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Review

FTIR studies of internal proton transfer reactions linked to inter-heme electron transfer in bovine cytochrome *c* oxidase

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Abstract

FTIR difference spectroscopy is used to reveal changes in the internal structure and amino acid protonation states of bovine cytochrome *c* oxidase (CcO) that occur upon photolysis of the CO adduct of the two-electron reduced (mixed valence, MV) and four-electron reduced (fully reduced, FR) forms of the enzyme. FTIR difference spectra were obtained in D_2O (pH 6–9.3) between the MV-CO adduct (heme a_3 and Cu_B reduced; heme a and Cu_A oxidized) and a photostationary state in which the MV-CO enzyme is photodissociated under constant illumination. In the photostationary state, part of the enzyme population has heme a_3 oxidized and heme a reduced. In MV-CO, the frequency of the stretch mode of CO bound to ferrous heme a_3 decreases from 1965.3 cm^{-1} at pH* ≤ 7 to 1963.7 cm^{-1} at pH* 9.3. In the CO adduct of the fully reduced enzyme (FR-CO), the CO stretching frequency is observed at 1963.46 ± 0.05 cm^{-1}, independent of pH. This indicates that in MV-CO there is a group proximal to heme a that deprotonates with a pK_a of about 8.3, but that remains protonated over the entire pH* range 6–9.3 in FR-CO. The pK_a of this group is therefore strongly coupled to the redox state of heme a. Following photodissociation of CO from heme a_3 in MV oxidases, the extent of electron transfer from heme a_3 to heme a shows a pH-dependent phase between pH 7 and 9, and a pH-independent phase at all pH's. The FTIR difference spectrum resulting from photolysis of MV-CO exhibits vibrational features of the protein backbone and side chains associated with (1) the loss of CO by the a_3 heme in the absence of electron transfer, (2) the pH-independent phase of the electron transfer, and (3) the pH-dependent phase of the electron transfer. Many infrared features change intensity or frequency during both electron transfer phases and thus appear as positive or negative features in the difference spectra. In particular, a negative band at 1735 cm^{-1} and a positive band at 1412 cm^{-1} are consistent with the deprotonation of the acidic residue E242. Positive features at 1552 and 1661 cm^{-1} are due to amide backbone modes. Other positive and negative features between 1600 and 1700 cm^{-1} are consistent with redox-induced shifts in heme formyl vibrations, and the redox-linked protonation of an arginine residue, accompanying electron transfer from heme a_3 to heme a. An arginine could be the residue responsible for the pH-dependent shift in the carbonyl frequency of MV-CO. Specific possibilities as to the functional significance of these observations are discussed.

Keywords: FTIR; Cytochrome oxidase; Glutamate; Mixed-valence

1. Introduction

The heme-copper oxidases are the terminal enzymes of the respiratory electron transfer chain and, by catalyzing the reduction of O_2 to H_2O, account a substantial fraction of the free energy made available by aerobic respiration [1,2]. The oxidases conserve this free energy by creating a proton electrochemical gradient (proton motive force) across the membrane in which they reside, in part by active transmem-

Abbreviations: CcO, cytochrome *c* oxidase; FR, fully reduced cytochrome *c* oxidase; MV, mixed valence (two-electron reduced) cytochrome *c* oxidase; MV-CO and FR-CO, refer to the carbon monoxide adducts with ferrous heme a_3 in the MV and FR forms of the enzyme, respectively; pH*, refers to the reading of the pH meter using D_2O buffer

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brane proton translocation. The microscopic mechanism of how the proton movements are coupled to electron transfer events and to the chemistry of dioxygen reduction at the enzyme active site is a key issue in bioenergetics. The atomic structures of several oxidases have been determined [3–11]. Although these structures provide essential information, additional experimental information is required to understand how changes in the redox state of the enzyme couple to changes in the protonation state of individual groups within the enzyme.

FTIR difference spectroscopy is a valuable method in this endeavor because it directly probes changes in the structure and functional state of the protein at the level of individual residues [12,13]. This technique is particularly valuable to reveal changes in the protonation state of acidic residues (aspartates or glutamates) [14–19]. In the specific case of the heme-copper oxidases difference spectra have provided information on changes in the protein upon CO photolysis from the fully reduced oxidase [12,13,20–23], from photolysis of CO from the two-electron (mixed valence) form of the enzyme [22] and upon reducing the fully oxidized enzyme [17,24–33]. Of particular interest are FTIR signals that originate from changes in a highly conserved, buried glutamate in subunit I of the oxidases (E242, bovine oxidase numbering). E242 is about 10 Å from the heme a_3-Cu_B binuclear center, which is the site where O_2 is reduced. This residue is at the upper (interior) end of the "D channel", a key element of the enzyme's proton translocation apparatus. The D channel starts with a conserved aspartate (D91) near the protein surface, about 25 Å from E242 [4,9,11]. The X-ray structures reveal more than 10 water molecules forming a pathway within the D channel that may facilitate proton transport from the mitochondrial matrix (or bacterial cytoplasm) to the heme-copper active site via E242. Mutagenesis studies show that E242, or its equivalent, is essential for enzyme function, and is involved in supplying both chemical protons (used to make H_2O at the active site) and pumped protons (transported across the membrane) during the steps in the catalytic cycle after oxygen has bound to the enzyme [34–37]. In some species variants, E242 (or an analogous E residue) is absent, but the functional role is played by other groups that have potentially labile protons and which occupy the same spatial location in the three-dimensional structure [38,39]. The critical feature appears to be that there is protonatable group with a high pK_a at this location in the enzyme [40,41]. FTIR difference spectroscopy of the *E. coli* and *R. sphaeroides* oxidases has shown that the residue equivalent to E242 is protonated in the fully reduced form of the enzyme [12,29], with a pK_a ≥ 9.5 [29].

