A pH-Dependent Polarity Change at the Binuclear Center of Reduced Cytochrome *c* Oxidase Detected by FTIR Difference Spectroscopy of the CO Adduct†

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ABSTRACT: A pH-dependent polarity change at the heme−copper binuclear center of the aa_3-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* has been identified by low-temperature FTIR difference spectroscopy. "Light"-minus-"dark" FTIR difference spectra of the fully reduced CO−enzyme adduct were recorded at a range of pH, and the dominance of different populations of bound CO, α and β, was found to vary with pH. An apparent pK_a of about 7.3 for the transition was obtained. The α and β forms are differentiated by different polarities at the heme−copper binuclear center of the enzyme, sensed by the stretching frequencies of CO bound either to the heme a_3 Fe or to Cu_B. Several site-directed mutants in the vicinity of the heme−copper center are shown to favor either the α or the β forms of the enyzme, suggesting that what is being monitored is an equilibrium between two conformations of the reduced form of the oxidase. Recent resonance Raman evidence has been presented demonstrating that the α and β forms of the *R. sphaeroides* oxidase exist at room temperature; therefore, the pH-dependent change in the polarity in the vicinity of the heme−copper center may be functionally significant.

Cytochrome *c* oxidase is an integral membrane metalloenzyme which contains four metal centers: two copper centers (Cu_A,Cu_B) and two iron porphyrins (heme *a*, heme a_3). Cytochrome *c* transfers electrons, via Cu_A and heme *a*, to a binuclear center consisting of heme a_3 and Cu_B, where dioxygen is reduced to water. Reduction of dioxygen is coupled to the translocation of one H^+ per electron across the lipid bilayer, creating a pH and voltage gradient across the membrane. The mechanism by which electron transfer within cytochrome oxidase is coupled to proton movement through the enzyme is still largely unknown. However, the recently reported structures of the oxidases from bovine (Tsukihara et al., 1995) and from *Paracoccus denitrificans* (Iwata et al., 1995) will greatly facilitate efforts to determine the catalytic mechanism.

Several authors have suggested that distinct redox-sensitive conformational states of the enzyme must exist in order to regulate proton input and output during active proton translocation (Chan & Li, 1990; Iwata et al., 1995; Larsen et al., 1992; Wikström, 1981; Wikström et al., 1994). This idea has led to several proposed mechanisms of proton−electron coupling, each linked to conformational changes near one of the metal centers (Chan & Li, 1990; Iwata et al., 1995; Rousseau et al., 1993; Wikström et al., 1994; Woodruff, 1993). Current opinion favors the heme−copper binuclear center as the most probable site of coupling (Iwata et al., 1995; Wikström et al., 1994).

Experimentally, there is ample evidence for groups whose pK_a values are coupled to the redox state of the metal centers of the enzyme. Such evidence comes from the pH dependence of the electrochemical properties of the metal centers (Moody & Rich, 1990), from the measured proton uptake accompanying reduction of the heme−copper center (Hallén & Nilsson, 1992; Mitchell & Rich, 1994; Oliveberg et al., 1991; Verkhovsky et al., 1995), and from proton release accompanying the reversed electron transfer from heme a_3 to heme *a* (Ädelroth et al., 1995; Hallén et al., 1994). Clearly, protonation/deprotonation events coupled to the chemistry occurring at the heme−copper center must be central to understanding how this enzyme functions (Iwata et al., 1995). Hence, it is to be expected that the properties of the heme−copper binuclear center may be sensitive to pH in the range of the pK_a values of groups coupled to or near the metal centers. pH-dependent changes in the characteristics of the fully oxidized form of the oxidase are well-known (e.g., slow/fast states) (Baker & Palmer, 1987; Brown et al., 1993; Moody et al., 1991; Palmer et al., 1987; Papadopoulos et al., 1991), though there is little understanding of the nature of these pH-induced changes in terms of the structure, or their relevance to catalysis. In the current study, a pH-dependent transition that affects the polarity in the vicinity of both the heme a_3 Fe and Cu_B is demonstrated using Fourier transform infrared (FTIR)[1] spectroscopy of CO ligated to the fully reduced oxidase.

