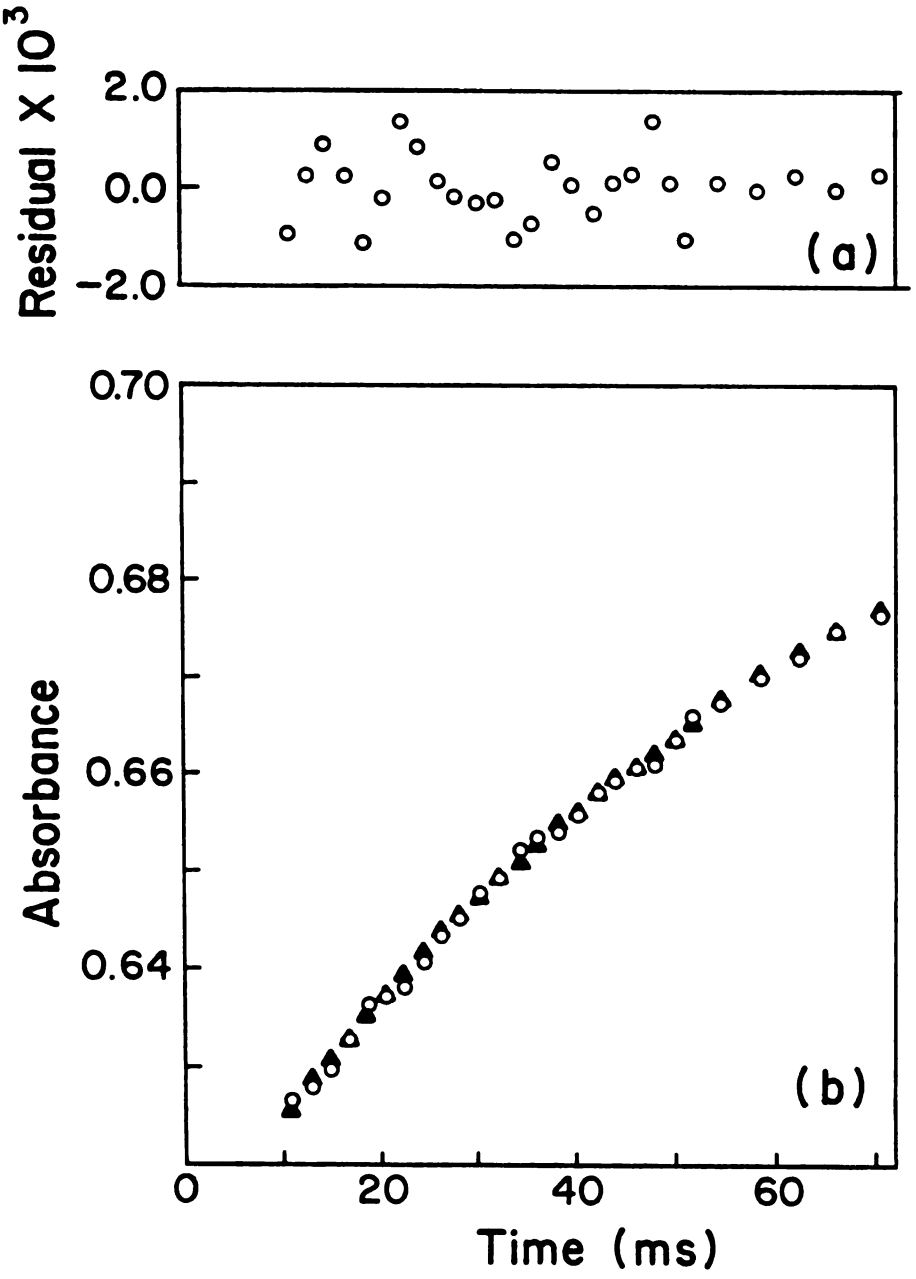


Figure III.10 The Calculated (Δ) and Experimental (o) Absorbances at 444 nm for the Presteady-State Reduction of Oxygenated Cytochrome Oxidase with MPH: Empirical One Exponential Equation. [aa_3]=3.8 μM, [MPH]=26.9 μM

A two exponential equation was fit to the data after the plateau:

$$A_t = A_\infty - \Delta_1 \exp(-k_1 t) - \Delta_2 \exp(-k_2 t)$$

where the absorbances are as defined above, Δ_1 and Δ_2 are parameters proportional to the fraction of the reaction described by each exponential, and k_1 and k_2 are rate constants. A typical fit to data collected in the scanning mode is shown in Figure III.11.

The values of Δ_1 and Δ_2 indicate that the reaction described by the second exponential (k_2) accounts for only one percent of the total absorbance change that occurs after the plateau. The rate constant found for this minor component of the reaction varies from 0.14±0.08 s^{-1} to 0.4±0.1 s^{-1} (Table III.3). The average value of 0.28 s^{-1} is close to the value of 0.2 s^{-1} found by Halaka (1981) for the first-order phase which accounted for the reduction of about fifteen percent of the cytochrome a_3 in the resting enzyme. Since one phase accounts for essentially all of the absorbance change and the rate constant for the minor component is similar to that found for a minor component of the resting enzyme indicate that essentially all of the heterogeneity characteristic of the resting enzyme is absent in the oxygenated enzyme of this experiment.

The rate constant for the predominant phase was found to vary linearly with the concentration of MPH at the end of the plateau. This is shown in Figure III.12. This implies

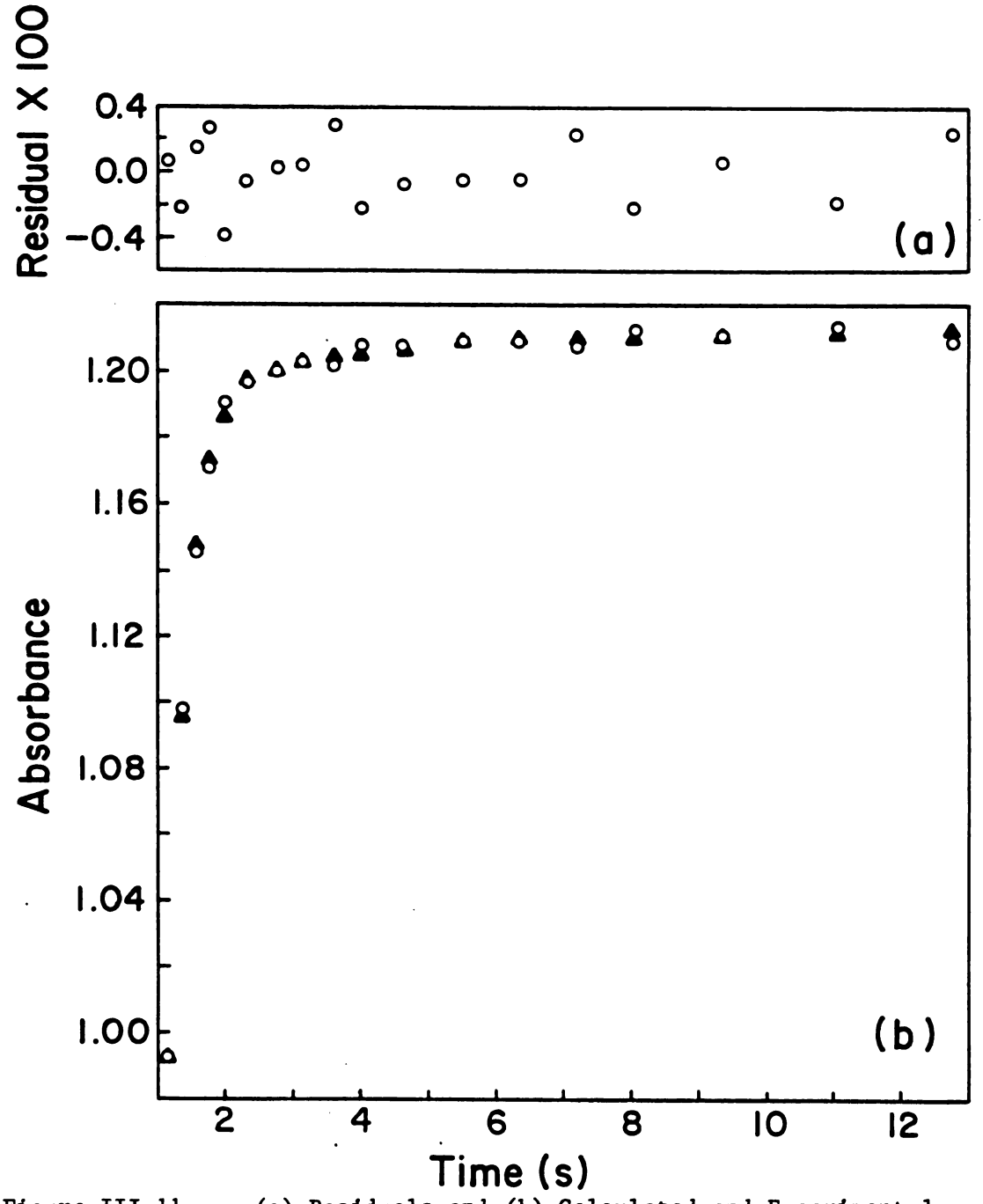


Figure III.11 (a) Residuals and (b) Calculated and Experimental Absorbances at 444 nm for the Poststeady-state Reduction of Oxygenated Cytochrome Oxidase with MPH: Empirical Two Exponential Equation. $[aa_3]$=3.8 μM

Table III.3 Rate Constants for the Reduction of Oxygenated Cytochrome Oxidase with MPH, Post Steady-State.