It has been suggested that E242 becomes transiently deprotonated and reprotonated during the catalytic cycle [12]. In the current study, it is demonstrated by FTIR difference spectroscopy that a carboxylic acid residue, arguably E242, undergoes pH-dependent deprotonation concomitant with electron transfer from heme a_3 to heme a. This "reverse electron transfer", so-called because the direction of electron transfer is the reverse of that which occurs during catalytic turnover, is obtained upon photolysis of the CO adduct of the two-electron (mixed valence) form of the oxidase (MV-CO).

In MV-CO, the heme a_3-Cu_B binuclear center is reduced while the other two metal redox centers in the enzyme, heme a and Cu_A, remain oxidized. CO binding to heme a_3 substantially raises the midpoint potential of the heme, thus stabilizing the reduced form of heme a_3. Photolysis of CO from heme a_3 in the MV-CO form of the enzyme results in a redistribution of the electrons among the four redox centers due to the lower midpoint potential of the unligated form of heme a_3. This electron redistribution has been examined previously by UV–Vis spectroscopy and been shown to be pH-dependent [42–46]. At all pH's, a rapid electron transfer phase is observed, with a characteristic time of about 3 μs [42–46] (an additional, faster pH-independent electron transfer phase has also been suggested [47] resulting in reduction of heme a in about 15% of the enzyme population). However, at pH values above 7 a slower (ca. millisecond) phase of electron transfer appears, the extent and rate of which are both pH-dependent [46]. This slow phase is accompanied by the release of a proton into the bulk solution from the enzyme. At pH 9, this accounts for an additional ca. 20% of the bovine enzyme. The simplest interpretation of the pH dependence of the reverse electron transfer suggests that moving an electron from heme a_3 to heme a causes the shift in the pK_a of a group from 9.7 to 8.5 in the bovine oxidase [43]. Hence, the FTIR difference spectrum resulting from photolysis of the MV-CO enzyme at pH ≤ 7 can be ascribed to a combination of CO dissociation from reduced heme a_3 (simple photolysis) and to the fast phase(s) of electron transfer not coupled to proton release into the bulk solution. Presumably, at pH ≤ 7 the group that releases the proton into the bulk solution is protonated both prior to and following photolysis and electron transfer. The FTIR difference spectrum at more alkaline pH values (pH>7), should show features that reflect the process coupled to the millisecond phase of proton-coupled electron transfer. Presumably, a pH-dependent subpopulation exists, comprising up to 20% of the total enzyme, in which the group is protonated prior to photolysis and becomes deprotonated in a rate-limiting step coupled to electron transfer to heme a.

The current work was motivated to obtain information from FTIR spectroscopy about structural changes in the bovine oxidase which accompany the pH-dependent phase of reverse electron transfer. The FTIR difference spectra were obtained by generating a photostationary state in which the MV-CO enzyme was under continuous laser illumination. Under these conditions, a steady state is established that is dependent on the intensity of illumination, the redox equilibria and kinetics, and the kinetics of CO rebinding. In this steady state a significant portion of the population has reduced heme a and oxidized heme a_3. Under

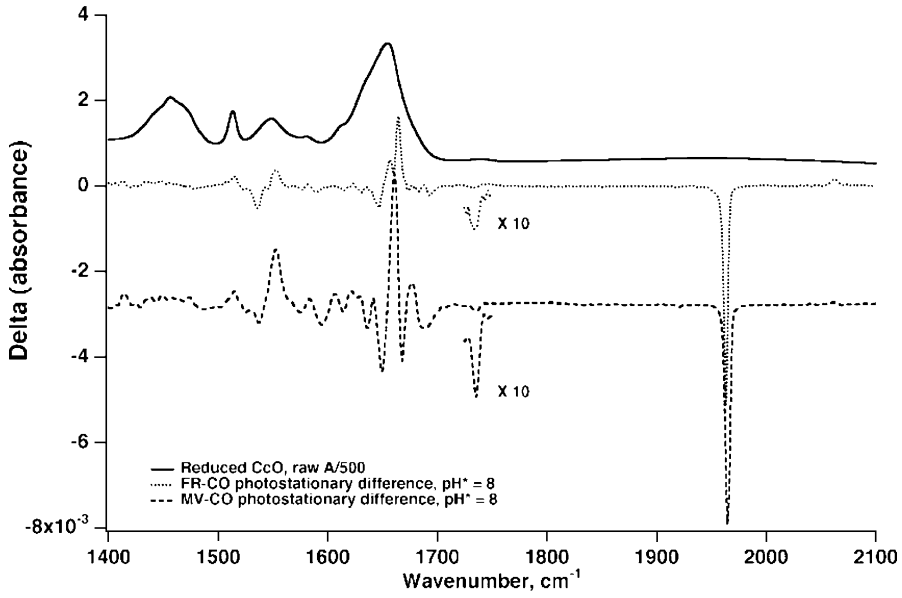

Fig. 1. Top trace: the raw FTIR absorbance spectrum of reduced bovine CcO, 300 μM, pathlength 25 μm; absorbance divided by 500 to scale with the difference spectra. Middle trace: the photostationary FTIR difference spectrum of FR-CO CcO (see text) at pH*= 8. Bottom trace: the photostationary FTIR difference spectrum of MV-CO at pH*= 8.

continuous illumination it is also likely that Cu_B will be oxidized in a portion of the enzyme [48]. At alkaline pH, the steady state population will also include the portion of the enzyme in which the electron transfer from the binuclear center to heme a is coupled to proton transfer and net deprotonation into the bulk solution [43,46].

The FTIR spectra (see Figs. 1 and 2) have four noteworthy characteristics. (1) The frequency of the absorption of CO bound to ferrous heme a_3 *prior to photodissociation* is pH-dependent (1965.3 cm^{-1} at low pH to 1963.7 cm^{-1} at high pH) with an apparent pK_a of 8.3; (2) a band at 1735 cm^{-1} decreases, and one at 1412 cm^{-1} increases, in intensity upon CO photodissociation, and further with the extent of the pH-dependent phase of the reverse electron transfer; (3) Amide I and II vibrations at 1661 and 1552 cm^{-1}, plus a series of peaks between 1600 and 1700 cm^{-1} increase in parallel with the extent of reverse electron transfer; and (4) most peaks in the difference spectra exhibit changes (particularly increases) in infrared transition moments in concert with the changes in intraprotein electric fields that accompany electron transfer. While definitive evidence for the identities of the specific residues responsible for the IR features requires mutant or isotope labeling studies [12,13], qualitative assignments can often be made with good reliability. Moreover, the data clearly show that reverse electron transfer changes the pK_a values of several residues, one of which is most plausibly E242.

