pH-Dependent Structural Changes at the Heme-Copper Binuclear Center of Cytochrome *c* Oxidase

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ABSTRACT The resonance Raman spectra of the aa_3 cytochrome *c* oxidase from *Rhodobacter sphaeroides* reveal pH-dependent structural changes in the binuclear site at room temperature. The binuclear site, which is the catalytic center of the enzyme, possesses two conformations at neutral pH, assessed from their distinctly different Fe-CO stretching modes in the resonance Raman spectra of the CO complex of the fully reduced enzyme. The two conformations (α and β) interconvert reversibly in the pH 6–9 range with a pKa of 7.4, consistent with Fourier transform infrared spectroscopy measurements done at cryogenic temperatures (D. M. Mitchell, J. P. Shapleigh, A. M. Archer, J. O. Alben, and R. B. Gennis, 1996, *Biochemistry* 35:9446–9450). It is postulated that the different structures result from a change in the position of the Cu_B atom with respect to the CO due to the presence of one or more ionizable groups in the vicinity of the binuclear center. The conserved tyrosine residue (Tyr-288 in *R. sphaeroides,* Tyr-244 in the bovine enzyme) that is adjacent to the oxygen-binding pocket or one of the histidines that coordinate Cu_B are possible candidates. The existence of an equilibrium between the two conformers at physiological pH and room temperature suggests that the conformers may be functionally involved in enzymatic activity.

INTRODUCTION

Cytochrome *c* oxidase (CcO), the terminal enzyme complex of the respiratory chain, has a binuclear (heme a_3-Cu_B) catalytic site where molecular oxygen is reduced to water. Electrons provided by cytochrome *c* enter into the enzyme through a copper homo-dinuclear center, designated as Cu_A, and then move, via the heme *a* center, to the catalytic site of dioxygen reduction. The dioxygen reaction is coupled to proton translocation across the mitochondrial membrane in the mammalian system and across the plasma membrane in prokaryotes (Babcock and Wikstrom, 1992). A wealth of information is available on the structural and functional properties of terminal oxidases from crystal structure determinations and various spectroscopic and biochemical studies.

Despite many important advances in the understanding of terminal oxidases, the mechanism by which the electron transfer events are coupled to proton translocation is unknown. The heme a_3-Cu_B center, where dioxygen reduction occurs, is a very crucial site for proton gating as the proton translocation has been shown to couple to the steps in the oxygen reduction pathway (Verkhovsky et al., 1999). Furthermore, when the two phases of the enzymatic cycle are considered (the oxidative phase when the enzyme becomes oxidized during turnover and the reductive phase when the enzyme is re-reduced), it was shown that approximately half of the charge is translocated in each of the phases (Verkhovsky et al., 1999). Therefore, the redox energy has to be stored in the protein after the dioxygen chemistry is completed in the oxidative phase, requiring the existence of metastable conformations (Verkhovsky et al., 1999; Rousseau, 1999).

There are two distinct functional processes in cytochrome *c* oxidase involving protons. Four scalar protons are utilized in the dioxygen reduction process to generate two molecules of water. These protons are taken up from the matrix side of the inner mitochondrial membrane in the eukaryotes. In addition, four vectorial protons are pumped from the matrix to the cytosol side of the membrane for each oxygen molecule that is used. Consequently, there are sites in the protein that must be redox sensitive (Das et al., 1999; Behr et al., 2000, 1998; Yoshikawa et al., 1998; Das and Mazumdar, 1994; Osborne et al., 1999; Ralle et al., 1999; Wittung and Malmstrom, 1996) and that must undergo protonation/deprotonation events associated with the catalytic activity. During proton translocation, conformational changes are also required so that the vectorial protons will cross the membrane rather than slipping back to the side from which they originated. Additional conformational changes are required to store the energy at the termination of the oxidative phase for the subsequent release in the reductive phase (Rousseau, 1999). Given the complexity of these processes, it is essential to determine any effect of changes in protonation state of groups near the catalytic center. Such groups are likely to play essential roles in these functionally important conformational changes.

