Time-Resolved FT-IR Studies on the CO Adduct of *Paracoccus denitrificans* Cytochrome *c* Oxidase: Comparison of the Fully Reduced and the Mixed Valence Form†

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ABSTRACT: The rebinding of CO to cytochrome *c* oxidase from *Paracoccus denitrificans* in the fully reduced and in the half-reduced (mixed valence) form as a function of temperature was investigated using time-resolved rapid-scan FT-IR spectroscopy in the mid-IR (1200−2100 cm^{-1}). For the fully reduced enzyme, rebinding was complete in approximately 2 s at 268 K and showed a biphasic reaction. At 84 K, nonreversible transfer of CO from heme a_3 to Cu_B was observed. Both photolysis at 84 K and photolysis at 268 K result in FT-IR difference spectra which show similarities in the amide I, amide II, and heme modes. Both processes, however, differ in spectral features characteristic for amino acid side chain modes and may thus be indicative for the motional constraint of CO at low temperature. Rebinding of photodissociated CO for the mixed-valence enzyme at 268 K is also biphasic, but much slower as compared to the fully reduced enzyme. FT-IR difference spectra show band features similar to those for the fully reduced enzyme. Additional strong bands in the amide I and amide II range indicate local conformational changes induced by electron and coupled proton transfer. These signals disappear when the temperature is lowered to 84 K. At 268 K, a difference signal at 1746 cm^{-1} is observed which is shifted by 6 cm^{-1} to 1740 cm^{-1} in ^2H_2O. The absence of this signal for the mutant Glu 278 Gln allows assignment to the COOH stretching mode of Glu 278, and indicates changes of the conformation, proton position, or protonation of this residue upon electron transfer.

Cytochrome *c* oxidase is the terminal enzyme in the respiratory chain of eukaryotes and many prokaryotes. It couples the transfer of four electrons for the reduction of O_2 to the generation of a transmembrane proton and charge gradient, which is the driving force for the synthesis of ATP in the cell. The formation of two water molecules during the catalytic cycle requires four protons ("consumed protons") which are taken up from the cytoplasmic side of the membrane. This process is coupled to the transfer of four protons across the membrane ("pumped protons"). Despite a large number of functional studies and the crystallographic characterization of the cytochrome *c* oxidase from the soil bacterium *Paracoccus denitrificans* (*1*) and of the bovine heart cytochrome *c* oxidase (*2*), the mechanism of coupling between electron and proton transfer is still not clear. Cytochrome *c* oxidase contains four metal centers involved in intramolecular electron transfer. The Cu_A center in subunit II, formed by two copper atoms with mixed charge ($Cu_A^{+1.5}$/$Cu_A^{+1.5}$) (*3*, *4*), is the first electron acceptor from which the electrons are transferred to heme *a* and further to the binuclear heme a_3/Cu_B metal center, which is capable of binding and reducing oxygen (for reviews, see refs *5*−*7*).

CO, CN^-, and others ligands compete with oxygen for its binding site to the heme a_3−Cu_B binuclear center. Binding of either of these ligands offers a convenient possibility to study the enzyme mechanism by inhibiting its catalytic cycle. These ligands can be photodissociated from the enzyme by an intense light flash which can photoexcite heme a_3. In contrast to oxygen, these ligands are IR-active and thus present excellent probes for spectroscopic investigations of cytochrome *c* oxidase. The negatively charged ligand CN^- was used to examine redox-linked conformational changes at the binuclear site (e.g., see refs *8*, *9*). But of the variety of possible ligands competing with oxygen, CO was most extensively used to probe the environment of the binuclear center. It tightly binds to the reduced heme a_3/Cu_B center and can be photolyzed with a quantum yield close to 100%. Ultrafast kinetic studies on the photolysis of CO in the visible and mid infrared spectral range (*10*, *11*, and references cited therein) have provided insight into the primary molecular steps of photodissociation and rebinding. In addition, the CO

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adduct of the cytochrome *c* oxidase has been studied with IR spectroscopy at cryogenic temperature (*12*−*15*).