2. Materials and methods

The bovine oxidase was purified as described by Palmer et al. [49], exchanged with D_2O, and prepared in a weak pH* 8 buffer. The MV state was prepared by exposure of the anaerobic enzyme to CO, and then the pH* was adjusted with 100 mM buffer (MES, HEPES, AMPSO, for pH* below 6.6, from 7 to 8, and above 8, respectively). 100 mM K_2SO_4 was added below pH* 7 to prevent aggregation of the enzyme. The FR state was prepared by reduction of half of the MV sample with minimal dithionite. The final enzyme concentration was 300 μM. The MV and FR samples were loaded onto the same split sample cell, between CaF_2 windows held 25 μm apart by Teflon spacers. UV–Vis spectra were acquired to ensure that the samples were reduced as required.

The sample cell was mated to a thermostatted brass block and equilibrated to 4 °C for 15 min before data were acquired using a Perkin-Elmer 1760x spectrometer. Typical acquisition runs lasted for 30 min, alternating between dark and illumination every 30 s. The photostationary state was created using either 440 nm HeCd, all-lines Ar^+ (458–514 nm), or single-line (514 nm) Ar^+ laser illumination, adjusted to provide 80% photolysis in the steady state as judged by the bleaching of the infrared absorbance due to the heme-bound C–O stretching vibration (approx. 80 mW). The minimal-power, multi-mode, Ar^+ illumination generally produced the maximal change in the Amide I region for a given Fe-CO bleach, because the other illumination regimes caused some photoreduction beyond the two-electron reduced MV state. When photoreduction occurred it was evident in the infrared spectra and the data were discarded. UV–Vis spectra also were acquired on each sample before and after each IR measurement to confirm that the samples had remained in the MV form. The sample prepared at pH* 9.3 showed a 20% reduction of heme a, easily

observed both as a shift in the Soret band of heme a and in the appearance of the IR difference spectra.

3. Results

Fig. 1 shows photostationary FTIR difference spectra of bovine cytochrome *c* oxidase (CcO) in D_2O generated by photolysis of the CO adducts of the fully reduced enzyme (FR-CO) and of the mixed valence enzyme (MV-CO) at pH* 8. The unsubtracted (raw) absorbance spectrum of the reduced enzyme, divided by 500, is included to show the major absorbance features and their absolute intensities relative to the features in the difference spectra. As we have demonstrated previously [12,13], IR peaks due to individual chemical bonds and discrete structures within this membrane protein of molecular weight ca. 200 kDa are easily discernible in the difference spectra. Fig. 2 compares the difference spectra of MV-CO obtained at pH* 6, pH* 7 and pH* 8, and also shows the double-difference spectrum pH* 8 minus 6. In each figure, the spectra have been normalized to the magnitude of the absorption band, ν(CO), from heme a_3 Fe-CO near 1960 cm^{-1}. This normalization quantifies the amount of enzyme from which CO has been photodissociated from the heme in the photostationary steady state. Fig. 1 shows the large differences between the difference spectrum of the FR enzyme, where CO is lost by heme a_3 but no electron transfer occurs, and that of MV where CO photodissociation initiates reverse electron transfer. Fig. 2 shows that the difference spectrum of the MV enzyme is strongly pH-dependent with the size of the changes of the Amide I band (1661 cm^{-1}) approximately doubling between pH* 6 and pH* 8. This correlates with the approximate doubling of the extent of reverse electron transfer observed at the more alkaline conditions [46,48] relative to pH* 7. The FTIR difference spectrum from the FR-CO enzyme, by comparison, is virtually independent of pH over the same range of pH*.

Fig. 3 shows the dependence of the frequency of the ν(CO) absorption band upon pH in the difference spectra of the FR and MV enzyme at pH* values between 6.3 and 9.3. As can be seen, the position of this band shifts to lower frequency in the MV enzyme as the pH* is increased, but that of the FR enzyme is essentially constant. The CO frequency of MV shifts from about 1965.3 cm^{-1} at pH* between 6.3 and 7 to 1963.7 cm^{-1} at pH* 9.3. It is critical to realize that, in both FR and MV, the Fe(C–O) frequency observed is due to CO bound to ferrous heme a_3 in the dark, before laser irradiation and any subsequent photoinduced chemistry. Thus any spectroscopic shifts represent changes in the environment sensed by the CO bound to the a_3 heme *prior to photolysis or electron transfer*. The frequency shift observed for MV-CO can be roughly fitted to the deprotonation of a single residue with a $pK_a \approx 8.3$. In contrast, the frequency of the ν(CO) band from FR-CO is independent of pH, with an average frequency of 1963.46 ± 0.05 cm^{-1}, which is approximately the same as the alkaline limit of the MV-CO frequency. Before photolysis, the heme a_3-bound CO is, to a first approximation, in an identical environment (Fe_{a3}^{2+}, Cu_B^+) whether the enzyme is in the FR (Fe_a^{2+}) or MV (Fe_a^{3+}) state. Apparently, oxidation of the heme a in the MV enzyme (Fe_a^{3+}) causes a residue near heme a to exhibit a pK_a^* near 8.3, while the pK_a^* of this residue in the FR (Fe_a^{2+}) enzyme is such that it does not undergo a change in