It has been demonstrated that, at cryogenic temperatures, CO bound to heme a_3 in fully reduced cytochrome oxidase

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[1] Abbreviations: FTIR, Fourier transform infrared; CO, carbon monoxide; PCR, polymerase chain reaction; MES, 2-(*N*-morpholino)-ethanesulfonic acid; BIS-TRIS, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane or 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TRIS, tris(hydroxymethyl)aminomethane; CHES,2-(*N*-cyclohexylamino)ethanesulfonic acid.

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can be photodissociated to form a stable Cu_B−CO adduct (Alben et al., 1981; Fiamingo et al., 1986). This phenomenon allows "light"-minus-"dark" difference spectra to be obtained in which both heme a_3 Fe−CO and Cu_B−CO stretching modes (ν_{C-O}) are clearly observed. In both mammalian and *R. sphaeroides* cytochrome *c* oxidases, multiple populations of CO bound to the fully reduced enzyme, referred to as α and β forms, have been identified (Fiamingo et al., 1986; Shapleigh et al., 1992a). Relaxation rates of the Cu_B−CO adduct back to heme a_3 following photodissociation previously demonstrated coupling between heme a_3 Fe−CO and Cu_B−CO in α or β forms (Fiamingo et al., 1986). The α form of Cu_B−CO (2061 cm^{-1}) relaxes to the α form of heme a_3 Fe−CO (1964 cm^{-1}), and the β form of Cu_B−CO (2037 cm^{-1}) relaxes to the β form of heme a_3 Fe−CO (1947 cm^{-1}). The differences in the stretching frequencies of CO reflect the polarity of the environment sensed by CO in each case, and clearly the polarities near both the heme a_3 Fe and Cu_B are equally influenced by the structural differences of the α and β forms of the oxidase. In the current study, it is shown that the α and β forms interconvert in a pH-dependent equilibrium. Furthermore, the α/β ratio at any given pH is altered by point mutations in the vicinity of the heme−copper center, suggesting that the local changes on polarity sensed at the binuclear center may be coupled to a more global protein conformational change.

MATERIALS AND METHODS

Sample Preparation. Wild-type and mutant cytochrome *c* oxidase, modified by a six-histidine affinity tag, was purified from *R. sphaeroides*, as described previously (Mitchell & Gennis, 1995). Purified enzyme, in 5% cholate, was reconstituted into proteoliposomes by extended dialysis in the presence of 40 mg/mL soybean phospholipids (asolectin) in 50 mM buffer at a range of pH (see Figure 1 for details). At pH 5.5 and below, proteoliposome preparations were unsuccessful, resulting in precipitation of lipids; hence, no data were collected below pH 6.0. The dialyzed samples were reduced with dithionite anaerobically in the presence of CO. The proteoliposomes were collected by centrifugation at 320000*g* for 2 h, and overlayed with CO-saturated glycerol for extraction of residual water. The extracted proteoliposome pellets were then pressed between two CaF_2 windows at a thickness of 40 μm for spectral analysis. The formation of the reduced CO complex was verified by observing a shift in the Soret peak in the visible spectrum to around 434 nm, as well as a broadening of the α band toward 590 nm. The mutants Thr-359-Ala, Phe-391-Gln, Gly-398-Ala, and Tyr-422-Phe were purified and reconstituted into proteoliposomes as described above whereas the mutants Pro-358-Ala, Phe-387-Ala, and Phe-420-Val were examined in cytoplasmic membrane preparations as previously described (Shapleigh et al., 1992a). More detailed studies of these mutants will be described separately.

FTIR Spectroscopy. FTIR spectroscopy was performed as previously described (Shapleigh et al., 1992a) using 0.5 cm^{-1} resolution and the average of 512 forward scans and 512 reverse scans without any further manipulation of the data.