Recently, we have shown that the binuclear site of CcO from *R. sphaeroides* possesses multiple conformations at neutral pH, as judged from their distinctly different Fe-CO stretching modes in the resonance Raman spectra (Wang et al., 1995a). The carbonmonoxy derivative of heme proteins is an extremely useful probe for studying the environment of the heme proximity. With resonance Raman spectros-

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copy, the frequencies of the Fe-CO stretching mode and other CO-related modes, which are particularly sensitive to interactions with neighboring groups, can be measured. CcO from *R. sphaeroides* has been shown to possess two distinct Fe-CO stretching modes, at 520 and 493 cm^{-1}, which are ascribed to two conformations of the binuclear center (termed α and β, respectively) at neutral pH (Wang et al., 1995a). The C-O frequencies of these two conformations have been shown to be pH sensitive at cryogenic temperatures by Fourier transform infrared (FTIR) spectroscopy (Mitchell et al., 1996). It is important to understand the pH dependence of the two Fe-CO stretching modes at room temperature as they may serve as a useful tool for studying the nature of the catalytic center and the dynamic changes coupled to its chemical reactions. Furthermore, the origin of a high Fe-CO stretching frequency in CcO has never been well understood. The steric and/or electronic structure of the binuclear site that is responsible for the unique Fe-C-O modes in cytochrome *c* oxidase may provide useful information on the catalytic mechanism of the enzyme.

A pH-dependent study of the redox centers in CcO is very useful as there is ample evidence that the redox chemistry of the enzyme is sensitive to the pK of one or more residues. The electrochemical properties of the metal centers have been shown to be pH dependent (Moody and Rich, 1990). In addition, proton uptake or release is induced by electron transfer through the redox centers (Hallén and Nilsson, 1992; Mitchell and Rich, 1994; Oliveberg et al., 1991; Verkhovsky et al., 1995; Adelroth et al., 1995; Hallén et al., 1994). Hence it is certain that some ionizable groups whose pKa values are near physiological pH are involved in the process. Understanding the conformational transition that is associated with such protonation/deprotonation events would be extremely useful in deciphering a proton gating mechanism.

In the present work, we have investigated the pH-dependent changes in the heme-copper binuclear site of the CO derivative of fully reduced cytochrome aa_3 from *R. sphaeroides* by resonance Raman scattering in solution at room temperature. We have observed the equilibrium between the α- and β-conformers that undergoes a pH-induced reversible population change in the pH range of 6–9. Structural changes in the binuclear site, which are associated with ionizable groups, may play a role in either the proton gating or the dioxygen chemistry during the enzymatic reactions.

MATERIALS AND METHODS

Purification of cytochrome *c* oxidase

The oxidase from *R. sphaeroides* was purified by Ni^{2+}-NTA agarose affinity chromatography with a modified six-histidine tag from *R. sphaeroides* (Mitchell and Gennis, 1995). Phosphate buffer at pH 8.0 was used instead of Tris for the Ni-nitrilotriacetic acid (Ni-NTA) column chromatography. The samples were concentrated to ~100 μM using a Centricon 100 (Amicon, Beverly, MA) apparatus. The PD-10 salt exchange columns (Pharmacia Biotech, Piscataway, NJ) were used to remove the Ni-column eluent that was replaced by a buffer of either 10 mM phosphate or Tricine, pH 7.0, and 5% glycerol. The protein samples were frozen in liquid nitrogen for storage until use.

Resonance Raman measurements

The excitation source for the Raman experiments was the 413.1-nm line of a krypton ion laser (Spectra Physics, Mountain View, CA). The sample cell (quartz, 2 mm path length, sample volume ~150 μl) into which a laser beam was focused was spun at 3000–6000 rpm to minimize local heating. The sample cells were custom designed for 1) strict anaerobic measurements and 2) recording optical spectra (UV-2100U spectrophotometer, Shimadzu, Kyoto, Japan) of the same sample on which Raman spectra are recorded. The Raman scattered light was focused onto the entrance slit (100 μm) of a polychromator (Spex, Metuchen, NJ) and was dispersed with a 1200-grooves/mm grating before its detection by a liquid-nitrogen-cooled charged-couple-device camera (CCD) (Princeton Instruments, Trenton, NJ). Holographic notch filters (Kaiser, Ann Arbor, MI) and narrow-band filters were used to eliminate from the spectrum the intense Rayleigh scattering and laser fluorescence lines, respectively. Typically, several 60-s spectra were recorded and averaged after removal of cosmic ray spikes by a standard software routine (CSMA, Princeton Instruments, Princeton, NJ). The spectral slit width was 5 cm^{-1}. Frequency shifts in the Raman spectra were calibrated using acetone-CCl_4 (for the 100-1000 cm^{-1} region), indene (for the 100-1700 cm^{-1} region), or acetone-potassium ferrocyanide (for the 1700–2400 cm^{-1} region) as references. The accuracy of the Raman shifts is $\sim\pm1$ cm^{-1}.

