FTIR Studies of the CO and Cyanide Adducts of Fully Reduced Bovine Cytochrome *c* Oxidase†

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ABSTRACT: Photolysis spectra of the CO and cyanide adducts of reduced bovine cytochrome *c* oxidase have been studied by FTIR difference spectroscopy. Bound CO is predominantly in a single 1963 cm^{-1} form whereas cyanide is bound in at least two forms (2058/2045 cm^{-1}). These forms are pH-independent between pH 6.5 and 8.5, indicating that there is no titratable protonatable group that influences significantly their binding in this pH range. Photolysis spectra of the cyanide adduct have a positive band around 2090 cm^{-1} in H_2O due at least in part to free HCN and at 1880 cm^{-1} in D_2O due to free DCN. The frequency of the positive band around 2090 cm^{-1}, and its persistence in D_2O media, raises the possibility that a transient cyanide−Cu_B adduct also contributes to this signal, equivalent to the CO−Cu_B species that is formed when CO is photolyzed. Photolysis produces changes throughout the 1000−1800 cm^{-1} region. Reduced *minus* (reduced + CO) photolysis spectra in H_2O exhibit a pH-independent and symmetrical peak/trough at 1749/1741 cm^{-1}. A related feature in homologous oxidases has been suggested to arise from a conserved glutamic acid. However, only around one-third of the feature is shifted to lower frequencies by incubation in D_2O media, and an additional fraction is shifted if catalytic turnover occurs in D_2O. Reduced *minus* (reduced + cyanide) photolysis spectra exhibit multiple features in H_2O in this region with peaks at 1752, 1725, and 1708 cm^{-1} and troughs at 1740, 1715, and 1698 cm^{-1}. Again, only a part of these features shift in D_2O, even with catalytic turnover. A variety of additional H/D-sensitive features in the 1700−1000 cm^{-1} region of the spectra can be discerned, one of which in cyanide photolysis spectra is tentatively assigned to a conserved tyrosine, Y244. Data are discussed in relation to the structure of the binuclear center and protonatable groups in its vicinity.

Introduction of electrons or anionic ligands into the binuclear center of cytochrome oxidase requires charge-compensating protonations within the protein in order to produce stable intermediates (*1*). These protonations are likely to be central to the protonmotive mechanism, and identification of their locations is a key factor than can differentiate proposed models of coupling (*2*). Various amino acid residues that could provide binding sites and possible pathways for protons are evident in the atomic models of cytochrome oxidase (*3*, *4*), and Fourier transform infrared (FTIR)[1] spectroscopy may be used to probe changes in individual amino acids caused by ligand and protonation binding changes.

Photolysis of the CO compound of fully reduced cytochrome oxidase from various sources has been studied in some detail already by FTIR. Two major forms of heme-bound CO that are in a pH-dependent equilibrium have been found in the *Rhodobacter sphaeroides* oxidase both by FTIR at cryogenic temperature (*5*) and by resonance Raman spectroscopy at room temperature (*6*). Cytochrome oxidase from *Paracoccus denitrificans* also appears to show pH-dependent behavior of CO binding (*7*). The major form of bound CO in bovine oxidase is at 1963 cm^{-1} at room temperature (*8*) with minor additional species evident (*8*, *9*), especially at low temperature (*10*). In contrast to the bacterial enzymes, however, little pH-dependency of forms was observed at room temperature (*9*, *11*). Photolysis induces a transfer of CO from heme a_3 to Cu_B that is stable at low temperatures (*12*). The Cu_B-bound state is transient at room temperature (*13*, *14*) and has a longer lifetime in D_2O (*11*). Changes in the 1000−1800 cm^{-1} region are associated with CO photolysis (*10*, *15*). Of particular interest are changes in the 1700−1750 cm^{-1} region that have been attributed to a conserved glutamic acid in subunit I (E242 in the bovine sequence). However, the features vary in oxidases from different sources. For fully reduced cytochrome *bo* from *E. coli* in D_2O, photodissociation of CO at 80 K results in a 3 cm^{-1} upward shift of the glutamic acid vibration to produce a peak/trough at 1731/1724 cm^{-1} in the photolysis spectrum (*15*). An equivalent shift is absent altogether when CO is

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[1] Abbreviations: FTIR, Fourier transform infrared spectroscopy; CO, carbon monoxide; CaF_2, calcium fluoride; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; MCT, mercury cadmium telluride.

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photolyzed from fully reduced *P. denitrificans* cytochrome oxidase at both room and low temperatures (*7*). Instead, an extinction coefficient decrease of a 1746 cm^{-1} band (1740 cm^{-1} in D_2O) occurs, resulting in a trough in the photolysis spectra, when CO is photolyzed from the half-reduced 'mixed-valence' form at room, but not at cryogenic, temperature (*7*). In contrast to both bacterial oxidases, photolysis of CO from the fully reduced form of bovine cytochrome oxidase (in D_2O and at room temperature) was reported to result in a 1737 cm^{-1} trough (*11*).

Vibrational modes of cyanide when bound to bovine cytochrome oxidase in various oxidation states have been investigated by infrared spectroscopy (*8*, *16*) although associated changes in the 1800−1000 cm^{-1} region have not been studied. In the oxidized enzyme, the major band is at 2151 cm^{-1} and has been assigned to a bridged Cu^{2+}−CN−Fe^{3+} structure (*8*, *16*). In fully reduced oxidase, bands occur at 2058 and 2045 cm^{-1}. Further bands at 2131 and 2093 cm^{-1} in partially reduced states of oxidase have been attributed to protonation differences (*8*) or 'conformational' changes associated with redox changes of individual metal centers (*16*). When high concentrations of cyanide are added to fully reduced enzyme, two additional bands can be formed at 2093 and 2037 cm^{-1} and have been attributed to binding of a second cyanide to Cu_B^{1+} (*16*).

In this report, FTIR photolysis difference spectra of the CO and cyanide compounds of fully reduced bovine cytochrome oxidase are compared, and the effects of pH and of H/D exchange are described. Results are discussed in relation to the bound ligand states and to protonation sites that may be influenced by ligand changes at the binuclear center.