[MPH]*, μM	k_1 s^{-1}	k_2 s^{-1}
21.4	4.2±0.2	0.27±0.05
20.4	3.9±0.2	0.14±0.08
18.4	3.3±0.2	0.3±0.1
15.4	2.6±0.1	0.37±0.06
14.8	2.6±0.1	0.2±0.2
13.5	2.03±0.09	0.4±0.1
13.0	1.98±0.05	0.29±0.05

* at the end of the plateau

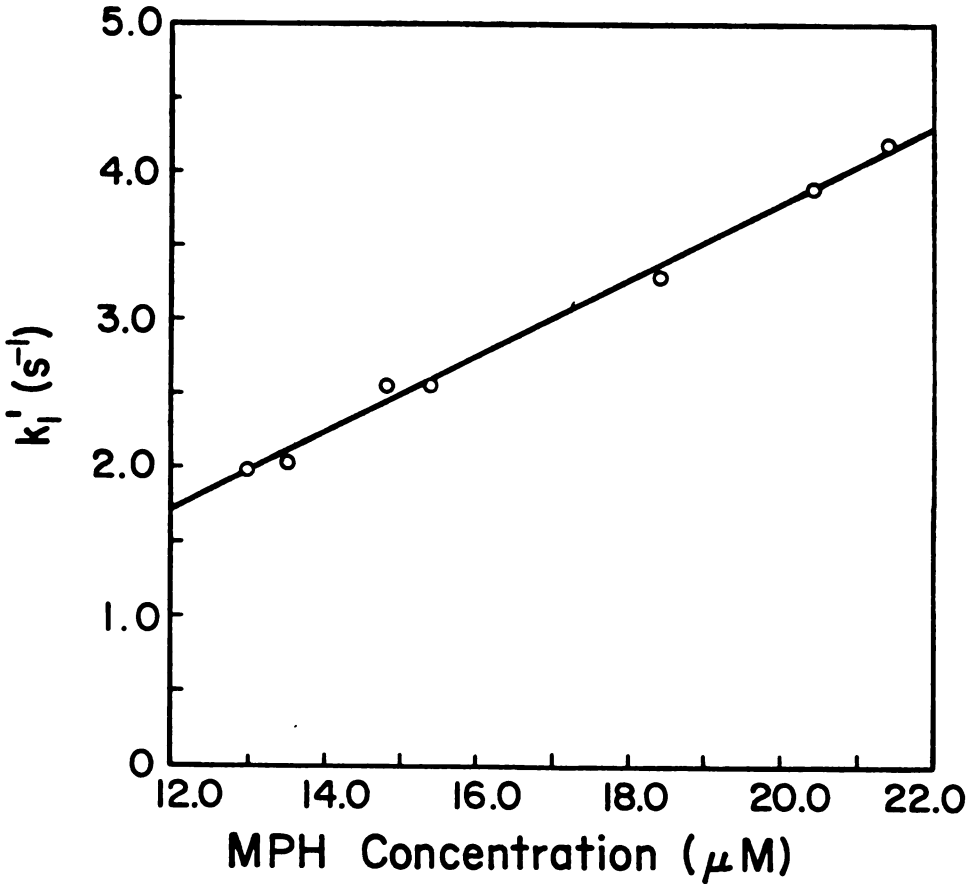


Figure III.12 The MPH Concentration Dependence of the Pseudo First-Order Rate Constant for the Poststeady-state Reduction of Cytochrome Oxidase

that the reaction is pseudo first-order in MPH. The slope of the graph corresponds to the bimolecular rate constant $2.6 \pm 0.1 \times 10^5\ M^{-1}\ s^{-1}$.

In order for the absorbance change after the plateau to be due to the reaction between MPH and cytochrome oxidase, this must be the rate-limiting step. If the intramolecular electron transfer were rate limiting the rate constant would not show an MPH concentration dependence. The rate constant for the intramolecular reaction must thus be greater than the largest pseudo first-order rate constant found, and so is larger than $4.2\ s^{-1}$. This is substantially greater than the value $0.002\ s^{-1}$ found for the intramolecular electron transfer for the dominant species of the resting enzyme (Halaka, 1981).

An attempt was made to find a more accurate value for the intramolecular rate constant. The equations were developed from

$$a^{2+}C^{R+} \xrightarrow{k_2} a^{3+}C^{(R-1)+}$$

$$MPH + a^{3+}C^{(R-1)+} \xrightarrow{k_1} a^{2+}C^{(R-1)+} + MP^{+}$$

It was assumed that the concentrations of completely oxidized enzyme and of reduced C were negligible at the end of the plateau and that the bimolecular reaction was pseudo first-order. The fraction of completely reduced enzyme at time t was defined as x:

$$x = [a^{2+}\ C^{(R-1)+}]_t / [a^{2+}C^{R+}]_o$$

Then x is given by

$$x = 1-k_1' \exp(-k_2 t)/(k_1'-k_2) - k_2 \exp(-k_1' t)/(k_1'-k_2) \quad \text{(III.3)}$$

where $k_1' = k_1[\text{MPH}]$. It may also be written in terms of absorbances:

$$x = (A_t - A_o)/(A_\infty - A_o)$$

where A_t, A_o and A_∞ are the absorbances at time t, time zero (defined as the end of the plateau) and at the end of the reaction, respectively. Then $A_t = (A_\infty - A_o)x + A_o$. Equation III.3 was substituted for x and the result was fit to the data after the plateau. Because the MPH reaction was pseudo order, the fixed wavelength files could be used. This equation did not fit the data as well as the empirical two exponential equation as the residuals were quite systematic (Figure III.13). The data were not sufficient to fit the intramolecular rate constant well. The program would converge for only one data file. The rate constants obtained were 2.42 $\pm$0.04 s^{-1} and 19$\pm$5 s^{-1}. Although the equation is symmetric in the rate constants and the oxygen concentration is not known for this file, the smaller of the two rate constants should be that for the MPH reaction; not only is the MPH reaction expected to be rate-limiting, but the smaller rate constant is in the range of that expected for the MPH reaction based on the results from the data analyses of the scanning files.

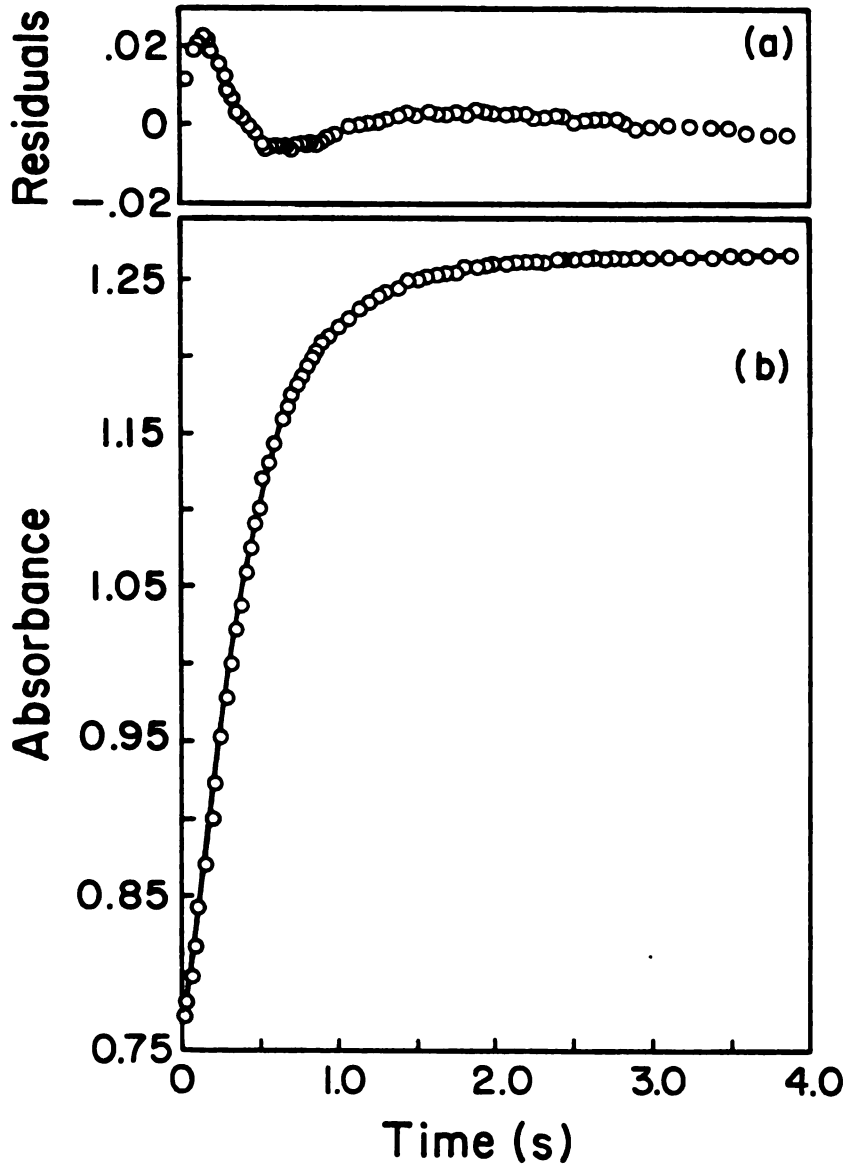


Figure III.13 The (a) Residuals and (b) Calculated ━ and Experimental (o) Absorbances at 444 nm for the Poststeady-state Reduction of Oxygenated Cytochrome Oxidase with MPH: Mechanistic Equation. $[aa_3]$=3.8 μM

C.4 Discussion

In order for the post steady state reaction to be limited by the reductant concentration, the intramolecular electron transfer must occur with a rate constant larger than the highest pseudo-order rate constant found for the post steady-state MPH reaction. That is, the intramolecular electron transfer must have a rate constant larger than 4.2 s^{-1} (Table III.3). The average number of electrons transferred can be calculated from the average rate of oxygen reduction during the plateau, 14 μM/s. The corresponding average electron transfer rate is 14 s^{-1}. The limiting intramolecular electron transfer thus has a rate constant between 4.2 and 14 s^{-1}.

This rate constant cannot be definitively assigned to a specific intramolecular step. If it corresponds to the reduction of cytochrome $\underline{a}_3^{3+}$, then it confirms the literature reports of an acceleration of that rate in the oxygenated enzyme. Peterson and Cox (1980) report a comparable value, 7.5 s^{-1}, for that reaction in the absence of oxygen. Hill and Greenwood (1984) and Greenwood (1967) report a value of 300 s^{-1} for the reduction of cytochrome $\underline{a}_3$ in the presence of oxygen. This large difference in rate constants indicates that the presence of oxygen somehow changes the nature of the $\underline{a}_3$ site.

If the intramolecular rate constant found in this experiment is for the reduction of Cu_A, then it is less than

the value 17.8 s^{-1} found for that reaction in the resting enzyme (Halaka, 1981).

This experiment also confirms that the rate constant for the reduction of cytochrome a in the oxygenated enzyme is not very different than that of the resting enzyme. Another interesting note is that the enzyme was oxidized more than 50% during the flow time, in spite of the presence of excess reductant; yet, by the beginning of the observation time, when the oxygen concentration had been lowered by a larger fraction than had the reductant concentration, the enzyme species present was being reduced by the MPH. This suggests that when the resting enzyme is reduced, an enzyme species is produced which loses its electrons more readily than does the oxygenated form of the enzyme.