The samples used for the Raman measurements had an enzyme concentration of ~50 μM and were placed in a desired buffer (pH 5–5.5, sodium acetate; pH 6.0–6.3, MES; pH 6.5–7.0, Bis-Tris; pH 7.2–7.8, HEPES or phosphate; pH 8.0–8.6, Tris; pH 9.0–9.5, CHES; pH 10.5–11.0, CAPS). Dithionite-reduced samples were exposed to CO in a tightly sealed Raman cell under anaerobic conditions to prepare the carbonmonoxy adduct. CO gas was obtained from Matheson (E. Rutherford, NJ) and isotopic CO ($^{13}C^{18}O$) was purchased from ICON (Mt. Marion, NY). Absorption spectra were recorded before and after the Raman measurements to ensure the stability of the CO adducts.

RESULTS

The resonance Raman spectra of the $^{12}C^{16}O$ derivative of *R. sphaeroides* CcO in the low-frequency region are shown in Fig. 1 as a function of pH. The lines at 494 and 517 cm^{-1} are assigned as Fe-CO stretching modes, as in the $^{13}C^{18}O$ adduct the lines shifted to 478 and 505 cm^{-1}, respectively (data not shown), the expected isotope shifts for Fe-CO stretching modes. The two Fe-CO stretching modes are assigned as originating from two different conformers (the 517-cm^{-1} line corresponds to the α-form and the 494-cm^{-1} line corresponds to the β-form) of the binuclear center (Wang et al., 1995a). Both Fe-CO stretching modes are observed in the pH 6–9 range. (Bovine CcO isolated from beef heart by the method of Yoshikawa et al. (1988) also showed pH dependence of the α- and β-conformers. However, the pH dependence was not reproducible in the bovine enzyme so no meaningful interpretation could be made. Similar observations of an irreproducible pH dependence in bovine oxidase (purified following Yoshikawa's method) were also made by Kitagawa and co-workers (personal communication with T. Kitagawa, Japan). We are currently

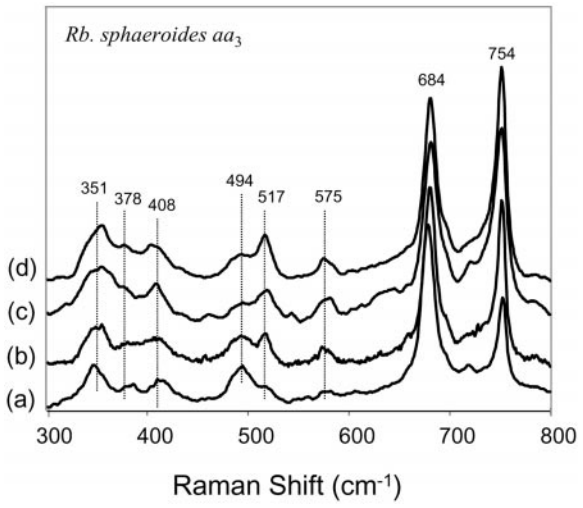

FIGURE 1 Low-frequency resonance Raman spectra of the CO derivative of the wild-type aa_3 cytochrome *c* oxidase from *R. sphaeroides* as a function of pH. (*a*) pH 6.0; (*b*) pH 7.0; (*c*) pH 8.3; (*d*) pH 9.0. Separate samples were made in the desired buffers for each pH value reported (see Materials and Methods) and were allowed to equilibrate for 2 min. The laser excitation wavelength was 413.1 nm.

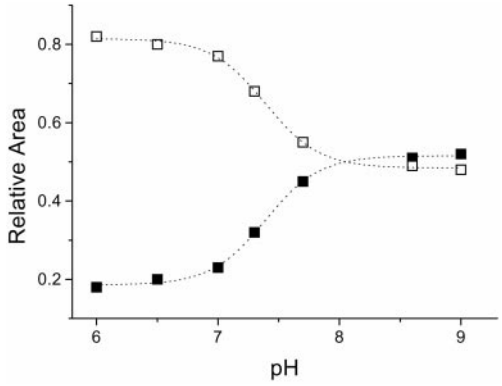

FIGURE 2 Titration profile of the two conformers (α and β) as a function of pH in the CO complex of the aa_3 cytochrome *c* oxidase from *R. sphaeroides*. The relative population of each conformer was calculated from the respective peak area.

