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Characterization of the Effects of Histidine Modification

by Diethylpyrocarbonate on the Functional Properties

of Bovine Heart Cytochrome c Oxidase

A Thesis

in

The Department

of

Chemistry & Biochemistry

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science at

Concordia University

Montreal, Quebec, Canada

June 1990

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Abstract

Characterization of the Effects of Histidine Modification by Diethylpyrocarbonate on the Functional Properties of Bovine Heart Cytochome c Oxidase

John D. Doran

The reaction of diethylpyrocarbonate (DEPC) with histidine residues of bovine heart cytochrome c oxidase is characterized and the consequences of the modification on some functional properties of the enzyme are investigated. Comparison of the oxidase's steady-state activity using Eadie-Hofstee plots for native and modified enzyme shows that increasing concentrations of DEPC cause progressive decreases in the high activity phase (TNmax1) and low affinity Michaelis constant (Km1), while the low activity, high affinity phase is unaffected (TNmax2 and Km2). Km1 and TNmax1 approach Km2 and TNmax2 with increasing DEPC, and the modified enzyme shows monophasic kinetics unlike the biphasic kinetics exhibited by the control. This suggests that the conformational transition postulated to link electron transfer with proton translocation has been abolished. Furthermore the respiratory control ratio of modified oxidase vesicles is diminished by 80 % relative to native vesicles while proton pumping is decreased by 40 %.

Difference Spectra between DEPC-treated and control enzyme shows perturbations of the UV-visible absorbance spectrum at 430 and 650 nm, while no change is detected in the 830 nm region. DEPC modification also causes an increase in the rate of cyanide

binding to the oxidized enzyme, similar to the rate enhancement seen with pulsed oxidase. These data suggest that the cytochrome a_3 heme environment is affected by modification.

The results are presented in the context of a two state proton pump model for cytochrome c oxidase. It is postulated that histidines essential for the efficient functioning of proton pumping and / or electron transfer are modified by the DEPC treatment.

Acknowledgements

I sincerely appreciate the numerous contributions that my supervisor, Dr. B. C. Hill made towards this thesis. He provided invaluable advice towards the design and execution of the experiments presented here, and he was actively involved in all facets of this project.

I thank Dr. O. Tee and Dr. R. Storms for participating in my committee, and I also thank Dr. L. Colebrook for being the external examiner for my committee.

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I must also thank Dr. P. Nicholls for helping me solve my RCR problems and for generously providing me with a sample of his enzyme.

Last but not least I acknowledge the contributions of P. Taslimi and T. Fox, whom provided much needed assistance for the operation of several software packages.

List of Abbreviations

DEPC - diethylpyrocarbonate

KPi - potassium phosphate

Abs. - absorbance

TN - turnover

EDTA - ethylenediaminetetraacetate

TMPD - N, N, N',N'-tetramethyl-p-phenylenediamine

DCCD - N, N'-dicyclohexylcarbodiimide

EPR - electron paramagnetic resonance

RCR - respiratory control ratio

CCCP - carbonylcyanide m-chlorophenyl hydrozone

e - electron

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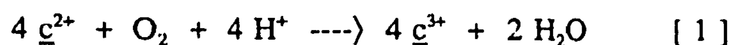
Introduction

Cytochrome c oxidase (ferrocytochrome c : O_2 oxidoreductase ; EC 1.9.3.1) is the terminal membrane bound enzyme complex of the mitochondrial respiratory chain. This enzyme catalyzes the transfer of 4 electrons from ferrocytochrome c to dioxygen according to equation 1 below:



$$4\ c^{2+} + O_2 + 4\ H^+ \longrightarrow 4\ c^{3+} + 2\ H_2O \qquad [1]$$

where c^{2+} and c^{3+} are reduced and oxidized cytochrome c respectively. Bovine heart cytochrome c oxidase has a molecular mass of 202 787 Daltons and it consists of 12 different polypeptide subunits in a 1:1 stoichiometry (Buse et al. , 1985).

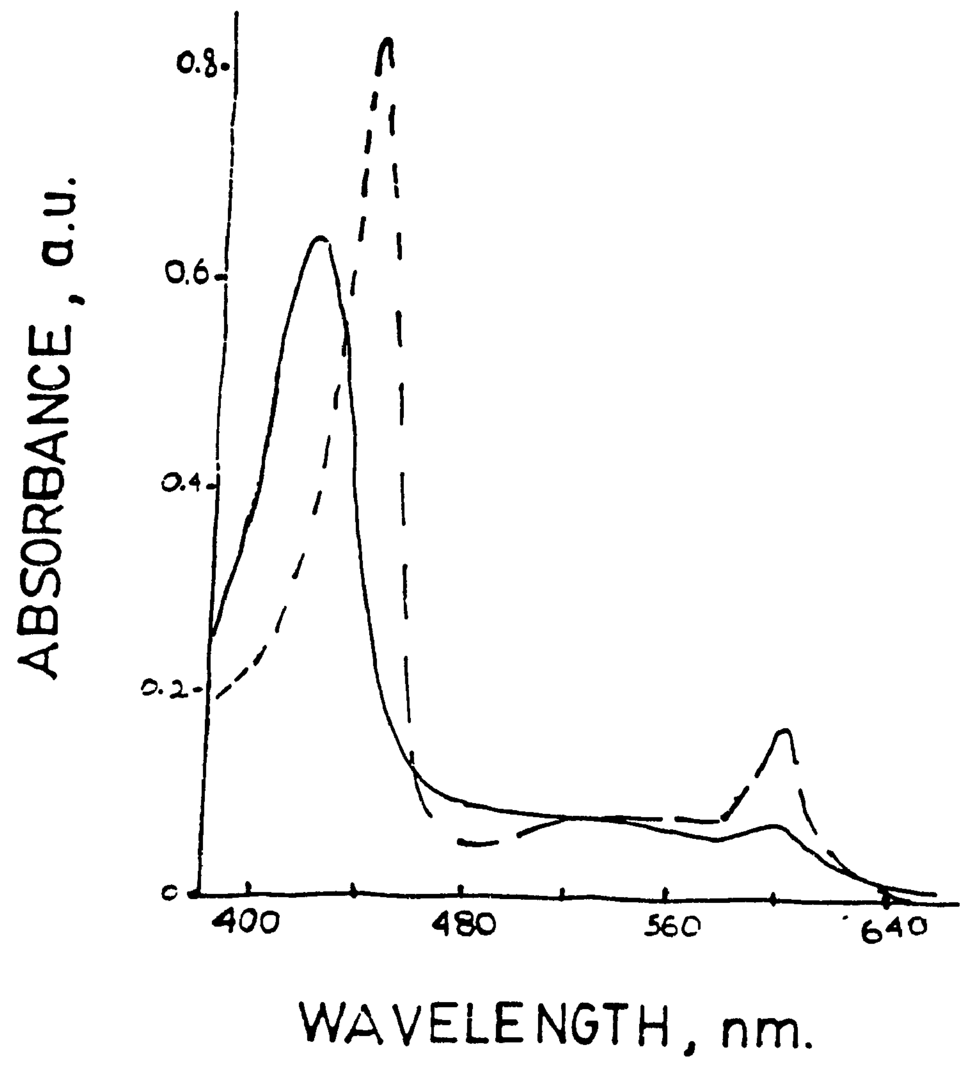
The oxidase contains 4 metal centers which are active in electron transfer , 2 heme A groups and 2 copper ions. Since these metal centers are in different protein environments each metal center has unique chemical characteristics allowing for their differentiation. The 2 heme groups in their protein environment are termed cytochrome a and cytochrome a_3 , while the two coppers are designated Cu_A and Cu_B. Cytochrome a and Cu_A are the initial acceptors of electrons from cytochrome c, whereas cytochrome a_3 and Cu_B form a pair which function as the oxygen binding and reduction site. Cytochrome a is six coordinate, low spin and does not react with ligands, while cytochrome a_3 is six coordinate, high spin and can bind to many ligands, such as cyanide, NO, CO, azide, and fluoride (Wikstrom et al. , 1981). Cu_A is called the "visible" copper

due to its detectability by EPR spectroscopy, while Cu_B is termed "invisible" since it is EPR silent (Wikstrom et al. , 1981). Figure A shows absolute absorbance spectra of fully oxidized and fully reduced oxidase as they appear in the visible spectral region. The peaks are predominantly due to contributions from both hemes. Upon reduction the Soret peak (420 nm region) shifts to 445 nm and the alpha band (600 nm) shifts to 605 nm. As well as the soret and alpha bands oxidized oxidase also exhibits a weak band at 655 nm believed to be due to cytochrome a_3 alone (Hartzell et al., 1973), and a weak band in the 820 - 840 nm region, which has been attributed to oxidized Cu_A (Wharton & Tzagoloff , 1964).

It has been well documented that cytochrome c oxidase isolated by different methods shows varying degrees of heterogeneity (Naqui et al., 1984 , Baker et al., 1987). Indeed even using the same preparation at different times may result in resting, oxidized enzyme with different properties. The source of this variability may be most simply interpreted as isolated enzyme existing in more than one distinct molecular form. There is ample evidence for heterogeneity of isolated oxidase. The soret peak can be found anywhere from 418 to 424 nm depending upon how the enzyme was isolated (Lemburg & Barrett, 1973), indicating that different preparations have different spectroscopic properties. The use of cyanide as a ligand for cytochrome a_3 has provided much insight into the heterogeneity problem. It was comprehensively demonstrated by Van Buuren et al. (1972) that the kinetics of cyanide binding to oxidized, resting enzyme is multiphasic. These workers reasoned that since the stoichiometry of cyanide

Figure A

Absolute Spectra of Oxidized and Reduced Oxidase



Oxidized Enzyme

Reduced Enzyme

binding was 1:1, the multiphasic kinetics must be due to isolated oxidase existing in more than 1 distinct conformation. Furthermore, Baker et al. (1987) demonstrated that the number and amplitude of these phases vary considerably in different preparations. Baker et al. show in a modification of one of the common preparative methods, that cyanide reacts with the oxidase in a single phase.

The oxidase can also exist in another conformation other than those found in the resting, oxidized enzyme. Orii and Okuniki (1963) discovered that oxidase that had been reduced and then reoxidized exhibited new properties compared to resting enzyme. This "pulsed" or "oxygenated" form is characterized by a soret shift to 428 nm and an increase in intensity of the alpha band. This variant conformation stimulates electron transfer from ferrocytochrome c to oxygen 4 - 5 fold faster than resting enzyme (Jones et al., 1984) and it also binds cyanide much faster and in a single phase (Naqui et al., 1984). It also reduces much faster than resting enzyme in the presence of excess dithionite (Lemburg & Gilmour, 1967), with the time lag required for the reduction of cytochrome a_3 in resting enzyme being abolished. In spite of these properties pulsed enzyme contains 4 oxidized metal centers, hence it may be considered to be another conformational variant of oxidized enzyme. Brudvig et al. (1981), Naqui et al. (1984), and Young (1988) have with reasonable success assigned identities to these various conformational substates using optical and EPR spectroscopy, cyanide binding kinetics, and X - ray absorption fine structure (EXAFS) data.

It has been conclusively demonstrated that oxidase is composed of many conformational substates, and that the kind and number of these states varies according

to the preparative procedure. Hence it is of interest to explore features of different preparations to characterize the reactivity of different conformational states of the oxidase.

One of the most interesting and controversial discoveries concerning the oxidase is that it functions as a redox - linked proton pump (Wikstrom et al., 1981). According to this concept electron transport from ferrocytochrome c to oxygen is coupled to translocation of protons from the matrix to the cytosol side of the mitochondrial membrane, with some of the free energy of the electron transfer reaction conserved via the generation of a transmembrane ion gradient. Convincing proof that the oxidase pumps protons was obtained through experiments in which the purified enzyme was incorporated into lipid vesicles. Reconstitution of membrane proteins into artificial phospholipid vesicles was pioneered by Racker (1973) and such reconstituted systems allow for the study of integral membrane bound proteins in a defined membrane environment without the complications that would be present in native membranes, such as intact mitochondria. Oxidase incorporated into artificial phospholipid membranes displays many of the properties associated with intact mitochondria, the most significant one being that of respiratory control. Respiratory control refers to the phenomenon whereby the activity of the oxidase is limited or "controlled" by the electrochemical gradient imposed by the membrane. During turnover 2 molecules of cytochrome c are oxidized on the outside and 2 protons are consumed to form water in the interior of the mitochondria or liposomes. The oxidase also pumps out protons with a stoichiometry of 1 H^+ : e (Casey et al., 1984

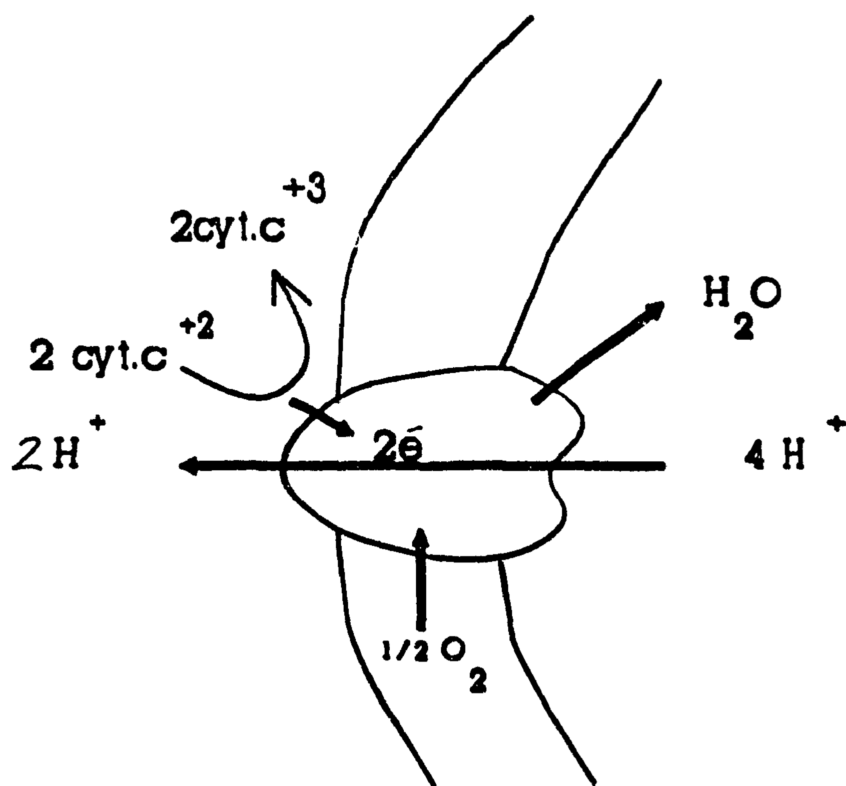
), resulting in the flow of positive charge to the outside. Figure B is a schematic model illustrating these processes. The model shows that there is a net separation of charge for each turnover, thus as turnover continues a prohibitive transmembrane potential is built up resulting in limited turnover. Addition of "uncouplers" that make the membrane permeable to particular ions relieves this stress and allows turnover to proceed at an "uncontrolled" (i.e maximal) rate. The ratio of oxidase activity in the presence of uncoupling agents (e.g the proton ionophore CCCP and the K^+ ionophore valinomycin) to that in the absence of uncouplers is referred to as the respiratory control ratio.

It was found by Wikstrom and Saari (1977) and Casey et al. (1979) that oxidase vesicles will cause an initial transient acidification of the external media due to the oxidation of its substrate, ferrocytochrome c. This had been interpreted by Wikstrom (1977) and Krab (1978) to mean that the oxidase is actively pumping out protons from the intravesicular medium. Other investigators viewed this acidification as an artifact caused by the reversible cleavage of salt linkages associated with the enzyme (Papa et al., 1983) or the membrane surface (Mitchell & Moyle, 198?) as a consequence of the binding and / or the oxidation of ferrocytochrome c. Strong support for the proton pumping hypothesis has been provided by Thelen et al. (1985). These investigators labelled the phospholipids with a fluorescent indicator capable of reliably monitoring the internal pH of the vesicles. They found that protons were consumed in the interior of the vesicles and in parallel experiments they detected acidification in the extravesicular medium. These experiments provided unequivocal proof that protons are transported from the inside to the outside of the vesicle membrane.

Figure B

Model Illustrating Stoichiometry of Proton Pumping

Coupled to Electron Transfer



In order for the oxidase to function as a redox - linked proton pump it is necessary for the enzyme complex to undergo some form of conformational transition. This transition is needed so that the oxidase can oscillate between proton input and proton output states during turnover. It is interesting that previous experimental results originally difficult to interpret, can now be adequately explained by invoking the conformational transition hypothesis. Two examples of such phenomena explained by this hypothesis are steady state biphasic kinetics observed for the oxidase and rapid inhibition of the enzyme by cyanide during turnover.

Investigators have noted that cytochrome oxidase displays nonhyperbolic kinetics when activity is plotted as a function of cytochrome c concentration (Ferguson - Miller et al., 1976, and Errede, Haight and Kamen, 1976). Such plots have been analyzed into two forms of enzymatic activity; one phase being the high affinity, low activity phase and the other the low affinity, high activity phase. Several proposals have been made to account for the biphasic kinetics. Nicholls (1974) proposed the hypothesis that the oxidase has two active sites for binding and oxididizing ferrocytochrome c. Others have suggested that there is a single catalytic site and a second regulatory site for cytochrome c interaction (Speck et al., 1984 , Sinjorgo et al., 1984). Still others envisioned a negative cooperativity model in which two catalytic sites on separate oxidase molecules are brought together in close enough proximity in a dimeric structure so that the binding of cytochrome c to one site would lead to inhibited binding at the other (Capaldi et al. 1982 , Nalecz et al., 1983). Malmstrom (1985) has proposed that the biphasic kinetics

are a consequence of a conformational transition mechanism, in which the binding of cytochrome c is tight and of low activity for one conformation and loose and of high activity for the other.

A conformational transition also provides an explanation for the puzzling observation first noted by Yonetani and Ray (1965), that the rate of cyanide binding to either oxidized or reduced oxidase is far too slow to account for the rapid rate of cyanide inhibition that occurs during turnover. It was first suggested by Nicholls et al. (1972), and subsequently verified by Jones et al. (1984) that it is the partially reduced form of the oxidase (i.e with cytochrome a and Cu_A reduced, and cytochrome a_3 and Cu_B oxidized) which is transiently formed during turnover that binds cyanide rapidly enough to account for the rate of inhibition during turnover. Reduction of cytochrome a and Cu_A supposedly triggers a conformational transition which converts the oxidase from a closed conformation (i.e cytochrome a_3 poorly accessible to cyanide) to an open conformation (cytochrome a_3 very accessible to cyanide). Since the two pairs of metal centers are quite far apart (≈ 25 Angstroms) this hypothesis requires that the protein undergoes a relatively large conformational change during the conversion from the closed to the open conformation.

Further evidence for a conformational transition was found when it was discovered that there are strong redox interactions between the two heme groups (Wikstrom et al. (1981). Transient kinetic studies of the reaction of cytochrome c and cytochrome oxidase in both the oxidized and reduced states lead to differing potentials for cytochrome a depending upon the redox state of cytochrome a_3. In the fully oxidized state cytochrome

a accepts an electron from ferrocytochrome c with a potential of ≈ 285 mV. However when cytochrome a transfers its electron to cytochrome a_3 at the same potential, the cytochrome a potential drops to 220 mV (Brzezinski and Malmstrom, 1986). It is thus apparent that the hemes interact in a cooperative manner in which the redox state of one heme affects the redox properties of the other. This interaction implies that the oxidase must exist in two different conformational states as evinced by the fact that it has two different affinities for an electron. Since as mentioned previously the hemes are relatively far apart, this redox cooperative interaction should encompass a relatively significant degree of structural rearrangement around these metal centers.

Thus the experimental results seem to point towards the existence of a conformational transition occurring in the enzyme during turnover. It has also been shown that all proteins that translocate ions across a membrane must involve some sort of conformational transition mechanism to alternate between input and output states (Tanford, 1983 and Malmstrom, 1985).

Assuming that both the conformational transition and the proton pumping hypothesis is correct, workers have tried to develop schemes in which the electron transfer can be coupled to proton pumping which are consistent with the available experimental evidence. Of particular significance is to what extent the conformational transition is involved in the coupling of electron transfer to proton translocation. One point of view is that the proton translocation is directly linked to the electron transfer. In this case the conformational change is restricted to the immediate vicinity of the metal centers with very little if any structural perturbations to the protein moiety surrounding the centers.

Another view is that the linkage is indirect with electron transfer inducing large conformational changes so that proton translocation may be initiated at some area relatively distant from the metal centers.

Experiments have been conducted to determine which metal centers are good candidates for initiating proton translocation. These models assume that it is the metal centers themselves which facilitate proton translocation and the role of the protein moiety is minimized. Two detailed models have emerged to explain direct metal center coupling to proton translocation, one involving cytochrome a and the other Cu_A.

Babock and Callahan (1983) have proposed on the basis of resonance Raman spectroscopy that cytochrome a is directly involved in proton translocation. They interpreted their spectra of cytochrome a in the reduced and oxidized state to indicate a change in hydrogen bonding strength of a formyl oxygen present as a peripheral group of the heme A macrocycle. This change in strength could be used as the primary switch for the transfer of protons along a relay of amino acids in close proximity to the formyl oxygen. Rousseau et al (1988) have also proposed a model involving cytochrome a. They interpreted resonance Raman spectroscopy to indicate the presence of bound water molecules which would be used as a source of protons as cytochrome a changes redox states.