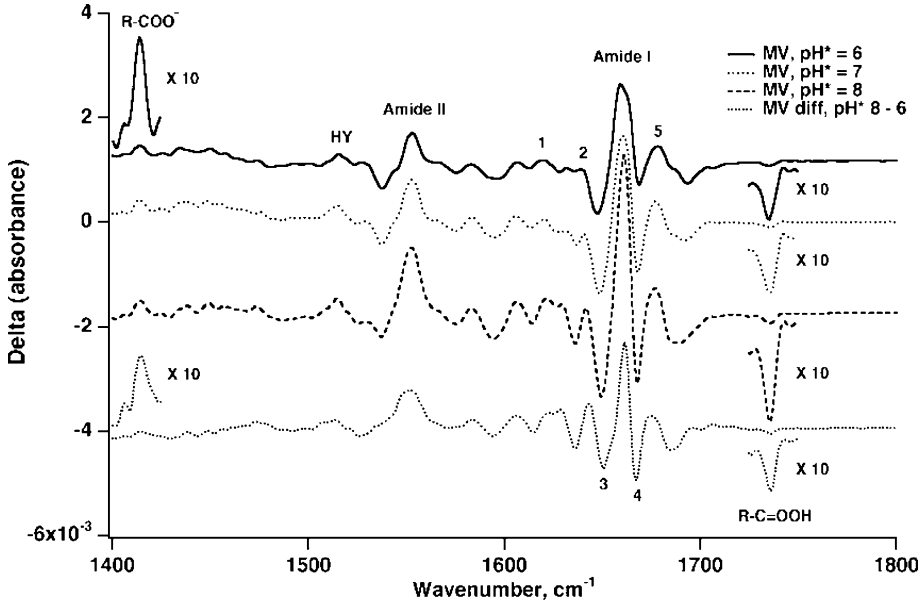

Fig. 2. Top three traces: photostationary FTIR difference spectra resulting from photolysis of CO from MV-CO CcO at pH* 6 (top trace), 7 (second trace from top), and 8 (third trace from top). Bottom trace: the double difference FTIR spectrum, photostationary spectrum at pH*=8 minus that at pH*=6.

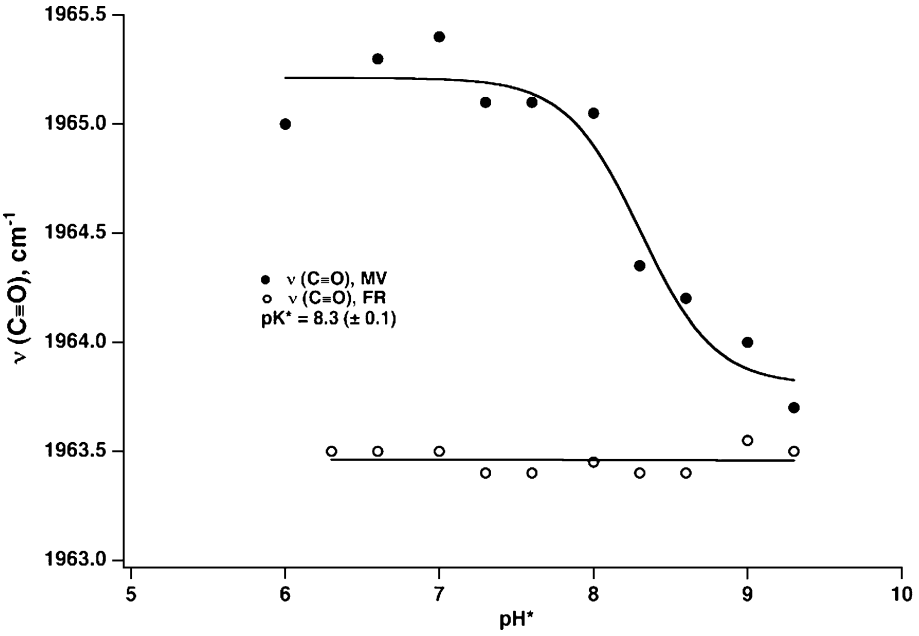

Fig. 3. Dependence upon pH* of the frequency of the FTIR band due to the stretching vibration of CO bound to ferrous heme a_3 before photodissociation of MV-CO and FR-CO CcO (see text).

protonation state over the pH range examined. Furthermore, the effects of the protonation state of this residue are reflected in a small shift in the CO stretching frequency of the remote Fe^{2+}_{a3}-CO moiety.

The FTIR difference spectra also reveal changes upon photolysis of both MV-CO and FR-CO that are due to the protein's polypeptide backbone and side chains, and also to the heme prosthetic groups. These are shown in Figs. 1 and 2. The changes in the FR-CO spectra have been discussed previously [20]. In MV-CO, even at pH* ≤ 7, the magnitudes of the changes in the Amide I (1661 cm^{-1}) and Amide II (1552 cm^{-1}) regions of the spectrum are larger than in FR-CO (Fig. 1). This reflects the fact that, in addition to the simple photolysis, at least 15% of the enzyme undergoes electron transfer from heme a_3 to heme a at pH ≤ 7 [43,45]. The positive Amide II feature may be shifted from the negative peak at ca. 1537 cm^{-1}, seen in both FR and MV with approximately the same intensity, which is also within the Amide II envelope (Fig. 1). The fact that the absorbances of the Amide I band at 1661 cm^{-1} and the Amide II band at 1552 cm^{-1} change in parallel as the pH is increased (Fig. 2) suggests that changes in the protein backbone conformation occur accompanying electron transfer. The intensities of the Amide I and II difference peaks compared to other identifiable amino acid residues (vide infra) suggest that several peptide bonds are within the perturbed structure [50]. Furthermore, the relative intensities of the Amide I and II peaks are near the 2:1 ratio expected for alpha-helical secondary structure [14], although the Amide I frequency is some 10 cm^{-1} higher than expected suggesting that the helix is in a low-dielectric environment [51]. It is also important to note that the Amide II components that are perturbed belong to peptide bonds that are protonated (frequencies ca. 1550 cm^{-1}) rather than exchanged for deuterium by exposure to D_2O solvent (Amide II′ frequencies ca. 1450 cm^{-1}; see Fig. 1). This demonstrates that the polypeptide backbone perturbations upon electron transfer occur within parts of the protein that are buried in hydrophobic regions.