Site-Directed Mutagenesis. Site-directed mutants were constructed using two separate methods. Pro-358-Ala, Thr-359-Ala, and Phe-420-Val were constructed by extension of a single mutagenic oligonucleotide on a single-stranded DNA template (Shapleigh et al., 1992b; Vandeyar et al., 1988). Phe-387-Ala, Phe-391-Gln, Gly-398-Ala, and Tyr-422-Phe were constructed using the two-step PCR method described by Landt et al. (1990). All mutations were verified by DNA sequencing.

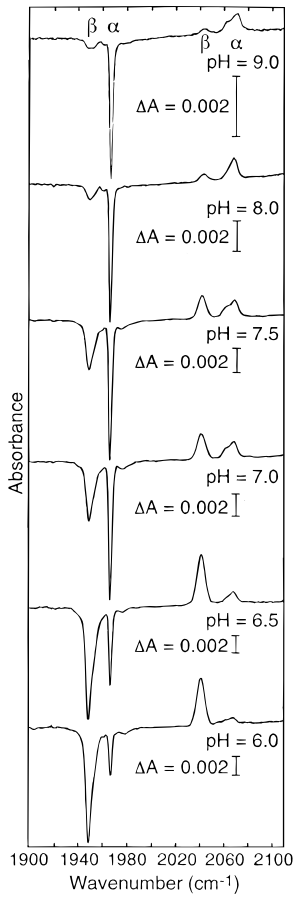

FIGURE 1: FTIR "light"-minus-"dark" difference spectra of fully-reduced enzyme−CO adduct after reconstitution of purified enzyme into phospholipid vesicles over a range of pH values. All spectra were recorded at 12 K, except that at pH 8.0, which was recorded at 35 K. For this reason, the band associated with the α-Cu_B C−O at pH 8.0 exhibits less structure. Buffers used were as follows: pH 6.0, MES; pH 6.5 and pH 7.0, BIS-TRIS; pH 7.5, HEPES; pH 8.0, TRIS; and pH 9.0, CHES. The positive and negative peaks represent the Cu_B C−O and Fe_{a3} C−O stretching frequencies, respectively. The spectra represent the average of 512 forward scans and 512 reverse scans, recorded at 0.5 cm^{-1} resolution.

RESULTS AND DISCUSSION

FTIR "light"-minus-"dark" difference spectra of purified cytochrome *c* oxidase reconstituted into phospholipid vesicles at pH 6.0, 6.5, 7.0, 7.5, 8.0, and 9.0 are shown in Figure 1. The spectra demonstrate clearly a pH-dependent shift between α (1964, 2061 cm^{-1}) and β (1947, 2037 cm^{-1}) forms. Band area ratios for heme a_3 Fe−CO and Cu_B−CO yield similar results, indicating that both metal centers are equally affected by the titration. Note that the extinction coefficients of the α and β forms may not be identical. An apparent pK_a of about 7.3 was obtained for the transition (Figure 2). This is an operationally defined pK_a value and reflects the pH of the samples at 4 °C prior to concentration and cooling. Furthermore, the fit of the data to a single titrating residue is far from perfect, perhaps reflecting the

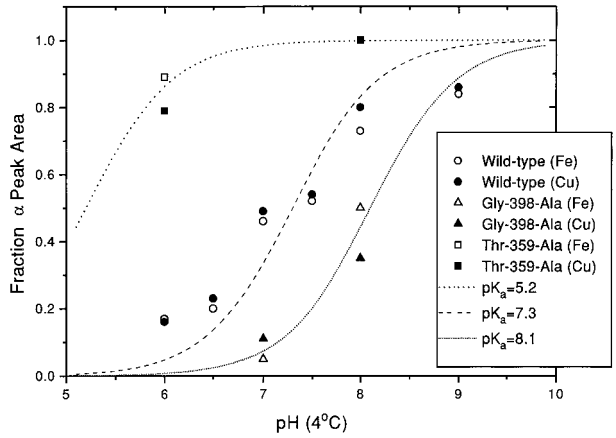

FIGURE 2: Plot of the pH dependence of α and β forms of the Cu_B and Fe_{a3} C−O stretching modes for wild-type cytochrome oxidase and the mutants Thr-359-Ala and Gly-398-Ala. Peak areas were obtained by cutting out and weighing peaks manually. The pK_a of each of the data sets was approximated with a Henderson−Hasselbach titration of a single protonatable group.