The thermodynamically favored binding site for CO is the iron of heme a_3. However, it has been demonstrated that the binding of CO to heme a_3 proceeds via a CO adduct to Cu_B (*10*). After photoexcitation of heme a_3, the Fe−CO bond is broken and CO is transferred to Cu_B, which is only 4.5 Å away from the heme a_3 center. The stability of this intermediate depends on temperature and on CO concentration in the surrounding medium. At ambient and moderately low temperature, this Cu_B −CO adduct is in equilibrium with free CO in the bulk phase, whereas for the enzyme at temperatures below 140 K the Cu_B-bound CO can be trapped; only transfer of CO within the binuclear center from heme a_3 to Cu_B after photoexcitation is observed (*16, 17*). The Cu_B binding site is relevant not only for CO but also for the physiological ligand O_2 (*18*).

Dissociation and rebinding of CO from the fully reduced protein (Cu_A^+, heme a^{2+}, heme a_3^{2+}−CO, and Cu_B^+) should influence mainly the surrounding of the heme a_3/Cu_B binuclear site whereas the rest of the protein should remain essentially unaffected. IR studies on the ubiquinol oxidase cytochrome bo_3 from *E. coli* (*15*) have shown that the binding of CO to Cu_B leads to a perturbation of the conserved amino acid Glu 286 at low temperature.

In contrast to the fully reduced enzyme, the half-reduced CO-poisoned cytochrome *c* oxidase (Cu_A^{2+}, heme a^{3+}, heme a_3^{2+}−CO, and Cu_B^+), termed mixed valence enzyme, shows a different behavior: photodissociation of CO leads to a drop of the midpoint potential of heme a_3. As a consequence, electron transfer from heme a_3 to heme *a* and further on to Cu_A is observed (*19*). In the case of the aa_3 cytochrome *c* oxidase from *R. sphaeroides*, this internal electron transfer was described by three phases (*20*): First, fast electron flow from heme a_3 to heme *a* ($\tau \cong 3\ \mu s$) is observed, which leads to approximately 40% reduction of heme *a*. This is followed by electron transfer to Cu_A ($\tau \cong 30\ \mu s$). Finally, a pH-dependent electron transfer ($\tau \cong 3$ ms, pH = 9) from heme a_3 to heme *a* is coupled to proton release to the medium (*21, 22*). Consequently, the rebinding of CO to heme a_3 induces the back-reaction of electron transfer from heme *a*/Cu_A to heme a_3 as well as the reverse proton-transfer reactions. These processes concomitant with rebinding of CO can be observed in the millisecond time domain accessible by rapid-scan FTIR spectroscopy.

Iwata et al. proposed two proton pathways in the cytochrome *c* oxidase structure of *P. denitrificans* (*1*), and similar pathways were also found in the structure of the bovine heart enzyme (*23*). Key residues in the K-pathway include Lys 354, Thr 351, and Tyr 280 and for the D-pathway residues Glu 278 and Asp 124 (numbering according to the *P. denitrificans* cytochrome *c* oxidase). The specific role of these two pathways for "pumped" and "consumed" protons and the coupling to the different steps in the catalytic cycle is still under debate (*7*).

Glu 278 as part of the D-channel is located nearby the binuclear center. The oxygen of its carboxyl group is 12.3 Å away from the iron atom of heme a_3, and the substitution by glutamine inhibits the catalytic function of the enzyme (*24*). Recent studies from our groups on the *P. denitrificans* cytochrome *c* oxidase combining FT-IR[1] spectroscopy and protein electrochemistry have shown that Glu 278 is involved in the molecular processes concomitant with the electrochemical oxidation and reduction of the cytochrome *c* oxidase (*25*). Ädelroth et al. have recently reported (*26*) that substitution of Glu 286 by Gln in the cytochrome *c* oxidase from *R. sphaeroides* (according to Glu 278 in *P. denitrificans*) does not affect the kinetic parameters of the proton-coupled electron transfer from heme a_3 to heme *a* after photoexcitation of CO from the mixed valence form. It was thus concluded that this residue is not involved in proton release to the medium during this process.

The COOH mode of Glu 278 in the protonated form exhibits a convenient IR signature in a spectral region easily accessible and almost devoid of other overlapping modes. Its identification by site-directed mutations (*25*), most evident for the mutant Glu 278 Gln, provides the possibility to follow internal proton-transfer reactions which could involve this residue, either directly or indirectly by electrostatic effects or conformational changes. Here we used the fully reduced and mixed valence enzyme to study CO photodissociation and rebinding over a wide temperature range by time-resolved rapid-scan FT-IR spectroscopy. Evidence is provided that Glu 278 undergoes changes coupled to electron transfer between heme *a* and heme a_3 upon rebinding of CO to the mixed valence cytochrome *c* oxidase.