Gelles et al. (1986) have proposed an alternate model using Cu_A as the trigger for proton translocation. They propose that upon reduction the coordination geometry of the copper becomes sufficiently distorted so that a nearby tyrosine residue replaces one of the cysteine ligands of Cu_A, causing a proton to move from tyrosine to cysteine in the

process. As the copper is reoxidized the cysteine residue gives up its proton down a proton channel leading to the cytosol as it binds to the copper, freeing the tyrosinate anion which subsequently picks up a proton from the matrix side of the mitochondria.

The above models require only local conformational changes. However, other investigators argue that the magnitude of the conformational transition is more global in nature. Brzezinski and Malmstrom (1986 , 1987) have reconciled the redox linked enhancement of cyanide binding and biphasic turnover kinetics into a model which predicts relatively large scale conformational changes. Sedimentation studies by Cabrel and Love (1972) have shown that the enzyme's volume is increased by 3 % when the oxidase is in its mixed valence (i.e partially reduced) state, indicating global changes in oxidase structure. Copeland et al. (1987) reported direct evidence for this transition by detecting fluorescence changes that occur after the oxidase is partially reduced, but these results have been shown to be artifactual (Ferreira-Rajabi, L. and Hill, B. C., 1989). The question of the degree of magnitude of the conformational transition thus remains a controversial one that has not yet been resolved.

It is perhaps more reasonable to speculate that the proton pumping process requires both the participation of the redox metal centers and some of the amino acids in the protein moiety that form the proton channel. Given the large size of the oxidase molecule and the necessity of protons traversing from one end of the protein to another it can be envisioned that the proton pumping process is relatively complex, requiring several amino acids to shuttle the protons through the proton channels, as well as a "control center" (i.e the redox metal centers) that regulates the flow of protons by

switching the system on and off. In general terms it can be envisioned that the partial reduction of cytochrome a and Cu_A trigger a local conformational change that changes the pKa's of nearby amino acids thus activating them for proton transport. Upon completion of the redox cycle the pKa's are again shifted so that the system can return to the initial state and thus be ready for another cycle. However the whole process probably requires the participation of several amino acids that compose the proton conducting channel. Nagle and Morowitz (1978) estimate that 20 amino acids would be required to translocate protons across the ≈ 40 Angstrom distance of a typical biological membrane. Figure C is a model of the oxidase illustrating a possible orientation of the proton channel as well as the location of the metal centers where they can function as a redox dependent conformational gate. It is evident from this figure that proton pumping is a complicated process that can involve both amino acids of the protein and metal centers directly. It is thus a major challenge for present and future investigators to precisely elucidate the structural features of the proton pumping apparatus and to correlate the electron transfer process with the control of this function.

Many investigators have tried to obtain structural information concerning the proton pumping mechanism through a variety of methods. The involvement of subunit III in proton pumping has been investigated extensively. Prochaska et al. (1981) were able to inhibit proton pumping by ≈ 50 % by modifying the oxidase with dicyclohexylcarbodiimide (DCCD), a hydrophobic reagent which specifically covalently modifies carboxylate groups that are present in the membrane domain. Evidence was

Figure C

Schematic Model Illustrating Orientation of the Proton Conducting Channel

and the Metal Centers

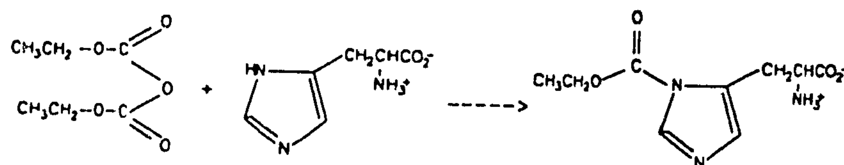
presented that the binding of DCCD to a specific glutamic acid residue on subunit III was responsible for this inhibition. It has also been shown that subunit III depleted oxidase reconstituted into vesicles can still pump protons, albeit with less efficiency (Saraste et al., 1981). Furthermore, it was shown by Puttner et al. (1983) that the oxidase from Paracoccus denitrificans which lacks subunit III can when incorporated into vesicles, still pump protons at 60 % efficiency relative to bovine cytochrome oxidase. Hence it is probable that subunit III is not the only component of the proton pathway.

Efforts have been made to modify Cu_A to see what effect this would have on the oxidase's proton pumping capabilities. Extensive work has been done modifying Cu_A either with heat treatment (Nilsson, Copeland, et al., 1988) or by using the reagent p - hydroxymercuri benzoate (pHMB) (Nilsson, Gelles, et al., 1988). These treatments disrupt the coordination sphere of Cu_A without affecting the other metal centers, and incorporation of this type of modified enzyme into vesicles leads to a $\approx$ 50 % reduction in proton pumping capabilities. This reduction is however accompanied by a decrease in the respiratory control ratio which was determined to be attributable to an increase in the permeability of the vesicles to protons. This could be due to the disruption of the proton pumping capabilities attributed to Cu_A or alternatively it could simply reflect the creation of a new proton conducting channel that is far removed from the true proton channel.

The goal of this thesis is to determine what role, if any, histidine residues in the oxidase play in the proton pumping mechanism. The imidazole side chain of histidine has a pKa of 6.0 making it a possible candidate to be one of the amino acids which

participates in the proton pumping apparatus. In order to answer this question bovine heart cytochrome oxidase was modified with the histidine specific reagent diethylpyrocarbonate (DEPC). The modification reaction was characterized and its effect on some of the functional properties of the oxidase was investigated, including proton pumping.

It was first demonstrated by Ovadi et al. (1967) that DEPC reacts fairly specifically with the imidazole ring of histidine, forming an N - carbethoxyhistidine derivative whose absorption at 240 nm allows for the quantification of histidine modification. The reaction sequence is shown in the scheme below.



The modification can often be reversed by treating the modified protein with hydroxylamine, which removes the carbethoxyl group and regenerates histidine. Hence DEPC is considered by many to be an ideal reagent for histidine modification and it is now probably the most commonly used reagent for this purpose.

The results from modification must nevertheless be interpreted with caution because there is the possibility of many undesirable side reactions. For example, high

concentrations of DEPC are capable of causing the formation of a disubstituted carbethoxyhistidine derivative (Miles, 1977). Treatment of this product with NH_2OH does not restore histidine, it instead causes a ring cleavage of imidazole (Avaeva & Krasnova, 1975). The disubstituted product also has a higher absorbance at 240 nm than the monosubstituted product, thus leading to the possibility of overestimating the number of histidines modified. It has also been shown that the disubstituted product is capable of being hydrolysed by water, resulting in a Bamburger cleavage of the imidazole ring (Grace et al., 1980) which is an irreversible modification of histidine.

The possibility of modification of other amino acids also must not be ignored. DEPC does not react solely with histidine, because it can react with most nucleophilic species. The only justification for DEPC specificity for histidine is that it reacts with it at a faster rate than the other amino acids (Osterman - Golkar et al., 1974). However there are several examples in the literature where DEPC has been shown to react with amino groups, serine, arginine, tyrosine, cysteine, and tryptophan (Melchoir & Fahrney 1970, Muhlrad et al., 1967, Rosen et al., 1970). Hence interpreting results on the assumption that only histidines are modified is a dangerous one unless the necessary controls are performed.

Experimental Materials

The following reagents were obtained from Sigma Chemical Company:

Potassium Chloride, Sucrose, DEPC, valinomycin, TMPD, CCCP, cytochrome c from horse heart (type VI), L - phosphatidylcholine (asolectin) from soyabean and egg yolk.

The following reagents were obtained from BDH Chemicals LtD:

L-ascorbic acid, potassium dihydrogen orthophosphate, dipotassium hydrogen phosphate, ammonium sulphate, potassium cyanide.

The following reagents were obtained from Fluka Biochemika:

DEPC, sodium hydrosulfite (sodium dithionite), cholic acid.

The following reagents were supplied from J.T. Baker Chemical Co. :

EDTA (disodium salt, dihydrate), L-histidine.

Dodecyl-beta-D-maltoside (lauryl maltoside) was obtained from Boehringer Mannheim, Sephadex G-25 (coarse grade) from Pharmacia Fine Chemicals, and sodium hydrosulfite from Fisher Scientific.

Experimental Methods

Preparation of Cytochrome Oxidase

The oxidase was isolated from beef hearts by combining the methods of Yonetani (1961) and Kuboyama et al. (1972). The submitochondrial particles were prepared according to Yonetani, while the rest of the isolation was done via the Kuboyama method.

The following procedure was done at 4 degrees celsius unless otherwise specified. Two fresh beef hearts were obtained from the slaughterhouse and packed in ice for the journey back to the lab. The fat and connective tissue was then removed from the hearts with a sharp knife and the muscle was cut into small cubes. The muscle was then minced using a mincer. The mince was washed repeatedly with 10 volumes of cold 0.02 M phosphate buffer pH 7.4 until the drained fluid was yellow instead of red. 300 gram portions of the mince were blended in a waring blender with 150 ml of 0.2 M phosphate buffer pH 7.4 and 150 ml of distilled water until a smooth homogenate was achieved (2 min). Pooled homogenates were diluted to 5 liters with distilled water for every 3 liters of homogenate. Homogenate was centrifuged on an IEC DPR-6000 centrifuge for 20 minutes at 3400 rpm. The supernatant was collected and the pellet was rehomogenized and recentrifuged as previously described. The resultant supernatant was combined with the supernatant obtained previously. 1 M acetic acid was added to the supernatant until the pH was 5.6. The supernatant was then recentrifuged at 3400 rpm for

10 minutes.The supernatant was discarded and the pellet was washed with 10 volumes of water and suspended. The suspension was centrifuged for 1 hour and the pellet was saved. The pellet was homogenized in 0.2 M phosphate buffer pH 7.4 with gentle stirring with a glass rod. The pH was then adjusted to 7.4 with 3 M NH2OH. At this point the concentration of total protein was determined via the bicinchoninic acid assay (Smith, 1985). After this was done the volume of the homogenate was adjusted with distilled water so that a protein concentration of about 20 mg/ml was achieved. This was then stored overnight. The subsequent steps of the isolation were done according to the method of Kuboyama. 10 % cholic acid pH 7.4 was added to give a final concentration of 1 %. Solid ammonium sulfate was added to 25 % saturation and the pH was adjusted to 8.0 with 1 M NaOH. This solution stood for 1 hour with occasional stirring. Then further ammonium sulfate was added to give 35% saturation and the solution was allowed to stand for 10 minutes with occasional stirring. This was then centrifuged for 60 minutes at 15000 rpm on an IEC Model B-20 centrifuge. The reddish supernatant was discarded and the pellet was resuspended in 0.1 M phosphate buffer to a volume of 300 ml with the aid of an homogenizer. To this suspension enough cholic acid was added to give a 2% solution. Ammonium sulfate was added to 25 % saturation and the pH was adjusted to 7.4. This was allowed to stand for 12 hours in the cold room. This was then centrifuged for 30 minutes at 15000 rpm and the pellet was discarded. Solid ammonium sulfate was then added to 40 % saturation and the suspension was allowed to stand for 10 minutes with occasional stirring. This was then centrifuged for 15 minutes and the resultant supernatant was discarded. The greenish pellet was dissolved in 100 ml 0.1 M phosphate

pH 7.4 containing 1.5 % cholate. To this solution a saturated (4.1 M) neutralized ammonium sulfate solution was added until a slight but persistent turbidity became apparent. The concentration required for this is usually 25 %. The mixture was allowed to stand for 30 minutes and was then centrifuged for 20 minutes at 15000 rpm. The pellet was discarded and the supernatant was brought up to 37 % saturation and centrifuged for 20 minutes at 15000 rpm. The resulting pellet was analyzed spectrophotometrically to determine the 280/420 nm ratio. A ratio of 2.5 or less indicates acceptable purity, in which case the pellet is solubilized in 0.1 M phosphate buffer, pH 7.4 containing either 1 % cholate or 1 mg/ml lauryl maltoside. If the ratio is greater than [illegible] then the ammonium sulfate fractionation procedure must be repeated until the desired level of purity is reached.

After an acceptable level of purity is achieved the enzyme was stored in liquid nitrogen. Prior to an experiment the enzyme was thawed and diluted to the desired concentration in a buffer containing either 1 % cholic acid or 1 mg/ml lauryl maltoside as the stabilizing detergent.

Three different preparations of enzymes were used for the following experiments. One preparation was isolated by the author and is designated enzyme type 1. Another preparation was isolated by a colleague and is called enzyme type 2. The last type of enzyme used was a preparation made in Dr. Nicholl's laboratory at Brock University, St. Catharines, Ontario and is designated enzyme type 3.

Enzyme concentration was determined using one of the following two methods. The most frequently used method relied upon the extinction coefficient developed by

Wrigglesworth (1988). This method was based on the subtraction of two wavelengths obtained from the oxidized spectrum of the oxidase as is shown in the equation below.

$$Abs_{(419\ nm)} - Abs._{(470\ nm)} = 139\ mM^{-1}cm^{-1} * c * l$$

where c = concentration of oxidase and l = path length in cm.

The other method is based upon the subtraction of two wavelengths from the difference spectrum of reduced - oxidized oxidase and is given in the following equation.

$$Abs._{(445\ nm)} - Abs._{(460\ nm)} = 148\ mM^{-1}cm^{-1}*c*l$$

<u>Modification of cytochrome oxidase with DEPC</u>

The DEPC is added as an ethanolic solution. Before use its concentration is checked by adding 5 ul to 3 ml of a 10 mM histidine solution, pH 6.0. A molar absorptivity coefficient of 3200 $M^{-1}cm^{-1}$ at 240 nm (Ovadi <u>et al.</u>, 1967) was used to calculate the concentration of DEPC. The DEPC was kept on ice before use.

The enzyme sample was diluted to the desired concentration and placed in a Shimadzu UV - visible recording spectrophotometer. The temperature was maintained at 20 degrees Celsius. The enzyme sample was allowed to equilibrate for 5 minutes following dilution

in order to come to 20 degrees Celsius. The desired concentration of DEPC is then added to the sample and the increase in absorbance at 240 nm due to histidine modification was followed for 30 minutes.

It was found that some samples of DEPC contained an impurity which gave rise to an absorbance band at 240 nm that quickly decayed to zero absorbance (t 1/2 ≈ 4 min). It was determined that pure DEPC and the contaminated samples both resulted in the same kinetics and magnitude of the absorbance increase providing that the absorbance contributions of the spectral impurity (determined in a separate experiment under identical conditions) were subtracted from the original absorbance values. It can thus be concluded that the impurity has no effect on the modification providing that its contribution to the 240 nm absorbance is corrected for.

Once a reaction profile of histidine modification was obtained the curve was digitized and entered into LOTUS 1-2-3 as a spread sheet and unformatted data files were created. F-CURVE software (Noggle, 1985) was used for curve fitting in order to obtain the pseudo first order rate constants for DEPC modification of the oxidase. The curve fitting program uses the simplex algorithm. In order to determine the number of histidines modified a molar absorptivity coefficient of 3600 M-1 sec-1 (Holbrook & Ingram, 1973) at 240 nm was used.

Determination of DEPC Hydrolysis Rate

The rate constants for DEPC hydrolysis in various solutions were determined by adding the same volume of DEPC to several test tubes containing 1 ml of the desired solution. At various time intervals the hydrolysis of DEPC was quenched by adding 1 ml of a 10 mM histidine solution pH 6.0 and the remaining DEPC was quantified spectrophotometrically at 240 nm as described previously. In order to determine the DEPC concentration at time 0 , the same volume of DEPC was added to a solution containing a 1/2 ml of histidine solution and a 1/2 ml of the solution being tested and the concentration was then determined spectrophotometrically. The data were then treated in accordance with pseudo first order kinetics as shown by the equation below.

$$\ln ([\text{DEPC}]_0 / [\text{DEPC}]_t) = kt$$

where; $[\text{DEPC}]_0$ = concentration of DEPC at time 0.

$[\text{DEPC}]_t$ = concentration of DEPC at time t.

k = observed pseudo first order rate constant

t = time

A plot of $\ln ([\text{DEPC}]_0 / [\text{DEPC}]_t$ vs time was constructed and the slope of the resulting line was equal to k.

Preparation of Reduced Cytochrome c

Approximately 10 uM cytochrome c (0.5 ml)was prepared in 79 mM Sucrose, 130 mM KCl, 1 mM EDTA, pH 7.3. Sufficient solid ascorbic acid was then added to give a final concentration of about 50 uM. The solution was allowed to stand for 15 minutes to ensure complete reduction of the cytochrome c. The solution was then passed through a Sephadex G - 25 column previously equilibrated with the sucrose solution mentioned above. The red fraction containing the cytochrome c was then collected from the column and its concentration was checked using the following equation.

$$\text{abs.}550\ nm_{(reduced)} - \text{abs.}540\ nm_{(reduced)} = 18.9\ mM^{-1}cm^{-1} * c * l$$

The % reduction was determined by adding an excess of sodium dithionite to the sample and redetermining the concentration of cytochrome c with the above equation. The concentration value obtained from ascorbate reduction was divided by the value from dithionite reduction to calculate the % reduction of the sample.

Binding of Cyanide to Native and Modified Enzyme

A 1 M stock solution of KCN in distilled water was prepared for the cyanide binding experiments. The oxidase (type 1) was modified as described previously, and after the reaction was complete the desired concentration of cyanide (usually 2 mM) was added. The increase in absorbance at 433 nm was then monitored (Van Buuren et al., 1972). The process was then repeated on a control of native enzyme and then the total changes in absorbance at various time intervals were compared. Other experiments were performed in which the cyanide was added at various time intervals during the modification reaction so that the DEPC effect on cyanide binding could be correlated to a specific time during the modification.

Pseudo first order rate constants for the binding of cyanide to native and modified oxidase were evaluated in the following manner. Type 1 oxidase was modified as described previously and after the reaction was complete sufficient KCN solution was added to give a final concentration of 2 mM. The reaction was monitored at 433 nm for 400 seconds and then the absorbance values were printed out every 20 seconds using the print command on the spectrophotometer. The process was repeated using native oxidase. Both samples were then allowed to sit for 24 hours at room temperature. The absorbance values at 433 nm were then recorded and assumed to be the final absorbance value (A_∞) signifying that the reaction had reached completion. A semilog plot was used to analyze the data using equation 2.

$$\mathrm{Ln}\ (A_t - A_\infty) = kt \qquad [2]$$

where: A_t = absorbance value at time t.

A_∞ = absorbance value at time ∞ .

k = pseudo first order rate constant.

t = time in seconds.

It was apparent during the analysis that the data was biphasic over the initial 400 second time interval, hence a "curve peeling" procedure was used to extract two rate constants, designated k1 and k2.

Polarographic Measurement of Oxidase Activity.

The measurement of oxygen consumption catalyzed by cytochrome oxidase was accomplished via a polarographic assay. The apparatus consisted of a 1.6 ml glass cup tightly fitted with a removable plastic assembly through which the necessary solutions may be injected with a hamilton syringe. There is an opening in the side of the cup through which a Clark electrode may be snugly fitted. The electrode was connected to a power box made by Concordia University, which in turn was connected to a Sargent Welch recorder model XKR. The recorder was set to a 10 mV span and the chart speed was 2 cm/min. For all experiments it was assumed that the concentration of O_2 in the buffer was equivalent to that of an air saturated solution, which corresponds to a value

of 270 uM O_2 at 20 degrees Celsius.

A typical experiment is as follows. 1.7 ml of 25 mM KPi, 1 mM EDTA, 1 mg/ml lauryl maltoside, pH 7.2 is added to the chamber. The plastic lid is carefully inserted into the chamber to insure that no bubbles are trapped inside. The excess buffer (0.1 ml) flows out through the opening used for injections of desired solutions. The stir bar is then activated by turning on the stirring motor, the same speed being used for all experiments reported. After a few minutes the system stabilizes resulting in a stable signal from the chart recorder. 10 uL of 0.67 M Ascorbate is then injected into the system, followed by 2 ul of 0.2 M TMPD. Then sufficient oxidase (either native or modified) to give a final concentration of 10 - 20 nM is injected. The system is allowed to run for a few minutes so that a reliable background rate due to the slow reduction of enzyme by TMPD may be obtained. Then the reaction is initiated by injecting cytochrome c, resulting in elevated O_2 consumption. The trace is allowed to continue until the O_2 supply is exhausted. The background rate is subtracted from the cytochrome c rate in order to get the rate due to cytochrome c alone. The rate is then converted from nM O_2 / sec to turnover number (units; e sec-1[aa3-1]) by multiplying by four (i.e the number of reducing equivalents necessary to convert O_2 to H_2O) and dividing by the aa3 concentration. The experiment was repeated at several cytochrome c concentrations to evaluate Km and TNmax values.

Preparation of cytochrome c oxidase vesicles

Three methods were used to prepare oxidase vesicles. A description of these

methods is as follows.