CHAPTER IV

THE MAGNETIC STATE OF THE a_3 CENTER IN CYTOCHROME c OXIDASE AND SOME OF ITS DERIVATIVES

A. Survey of the Literature

The current literature concerning the magnetic state of the a_3 center in the resting and oxidized cyanide-bound forms of cytochrome oxidase is conflicting. While it is relatively uniform in stating that the iron and copper ions are magnetically coupled, there is disagreement over the nature of that coupling.

Magnetic circular dichroism (MCD) data (Thomson et.al., 1981) indicate that the ions of the a_3 center of the oxidized cyanide-bound bovine enzyme are ferromagnetically coupled with $J>10\ cm^{-1}$. Mössbauer data for both bacterial and bovine cytochrome oxidase (Kent et.al., 1982) also indicate ferromagnetically coupled ions in the a_3 center of the cyanide-bound enzyme.

Thomson could not comment on the magnetic state of the a_3 center of the resting enzyme as it is MCD silent. Kent found preparation-dependent results for the oxidized bacterial enzyme: the ground state sometimes appeared to be S=0 and sometimes S=1. Mössbauer data for the bovine resting enzyme are somewhat ambiguous due to the small signal-to-noise ratio.

By using magnetic susceptibility measurements, Tweedle et.al. (1978) found that the iron and copper ions of the a_3 center are antiferromagnetically coupled in both the oxidized cyanide-bound and the resting enzyme.

There are several problems with trying to compare the results of these MCD, Mössbauer, and magnetic susceptibility experiments:

(1) Each type of measurement was performed on cytochrome oxidase isolated by a different technique. Yonetani, Yoshikawa and Hartzell-Beinert isolation procedures were used for the enzyme for MCD, Mössbauer, and magnetic susceptibility measurements, respectively. In some cases enzyme isolated by different techniques has shown different reduction (Halaka, 1981; Jones et.al., 1983) and ligand binding properties (Naqui et.al., 1984) at the a_3 center. It is not known if the magnetic properties are affected by the isolation procedure used.

(2) While both Thomson and Tweedle used the dispersing detergent Tween-20, Kent used either Triton X-100 or deoxycholate with the bacterial enzyme and does not report what detergent was used with the bovine oxidase. MCD samples must be optical glasses, so the MCD sample was in a 50% ethylene glycol solvent. Enzyme activity differs with the detergent used (Robinson and Capaldi, 1977; Rosevear et.al., 1980;

Robinson et.al. 1985). For simpler transition metal compounds, small changes in bond angle can strongly influence the nature of the coupling between the metals (Hoard, et.al., 1967). Also, the coupling of the ions in a compound prepared as a model for the a_3 center depended on the solvent used (Gunter et.al., 1984). Thus it can be seen that the environment provided in solution may change the way that the enzyme behaves. Whether the changes in the environment caused by different detergents or by different solvents change the magnetic properties of the enzyme is not known.

(3) To make susceptibility measurements, dissolved oxygen must be removed from the sample. Tweedle et.al. removed it by adding the oxygen scavengers glucose oxidase and glucose to the sample. These scavengers produce hydrogen peroxide, which is known to bind to the enzyme (Bickar et.al., 1982). It is not known if the susceptibility samples prepared in this way are peroxide bound or whether peroxide binding affects the magnetic susceptibility of the enzyme.

The experiments described here were designed to see if the magnetic susceptibility was influenced by the procedural differences described above. Magnetic susceptibility measurements were made on enzyme isolated by

two techniques (Hartzell-Beinert and Yonetani) and in different detergents (Tween-20 and lauryl maltoside), as well as in a 50% ethylene glycol solvent. To check the reproducibility, samples were exchanged with L. Wilson at Rice University, Houston, Texas.

B. Susceptibility Experiments

B.1 Resting Cytochrome Oxidase

The magnetic susceptibility of resting cytochrome oxidase was measured at temperatures from about 2 to 180 K. Above 20 K the susceptibility is inversely dependent on the temperature; that is, it conforms to the Curie Law

$$\chi = \frac{N g^2 \beta^2 S(S+1)}{3kT} = \frac{N \beta^2 \mu_{eff}^2}{3kT} = \frac{\mu_{eff}^2}{7.95\ T}$$

where β is the Bohr magneton number and μ_{eff}^2 is the square of the effective magnetic moment, given by $\mu_{eff}^2 = (\bar{g})^2\ S\ (S+1)$. The χ vs 1/T plot of resting cytochrome oxidase in maltoside, shown in Figure IV.1, has a slope at higher temperatures which corresponds to a μ_{eff}^2 of 32.0 ± 0.5.

Each of the four metal ions in resting cytochrome oxidase contributes additively to the susceptibility, and

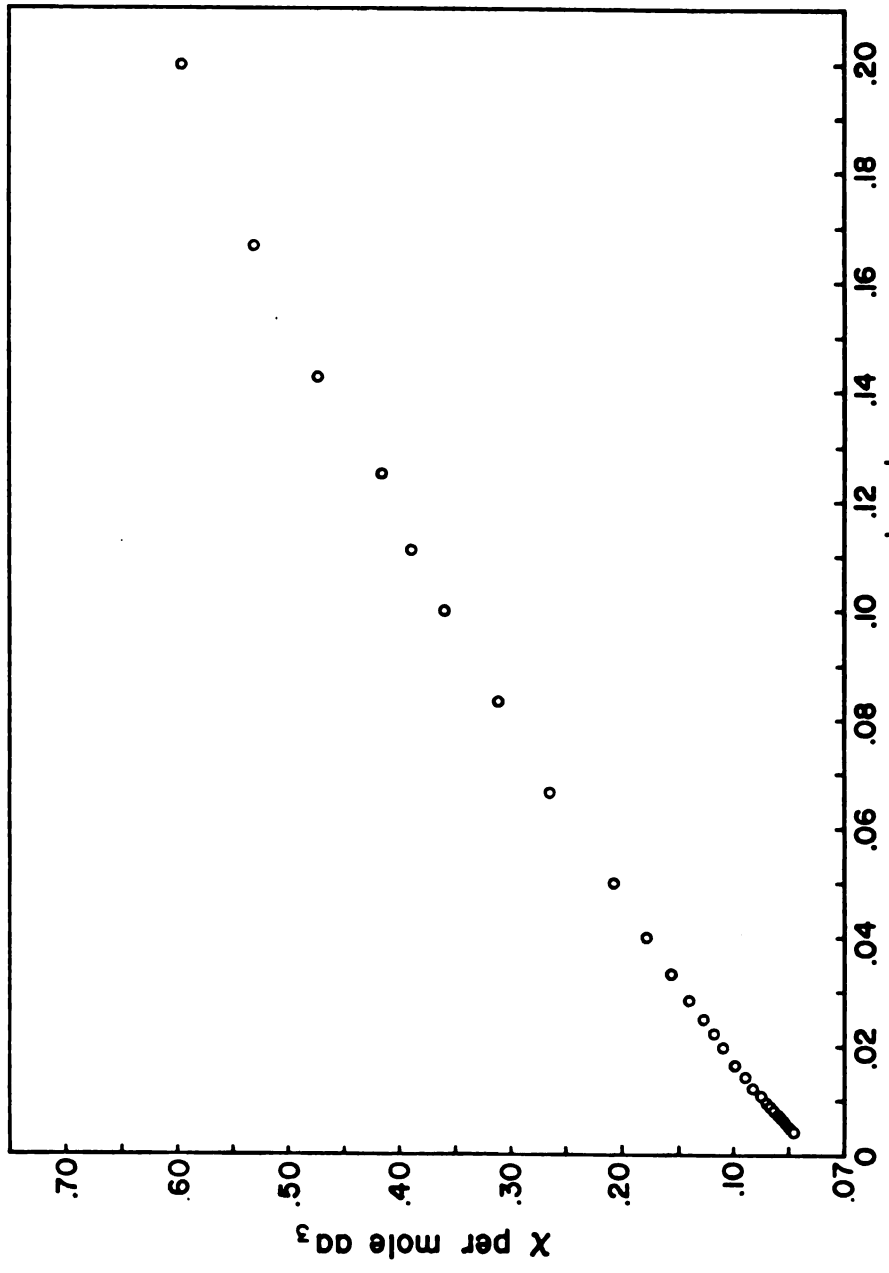


Figure IV.1 Magnetic Susceptibility vs Reciprocal Temperature for Resting Cytochrome Oxidase

so to the square of the effective magnetic moment. The contribution of cytochrome $\underline{a}^{3+}$ and Cu_A can be calculated from their spin states (S=1/2 for both) and their g values: g_z, g_y, and g_x are 3.03, 2.21, and 1.45 for cytochrome $\underline{a}^{3+}$ and 2.18, 2.02, and 1.99 for Cu_A^{2+} (Aasa,et.al.,1976). The mean g values are then $(\bar{g})^2 = (1/3)(g_x^2 + g_y^2 + g_z^2)$. The effective magnetic moments squared that are found from these values are 4.0 for cytochrome $\underline{a}^{3+}$ and 3.2 for Cu_A. Thus the contribution of the $\underline{a}_3$ center to μ_{eff}^2 is 7.2 and the μ_{eff}^2 for the $\underline{a}_3$ center is then 32.0-7.2 = 24.8.

Comparing this with the values of the μ_{eff}^2 for the possible magnetic states of the $\underline{a}_3$ center (Table IV.1) permits a determination of the actual magnetic state. Assuming the iron ion is in the +3 state, there are only two states which give a μ_{eff}^2 value of about 24 for the $\underline{a}_3$ center. One is an intermediate spin cytochrome coupled ferromagnetically to the copper, the other is a high spin cytochrome coupled antiferromagnetically to the copper. The iron of cytochrome $\underline{a}_3$ is generally believed to be high spin (Babcock et.al., 1976), so the ions of the $\underline{a}_3$ center must be antiferromagnetically coupled.

The fact that the χ vs 1/T plot remains linear at high temperature shows that the coupling factor, J, must be large enough that the higher energy levels are not substantially populated at high temperature, that is, $-2J > 126\ cm^{-1}$.

Table IV.1 Squares of Effective Magnetic Moments for the Possible Magnetic States for the a_3 Center of Cytochrome Oxidase.