involvement of more than one group being titrated. This will require further study. The main point is that at pH 8.0 the oxidase is predominantly in the α form, whereas at pH 6.0 the enzyme is predominantly in the β form. The above results clearly identify two distinct, pH-dependent states (α and β) of the CO-bound binuclear center. It should also be noted that there is a clear splitting of the α-Cu_B C−O peak in all spectra recorded at 12 K, indicating additional heterogeneity at this site. This splitting does not, however, appear to change with pH.

In addition to the wild-type *R. sphaeroides* oxidase, several site-directed point mutants have been examined. The resulting spectra are presented in Figure 3. Peak positions and turnover numbers for all mutants are summarized in Table 1. In panels A and B of Figure 3, the spectra of Thr-359-Ala and Gly-398-Ala are presented at two representative pH values. The Thr-359-Ala mutant is partially active (20−35% of wild-type rate), and this residue is within one of the proton-conducting channels leading to the heme−copper center (Iwata et al., 1995). Thr-359 is located below the hydroxyl group of the hydroxyethylfarnesyl side chain of heme a_3, and is postulated to facilitate proton transfer to the oxygenated species during turnover (Iwata et al., 1995). The Thr-359-Ala mutant at pH 8.0 is almost entirely in the α form, and this predominance of the α form persists even at pH 6.0 where the wild-type oxidase is mostly in the β form. Hence, this mutation shifts the equilibrium in favor of the α form of the oxidase with an approximate pK of 5.2 (Figure 2). The opposite pattern is observed for Gly-398-Ala, which at pH 7.0 is predominantly in the β form, and shifts the apparent pK to around 8.1 (Figure 2). In the structure of the *Paracoccus* oxidase (Iwata et al., 1995), the residue corresponding to Gly-398 is near heme a_3.

It is also clear that both mutants demonstrate unique band splittings when compared to wild type. For Thr-359-Ala, the pH 8.0 sample has a strongly split α-Cu_B C−O band, and the spectra of Gly-398-Ala show a clear splitting of both β peaks. These changes suggest that the mutations have introduced more subtle heterogeneity at the active site, in addition to shifting the pK of the α/β transition.

Preliminary results from five additional mutants which alter the α/β states are also presented in Figure 3 (panels C and D). The mutants in panel C, Tyr-422-Phe and Phe-391-Gln, were studied using purified enzyme, as in the previous spectra. Tyr-422-Phe shows an increased degree of the β-form at pH 8.0 relative to wild type as well as a splitting of the β-Cu_B signal. In contrast, Phe-391-Gln lacks any indication of the α-form, and it demonstrates a sharply split α-Cu_B signal.

The spectra in panel D, Phe-387-Ala, Phe-420-Val, and Pro-358-Ala, were obtained from inner membrane preparations. It is important to note that the inner membranes of