MATERIALS AND METHODS

Preparation of Bovine Heart Oxidase. Cytochrome *c* oxidase was prepared from beef heart by a procedure (*17*) that yields 'fast' enzyme with monophasic cyanide binding kinetics and a characteristic Soret maximum at 424 nm. The final preparation was dissolved in 0.1 M potassium phosphate, 0.1 M potassium borate, 0.1% (w/v) Tween-80, pH 8.5, to a concentration of 150−350 μM, as quantitated from the dithionite-reduced *minus* oxidized optical difference spectrum using an extinction coefficient of $\Delta\epsilon_{606-621nm} = 25.7\ mM^{-1}\ cm^{-1}$ (*18*). Aliquots were stored in liquid nitrogen until required.

Preparation of FTIR Samples. For the cyanide compound, an oxidase sample (5−20 μL, equivalent to 2−3 nmol of cytochrome oxidase) was partially dried under argon on the face of a 25 mm diameter CaF_2 window. After rehydration with 2 μL of 0.5 M sodium dithionite and 1 μL of 0.2 M KCN (both in 1 M potassium phosphate, pH 8.5), a second window was placed on top, and the sample was spread by squeezing. When spread to provide an optimal thickness (giving an absorbance of around 1.0 at the 1650 cm^{-1} peak), the gap around the sample was filled with the buffered dithionite solution and sealed with silicon vacuum grease. Formation of the ferrous−cyanide adduct was confirmed by optical spectroscopy.

For the CO compound, an oxidase sample (5−20 μL, equivalent to 2−3 nmol of cytochrome oxidase) was partially dried as above. The sample was then rehydrated with 2.5 μL of 0.1 M sodium dithionite (in 1 M potassium phosphate, pH 8.5). After exposure to a stream of water-saturated CO for 1 min, the second window was placed on top, and the sample was treated as above. Stoichiometric formation of the reduced CO compound was confirmed by optical spectroscopy and by the presence of the characteristic 1963 cm^{-1} band of bound CO.

D_2O Exchange. Cytochrome oxidase was diluted 20-fold with 0.1 M potassium borate and 0.1% (w/v) Tween-80 in D_2O at a pH meter reading of 8.1 [equivalent to pD 8.5 (*19*)]. The oxidase was pelleted by centrifugation at $100000g_{av}$ for 60 min, and the process was repeated. Samples were then prepared as for H_2O solutions, but with all re-wetting solutions prepared with equivalent D_2O buffers at pD 8.5 and with a CO stream saturated with D_2O vapor.

To further promote H/D exchange within buried proton channels, oxidase was also incubated in D_2O media while catalytic turnover was occurring. Enzyme was diluted 20-fold into D_2O buffer as above, but containing 2.5 mM sodium ascorbate, 25 μM TMPD, and 2.5 μM cytochrome *c*. This provided a continuous source of reducing equivalents so that the enzyme turned over many times in D_2O while it was pelleted by centrifugation as above. The pellet was washed twice in D_2O buffer without the turnover reagents.

FTIR Photolysis Difference Spectroscopy. FTIR spectra were recorded in Saclay on Nicolet 860 and Nicolet 60SX spectrometers, and in London on a Bruker ISF 66/S machine, fitted with liquid nitrogen-cooled MCT-A detectors. Actinic light was provided by 100−250 W quartz−iodine lamps, filtered with glass heat filters, water, and BG39 or 520 nm cutoff filters, and delivered to the sample via a light pipe. Typically, 100 interferograms at 4 cm^{-1} resolution were averaged over 15 s to provide an initial dark baseline, and the light was then switched on and recording was repeated after 1 s. Finally, the light was switched off, and the recording was repeated after 2 s dark relaxation in order to provide an indication of relaxation rate and sample baseline drift. For nonfrozen samples, light/dark cycles were repeated up to 4000 times to enable detection of signals of 10^{-6} ΔA. No significant deterioration of the sample occurred during these cycles. Spectra shown are unligated *minus* ligated difference spectra, i.e., light *minus* dark difference spectra. In most cases (stated in the figure legends), the initial dark *minus* final dark spectrum has been subtracted to counteract baseline drift. All frequencies have an accuracy to ±1 wavenumber.

RESULTS AND DISCUSSION

Cyanide and CO Photolysis Spectra. Photolysis data with CO and cyanide complement each other. Although cyanide binds strongly to the net-positive ferric heme, binding to (overall neutral) ferrous heme is much weaker. In contrast, neutral CO binds strongly only to ferrous heme because of additional bonding energy from interaction of its antibonding p orbital with the iron d orbitals. Also, whereas CO has a small dipole moment, cyanide is more hydrophilic, has a large dipole moment, and binds to oxidase with an accompanying proton (*1*). The proton must presumably bind to the nitrogen of the Fe^{2+}−C−N^- species, to a nearby amino acid or, to both. These differences between the ligands lead to the more extensive perturbations of protein caused by cyanide and, probably, to the finding that cyanide but not CO has several major modes of binding.