investigating the factors that could be responsible for such anomalies.) However, their relative intensities vary as a function of pH (Fig. 1). At pH ~6, the line at 494 cm^{-1} is predominant whereas at pH ~9, the 517-cm^{-1} line dominates. At neutral pH both of these equilibrium conformers are present. At very high pH (>10.5), the enzyme converts irreversibly to a Schiff base (Han et al., 1991; Callahan and Babcock, 1983) and shows a single Fe-CO line at ~495 cm^{-1}, with virtually no line at 576 cm^{-1} (spectrum not shown). The Fe-CO frequency at highly alkaline pH is assigned to a δ-form of the enzyme that has a very poor catalytic activity (unpublished results). In contrast to the behavior of the stretching mode, only a single bending mode ($\delta_{Fe\text{-}C\text{-}O}$) has been detected at ~574 cm^{-1}. The α-conformer makes a strong contribution to the bending mode whereas the contribution to the bending mode from the β-form is very small.

The pH-dependent changes in the Fe-CO stretching modes observed here at room temperature correlate well with the pH-dependent changes in the C-O stretching frequencies observed by low-temperature FTIR in the CO derivative of aa_3 from *R. sphaeroides* reconstituted into vesicles (Mitchell et al., 1996). The relative intensities (peak area) of the two Fe-CO lines are shown in Fig. 2 as a function of pH. An apparent pKa of 7.4 (±0.1) was calculated from the titration curve, which is similar to that determined from FTIR studies on the pH dependence of C-O stretching frequency (Mitchell et al., 1996).

DISCUSSION

The presence of two distinct frequencies for the Fe-CO stretching mode in CcO from *R. sphaeroides* signifies two conformations (α and β) of the binuclear center. In the α-conformer, the Fe-CO stretching mode from the resonance Raman spectra is located at 517 cm^{-1}, and from the FTIR measurements (Mitchell et al., 1996) the C-O stretching mode is located at 1964 cm^{-1}, whereas in the β-conformer the two modes are located at 494 and 1947 cm^{-1}, respectively. The origin of a high frequency of the Fe-CO stretching mode in the α-conformation of CcO has been debated extensively (Wang et al., 1995a,b,c, 1993; Argade et al., 1984; Ray et al., 1994). The major anomaly is that the frequencies of the α-conformer do not fall on the well-recognized inverse correlation line between the Fe-CO and the C-O frequencies shown in Fig. 3. The unique position on the correlation line of the CcO frequencies in the α-conformation suggests an anomalous binding geometry of the histidine-Fe-CO complex (Argade et al., 1984). We consider both proximal and distal effects on the origin of the two conformations of the heme pocket.

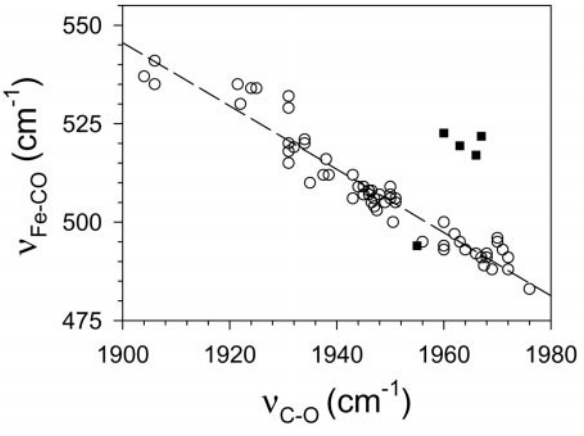

FIGURE 3 Correlation between Fe-CO ($\nu_{Fe\text{-}CO}$) and C-O ($\nu_{C\text{-}O}$) stretching frequencies for various heme proteins that have histidine as the proximal ligand. ○, stretching frequencies of globins and peroxidases; ■, frequencies of oxidase (from Wang et al., 1995b, and this work).