Cyt a_3	Cu_B	Coupling	μ^2_{eff}*
S=1/2	S=1/2	antiferromagnetic	0(strong),6(weak)
S=1/2	S=1/2	none	6
S=1/2	S=1/2	ferromagnetic	8(strong),6(weak)
S=3/2	S=1/2	antiferromagnetic	8(strong),18(weak)
S=3/2	S=1/2	none	18
S=3/2	S=1/2	ferromagnetic	24(strong),18(weak)
S=5/2	S=1/2	antiferromagnetic	24(strong),38(weak)
S=5/2	S=1/2	none	38
S=5/2	S=1/2	ferromagnetic	48(strong),38(weak)

* $\mu^2_{eff} = \bar{(g)}^2 S (S+1), \quad \bar{(g)}^2 = 2.0$

At lower temperatures the susceptibility no longer follows the Curie Law. This may be explained by assuming that the spin degeneracy of the ground state is lifted by spin-orbit interaction. This phenomenon is commonly called zero-field splitting because it occurs in the absence of an applied magnetic field. This splitting is generally small enough that it does not influence the susceptibility at high temperatures, but it can influence the susceptibility when the temperature is low enough for kT to approximate the energy difference between the split levels.

Griffith (1971) has derived the equation that describes the temperature behavior of the square of the effective magnetic moment of a coupled ion pair consisting of an S=5/2 ion and an S=1/2 ion. This equation includes the axial zero-field splitting parameter, D, but assumes that the rhombic parameter, E, is zero. This assumption is reasonable for cytochrome oxidase because for heme proteins E is often small compared to D (Unenoyama et.al., 1968; Nakano et.al., 1972).

Griffith's equation for μ^2_{eff} may, after modification, be used to find the temperature dependence of the susceptibility of all the metal centers in the enzyme. The contribution of cytochrome $\underline{a}^{3+}$ and Cu_A is accounted for by adding 7.2 to Griffith's μ^2_{eff}:

$$\mu^2_{eff} = \frac{\mu^2(2) + 7\mu^2(3)\exp(-x)}{5 + 7\exp(-x)} \qquad \text{(IV.1)}$$

where

$$\mu^2(2) = \frac{4[9+(2y-7)\exp(-4y/3)+(8y-2)\exp(-16y/3)]}{y[1+2\exp(-4y/3)+2\exp(-16y/3)]}$$

$$\mu^2(3) = \frac{8[90+(5y-65)\exp(-2y/3)+(20y-16)\exp(-6y)+(45y-9)\exp(-6y)]}{5y[1+2\exp(-2y/3)+2\exp(-8y/3)+2\exp(-6y)]}$$

and

$$x = 3J/kT \qquad \text{and} \qquad y = D/kT.$$

Theoretical values of the magnetic susceptibility were calculated by using a large value of J and various values of D. The results, shown along with the experimental data in Figure IV.2, indicate that a D value between 5 cm^{-1} and 10 cm^{-1} is adequate to explain the deviation from the Curie Law behavior that occurs at lower temperatures. This is within the range of D's that has been found for other heme proteins (Tasaki et.al., 1966; Uenoyama et.al., 1968; Behere et.al., 1979).

B.2 Peroxide-bound Cytochrome Oxidase

When resting enzyme samples were made anaerobic by the use of glucose and glucose oxidase, the susceptibilities found were different than when the oxygen was removed from the samples by evacuations followed by equilibrations with argon. Typical data for the samples made with the oxygen

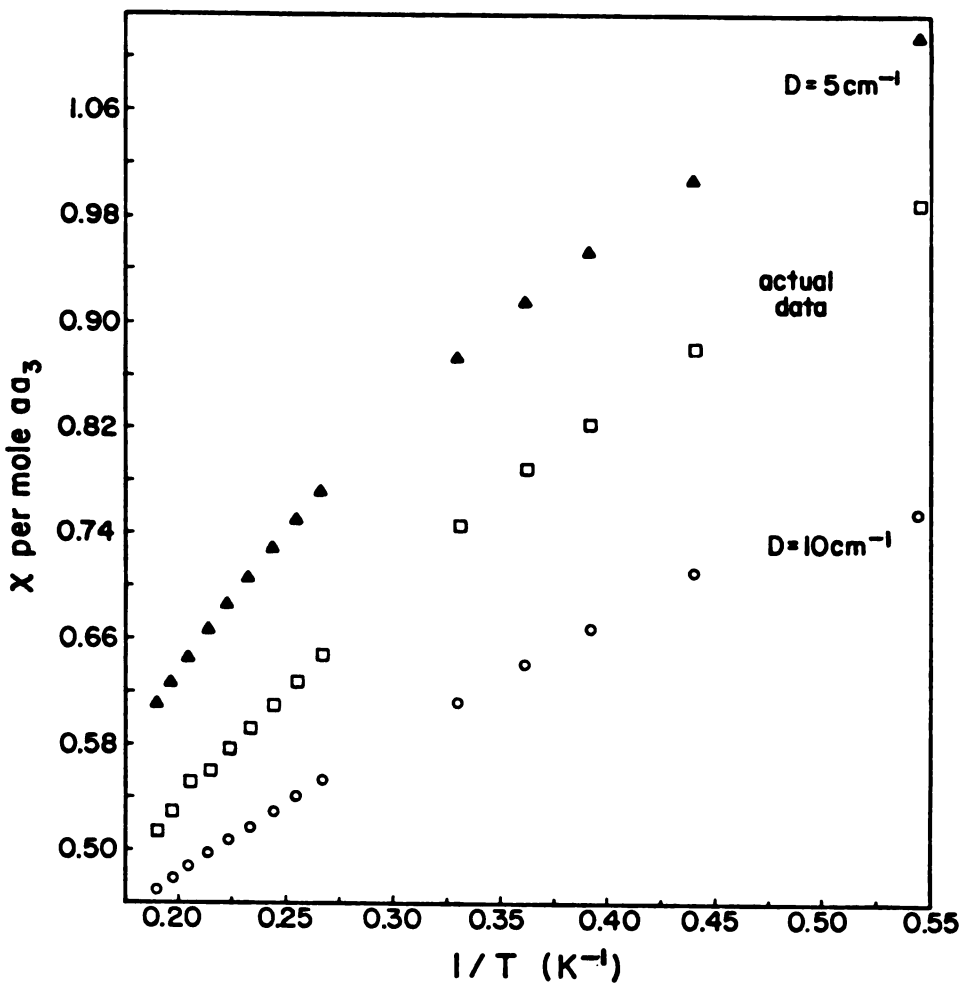


Figure IV.2 Experimental and Calculated Magnetic Susceptibilities vs Reciprocal Temperature for Resting Cytochrome Oxidase

scavengers are shown in Figure IV.3. Above 15 K the susceptibility shows a Curie Law behavior. The high temperature slope corresponds to a μ^2_{eff} of 24.8, and thus an a_3 center contribution of 17.6. Comparing this with the calculated values in Table IV.1 indicated three possible magnetic states for the a_3 center: an S=3/2 cytochrome weakly coupled, either ferromagnetically or antiferromagnetically, to the S=1/2 Cu_B, or an isolated S=3/2 cytochrome and S=1/2 Cu_B. An epr spectrum of the sample did not indicate the presence of an isolated S=3/2 center, so the ions must remain coupled in some way.

An optical spectrum of the sample was taken after the susceptibility determination had been made. A near-UV peak at 428 nm indicated that the enzyme was peroxide-bound (Kumar et.al., 1984). It is postulated that the oxygen scrubbers produced enough peroxide to lead to this peroxide binding. To ensure that all of the enzyme capable of binding peroxide did so, twenty equivalents of hydrogen peroxide were added to another resting enzyme sample which was then made anaerobic with the oxygen scrubbers. The susceptibility was then measured. The resulting data correspond to a μ^2_{eff} of 26.0 (aa_3), indicating no increase in the concentration of peroxide-bound enzyme.

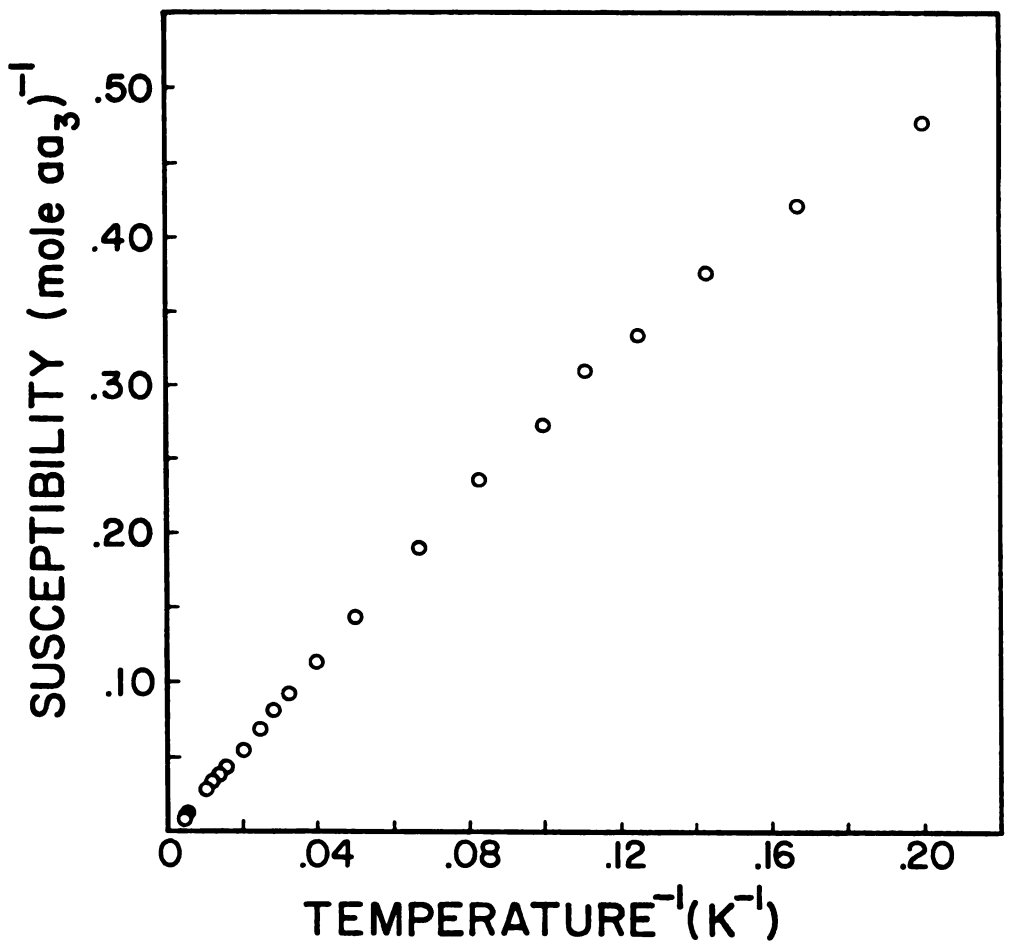


Figure IV.3 Magnetic Susceptibility vs Reciprocal Temperature for Peroxide-bound Cytochrome Oxidase

B.3 Oxidized Formate-bound Cytochrome Oxidase

Formate-bound cytochrome oxidase was prepared by incubating the enzyme overnight with a 60- to 100-fold excess of sodium formate. Binding was complete as indicated by the shift of the near-UV optical band to 417 nm (Nicholls, 1976).