Protonated forms of aliphatic carboxylic acids show the carbonyl stretch of their COOH group in the 1700–1800 cm^{-1} range, while deprotonated carboxylates have IR-active vibrations of the COO^- group near 1560 cm^{-1} (asymmetric stretch) and 1410 cm^{-1} (symmetric stretch) [14]. Accordingly, the negative peak in the difference spectra (Fig. 2) at 1735 cm^{-1} clearly belongs to a protonated carboxylic acid side chain (aspartic or glutamic acid) and the positive peak at 1412 cm^{-1} can be ascribed to deprotonated carboxylate. The carboxylate feature expected near 1560 cm^{-1} is at least partially masked by the stronger Amide II feature. In the FR-CO bovine enzyme, the 1735 cm^{-1} feature has been attributed to a perturbation resulting in a change of intensity or frequency of the protonated form of E242, by analogy to definitive assignments in the bacterial enzymes [12,25]. Deprotonation is excluded in the FR case because no concomitant positive carboxylate peaks are observed. At pH* ≤ 7 in the MV spectra (Fig. 2), the change in the 1735 cm^{-1} intensity is only slightly greater than that observed in the spectrum from the FR-CO enzyme under the same conditions (Fig. 1). In MV-CO, however, a positive peak of comparable intensity appears at 1412 cm^{-1}, suggesting that E242 is at least partially deprotonated during the 3 μs, pH-independent phase of electron transfer. Because of uncertainties as to the values

of intraprotein IR extinction coefficients compared to those measured in water [14], it has not previously been possible to quantitate accurately the fraction of deprotonation of E242 from the infrared intensity changes. However, we have measured infrared transition moments of monomeric carboxylic acids in nonaqueous solvents, mimicking the protein environment, and the results show that the peak intensities at 1412 and 1735 cm^{-1} (normalized to the CO peak intensity change and transition moment) [52,53] change by approximately the same percentage as the extent of the 3 μs electron transfer, ca. 15%.

Previous studies have shown that at pH 9, an additional ca. 20% of the bovine enzyme undergoes internal electron transfer on the time scale of milliseconds [43] and that this additional electron transfer is coupled to proton release on the same time scale [43,44,46]. As expected, therefore, the FTIR difference spectrum of the MV enzyme is strongly pH-dependent (Fig. 2). Comparing the spectra at pH* 8 with that at pH* 7 shows that the negative band at 1735 cm^{-1} approximately doubles in magnitude, as does the peak at 1412 cm^{-1}. This observation is consistent with additional deprotonation of E242 during the millisecond phase of electron transfer, again with approximately the same percent deprotonation as the percent electron transfer from heme a_3 to heme a.

One other feature in the difference spectra can be assigned by inspection. The peak at 1515 cm^{-1} is characteristic in frequency and intensity of the most intense IR-active ring mode of protonated tyrosine (labeled HY in Fig. 2) [14]. This peak also appears in the FR difference spectrum in Fig. 1, which suggests that it must be due to a tyrosine close to the heme a_3/Cu_B binuclear site. The only tyrosine in the bovine enzyme that meets this criterion is Y244, which is covalently bonded via its epsilon-carbon to the delta-nitrogen of H240, a histidine that is one of the ligands of Cu_B. Thus it appears that many of the IR observables in the difference spectra, including at least some of the amide intensity, arise from the "macrocyclic" turn of helix at the binuclear heme-copper site, comprising residues H240 to Y244. It is also noteworthy that the intensity of the Y244 peak seems to respond to CO photodissociation but not to electron transfer. Its intensity is approximately the same in the FR and MV spectra, and virtually independent of pH.

In Fig. 2, a set of positive and negative peaks are numbered 1 through 5. Tentative assignments for these features can be suggested. In the FR spectrum (Fig. 1), the sharp band at 1664 cm^{-1} in FR is at the frequency expected for the formyl group of ferrous heme a_3-CO [20]. However, following electron transfer (i.e. in all the MV spectra) the amount of reduced heme a_3 is diminished, which should result in a negative band in the MV spectrum. This may be observed as feature 4 in Fig. 2, the trough at 1667 cm^{-1}, which could be shifted to higher frequency by overlap with the strong positive Amide I peak at 1661 cm^{-1}. When the a_3 heme is oxidized, its formyl frequency shifts to ca. 1676 cm^{-1}. This should appear as a positive peak after electron transfer. Feature 5 in Fig. 2 appears between 1675 and 1678 cm^{-1}, and therefore is a good candidate for the formyl mode of ferric heme a_3. However, variability of the apparent frequency of this feature among the MV spectra suggests that the observed peak may have a contributor other than formyl, at a slightly different frequency (vide infra).