MATERIALS AND METHODS

Sample Preparation. Cytochrome *c* oxidase from *P. denitrificans* was prepared as described for the crystallization (*1, 27*). The Glu 278 Gln mutant was generated as detailed in (*24*), expressed in a *P. denitrificans* host strain, and purified as described in (*27*). The activity for the Glu 278 Gln mutant was found to be 2% of that of the wild type (*24*). For IR spectroscopy, the enzyme was taken up in 200 mM buffers with 3.2 mM *n*-decyl-*β*-D-maltopyranoside as detergent. At pH 7, a potassium phosphate buffer was used; for pH 5.3 and 8.7, cacodylate/HCl and sodium borate were used, respectively. For measurements in ^2H_2O, the protein was taken up in the corresponding ^2H_2O buffer and kept for at least 1 week at 4 °C, concentrated, and rediluted in the ^2H_2O buffer several times. The extent of $^2H/^1H$ exchange was verified by the shift of the amide II band in the IR absorption spectra and was found to be better than 70% (data not shown).

The fully reduced enzyme was prepared by adding an excess of a potassium phosphate-buffered $Na_2S_2O_4$ solution (pH 7) under an atmosphere of Ar and waiting for at least 2 h. After reduction, the cytochrome *c* oxidase was poisoned by exposing the sample for several minutes to a CO gas stream. The partially reduced protein can be prepared by exposing the protein solution for at least 2 h to CO gas (*28*). Upon oxidizing CO to CO_2, the enzyme becomes reduced to the mixed valence form. To obtain a higher concentration of the mixed valence form, the reduction was done by addition of small amounts of $Na_2S_2O_4$ and poisoning the sample with CO gas afterward. The partially reduced enzyme is stabilized by bound CO. Both preparation methods result in nearly the same state of the enzyme; reduction with $Na_2S_2O_4$ may lead to a small part of fully reduced enzyme, which is not significant, however, for the resulting spectra

[1] Abbreviation: FT-IR, Fourier transform infrared.

(see below). The samples were transferred under argon into a gastight thin-layer CaF_2 cell; the final concentration of the solution was in the range of 0.5−1 mM. The path length of the cell was chosen to be between 5 and 8 μm for H_2O samples and 15 μm for ^2H_2O samples. The oxidation states were determined by size and position of the Soret band and α-band in the visible spectral range and by analysis of the position of the CO peaks in the IR difference spectra (*29*).

Spectroscopy. FT-IR spectra were recorded with a modified Bruker IFS-66 spectrometer which was used in the rapid-scan mode in the spectral range from 1200 to 2100 cm^{-1}. This enables a time window from <30 ms up to several minutes with a spectral resolution of 4 cm^{-1}. The instrument was purged with dry air to avoid contributions of water vapor absorption to the spectra. For photoexcitation at 532 nm, a 7 ns, 100 mJ pulse of a frequency-doubled ND-YAG laser (Spectron Laser Systems SL454G) was used which was triggered by the mirror movement of the spectrometer. Photolysis and rebinding at 268 K were repeated several hundred times, and the resulting data were averaged to obtain a sufficient signal/noise ratio. Low-temperature experiments at 83 K, where no CO rebinding can be seen after photoexcitation, were performed with a home-built liquid N_2-cooled cryostat. Difference spectra (after flash minus before flash) were recorded at 84 K, and the sample was warmed to room temperature to allow rebinding of CO to heme a_3. The enzyme was stable enough to repeat this procedure several times. The IR spectrometer was additionally equipped with a home-built UV−vis−NIR spectrometer [based on a Zeiss MMS1 charge-coupled-device (CCD) module for the spectral range from 310 to 1130 nm] which allowed the control of the oxidation state of the enzyme in situ by UV−vis spectroscopy. All shown IR spectra are difference spectra of the photodissociated protein **after** the laser flash minus the CO-bound enzyme **before** the flash. This results in positive bands corresponding to the state after photolysis and negative bands corresponding to the CO-bound form.

Data Processing. IR data were collected, averaged, and further processed with the spectroscopy software Opus from Bruker. Overlapping CO modes were resolved by deconvolution and second-derivative spectra; peak shapes were assumed to be a mixture of Gaussian and Lorentzian functions. Kinetic data were collected by integration of single bands or overlapping peaks and fitting the results with the commercial program Origin (Microcal Software Inc.).