When the oxygen in the sample was removed by cycles of evacuations and equilibrations with argon, the susceptibility behavior was very similar to that of the resting enzyme. Figure IV.4 shows that at higher temperatures the susceptibility followed the Curie law ; the μ^2_{eff} was 31.2 (24.0 for the a_3 center). Since the iron of formate-bound cytochrome a_3^{3+} is high spin (Nicholls,1976; Babcock et.al., 1976), an antiferromagnetically coupled cytochrome a_3-Cu_A center is indicated. The linearity of the plot to 180 K indicates that $-2J > 126\ cm^{-1}$.

At low temperatures the susceptibility was less than predicted by the Curie Law. As with the resting enzyme, this deviation can be explained by zero-field splitting. Equation IV.1 can be used to calculate theoretical values of the susceptibility at various D values. As shown in Figure IV.5, a D value between 5 cm^{-1} and 10 cm^{-1} adequately explains the lower than predicted susceptibility values.

It has been suggested (Babcock et.al, 1981) that the magnetic behavior of the oxidized formate-bound enzyme could

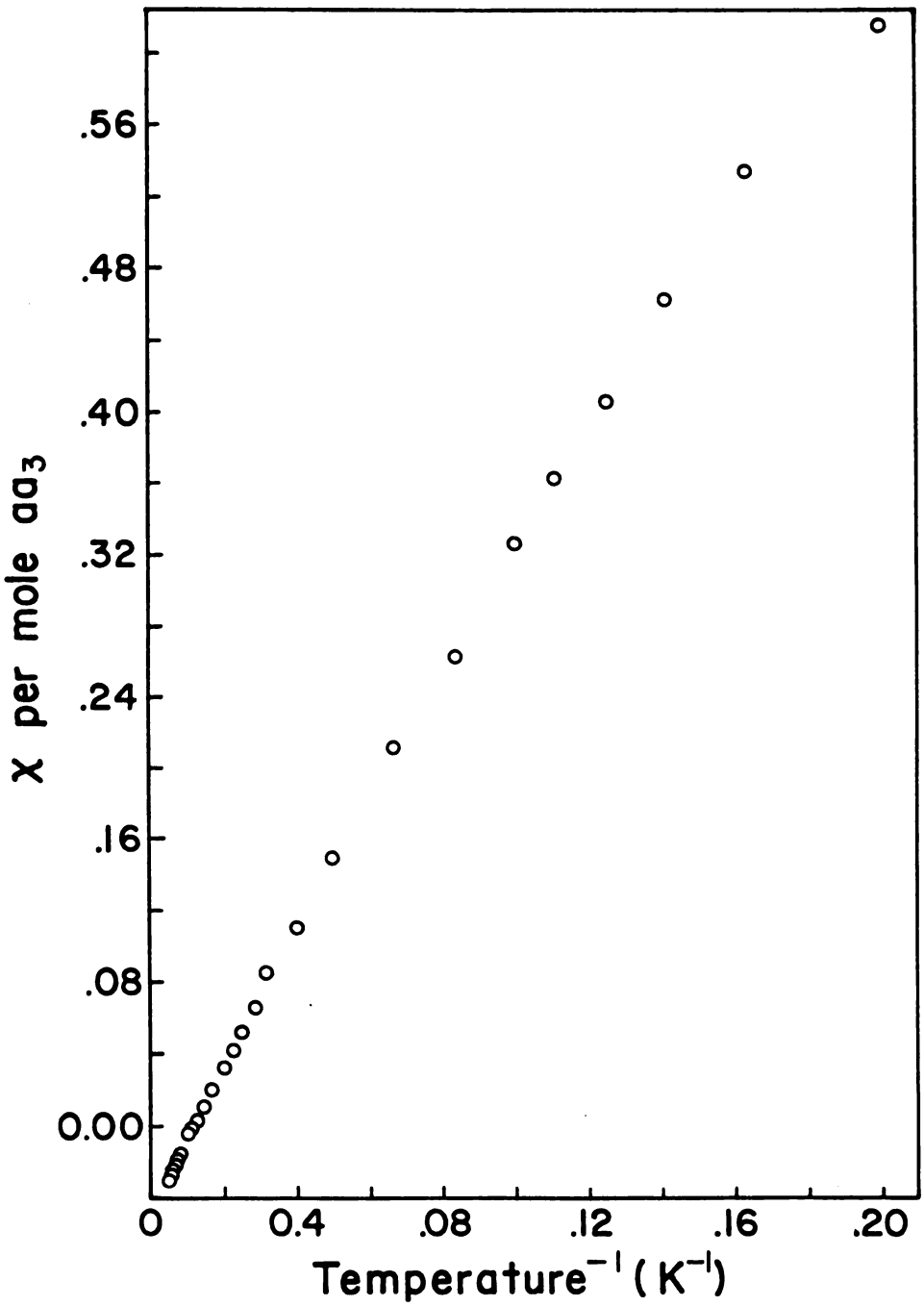


Figure IV.4 Magnetic Susceptibility vs Reciprocal Temperature for Formate-bound Cytochrome Oxidase

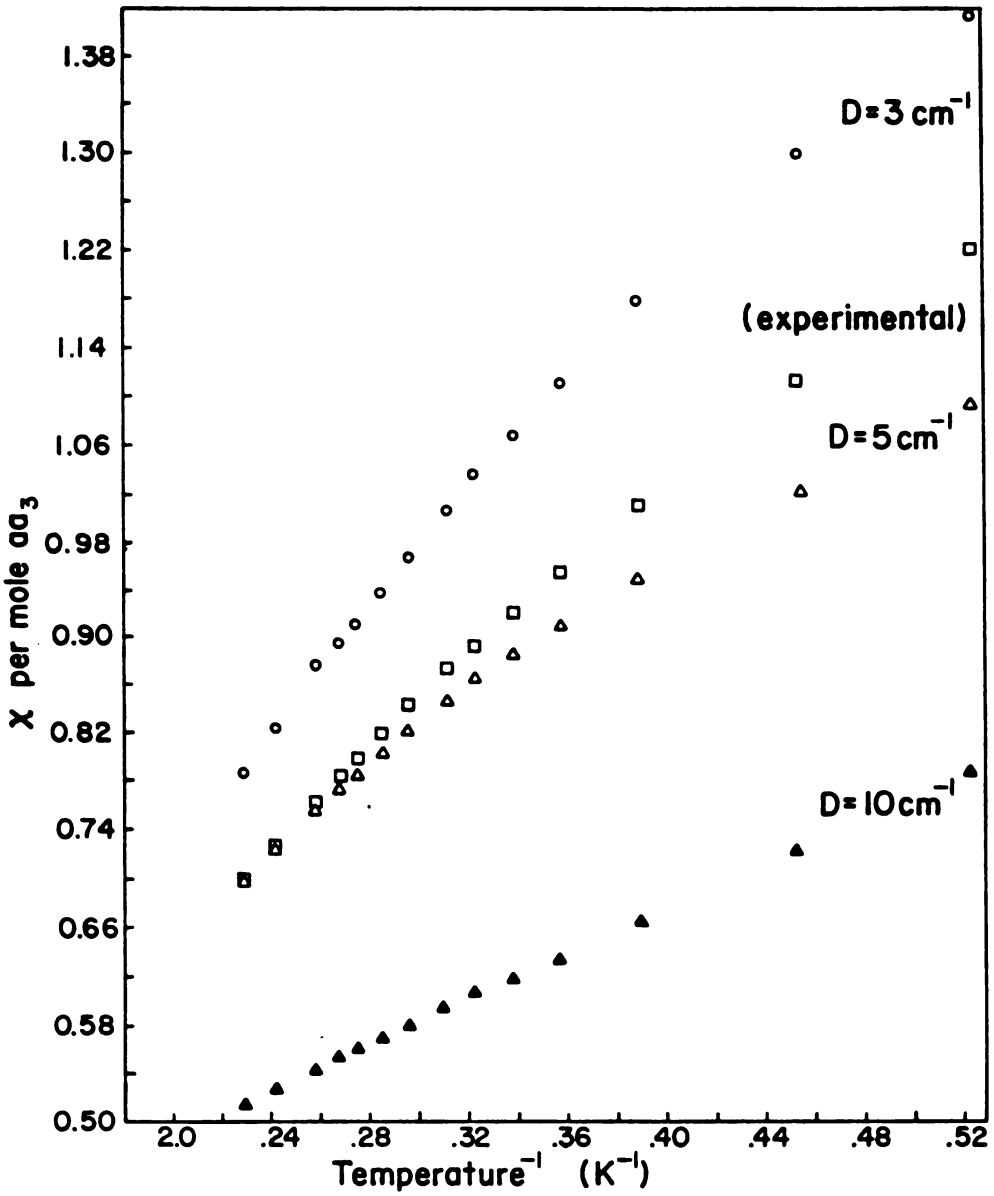


Figure IV.5 Experimental and Calculated Magnetic Susceptibilities vs Reciprocal Temperature for Formate-bound Cytochrome Oxidase

help distinguish between two modes by which exchange coupling may occur. The models for these, illustrated in Figure IV.6, may be classified as "back-side" bridging or as "front-side" bridging. More specific models, also shown, have been proposed by Palmer et.al. (1976) and by Blumberg and Peisach (1976). Formation of the formate-enzyme complex would perturb the structure shown in (c) only slightly and likely cause little change in the susceptibility. Displacement of the μ-oxo bridge in the structure shown in (d) would likely cause a large change in susceptibility. Since the susceptibility of the formate bound enzyme is essentially the same as that of the resting enzyme, the back-side bridging model is indicated. The above discussion assumes that formate binds to the iron of cytochrome a_3. If it binds elsewhere, this discussion of the geometry of the binding does not apply.