Method 1

Soyabean asolectin was suspended at 50 mg/ml in 25 mM KPi, 1 mM EDTA, pH 7.2 overnight. The next day 1 ml of ~ 20 uM enzyme prepared in the above buffer plus 1 % cholic acid was gently mixed with two ml of the lipids in a 10 ml beaker. The above mixture was then sonicated with an MSE sonicator. The beaker was clamped under the probe so that 1.8 cm of it was submerged into the lipids. The beaker was immersed in an ice water bath to prevent heating of the sample during sonication. The sample was sonicated for five 10 second intervals with 20 seconds between each sonication. The sonicated solution was then centrifuged at 13000 rpm for 10 minutes in a MSE MicroCentaur centrifuge to remove any titanium dust from the probe and excess asolectin. The oxidase vesicles were then separated from the pellet and kept on ice or stored in the refrigerator.

Method 2

The following method is a modification of the method first proposed by Racker (1973), and used by Casey (1986). 1 gram of egg yolk asolectin was washed three times with 25 ml of anhydrous acetone. The duration of the washes was one hour, overnight, and three hours. The washing was done in the presence of 1 mM dithiothreitol,

an antioxidant which is present to prevent extensive lipid peroxidation. The lipids were then solubilized in 10 ml of ether. The ether was then evaporated using a rotovap followed by blowing nitrogen gas onto the dried lipids. The dried lipids were then suspended overnight in 25 mM KPi, 1 mM EDTA pH 7.2 at a concentration of 10 mg/ml. The next day 1 ml of ≈ 2 uM type 2 enzyme in the above buffer plus 1 % cholic acid was gently mixed with 3 ml of the lipid suspension. It was then sonicated under the same conditions described in method 1 except that the sonications were done fifteen times for a duration of 4 seconds with a 30 second delay between intervals. The depth of the probe was also deeper (2.4 cm) due to the greater volume of liquid. The vesicles were then prepared as described in method 1.

Method 3

Oxidase vesicles were also prepared via the dialysis method. This method consists of diluting oxidase to a final concentration of 6.5 uM in 5 ml of a mixture containing 250 mg of soyabean asolectin, 1.5 % sodium cholate, and 100 mM Potassium Hepes, pH 7.4. The suspension is sonicated to clarity and then centrifuged at 13000 RPM for 10 minutes. Then the sample is then dialysed against 500 ml of 100 mM K^+ - Hepes, pH 7.4 for four hours. The sample is then changed to 1 L of 10 mM K^+ - Hepes, 40 mM KCl, 50 mM sucrose, pH 7.4 and allowed to dialyse overnight. The medium is then changed twice the next day using the same type of fresh solution at 4 hour intervals. This is followed by a final change of solution that continues overnight. The resultant vesicles are then kept on

ice or stored in the fridge until needed.

Determination of the Respiratory Control Ratio (RCR)

The oxygen electrode was set up as described previously. The assay buffer was 25 mM KPi, 1 mM EDTA, pH 7.4 . The procedure is similar to that described previously, except that vesicles (native or modified) are substituted for free enzyme. When cytochrome c is added one obtains the coupled rate of respiration. Addition of 1 uL of 2 mg/ml valinomycin followed by the addition of 1 uL of 0.4 mg/ml of CCCP results in the uncoupled rate of O_2 consumption. The RCR may be evaluated by the following equation.

RCR = uncoupled rate / coupled rate.

Determination of oxidase orientation in vesicles

A sample of oxidase vesicles in KPi buffer had its spectrum recorded from 400 to 700 nm and it was stored in memory. 10 mM ascorbate and a catalytic amount of cytochrome c was then added and the increase in absorbance at 445 nm was monitored until no further increases were observed. The spectrum was then recorded and stored in memory. The reduced vesicles spectrum is subtracted from the oxidized one and the

difference values of 445 nm and 460 nm are used to calculate the concentration of oxidase vesicles accessible to ascorbate. Then 1 mM of TMPD is added and the increases in absorbance in the 445 nm region are monitored until no further increases are seen. The spectrum is then recorded, stored in memory, and then the oxidized spectrum subtracted from it. The concentration of total active enzyme in the vesicles is then determined from the 445 and 460 nm values from the difference spectra. The percentage of enzyme molecules oriented outwards may then be calculated with the following equation.

$$\% \text{ facing outward} = \frac{[\text{ enzyme reducible by ascorbate }]}{[\text{ total enzyme }]}$$

<u>Determination of proton pumping by oxidase vesicles</u>

Enzyme type 3 (2 uM) was modified with 0.75 mM DEPC. After modification was complete the enzyme was concentrated to ≈ 20 uM with a Amicon ultrafiltration cell model M - 3 using a YM100 Diaflo ultrafiltration membrane. The same procedure was repeated with native enzyme. Vesicles were then prepared according to method 1 described previously. The resultant vesicles were then passed through a G-25 column that had been previously equilibrated with 79 mM sucrose, 130 mM KCl, 1 mM EDTA pH 7.3. This step was necessary to remove the external buffer from the vesicles. The width of the column was 1 cm and the length of the bed was 42 cm. After the vesicles were eluted from the column the enzyme concentration was determined from the reduced -

oxidized spectrum as described previously. The vesicles were then diluted to 0.42 uM with the sucrose solution. The apparatus used to detect proton pumping was a pH electrode connected to a Corning pH / ion analyzer model 250. The signal from the pH meter was fed through a tuneable lowpass filter instrument model 901F from Frequency Devices to eliminate high frequency noise. The filter box was connected to the Sargent Welch recorder which was set at a 2 mV span and a chart speed of 10 cm/sec. Reduced Cytochrome c (1 mM) was prepared as described previously. Before experimental readings were taken the pH of both the cytochrome c solution and the vesicles were carefully adjusted so that they both had the same initial pH of 7.3. The vesicles contained 3.6 uM valinomycin and for some experiments also contained 2.6 uM CCCP.

The experiment was done as follows. 1.5 ml of vesicles was pipetted into a glass chamber with magnetic stirring capabilities. The pH electrode was inserted into the vesicles and the stirrer was turned on at a certain speed that was used for all subsequent experiments. After the recorder reading had stabilized 5 nmoles of cytochrome c was added. The delta pH reaction was allowed to go to completion after which 5 nmoles of HCl was added for calibration purposes. This procedure was repeated 3 or 4 times for each sample. Then the chamber was rinsed several times with water followed by 2 rinses with sucrose solution. The experiment was then repeated with a fresh sample.

Fluorescence Experiments

All fluorescence experiments were done on a Shimadzu Spectrofluorophotometer model RF - 5000. The following parameters were used for the measurements. The excitation wavelength was 280 nm and the emission range was from 280 to 500 nm. The band width for both the excitation and emission was 10 nm. The scan speed was set to medium speed and the sensitivity was put on high.

Comparison of the fluorescent intensities of native and modified enzyme was done as follows. Type 2 oxidase was modified as described previously. After modification was complete it was diluted in 25 mM KPi, 1 mM EDTA, pH 7.2 with 1 mg/ml lauryl maltoside to a final concentration of 22.9 nM. The emission spectrum was then recorded, and stored in memory. The emission spectrum of the buffer solution alone was also recorded and stored in memory. The buffer spectrum was subtracted from the modified enzyme spectrum to give the corrected spectrum. Native enzyme to which was added the same volume of ethanol as the modified enzyme was diluted to the same concentration and was measured and stored in memory the same way. Difference spectra were obtained by subtracting the corrected spectrum of the modified enzyme from the corrected spectrum of the native one.

Results

1) Characterization of the Reaction of DEPC with Cytochrome c Oxidase

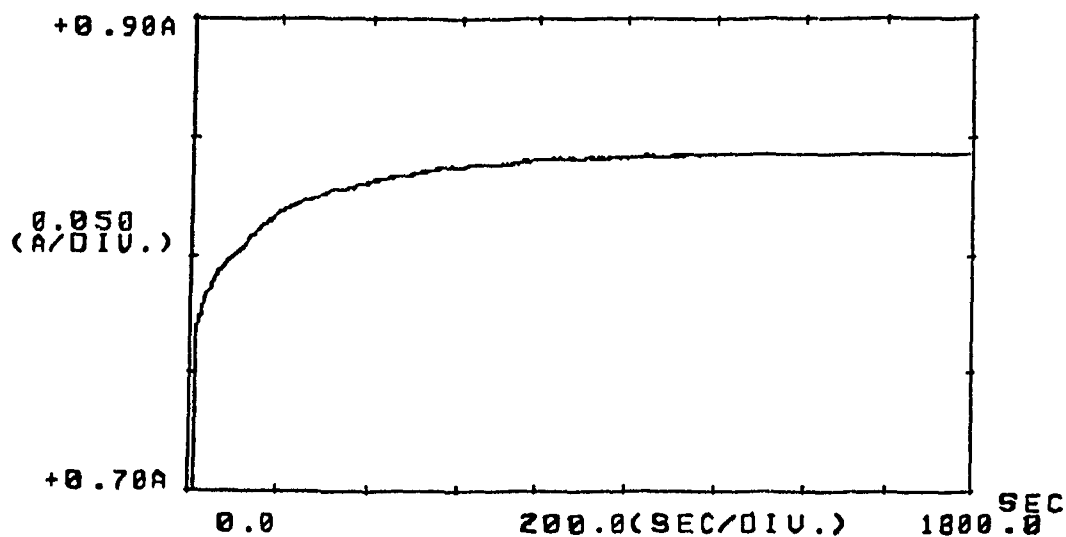
A) Spectroscopic Characterizations

Figure 1 is typical reaction profile of DEPC reacting with the oxidase illustrating the increase in absorbance with time at 240 nm indicating the formation of ethoxyformylhistidines. The initial sharp jump in the profile is not part of the reaction, it is a consequence of the cuvette being placed in the spectrophotometer while it is actively scanning. After this initial jump follows the reaction curve of histidine modification. The curve to a first approximation is hyperbolic which is typical of pseudo first order reactions. A difference spectrum obtained by subtracting the modified spectrum from the native one is given in figure 2. This figure shows an absorption band with a peak at 240 nm. The difference spectrum shows no increase in absorbance in the vicinity of 280 nm (see figure 3), indicating that DEPC has not modified tyrosine or tryptophan residues. However there is a decrease in the Soret band (see figure 4) of the DEPC reacted enzyme in the region of 428 - 432 nm. This small but reproducible trough was present on every modification monitored. This spectral perturbation indicates that one or both of the heme environments are altered.

Figure 1

Reaction Profile of the Modification of

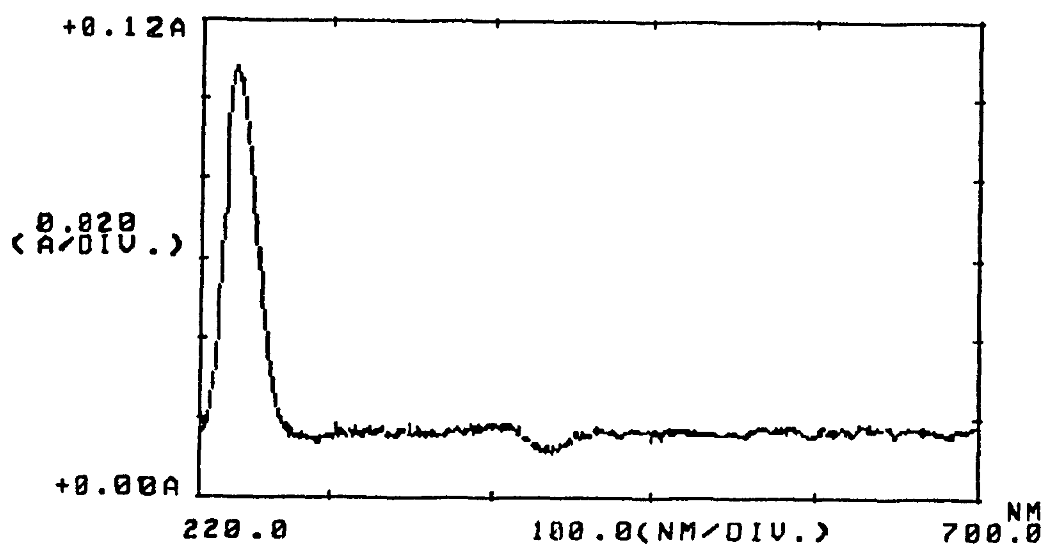
Cytochrome c oxidase with DEPC



Type 2 enzyme was diluted to a final concentration of 2.29 uM in 25 mM KPi, pH 7.2 with 1 mg/ml lauryl maltoside. The oxidase was allowed to sit in the cuvette holder for 5 minutes to allow it to reach 20 degrees Celsius. Sufficient DEPC was then added to give a final concentration of 0.75 mM and the reaction was monitored at 240 nm for 30 minutes.

Figure 2

Difference Spectrum of Modified Oxidase - Native Oxidase



The spectrum of the native oxidase was recorded from 220 to 700 nm and was then stored in memory 2. The oxidase was then modified with 0.53 mM DEPC for 30 minutes. The spectrum of the modified oxidase was then recorded from 220 to 700 nm and stored in memory 1. Subtraction of the contents in memory 1 from that in memory 2 resulted in the difference spectrum of modified oxidase - native oxidase and is shown in the above figure.

Figure 3

Expansion of the 280 nm Region of the Difference Spectrum

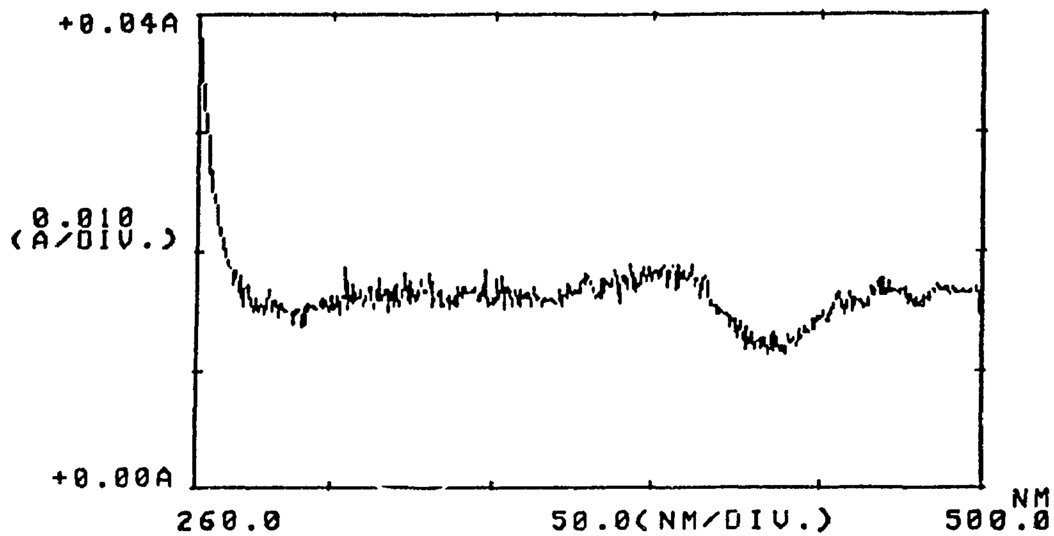
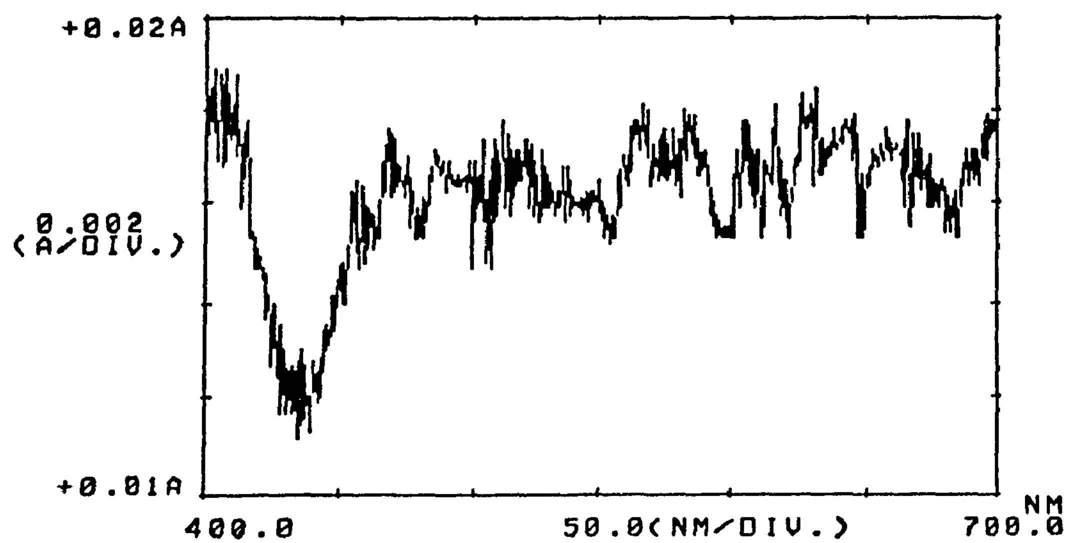


Figure 4

Expansion of the 430 nm Region of the Difference Spectrum



The spectrum obtained in figure 2 was manipulated with the expansion function on the spectrophotometer to obtain figures 3 and 4.

The magnitude of this trough is dependent upon the concentration of DEPC used for modification. Figure 5 depicts the dependence of the magnitude of the trough upon DEPC concentration. The magnitude increases approximately linearly as the concentration of DEPC increases.

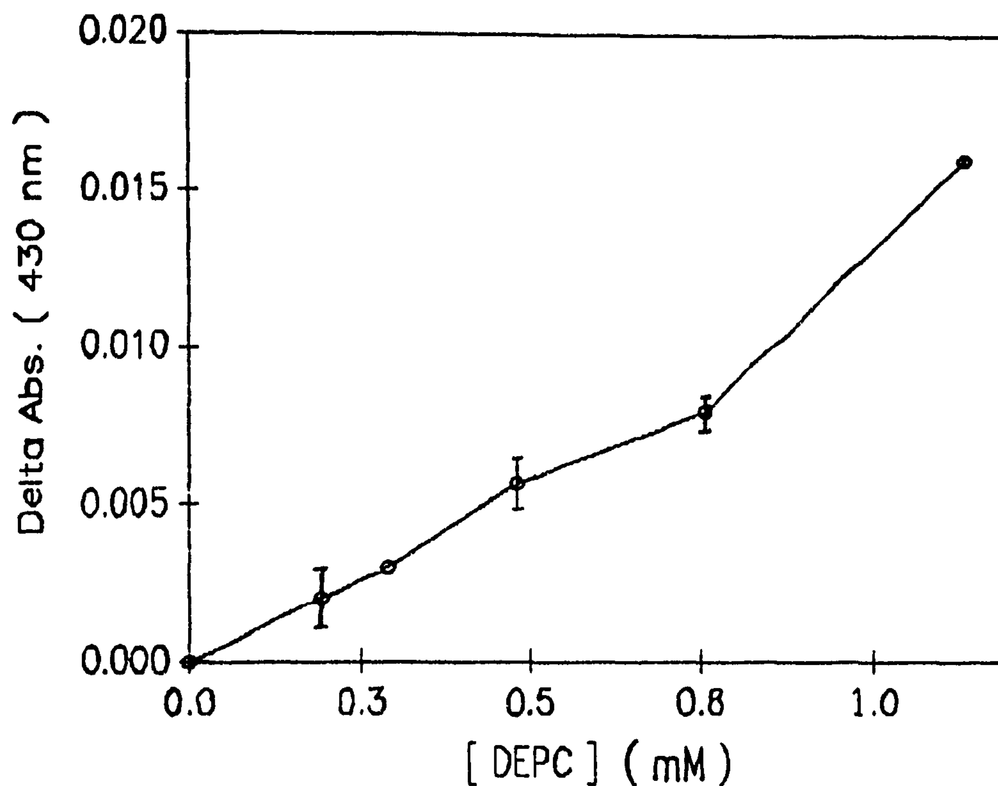
At higher enzyme concentration other spectral perturbations in the visible region are apparent. Figure 6 is a difference spectrum obtained after modifying 11 uM enzyme with 11.2 mM DEPC. This spectrum shows the previous spectroscopic trough at 430 nm although in this case the magnitude of the perturbation is larger due to the higher concentration of enzyme. The figure illustrates a new spectral perturbation in the vicinity of 600 - 660 nm. Hence modification under these conditions gives rise to two distinct perturbations of the visible spectrum.

The effect of modification on the reduced - oxidized difference spectrum was also investigated. Figures 7 and 8 show difference spectra that were obtained for native and modified oxidase respectively. The positions of the main peaks and troughs for the native and modified enzyme are at the same position and are of the same magnitude. However a difference spectrum taken of the two reduced - oxidized spectra (figure 9) shows a 5 nm red shift of the Soret and alpha bands. A separate control experiment was done in which two separate reduced - oxidized spectra of native enzyme were stored and subtracted.

