Proton Translocation in Cytochrome *c* Oxidase: Redox Linkage Through Proximal Ligand Exchange on Cytochrome a_3

Denis L. Rousseau,[1] Yuan-chin Ching[1] and Jianling Wang[1]

An analysis of resonance Raman scattering data from CO-bound cytochrome *c* oxidase and from the photodissociated enzyme indicates that histidine may not be coordinated to the iron atom of cytochrome a_3 in the CO-bound form of the enzyme. Instead, the data suggest that either a water molecule or a different amino acid residue occupies the proximal ligand position. From these data, it is postulated that ligand exchange on cytochrome a_3 can occur under physiological conditions. Studies of mutant hemoglobins have demonstrated that tyrosinate binds preferentially to histidine in the ferric forms of the proteins. In cytochrome *c* oxidase tyrosine residues are located near the histidine residues recently implicated in coordination to cytochrome a_3 (Shapleigh *et al.*, 1992; Hosler *et al.*, this volume). Expanding on these concepts, we propose a model for proton translocation at the O_2-binding site based on proximal ligand exchange between tyrosine and histidine on cytochrome a_3. The pumping steps take place at the level of the peroxy intermediate and at the level of the ferryl intermediate in the catalytic cycle and are thereby consistent with the recent results of Wilkstrom (1989) who found that proton pumping occurs only at these two steps. It is shown that the model may be readily extended to account for the pumping of two protons at each of the steps.

INTRODUCTION

One of the central themes of bioenergetics is the mechanism by which the oxidation of substrates or the absorption of light generates ATP. A major advance in the understanding of energy transduction occurred several years ago when Peter Mitchell proposed the chemiosmotic hypothesis (Mitchell, 1961). The "central dogma" of this hypothesis states that ATP synthesis is coupled to respiration or light absorption by a proton electrochemical potential. In aerobic organisms key steps in this process involve linking electron transport to proton translocation across membranes, thereby transducing the redox energy to the proton gradient which drives the synthesis of ATP. Despite many studies on proteins involved in the respiration-linked proton translocation, the molecular mechanism of the coupling is not known.

Cytochrome *c* oxidase is a membrane-bound enzyme which forms the terminal site in the electron transport chain in eukaryotic species (Wikstrom *et al.*, 1981). As such it reduces oxygen to water and translocates protons across the inner mitochrondrial membrane. Four electrons are required to fully reduce O_2 to H_2O. In this process four protons are taken up from the matrix side of the mitochondrial membrane for the formation of H_2O and four more protons are vectorially translocated from the matrix to the cytosol side of the membrane. The mammalian enzyme consists of thirteen subunits and four redox active metal centers (Capaldi, 1990; Saraste, 1990). Additional metal atoms have been shown to be present but their

[1] AT&T Bell Laboratories, Murray Hill, New Jersey 07974.

functional role remains to be determined (Steffans et al., 1987; Yewey and Caughey, 1988). Two of the metal centers, cytochrome a and Cu_A, transfer electrons from cytochrome c to the O_2 binding site formed by the two other redox active metal centers, cytochrome a_3 and Cu_B. Until recently it had been assumed that each proton translocation was linked to a single electron transfer and the low potential centers (cytochrome a and Cu_A) were postulated as the sites for the redox-linked proton translocation (Wikstrom et al., 1981). This led to some elegant models for proton translocation (Chan and Li, 1990) such as that involving ligand exchange at Cu_A (Gelles et al., 1986) and that involving modulation of the hydrogen bond strength of the formyl group on cytochrome a (Babcock and Callahan, 1983).

Recently, Wilkstrom (1989) reported reverse electron transfer measurements which indicate that the translocation of *two* protons is associated with each of *two* of the redox steps and no proton translocation is associated with the other two steps. This finding makes it more likely that redox linkage is associated with the O_2-binding site, the binuclear center formed by the iron atom of cytochrome a_3 and the nearby Cu_B, rather than by the low potential centers. Consequently, the possibility that proton translocation is linked to electron transfer steps at the binuclear O_2 binding site is now being considered by several groups. Along these lines, Cu_B was postulated by Babcock and Wikstrom (1992) to be the site of the linkage based on evidence suggesting that Cu_B is the electron entry point to the binuclear center. This model requires that when a ligand is bound to cytochrome a_3 all the electrons pass through Cu_B. Larsen et al. (1992) also considered Cu_B as the site of redox linkage and concluded that several drawbacks make it an unlikely possibility. Specifically, they pointed out that the reported (Han et al., 1990c; Varotsis and Babcock, 1990) rapid electron transfer from cytochrome a to cytochrome a_3 would uncouple the redox linkage by bypassing Cu_B; the close proximity of cytochrome a_3 and Cu_B would not allow easy changes between input and output states; and the position of Cu_B in the membrane and in relation to cytochrome a_3 makes it difficult to obtain respiratory control. Woodruff et al. (1991) have proposed another model for redox coupling involving an endogenous ligand shuttle between the iron atom of cytochrome a_3 and Cu_B. However, this shuttle will be blocked when the oxo-ligands are bound to cytochrome a_3 making it difficult to postulate how the ligand shuttle will be able to couple to the redox events.

The redox events that occur in cytochrome c oxidase during O_2 reduction have been studied for many years in an effort to understand the catalytic mechanism on a molecular level. Considerable progress has been made from biochemical studies (Brunori et al., 1979; Wikstrom, 1981; Jones et al., 1984; Wikstrom, 1987, 1988; Cooper, 1990; Antonini et al., 1991; Oliveberg and Malmstrom, 1991) and many types of spectroscopic studies (Gibson and Greenwood, 1963; Greenwood and Gibson, 1967; Chance et al., 1975; Clore et al., 1980; Hill et al., 1986; Baker et al., 1987; Einarsdottir et al., 1988; Chan and Li, 1990). Most recently, resonance Raman scattering has been particularly valuable in determining the properties of cytochrome a_3 in various equilibrium states of the enzyme (Babcock, 1988), the intermediates in the redox cycle (Babcock et al., 1984, 1985; Han et al., 1990a-e, 1992; Varotsis et al., 1989, 1990a,b; Varotsis and Babcock, 1990; Ogura et al., 1990a,b, 1991), and the effect of carbon monoxide photodissociation on the heme (Findsen and Ondrias, 1984; Findsen et al., 1987; Sassaroli et al., 1988). These studies have led to a high level of understanding of the redox process but have shed little light on the mechanism of proton translocation.

In this paper we propose a new model for proton translocation based on redox linkage at cytochrome a_3. Cytochrome a_3 does not appear to suffer from many of the limitations noted above for Cu_B. Furthermore, there is substantial evidence that electron transfer from cytochrome a to ligand-bound cytochrome a_3 may be very rapid (Han et al., 1990c; Varotsis and Babcock, 1990). The model we propose involves ligand exchange at cytochrome a_3 and draws from the ligand exchange ideas put forward by Chan and co-workers for a model proposing Cu_A as the site for redox linkage (Gelles et al., 1986; Chan and Li, 1990). Evidence for ligand exchange at cytochrome a_3 is supplied by resonance Raman scattering measurements of carbon monoxide-bound cytochrome c oxidase and from experiments on the product of CO photodissociation (Woodruff et al., 1991).