When the oxygen was removed from the formate-bound enzyme sample by use of the oxygen scavengers glucose oxidase and glucose, a different susceptibility behavior was observed, though the near-UV peak remained at 417 nm, indicating that the formate remained bound. The value of μ^2_{eff} was then 45.3, so the a_3 center contribution to it was 38.1. Comparing this with the calculated values of μ^2_{eff} for the possible magnetic states of the a_3 center (Table IV.1) seemed to indicate that the coupling was either weakened or broken altogether when the enzyme sample was prepared in this way. Since the resting enzyme was shown to bind peroxide when

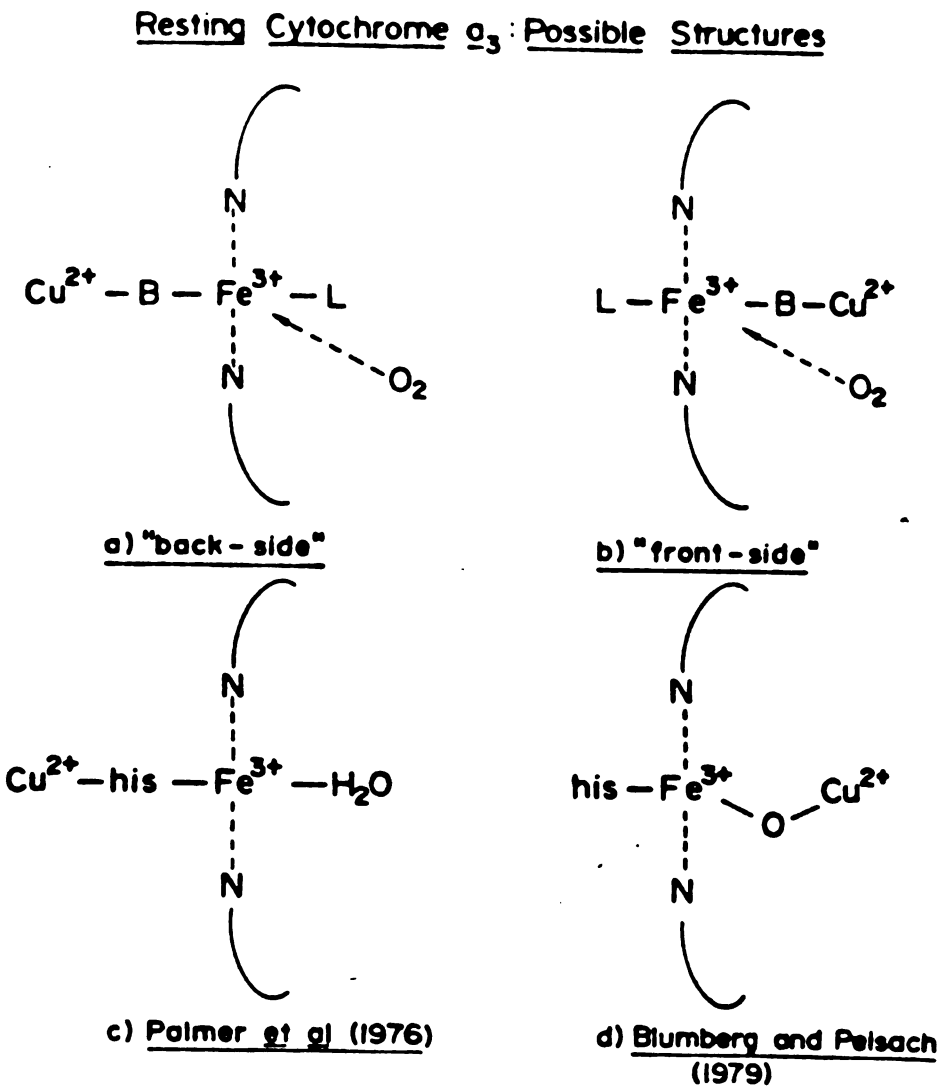


Figure IV.6 Possible Structures for the Cytochrome a_3 Oxygen Reducing Site (from Babcock et.al., 1981)

oxygen scavengers were used, it is reasonable to suppose that it may have also occurred with the formate-bound enzyme, though this requires that the peroxide and formate bind at different locations. To test whether the enzyme was completely peroxide bound, four equivalents of peroxide were added to this formate-bound enzyme, the oxygen was removed with scavengers, and measurements were repeated. The μ^2_{eff} was then found to be 51.9, indicating an a_3 center contribution of 44.7. This does not match any of the values on Table IV.1. If it is assumed that the enzyme was closer to completely peroxide bound than it was when no peroxide was added, but not yet completely bound, ferromagnetic coupling between the high-spin cytochrome and the copper is indicated. In any case it is clear that removing the oxygen by the use of glucose oxidase perturbs the magnetic state of the formate-bound enzyme in some way, presumably by peroxide binding.

B.4 Oxidized Cyanide-Bound Cytochrome Oxidase

When the oxidized enzyme binds cyanide, cytochrome a_3 goes from high to low spin (Babcock et.al.,1978). The a_3 center still has no epr signal, so the metals remain coupled. Most of the disagreement in the literature about the magnetic state of the a_3 center concerns the nature of the coupling in the cyanide-bound enzyme.

If antiferromagnetic coupling with a large 2J value is retained then the a_3 center would not contribute to μ^2_{eff}

since the populated spin state would have S=0. The contribution of cytochrome a and Cu_A would remain at 7.2 since their spin states are unchanged from those in the resting enzyme (Babcock et.al., 1976). The susceptibility would follow the Curie Law.

If 2J is small compared to kT then the a_3 center would be a thermally randomized S=1 manifold whose contribution to μ^2_{eff} would be 8.0. With the value of 7.2 from the other metal ions, the total μ^2_{eff} would be 15.2, and the susceptibility would follow the Curie Law.

If 2J has an intermediate value, then at high temperatures the susceptibility would show behavior typical of $2J<kT$ while at low temperatures the behavior would be that observed when $2J>kT$. Thus, μ^2_{eff} would be 15.2 at high temperatures and 7.2 at low temperatures and the susceptibility would not follow the Curie Law.

The observed behavior is that expected from an intermediate value of J (Figure IV.7). Below $\approx$ 3 K the value of μ^2_{eff} is 7.4 while at $\approx$ 180 K it is 16.0. Tweedle et.al. (1978) also observed limiting values of μ^2_{eff}, but they reached the values of 7.8 below 50 K and 15.1 at high temperatures.

The equation that describes the temperature dependence of the susceptibility of two antiferromagnetically coupled spins is

$$\chi = \frac{2 g^2 N \beta^2}{3kT} \left[1 + \frac{1}{3} \exp\ (-2J/kT)\right]^{-1}$$

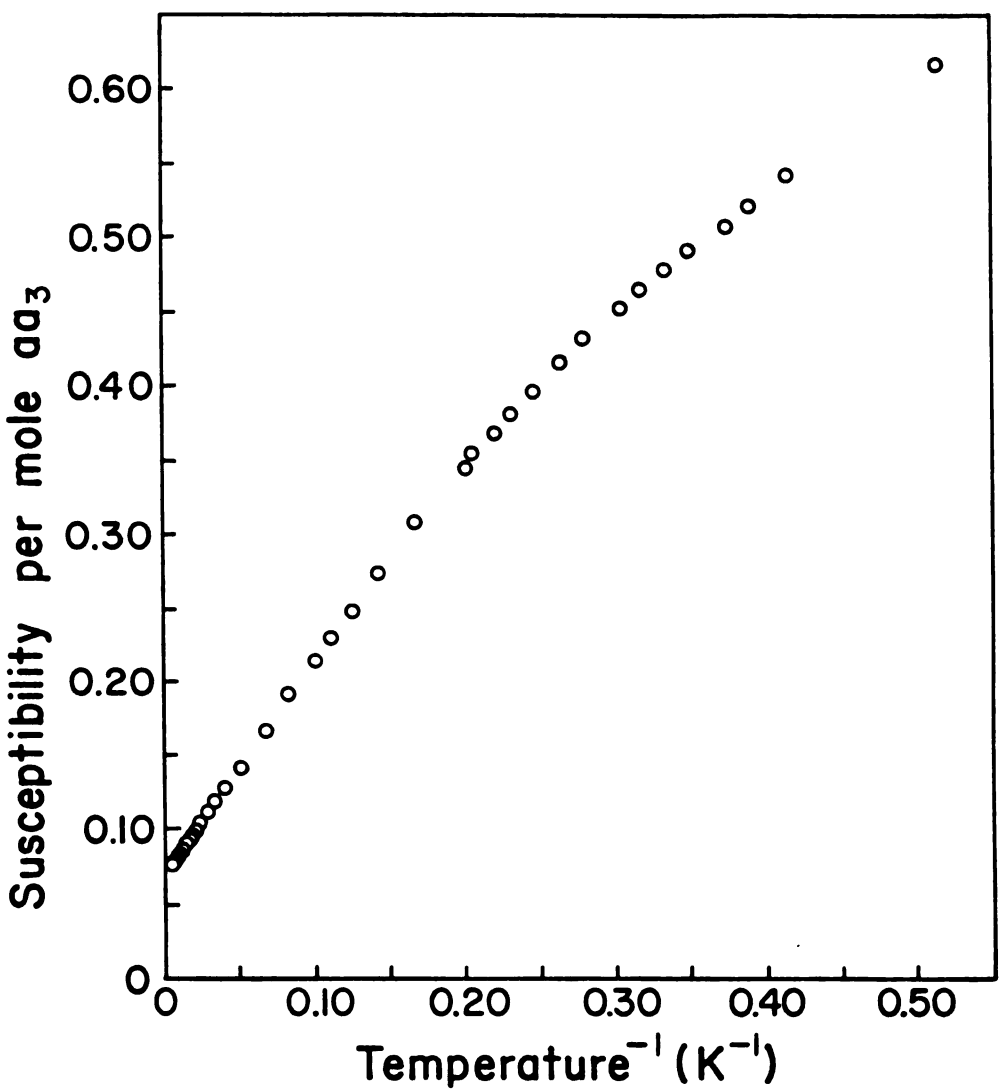


Figure IV.7 Magnetic Susceptibility vs Reciprocal Temperature for Cyanide-bound Cytochrome Oxidase

where 2J is the energy difference between the two spin states. In order to fit this equation to the cytochrome oxidase data, a Curie Law term, C/T, and a temperature independent term were added for the contribution of the a-center and the diamagnetism of the protein, respectively.

When the modified equation was fit to the data collected between 5 and 180 K the value of -J that was found ranged from 29.6 $\pm$ 11.6 cm^{-1} to 56.5 $\pm$ 5.7 cm^{-1} (Table IV.2). These values bracket the -J value of 38.5 $\pm$ 1.3 cm^{-1} found by Tweedle et.al., but have a much larger standard deviation. Substantially different J's were found when the equation was fit to data collected between 2 and 180 K. The J value then ranges from 1.26 $\pm$ 0.13 cm^{-1} to 1.31 $\pm$ 0.11 cm^{-1}.