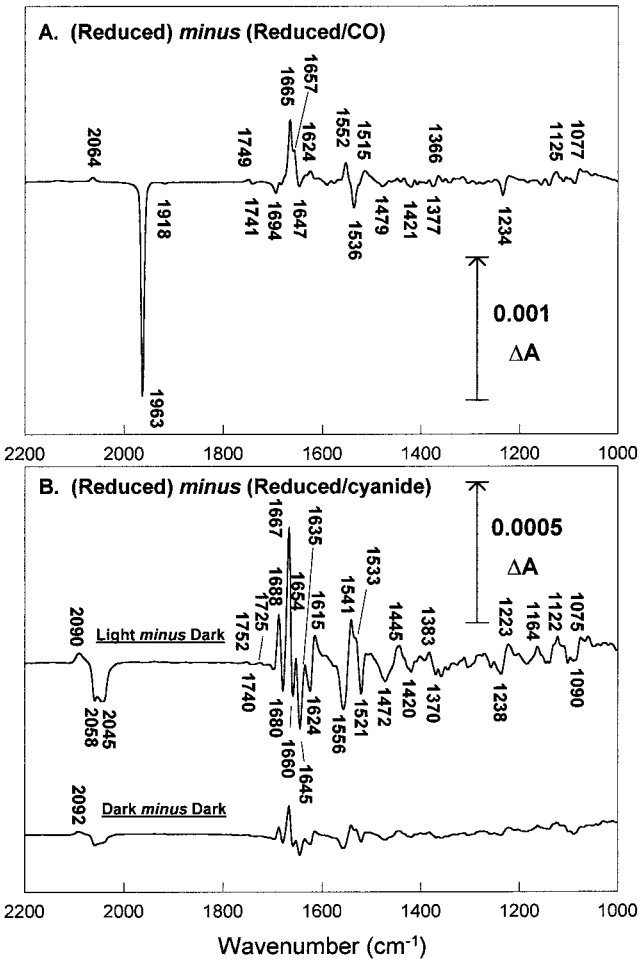

FIGURE 1: Light-induced FTIR difference spectra of cyanide and CO photolysis in H_2O at pH 8.5 and 283 K. Samples of the CO or cyanide adducts of fully reduced bovine cytochrome oxidase were prepared in H_2O buffers at pH 8.5 as described under Materials and Methods. After sufficient time for equilibration and settling at 283 K, repetitive light/dark cycles were recorded and averaged. Spectra shown are: for CO (A), light *minus* dark difference spectra from which the dark *minus* dark control has been subtracted; for cyanide (B), light *minus* dark and dark *minus* dark spectra separately. In all cases, each scan consisted of 100 averaged interferograms at 4 cm^{-1} resolution, and scans were averaged in blocks of 50. The CO photolysis spectrum is an average of 2800 scans using a 100 W lamp protected by a BG39 filter. The cyanide photolysis spectra are averages of 3273 scans using a 250 W lamp protected by a 520 nm cutoff filter. In the case of the cyanide sample, the oxidase was been turned over catalytically before preparation of the FTIR sample, a treatment that makes very small differences in the details of the carboxylic acid region. For CO photolysis spectra, no significant effects of turnover were found.

Figure 1 compares the static a_3^{2+} *minus* (a_3^{2+}-CO) and a_3^{2+} *minus* (a_3^{2+}-cyanide) (i.e., light *minus* dark) difference spectra of bovine cytochrome *c* oxidase in H_2O at pH 8.5 and 283 K. Positive peaks correspond to features of the unligated enzyme and troughs to those of the ligand-bound proteins. Changes caused by ligand photolysis occur primarily at wavenumbers above 1900 cm^{-1} while protein/prosthetic group changes are seen in the 1800−1000 cm^{-1} range. Spectra are highly reproducible with different samples and did not alter significantly in the same sample after light/dark cycles over several days, hence allowing several thousand photolysis spectra from the same sample to be signal-averaged. By comparison of the size of the 1963 cm^{-1} trough due to loss of bound CO with the 1963 cm^{-1} peak height in the absolute spectrum, the CO photolysis yield at steady state was estimated to be 25 ± 5%. Recombination of CO is sufficiently fast [$k = 70\ s^{-1}$ at 1 mM CO (*20*)] that no photolysis features remained in the 'dark *minus* dark' control spectrum and this spectrum was routinely subtracted to give the spectra shown. Cyanide is photolyzed less readily than CO (*21, 22*), and more intense photolysis illumination was generally used. Bands of bound cyanide are broader and are partially overlapped by the broad 2130 cm^{-1} combination band of water and by the 2093 and 2079 cm^{-1} bands of free HCN and CN^- (*23*), making quantitation of photolysis yield by comparison with absolute spectra less precise; 10−25% photolysis of the cyanide compound was estimated in the photostationary state under the stronger illumination conditions used for Figure 1B. Recombination of cyanide is relatively slow [$k = 0.235\ s^{-1}$ at 1 mM cyanide (*24*)], so that the dark *minus* dark control spectrum begun 2 s after cessation of illumination still contained a few percent of photolysis products (see Figure 1B). All major features of the photolysis spectrum in the 1700−1000 cm^{-1} region are equally diminished in this dark *minus* dark control spectrum, which indicated that all are reversible and decaying at the same rate, rather than any being associated with metal contaminants [cf. (*8*)]. Some small differences seen in the cyanide and 1800−1700 cm^{-1} regions are discussed below.

Ligand Vibrations and Their pH Dependencies. For CO photolysis, the dark *minus* dark spectrum after illumination has been subtracted in order to remove any baseline changes, although this has little effect on resultant spectra. Photolysis of bound CO at 283 K (Figure 1A) results in a trough at 1963 cm^{-1} due to loss of heme a_3-bound CO (*8, 10, 11*). Minor (β) populations of the fully reduced CO-adduct have been observed with frequencies between 1944 and 1959 cm^{-1} (*8*−*10*), but these are barely detectable in our preparation at room temperature. The small trough at 1918 cm^{-1} arises from photolysis of the naturally occurring ^{13}C isotope of CO (*12*). No positive signal of free, photolyzed CO is observed since it is broadened and, due to its low dipolar moment, has a very low extinction coefficient. Also observable in Figure 1A is a small positive peak at 2064 cm^{-1} that is due to a small steady-state concentration of the Cu_B−CO intermediate of CO photolysis/recombination (*12, 14*).

Since recombination of the cyanide compound is not complete in dark *minus* dark spectra, and since some subtle differences are evident in the residual photolysis spectra, these spectra are plotted separately in Figure 1B. Photolysis of the cyanide compound produces major negative bands at 2058 and 2045 cm^{-1}, indicating photolysis of multiple populations of heme a_3^{2+}-bound cyanide. A 2046 cm^{-1} signal was initially interpreted as due to the cyanide compound of a contaminating metal (*25*), but both 2058 and 2046 cm^{-1} bands were subsequently ascribed to a_3^{2+}-CN (*8, 16*). This is confirmed by their similar (though not identical, see Figure 1B) relaxation kinetics [cf. (*26*)] and by their sensitivity to CO (*27*). It has previously been noted that a 2038 cm^{-1} feature appears at high cyanide levels (>5 mM) and may be due to Cu^+CN (*16*); only minute traces of this are evident in our samples. Photolysis also results in a positive band at 2085−2090 cm^{-1}, the frequency and bandwidth of which could be consistent with formation of free HCN + CN^- (*23*). However, the product at this pH is expected to be primarily HCN at 2093 cm^{-1}, and the frequency is always significantly