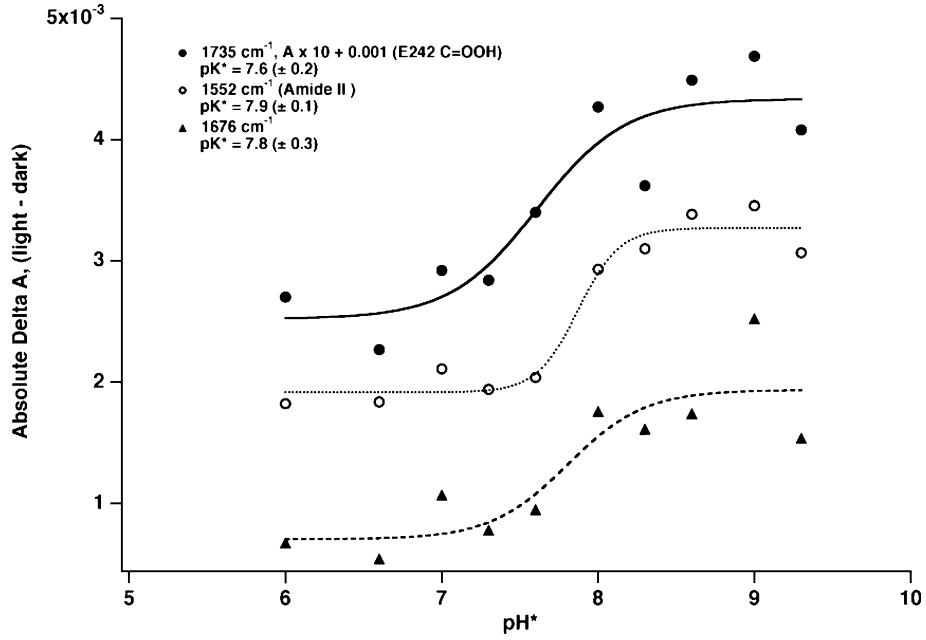

Fig. 4. Dependence upon pH* of the absolute magnitude of features in the photostationary difference spectra of MV-CO CcO: (top trace) the negative band at 1735 cm^{-1} assigned to E242 (multiplied by 10 to scale with the other traces); (middle trace) the Amide II peak at 1552 cm^{-1}; (bottom trace) the peak at 1676 cm^{-1}, see text for discussion of possible assignments.

The heme a formyl frequencies are considerably lower than those of heme a_3, because of hydrogen bonding of the heme a formyl [54,55] to R38 (in bovine CcO). Resonance Raman studies [55,56] show that the oxidized heme a formyl vibration should appear near 1650 cm^{-1} and that reduction should shift the frequency to ca. 1620 cm^{-1}. Upon reverse electron transfer, resulting in reduction of heme a, the difference spectra should show a positive feature near 1620 cm^{-1} and a negative one near 1650 cm^{-1}. In the MV difference spectra in Fig. 2, the positive feature 1 is at 1619 cm^{-1} and the negative feature 3 is at 1650 cm^{-1}. These are tentatively assigned as the formyl modes of reduced and oxidized heme a, respectively.

Two peaks in the 1600–1700 cm^{-1} range may also correspond to absorptions expected from the guanidinium side chain of protonated arginine [57–59]. The frequencies expected of these modes are quite medium-dependent, but in H_2O solution are generally between 1630 and 1640 cm^{-1} and between 1670 and 1680 cm^{-1} [58]. In D_2O solution, however, these modes (which involve large contributions from proton motions) shift to ca. 1580 and 1605 cm^{-1}. In Fig. 2, peak 2 is at 1642 cm^{-1} and peak 5, as previously stated, is at ca. 1676 cm^{-1} (and may consist of two overlapping peaks). These might be ascribed to a protonated arginine close to heme a, which protonates to conserve electroneutrality as heme a is reduced (see Discussion). For this to be possible, however, the proton transfer in question would have to be between groups buried within the protein and unavailable for H/D exchange with solvent D_2O.

The pH dependence of the intensities of the absorption changes of the E242 feature at 1735 cm^{-1}, the peak at 1676 cm^{-1}, and the Amide II band at 1552 cm^{-1} are shown in Fig. 4. All three curves have low-pH* and high-pH* limiting values with midpoints near pH* 7.8.

4. Discussion

The FTIR difference spectra provide information about the changes at protonatable (and other) residues that are sensitive to the electron distribution in the enzyme. Since the oxidase function is critically dependent on the timely movement of protons, both to the active site and through the pumping pathway, this information approaches the heart of the mechanism. The current work is the first to use FTIR to examine the pH-induced phase of the reverse electron transfer which follows photolysis of the MV-CO form of the oxidase. Several important questions are raised by these data and their possible interpretations.

4.1. Does E242 deprotonation accompany the pH-dependent phase of reverse electron transfer?

The trough observed at 1735 cm^{-1} is assigned to E242 based on previous observations [12,25]. The COOH group of E242 has been shown by FTIR difference spectroscopy to be perturbed upon full reduction of the bovine oxidase and upon photolysis of the FR-CO form of the enzyme [21,29,31]. Studies with mutants of bacterial oxidases have definitively shown this critical glutamic acid is the origin of the spectroscopic changes in the FTIR redox difference spectra and in the photolysis difference spectra of the FR-CO enzymes [12,24,25,29]. It is noted that photolysis of the FR-CO oxidase results in perturbation to the E242 environment in some oxidases but not in others [22]. Full reduction of the oxidase does not result in deprotonation of E242 (or the equivalent residues in bacterial oxidases) but cause a shift in the absorption peak due to the COOH group. This indicates a change in local conformation which influences the hydrogen bonding of E242 that is observed in the difference spectrum as a bi-lobed feature with equal positive and negative components. This is what is observed, for example, in the spectroscopic perturbations of E242 with the photolysis of the FR-CO bovine oxidase [21,29]. In contrast, observations presented in the current work are most readily ascribed to deprotonation ($COOH \rightarrow COO^-$) of E242. An alternative argument could be that there is a change in the environment of E242 resulting in a lower extinction coefficient and, therefore, the loss of absorbance upon reverse electron transfer, but this does not account for the appearance of the positive carboxylate peak at 1412 cm^{-1}, which is missing in the FR spectra.

Photolysis of the MV-CO form of the *P. denitrificans* oxidase at pH 7 has previously been studied by rapid scan time-resolved FTIR difference spectroscopy [22,60]. It was concluded that the electron transfer from the binuclear center to heme a is coupled to a reorganization of E278 (equivalent to bovine E242). Assignment of the spectroscopic perturbation at 1746 cm^{-1} was definitively made by comparison with the E278Q mutant. Changes in the E278 conformation, proton position, or loss of the proton were all considered as possible explanations. It is important to note that the studies on the *P. denitrificans* enzyme were not extended to alkaline pH, and would not capture changes related to the pH-dependent phase of reverse electron transfer.