The frequencies of the Fe-C-O modes in the α-conformation of CcO lie close to those in cases where the proximal histidine bond is unusually weak. This led to a suggestion (Ray et al., 1994) that the behavior of the Fe-C-O modes may result from a weak Fe-His bond as CcO displays a low (213-cm^{-1}) Fe-His stretching frequency. However, this possibility may be ruled out by noting that many hemoglobins also have low frequencies for their Fe-His stretching modes but lie on the correlation curve. For example, a clam hemoglobin (*Scapharca inaequivalvis*) has a very low Fe-His stretching frequency ($\sim$200 cm^{-1}), yet its Fe-CO and C-O frequencies fall on the correlation line of the proximal histidine-containing heme proteins (Guarrera et al., 1998). Similarly, the Fe-C-O frequencies of a nematode hemoglobin (*Ascaris suum*) lie on the correlation curve, although the frequency of the proximal histidine stretching mode is very low (Das et al., 2000). In a study with the CO complex of the aa_3-type quinol oxidase from *Bacillus subtilis*, it was proposed that the Fe-CO frequency is controlled by H-bonding interactions with the proximal histidine (Varotsis and Vamvouka, 1998). It also has been argued that an inverse linear correlation exists between the strength of the Fe-His and the Fe-CO bonds in oxidases (Varotsis and Vamvouka, 1998). However, the Fe-His stretching frequency at $\sim$213 cm^{-1} in the bacterial aa_3 oxidase (in the ligand-free reduced form) does not show any significant pH-dependent change (data not shown) in the same pH range where the CO conformer shows a population change. This strongly suggests that the strength of the Fe-His bond has no direct control on the Fe-CO stretching frequency in our case. In the cbb_3 oxidase, a low Fe-CO stretching frequency ($\sim$495 cm^{-1}) is very unlikely to originate from an imidazolate character of the proximal histidine, as was suggested (Varotsis and Vamvouka, 1998), because many peroxidases (Dasgupta et al., 1989; Uno et al., 1987; Mylrajan et al., 1990; Kitagawa, 1988; Spiro et al., 1990; Hu et al., 1993) that display an imidazolate nature of their proximal histidine show both the Fe-His and Fe-CO stretching modes at high frequencies ($\sim$235–270 cm^{-1} and $\sim$530–548 cm^{-1}, respectively). Alternatively, the low Fe-CO frequency in cbb_3 has been proposed to be due to a less tightly structured binuclear center (open pocket) because of the absence of the farnesyl chain that otherwise provides stabilization to the binuclear center (Wang et al., 1995c).

As the behavior of the Fe-C-O modes in the two conformations cannot be attributed to proximal effects, we consider distal interactions. The electrostatic interactions between heme-bound CO and polar residues in the distal pocket have been extensively studied and reasonably well understood. Specifically, polar interactions between residues in the distal pocket and the bound CO modulate the π-electrons of the Fe-C-O moiety resulting in an inverse correlation between the Fe-CO and C-O stretching modes (Fig. 3). However, contributions from steric effects are less well characterized. Large steric factors are anticipated in the cytochrome *c* oxidase case because the distance between the iron atom of heme a_3 and Cu_B is very short. As discussed below, these factors could cause either the bonds to be contracted and/or the Fe-C-O moiety to adopt a bent structure. These changes affect the π^* electron density in both the Fe-C and the C-O bonds as well as the normal mode composition because with a bent structure the stretching and bending modes become mixed. Under such conditions the Fe-C versus the C-O frequency correlation would be expected to break down.

We attribute the two conformations to a difference in the Fea_3-Cu_B distance. The crystal structure of the CO-bound form of the bovine enzyme (Yoshikawa et al., 1998) shows that the Fe-C-O unit is bent at an angle of 146° (See Fig. 4 *A*). Simulations were carried out to determine how much the Fe-Cu distance has to change to affect the Fe-C-O angle. In these simulations the position of Cu_B was varied and the energy was minimized by allowing the position of the CO to vary using the CHARMm force field. The positions of the other atoms were held fixed. The results of the simulation at various Fe$\cdots$Cu distances are presented in Fig. 4 *B*. It is evident from this figure that when the Fe$\cdots$Cu distance is significantly increased, the Fe-CO unit becomes almost upright. A movement of Cu_B 0.7 Å away from the CO oxygen results in an increase of $\sim$35° in the Fe-C-O bending angle. Such a variation in distance is within that observed in the various crystal structures. In the various structures reported the Fe-Cu distance ranges from 4.5 to 5.3 Å (Tsukihara et al., 1995, 1996; Yoshikawa et al., 1998; Iwata et al., 1995; Ostermeier et al., 1997; Harrenga and Michel, 1999). In the absence of copper, the simulation results in a nearly linear structure of Fe-CO. In summary, these results show that the Fe-CO unit is indeed bent, and the bending angle is a sensitive function of the Fe-Cu_B distance. This supports the hypothesis that steric factors may govern the Fe-CO and C-O frequencies in oxidases.