Figure 5

Effect of Increasing DEPC concentration on the Magnitude

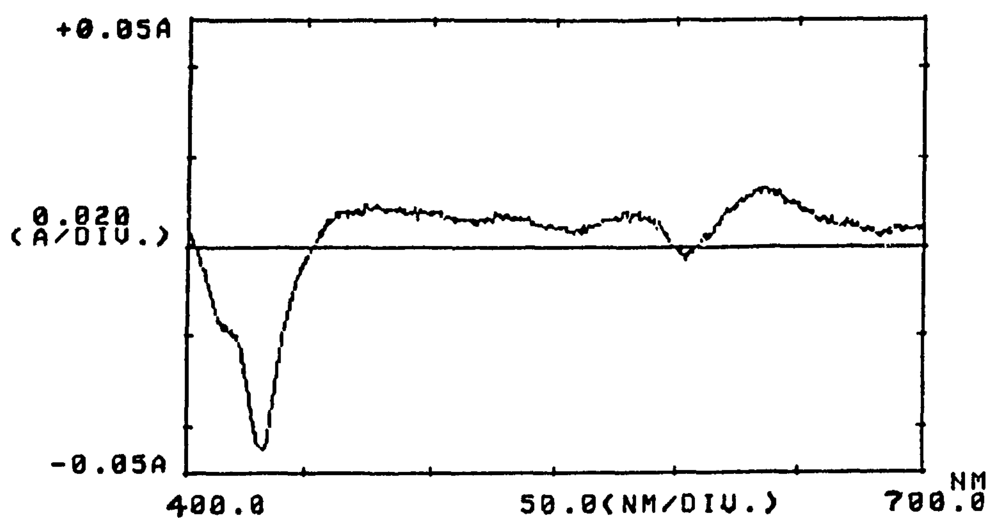
of the 430 nm spectroscopic Trough



Type 1 oxidase was diluted to 1 uM in 0.1 M KPi, pH 7.2 with 1 mg/ml lauryl maltoside. The native oxidase had its spectrum recorded from 220 to 700 nm and it was stored in memory 2. Several modification experiments were then performed with varying concentrations of DEPC, with each modification being permitted to run for 30 minutes. The spectrum of the modified oxidase was then recorded from 220 to 700 nm and stored in memory 1. The 430 nm was obtained by subtracting the modified spectrum from the native one. The magnitude of the dip was then measured by visually drawing a line of best fit through the base line. The distance was then measured in mm from this base line to the maximal point of decrease exhibited by the dip with a ruler. The distance in mm was then converted to absorbance units by using the scale on the absorbance (i.e y) axis.

Figure 6

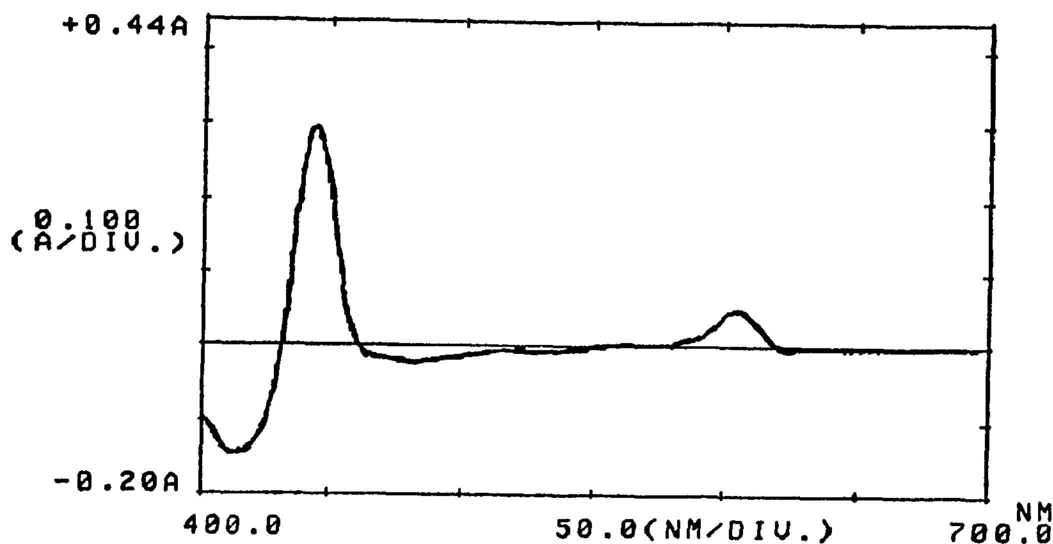
Difference Spectrum of Modified Oxidase - Native Oxidase



Type 1 oxidase was diluted to a concentration of 11.0 uM in 0.1 M KPi, 1 mg/ml lauryl maltoside, pH 7.2. The native oxidase spectrum was recorded from 400 to 700 nm and was then stored in memory 2. The oxidase was then modified with 11.2 mM DEPC for 30 minutes. The spectrum of the modified oxidase was then recorded from 400 to 700 nm and was subsequently stored in memory 1. The difference spectrum of modified oxidase - native oxidase was then obtained by subtracting memory 1 from memory 2.

Figure 7

Reduced - Oxidized Spectrum of Native Oxidase

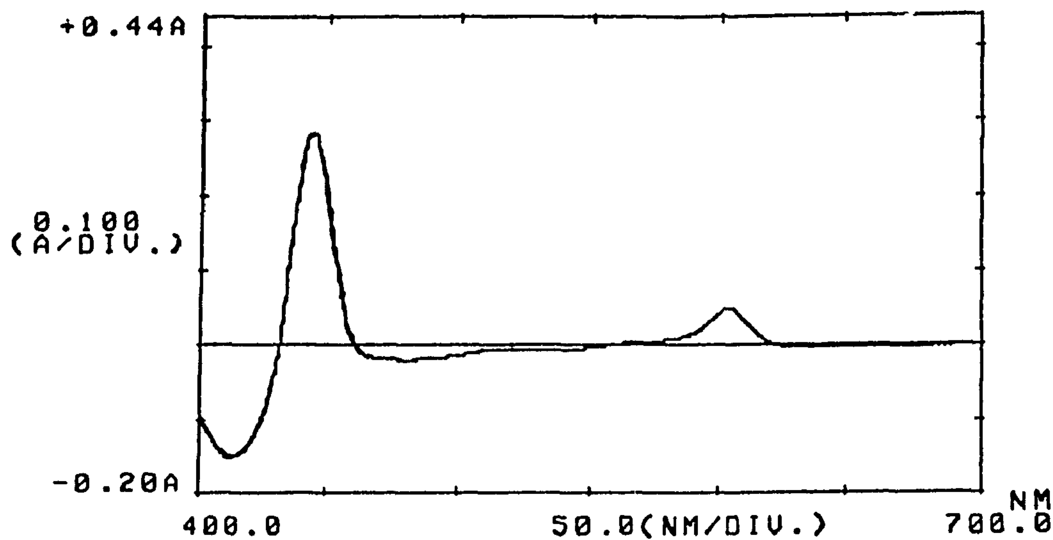


λ max values (nm)
445 (peak)
605 (peak)
412 (valley)

Type 2 oxidase was diluted to 2.1 uM in 25 mM KPi, 1 mM EDTA, 1 % cholic acid, pH 7.2. A 1 ml sample of the oxidized enzyme was placed in the reference compartment of the spectrophotometer. A separate 1 ml sample was placed in a matched cuvette and a few grains of sodium dithionite were added. The sample was then placed in the cell compartment and the live difference spectrum was monitored from 400 to 700 nm. After 15 minutes the magnitude of the 445 nm peak ceased to change, indicating that the reduction of the oxidase was complete. Figure 7 is a picture of this difference spectrum. It was then stored in memory 1 so that it could be manipulated for further analysis.

Figure 8

Reduced - Oxidized Spectrum of Modified Oxidase



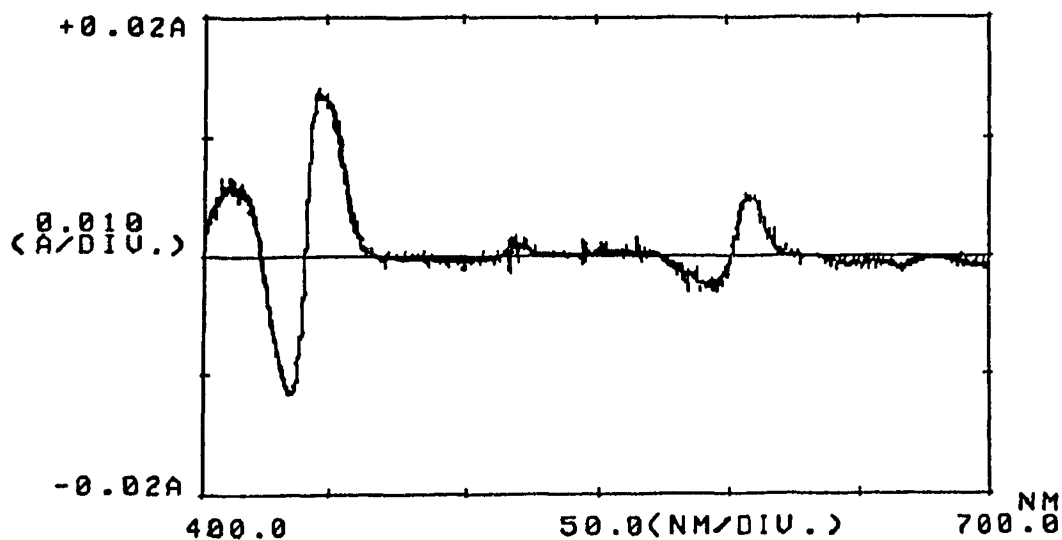
λ max. values (nm)
445 (peak)
605 (peak)
413 (valley)

Type 2 Oxidase (2.1 uM) was modified with 0.75 mM DEPC for 30 minutes. A 1 ml sample was then placed in the reference cuvette holder of the spectrophotometer. Another 1 ml sample was placed in a matched cuvette and a few grains of sodium dithionite were added. The sample was then placed in the cell compartment and the live difference spectrum was monitored from 400 to 700 nm. After 15 minutes the magnitude of the 445 nm peak ceased to change, indicating that reduction was essentially complete. Figure 8 is a picture of this difference spectrum. It was then put in memory 2 so that it could be manipulated for further analysis.

Figure 9

Native (Reduced - Oxidized) Difference Spectrum -

Modified (Reduced - Oxidized) Difference Spectrum



λ max. values (nm)
448 nm (peak)
610 nm (peak)
435 nm (valley)

The modified difference spectrum in memory 2 was subtracted from the native difference spectrum in memory 1. The result of this subtraction is illustrated in the above figure.

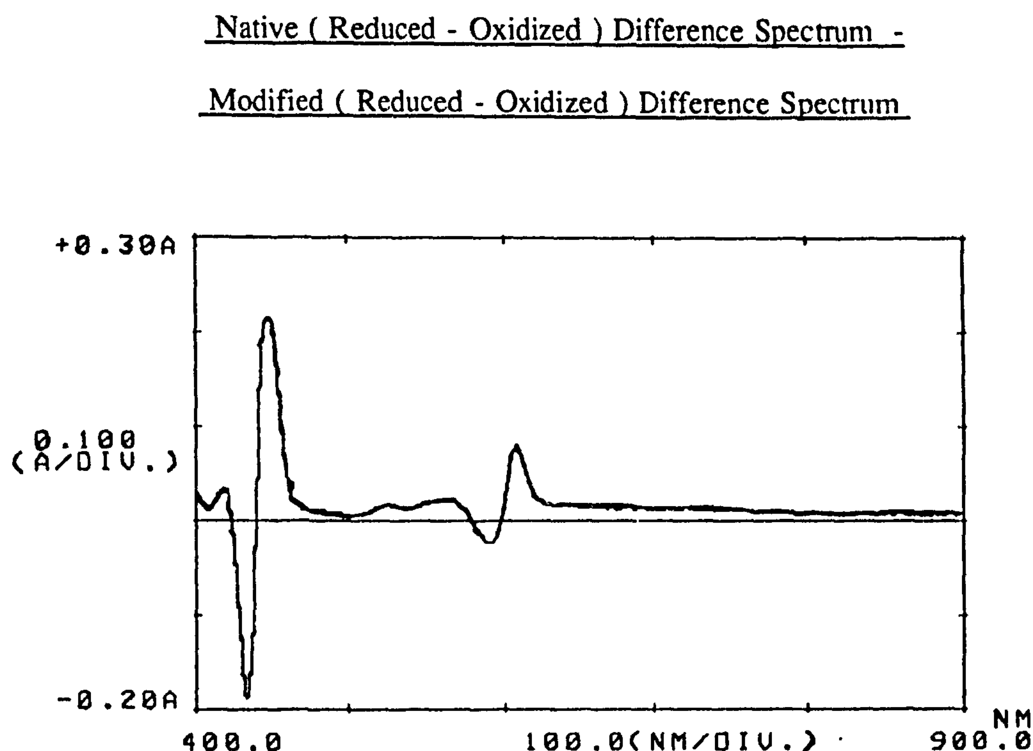
This was done to determine if the peak shifts and their magnitude were due to subtracting difference spectra in which the two samples had varying degrees of reduction. This control experiment showed no shift in the peaks or troughs. Hence it can be concluded that differences in reduction levels cannot account for the small difference between the native and modified enzyme. It may also be concluded that the peak and trough shifts are a direct consequence of the modification.

The above experimental protocol was repeated, except that higher concentrations of enzyme and DEPC were employed. The rationale behind this experiment was to ascertain whether the reduction of Cu_A is affected by the modification. Cu_A exhibits a weak band at 820 - 840 nm with an extinction coefficient of 2.15 mM^{-1} cm^{-1} (Wikstrom, et al., 1981). Figure 10 illustrates a difference spectrum obtained by subtracting the reduced - oxidized spectrum of modified oxidase (9.32 uM enzyme modified with 2 mM DEPC) from the reduced - oxidized spectrum of native enzyme. Figure 11 is a control difference spectrum similar to the one previously described. Comparing figures 10 and 11 leads to the same results as was found for figure 9 at a lower oxidase and DEPC concentration. The control difference spectrum (figure 11) shows a peak of 443 nm in the Soret region with a magnitude of 0.038 absorbance units. It also has a valley of 414 nm with a magnitude of 0.034 absorbance units.

Figure 10

Native (Reduced - Oxidized) Difference Spectrum -

Modified (Reduced - Oxidized) Difference Spectrum



λ max. values (nm)	Δ Absorbance
447 (peak)	0.21
609 (peak)	0.074
444 (valley)	-0.169

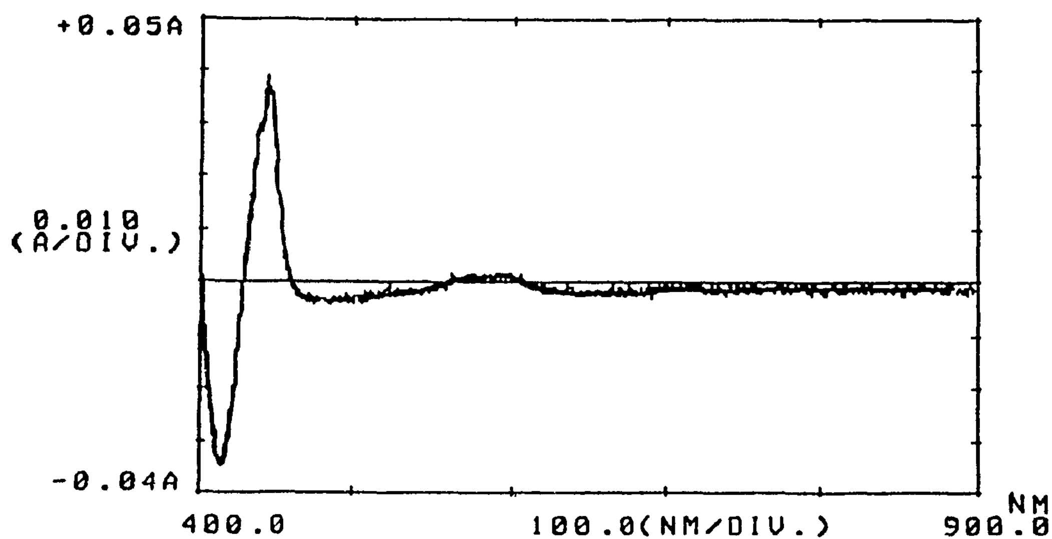
Type 2 oxidase was diluted to a concentration Of 9.32 uM in 25 mM KPi, 1mM EDTA, 1 % cholic acid, pH 7.2. A 1 ml sample was placed in the reference compartment. A second 1 ml sample was placed in a matched cuvette and had a few grains of dithionite added. It was then placed in the sample compartment and a live difference spectrum was recorded from 400 to 900 nm. After reduction was complete (15 minutes) the difference spectrum was stored in memory 1. This process was repeated for oxidase that had been previously modified with 2.0 mM DEPC for 30 minutes. The resulting difference spectrum was then stored in memory 2. The modified difference spectrum was subtracted from the native one and the above figure was generated.

Figure 11

Control Difference Spectrum

Native (Reduced - Oxidized) spectrum -

Native (Reduced - Oxidized) Spectrum



λ max values (nm)	Δ Absorbance
443 nm (peak)	0.038
413 nm (valley)	-0.034

The experiment was repeated as described in figure 10 except a second native reduced - oxidized oxidase sample was run and stored in memory 2 instead of modified oxidase.

The difference spectrum obtained using modified enzyme shows that the peak and the valley in the Soret region shifts to 447 and 444 nm respectively, consistent with the previous experimental results exemplified in figures 7, 8, and 9. This indicates that their difference spectrum is not due simply to a difference in reducibility of the native and modified samples, but arises from a spectral perturbation of the modified protein.

The results of this experiment also provides evidence that Cu_A is not affected by the modification. Figure 10 shows no distinct spectral perturbations in the vicinity of 820 - 840 nm. Hence it can be concluded that modification induces no detectable spectroscopic changes in the reduced - oxidized Cu_A band.

B) Characterization of the Fluorescence of Modified Oxidase.

It has been previously demonstrated that the oxidase has an emission spectrum with a maximum peak at about 330 nm using an excitation wavelength of 280 nm (Hill et al., 1986). This fluorescence is predominantly due to the tryptophans present in the oxidase. Hence experiments were performed to compare the fluorescence intensity of modified oxidase with native oxidase. Figure 12 shows that the emission spectrum of oxidase modified with 0.75 mM DEPC exhibits a small drop in fluorescence intensity relative to the native oxidase.

The difference spectrum illustrated in figure 13 shows the magnitude of this difference. The maximum value of the native fluorescence magnitude is about 300

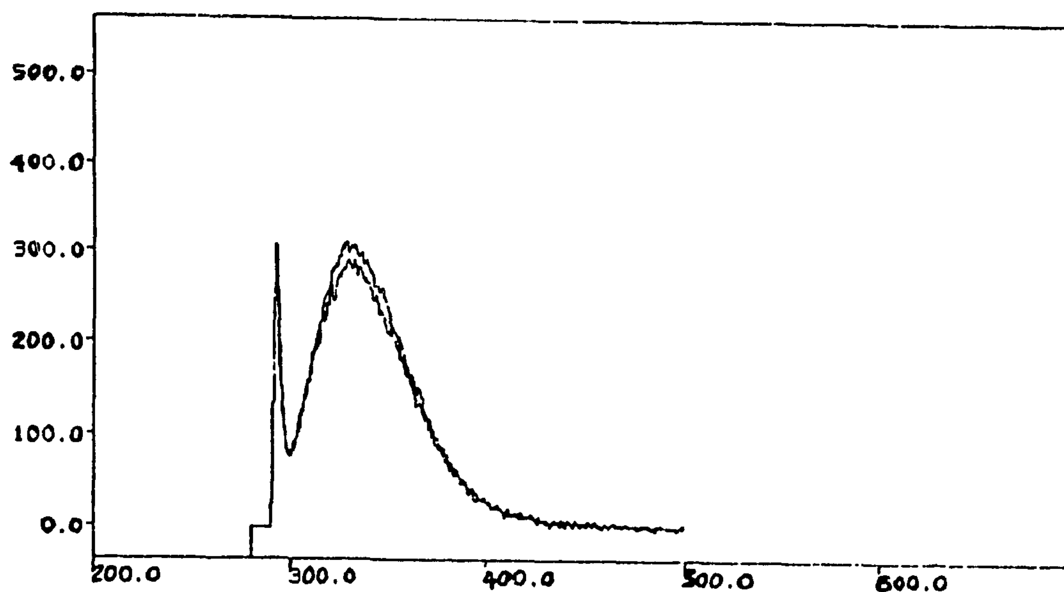
absorbance units, while the difference spectrum shows a drop of about 25 absorbance units. Hence modification of oxidase with 0.75 mM DEPC leads to an approx. 10 % drop in the fluorescence intensity relative to the native oxidase. The experiments were repeated for oxidase that had been modified with lower concentrations of DEPC (0.53, 0.4, and 0.25 mM) and it could be qualitatively discerned that this drop in fluorescence intensity became smaller until it approached zero for the lowest concentration of DEPC tested (0.25 mM). The data were however too noisy to reliably extract numbers so that the decrease in intensity could be plotted as a function of DEPC concentration. However it can be concluded that for high concentrations of DEPC (0.53 mM and greater) there is a small but noticeable drop in fluorescence intensity relative to the native oxidase. Since there is no corresponding change in the absorbance spectrum of the tryptophans at 280 nm, this fluorescence change probably arises from a small alteration in the trytophan environments.