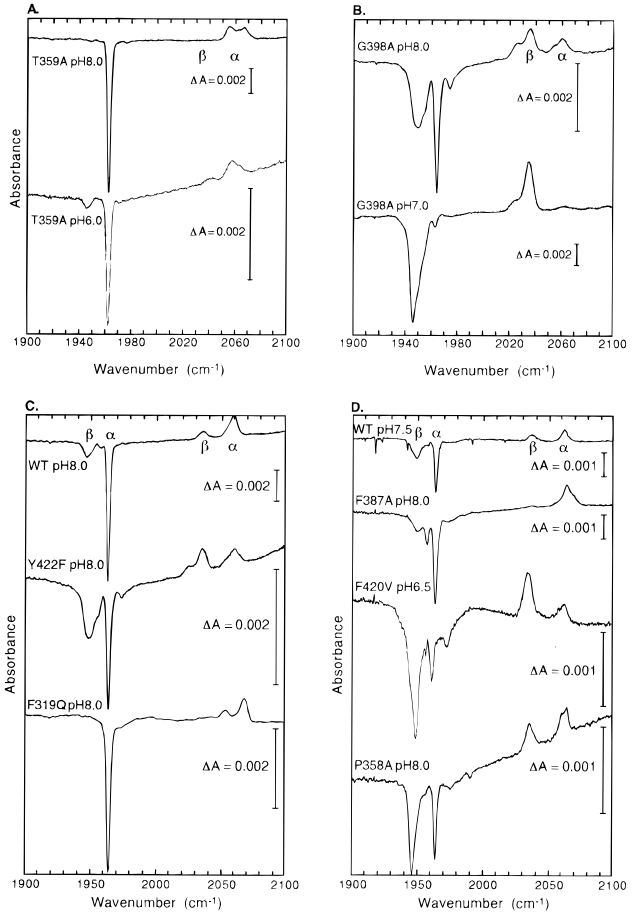

FIGURE 3: FTIR "light"-minus-"dark" difference spectra of fully-reduced enzyme−CO adducts of Thr-359-Ala and Gly-398-Ala cytochrome oxidase mutants reconstituted into proteoliposomes at the indicated pH. Panel A, Thr-359-Ala proteoliposomes at pH 6.0, MES, and pH 8.0, TRIS; panel B, Gly-398-Ala proteoliposomes at pH 8.0, TRIS, and pH 6.5, BIS-TRIS; panel C, wild-type, Tyr-422-Phe, and Phe-391-Gln proteoliposomes at pH 8.0, TRIS; Panel D, wildtype, Phe-387-Ala, Phe-420-Val, and Pro-358-Ala inner membrane samples at the indicated pH. The spectra represent the average of 512 forwards scans and 512 reverse scans, recorded at 0.5 cm^{-1} resolution. Spectra were recorded at 12 K.

Table 1: Summary of the ν_{C-O} Frequencies (cm^{-1}) Observed for Mutants Which Alter the pK_a of the Transition between α and β Conformers, as Well as Their Turnover Numbers

mutant	α(Fe)	β(Fe)	α(Cu)	β(Cu)	turnover (s^{-1})
wild type	1963.5	1947.0	2062.5/∼2056	2036.0	1500
P358A	1964.5	1947.0	∼2062	2037	600
T359A	1963.0	1946.0	2055/2067	∼2041	250
F387A	1964.0	1950.0	2064.5	∼2037	NA
F391Q	1963.0	NA	2053/2065.0	NA	250
G398A	1964.0	1947.5	∼2061	2034.5/2025	1500
F420V	1963.0	1949	∼2064	2036	1100
Y422F	1963.5	1948.0	∼2061	2035/2026	600

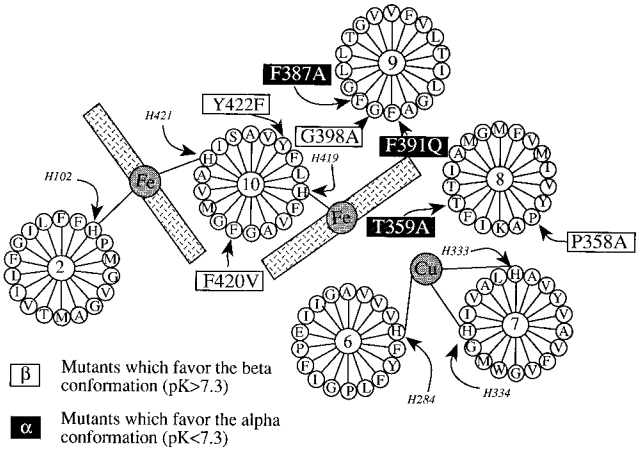

FIGURE 4: Helical wheel diagram of a portion of subunit I showing the positions of residues serving as metal ligands (small italics) and the relative positions of mutations which affect the pK_a of the α/β transition. Mutants which decrease the apparent pK_a are shown in white, whereas mutants which increase the apparent pK_a are shown in black.