LIGAND BINDING TO CYTOCHROME a_3

To obtain a complete understanding of the structure and properties of the ligand binding site and how
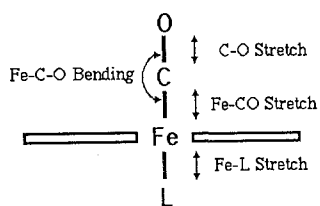
Fig. 1. Possible vibrational modes studied in heme carbonyl complexes with a proximal base L. In the absence of the bound CO or another ligand at that position, the iron-proximal base stretching mode, Fe-L (e.g., Fe-histidine), may be observed in the resonance Raman spectrum. When CO is bound, the Fe–CO and the C–O stretching modes and the Fe–C–O bending mode may be observed in the Raman spectrum. The C–O stretching mode is also detected in the infrared absorption spectrum. For the same type of axial ligand (e.g., nitrogenous) there is a correlation between the Fe–CO stretching mode frequency and the C–O stretching mode frequency.

these could couple to proton translocation, we draw inferences from ligand bound forms of the enzyme which can be stabilized and thereby studied in detail. Carbon monoxide-bound cytochrome *c* oxidase is especially powerful in this regard because it forms a very stable complex. In addition, the CO can be photodissociated to yield additional information concerning the transient intermediates present before the protein structure can relax back to the ligand-free form. Spectroscopic studies, especially vibrational spectroscopy, of CO-bound cytochrome oxidase have elucidated many properties of the ligand binding site. Vibrational modes involving the bound CO (Fig. 1) are present in infrared (IR) absorption and Raman scattering spectra. The Fe–CO and the C–O stretching modes and the Fe–C–O bending mode have all been identified in the spectra (Yoshikawa *et al.*, 1977; Argade *et al.*, 1984). The frequencies and intensities of these modes have been useful for determining the local structure in the heme-CO complex. The vibrational modes of the heme group itself are also sensitive to the properties of the bound ligand and yield information concerning the distribution of electrons between the heme and its ligands (Babcock, 1988).

The spectroscopic properties of cytochrome *c* oxidase in which CO is bound to cytochrome a_3 are well established. The C–O stretching mode has been examined in several IR absorption studies including studies of the mode as a function of the degree of enzyme reduction in preparations that ranged from being reduced by only one electron to those in which the enzyme was fully reduced (four electrons) (Yoshikawa and Caughey, 1982). In those experiments it was found that the C–O stretching mode of CO-bound

cytochrome a_3 is located at 1963.5 cm^{-1} and shifts by no more than 2 cm^{-1} as the other redox centers change their oxidation state. This indicates that the change in oxidation state of the nearby Cu_B has very little effect on the Fe–C–O moiety even though it is well established that the polarity of the local environment near a bound CO influences the frequency of the CO stretching mode (Caughey, 1980; Maxwell and Caughey, 1976). Thus, the interaction between the CO and the nearby copper atom must be very small. The Fe–CO stretching mode and the Fe–C–O bending mode were detected in the resonance Raman spectrum by Argade *et al.* (1984) and confirmed by the data presented here. In the data shown in Fig. 2, the stretching mode displays a very high frequency (520 cm^{-1} at neutral pH) which Argade *et al.* (1984) attributed to a weak bond trans to the CO bond. They postulated that the weak proximal ligand was a strained histidine residue since in the ligand-free preparation the frequency of the iron-histidine (Fe–His) stretching mode (214 cm^{-1}) is low, consistent with considerable strain (Kitagawa, 1988).

A strong negative correlation (presented in Fig. 3) has been noted between the Fe–CO stretching frequency and the C–O stretching frequency in many heme proteins and model compounds when the axial base has similar bonding characteristics (Yu *et al.*, 1983; Tsubaki *et al.*, 1986; Uno *et al.*, 1987; Smulevich *et al.*, 1988; Kerr and Yu, 1988; Morikis *et al.*, 1989; Spiro *et al.*, 1990; Nagai *et al.*, 1991). For example, in Fig. 3 all of the proteins and model compounds with a nitrogenous base (histidine, substituted imidazoles, or pyridine) lie on the same line. Those compounds with a cysteinal sulfur lie on a different line with a weaker iron-carbon bond, and the one compound with a very weak proximal ligand is found well above the nitrogenous base correlation line, thereby indicating a stronger iron-carbon bond. The reasons for these corrrelations and the relative positions are reasonably well understood (Kerr and Yu, 1988). Carbon monoxide binds to the iron atom through a σ-bond formed by donation of a nonbonded pair to the iron d_{z^2} orbital. In addition, π-bonding takes place by donation from the occupied d_π-orbitals of the iron atom to the empty antibonding π^*-orbitals of the CO. Thus, the π-bonding strengthens the Fe–C bond and weakens the C–O bond. For hemes with the same proximal base (e.g., nitrogenous), changes in the Fe–C–O geometry or changes in geometry of the proximal ligand affect the π-electron distribution between the Fe–C bond and the C–O bond, thereby moving the
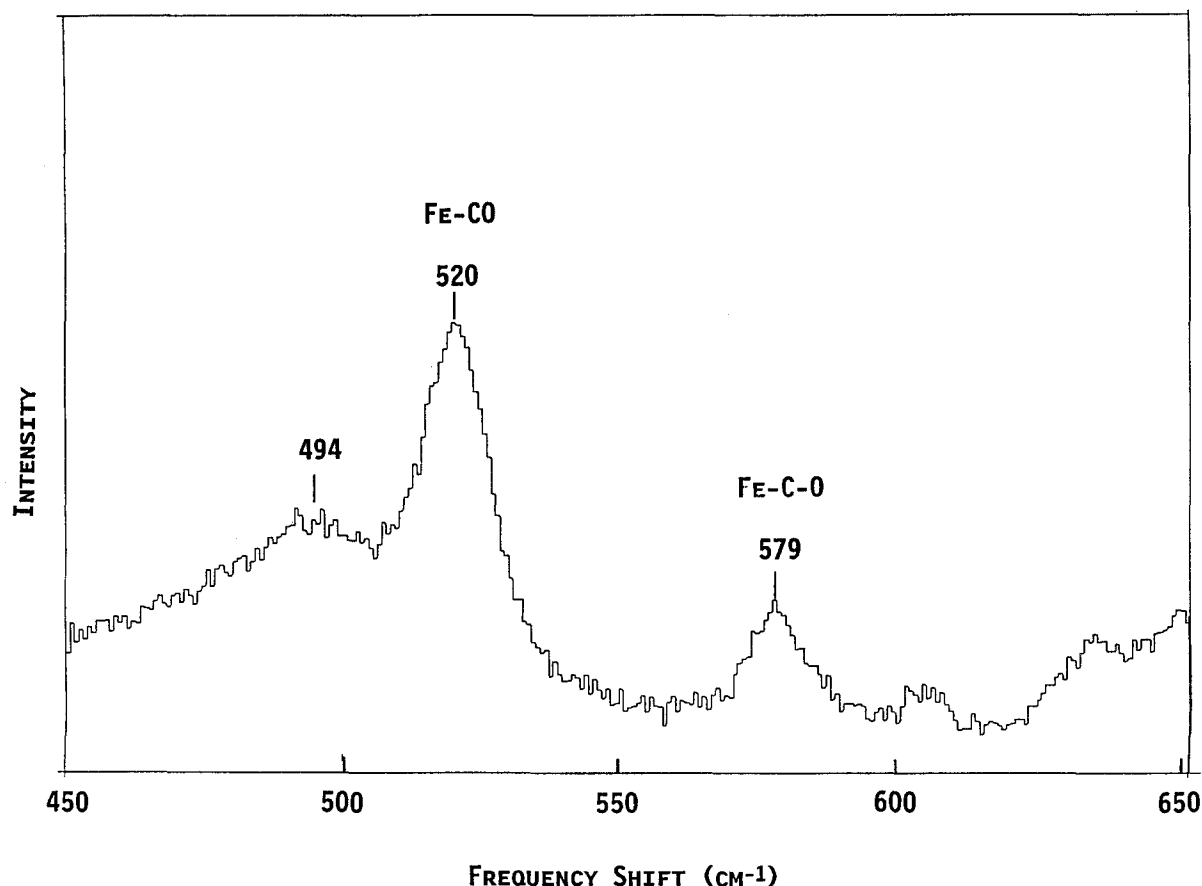
Fig. 2. Resonance Raman spectrum of CO-bound cytochrome c oxidase at pH 7.0. The major Fe–CO stretching mode appears at 520 cm^{-1} and the bending mode is at 579 cm^{-1}. The spectrum was obtained on a previously described Raman difference apparatus (Rousseau, 1981) excited at 413.1 nm from a defocussed krypton ion laser beam with a scanning speed of 50 cm^{-1}/min. The heme concentration of the enzyme was about 100 μM. The bovine cytochrome c oxidase was isolated by a modified Yonetani procedure (Yonetani, 1960) and solubilized in 100 mM phosphate buffer containing 0.4% dodecyl β-D-maltoside. The sample was deoxygenated, reduced with minimal dithionite, and saturated with CO prior to obtaining the Raman data.