The different J values can be explained if the enzyme is heterogeneous with two forms of the enzyme present. One form has a moderate value of J and the other form has a low value of J. When high temperature data are used, the moderate J value is found because the enzyme with a low J value contributes to the signal in a Curie manner, and so does not contribute to the fit of the coupling term in the equation. At low temperatures the signal of the enzyme with a moderate J value is zero because saturation to the S=0 level has occurred; thus the low J value is obtained when the low temperature data are used to fit the equation. This is supported by the fact that the Curie term found from high

Table IV.2 Coupling Factors for Cyanide-Bound Cytochrome Oxidase

Preparation	Temperature Range	J Value
Yonetani	5-190 K	-29.6 ± 11.6 cm^{-1}
(in Tween-20)		
	2-190 K	-1.26 ± 0.13 cm^{-1}
Hartzell-Beinert	5-180 K	-30.1 ± 6.4 cm^{-1}
(in Tween-20)		
Hartzell-Beinert	20-201 K	-56.5 ± 5.7 cm^{-1}
(in Lauryl Maltoside)		
	2-201 K	-1.31 ± 0.11 cm^{-1}

temperature data is larger than the value 0.90 expected from the cytochrome a and the Cu_A (Table IV.3).

An equation with two coupling terms, a Curie term, and a diamagnetic term was fit to the data collected between 2 and 180 K. The coefficients of the coupling terms were allowed to vary and the Curie constant was fixed at 0.9 to account for the contributions of the cytochrome a and Cu_A. The coefficients thus represented the fraction of enzyme present in each form. It was found that 78±1 percent of the enzyme had the J value of lower magnitude.

It must be noted that when the equation is fit in this way, the accuracy of one J value is increased at the expense of the accuracy of the other J value, that is the J values found were -1.70 ± 0.03 cm^{-1} and -44 ± 28 cm^{-1}. This is because the equation used does not describe the system perfectly, particularly at low temperature where zero-field splitting would have an effect. Because the errors in measurement of the high temperatures are greater, the program assumes most of the error is in the high temperature data. As a result, the equation is fit such that the J value obtained from the high temperatures is most affected by the deviations from the equation.

B.5 Cyanide-Bound Cytochrome Oxidase In Ethylene Glycol

In order to reproduce the enzyme environment in the sample used for the MCD studies done by Thomson (1981), a

Table IV.3 Curie Law Constants for Cyanide-Bound Cytochrome Oxidase

Preparation	T Range	C
Yonetani (in Tween-20)	5-190 K	1.61±0.04
	2-190 K	0.81±0.4
Hartzell-Beinert (in Tween-20)	5-180 K	1.49±0.11
Hartzell-Beinert (in Lauryl Maltoside)	20-201 K	1.60±0.04
	2-201 K	0.69±0.03

sample of cyanide-bound enzyme was dissolved in a solution that was fifty percent ethylene glycol and fifty percent HEPES buffer. The detergent used was lauryl maltoside.

Direct addition of pure ethylene glycol to an enzyme sample previously dissolved in a buffer caused precipitation of the enzyme due to local concentration effects. Consequently, the cyanide-bound enzyme was prepared in the buffer, precipitated with ammonium sulfate, and then redissolved in the solution of ethylene glycol, buffer and detergent. This method was chosen as ammonium sulfate precipitation does not harm the resting enzyme (note, however, that it was the cyanide-bound enzyme that was precipitated). It has the added benefit of avoiding the dilution of the enzyme that occurs with direct addition of ethylene glycol.

The susceptibility data for the cyanide-bound enzyme in the ethylene glycol/buffer solution are shown in Figure IV.8. A feature immediately apparent is the peak in the susceptibility values, which indicates an antiferromagnetic signal. What is surprising is that this peak shows up in the susceptibility data before the contributions of the cytochrome a and Cu_A are subtracted. The equation fit to this data indicates that the Curie contributions of cytochrome a and Cu_A that were present in the other forms of the enzyme are not present in this sample. What has caused their loss is not known, but precipitation of the cyanide-bound enzyme

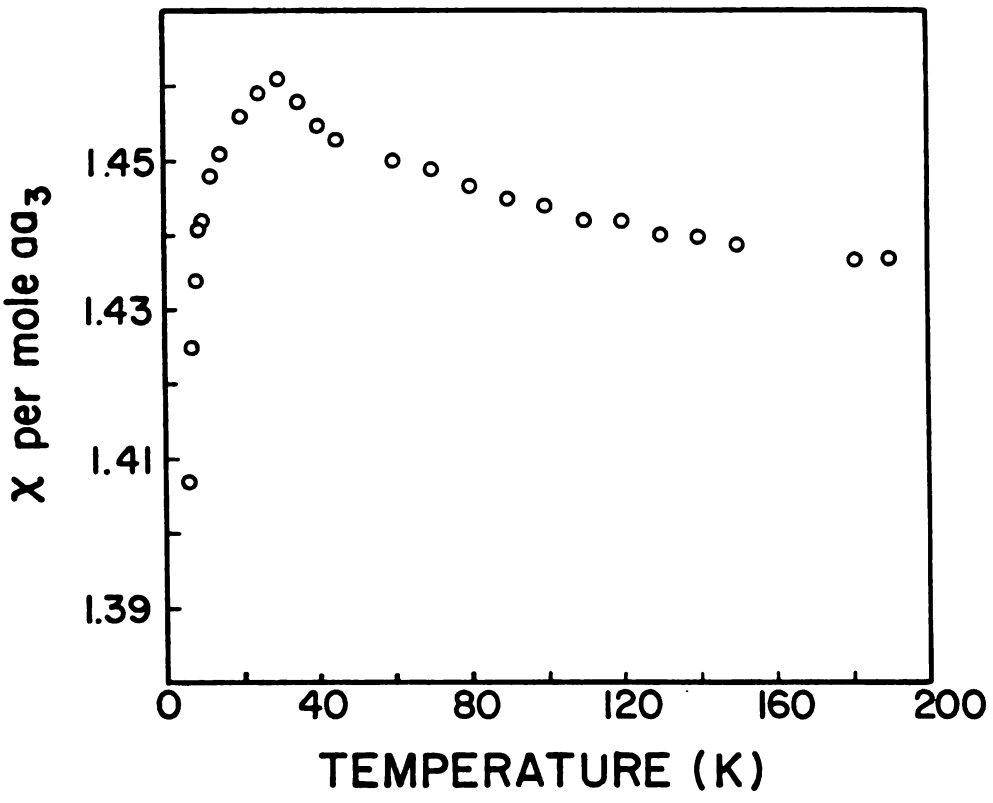


Figure IV.8 Magnetic Susceptibility vs Reciprocal Temperature for Cyanide-bound Cytochrome Oxidase in 50:50 Ethylene Glycol/HEPES

has not been tried previously and it may affect the enzyme in some way. The most likely occurance would be auto-reduction.

B.6 Isolation Procedure and Detergent Effects

Table IV.4 shows the μ^2_{eff} values obtained for resting cytochrome oxidase that was prepared by two isolation procedures (Hartzell-Beinert and Yonetanti) and that was dissolved in two types of detergent (lauryl maltoside and Tween). The constancy of the effective magnetic moments indicates that neither the isolation procedure nor the detergent influences the magnetic state of the a_3 center of the resting enzyme.

The possibility of isolation procedure or detergent effects on the magnetic state of the a_3 center of the low J and intermediate J forms of the cyanide-bound enzyme may be considered by examining their J values (Table IV.2). The isolation procedure did not change the J value of either form of the enzyme. Thus the choice of isolation procedure did not influence the a_3 center magnetic state in the cyanide-bound enzyme.

The choice of detergent did not influence the antiferromagnetic coupling factor of the low J form of the enzyme, again indicating no change in the magnetic state of its a_3 center. However, the intermediate J form of the cyanide-bound enzyme had a larger coupling factor when in lauryl maltoside than it had when in Tween. This indicates a change in the bond overlap or bond angles at the a_3 center

Table IV.4 Effective Magnetic Moments Squared for Resting Cytochrome Oxidase

Preparation	Temperature Range	μ^2_{eff}
Yonetani (in Tween-20)	12-169 K	33.1±2.5
Hartzell-Beinert (in Tween-20)	20-201 K	30.2±1.1
Hartzell-Beinert (in Lauryl Maltoside)	30-180 K	32.0±0.5

of the enzyme. This change is probably not dramatic since the J value is still moderate enough to result in non-Curie Law behavior when the enzyme is in a lauryl maltoside solution.

B.7 Discussion

The magnetic susceptibility behavior of resting oxidized cytochrome oxidase clearly indicates that the S=5/2 cytochrome a_3 is antiferromagnetically coupled to the S=1/2 Cu_B ion. The Curie Law behavior that extends to temperatures as high as 180 K means that the energy separation between the two lowest energy levels exceeds 126 cm^{-1}. The deviation from Curie Law behavior at low temperatures is readily explained by a zero-field splitting whose magnitude is typical for that of heme proteins. These results confirm the conclusions reached by Tweedle et.al. (1978). In addition, this work shows that the magnetic state of the a_3 center of the resting oxidized enzyme does not depend on the isolation procedure or on the detergent used. This eliminates one of the possible reasons for the conflicting reports of the type of coupling present in resting cytochrome oxidase.

The susceptibility behavior of the peroxide-bound enzyme indicates that an S=3/2 cytochrome a_3 is present. Such a spin state is extremely unusual in a heme protein. Peroxide binding studies done by Bickar et.al. (1982) indicated that some preparations of cytochrome oxidase may not bind peroxide completely, even in the presence of a large excess of peroxide,

but the 428 nm near-UV band of these samples means all of the enzyme in this sample was peroxide bound. The linearity of the susceptibility vs reciprocal temperature graphs also indicates that there is no shift in equilibrium occuring between spin states and that the coupling factor remains large. It thus appears that the peroxide-bound enzyme actually has an S=3/2 iron ion. The lack of a cytochrome $\underline{a}_3$ MCD signal from what was formerly believed to be the oxygenated enzyme (but is now believed to be peroxide-bound) (Babcock et.al., 1976) confirms that the cytochrome $\underline{a}_3$ is not low spin.