Photolysis of the MV-CO form of the *R. sphaeroides* oxidase has been recently studied by rapid-scan FTIR difference spectroscopy at both pH 6.5 and 8.5 [61]. The spectra of the bacterial enzyme are similar to those reported in the current work for the bovine oxidase. Examination of site-directed mutants confirmed the assignment of the negative band near 1745 cm^{-1} as coming from E286 (*R. sphaeroides* numbering). It was concluded that E286 (equivalent to bovine E242) becomes deprotonated at pH 8.5 upon electron transfer from the binuclear center to heme a. This is additional evidence that the assignment of the 1735 cm^{-1} in the current work to E242 is correct. It is important to note that our results suggest that this residue undergoes partial deprotonation *both* during the pH-independent (3 μs) and pH-dependent (millisecond) phases of reverse electron transfer.

The pH dependence of the change in the 1735 cm^{-1} absorbance (Fig. 4) shows that the apparent pK_a of E242 is about 7.8 when heme a is reduced and heme a_3 is oxidized and that the pK_a is >9.5 in the MV-CO form of the enzyme [19], as it is in the fully reduced and fully oxidized forms of the oxidase [29]. However, the pH dependence of the spectroscopic changes are very likely due to the pK_a of a group controlling the extent of reverse electron transfer and not the actual pK_a of the groups being observed in those spectra. Hence, the pK_a of E242 can simply be estimated to be low enough to result in partial deprotonation in that population of the enzyme in which reverse electron transfer has occurred.

It is noteworthy that a recent study [31] of the FTIR redox difference spectra of the bovine oxidase indicated a pair of troughs at 1748 and 1737 cm^{-1} suggesting the deprotonation of carboxylic residues specifically associated with the reduction of Cu_A/heme a/Cu_B (i.e. not heme a_3). This might also suggest that the reduction of heme a results in deprotonation of E242, implying in turn that electroneutrality requirements in the vicinity of heme a can trigger proton transfers over relatively long distances.

An alternative assignment of the 1735 cm^{-1} band is that it originates from the deprotonation of residue D51, at the outer surface of the bovine oxidase. This residue has been shown to become exposed to solution in the fully reduced bovine enzyme, whereas it is hydrogen bonded within the protein and presumed to be protonated in the fully oxidized enzyme. These structural data have provided the impetus for Yoshikawa et al. [5] to propose a proton pumping mechanism based in part of changes in D51 as a proposed exit residue. Hence, the observation of a trough at 1735 cm^{-1} would be consistent with deprotonation of D51 upon reduction of heme a. Unfortunately, the equivalent residue to D51 does not exist in the bacterial oxidases. The observation of similar features in the FTIR difference spectra of the *R. sphaeroides* enzyme and the bovine enzyme argues that it is likely that they originate from the same source, which could not be D51. However, it cannot be ruled out that the mammalian enzyme behaves differently from the bacterial oxidase, as has been suggested [5].

4.2. What is the source of the released proton?

The E286Q mutant of the *R. sphaeroides* oxidase exhibits the pH-dependent phase of reverse electron transfer along with proton release [35]. Hence, *R. sphaeroides* E286 (equivalent to bovine E242) cannot be the source of the released proton. Therefore, the deprotonation of E242 described above must result in intraprotein proton transfer to another basic group, rather than release of a proton to the surroundings.

It has been shown that proton release to (or, equivalently, hydroxide ion uptake from) the surroundings and the slow phase of reverse electron transfer are dependent on an intact K channel and not on the D channel, of which E242 is a component. This is part of the phenotype that distinguish mutants in the K channel from those in the D channel [36,62–64]. The pH-dependent phase of reverse electron transfer is eliminated in the K362M and T359A mutants [62] and these mutants eliminate the proton release associated with this phase of the reverse electron transfer. Recent work has demonstrated that there is a glutamic acid in subunit II of the oxidase ($E101^{II}$ in the *R. sphaeroides* oxidase) which is at the entrance of the K channel on the protein surface. Mutations at this site of the *R. sphaeroides* oxidase slow down but do not eliminate proton release [64]. Hence, there are considerable data showing that the released proton must exit (or the equivalent hydroxide ion enter) through the K channel, with some control exerted "upstream" of T359.

If there is a proton associated with the hydroxyl substituent of the farnesyl side chain of heme a_3, as has been postulated [65], this would be one candidate. However, protonation of the hydroxyfarnesyl group appears superficially unlikely because ROH_2^+ in aqueous medium is a stronger acid than HNO_3 [66], and would be expected to be stronger yet as a positively charged conjugate acid in the low-dielectric protein medium. Another candidate is the hydroxyl group of the cross-linked H240-Y244 at the enzyme active site. Phenols are poor acids in aqueous solution and, having a charged conjugate base, would be much poorer in the protein. Moreover, in either case, the FTIR difference spectra at alkaline pH (Fig. 2) would have features diagnostic of the deprotonation of the endogeneous proton donor, e.g. for tyrosine to tyrosinate, the phenyl stretch at 1515 cm^{-1} downshifts to ca. 1498 cm^{-1} upon formation of tyrosinate [14]. This is not observed, however. Another suggestion is that the proton originates from a water molecule at the active site that is converted to (heme Fe or Cu_B-bound) ^-OH. This is reasonable, though it is usually considered that the one-electron reduced form of the binuclear center has water and not a hydroxyl group associated with the binuclear center [2]. In summary, the current work suggests that the released proton does not originate from the deprotonation of the active-site tyrosine or protonated hydroxyfarnasyl, but provides no positive evidence as to the origin the proton.

4.3. Is there a neutral arginine in the MV-CO oxidase that is protonated upon reverse electron transfer?

In the absence of isotopic substitutions or mutants, the attribution of the 1638 cm^{-1} and (a partial contribution to the) 1676 cm^{-1} bands as being due to arginine is quite tentative. These are strong FTIR bands that correspond to the major stretching modes of the protonated guanidinium ($CN_3H_5^+$) side chain [57–59]. Convincing evidence for arginine bands appearing in FTIR spectra of proteins has been presented for the bacteriorhodopsin photocycle [67]. Arginine bands have also been previously noted in the fully reduced-minus-oxidized FTIR difference spectrum of the *P. denitrificans* oxidase [68]. These bands are much more

prominent in the spectra from the CO photolysis of the MV-CO oxidase observed in this work (Fig. 2) and also in the spectra reported from the *P. denitrificans* oxidase [60] and from the *R. sphaeroides* oxidase [61]. These bands are not present in the deprotonated guanidinium group [57]. A rough estimate is that the intensity of the bands is of the magnitude expected for the appearance of about 30% of one residue. Conceivably, these bands could originate from a substantial increase in the extinction coefficients of one or more *protonated* arginines upon reduction of heme a (note that deuterated arginine does not have the observed spectral signature, see Results).