vibrational frequencies along the correlation line. However, when the axial ligand is changed, the σ-bond between the iron and the carbon is affected. Since it is formed by donation of a lone pair into the d_{z^2} orbital, strengthening the bond on the proximal side weakens the iron-carbon bond on the distal side and *vice versa* establishing a different correlation line. Thus, the stronger σ-bond from the sulfur ligand causes the correlation to move to a lower line in Fig. 3 than the nitrogenous line, and the iron porphyrin in which the proximal ligand is tetrahydrofuran (THF) occupies a position above the nitrogenous-ligand correlation line.

It has been noted previously that carbon monoxide-bound cytochrome c oxidase does not properly fit on the stretching frequency correlation curve for a

normal nitrogenous base (Kerr and Yu, 1988; Nagai *et al.*, 1991). Indeed, as may be seen in Fig. 3, it lies more than 10 cm^{-1} away from any point other than that of the model heme with THF as the proximal base. In addition, for myoglobin at low pH where the Fe–His bond is absent, the Fe–CO stretching mode is detected in the same region as that of cytochrome c oxidase (Sage *et al.*, 1991). When the Fe–CO stretching mode was first reported by Argade *et al.* (1984), its very high frequency was attributed to an anomalously weak Fe–His bond. However, with the extensive data set now available relating the frequencies of the Fe–CO stretching mode to the C–O stretching mode shown in Fig. 3, it is clear that independent of proximal strain the correlation still holds. Thus, the analysis suggests that *histidine is not the proximal*
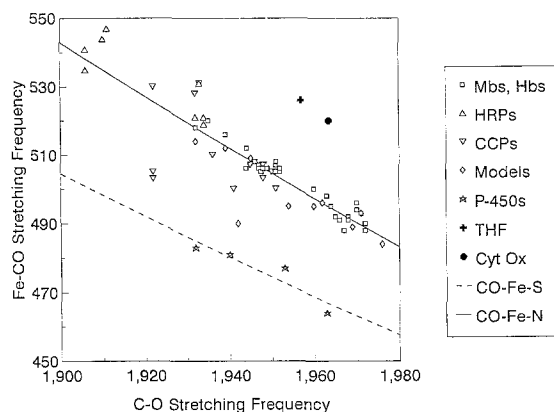
Fig. 3. Correlation of the Fe–CO stretching mode frequency and the C–O stretching mode frequency of CO-bound heme proteins and prophyrin complexes. Different correlation lines are obtained for different proximal axial ligands. The dashed line is the corrrelation for thiolate axial binding and the solid line is for nitrogenous binding. The point designated by the darkened cross is for a porphyrin coordinated with tetrahydrofuran, a weak proximal ligand. CO-bound cytochrome *c* oxidase at pH 7, designated by the darkened octagon, does not lie on the nitrogenous correlation curve but instead is near the weak ligand point.

ligand in CO-bound cytochrome c oxidase. We postulate that the proximal histidine bound has been broken in the CO-bound form of the enzyme. The spectrum of the CO-bound enzyme does not allow determination of whether or not another ligand has replaced the histidine. However, CO photolysis measurements reported by others and discussed below give additional insights.

Breaking of the proximal histidine bond in heme proteins by protein structural forces and replacement by another ligand are not without president. Several examples (Table I) have been identified in naturally occurring mutants of hemoglobin (Nagai *et al.*, 1983, 1989, 1991). Furthermore, in the α-subunits of nitrosyl human hemoglobin the addition of inositol hexaphosphate ruptures the Fe–His bond, leading to a 5-coordinate structure (Maxwell and Caughey, 1976; Perutz *et al.*, 1976). In several mutant hemoglobins, listed in

Table I, either the proximal or distal histidine is replaced by tyrosine. In those cases the *tyrosine is always one of the axial ligands* or the only axial ligand in the ferric form of the protein. This is in contrast to the ferrous form of the protein in which a *histidine is always an axial ligand.* These data illustrate that ligand exchange can take place in stable heme proteins and that the coordination depends on the heme oxidation state and on protein structural forces. It is noteworthy that whenever tyrosine is available in the proximal or the distal pocket it binds to the ferric heme.

In the ligand-free form of cytochrome *c* oxidase histidine occupies the fifth coordination site on cytochrome a_3. This has been proven from resonance Raman studies where the Fe–His stretching mode has been identified at 210–215 cm^{-1} region (Ogura *et al.*, 1983; Kitagawa, 1988). In NO-bound reduced cytochrome *c* oxidase at cryogenic temperatures, EPR studies have demonstrated that histidine occupies the fifth coordination site (Stevens *et al.*, 1979a,b; Stevens and Chan, 1981). If histidine does not occupy the fifth coordination site in the CO-bound form of the protein under our experimental conditions, then there may be conditions under which it does rebind and a second Fe–CO stretching mode should appear.

To test whether or not additional Fe–CO stretching modes are present in cytochrome *c* oxidase, we have measured the resonance Raman spectra as a function of pH. As the pH was increased, a new line developed in the spectrum at 494 cm^{-1}, as may be seen in Fig. 4. Several isotopic substitution studies confirmed that this line was a Fe–CO stretching mode. Thus, the enzyme displays more than one Fe–CO stretching mode, consistent with the concept that the proximal histidine bond may be broken in some forms of the enzyme. A titration study comparing the formation of the line at 494 cm^{-1} with the formation of the Schiff base at the formyl group (Han *et al.*, 1991) demonstrated that the axial ligation change was independent of the Schiff base formation. However, we recognize that there may be other explanations for

Table I. Ligand Coordination in Mutant Hemoglobins. Coordination for the Mutated Subunits Shown for the Deoxy and the Ferric Forms