The α- and β-conformers have the C-O frequencies at 1964 and 1947 cm^{-1}, respectively (Mitchell et al., 1996) and Fe-CO frequencies of 517 and 494 cm^{-1}, respectively. Consequently, the α-conformer lies off the Fe-CO versus the C-O correlation curve whereas the β-conformer lies on the curve (Fig. 3). Due to the steric constraints in the binuclear center resulting from Cu_B, in the α-conformer the Fe-C-O moiety in CcO is significantly bent and possibly the Fe-C bond is compressed. Steric factors would be expected to compress the low-energy Fe-C bond ($\sim$500 cm^{-1}) more than the high-energy C-O bond ($\sim$1950 cm^{-1}). The resulting increase in the interaction of the electronic orbitals between Fe and C causes an increase of the Fe-C bond order, but this does not lead to a simultaneous decrease of the C-O bond order. In addition, the change in the normal mode structure due to the bent Fe-C-O moiety mixes the bending and the stretching modes, thereby resulting in the strong bending mode that we observe in the α-conformer.

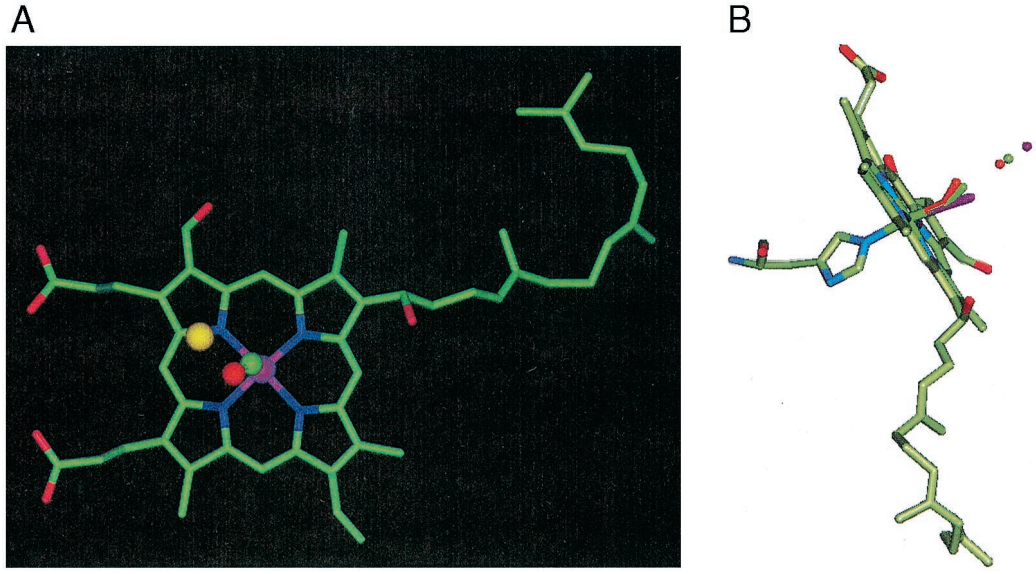

FIGURE 4 (*A*) Structure of CO-bound heme a_3 in bovine CcO (1oco.pdb). Color-coding is as follows: iron (pink), carbon (green), oxygen (red), nitrogen (blue), and Cu_B (yellow). (*B*) Simulation of the effect of the Fe-Cu distance on the bending angle of the Fe-C-O moiety. The best orientation of the heme a_3 bound CO was calculated by energy minimization using CHARMm (MSI) of a CO adduct of the structure reported by Tsukihara et al. (1995). The view shown is taken along the heme a_3 plane. Three orientations of the Fe-CO unit are shown for three Fe· · ·Cu distances. In a given orientation, CO (stick) and Cu_B (sphere) are shown in the same color. As the Fe· · ·Cu distance changes from 4.7 Å to 6.0 Å, the Fe-C-O angle changes from 143° to an orientation nearly perpendicular (178°) to the heme.