Alternatively, it should be considered that these prominent bands are due to an unusual deprotonated arginine in the MV-CO form of the oxidase that becomes protonated following photolysis. This would require a local environment in which the pK_a of an arginine is about 4 to 5 pH units lower than the pK_a in water (pH 12) [57]. Changes in the pK_a's of carboxyl residues by this amount and more, induced by dielectric effects on the stability of charged conjugate acids or bases, are not unusual in proteins, as illustrated by the high pK_a of E242 in the bovine oxidase. Hence, from an energetics viewpoint, it is not unreasonable that an arginine could exist in a neutral form buried within the enzyme.

Two arginines (bovine R438 and R439) are involved in salt bridges with the heme propionates which have already been implicated as being involved in the proton exit pathway [69]. These two arginines would be in a good position to receive a proton from E242 via one of the heme propionates (12 Å from E242) if there were a conformational change to create a pathway for proton translocation. However, the structures of the oxidases show that these arginines are in a region of the protein with considerable water and it seems unlikely that either of these would (1) exist as a neutral species, or (2) fail to exchange their protons with solvent D_2O. A third candidate is the arginine (bovine R38) that is hydrogen bonded to the formyl group of heme a. This residue is in a more hydrophobic environment and its protonation has been proposed to be part of the mechanism for proton pumping in the bovine enzyme that also includes D51. The appearance of a protonated arginine upon reduction of heme a would be consistent with this proposed mechanism, which does not involve E242 [5]. R38 is located about 25 Å from E242 with no evident pathway for proton transfer between them. Nevertheless, if the 1638/1676 cm^{-1} features are actually due to protonated arginine, the IR results are consistent with proton transfer from E242 to R38 linked to both the 3 μs and millisecond phases of reverse electron transfer. This scenario may make sense in terms of electroneutrality requirements in the vicinity of both hemes a and a_3.

4.4. What group is responsible for the pH dependence of the CO stretching frequency in the MV-CO oxidase?

The data presented in this study demonstrate that the frequency of the bound CO in the MV form of the oxidase is slightly altered as the pH is increased. The simplest interpretation is that there is a single group (X) that titrates with an apparent pK_a of 8.3 in the MV-CO form of the oxidase. Since the pH dependence is not observed for CO bound to the fully reduced enzyme, it is reasonable to postulate that the pK_a of this group is out of the measurement range when heme a is reduced (FR-CO), but is pH 8.3 when heme a is oxidized (MV-CO), and implying that X is close to heme a. We propose that when heme a is reduced, the protonated form of this group (XH^+) is stabilized by a simple electrostatic interaction with heme a. We also propose that it is the net charge around heme a, including XH^+, that influences the frequency of CO bound to heme a_3. This could occur by an inductive effect between the two heme edges, which are separated by only a few angstroms. The net effective charge associated with ferrous heme a in the FR enzyme (ferrous heme plus XH^+) will be the same as in the MV-CO form of the enzyme at high pH (ferric heme a plus X) This explains why the limiting frequency observed for the MV-CO enzyme at high pH (ferric heme plus X) is the same as for FR-CO enzyme (ferrous heme plus XH^+) (Fig. 3).

With these postulates, group X must be strongly coupled to the redox state of heme a. Electrochemical studies show that net proton uptake associated with the reduction of heme a is dependent on the state of the binuclear center [70] and it is not evident what to expect when one electron remains at the heme/copper center. Net proton uptake would also depend on whether other groups, such as E242, release protons, effectively resulting in internal proton rearrangements. The identification of group X cannot be made with any certainly, but one possibility is that the protonatable group coupled to heme a could be the hypothetical neutral form of R38 that is hydrogen bonded to the formyl group of heme a.

5. Conclusions

(1) The reduction of heme a, at least when there is one electron in the binuclear center, is associated with the change in the pK_a of a group from a value near 8.3 (ferric heme a, as in MV-CO) to a high value (i.e. it is fully protonated) when heme a is reduced. It is possible that this situation is equivalent to what one might observe during steady state turnover.

(2) The pK_a of E242 can be substantially lowered from the estimated value of ≥ 9.5 (fully oxidized/fully reduced) and is under control of the redox status of the metal centers. The data show that E242 deprotonates in the state of the enzyme in which heme a is reduced and a_3 is oxidized, as observed following reverse electron transfer. The question whether this situation readily applies to situations obtained during steady state turnover is very intriguing.

(3) The possibility that there is a neutral, unprotonated arginine that plays a role in oxidase function should be considered worthy of further examination. The FTIR data

are not definitive on this point, but the possibilities are easily testable using mutants of the bacterial enzymes.

(4) The possibility that there is an internal proton movement from E242 to another residue within the enzyme concomitant with reverse electron transfer under select circumstances is also worthy of consideration based on these studies. Aside from arginines, another candidate is the ring D propionate of heme a. Based on analysis of the FTIR redox difference spectrum, it has already been proposed that this ring D propionate becomes protonated upon the reduction of the *P. denitrificans* oxidase [21]. Among other things, obligatory redox-linked transfer from E242 to the vicinity of heme a, of a proton that (at least under some circumstances) does not exchange with the extraprotein medium, potentially explains both the "slow" 3 μs rate of the reverse electron transfer reaction (relative, for example, to Dutton's predictions, see Ref. [71]) and the absence of a deuterium isotope effect on this reaction rate.

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