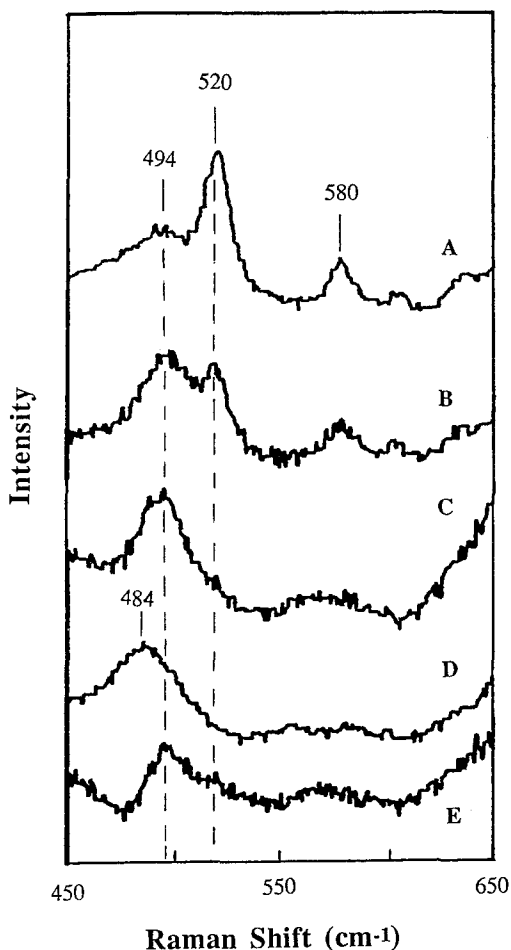|	HbA (Prox)F8 = His (Dis)E7 = His	Boston αE7 = Tyr	Saskatoon βE7 = Tyr	Iwate αF8 = Tyr	Hyde Park βF8 = Tyr
Deoxy	5c (F8)His–Fe	5c (F8)His–Fe	5c (F8)His–Fe	5c Fe–His(E7)	5c Fe–His(E7)
Ferric	6c His–Fe–H$_2$O	5c Fe–Tyr(E7)	6c His–Fe–Tyr(E7)	5c (F8)Tyr–Fe	5c (F8)Tyr–Fe

Fig. 4. The pH dependence of the Fe–CO stretching mode frequency in CO-bound cytochrome c oxidase. A. The sample at pH 7.0 has a strong Fe–CO stretching mode at $520\,\text{cm}^{-1}$. B. The sample at pH 8.0 has Fe–CO stretching modes at 494 and $520\,\text{cm}^{-1}$. C. In this sample at pH 10.0 the Fe–CO stretching mode is at $494\,\text{cm}^{-1}$. D. The pH is the same as that in spectrum C (10.0) but the enzyme was exposed to $^{13}\text{C}^{18}\text{O}$ rather than the natural abundance isotopes. E. Difference spectra of C–D demonstrating the isotope shift, confirming that the mode at $494\,\text{cm}^{-1}$ is an Fe–CO stretching mode. All conditions for sample preparation and data acquisition are the same as in Fig. 2.

the data that do not require proximal ligand exchange. Indeed, many pH studies of various properties of cytochrome c oxidase have been reported and a large number of pK's have been identified (Callahan and Babcock, 1983; Ogura *et al.*, 1984; Thornstrom *et al.*, 1984; Sone *et al.*, 1986; Baker and Palmer, 1987; Oliveberg *et al.*, 1989; Maison-Peteri and Malmstrom, 1989; Harmon and Stringer, 1990; Moody and Rich, 1990; Papadopoulos *et al.*, 1991). Additional studies are needed to make a firm assignment of the origin of

the modes which we detect and relate the changes to the other pH-induced changes that have been reported.

An additional test of whether or not the Fe–His bond is intact in CO-bound cytochrome c oxidase comes from photodissociation experiments. The schematic process for this type of measurement is illustrated in Fig. 5. It is well established that in the ligand–free form of the enzyme cytochrome a_3 is five-coordinate and histidine is the proximal base (Ogura *et al.*, 1983; Kitagawa, 1988). As discussed above, we propose that binding CO causes ligand exchange and thereby a different proximal base. If our analysis is correct and the histidine is replaced in the CO-bound preparation by a different ligand, then when CO is photodissociated the Fe–His stretching mode may or may not reappear in the Raman spectrum depending on the rate at which it is re-established. Several experiments reporting the photodissociation of CO have appeared and the Fe–His stretching mode was present (Findsen and Ondrias, 1984; Findsen *et al.*, 1987; Sassaroli *et al.*, 1988). However, Woodruff *et al.* (1991) have shown that a *five-coordinate species* appears subsequent to photodissociation in which *the Fe–His stretching mode is completely absent* from the resonance Raman spectrum. In order to find this species, the laser power had to be kept very low since the coordinated ligand was photolabile and, once dissociated, would allow for the return of the proximal histidine. The identity of this ligand could not be determined, but it was proposed to be an endogenous ligand originally bound to Cu_B. The absence of the histidine in the transient photoproduct was confirmed recently by Lou *et al.* (1993).

Taken together, the independent results on the properties of the CO-bound form of the enzyme and the product of photodissociation lead to the following consistent interpretation: In the CO-bound form of the enzyme, histidine does not occupy the fifth coordination site on the iron atom of cytochrome a_3. Instead the position is occupied by a weaker ligand. Upon photodissociation the Fe–CO bond breaks, leaving the iron five-coordinated, with the proximal ligand being the same as that in the CO-bound form. This ligand is very photolabile and when it is photodissociated it is replaced by the proximal histidine.

From the available data it is not possible at present to determine the identity of the putative weak ligand bound to cytochrome a_3 at the fifth coordination stie. The ligand could be exogenous, such as H_2O for example, or it could be an endogenous amino acid
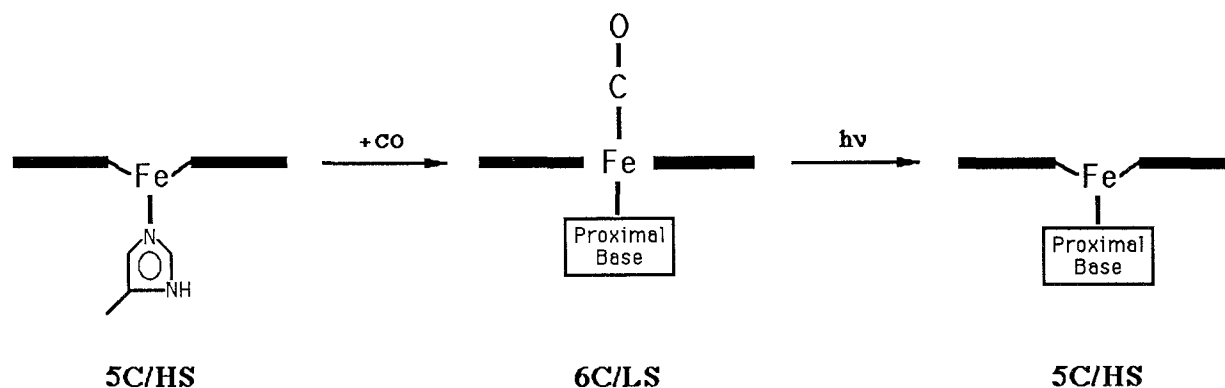
Fig. 5. Schematic concept of the comparison among the deoxy form of the enzyme, the CO-bound form, and the photoproduct. When CO is bound to the enzyme, the proximal base is different from a normal histidinal ligand. Photodissociation leads to a transient photoproduct with the same proximal base as in the CO-bound form. Experiments first reported by Woodruff _et al._ (1991) demonstrated that this proximal base was very photolabile.

side chain. Studies of transient forms of myoglobin have led to the proposal that in the CO-bound form of the protein, H_2O replaces the proximal histidine at low pH (Han _et al._, 1990f). While we are unable to exclude H_2O, endogenous ligands such as tyrosine, lysine, arginine, or an acidic residue are other candidates. Studies are ongoing to determine the identity of the ligand.

Carbon monoxide plays no known role in the enzymatic function of cytochrome _c_ oxidase. However, the important implication of the results presented above is that the histidine binding to the iron atom is labile. We postulate that the histidine may be replaced by an exogenous water molecule or an endogenous protein amino acid residue in the physiological function of cytochrome _c_ oxidase. However, it is well established that in some forms of the enzyme histidine is the fifth ligand (Ogura _et al._, 1983; Kitagawa, 1988; Stevens _et al._, 1979a,b; Stevens and Chan, 1981). Based on these results, ligand exchange on cytochrome a_3 in cytochrome _c_ oxidase is likely and may play a central role in proton translocation. The specific ligand bound at the fifth coordination site on cytochrome a_3 can be controlled by both the electron transfer events at cytochrome a_3 during the reduction process and the nature of the oxo ligand on cytochrome a_3. In the next section we present a possible model for redox coupling through ligand exchange at cytochrome a_3.