Figure 12

Effect of DEPC Modification on the Fluorescence

Intensity of the Emission Spectrum

Arbitrary Fluorescence Intensity units vs Wavelength Graph



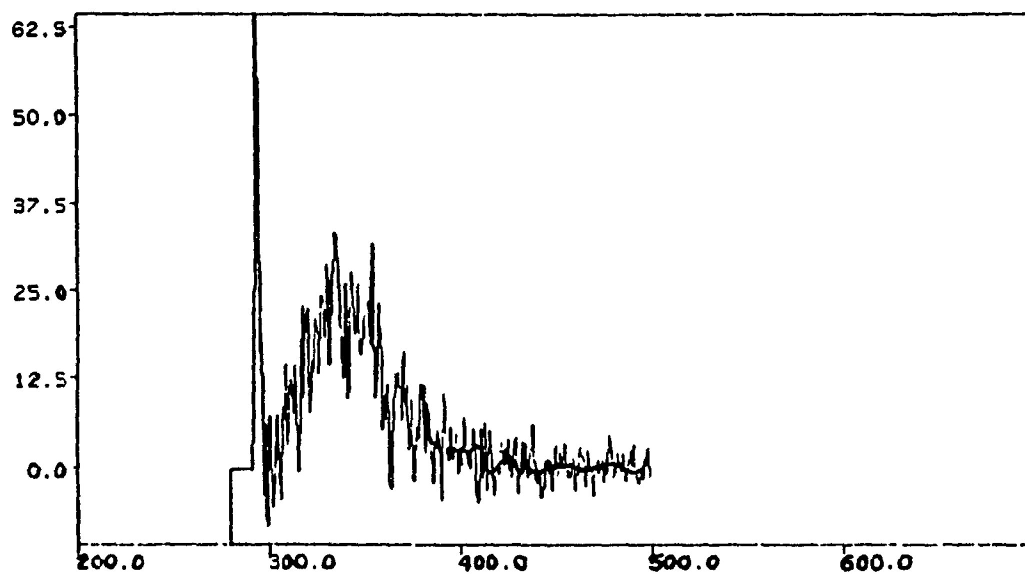
Type 2 enzyme was diluted to a concentration of 2.29 uM in 25 mM KPi, 1 mM EDTA, 1 mg/ml lauryl maltoside, pH 7.2. A sample was then modified with 0.75 mM DEPC for 30 minutes. An aliquot of modified enzyme was then diluted to 22.9 nM in the above buffer and the emission spectrum was then recorded from 280 to 500 nm using an excitation wavelength of 280 nm. This is represented by the smaller of the above two traces. A sample of native oxidase treated with the same concentration of ethanol that was added to the modified sample was also diluted to 22.9 nM and the emission spectrum was recorded. This is represented by the trace that shows the greatest intensity. Both of the above traces were corrected for the spectral contribution of the buffer by subtracting out the emission spectrum of the buffer alone. The above two traces were stored in separate memory files for further analysis.

Figure 13

Fluorescence Emission Difference Spectrum of

Native Oxidase - Modified Oxidase

Arbitrary Fluorescence units vs wavelength Graph



The emission spectrum of the modified oxidase was subtracted from the emission spectrum of the native oxidase and the resultant difference spectrum was obtained.

C) Evaluation of the Kinetic Parameters and the Number of Histidines Modified.

Table 1 summarizes the rate constants and the number of histidines modified for various concentrations of DEPC. Both type 2 and type 3 oxidase are evaluated. It is determined that obtaining the best fit using the F - curve program required the use of a double exponential (i.e the curve is biphasic). Fitting the curves to a single exponential was attempted and it was found that the resultant fitted line had an unacceptable standard deviation in the range of 40 - 60 % and a non random distribution of residual differences between the fitted and observed data. The modification done at 0.53 mM was done 6 times so that an idea of the error associated with these measurements could be obtained. It is apparent that the error ranges from 5 to 10 per cent.

The following trends are discernable from the data in table 2. In general both k1 and k2 increase with increasing DEPC concentration. The rate constant k1 shows a much larger increase in rate relative to k2. The ratio of k1 to k2 does not vary systematically, with k1 being approximately 5 fold faster than k2 for all concentrations of DEPC tested. The number of histidines modified reaches a plateau of ≈ 32 in the DEPC range of 0.53 to 0.75 mM for type 2 oxidase, with about one third of the total number of histidines modified reacting with the rate constant k1.

Table 1

Kinetic Parameters and the number of Histidines Modified

for Various Concentrations of DEPC

[DEPC] (mM) Type 2 Enz.	k1 (sec^{-1}) ($x10^{-3}$)	k2 (sec^{-1}) ($x10^{-3}$)	k1/k2	# His. modified	# His. that react at k1
0.40 (1)	7.83	1.62	4.8	28.3	11.0
0.53 (6)	8.47 (± 0.8)	1.62 (± 0.02)	5.2	32.6 (± 1.5)	13.3 (± 1.2)
0.64 (1)	10.8	1.67	6.5	32.0	13.4
0.75 (2)	11.9 (± 0.5)	2.15 (± 0.05)	5.5	32.3 (± 2.9)	14.9 (± 0.6)
0.75 (2) Type 3 Enz	21.8 (± 2)	4.90 (± 1.4)	4.4	27.7 (± 0.7)	12.3 (± 1.2)

All of the above experiments were done on type 2 oxidase that had been diluted to ≈ 2.1 uM in 25 mM KPi, 1 mM EDTA,
1 % cholic acid, pH 7.2. The specified concentration of DEPC was added and the reaction was allowed to proceed for 30 minutes. After the reaction was complete the rate constants for modification and the number of histidines modified were evaluated as described in the methods. The bracketed number beneath the concentration of DEPC in column 1 represents the number of trials that were performed at that concentration.
The last row was done under the same conditions except type 3 oxidase was used.

Abbreviations:

k1 = fast pseudo first order rate constant
k2 = slow pseudo first order rate constant
k1/k2 = ratio of the two pseudo first order rate constants.
His. = Number of histidines

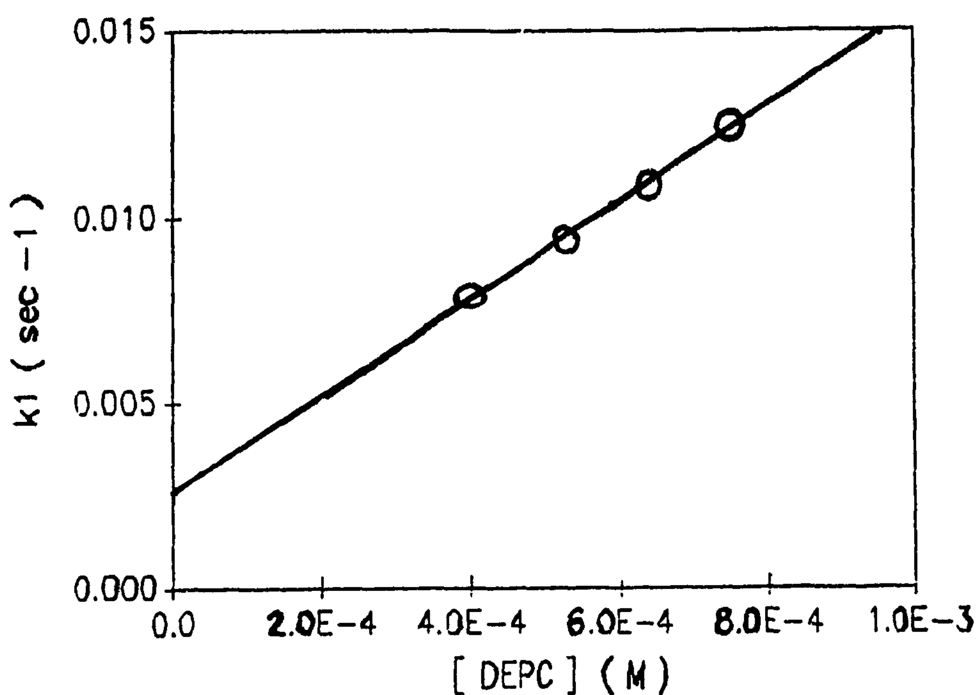
It is also evident that type 3 oxidase seems to react slightly differently although the experimental conditions are identical. Both k1 and k2 are ≈ 2 fold faster than that obtained for type 2 oxidase using the same concentration of DEPC. There also appears to be slightly fewer histidines modified (≈ 4) with type 3 oxidase.

A graph plotting k1 as a function of DEPC concentration is found in figure 14. The slope of this line may be regarded as an approximate value for the bimolecular rate constant, determined to be 13.0 M^{-1} sec^{-1}. This value is approximate because the rate of DEPC hydrolysis is not corrected for. The bimolecular rate constant for the reaction of free imidazole with DEPC is ≈ 53 M^{-1} sec^{-1} (Osterman - Golkar et al., 1974), indicating that the oxidase histidines are less reactive than free imidazole.

Figure 14

Evaluation of the Bimolecular Rate Constant

For DEPC Reacting With Cytochrome Oxidase



Slope = bimolecular rate constant = 13.0 M^{-1} sec^{-1}

The value of k1 was determined for four different DEPC concentrations. All of the experiments were done on the same day to eliminate errors due to the uncertainties inherent in enzyme and DEPC preparation. The values of k1 were plotted against DEPC concentration as shown above and the approximate bimolecular rate constant was evaluated by taking the slope of the above line.

D) Evaluation of DEPC HYdrolysis Rates in Various Buffers and in Distilled Water

It has been demonstrated that DEPC is hydrolysed in aqueous solutions at rates which are dependent on temperature and the concentration and type of nucleophiles present in the aqueous system (Osterman - Golkar et al., 1974). Table 2 summarizes the hydrolysis rates that were determined for the various buffer conditions employed during the oxidase modification experiments.The hydrolysis rate in distilled water alone was also evaluated for comparative purposes.

The rate constants given in the table are in agreement with similar values given in the literature. Osterman - Golkar et al (1974) report a hydrolysis half life of 28 minutes in water at 25 degrees Celsius, and Dominici et al (1985) determined a half life of 9 minutes in 0.1 M phosphate buffer, pH 6.8 at 25 degrees Celsius. The phosphate buffer half life in table 1 is significantly higher than the literature value due to the lower concentration of phosphate buffer. It is apparent that the presence of cholic acid or lauryl maltoside has no effect on the rate of DEPC hydrolysis.

Table 2

DEPC Hydrolysis Rate Constants for Various Solutions

Solution	k (sec^{-1})	half life (minutes)	r^2 (5 points)
Distilled Water	3.16×10^{-4}	36.6	0.999
25 mM KPi	6.67×10^{-4}	17.3	0.999
25 mM KPi +1 mg/ml L.M	6.93×10^{-4}	16.7	0.999
25 mM KPi +1 % cholic acid	6.72×10^{-4}	17.2	0.999

Abbreviations:

KPi = potassium phosphate buffer, pH 7.4
L.M = lauryl maltoside
k = observed pseudo first order hydrolysis rate constant
r^2 = correlation coefficient

Conditions:

5 uL of an ethanolic DEPC solution was added to a series of test tubes containing the designated solution and the hydrolysis reaction was stopped with a 10 mM histidine solution at various time intervals. The concentration of DEPC at the time intervals was determined spectrophotometrically and the pseudo first order rate constants were evaluated as described in the methods.

E) Reversal of Modification with Hydroxylamine

It has been previously established that hydroxylamine under the right conditions can reverse the modification of histidine residues by DEPC (Ovadi et al. (1967). An attempt was thus made to reverse the modification of the oxidase by incubating it with hydroxylamine and monitoring the disappearance of the 240 nm band. Table 3 shows that it was possible to reverse the modification of ≈ 12 of 33 histidines (37 %) under the conditions described in the legend. It is conceivable that complete reversal could have been achieved if different conditions were tried. Ovadi et al. (1967) report that the time and hydroxylamine concentration necessary for reactivation varies for different proteins.

It is also apparent that hydroxylamine has a significant effect on the visible spectrum of the oxidase, as can be seen from the absolute spectra of modified and hydroxylamine treated modified oxidase in figure 15. The hydroxylamine treated sample shows a significant red shift in the Soret region as well as an increase in amplitude of the alpha band relative to the native spectra. These effects are more clearly seen in the difference spectrum shown in figure 16. The difference spectrum shows peak maxima of 445 and 603 nm and a valley of 415 nm. This resembles the reduced - oxidized difference spectrum of native enzyme and it could be speculated that hydroxylamine functions as a reductant.

Table 3

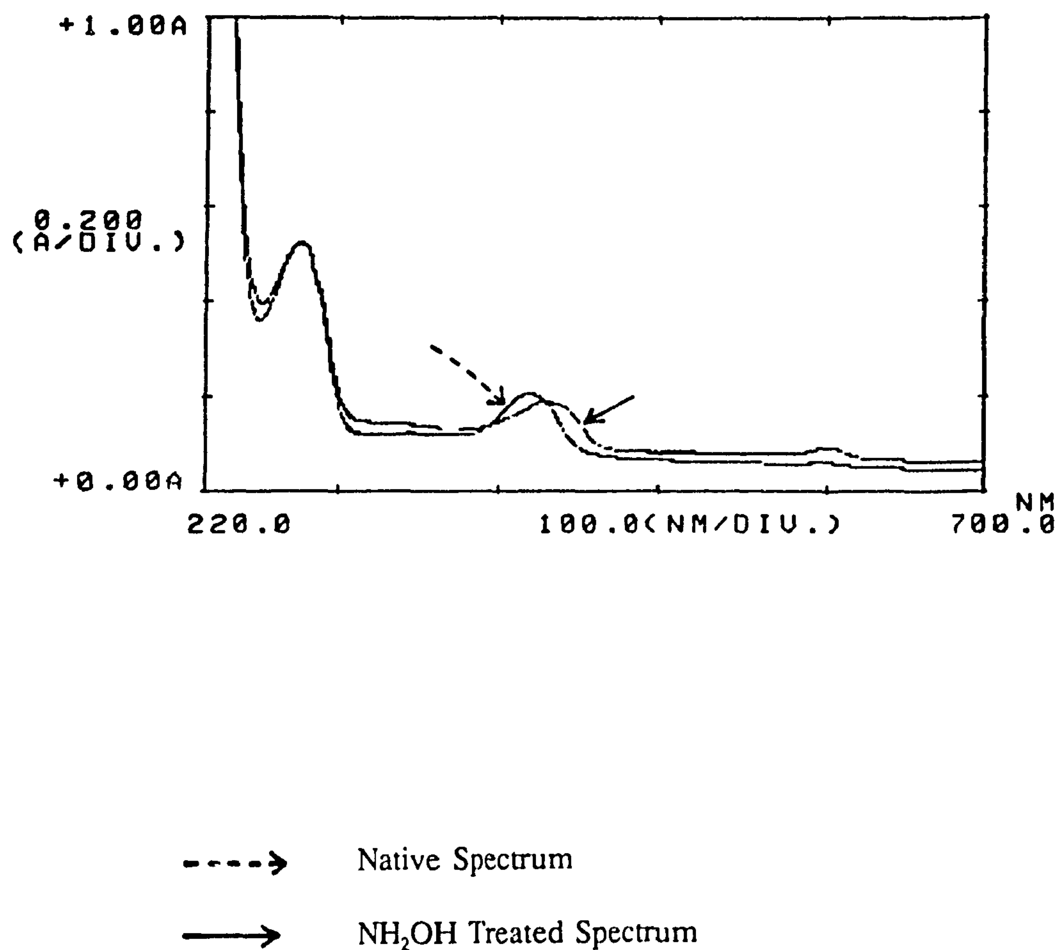
Reversal of the Ethoxylformylation of Histidines

with Hydroxylamine

Δ Absorbance at 240 nm for mod. oxidase.	Δ Absorbance at 240 nm after NH_2OH addition.	Number of histidines unmodified
0.113 (32.9 his.)	0.071 (20.7 his.)	12.2 (37 % of total)

Type 2 enzyme was diluted to a concentration of 2.29 uM in 25 mM KPi, 1 mM EDTA, 1 mg/ml lauryl maltoside, pH 7.2. A 1.7 ml sample was then modified with 0.53 mM DEPC for 30 minutes. Sufficient 2.7 M hydroxylamine solution pH 7.2 was then added to give a final concentration of 350 mM. The decrease in absorbance was then monitored at 240 nm for 90 minutes. A sample of both native and modified oxidase was then diluted with buffer solution to give the same enzyme concentration as that of the hydroxylamine treated solution. The 240 nm absorbance of the native oxidase was subtracted from the absorbance of the modified one and the total number of histidines was calculated (results in column 1). The process was repeated except hydroxylamine treated oxidase was substitu ed for modified (results in column 2). Column 3 shows the number of histidines that were regenerated from the modified ones as a result of this treatment.

Figure 15 Absolute Spectra of Hydroxylamine Treated and Native Oxidase



- - - -> Native Spectrum

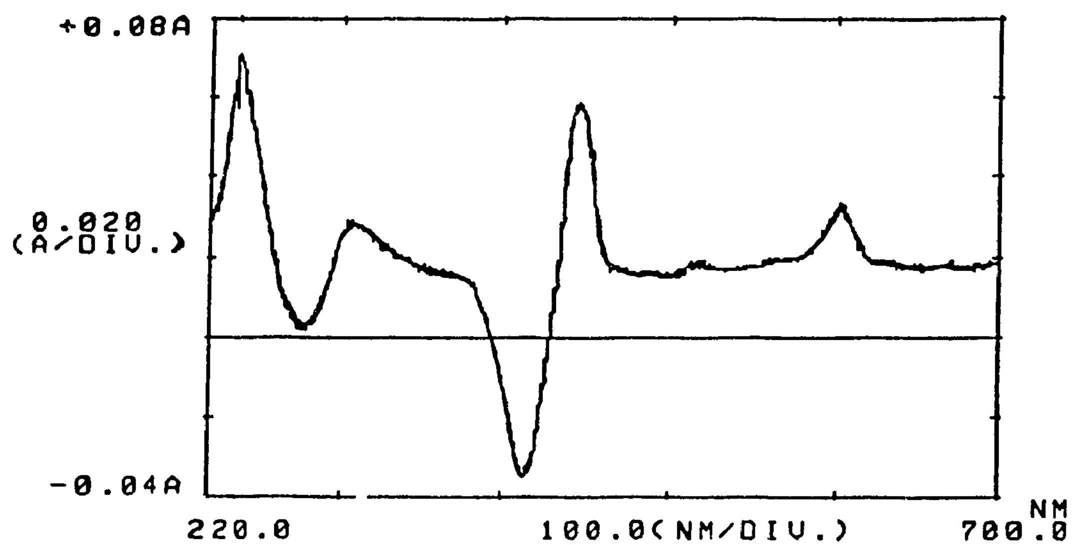
———> NH_2OH Treated Spectrum

The native and hydroxylamine treated oxidase was prepared as described in the table 3 conditions and the spectra of both were recorded from 220 to 700 nm. They were both stored in memory so an NH_2OH treated oxidase - native oxidase difference spectrum could be generated for figure 16.

Figure 16

Hydroxylamine Treated - DEPC Modified Oxidase

Difference Spectrum



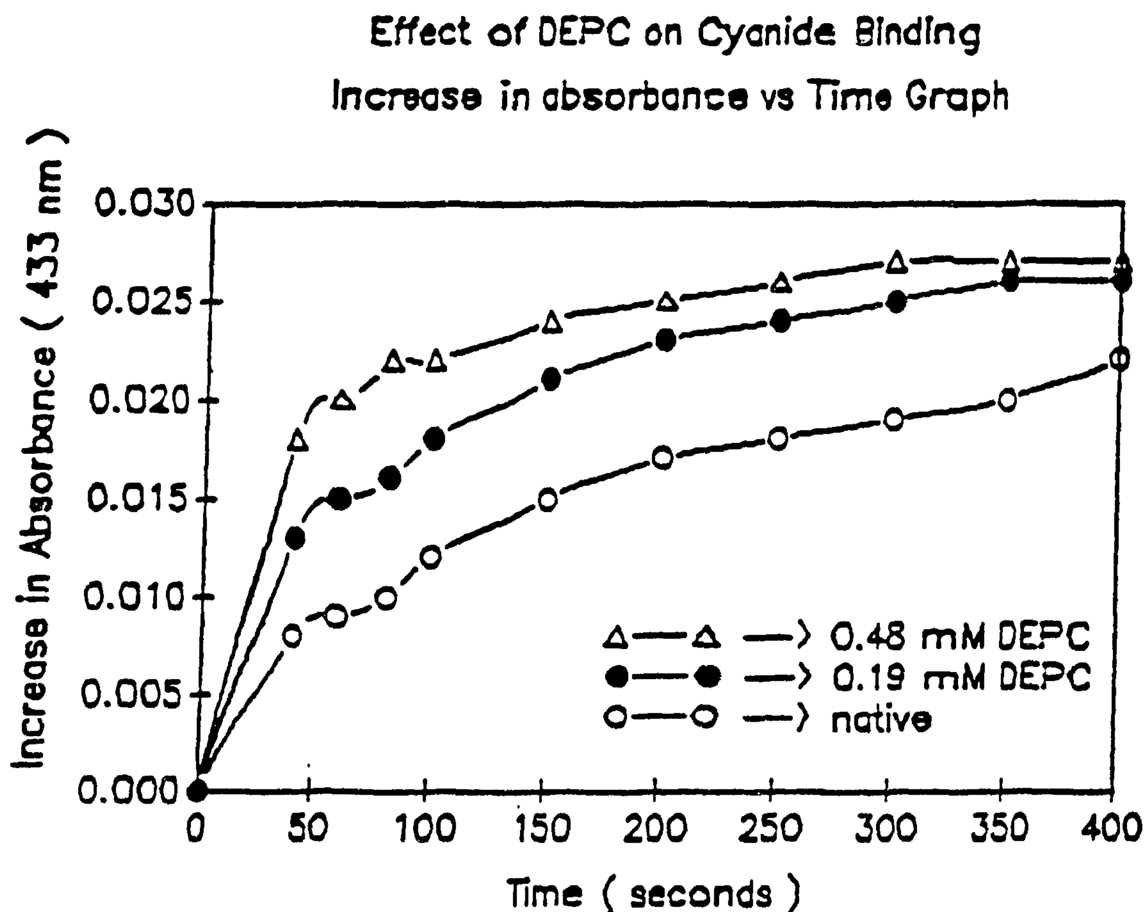
λ Maximum
239 nm (peak)
445 nm (peak)
603 nm (peak)
415 nm (valley)

2) The Binding of cyanide to Modified Oxidase

Cyanide can bind to the heme moiety of several proteins, including cytochrome c oxidase. The kinetics of cyanide binding to the resting, oxidized oxidase was comprehensively investigated by Van Buuren et al (1972), and their results were verified by Jones et al. (1984). It was determined that for the resting, oxidized form of the oxidase cyanide binds to the a_3 heme very slowly, with the reaction taking 1 or more days to reach completion. The binding of cyanide is however very tight, with a Kd estimated in the range of 1 uM. Oxidized, resting oxidase with cyanide bound to it shows a Soret red shift to 428 nm as well as an increase in intensity. This is manifested in a difference spectrum ([ferric aa_3 - cyanide complex] minus [oxidized aa_3]) by showing a maximum peak at 433 nm and a valley at 410 nm. The kinetics of binding are multiphasic, which is believed to be a consequence of isolated oxidase existing in a number of different conformations (Baker et al., 1987).