The structural changes that modulate the position of the copper with respect to the CO are coupled to protonation/deprotonation events of one or more residues. In the ligand-free ferrous form of the protein no pH-dependent changes were detected in the Fe-histidine stretching mode. In addition, in the CO-bound form of the enzyme only the Fe-C-O modes have a pH dependence; no variation was detected in the heme a_3 modes. Thus, we conclude that there is no global pH-dependent structural change, but only a local change close to the catalytic site. The residues most likely to affect the Cu_B position are the histidines that are coordinated to it and Tyr-244 (bovine numbering). The tyrosine residue has been shown to be covalently linked to His-240, a Cu_B ligand, in the bovine oxidase (Yoshikawa et al., 1998) as well as in the oxidase from *P. denitrificans* (Ostermeier et al., 1997), a result of post-translational modification. His-290 and His-291 are the other Cu_B ligands. Based upon redox-induced changes seen in the nearby heme a_3 formyl group, His-290 has been proposed to undergo protonation/deprotonation events (Das et al., 1999).

It is generally believed that Tyr-244 is critically involved in the catalytic functioning of CcO owing to its close proximity to the heme a_3-Cu_B center. Evidence supporting its role as supplying a redox equivalent during its catalytic cycle was recently reported (Proshlyakov et al., 2000). Mutation of this tyrosine to a nonpolar residue (Phe) produces an inactive enzyme and also results in a collapse of the heme-copper binuclear site. Thus, the post-translational modification in the wild-type enzyme is necessary to establish a functional catalytic site (Das et al., 1998). However, the role of Tyr-244 in proton gating/transfer is not known. The hydroxyl group of Tyr-244 is located close enough to form a hydrogen bond with a diatomic ligand (CO or O_2) bound to Fea_3 (Yoshikawa et al., 1998). The pKa of such post-translationally modified tyrosine-OH protonation would be expected to be less than that for unmodified tyrosine (Yoshikawa et al., 1998; McCauley et al., 2000). Indeed, in a model compound study of a phenol bound to an imidazole, the pKa of the phenol was reduced by 1.5 pH units to 8.6 (McCauley et al., 2000). An additional reduction could be expected when the imidazole was coordinated to a copper atom as in the oxidase. Hence, it is possible that the observed pKa of 7.4 that is related to the interconversion between two conformers of the binuclear site is coupled to the protonation event of Tyr-244. Its covalent linkage to His-240 could affect the position of the Cu_B atom, especially as it is established that the absence of Tyr at this position completely destabilizes the binuclear center. On the other hand, the proximity of the reduced heme to the tyrosine could serve to counteract the pKa reduction. Therefore, other possibilities should be considered.

The other candidates with labile protons that could affect the position of Cu_B are the remaining two histidine ligands to the copper. (His-240 is excluded because it is covalently linked through its nitrogen to Tyr-244.) Recently, based on resonance Raman scattering experiments in an aa_3 quinol oxidase from an acidophilic archaeon, Das et al. (1999) reported that there are changes in the heme a_3 formyl C-O

stretching mode upon heme reduction that indicated a change in H-bonding to the formyl group. The closest residue to the formyl oxygen atom is His-290, one of the Cu_B ligands. The Raman experiments suggest that the proton on the His-290 is very labile so it is a possible candidate for the pH-dependent changes reported here. Changes of the protonation state of this histidine could modulate the position of Cu_B. Lability of the ligands to the copper has been demonstrated in the cytochrome bo_3 quinol oxidase from *Escherichia coli*, also a member of the oxidase superfamily. In two independent Cu x-ray absorption spectroscopy studies it was found that in the reduced enzyme one of the histidine ligands was either significantly weakened (Ralle et al., 1999) or lost (Osborne et al., 1999) in comparison with the oxidized state. Additional structural changes occurred when CO was coordinated to the Cu (Ralle et al., 1999). Thus, it is possible that the chemical environment could alter the coordination of the copper and concomitantly the Fe-Cu distance.

CONCLUSIONS

The observation of pH-dependent conformational changes in the catalytic center of CcO is a step toward the identification of groups that may be operational in proton gating or oxygen protonation in the heme-copper oxidases. The presence of equilibrium populations of both α- and β-conformers at physiological pH suggests that related conformers may play a role in the catalytic cycle of the enzyme. What that role is remains to be determined. However, it is tempting to speculate that a change in the protonation state of an ionizable group in the vicinity of the binuclear site may be functioning as a proton switch during the enzymatic cycle. Indeed, His-290 has already been proposed to play such a role (Das et al. 1999). On the other hand, the proximity of Tyr-244 to the oxygen-binding site makes it plausible that it may undergo deprotonation events that are associated with the delivery of protons for the dioxygen reduction chemistry.

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