A MODEL FOR REDOX LINKAGE

Facile ligand exchange on the proximal side of

the heme which is serving as the O_2 reduction site, is an attractive mechanism that may play a pivotal role in proton translocation. We propose a model for redox linkage based on a cytochrome a_3 ligand exchange mechanism. This model directly couples the electron transfer events occurring at the oxygen binding site to the proton translocation. The essential features of the model are:

1. Histidine or tyrosine may occupy the fifth ligand coordination site on cytochrome a_3.
2. Histidine is the favored ligand in the ferrous form of the heme and tyrosine is favored in the ferric form.
3. Electron entry to the binuclear site follows a pathway through the heme macrocycle of cytochrome a_3.

In this model, ligand exchange between histidine and tyrosine is driven by the redox events at cytochrome a_3. Tyrosine is proposed as an active ligand for coordination to cytochrome a_3 along with histidine since it exchanges with histidine in mutant hemoglobins as summarized in Table I. In addition, tyrosine is the native proximal ligand in catalase (Murthy _et al._, 1981). As already pointed out, _whenever tyrosine is in the proximal or distal pocket of hemoglobin it binds to the heme in the ferric form whereas for the same proteins histidine binds in the ferrous form_ (Nagai _et al._, 1983, 1989, 1991). In the mutant hemoglobins and catalases tyrosine binds in the tyrosinate form and stabilizes the ferric oxidation state. Thus, tyrosine should be a viable ligand for cytochrome a_3 capable of

displacing the histidine under some conditions if tyrosine residues are located in the heme pocket.

In cytochrome c oxidase advances have been made recently in determining the ligands which coordinate to the metal centers by site-directed mutagenesis experiments on bacterial terminal oxidases. Examination of the amino acid sequences of available terminal oxidases (Cao et al., 1991) coupled with the site-directed mutagenesis experiments has led to an assignment of the proximal histidine that is coordinated to cytochrome a_3. The proximal histidine was originally suggested to be His[284] in helix VI by these site-directed mutagenesis studies of an aa_3 type of oxidase from Rhodobacter sphaeroides (Shapleigh et al., 1992) and a structurally related bo oxidase from Escherichia coli (Minagawa et al., 1992; Lemieux et al., 1992). A totally invariant residue (Tyr[288]) lies one turn of the helix away in the same trans membrane helix (VI) as His[284]. A recent refinement of the analysis of the data, however, indicates that the more likely histidine linkage to the heme of cytochrome a_3 is His[419] in helix (X) (Hosler et al., this volume). In this transmembrane helix, another tyrosine (Tyr[422]) is a highly conserved residue located one turn away from His[419] and thereby capable of binding to the heme as well. Thus, both histidines which have been proposed to coordinate to cytochrome a_3 have nearby tyrosines. From these assignments and the amino acid sequences it is very likely that tyrosine should be an axial ligand to cytochrome a_3 under some conditions.

A model through which these elements come together in proton translocation is illustrated in Fig. 6. In the oxy intermediate (stage 1) histidine occupies the fifth coordination site of cytochrome a_3. The proton on the N_δ atom of the histidine is hydrogen bonded as in essentially all heme proteins (Rousseau and Rousseau, 1992). We propose that the hydrogen bond acceptor is a carboxylate group as it is in cytochrome c peroxidase (Finzel et al., 1984). Upon formation of the peroxy intermediate (stage 2), the iron atom becomes ferric, driving the exchange of the histidine for a tyrosine. When the tyrosine binds to the ferric iron atom (stage 3) it becomes deprotonated. The proton is transferred to the N_ε atom of the released histidine. This weakens the N_δ-H bond, thereby allowing that proton to be transferred to the carboxylate group. When the next electron is accepted by the heme of cytochrome a_3 (stage 4), the iron atom transiently becomes ferrous (stage 5), causing the tyrosine to be released and the histidine to rebind to the iron atom (stage 6). This is the "power stroke" in the pumping

mechanism. For the histidine to re-coordinate to the iron atom of the heme, the proton from N_ε transfers to N_δ and the proton on the carboxylate is translocated to the cytosol side of the membrane. The hydrogen bond between the proton on the N_δ atom and the carboxylate group is then re-established. Once the tyrosinate is released, it immediately becomes protonated by a proton from the matrix side of the enzyme. As the electron is transferred from the heme iron to the oxo atoms, raising the oxidation state of the iron atom, the histidine is once again replaced by the tyrosine as the fifth ligand on cytochrome a_3 (stage 7) and the same sequence of events pumps another proton (stages 8 and 9).

In the model outlined above and illustrated in Fig. 6 one proton is translocated at the peroxy level and one at the ferryl level in the catalytic mechanism. However, Wikstrom (1989) has pointed out that the redox energy is available for the translocation of two protons at each of these steps. Two protons per step may be accommodated readily into our model if it is assumed that the proton released by the tyrosine does not transfer to N_ε of the histidine, as illustrated in Fig. 7. In this mechanism for the translocation of two protons per step, the proton given up by the tyrosine moves to a carboxylate group directly rather than to the proximal histidine. In such a case, an additional proton originating from the matrix side is picked up by the N_ε atom of the histidine after the iron-histidine bond ruptures and is translocated as illustrated in the model for a single proton translocation per step. Thus each step would translocate two protons, one from the tyrosine and one from the histidine.