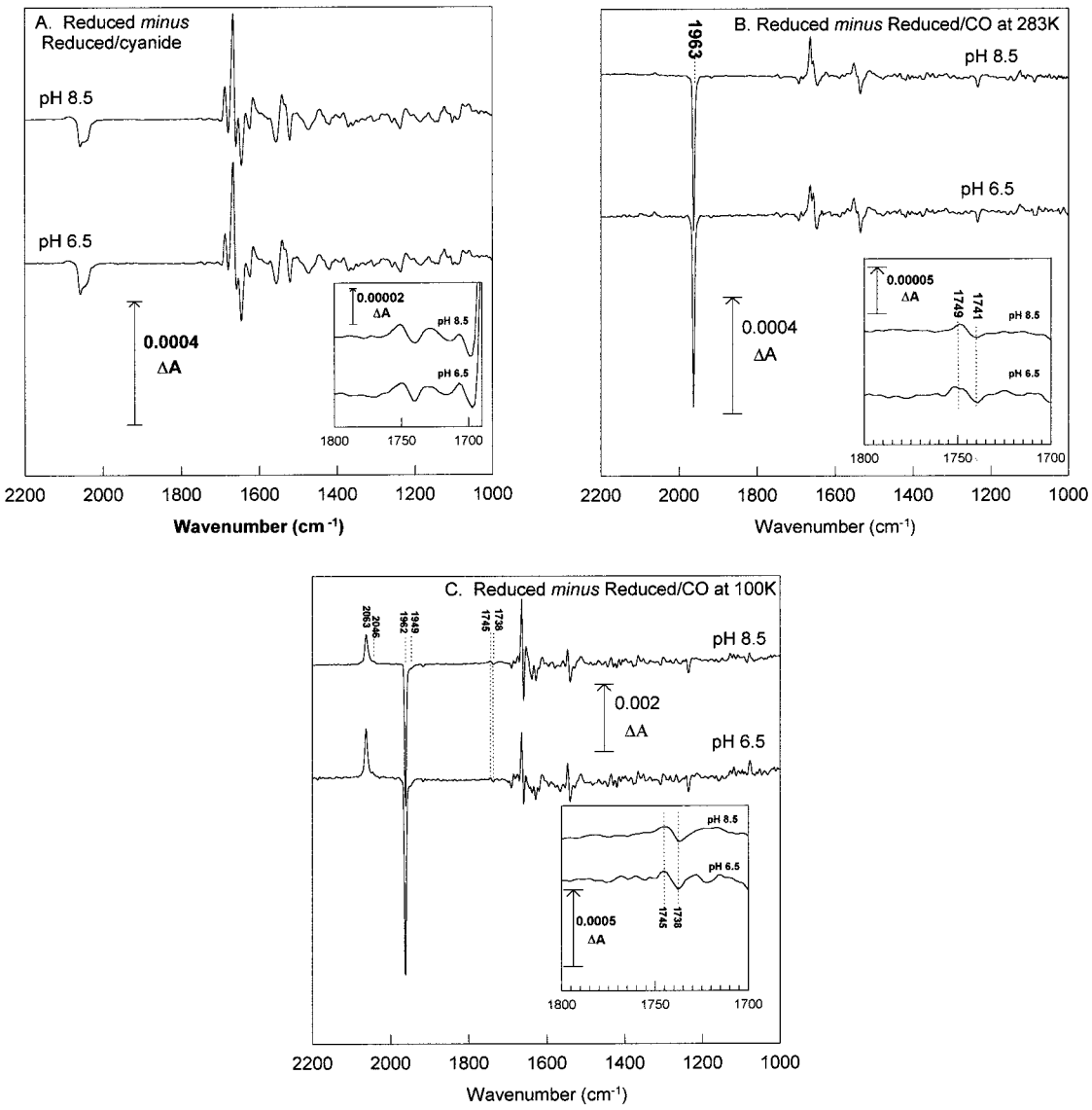

FIGURE 2: pH dependency of the CO and cyanide FTIR photolysis difference spectra. Samples were prepared in H_2O media, and spectra were recorded as in Figure 1. For pH adjustment, stock oxidase was diluted 20-fold into 50 mM Na-Bicine buffer at the required pH, concentrated to the original volume in a Microcon 100 concentrator, and used as above. Reagents were added in 1 M potassium phosphate buffer at the required pH. Spectra shown are: (A) reduced *minus* (reduced/cyanide) at pH 6.5 and 8.5 and 283 K (averages of 3285 and 2278 scans); (B) reduced *minus* (reduced/CO) at pH 6.5 and 8.5 and 283 K (averages of 1000 and 1200 scans); (C) reduced *minus* (reduced/CO) at pH 6.5 and 8.5 and 100 K (each is the average of 4 sets of 128 interferograms). In all cases, dark *minus* dark spectra have been subtracted from light *minus* dark spectra to give the traces shown. The insets show the same spectra expanded in the 1800−1700 cm^{-1} regions.

below 2093 cm^{-1} even at pH 6.5 (Figure 2A), although it does get closer to 2093 cm^{-1} in the residual dark *minus* dark spectra (Figure 1B). Furthermore, a positive band around 2090 cm^{-1} is also found in D_2O media (see Figure 5 and below) even although free DCN absorbs at 1887 cm^{-1} (*23*). Hence, it seems possible that a bound cyanide intermediate that is equivalent to the 2064 cm^{-1} Cu_B^+−CO intermediate (*12*, *14*) contributes to the positive band around 2090 cm^{-1}. This is consistent with the assignment of a similar band in (*16*), but, because of its similarity to the 2093 cm^{-1} band of free HCN, further work will be required to establish definitively its nature.

Effects of pH on ligand vibrations were investigated. Figure 2A shows that the two major bound cyanide vibrations are not altered significantly between pH 6.5 and 8.5. Again, the positive band at 2090 cm^{-1} is below that of free HCN even at pH 6.5. Very small variations in the relative intensities of the bound cyanide signals were found between different oxidase preparations, and their significance, if any, is unclear. Some variation in ratios of bound cyanide frequencies has been noted previously (*25*). All of these data indicate, however, that the major forms of bound cyanide are not in a pH-dependent equilibrium between pH 6.5 and 8.5.

The pH-dependency of bound forms of CO in the bovine enzyme at room temperature has already been reported, and neither the dominant 1963 cm^{-1} form nor the minor β forms (1944−1959 cm^{-1}) of heme a_3-bound CO show pH-sensitive relative amplitudes (*9*, *11*). This is confirmed in Figure 2B where spectra of samples at pH 6.5 and 8.5 and 283 K show that the 1963 cm^{-1} band remains unchanged. This is in contrast to the behavior of the homologous *Rb. sphaeroides* oxidase where two dominant α and β forms of bound CO are in a pH-dependent equilibrium with an apparent pK_a of

7.3 (*5*, *28*). These findings have been interpreted as reflecting pH-dependent conformers with slight differences in the polarity of the environment sensed by the CO (*14*). However, the pH dependency of the CO bands in *Rb. sphaeroides* oxidase has been reported predominantly at cryogenic temperature. Hence, for definitive comparison, CO photolysis spectra of the bovine oxidase were repeated at 100 K (Figure 2C). At this temperature, no recombination occurs on a minutes time scale (*13*), and the sample was warmed to room temperature to achieve recombination so that photolysis could be repeated. The trough due to loss of bound CO is shifted to 1962 cm^{-1} [cf. (*9*)]. In addition, minor components around 1949 cm^{-1} become more evident at 100 K, and a minor additional Cu_B−CO population is seen at 2046 cm^{-1} (*10*), but again none of these are altered with pH. Hence, there is a difference between the bovine and *Rb. sphaeroides* oxidases that warrants further investigation, since it implies that a titratable protonation site that influences bound CO in the binuclear center of the bacterial enzyme is absent in the otherwise structurally very similar bovine oxidase.