R. sphaeroides contain an additional cytochrome *c* oxidase, cytochrome *cbb*$_3$, which is present at relatively low levels in aerobically grown cultures (Garcia-Horsman et al., 1994). This alternate oxidase also has a heme−copper binuclear center and demonstrates ν_{C-O} frequencies at 1950 cm^{-1} and 2065 cm^{-1} in light-minus-dark FTIR spectra. Spectroscopic contributions of cytochrome *cbb*$_3$ can be seen in the spectrum of Phe-387-Ala: a negative peak at 1950 cm^{-1} and shoulder at 2065 cm^{-1}. It is also important to note that the ratio of α and β forms observed in inner membrane samples is less responsive to changes in pH than in purified samples. Thus, although the spectrum of Phe-420-Val at pH 6.5 in inner membranes shows an α/β ratio similar to that of the purified enzyme at this pH, a wild-type inner membrane sample prepared at pH 6.5 exhibits less β form than does the purified enzyme (not shown). Therefore, the Phe-420-Val mutant is classified as favoring the β-form. Further, Pro-358-Ala also favors the β form, whereas Phe-387-Ala lacks the β form completely. With over 30 mutants examined using inner membranes and several examined in both purified and inner membrane states, the deviations in the α/β ratios of the mutants presented are reproducible and qualitatively correct, although further study is warranted. The discrepancy between inner membrane samples and purified enzyme reconstituted into proteoliposomes is perhaps due to differences in sample preparation. The proteoliposome samples were prepared by 24-h dialysis at the pH of interest, whereas inner membrane samples were homogenized in buffer at the indicated pH prior to reducing and forming the CO adduct.

A summary of the effects of all mutants that alter the α/β ratio is shown in Figure 4. The results demonstrate that numerous mutants shift the α/β ratio, and these mutants are widely distributed around the heme−copper center and are in several different transmembrane spans. Although there is no clear correlation between the location of the mutations and the direction of the α/β shift, it is important to note that for the majority of examples the center frequencies and band shapes of the α and β forms are consistent throughout this set of mutants, and primarily the α/β ratio changes. Hence, the conformational differences that manifest themselves as a difference in the polarity at the heme−copper center appear to be widespread and involve the interactions of numerous residues in the protein.

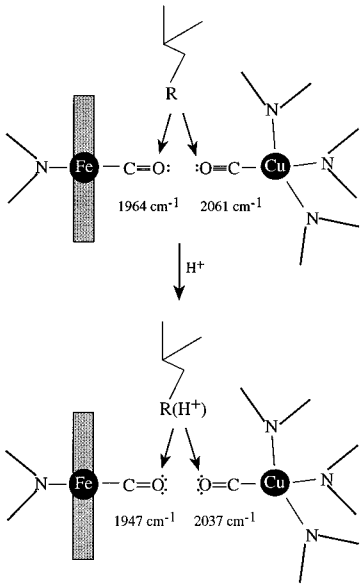

FIGURE 5: Schematic model of possible pH-dependent interactions with CO at the binuclear center that could cause the shift between α and β forms. The position of the protonatable group (R), the specific interaction with CO, and the geometry of CO bound to the metals are drawn for illustrative purposes only. Electron pairs, represented by dots on the oxygen atom, are shown to redistribute in response to a change in the polarity of the pocket, decreasing the bond order and the stretching frequency of CO. Note that only one molecule of CO is present at the binuclear center at a given time bound either to the heme a_3 Fe or to Cu$_B$, although two are shown in the figure.