This model for proton translocation differs significantly from that recently proposed by Babcock and Wikstrom (1992). In their model, in all of the electron transfer steps the incoming electron passes through the Cu_B atom. In the model proposed here the electrons all pass through the heme group of cytochrome a_3 as a conduit to the bound ligand. We justify this pathway from the rapid kinetic studies of the reaction of oxygen with the fully reduced enzyme. In those studies it was shown that even when Cu_B is reduced, electron transfer to the bound di-oxygen takes place directly from cytochrome a, presumably through the heme of cytochrome a_3, to the bound oxygen rather than from Cu_B to the oxygen (Han et al., 1990c; Varotsis and Babcock, 1990). Indeed, there is no evidence for any interactions between the oxo ligands and Cu_B. The rate of the decay of the primary intermediate in the fully reduced enzyme as deter-
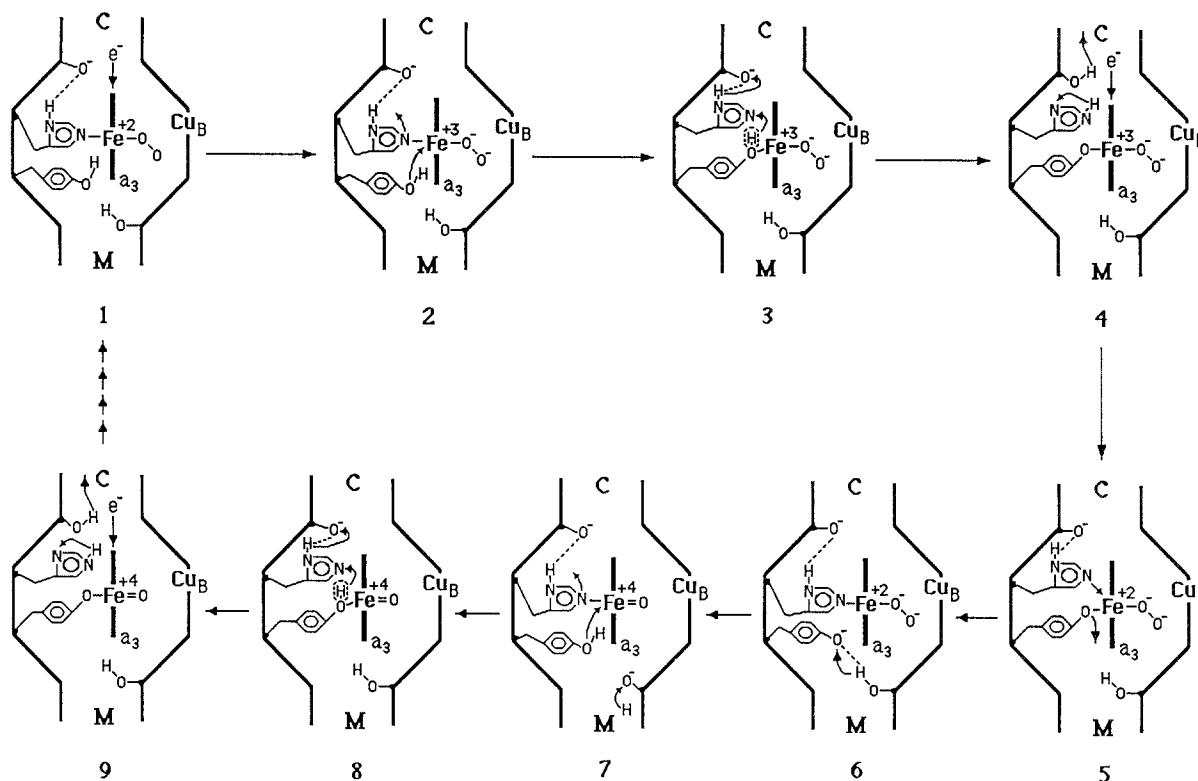
Fig. 6. A model for single proton translocation per step based on ligand exchange at cytochrome a_3. In stage 1, di-oxygen has bound to the heme and histidine is the proximal ligand. An electron enters the binding site through the heme, resulting in stage 2, a peroxy intermediate. Since the iron atom has become ferric, the histidine ligand is released and a nearby tyrosine becomes the proximal ligand. The tyrosine deprotonates by transferring its proton to the N_ε atom of the histidine (stage 3). This causes the proton on the N_δ atom to be transferred to a carboxylate. When the next electron enters the heme (stage 4), the proton on the N_ε atom is transferred to the N_δ position, releasing the proton from the carboxylate. The entry of the electron transiently reduces the heme, causing the histidine to recoordinate to the heme replacing the tyrosine (stages 5 and 6). The tyrosine is reprotonated from the matrix side of the membrane. When the electron moves from the heme to the bound ligand generating the ferryl species (stage 7), the histidine is released and the tyrosine binds again. Stages 8 and 9 are fully analogous to stages 3 and 4.

mined in the kinetic measurements is 3.5×10^4/sec (Han _et al._, 1990b). If it assumed that the electron transfer from cytochrome _a_ to cytochrome a_3 is the rate-limiting step in this process, then it has this same rate constant. If under physiological conditions, the electron were to pass through Cu_B and then to the bound ligand, its electron transfer rate would have to be much faster to compete with the electron transfer rate from cytochrome _a_ to cytochrome a_3. However, the latest analysis of the structure (Hosler _et al._, this volume) places cytochrome a_3 between Cu_B and cytochrome _a_ since the two hemes share ligands from the same helix. With such a structure, the electron transfer from cytochrome _a_ to cytochrome a_3 should be significantly faster than that from cytochrome _a_ to Cu_B.

CONCLUSIONS

In this paper evidence has been presented suggesting that histidine is not the proximal ligand in cytochrome a_3 in cytochrome _c_ oxidase for all forms of the enzyme. Specifically, the Fe–CO and the C–O stretching frequencies do not fit on the vibrational frequency correlation curve for imidazole coordination at the fifth ligand position for the CO complex. In addition, some CO-photolysis experiments yield a five-coordinate species and yet the iron-histidine stretching mode is absent from the resonance Raman spectrum, indicating that an axial ligand other than histidine is bound to the heme in the photoproduct (Woodruff _et al._, 1991). The new resonance Raman data presented here show changes in the Fe–CO stretching mode as a function of pH that are consistent with ligand exchange between histidine and some
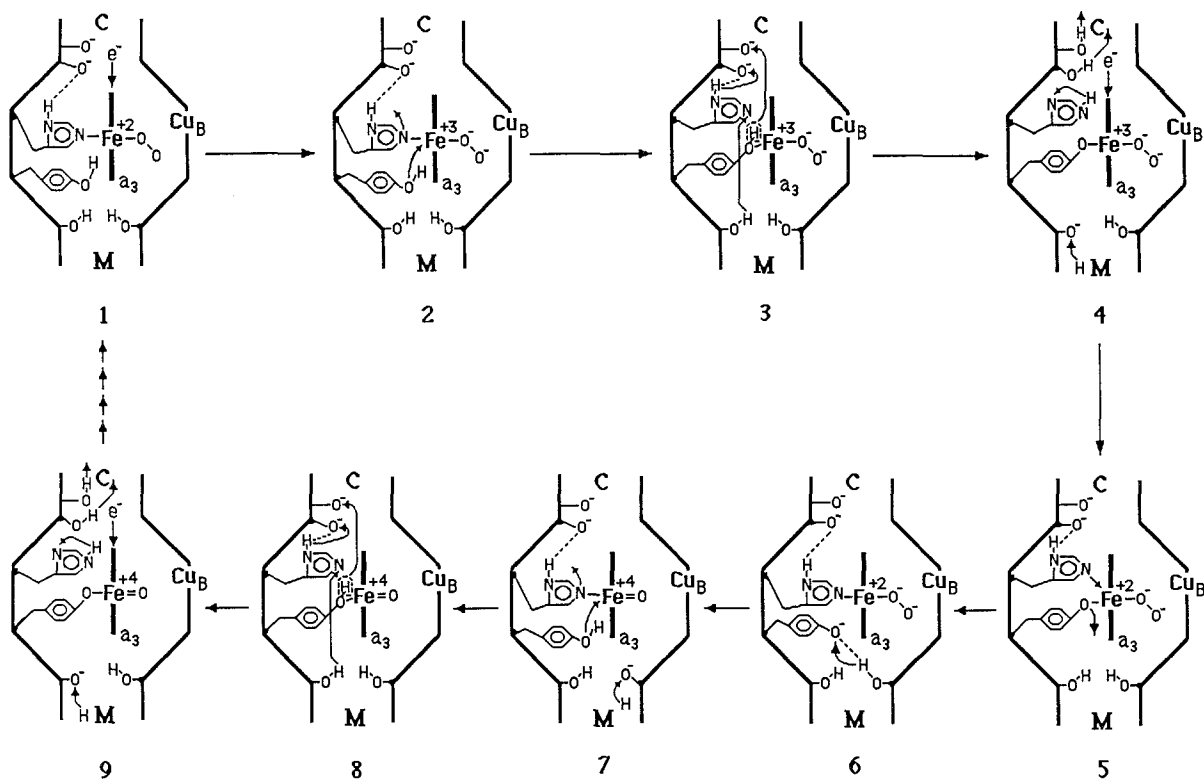
Fig. 7. A model for double proton translocation analogous to that presented in Fig. 6 except two protons are pumped at each of the two steps rather than just one. The first difference occurs at stage 3 where the proton from the tyrosine transfers to a carboxylate ratheer than to the released histidine. The histidine in turn picks up a proton from the matrix side of the membrane. Stage 8 is analogous to stage 3. The other stages proceed as in Fig. 6. The mechanism presented in this figure yields two protons at the peroxy level and two more at the ferryl level.