RESULTS AND DISCUSSION

Fully Reduced Enzyme at 268 and 84 K. The difference spectra at 268 K of the fully reduced enzyme (cf. Figure 1) show at least three different heme-bound CO stretching frequencies. A small shoulder at 1975.0 cm^{-1} and the two main bands at 1965.5 and 1955.5 cm^{-1} correspond to the so-called α- and β-forms recently described (*17*, *30*, *31*). At the high concentration of the sample used (0.5−1 mM), a precise determination of the pH value of the sample is difficult because of the buffering groups of the enzyme. Nevertheless, it was found that the β-form (CO mode at 1955.5 cm^{-1}) is favored at low pH (<7), whereas the α-form (CO mode at 1965.5 cm^{-1}) becomes dominant at higher pH (data not shown). This is in accordance with the pH dependence of the CO adduct to the aa_3 cytochrome *c* oxidase from *R. sphaeroides* previously described (*31*). The different stretching frequencies of the CO mode should correspond to different conformers of the heme−copper pocket. The rebinding of CO to heme a_3 reveals biphasic kinetics; identical parameters are observed for the most prominent bands in the difference spectrum at 268 K. Two representative time courses, one for CO bound to the α-form (1965.5 cm^{-1}) and one for the major signals in the amide I spectral region (1662.0 cm^{-1}), are shown in Figure 4A. The presence of a common time constant for CO rebinding and signals corresponding to heme/amide I perturbation indicates that under these conditions CO rebinding and the corresponding structural changes occur in a concerted action, with all local structural changes of the enzyme adapting rapidly to the CO-bound form. The entire reaction is complete after approximately 2 s.

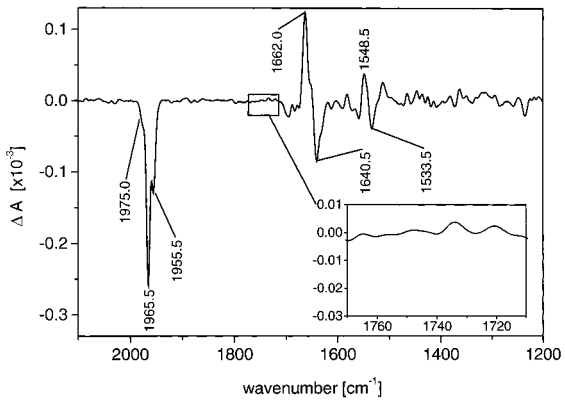

FIGURE 1: Difference spectrum of the fully reduced WT cytochrome *c* oxidase at 268 K (^1H_2O solution, pH 7) averaged over a time period from 33 to 260 ms after photolysis. Inset: enlarged view for the spectral range from 1710 to 1770 cm^{-1}.

The situation is different at temperatures around 200 K (data not shown). The different conformers of the CO adduct reveal different kinetic parameters for the rebinding which can be separated within the time resolution of rapid-scan FT-IR spectroscopy.

At 268 K the Cu_B−CO intermediate has a lifetime in the range of microseconds (*32*) which is not detectable due to the time resolution of 30 ms. In samples with a higher CO concentration in the medium, the equilibrium between free and Cu_B-bound CO is shifted toward the bound state, and the spectra reveal a small positive CO stretching band at 2062 cm^{-1} corresponding to the α species (data not shown). According to that, the modes in the difference spectra correspond not only to a change in the position of CO within the binuclear center but also to the dissociation of CO from the protein into the solution. Bands of CO in the bulk phase could be detected, since they are considerably broadened and weak.

Photolysis at cryogenic temperature (Figure 2) results in the binding of CO to Cu_B but not in dissociation of CO from the protein. At least two positive peaks at 2061.0 and 2038.0 cm^{-1} indicate CO bound to Cu_B.