Photolysis Spectra in the 1800−*1000 cm*$^{-1}$ *Range.* Figure 1 also illustrates the changes that occur on ligand photolysis in the 1800−1000 cm^{-1} region. The bovine CO photolysis spectrum (Figure 1A) is in outline comparable to the equivalent spectrum reported for the *P. denitrificans* oxidase (*7*), but cyanide photolysis spectra of a bacterial oxidase are not yet available for comparison. For both ligands, the most prominent changes occur in the amide I region (1700−1620 cm^{-1}). These represent predominantly changes of vibrations of groups associated with the polypeptide backbone (primarily C═O stretching). At a maximum Δ*A* (peak to trough) of around 0.0005 in both cases, the absorbance differences are several orders of magnitude lower than the absolute spectrum, which has an absorbance of around 1.0 at the 1656 cm^{-1} amide I/water peak in these samples (of which around 50% is attributable to protein absorbance). This suggests that only a small number of bonds are involved in the photolysis-induced conformational changes. The changes induced by cyanide photolysis in the amide I region are more complex than the equivalent changes induced by CO photolysis. This presumably arises from the fact that cyanide is more polar than CO and so is likely to cause greater perturbation of local vibrational frequencies and extinction coefficients.

The 1700−1620 cm^{-1} range may also contain C═O contributions from heme formyl and propionate groups [cf. (*10*, *29*)]. In particular, a 1676 cm^{-1} feature in the redox difference spectra of *P. denitrificans* oxidase has been assigned to a protonated heme propionate, at least in part, based on specific ^{13}C labeling (*30*). A large peak/trough at 1688/1680 cm^{-1} can be seen in the cyanide photolysis spectrum that is absent from the CO photolysis spectrum. This suggests that the binding of cyanide, but not CO, might perturb one or more heme propionic acids. However, some amino acid side chains and amide I C═O stretch can also contribute to this region (*31*), and more detailed studies are required to enable reliable assignments in this region.

Strong signals also occur in the amide II (1560−1520 cm^{-1}) region of both photolysis spectra where contributions from polypeptide CN stretching and NH bending modes, aromatic amino acids, and heme C═C groups are expected [cf. (*29*)]. The changes in this region are more complex in the cyanide photolysis spectrum where several overlapping signals are evident. Signals due to ionized carboxylate groups of amino acids and heme propionates can also contribute, as can vibrational modes of several other amino acids such as the strong ring-stretching mode of tyrosine (*32*−*34*). A conserved tyrosine (Y244 in bovine subunit I) that is covalently linked to a histidine ligand to Cu_B is present in the binuclear center (*4*) and has been implicated functionally both in binding of intermediates and in proton transfer (*35*, *36*). This tyrosine might well be expected to be influenced by binding of the polar cyanide within the binuclear center. Data with D_2O (see below) indicate that the region is sensitive to H/D exchange, implying that an exchangeable residue does indeed contribute to this region.

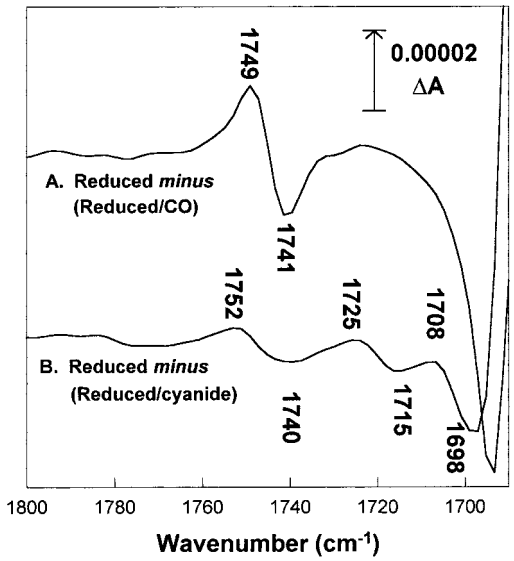

FIGURE 3: Light-induced FTIR difference spectra of cyanide and CO photolysis in H_2O at pH 8.5 and 283 K in the 1800−1700 cm^{-1} region. The 1800−1700 cm^{-1} regions of the photolysis difference spectra shown in Figure 1 have been plotted in expanded form to show features attributable to carboxylic acid changes.

The 1700−1750 cm^{-1} range is of particular interest since the only protein-associated bands arise from protonated aspartic or glutamic acid residues. A prominent and symmetrical peak/trough at 1749/1741 cm^{-1} is evident in the CO photolysis spectrum (shown in expanded form in Figure 3A). Previously, only a negative signal at 1737 cm^{-1} has been reported following photolysis of the CO compound of bovine oxidase in D_2O and at room temperature (*11*), although the signal/noise ratio was low. The photolysis spectrum of the CO compound of fully reduced *E. coli bo*-type oxidase at 80 K exhibits a similar peak/trough at 1731/1724 cm^{-1} that has been assigned, on the basis of site-directed mutagenesis, to a conserved glutamic acid in subunit I (E286 in *E. coli* numbering) (*15*). It is likely that at least part of the bovine 1749/1741 cm^{-1} feature may be attributed to the equivalent glutamic acid E242 in the bovine sequence. However, it is evident from H/D exchange data that the change arises from several different populations (see below).

Changes in the carboxylic acid region are more complex in cyanide photolysis spectra where at least three features are evident (Figure 3B). The first is a symmetrical peak/trough at 1752/1740 cm^{-1} (1753/1742 cm^{-1} if the dark *minus* dark spectrum is subtracted) that is similar to the feature seen in the CO photolysis spectrum albeit at significantly lower intensity if normalized in relation to changes in the

amide I region. Additional features are evident with peaks at 1725 and 1708 cm^{-1} and troughs at 1715 and 1698 cm^{-1}. The 1725/1715 cm^{-1} features likely arise from a shift of a second carboxylic acid, whereas the 1708 cm^{-1} peak could arise from a further carboxylic acid or heme propionic acid. Whether the 1708 cm^{-1} peak has an associated trough cannot be assessed since the amide I features overlap on the lower frequency side. These features are discussed in more detail below.