other residue. Each of these experiments has alternate interpretations which do not require ligand exchange. However, when viewed together they yield a consistent picture strongly suggesting that histidine is not always coordinated to cytochrome a_3. On the other hand, there are clear experiments demonstrating the presence of coordinated histidine. CO-photolysis experiments at high laser powers do yield an Fe–His stretching mode, confirming the presence of histidine coordination in the photoproduct under these conditions (Findsen and Ondrias, 1984; Findsen et al., 1987; Sassaroli et al., 1988) just as in the equilibrium ligand-free form of the enzyme. Furthermore, in the NO-bound form of the enzyme it has been demonstrated that histidine is the proximal ligand in a series of careful EPR investigations (Stevens et al., 1979a,b; Stevens and Chan, 1981). It is clear that additional experiments are necessary to reconcile this diversity of results and thereby clarify the axial coordination in cytochrome a_3 under all conditions. Clarification of

the axial binding not only has importance for the proton pumping model proposed here but also for studies of CO binding and photolysis which have been used to identify the heme axial ligands in the mutants generated by site-directed mutagenesis (Hosler et al., this volume).

Based on the available evidence and the limitations discussed above, we assume that proximal ligand exchange in cytochrome a_3 can take place. The site-directed mutagenesis experiments have identified two possible histidine residues which could coordinate to the heme of cytochrome a_3 (Shapleigh et al., 1992; Hosler et al., this volume). For each of these, there is a nearby tyrosine residue. By analogy to mutant hemoglobins, the presence of such a tyrosine should lead to tyrosinate coordination in the ferric form of cytochrome a_3 and, consequently, a heme that can bind either histidine or tyrosine depending on the iron oxidation state and the exogenous ligand. Ligand exchange and the associated protonation and

deprotonation events driven directly by the redox process form the foundation of a model for a proton pump which is capable of translocating one or two protons per step at the peroxy and ferryl intermediate levels of the catalytic reduction of O_2.

An attractive feature of the mechanism postulated here is that it can be tested owing to the application of site-directed mutagenesis to bacterial terminal oxidases. The properties of the enzyme in which the conserved tyrosines, that have been proposed here to be essential for proton translocation, have been mutated will be a stringent test of our model. In addition, if tyrosine binding to cytochrome a_3 does occur, then in the mutants lacking the tyrosine significant differences would be expected in the proton pumping efficiency and in the spectroscopic properties in some of the ferric forms of the enzyme. Until such experiments are completed, the mechanism we propose should serve as a testable working model for proton translocation.

ACKNOWLEDGMENT

REFERENCES

Antonini, G., Malatesta, F., Sarti, P., and Brunori, M. (1991). *J. Biol. Chem.* **266**, 13193.

Argade, P. V., Ching, Y.-C., and Rousseau, D. L. (1984). *Science* **255**, 329.

Babcock, G. T. (1988). In *Biological Applications of Raman Spectroscopy* (Spiro, T. G., ed.), Wiley, New York, Vol. 3, p. 293.

Babcock, G. T., and Callahan, P. M. (1983). *Biochemistry* **22**, 2314.

Babcock, G. T., and Wikstrom, M. (1992). *Nature* **356**, 301.

Babcock, G. T., Jean, J. M., Johnson, L. N., Palmer, G., and Woodruff, W. H. (1984). *J. Am. Chem. Soc.* **106**, 8305.

Babcock, G. T., Jean, J. M., Johnson, L. N., Woodruff, W. H., and Palmer, G. (1985). *J. Inorg. Biochem.* **23**, 243.

Baker, G. M., and Palmer, G. (1987). *Biochemistry* **26**, 3038.

Baker, G. M., Noguchi, M., and Palmer G. (1987). *J. Biol. Chem.* **262**, 595.

Brunori, M., Colosimo, A., Rainoni, G., Wilson, M. T., and Antonini, E. (1979). *J. Biol. Chem.* **254**, 10769.

Callahan, P. M., and Babcock, G. T. (1983). *Biochemistry* **22**, 452.

Cao, J., Shapleigh, J., Gennis, R., Revzin, A., and Ferguson-Miller, S. (1991). *Gene* **101**, 133.

Capaldi, R. A. (1990). *Annu. Rev. Biochem.* **59**, 569.

Caughey, W. S. (1980). In *Methods for Determining Metal Ion Environments in Proteins: Structure and Function of Metalloproteins* (Darnall, D. W., and Wilkins, R. G., eds.), Academic Press, New York, p. 95.

Chan, S. I., and Li, P. M. (1990). *Biochemistry* **29**, 1.

Chance, B., Saronio, C., and Leigh J. S., Jr. (1975). *Proc. Natl. Acad. Sci. USA* **72**, 1635.

Clore, G. M., Andreasson, L.-E., Karlsson, B., Aasa, R., and Malmstrom, B. G. (1980). *Biochem. J.* **185**, 139.

Cooper, C. E. (1990). *Biochim. Biophys. Acta* **1017**, 187.

Einarsdottir, O., Choc, M. O., Weldon, S., and Caughey, W. (1988). *J. Biol. Chem.* **263**, 13641.

Findsen, E. W., and Ondrias, M. R. (1984). *J. Am. Chem. Soc.* **106**, 5736.

Findsen, E. W., Centeno, J., Babcock, G. T., and Ondrias, M. R. (1987). *J. Am. Chem. Soc.* **109**, 5367.

Finzel, B. C., Poulos, T. L., and Kraut, J. (1984). *J. Biol. Chem.* **259**, 13027.

Gelles, J., Blair, D. F., and Chan, S. I. (1986). *Biochim. Biophys. Acta* **853**, 205.

Gibson, Q. H., and Greenwood, C. (1963). *Biochem. J.* **86**, 541.

Greenwood, C., and Gibson, Q. H. (1967). *J. Biol. Chem.* **242**, 1782.

Han, S., Ching, Y.-C., and Rousseau, D. L. (1990a). *Biochemistry* **29**, 1380.

Han, S., Ching, Y.-C., and Rousseau, D. L. (1990b). *Proc. Natl. Acad. Sci. USA* **87**, 2491.

Han, S., Ching, Y.-C., and Rousseau, D. L. (1990c). *Proc. Natl. Acad. Sci. USA* **87**, 8408.

Han, S., Ching, Y.-C., and Rousseau, D. L. (1990d). *J. Am. Chem. Soc.* **112**, 9445.

Han, S., Ching, Y.-C., and Rousseau, D. L. (1990e). *Nature (London)* **348**, 89.

Han, S., Rousseau, D. L., Giacometti, G., and Brunori, M. (1990f). *Proc. Natl. Acad. Sci. USA* **87**, 205.

Han, S., Ching, Y.-C., Hammes, S. L., and Rousseau, D. L. (1991). *Biophys. J.* **60**, 45.

Han, S., Song, S., Ching, Y.-C., and Rousseau, D. L. (1992). In *Time-Resolved Vibrational Spectroscopy V*, Springer Verlag, Tokyo, p. 20.

Harmon, H. J., and Stringer, B. K. (1990). *FEBS Lett.* **267**, 167.

Hill, B. C., Greenwood, C., and Nicholls, P. (1986). *Biochim. Biophys. Acta* **853**, 91.

Hosler, J. P., Ferguson-Miller, S., Calhoun, M. W., Thomas, J. W., Hill, J., Lemieux, L., Ma, J., Georgiou, C., Fetter, J., Shapleigh, J., Tecklenburg, M. M., Babcock, G. T., and Gennis, R. B. (1993) *J. Bioen. Biomem.* **25**, 121.