The effect of modifying oxidase with DEPC prior to the addition of cyanide was investigated. Figure 17 shows that modified oxidase exhibits a greater change in absorbance relative to native oxidase during the early stages of the reaction, indicating that modified oxidase is capable of binding cyanide more quickly than the control.

Figure 17



Type 1 oxidase was diluted to a concentration of 1.06 uM in 0.1 M KPi, 1 mg/ml lauryl maltoside, pH 7.2. Sufficient ethanolic DEPC was added to a sample to give a final concentration of 0.19 mM and the reaction was allowed to proceed for 20 minutes. Sufficient KCN solution was then added to give a final concentration of 2 mM and the increase in absorbance at 433 nm was monitored for 400 seconds. This experimental protocol was repeated using a DEPC concentration of 0.48 mM. A sample of native oxidase was also treated with cyanide as a comparative control.

The resultant curves were then digitized so that all three curves could be placed on the same graph as shown above.

It was also found that this enhancement of rate increased with increasing DEPC concentration, as is shown by figure 17 and also in table 4. Table 4 also demonstrates that the maximum absorbance achieved at 433 nm is not stable for the higher concentrations of DEPC, and proceeds to decay with time.

The wavelength used to monitor the cyanide binding of the modified oxidase (433 nm) is in the same region as the spectroscopic perturbation induced by modification (e.g figure 4). An experiment was conducted to see what kind of a relationship exists between the two phenomena and the results are presented in table 5. These data show that during the modification reaction there is a steady decrease in the Ao value consistent with the previously characterized drop in absorbance in this region. The table also shows that the enhanced cyanide binding effect does not manifest itself until the middle stages of the reaction and is not fully expressed until the modification reaction is virtually complete.

The occurrence of the enhanced cyanide binding effect was also investigated for its temporal manifestation as a function of the number of histidines modified. Figure 18 shows that the enhanced binding does not occur until nearly all of the reactive histidines are modified and the reaction is close to completion. This result is in agreement with those obtained in table 5 where the enhanced cyanide binding effect does not significantly occur until the latter stages of the reaction.

Table 4

Effect of Increasing DEPC Concentration on the

Stability of the Maximal Δ Absorbance Value at 433 nm

[DEPC] (mM)	Δ Abs. total (24 hrs)	Δ Abs. Maximum	Time to reach Δ Abs. Maximum
-	0.033	0.033	24 hrs.
0.19	0.035	0.035	24 hrs.
0.29	0.036	0.036	24 hrs.
0.48	0.024	0.028	300 sec.
0.76	0.014	0.029	300 sec.
1.14	0.015	0.035	300 sec.

Abbreviations: Abs. = Absorbance
hrs. = hours
sec. = seconds

Type 1 oxidase was prepared and modified using the same conditions as those in figure 17. 2 mM KCN was then added and the reaction was monitored at 433 nm. The samples were allowed to sit at room temperature for 24 hours and then the absorbance value at 433 nm was recorded using the same cuvette. The initial absorbance value prior to the addition of cyanide was subtracted from these values to generate the Δ absorbance values listed in column 2. For the three highest concentrations of DEPC the maximum absorbance value occurred during the first 5 minutes of the reaction and the initial absorbance value was subtracted from these values to give the Δ absorbance values in column 3.

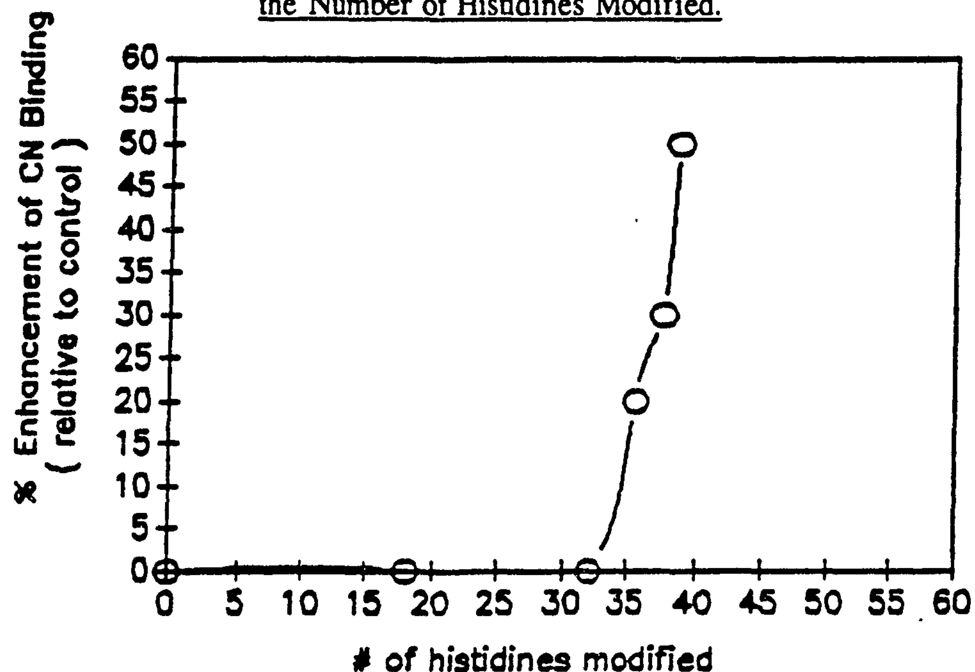
Table 5

Determination of the Temporal Relationship Between the Loss in absorbance at ≈ 430 nm Due to Modification and the Enhanced Cyanide Binding Effect.

Time After Addition of DEPC (min)	(Abs.)o	At 100 seconds after the addition of 2 mM KCN	Δ Abs. Extent of cyanide Reaction
0	0.293	0.302	0.009
1	0.276	0.286	0.010
5	0.272	0.282	0.010
9	0.270	0.282	0.012
15	0.270	0.283	0.013
25	0.268	0.283	0.015

Type 1 oxidase was diluted to 1.7 uM in 0.1 M KPi, pH 7.2. DEPC was added to a final concentration of 0.77 mM to 6.0 ml of oxidase solution. At the specified time intervals indicated in column 1, 1 ml was removed and 2 mM KCN was added. Prior to the cyanide addition the initial absorbance at 433 nm ((abs.)o) was recorded. After the cyanide reaction had proceeded for 100 seconds, the absorbance value (At) was recorded. The Δ absorbance between two values was then calculated. The values at t = 0 represents native enzyme parameters.

Figure 18



Type 1 oxidase was diluted to a concentration of 1.7 uM in 0.1 M KPi, pH 7.2. 6 ml of this solution was treated with 0.77 mM DEPC and as the modification proceeded 1 ml aliquots were withdrawn and treated with 2 mM cyanide at various time intervals. After 100 seconds of the cyanide reaction had passed, the Δ Absorbance was calculated for each interval. The 100 seconds of reaction was repeated for a sample of native oxidase as a control. The degree of enhancement of cyanide binding was calculated using the equation;

$$\text{deg. enh.} = \frac{\Delta \text{ Abs (modified)} - \Delta \text{ Abs (native)} \times 100}{\Delta \text{ Abs (native)}}$$

The number of histidines modified at the various time intervals was determined in a separate experiment using the same experimental conditions. The degree of enhancement was then plotted as a function of the number of histidines modified.

The pseudo first order rate constants of cyanide binding were also evaluated for native and modified oxidase and these results are listed in table 6. The reaction was monitored at 433 nm for 400 seconds and the resultant curve was analyzed for two rate constants due to its biphasicity as described in the methods. In reality there are at least 3 and perhaps more rate constants that are necessary to describe the complete reaction, since it takes several hours to reach completion. Therefore the two rate constants generated from the analysis are only approximate values since they have not been corrected for the contributions of the other slower phases. However they are suitable for comparing the effects of modification on the cyanide binding rate. The data in table 6 show that prior modification of the oxidase with 0.77 mM DEPC leads to an approximate 3 fold increase in the fast k1 rate and an approximate 2 fold increase in the slow k2 rate. Hence the increase in absorbance of modified oxidase relative to native noted in figures 17 and 18 and in tables 4 and 5 is at least partly due the acceleration of two or more of the pseudo first order rate constants.

Table 6

Comparison of the Two Pseudo First Order Rate Constants

Between Native and Modified Oxidase

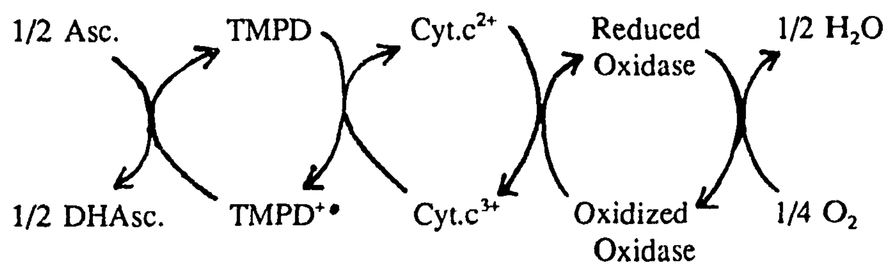
Oxidase Type	k1 (fast) ($sec.^{-1}$) (x 10^{-3})	k2 (slow) ($sec.^{-1}$) (x 10^{-3})
Native	9.7 (± 0.8)	1.05 (± 0.005)
Modified	27.5 (± 7.5)	2.20 (± 0.50)

Type 1 oxidase was diluted to a final concentration of 1.70 uM in 0.1 M KPi, 1 mg/ml lauryl maltoside, pH 7.2. To the native sample KCN solution was added to a final concentration of 2 mM and the reaction was monitored at 433 nm for 400 seconds. Another sample was modified with 0.77 mM DEPC for 20 minutes, and then the cyanide addition was repeated. The experiment was done in duplicate so that an error range could be obtained. The samples were allowed to sit for 24 hours at room temperature and then the A_{∞} was recorded. It was decided that the A_{∞} value from the native sample would be used for the modified sample as well since modified samples eventually show a drop in absorbance at 433 nm as documented previously. The data were then subjected to the curve peeling procedure outlined in the methods so that two pseudo first order rate constants could be extracted from each curve.

3) Evaluation of Km and TNmax for Native Oxidase and for Oxidase Modified With Various DEPC Concentrations

It is a well known fact that the oxidation of cytochrome c catalyzed by cytochrome c oxidase does not obey classical Michaelis - Menten kinetics (Ferguson - Miller et al., 1976). Graphical presentation methods of kinetic data such as the Eadie - Hofstee plot reveal biphasic kinetics when a wide enough range of cytochrome c concentrations is used. From this biphasic curve two values of Km and TNmax may be obtained by extrapolating the linear portions at each end of the curve. One set of Km and TNmax values is commonly called the tight binding, low activity phase, while the other is termed the loose binding, high activity phase.

The turnover numbers for various concentrations of cytochrome c were determined with a polarographic assay for oxygen as described in the methods. The principle behind the assay system is illustrated in the scheme below.



Where : Asc. = Ascorbate

DHAsc. = dehydroascorbate TMPD$^{+\bullet}$ = TMPD radical

cyt.c = cytochrome c

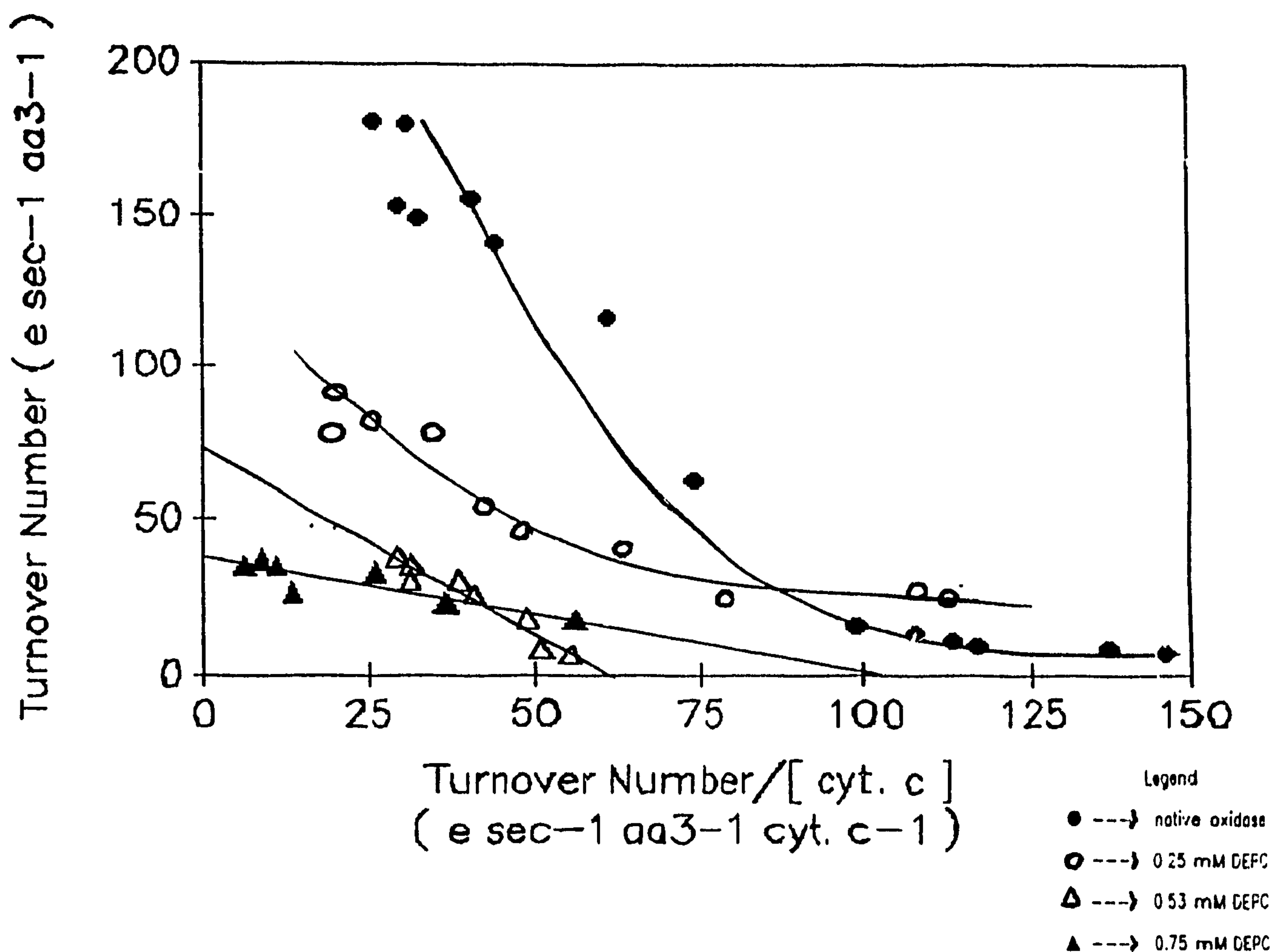
Excess ascorbic acid (≈ 4.2 mM) is present in the system to ensure the continuous reduction of TMPD (≈ 0.25 mM). The TMPD in turn is in excess of the cytochrome c concentration (range = 0.01 - 10 uM), thus ensuring that the cytochrome c in the system is continually reduced after it has been oxidized by cytochrome c oxidase(≈ 10 - 20 nM). Hence the system is able to continuously supply the enzyme with a reasonably constant supply of reduced substrate over the time range of the assay.

Figure 19 shows 4 Eadie - Hofstee plots, one being the normal biphasic curve of native oxidase, and the other three being oxidase samples that had been previously modified with 0.25, 0.53, and 0.75 mM DEPC. The curves shift from biphasic to monophasic kinetics as the DEPC concentration increases. This is accompanied by corresponding successive decreases in activity. The Km and TNmax values were calculated by taking the slope (equal to - Km) and the Y intercept (equal to TNmax) of the straight line of best fit through the data points. For the native and the 0.25 mM DEPC biphasic curves two values of Km and TNmax were determined. The best straight line was drawn through the beginning of the curve, corresponding to the high activity, low affinity phase (TNmax1 and Km1); and the best straight line was also drawn through the latter portion of the curve, corresponding to the low activity, high affinity phase (TNmax2 and Km2). Since the data for oxidase modified with 0.53 and 0.75 mM are best represented by a straight line only a single TNmax and Km value was calculated. These parameters are listed in tables 7 A and B.

Figure 19

Effect on the Activity and the Biphasicity of

Eadie - Hofstee Plots by Prior Modification with DEPC



Type 2 oxidase was diluted to a final concentration of ≈ 2.1 uM in 25 mM KPi, 1 mM EDTA, 1 % Cholic acid, pH 7.2 for each of the above plots. For native oxidase turnover numbers were calculated for each cytochrome c concentration tested as described in the methods. The data were then expressed in the form of a Eadie - Hofstee plot as shown above. This process was repeated for samples of oxidase that had been previously modified with 0.25, 0.53, and 0.75 mM DEPC.

Table 7 (A)

TNmax and Km Values Evaluated From the Plots in Figure 19

Evaluation of TNmax1, TNmax2, Km1, and Km2 for native oxidase and for oxidase modified with 0.25 mM DEPC.

Oxidase Type	TNmax1	Km1	TNmax2	Km2
Native	234.3	2.16 (0.946)	30.8	0.166 (0.916)
0.25 mM DEPC	114.3	1.34 (0.913)	50.7	0.234 (0.74)

Abreviations:

TNmax1 = Maximal velocity for the low affinity, high activity phase. (units = $e * sec^{-1} * [aa_3]^{-1}$)

Km1 = Michaelis - Menten constant for the low affinity, high activity phase. (units = uM cytochrome c)

TNmax2 = Maximal velocity for the high affinity, low activity phase.

Km2 = Michaelis - Menten constant for the low affinity, high activity phase.

The best straight line was fit through the first 8 points of the native curve and the Km2 and TNmax2 were evaluated as described previously. A second best line was drawn through the latter 6 points to obtain Km1 and TNmax1. A similar procedure was done on the 0.25 mM curve with best lines drawn through the first 6 and latter 4 points to get Km2, TNmax2, and Km1,TNmax2 respectively. The correlation coefficients for each line segment are listed below the Km values in brackets.

Table 7 (B)

Evaluation of Km and TNmax for Oxidase

Modified With 0.53 and 0.75 mM DEPC

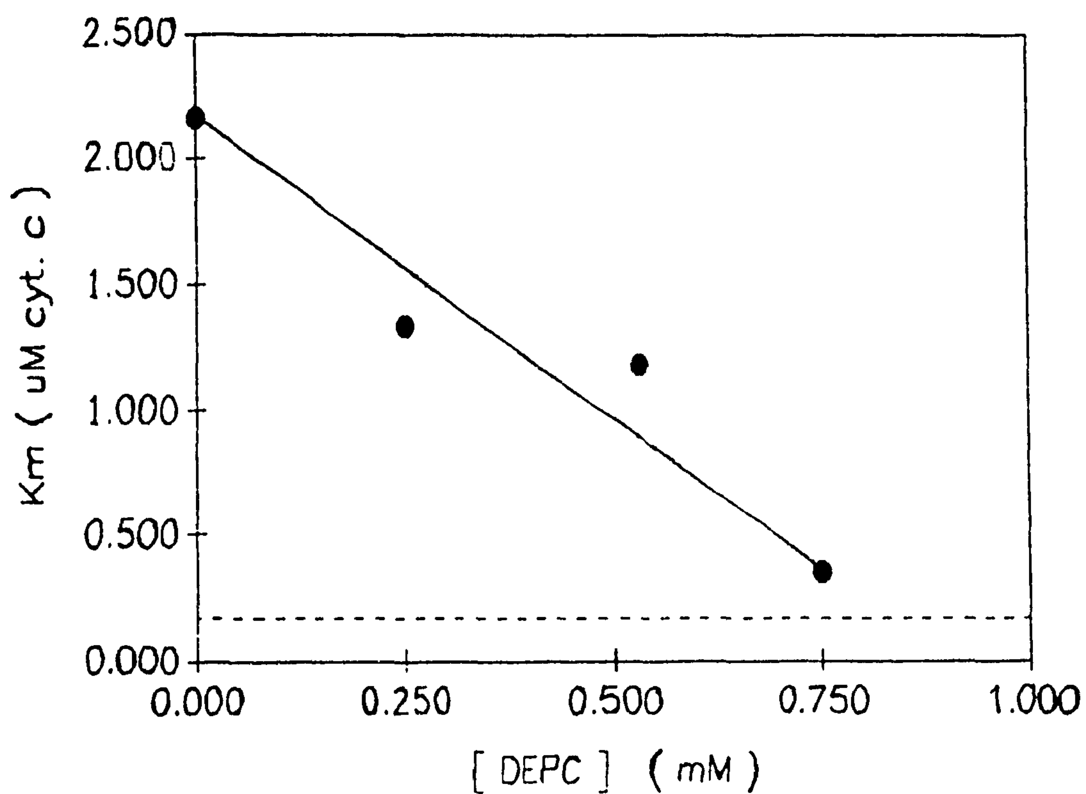
Oxidase Type	TNmax	Km
0.53 mM DEPC	72.6	1.18 (0.964)
0.75 mM DEPC	37.7	0.35 (0.878)

The best straight line was drawn through all of the data points for both the 0.53 and the 0.75 mM DEPC data illustrated in figure 19. A TNmax and Km value was determined for each concentration. The correlation coefficient for each line is listed beneath the Km value in brackets.