Little or no pH dependency was found for the intensity or characteristics of the carboxylic regions of either the cyanide (inset to Figure 2A) or the CO (insets to Figure 2B,C) photolysis spectra (the small apparent difference in the Figure 2B inset is within the experimental noise and was not observed reproducibly). Hence, it is apparent that the protonated groups that contribute to these regions must all have p*K* values above 8.5.

Temperature Dependency of the CO Photolysis Spectrum in the 1800−1000 cm^{-1} Region. The carboxylic acid signal associated with photolysis of the CO-ligated state of the mixed-valence form of the *P. denitrificans* enzyme was entirely abolished on transition from 268 to 84 K (*7*). An indication of the temperature dependencies of features of the bovine CO photolysis spectrum in the 1800−1000 cm^{-1} region can be assessed by comparison of spectra in Figures 2B and 2C. It may be seen that bandshifts and narrowing occur throughout this range as temperature is lowered, especially in the amide I and amide II regions. In the carboxylic acid region, the 1749/1741 cm^{-1} feature at 283 K shifts to 1745/1738 cm^{-1} at 100 K, and its intensity is diminished relative to the bound CO feature by about 50%. This behavior presumably reflects some freezing out of structural movements that must be required for the frequency shift to occur.

Effects of D_2O Exchange on Photolysis Spectra. To shed further light on the numbers and types of protonated residues that are influenced by ligand photolysis at the binuclear center, photolysis spectra were compared in D_2O and H_2O media. These spectra, together with H_2O *minus* D_2O double difference spectra, are shown in Figures 4−7. The changes induced by H/D exchange are limited for both ligands. This indicates that the bulk of amide protons in the tightly packed, hydrophobic protein are very resistant to H/D exchange, as has been observed in some other membrane proteins such as bacteriorhodopsin (*37*). Hence, H/D exchange occurs only with surface residues and those buried residues that are accessible to solvent. In the case of CO photolysis at 283 K, only slight differences in the shape of the 1749/1741 cm^{-1} peak/trough and small changes in the amide I region are evident in the photolysis spectra (Figure 4). The changes that have occurred when oxidase undergoes catalytic turnover in D_2O media are clearer in the H_2O *minus* D_2O/turnover double difference spectrum (i.e., trace 4A *minus* 4C). A frequency decrease of the bound CO band in D_2O results in the 1965/1959 cm^{-1} peak/trough, an effect that has been observed previously (*9*). This small peak shift, less than 0.2 cm^{-1}, may well arise from extremely small changes of the overall conformation of the protein around the CO binding site that are induced by H/D exchange more globally. Further features are consistently observed in double difference spectra in the protein region. These include changes in the 1700−1750 cm^{-1} carboxylic acid region (see below), negative bands

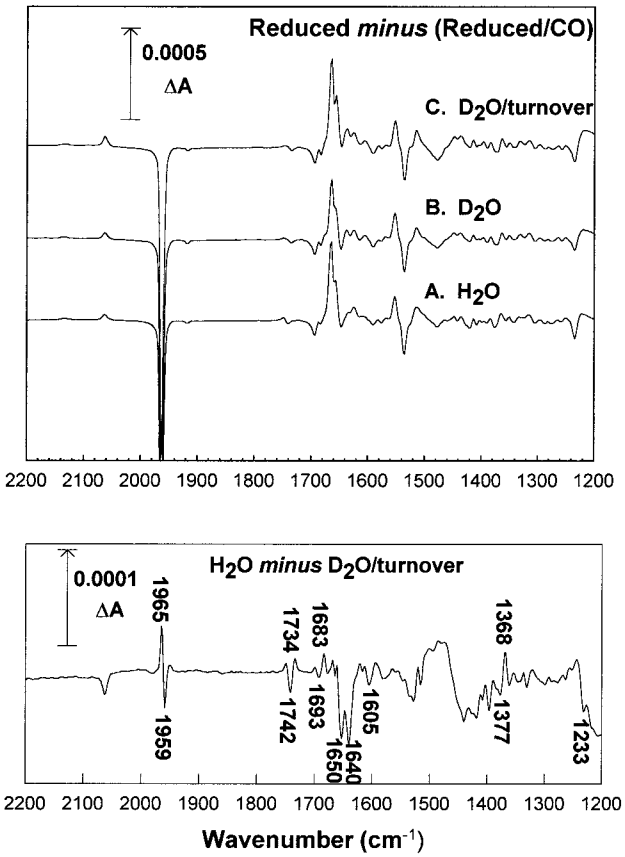

FIGURE 4: Comparison of CO photolysis difference spectra at pH 8.5 and 283 K in H_2O and D_2O. Samples of the CO adduct of fully reduced bovine cytochrome oxidase were prepared in H_2O or D_2O buffers at pH 8.5 or pD 8.1 as described under Materials and Methods. After sufficient time for equilibration and settling at 283 K, repetitive light/dark cycles were recorded and averaged. Spectra shown are light *minus* dark difference spectra from which the dark *minus* dark controls have been subtracted. Each scan consisted of 100 averaged interferograms at 4 cm^{-1} resolution, and scans were averaged in blocks of 50 illumination cycles using a 100 W lamp protected by a BG39 filter. The photolysis spectra are: (A) H_2O media (average of 2800 cycles); (B) D_2O media (average of 2587 cycles); (C) D_2O media with catalytic turnover (average of 1000 cycles); (bottom trace) H_2O *minus* D_2O/turnover, i.e., trace A *minus* trace C.

at 1650, 1640, and 1605 cm^{-1}, a peak/trough at 1683/1693 cm^{-1} that might arise from a heme propionic acid, a peak/(possible trough) at 1368/(1377) cm^{-1}, and sharp negative band at 1233 cm^{-1}. All of these features are likely to arise from exchangeable protonated sites that are influenced by ligand binding and provide a first step to identification of the number of such groups that might be affected.