Jones, M. G., Bickar, D., Wilson, M. T., Brunori, M., Colosimo, A., and Sarti, P. (1984). *Biochem. J.* **220**, 57.

Kerr, E. A., and Yu, N.-T. (1988). In *Biological Applications of Raman Spectroscopy* (Spiro, T. G., ed.), Wiley, New York, Vol. 3, p. 39.

Kitagawa, T. (1988). In *Biological Applications of Raman Spectroscopy* (Spiro, T. G., ed.), Wiley, New York, Vol. 3, p. 97.

Larsen, R. W., Pan, L.-P., Musser, S. M., Li, Z., and Chan, S. I. (1992). *Proc. Natl. Acad. Sci. USA* **89**, 723.

Lemieux, L. J., Calhoun, M. W., Thomas, J. W., Ingledew, W. J., and Gennis, R. B. (1992). *J. Biol. Chem.* **267**, 2105.

Lou, B.-S., Larsen, R. W., Chan, S. I., and Ondrias, M. R. (1993). *J. Am. Chem. Soc.*, in press.

Maison-Peteri, B., and Malmstrom, B. G. (1989). *Biochemistry* **28**, 3156.

Maxwell, J. C., and Caughey, W. S. (1976). *Biochemistry* **15**, 388.

Minagawa, J., Mogi, T., Gennis, R. B., and Anraku, Y. (1992). *J. Biol. Chem.* **267**, 2096.

Mitchell, P. (1961). *Nature (London)* **191**, 144.

Moody, A. J., and Rich, P. R. (1990). *Biochim. Biophys. Acta* **1015**, 205.

Morikis, D., Champion, P. M., Springer, B. A., and Sligar, S. G., (1989). *Biochemistry* **28**, 4791.

Murthy, M. R. N., Reid, T. J., Sicignano, A., Tanaka, N., and Rossmann, M. G. (1981). *J. Mol. Biol.* **152**, 465.

Nagai, K., Kagimoto, T., Hayashi, A., Taketa, F., and Kitagawa, T. (1983). *Biochemistry* **22**, 1305.

Nagai, M., Yoneyama, Y., and Kitagawa, T. (1989). *Biochemistry* **28**, 2418.

Nagai, M., Yoneyama, Y., and Kitagawa, T. (1991). *Biochemistry* **30**, 6495.

Ogura, T., Hon-nami, K., Oshima, T., Yoshikawa, S., and Kitagawa, T. (1983). *J. Am. Chem. Soc.* **105**, 7781.

Ogura, T., Sone, N., Tagawa, K., and Kitagawa, T. (1984). *Biochemistry* **23**, 2826.

Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1990a). *J. Am. Chem. Soc.* **112**, 5630.

Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1990b). *J. Biol. Chem.* **265**, 14721.

Ogura, T., Takahashi, S., Shinzawa-Itoh, H., Yoshikawa, S., and Kitagawa, T. (1991). *Bull. Chem. Soc. Jpn.* **64**, 2901.

Oliverberg, M., and Malmstrom, B. G. (1991). *Biochemistry* **30**, 7053.

Oliveberg, M., Brzezinski, P., and Malmstrom, B. G. (1989). *Biochim. Biophys. Acta* **977**, 322.

Papadopoulos, P. G., Walter, S. A., Li, J., and Baker, G. M. (1991). *Biochemistry* **30**, 840.

Perutz, M. F., Kilmartin, J. V., Nagai, K., Szabo, A., Simon, S. R. (1976). *Biochemistry* **15**, 378.

Rousseau, D. L. (1981). *J. Raman Spectrosc.* **10**, 94.

Rousseau, D. G., and Rousseau, D. L. (1992). *J. Struct. Biol.* **109**, 13.

Sage, J. T., Morikis, D., and Champion, P. M. (1991). *Biochemistry* **30**, 1227.

Saraste, M. (1990). *Q. Rev. Biophys.* **23**, 331.

Sassaroli, M., Ching, Y.-C., Argade, P. V., and Rousseau, D. L. (1988). *Biochemistry* **27**, 2496.

Shapleigh, J. P., Hosler, J. P., Tecklenburg, M. M. J., Kim, Y., Babcock, G. T., Gennis, R. B., and Ferguson-Miller, S. (1992). *Proc. Natl. Acad Sci. USA* **89**, 4786.

Smulevich, G., Mauro, J. M., Fishel, L. A., English, A. M., Kraut, J., and Spiro, T. G. (1988). *Biochemistry* **27**, 5486.

Sone, N., Ogura, T., and Kitagawa, T. (1986). *Biochim. Biophys. Acta* **850**, 139.

Spiro, T. G., Smulevich, G., and Su, C. (1990). *Biochemistry* **29**, 4497.

Steffans, G. C. M., Biewald, E., and Buse, G. (1987). *Eur. J. Biochem.* **164**, 295.

Stevens, T. H., and Chan, S. I. (1981). *J. Biol. Chem.* **256**, 1069.

Stevens, T. H., Bocian, D. D., and Chan, S. I. (1979a). *FEBS Lett.* **97**, 314.

Stevens, T. H., Brudvig, G. W., Bocian, D. F., and Chan, S. I. (1979b). *Proc. Natl. Acad. Sci. USA* **76**, 3320.

Thornstrom, P.-E., Soussi, B., Arvidsson, L., and Malmstrom, B. G. (1984). *Chem. Scr.* **24**, 230.

Tsubaki, M., Hiwatashi, A., and Ichikawa, Y. (1986). *Biochemistry* **25**, 3563.

Uno, T., Nishimura, Y., Tsuboi, M., Makino, R., Iizuka, T., and Ishimura, Y. (1987). *J. Biol. Chem.* **262**, 4549.

Varotsis, C., and Babcock, G. T. (1990). *Biochemistry* **29**, 7357.

Varotsis, C., Woodruff, W. H., and Babcock, G. T., (1989). *J. Am. Chem. Soc.* **111**, 6439.

Varotsis, C., Woodruff, W. H., and Babcock, G. T. (1990a). *J. Am. Chem. Soc.* **112**, 1297.

Varotsis, C., Woodruff, W. H., and Babcock, G. T. (1990b). *J. Biol. Chem.* **265**, 11131.

Wikstrom, M. (1981). *Proc. Natl. Acad. Sci. USA* **78**, 4051.

Wikstrom, M. (1987). *Chem. Scr.* **27B**, 53.

Wikstrom, M. (1988). *Chem. Scr.* **28A**, 71.

Wikstrom, M. (1989). *Nature (London)* **338**, 776.

Wikstrom, M., Krab, K., and Saraste, M. (1981). In *Cytochrome Oxidase: A Synthesis*, Academic Press, London.

Woodruff, W. H., Einarsdottir, O., Dyer, R. B., Bagley, K. A., Palmer, G., Atherton, S. J., Goldbeck, R. A., Dawes, T. D., and Kliger, D. S. (1991). *Proc. Natl. Acad. Sci. USA* **88**, 2588.

Yewey, G. L., and Caughey, W. S. (1988). *Ann. N.Y. Acad. Sci.* **550**, 22.

Yonetani, T. (1960). *J. Biol. Chem.* **235**, 845.

Yoshikawa, S., and Caughey, W. S. (1982). *J. Biol. Chem.* **257**, 412.

Yoshikawa, S., Choc, M. G., O'Toole, M. C., and Caughey, W. S. (1977). *J. Biol. Chem.* **252**, 5498.

Yu, N.-T., Kerr, E. A., Ward, B., and Chang, C. K. (1983). *Biochemistry* **22**, 4534.