The Michaelis - Menten binding constant becomes increasingly smaller as the concentration of DEPC is raised and the maximal turnover decreases with increasing DEPC. Figure 20 is a plot showing the decrease in Km values as a function of the DEPC concentration. This plot shows that the Km values of the three modified oxidases approach the Km2 value of the native oxidase. A similar plot was made using the TNmax values in figure 21. Once again the TNmax values of the three modified samples approach the TNmax2 value of the native sample An attractive hypothesis is that increasing DEPC concentrations successively eliminate more and more of the high activity, low affinity phase while the other low activity, high affinity phase is unaffected. This hypothesis is consistent with the appearance of monophasic kinetics at the higher concentrations of DEPC as well as the decreases in the Km and TNmax values.

Figure 20

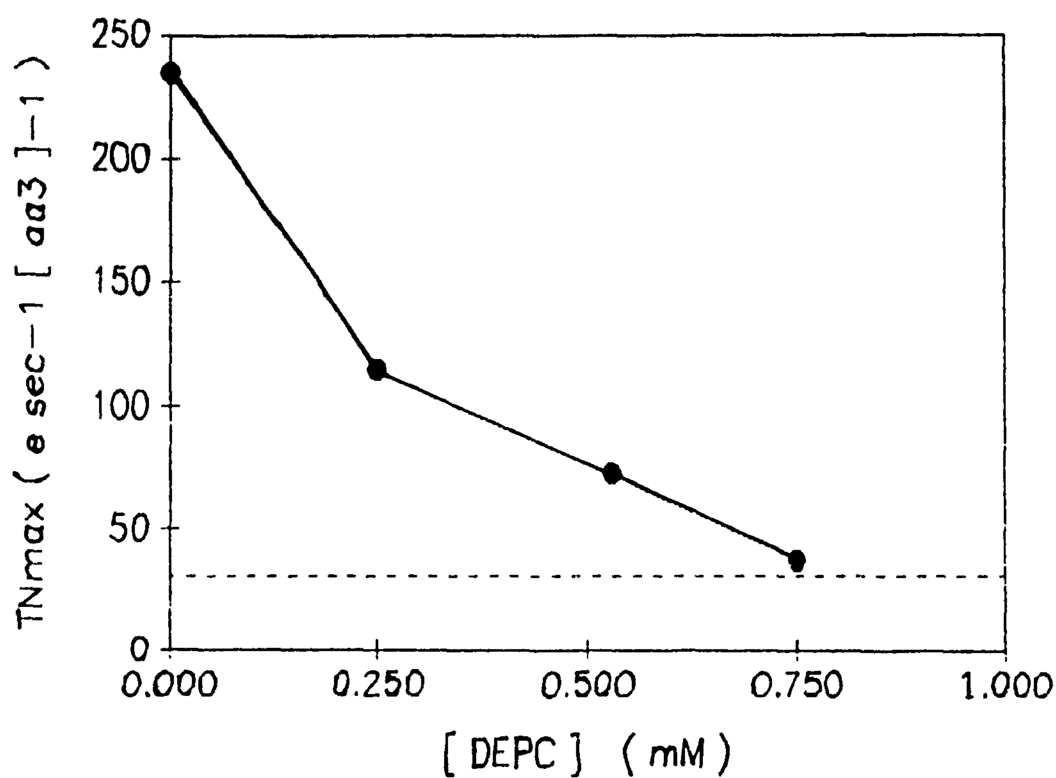
Effect of DEPC Modifications on Km Values



The Km1 values of both native oxidase and the sample modified with 0.25 mM DEPC are represented in the above graph along with the Km values of samples modified with 0.53 and 0.75 mM DEPC. The dashed line along the bottom of the graph represents the Km2 value of native oxidase.

Figure 21

Effect of DEPC Modifications on TNmax Values



The TNmax1 values of both native oxidase and the sample modified with 0.25 mM DEPC are represented in the above graph along with the TNmax values of samples modified with 0.53 and 0.75 mM DEPC. The dashed line along the bottom of the graph represents the TNmax2 value of native oxidase.

4) Characterization of the RCR for Native and Modified Oxidase Incorporated in Asolectin Vesicles

It is possible to incorporate cytochrome c oxidase into phospholipid vesicles to provide a simple model system that mimics many of the properties of the oxidase in its natural membrane bound state in the mitochondria. In particular oxidase in artificially reconstituted membrane systems is capable of demonstrating some of the respiratory control effects seen with mitochondria. The ratio of the uncontrolled rate to the controlled rate leads to a respiratory control ratio (RCR) which may be calculated as described in the methods. A good ratio is 6 - 8, indicating a low degree of membrane leakiness to protons in the absence of uncouplers.

Initially the incorporation of type 2 oxidase into vesicles using method 1 was unsuccessful, resulting in RCR values of 1.2 or less. Hence the method was modified by treating the lipids according to the method of Racker (1973). Asolectin from egg yolk was also tried and compared to the asolectin from soyabeans. Table 8 illustrates the RCRs obtained for both egg yolk and soyabean vesicles which were prepared under identical conditions. Vesicles prepared with egg yolk asolectin give a better RCR value (32 % greater) than those prepared with soyabean asolectin. However the RCR of 2.6 is still much lower than the 6 - 8 reported in the literature. It was subsequently determined that it was the oxidase itself which was the source of uncoupling leading to the low RCR

Table 8

Comparison of the RCRs for Vesicles Prepared in

Either Egg yolk Asolectin or in Soyabean Asolectin

Type of Asolectin	RCR
Egg Yolk (3 trials)	2.60 (± 0.14)
Soyabean (3 trials)	1.77 (± 0.26)

Type 2 oxidase was diluted to a final concentration of 2.1 uM in 25 mM KPi, 1 mM EDTA, 1 % cholic acid, pH 7.2. Vesicles were then prepared according to method 2 and the RCRs were evaluated as described in the methods. Both systems were treated identically so that the only varying parameter was the type of asolectin.

ratio. This was accomplished by comparing the RCRs of type 2 oxidase and type 3 oxidase prepared under identical conditions. Table 9 shows that type 3 oxidase has an RCR of 7.05 while type 2 oxidase was 2.3, consistent with the previous RCRs in table 8. Hence type 2 oxidase probably has some sort of contaminant not present in type 3 oxidase which acts as an uncoupler leading to an inherently low RCR.

The effect of prior modification of the oxidase with 0.53 mM DEPC on the RCR was nevertheless tested on both type 2 and type 3 oxidase. Table 10 illustrates the effect on the RCR using vesicles made with type 2 oxidase via method 2 while table 11 depicts type 3 oxidase vesicles prepared via method 1. It is apparent from both of these tables that prior modification with 0.53 mM DEPC leads to an appreciable decrease in the RCR value. It is significant to note that although the magnitudes of the RCR ratios are very different between the two tables, the percentage of inhibition of the RCR as a consequence of modification is virtually the same for both types of oxidase vesicles. This indicates that modification induces a common effect that reduces the RCR to the same extent regardless of the initial degree of coupling exhibited by the vesicles.

Further analysis of the data in table 10 resulted in the conclusion that the diminution of the RCR is due to a large decrease in the uncoupled rate, with the coupled rate being diminished to a much smaller extent.

Table 9

Comparison of the RCRs of Type 2 and Type 3

Oxidase incorporated into Vesicles

Oxidase Type	RCR
Type 2 (2 readings)	2.3 (± 0.1)
Type 3 (2 readings)	7.05 (± 0.05)

Two dialysis preparations were performed in parallel using method 3 described in the methods section. One preparation was performed using type 3 oxidase while the other was done with an identical concentration of type 2 oxidase. After the dialysis was complete both preparations had their RCRs evaluated using the assay procedure reported in the methods section. The assay was done twice for each preparation.

Table 10

Comparison of the RCRs of Native and Modified

Type 2 Oxidase in Asolectin Vesicles

Oxidase Type	RCR (9 readings)	% Inhibition of RCR relative to native
Native	2.6 (± 0.43)	81 %
Modified (0.53 mM DEPC)	1.3 (± 0.10)	

Native oxidase and oxidase which had been previously modified with 0.53 mM DEPC were incorporated into egg yolk asolectin vesicles via method 2. The RCRs were determined using the assay described in the methods. The % inhibition was calculated the following way. An RCR of 1 indicates 100 % inhibition (i.e the coupled rate equals the uncoupled rate). Therefore it was necessary to subtract 1 from both of the RCR values. The equation below was used to calculate % inhibition.

$$\frac{(\text{RCR}(\text{modified}) - 1)}{(\text{RCR}(\text{native}) - 1)} \times 100 - 100 \times -1 = \%\ \text{Inhibition}$$

Table 11

Comparison of the RCRs of Native and Modified

Type 3 Oxidase in Asolectin Vesicles

Oxidase Type	RCR (2 readings)	% Inhibition of RCR relative to native
Native	5.7 (± 0.4)	78 %
Modified (0.53 mM DEPC)	2.05 (± 0.15)	

Type 3 oxidase was incorporated into soyabean asolectin via method 1 and the RCRs of both native and modified oxidase vesicles was evaluated as described in the methods. The % inhibition of the RCR caused by prior modification with 0.53 mM DEPC was calculated using the equation shown in table 10.

The data in table 12 express the % decrease of the modified oxidase vesicles rate relative to the native rate both in the presence and absence of the uncouplers valinomycin and FCCP. In the presence of the uncouplers the rate is diminished by ≈ 32 % while in their absence the rate is only decreased by ≈ 6 % .

Table 12

Comparison of Modified and Native Vesicles Rate

Differences in the Absence and Presence of Uncouplers

Presence or Absence of Uncouplers	Native Rate (nM O_2 / sec)	Modified Rate (nM O_2 / sec)	% Difference of modified Rate From Mean
Absent	203	205	0.5 %
Present	397	266	20 %
absent	151	189	11 %
Present	472	231	34 %
Absent	191	153	11 %
Present	508	220	40 %
Absent	195	194	0.3 %
Present	510	260	32 %

The rates of cytochrome c oxidation by native and modified vesicles in the presence or absence of uncouplers are listed above using the same conditions as shown in table 10. The % difference of the modified rate from the average of the native and modified rates was calculated in order to determine which rate (coupled or uncoupled) was mostly responsible for the decrease in the RCR. Two rows of the above table each represent a separate experiment with the modified and the native oxidase rates compared in the presence and absence of uncouplers. The % difference was calculated using the following equation;

$$\% \text{ Difference} = \frac{|\text{avg. rate} - \text{mod. rate}| \times 100}{\text{avg. rate}}$$

where: avg. rate = The mean of the modified and native rates
(either for in the absence or in the presence of uncouplers)

mod.rate = Modified oxidase rate (either with or without uncouplers)

5) Determination of the orientation of Native and Modified Oxidase in Asolectin Vesicles

The determination of the orientation of the oxidase in vesicles was done on a sample of type 2 oxidase vesicles. Oxidase molecules which are oriented in the wrong direction are inactive since cytochrome c cannot diffuse across the lipid bilayer. The principle of the assay method described in the methods is that only oxidase molecules in the correct orientation (i.e cytochrome c binding site facing outwards) are capable of being reduced since the membrane is impermeable to both cytochrome c and ascorbate. Subsequent addition of TMPD leads to the complete reduction of all of the oxidase since the lipid bilayer is permeable to TMPD. The percentage of oxidase molecules facing outward can then be easily calculated by dividing the concentration of oxidase accessible to ascorbate by the total concentration of oxidase determined with TMPD. Using the procedure described in the methods it was determined that a sample of native oxidase vesicles had 79 % of its oxidase molecules facing outward, while a sample of vesicles made with oxidase previously modified with 0.53 mM DEPC had a value of 73 % . These values are in agreement with those reported in the literature (Nicholls et al., 1980), where the % orientation is in the range of 70 - 80 % . This result demonstrates that any observed differences in the properties of modified enzyme vesicles is not due to their orientation being different from native vesicles.

6) Comparison of the Proton Pumping Capabilities of Native and Modified Oxidase Incorporated Into Asolectin Vesicles

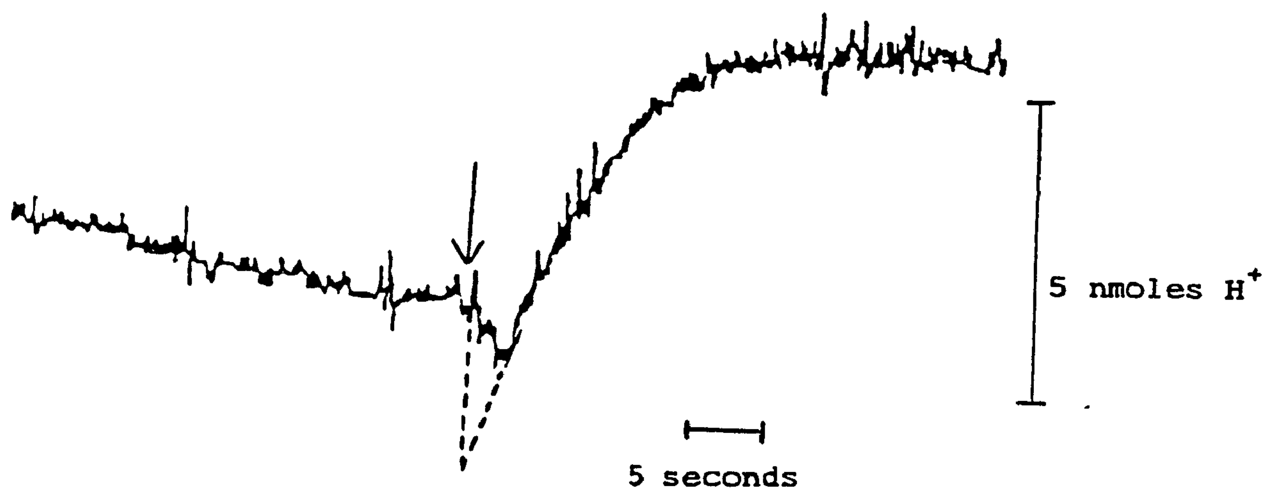
It was originally proposed by Wikstrom (1977) that cytochrome c oxidase functions as a redox - linked proton pump. According to this theory the redox energy liberated during the transfer of electrons to oxygen is partly conserved via the vectorial translocation of protons across the mitochondrial membrane, creating an electrochemical proton gradient. The main experimental result that supports this hypothesis is the appearance of an initial acidification when the pH of the vesicle medium is monitored. This acidification is followed by an alkalinization phase which is due to the overall stoichiometry of the reaction as shown in equation 1.

Experiments were conducted to see what effects prior modification of the oxidase with 0.75 mM DEPC followed by incorporation into vesicles had on the extent of acidification and the alkalinization reaction. Figure 22 (A) illustrates the acidification followed by the alkalinization phase of native oxidase vesicles in the presence of valinomycin. Valinomycin is included so that the duration and magnitude of the acidification phase is enhanced due to the transport of potassium ions into the vesicles as protons are pumped out. This K^+ transport relieves the stress due to positive charge translocation and thus the proton pumping is only limited by the Δ pH gradient.

Figure 22 (A)

Extravesicular pH Changes with Native Oxidase Vesicles

in the Presence of Valinomycin



Type 3 oxidase vesicles were prepared as described in the methods under the proton pumping section. The reaction was initiated by the addition of 5 nmoles of cytochrome c to the media containing valinomycin and native vesicles and the changes in pH were recorded. After the reaction was complete 5 nmoles of HCl was added so that the trace could be calibrated. The true magnitude of the proton pulse was determined by extrapolating the trace down to zero time (i.e at the time of cytochrome c addition shown by arrow above). This is necessary to compensate for the part of the proton pulse that is lost as a consequence of the competing alkalinization phase occurring at the same time.

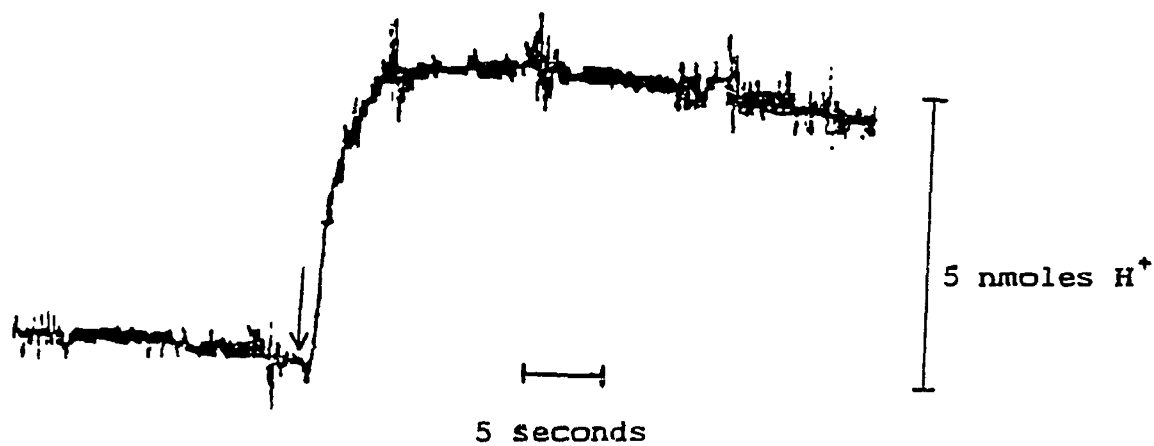
Figure 22 (A) clearly shows the acidification phase followed by the alkalinization phase as expected. Figure 22 (B) is the same experiment except CCCP is now present. Since CCCP makes the membrane freely permeable to protons the acidification phase is abolished. It is also apparent that the alkalinization phase is much faster which is in accordance with the oxidase turning over without the respiratory control restraints. The effects of prior modification of the oxidase is depicted in figures 23 (A) and (B). Figure 23 (A) shows a marked decrease in the magnitude of the proton pulse relative to the native one in figure 22 (A). Once again this acidification phase is abolished with the addition of CCCP as shown in figure 23 (B).

Several trials of each type of trace were obtained and the magnitude of the proton pulse (expressed as H^+ / e ratio) and the half lives of the alkalinization reaction are summarized in tables 13 and 14. From table 13 it is apparent that modification diminishes the magnitude of the proton pulse by about 41 % . Hence modification probably somehow impairs the proton pumping process leading to the decrease in magnitude. Table 14 shows that the alkalinization rate of modified oxidase is slowed down to a much greater extent in the presence of CCCP relative to the native rate than it is in the presence of valinomycin alone. This result illustrates the same trend that was found in table 11, hence it is evident that the rates determined polarographically behave in a similar way as the alkalinization phase determined potentiometrically.

Figure 22 (B)

Extravesicular pH Changes with Native Vesicles

in the Presence of Valinomycin and CCCP

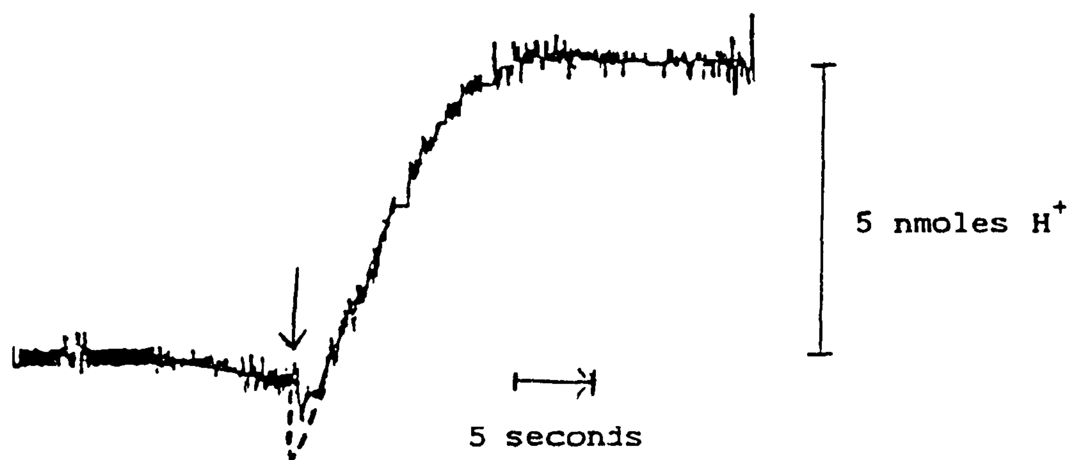


The experiment was repeated using the same conditions in figure 22 (A) except that CCCP was present in the media as well.