Effects on the cyanide photolysis spectrum of catalytic turnover while in D_2O media are shown in Figure 5 (in these spectra, dark *minus* dark controls have been subtracted). Both control and D_2O samples were subjected to identical dilution/turnover manipulations, since it was found that turnover subtly affected some features of the spectrum. In the D_2O spectrum, only features that are different from the H_2O sample are labeled, and asterisks indicate two features that are missing. Again, the changes are remarkably limited and are most easily highlighted in an H_2O *minus* D_2O/turnover double difference spectrum. Easiest to assign is the appearance in D_2O of a broad band around 1880 cm^{-1} due to free DCN (*23*). Surprisingly, a positive band is still evident

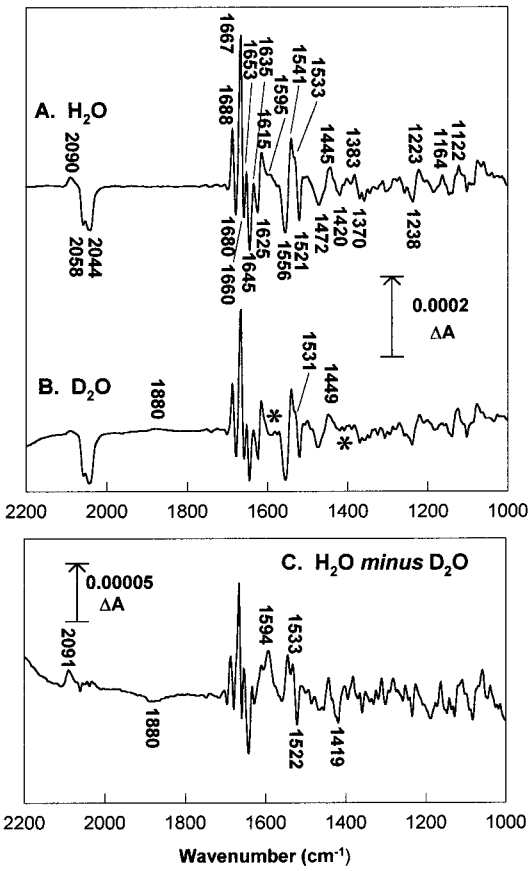

FIGURE 5: Comparison of cyanide photolysis difference spectra at pH 8.5 and 283 K in H_2O and D_2O. Samples of the cyanide adduct of fully reduced bovine cytochrome oxidase were prepared in H_2O or D_2O buffers at pH 8.5 or pD 8.1 as described under Materials and Methods. In this case, both samples underwent dilution and catalytic turnover in an identical manner. Spectra shown are light *minus* dark difference spectra from which the dark *minus* dark controls have been subtracted. In all cases, each scan consisted of 100 averaged interferograms at 4 cm^{-1} resolution, and scans were averaged in blocks of 50 illumination cycles using a 250 W lamp protected by a 520 nm cutoff filter. The photolysis spectra are: (A) H_2O media (average of 3273 cycles); (B) D_2O media (average of 4252 cycles); (C) H_2O *minus* D_2O, i.e., trace A *minus* trace B. For trace B, only those features that are significantly different from the H_2O sample have been labeled, and the two asterisks indicate features that are absent in D_2O.

around 2090 cm^{-1} though with lower intensity that that seen in H_2O, and provides additional evidence for a bound cyanide intermediate equivalent to the Cu_B−CO species (*12*). A very small change in the ratio of bound cyanide states was also observed, with the lower frequency form slightly more favored in D_2O. Small changes are evident in the amide I region, presumably reflecting the different perturbation caused by the binding of DCN versus HCN [since cyanide is known to bind with an accompanying charge-counterbalancing proton (*1*) or deuteron] and by H/D exchange of sites within the protein and, possibly, of heme propionic acids, but specific assignments in this region are not possible. Other consistent features seen in the H_2O *minus* D_2O double difference spectrum of Figure 5 are changes in the carboxylic acid region (discussed below), a peak at 1594 cm^{-1}, a trough at 1419 cm^{-1}, and a difference in the amide II region that produces a sharp peak/trough at 1533/1522 cm^{-1}. A strong ring-bending mode of free tyrosine occurs in water at 1516 cm^{-1} (*32*, *34*) and should be influenced by H/D exchange

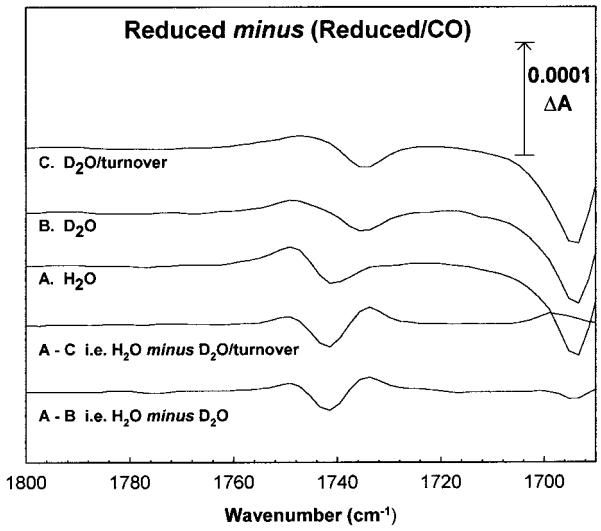

FIGURE 6: CO photolysis difference spectra at pH 8.5 and 283 K in H_2O and D_2O in the 1800−1700 cm^{-1} region. The 1800−1700 cm^{-1} regions of the photolysis difference spectra shown in Figure 4 have been plotted in expanded form to show features attributable to carboxylic acid changes.

of the hydroxyl group (*33*). Hence, the presence of the 1533/1522 cm^{-1} feature in the H_2O *minus* D_2O/turnover double difference spectrum lends further support to the proposal that a tyrosine (presumably Y244) has an H/D-sensitive band in the 1530 cm^{-1} region that is perturbed by cyanide photolysis.

Complexity of the Carboxylic Acid Changes. Effects of H/D exchange in the 1800−1690 cm^{-1} region of the CO photolysis spectra are shown in expanded form in Figure 6. The symmetrical 1749/1741 cm^{-1} peak/trough in the H_2O sample (trace A) becomes distorted in oxidase that has been incubated in D_2O media (trace B). The H_2O *minus* D_2O double difference spectrum (A − B in Figure 6) shows that around one-third of the 1749/1741 cm^{-1} peak/trough has shifted to lower frequency, with the remainder unchanged. Exposure to D_2O for several days failed to cause further change. By promoting catalytic turnover of the oxidase while in D_2O media, which presumably aids exchange of residues lining the proton channels, more of the signal was observed to shift (trace 6C and the H_2O *minus* D_2O/turnover double difference i.e., A − C). However, even after turnover in D_2O media, it is evident in trace 6C that a significant fraction (up to one-half) of the original 1749/1741 cm^{-1} remained unchanged. For the fraction that does shift, the frequency decrease is 4−5 cm^{-1} for both peak and trough and is within the range expected for an H/D exchange effect on a carboxylic acid group.