Both α and β populations of the heme a_3 Fe−CO adduct have recently been observed at room temperature using resonance Raman spectroscopy of the *R. sphaeroides* oxidase (Wang et al., 1995), indicating that these two states of the enzyme do exist at physiological temperatures and are not an artifact of low temperature. In addition, the resonance Raman analysis (Wang et al., 1995) suggests that in the α form the CO in the distal pocket of heme a_3 is conformationally constrained. This is consistent with the extremely narrow bandwidth of the heme a_3 Fe−CO (2.5 cm^{-1}) observed in FTIR, which represents a highly ordered CO population. It was suggested (Wang et al., 1995) that in the α form Cu$_B$ may be closer to heme a_3, resulting in conformational constraints on bound CO, whereas in the β form Cu$_B$ may be further away from heme a_3 as a result of a conformational change, resulting in a larger bandwidth. The current work extends this conclusion by indicating that this conformational equilibrium is pH-dependent, and affects the Cu$_B$−CO adduct as well as the heme a_3 Fe−CO adduct.

That variations in pH affect equally the heme a_3 Fe−CO and Cu$_B$−CO states indicates that the α/β transition is the result of a local polarity change influencing both heme a_3 and Cu$_B$. The simplest model of this transition would involve the protonation, at low pH, of an amino acid that interacts with CO on both metals. The protonated residue would withdraw electrons from the C−O bond, decreasing its bond order, and shifting its stretching frequency to lower frequencies, as is the case for the β form. A schematic model of this interaction is presented in Figure 5. Studies of pH-dependent shifts in the C−O stretching frequencies in myoglobin−CO and leghemoglobin−CO complexes have led to similar considerations of polarity effects within the heme pocket as well as global protein dynamics (Fuchsman & Appleby, 1979; Shimada & Caughey, 1982).

Such a protonatable residue that can interact with CO bound to either heme a_3 or Cu_B would be undoubtedly near enough to influence the reaction of oxygen at the same site. In examining the structure of the active site for possible residues performing this role, the highly-conserved Tyr-288 appears as a reasonable candidate. The two crystal structures of the cytochrome oxidase indicate that Tyr-288 is hydrogen-bonded to the hydroxy group of the hydroxyethylfarnesyl group of heme a_3 in the oxidized form of the enzyme. FTIR spectra recorded for the mutant Tyr-288-Phe show a severely disrupted binuclear center. Alternatively, one of the histidine ligands to Cu_B could be involved. Indeed, it has been suggested that one of the Cu_B ligands, His-333 (*R. sphaeroides* numbering), might become dissociated from Cu_B and become protonated during the catalytic cycle as part of the proton pumping mechanism (Iwata et al., 1995). Perhaps the conformational change, due to protonation of this histidine ligand, is related to the α/β transition.

It is worth pointing out that such a strong pH dependence of the C−O stretching frequency was not observed in a previous FTIR investigation of cytochrome oxidase from bovine heart (Einarsdóttir et al., 1988). In addition, the homologous cytochrome bo_3 quinol oxidase has only been observed in the α form (Hill et al., 1992). More detailed studies of these and other heme−copper oxidases will be required to determine if the structural features which cause the pH dependence observed in the *R. sphaeroides* oxidases are conserved among different species of cytochrome oxidase.

In summary, this work reports for the first time a pH-dependent conformational change in cytochrome *c* oxidase in the fully reduced form of the protein bound to CO which significantly alters the polarity at the heme−copper center. Clearly, the protonation of one or more groups in the enzyme is coupled to changes in the physical state of the reduced form of the heme−copper center. The apparent pK_a of this pH-induced transition is 7.3 under the conditions of these experiments. It will be particularly interesting to pursue the nature of this α/β transition at room temperature by resonance Raman spectroscopy and to identify the group(s) being titrated that is (are) responsible for these observations.

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