This work shows that the formate bound enzyme has an $\underline{a}_3$ center which is composed of an S=5/2 cytochrome $\underline{a}_3$ coupled to an S=1/2 Cu_B. The adherence to the Curie Law at temperatures as high as 180 K indicates that the energy difference between the two lowest energy levels is at least 126 cm^{-1}. As with the resting enzyme, the deviation from the Curie Law behavior that occurs at low temperatures can be explained with reasonable values of a zero-field splitting. The relative deviations from the Curie Law of the resting and formate-bound enzyme suggest that the zero-field splitting of the formate-bound enzyme may be less than that of the resting enzyme. It must be remembered, however, that the average susceptibility is not very sensitive to the zero-field splitting. Indeed, the values of the zero-field splitting obtained from the average susceptibilities have sometimes been found to be in significant error when compared to the values obtained later

from the more dependable anisotropy measurements (Mitra, 1977). The calculations of the zero-field splitting shown here were undertaken to explain the low temperature deviation from the Curie Law, not to pinpoint the value of D.

The magnetic susceptibility of oxidized cyanide-bound cytochrome oxidase indicates that this form of the enzyme is magnetically heterogeneous. Twenty-two percent of the cyanide-bound enzyme was in a form with a moderate J value while the rest was in a form with a low J value. The percent heterogeneity matches that found by Jensen et.al. (1984), who found a heterogeneity in partially reduced cyanide-bound cytochrome oxidase with twenty percent of the molecules showing an epr signal from the cytochrome a_3^{3+}-HCN complex.

The behavior of the enzyme form with a moderate J value matches that found by Tweedle et.al. (1978). The magnitude of the coupling factor found in these experiments did not depend on the enzyme isolation procedure used. It did depend somewhat on which detergent was used, but the variation was not sufficient to explain the conflicts in the magnetic susceptibility literature.

While the form of the cyanide-enzyme with a moderate J value clearly shows antiferromagnetic coupling behavior, there remains some doubt about the behavior shown by the low J form of the cyanide-bound enzyme. Equation fitting to the data gave a small negative coupling factor with a magnitude of one to two reciprocal centimeters, but this

does not mandate true antiferromagnetic coupling. Another possibility is weak ferromagnetic coupling with a zero-field splitting that results in a lowest energy level with M_s of zero.

As shown in Figure IV.9, neither ferro- nor antiferromagnetic coupling can result in an ordering of energy levels that would give a unique susceptibility behavior if the magnitude of the zero-field splitting is unknown. Hence the determination of the field dependence of the saturation temperature cannot permit the determination of the type of coupling.

To check the reproducibility, samples of the resting and cyanide-bound enzyme were exchanged with L. Wilson of Rice University, Houston, Texas. The enzyme received from Rice was isolated with the Hartzell-Beinert technique and the oxygen had been removed by using glucose and glucose oxidase and catalase. This sample of resting enzyme gave the same susceptibility behavior as those we made that were deoxygenated by evacuations and argon equilibrations, that is, the use of the oxygen scrubbers had no effect on the susceptibility. When glucose and glucose oxidase were added to a sample of their enzyme (that had no catalase) in the presence of air, the near-UV band shifted to 428 nm, but it did so over the course of several minutes. In contrast, when the oxygen scrubbers were added to our enzyme, also with no added catalase, its near-UV band shifted to 428 nm

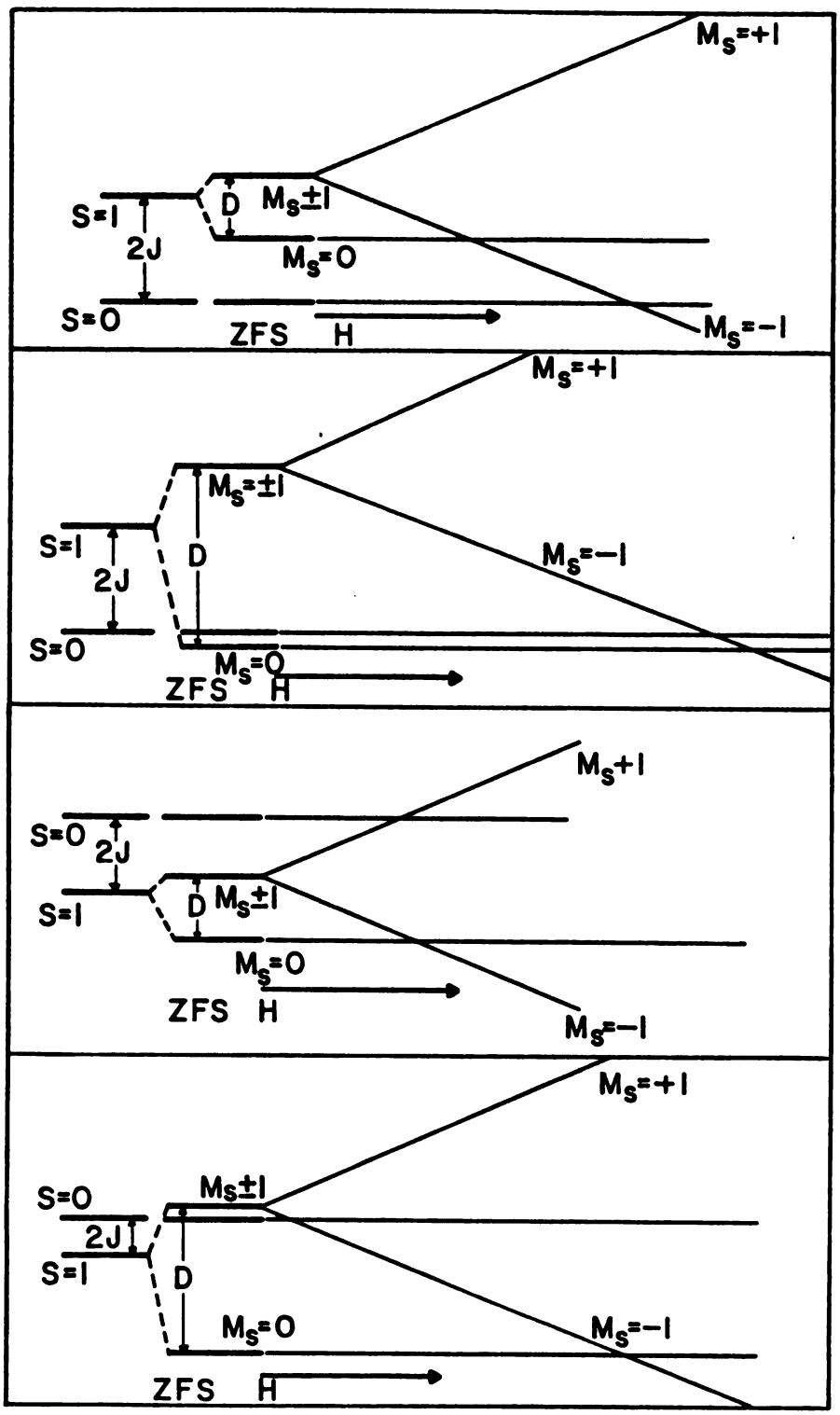


Figure IV.9 A Diagram of the Energy Levels for Ferromagnetically and Antiferromagnetically Coupled Systems Composed of Two S=½ Ions.

within 15 seconds. It is suggested that the samples from Rice were frozen quickly enough after addition of oxygen scrubbers to prevent the binding of peroxide, or their added catalase kept peroxide from reaching a high concentration when the glucose oxidase was added in the helium-flushed glovebag.

The results obtained for the cyanide-bound enzyme sent from Rice were consistent with those obtained for the enzyme made in our laboratory. This is important because the people in the laboratory at Rice obtained results consistent with those reported by Tweedle et.al. (1978), that is, antiferromagnetic coupling with a -J value around 40 cm^{-1} and no heterogeneity present. While they can collect data only to 20 K, the standard deviations of the J values they obtained by data fitting are much smaller than those obtained in our laboratory, indicating closer adherence to the coupling equation with one J factor. However, the Curie factors they obtain are approximately 1.2 instead of the 0.9 that is expected from the a center. The deviation from 0.9 indicates the presence of a species (besides the a center) that contributes in a Curie fashion in the temperature range used. The value consistently obtained in our laboratory for the high temperature (5-180 K) Curie factor was approximately 1.5. Since all of these results were obtained consistently in both laboratories, it appears that there is a difference, as yet unknown, in the way the enzyme is handled

in the two laboratories which affects the susceptibility. Whether there could have been a smaller amount of heterogeneity in their enzyme that was hard to detect above 20 K is open to question, but even if there was, the amount of heterogeneity changed with sample handling. This sensitivty to handling procedure is important when interpreting the results obtained in different laboratories. When these results differ, it may be due to different procedures.

CHAPTER V

Future Work

A. Magnetic Susceptibility

The effect of the presence of peroxide on the magnetic susceptibilities of the resting and formate-bound enzyme raised some questions. It appears that the peroxide binding changes the spin states of the iron ion to 3/2. The lack of heterogeneity of peroxide binding could be confirmed by finding the spin state of the peroxide-bound oxygenated cytochrome oxidase, which shows no peroxide-binding heterogeneity. It also appears that the formate binds in a different place than peroxide does, but where is not known. This needs to be known before the susceptibility for the formate-bound enzyme can be interpreted in terms of a back- or front-binding model.

It has been suggested (Palmer et.al., 1976) that there is a structural change that occurs upon reduction of the enzyme which makes cytochrome a_3 more accessible. For instance, the reaction of resting oxidase with sodium azide is rapid and the changes in the spectrum are small (Wever et.al., 1973), making it unlikely that the azide is binding at the iron ion. However, the partially reduced enzyme reacts with azide in a way which causes the conversion of a high-spin epr signal to a low-spin signal, demonstrating

that under those conditions the azide is reacting with the iron ion. The magnetic susceptibility behavior of these two azide derivatives would test the hypothesis that the azide binds in a different place in the resting and partially reduced enzyme forms.

B. Stopped-flow Kinetics

The experiment detailed above indicated that if the resting enzyme is more active under anaerobic conditions, it is due to a step which cannot be observed at 444 nm. This would suggest that the reaction should be followed, under the same conditions, by observation of the 830 nm band to see if a difference in the Cu_A reaction is evident. Another possibility is that the detergent effect on the activity occurs with the oxygenated enzyme rather than the resting enzyme. This suggests that a comparison of the reactions of the oxygenated enzyme in different detergents needs to be carried out, with observation in the near-IR as well as the near-UV and visible spectral regions.

Given previous suggestions that different aggregation states may be responsible for the difference in enzyme activities, the question of whether the aggregation states of the enzyme in the two detergents is the same for the oxygenated and resting enzyme becomes important.

The magnetic susceptibility results indicate that the formate ion and peroxide bind at different places on the

enzyme molecule. This could be confirmed if formate-bound enzyme is able to use peroxide as an electon acceptor, as the resting enzyme is able to do.

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