Effects of H/D exchange with catalytic turnover on the 1800−1690 cm^{-1} region of the cyanide photolysis spectra are shown in Figure 7 (again with dark *minus* dark controls subtracted). A small change occurred in the 1753/1742 cm^{-1} peak/trough (this signal is shifted slightly compared to that of Figure 3, due to subtraction of the dark *minus* dark control), with at most around one-third of the signal shifting downward by 2−3 cm^{-1}. Minor changes occurred in the 1725/1715 cm^{-1} peak/trough, again possibly consistent with a 2−3 cm^{-1} shift downward of a fraction of the signal. The 1708 cm^{-1} peak became far less prominent in the D_2O sample, consistent with a shift downward by around 4 cm^{-1} and/or a decrease of extinction coefficient. The effects of

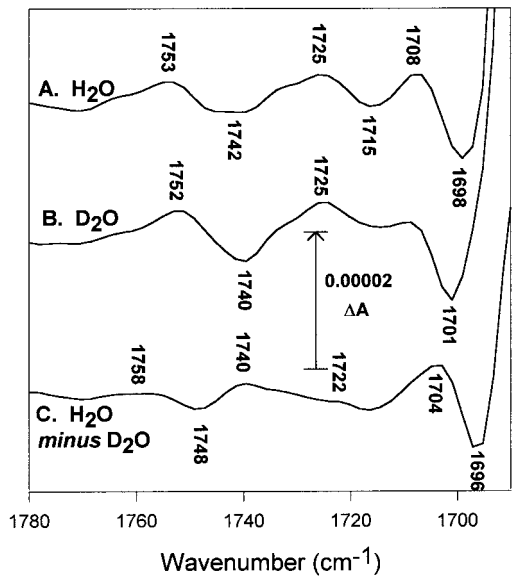

FIGURE 7: Cyanide photolysis difference spectra at pH 8.5 and 283 K in H_2O and D_2O in the 1800−1700 cm^{-1} region. The 1800−1700 cm^{-1} regions of the photolysis difference spectra shown in Figure 6 have been plotted in expanded form to show features attributable to carboxylic acid changes.

H/D exchange for all of these features are consistent with assignment to carboxylic acid species, although the magnitudes of the changes are small and show that a large fraction is resistant to H/D exchange.

These data indicate that multiple carboxylic acid residues, or the same residue in different states, respond to the photolysis of CO and cyanide in bovine oxidase. That changes in this region are complex may be suspected from the fact that very different signals have been observed when CO is photolyzed from oxidases derived from different organisms. The present data show that CO photolysis from the bovine enzyme affects three populations that undergo H/D exchange either easily, only with catalytic turnover, or not at all. Whether these represent different residues, or the same residue in different conformations, remains to be established. For those species that do not shift in D_2O media even with turnover, further studies are required to establish whether they arise from carboxylic acids that are difficult to exchange or from other species. A component of the 1749/1741 cm^{-1} feature of the CO photolysis spectrum is presumably also responsible for the 1752/1740 cm^{-1} component of the cyanide photolysis spectrum (Figure 3). However, two or more further carboxylic or propionic acid species may be responsible for the additional changes in the 1725−1698 cm^{-1} region of the cyanide photolysis spectrum.

Multiplicity of components in this region is also suggested by data on redox-induced FTIR changes of related oxidases. Initial studies identified a major feature attributable to the conserved glutamic acid equivalent to bovine E242 (*38*−*40*), but subsequent studies have indicated greater complexity. The most detailed report is that of Lübben et al. on cytochrome *bo* from *E. coli* (*41*). A 1735/1745 cm^{-1} peak/trough was observed in reduced *minus* oxidized difference spectra, which could be attributed from mutant studies to E286 (equivalent to bovine E242), together with weaker peaks at 1720 and 1704 cm^{-1} whose origin was not determined. After H/D exchange, the 1735 cm^{-1} was shifted down, whereas the 1745 cm^{-1} trough remained unchanged, and it was suggested that the proton on E286 could exchange only in the reduced enzyme. Carboxylic acid components additional to the components attributable to the equivalent of E242 are also evident in the redox difference spectra of oxidase from *P. denitrificans*, but again could not be assigned (*42*). Hellwig et al. (*43*) have noted multiple carboxylic acid features in the redox FTIR difference spectrum of bovine oxidase with two bands at 1748 and 1736 cm^{-1} that decrease on reduction. It is quite possible that the 1748 cm^{-1} band in the oxidized enzyme (*43*) and the 1749 cm^{-1} band associated with the reduced enzyme that is described in the present report are both attributable to E242, but with different extinction coefficients or degrees of protonation between the two states. It was suggested that the 1736 cm^{-1} band might arise from an aspartic acid, D51, that has been proposed from crystallographic data to undergo redox-induced protonation change (*44*). If the amplitude decreases on reduction are indeed due to protonation changes, then both D51 and E242 would have the unusual property of deprotonation on reduction.

Overall, therefore, it is apparent that this region of the oxidase FTIR spectra contains significant information on multiple carboxylic acid residues that respond to ligand and redox state changes. The conserved glutamic acid, E242, can confidently be implicated as a major contributor in both ligand and redox spectra. However, major questions remain as to whether it exists in several conformations that have different H/D exchange characteristics, as do questions as to the nature of the other residues that might produce signals in this region. Candidates include other carboxylic acids associated with the magnesium binding region above heme a_3 or heme propionic acids, all of which are reasonably close to the metal centers. However, more remote residues might also be considered, for example, other carboxylic acids associated with the 'D' channel (*3*, *45*) or around the conformationally flexible E51 of the bovine enzyme (*44*) or even residues in other subunits such as a glutamic acid residue in subunit II that has recently been implicated in K channel function (*46*).

The data here and elsewhere show that the pattern of responses of protonatable groups to ligand and redox changes in otherwise highly homologous oxidases may differ considerably. This in turn suggests that the detailed atomic mechanisms of proton translocation may not necessarily be identical in different oxidases. Such differences could well lead to variations in different oxidases as to which steps are coupled to proton translocation, even although the overall cycle remains 'robust' in being coupled to four proton translocations per cycle in all cases.

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