Figure 23 (A)

Extravesicular pH Changes with Modified Vesicles

in the Presence of Valinomycin

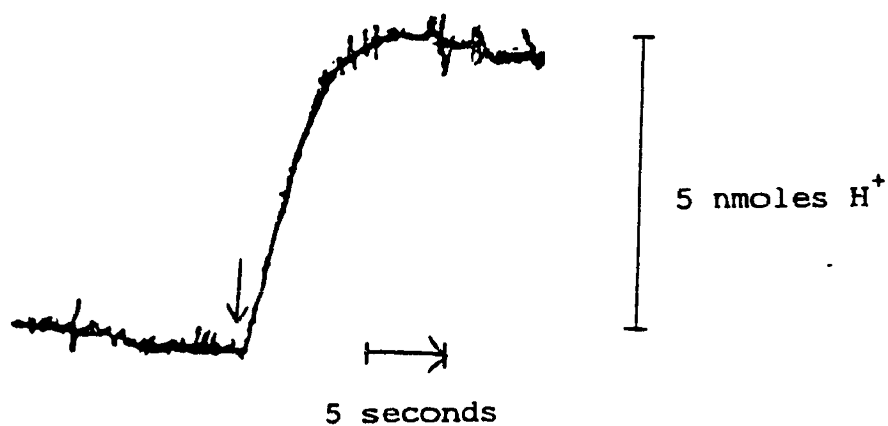


The proton pumping experiments in the presence of valinomycin were repeated except this time type 3 oxidase was previously modified with 0.75 mM DEPC.

Figure 23 (B)

Extravesicular pH Changes with Modified Vesicles

in the Presence of Valinomycin and CCCP



The experiments were repeated except this time CCCP was present in the media.

Table 13

Comparison of the H^+ / e Stoichiometry Generated

by Native and Modified Oxidase

Oxidase Type	H^+ / e Stoichiometry
Native (8 readings)	0.44 ± 0.06
Modified (9 readings)	0.26 ± 0.02

% Decrease in H^+ / e due to modification = 41 %

Several traces similar to the ones shown in figures 22 (A) and 23 (A) were analyzed for the magnitude of the proton pulse and the average of the results is shown above. The magnitude was calculated by measuring the distance from zero time to the extrapolated peak of the proton pulse. The measurement was then converted to nMoles H_+ by setting the magnitude of the standard HCl pulse equal to 5 nMoles.

Table 14

Comparison of the Half Lives of the Alkalinization

Reaction Between Native and Modified Oxidase in the

Presence and Absence of CCCP

Oxidase Type	Type of Uncouplers Present	t 1/2 (seconds)	% Decrease in t 1/2 of Modified Oxidase
Native (9 traces)	Val.	5.1 (± 0.9)	18 %
Modified (9 traces)	Val.	4.2 (± 0.6)	
Native (4 traces)	Val. + CCCP	1.3 (± 0.15)	46 %
Modified (4 traces)	Val. + CCCP	2.4 (± 0.5)	

Several traces similar to the ones in figures 22 (A and B) and 23 (A and B) were obtained and the half lives for the alkalinization reaction were calculated. This was done by taking an arbitrary Δ Absorbance, dividing it by two, and then reading off the corresponding time value from the trace.

It is also significant to note that type 2 oxidase with a poorer RCR (used for table 10) nevertheless manifests a similar behaviour as type 3 oxidase with a better RCR (table 14).

The data in table 14 are also consistent with previous data in which RCRs were determined. An approximate RCR may be calculated using the ratio of the alkalinization reaction in the absence and presence of CCCP for both native and modified oxidase. An RCR of 3.9 is calculated for native oxidase (i.e 5.1 / 1.3) and 1.8 for modified oxidase (i.e 4.2 / 2.4). Hence these ratios reflect the same relationship as was seen previously, namely that modification induces a decrease in the RCR.

In the presence of CCCP the alkalinization reaction should correspond to 1 proton consumed per electron transferred according to the stoichiometry shown in equation 1. This stoichiometry entails that an arbitrary concentration of HCl will give the same Δ pH response as an equivalent concentration of cytochrome c when a trace is measured in the presence of CCCP. Figures 22 (B) and 23 (B) show that the standard HCl pulse has close to the same magnitude as the alkalinization reaction due to oxidation of reduced cytochrome c. Careful measurements of all the HCl standard pulses and the CCCP alkalinization reactions resulted in a 1:1 stoichiometry, hence the results shown here are consistent with the results shown by others (Casey et al. (1984), Casey, 1986).

Discussion

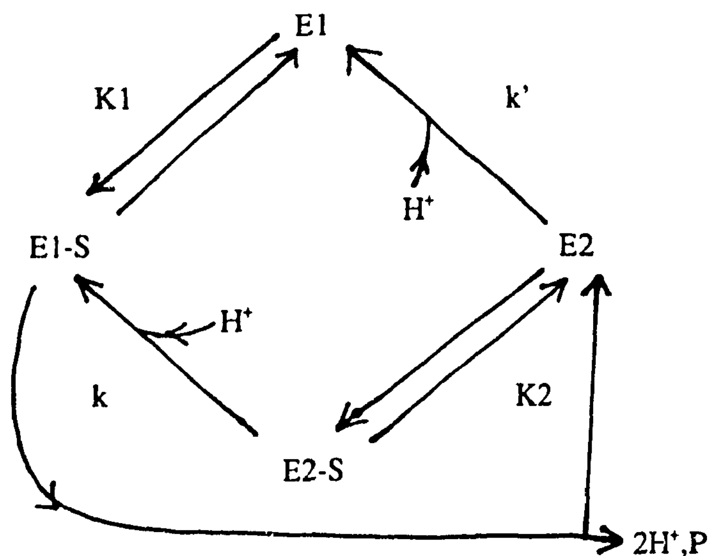
The proton pumping and activity results show that prior modification of the oxidase with DEPC results in both an impairment of proton pumping (table 13) and an inhibition of the steady state activity (figure 19). Some of the histidines that are modified are therefore directly or indirectly essential for the integrity of the proton pumping apparatus. This is assuming that the decrease in proton pumping is not due to general modification induced conformational changes that nonspecifically impede the proton pumping function. The fluorescent studies show a small 10 per cent drop in fluorescent intensity using 0.75 mM DEPC for modification (figures 13 & 14), and there were no detectable changes in absorbance at 280 nm (figure 2). This indicates that there are only small scale, nonspecific conformational changes induced by modification. It is therefore unlikely that the disruption of proton pumping is due to gross conformational changes.

It is possible that modification causes the oxidase to become inherently leaky to protons after it is incorporated into vesicles. This would explain both the decreased proton pumping (table 13) and the reduction in the RCR (tables 10 & 11). There is indirect evidence that this is not the case. The decrease in the RCR is due to a large decrease in the uncoupled rate, with the coupled rate being diminished to a much smaller extent as shown in table 12. In the coupled state ferrocytochrome c turnover is limited by the transmembrane potential which in turn is governed by the inherent permeability of the membrane to protons. Since the rate of turnovers in the coupled state for both native and modified oxidase in vesicles is very similar it follows that their permeability to protons

must also be similar. The rates of alkalinization of native and modified oxidase vesicles that were measured in the presence and absence of CCCP (table 14) illustrates the same trend and the results are consistent with the rates obtained polarographically in table 12. The alkalinization rates of modified and native vesicles were very similar in the absence of CCCP, and the native rate was faster than the modified rate in the presence of CCCP. This means that the loss in proton pumping efficiency is caused by a direct perturbation of its chemical constituents and is not an artifact caused by increased leakiness. It also implies that the reduction in RCR and the decrease in alkalinization rate in the presence of CCCP is due to a decrease in the catalytic efficiency of the oxidase caused by modification.

It is thus probable that it is the modification of histidines essential for proton pumping and / or electron transfer which results in the observed loss in proton pumping and activity. The appearance of monophasic kinetics at higher concentrations of DEPC is interesting because of the implications this has on a model used to explain proton pumping and of the consequences manifested by its elimination. Michel and Bosshard (1989) have proposed an attractive model which is based on their own findings and some earlier models developed by Brzezinski and Malmstrom (1986, 1987). Scheme 1 illustrates the salient features of the model.

Scheme 1



where :

E1 = oxidase prior to the conformational transition.

E2 = oxidase after the conformational transition.

E1-S = E1 oxidase with bound cytochrome c

E2-S = E2 oxidase with bound cytochrome c

K1 = dissociation constant between E1 and E1-S

K2 = dissociation constant between E2 and E2-S

k = rate constant with which E2-S reverts to E1-S while simultaneously picking up a proton.

k' = rate constant with which E2 reverts to E1 while simultaneously picking up a proton.

An explanation of the model is as follows. The oxidase can exist in two different conformations, called E1 and E2. The transition from E1 to E2 is accompanied by the release of two protons as shown in the model. The transition from E1-S to E2 requires the oxidation of two cytochrome c molecules, because as mentioned previously it is believed that it is the reduction of cytochrome a and Cu_A that triggers the conformational transition. It is important to note that the release of one or more protons is essential for the transition to E2, making the processes of electron transfer and proton translocation tightly coupled. Once the oxidase is in state 2 it has two possible paths. It can revert directly to E1 and another cycle of cytochrome c oxidation begins. Or E2 can bind another substrate molecule before the conformational transition to E1, and hence it would revert to E1 with the substrate already bound.

By assuming that the dissociation constant K1 is much less than K2, it is possible to reproduce the biphasic kinetics seen in steady state assays of cytochrome oxidase activity. The low activity, high affinity phase corresponds to the pathway where E2 reverts directly to E1, while the high activity, low affinity phase corresponds to E2 binding to substrate before the conformational transition. Since K1 $<$ K2, the high activity phase only becomes important at high concentrations of cytochrome c, resulting in the appearance of biphasic kinetics.

The model also explains the ionic strength dependence observed for the steady state kinetics. The biphasic pattern is more pronounced at low ionic strength, while at high ionic strength only a single phase is observed. As the ionic strength increases K1 increases and approaches the value of K2. It is known that the second order rate constant for cytochrome

c oxidation decreases with increasing ionic strength (Veerman et al., 1983). Hence the kinetics become monophasic when this condition is reached.

According to the model the oxidase will pump protons to reach the E2 state. This is a consequence of the pathways from E2 to E1 and E2-S to E1-S being unidirectional as indicated in the model scheme. Elimination of the conformational transition entails the elimination of proton pumping and vice versa, with the effect that the kinetics should become monophasic. If the oxidase is trapped in its E1 conformation then it can only oxidize cytochrome c via the low activity, high affinity pathway.

The steady state kinetics presented previously (figures 19, 20, & 21) show that the modified oxidase becomes monophasic and the Km and TNmax values approach the high affinity, low activity phase parameters of the native oxidase. A hypothesis is that increasing concentrations of DEPC used for modification results in the successive elimination of the conformational transition. The elimination of the conformational transition and the appearance of monophasic kinetics predicts that proton pumping should be abolished as well. The proton pumping results of native and modified oxidase (table 13) demonstrate that proton pumping is indeed inhibited by 40 per cent. This result is in agreement with some preliminary work done with DEPC by Papa et al. (1989). These workers modified oxidase vesicles with DEPC and found that the H^+ / e stoichiometry decreased by 40 % relative to native oxidase vesicles.

A discrepancy requiring comment is the fact that modified oxidase still retains 60 per cent of its proton pumping capacity. Ideally this function should be completely eliminated if the conformational transition is abolished, unless the oxidase can pump

protons with reduced efficiency in the absence of a conformational transition. This however would be contrary to the hypothesis that the conformational transition and the proton pumping are tightly linked.

Nevertheless Michel and Bosshard (1989) also report monophasic kinetics with oxidase that has been briefly treated with chymotrypsin, and the proton pumping is only inhibited by varying degrees without being completely eliminated. In fact a complete abolishment of proton pumping has not been demonstrated with any sort of modification treatment. Prochaska et al. (1981) achieved a 50 % inhibition of proton pumping when the oxidase was treated with a large excess of DCCD, which reacts predominantly with carboxyl groups on proteins. Nilsson et al. (1988) also achieved 50 % inhibition by modifying Cu_A with p - hydroxymercuribenzoate. Papa et al. (1989) were able to reduce proton pumping by ≈ 70 % by modifying the oxidase with a large excess of succinic anhydride, a reagent that preferentially reacts with lysine residues. Saraste et al. (1981) found that subunit III depleted oxidase incorporated into vesicles could still translocate protons at a reduced proton to electron stoichiometry. These results show that a wide variety of treatments all result in diminished but not completely abolished proton pumping. This illustrates the complexity of the proton pumping process, in that treatments that target different components of the oxidase structure all result in reduced proton pumping efficiency. A hypothesis is that all the modification treatments described impair some part of the proton pumping mechanism, but the apparatus as a whole operates at an impaired (as opposed to zero) efficiency due to the large spatial domain occupied by the proton pumping machinery.

The complexity of the proton pumping mechanism allows for the hypothesis that the monophasic kinetics and the impaired proton pumping are reconcilable if the link between electron transfer and proton translocation is indirect as a consequence of a large conformational change. Treatments may disturb the conformational change that relates to cytochrome c binding, leading to monophasic kinetics. However if the conformational change is large there is the possibility that the transition related to proton pumping would be only indirectly affected, and could still occur at impaired efficiency.

The decrease in proton pumping could be due to the modification of essential histidines that directly participate in proton transfer. It is also possible that some of the modified histidines are involved in electron transfer, which would account for the depression in activity and could also be indirectly responsible for the impairment of the proton pump. Electrons have to travel a considerable distance to get from cytochrome c to the oxygen reduction site and the efficient transfer is probably mediated by some amino acids. Ideal molecules for this function are ones containing conjugated π bond systems, hence the imidazole ring of histidine could participate in electron transfer. The role of histidine residues in electron transfer has been demonstrated in another heme containing protein, cytochrome c peroxidase by Bosshard et al. (1984). These workers provided evidence for a single histidine being essential for the transfer of an electron from cytochrome c to the heme in the peroxidase through chemical modification studies.

The spectroscopic results in this thesis show that modification induces spectral changes in the visible region of the spectrum. The reproducible small drop in absorbance

in the vicinity of 430 nm (figure 4) provides evidence that the heme centers are perturbed as a consequence of modification. Alterations of the heme environments are also apparent from the 5 nm shifts in the reduced - oxidized spectra (figures 9 & 10). These spectra show no change in the 830 nm region, hence on the basis of spectroscopy it can be concluded that Cu_A is unaffected by modification. It follows that the source of proton pumping inhibition is different than the inhibition induced by Cu_A modification. When higher concentrations of DEPC and enzyme were employed a spectral perturbation is also evident in the 650 nm region (figure 6). It has been previously proposed on the basis of EPR studies (Hartzell et al., 1973) that the small absorption band at 655 nm is the most unambiguous manifestation of the heme a_3 component. This provides specific evidence for alterations of the cytochrome a_3 environment.

The spectroscopic changes induced by modification could be indicative of heme environmental changes that result in an impairment of efficient electron transfer through the metal centers, which in turn could be a factor in the observed decrease in the proton to electron stoichiometry. Increasing concentrations of DEPC for modification results in successive decreases in the TNmax and Km values (figures 21 & 20 respectively) and also gives rise to consecutive increases in the magnitude of the trough at 430 nm (figure 5). Although no direct correlation was done, these data suggest an increase in the spectroscopic perturbation is accompanied by decreases in TNmax and Km. A hypothesis is that increasing concentrations of DEPC induces larger alterations in the heme environments which is followed by corresponding decreases in efficient electron transfer, manifested by the observed reductions in TNmax and Km.

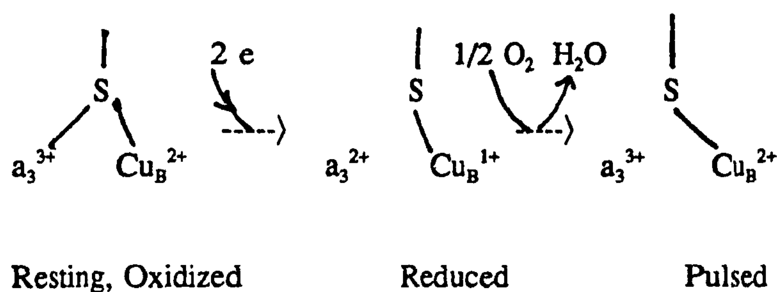
Evidence for specific alterations in cytochrome a_3 was also found from the enhanced rate of cyanide binding determined in several experiments (figures 17 & 18, tables 4, 5, & 6). Magnetic circular dichroism studies done by Thomson et al. (1985) have provided evidence that cyanide binds to resting, oxidized oxidase by forming a linear bridge between cytochrome a_3^{3+} and Cu_B^{2+}. It follows that the changes in cyanide binding to modified oxidase reflect perturbations in the cytochrome a_3 and / or the Cu_B environments. The occurrence of the enhanced cyanide binding rate does not manifest itself until the modification reaction is near completion (figure 18 & table 5), thus the cytochrome a_3 environment experiences a relatively abrupt change leading to increased accessibility of the site to cyanide. It is possible that modification of most of the histidines accessible to DEPC leads to a sudden conformational change resulting in cytochrome a_3 being more accessible to cyanide, or alternatively it is the modification of the last 2 to 3 histidines which directly causes the increased accessibility.

It is possible that the acceleration of the rate of cyanide binding is because modification induces changes in the oxidase to cause it to resemble the pulsed form of the enzyme. Jones et al. (1984) show that pulsed oxidase binds cyanide 4 - 5 fold faster than resting, oxidized enzyme, and Naqui et al. (1984) show that the pulsed enzyme reacts with cyanide in a single phase. Table 6 demonstrates that prior modification of the oxidase with 0.75 mM DEPC results in a $\approx$ 3 fold enhancement of the fastest phase of the cyanide binding rate, which is similar to the rate enhancement due to making the enzyme pulsed.

A model has been proposed by Chance and Kumar (1983) to account for the altered cyanide binding kinetics exhibited by the pulsed enzyme. On the basis of X - ray

absorption spectoscopy studies these workers found evidence that the pulsed form of the oxidase lacks a sulfur bridge between cytochrome a_3 and Cu_B which is present in the resting, oxidized enzyme. Scheme 2 illustrates a simplified version of the transition from resting to pulsed oxidase.

Scheme 2



When the oxidase is in its resting, oxidized form there is ia a sulfur bridge between the two metal centers which hinders the accessibiltiy of cyanide. During turnover this bridge is broken and it remains broken for some time even after turnover is finished, resulting in the pulsed form of the oxidase. Modification with DEPC could result in local conformational changes around the bridging sulfur causing the bond between cytochrome a_3 and the sulfur to break, resulting in an oxidase molecule with similar properties as the pulsed enzyme. The relatively abrupt appearance of the enhanced cyanide binding effect is consistent with a certain degree of modification causing the sudden breakage of the sulfur cytochrome a_3 bond. The kinetics of cyanide binding to modified oxidase however is not monophasic like it is with pulsed form. Perhaps a higher concentration of DEPC would

result in monophasic cyanide binding kinetics, since as shown in table 4 the extent of the cyanide binding reaction becomes larger over a given unit of time with increasing concentrations of DEPC. Unfortunately it is difficult to interpret the cyanide binding to oxidase that has been modified with high concentrations of DEPC since the maximal absorbance at 433 nm starts to decay after a certain point of the reaction (table 4). It is possible that the maximum absorbance peak for the cyanide - a_3 complex is being shifted as the binding reaction occurs. Further experiments are necessary before a more definitive explanation on the cyanide binding to modified oxidase can be proposed.

The perturbations of cytochrome a_3 as evinced by the enhanced cyanide binding kinetics and the spectral change noted at 650 nm could be partially responsible for the decreases in TNmax and Km noted previously. The magnitude of the enhanced cyanide binding effect increases with increasing concentrations of DEPC as shown in figure 17 and table 4. The magnitude of the 430 nm trough also increases with increasing DEPC, and the values of TNmax and Km also successively decrease as the DEPC concentration is raised. The effects on these three separate parameters of the oxidase could all be a reflection of a common cause arising from the modification of certain histidines that are essential for efficient electron transfer. Successive increases in the DEPC concentration induces larger alterations of the heme environments, in particular to cytochrome a_3. This effect is translated into consecutive decreases of electron transfer efficiency and the creation of a cytochrome a_3 site that becomes more accessible to cyanide. The partial inhibition of proton pumping could be an indirect effect of these changes. Impairment of electron

transfer efficiency could affect the integrity of the conformational transition resulting in the observed monophasic kinetics and the decrease in the proton to electron stoichiometry. The possibility also exists that histidines which directly participate in proton translocation are modified as well, resulting in the observed inhibition. In principle any amino acid with a polar side chain containing either nitrogen or oxygen can participate in proton transfer. However histidine could be a better candidate than the other amino acids since its imidazole side chain pKa of 6 makes it particularly well suited for proton transfer at physiological pH. If histidines essential for proton translocation are modified then they are probably located in the proton channel relatively far removed from the metal centers. This is because modification of one or more hypothetical essential histidines that function as the primary proton translocating switch triggered by the changes in redox state of the metal centers would result in a much larger inhibition.

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