Figure 3 shows a comparison of the different CO modes at three temperatures, 84, 183, and 268 K. The amplitudes of the bands attributed above to the α- and β-forms indicate that the α-to-β-ratio is dependent not only on the pH of the system, but also on the temperature. The intensities of the modes obtained in the time-resolved measurements of liquid

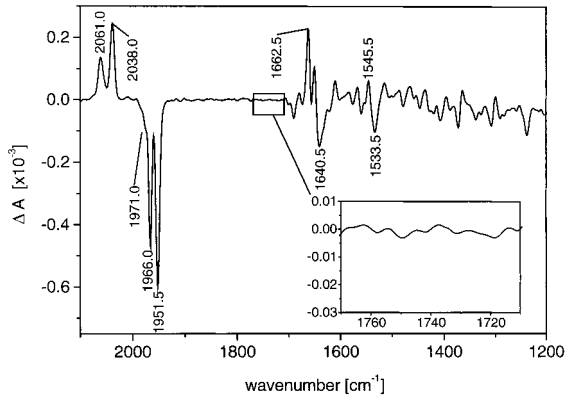

FIGURE 2: Difference spectrum (after flash minus before flash) of the fully reduced WT cytochrome *c* oxidase at 84 K (^1H_2O solution, pH 7). Inset: enlarged view for the spectral range from 1710 to 1770 cm^{-1}.

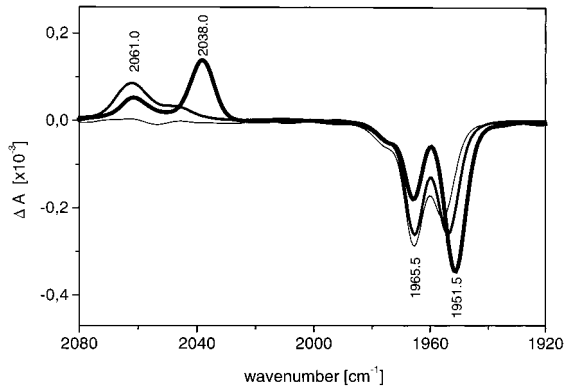

FIGURE 3: CO modes of the fully reduced WT cytochrome *c* oxidase (^1H_2O solution, pH 7) at different temperatures in the range from 1920 to 2080 cm^{-1}. Thick line: spectrum at 84 K; main CO modes are at 2061.0, 2038.0, 1965.5, and 1951.5 cm^{-1}. Medium line: spectrum at 183 K; main CO modes are at 2061.0, 1965.5, and 1953.0 cm^{-1} with a shoulder at 2045.0 cm^{-1}. Thin line: spectrum at 268 K; main CO modes are at 1965.5 and 1955.5 cm^{-1}.

samples at 268 K are not directly comparable to those obtained from frozen samples at 84 and 183 K. However, the ratio of the α-form to the β-form (see above) is changed in favor of the β-form at low temperature. Due to the temperature independence of the band position, the mode at 1965.5 cm^{-1} seems to be caused by a more rigid environment of the CO binding site, whereas that at 1955.5 cm^{-1} seems to belong to more flexible conformers. This CO mode is strongly temperature-dependent; the position of the 1955.5 cm^{-1} mode at 268 K shifts to 1953.0 cm^{-1} at 183 K and to 1951.5 cm^{-1} at 84 K.

The band with a maximum at 2061.5 cm^{-1} is independent of temperature as is the mode at 1965.5 cm^{-1}. These two modes thus seem to belong to the same conformer. On the other hand, the 2038.5 cm^{-1} peak seems to be associated with the one at 1955.5 cm^{-1}; its size and position is also strongly dependent on temperature; it shifts from 2038.0 cm^{-1} at 84 K to a small shoulder at 2045.0 cm^{-1} at 183 K.

To obtain information on possible conformational changes in the course of CO photolysis and rebinding, as well as on the role of individual amino acid side chains, an analysis of the spectral region from 1800 cm^{-1} to approximately 1200 cm^{-1} is necessary. In contrast to earlier IR studies of CO photolysis from oxidases, where analysis was limited to the 2200−1800 cm^{-1} range, the entire frequency range from 1200 to 2200 cm^{-1} is equally accessible, even in the range of strong water absorbance around 1650 cm^{-1}. This is due to the small path length set to approximately 5−8 μm in order to keep the absorbance around 1650 cm^{-1} (predominantly water O−H deformation and amide I modes) below 1. Path lengths up to 50 μm as used in previous work would result in a higher noise level and uncertainties in band amplitudes in this range. In the spectra presented here, even minor bands have been found to be highly reproducible.

In the spectral region from approximately 1700 to 1750 cm^{-1}, the CO modes from protonated Asp and Glu side chains provide signatures for protonation and deprotonation processes as well as for perturbation of COOH groups by changes of the local environment and/or interactions (*25*, *33*, *34*). In a recent publication on CO photolysis at low temperature from the bo_3 oxidase from *E. coli* (*15*), a difference band at 1724 cm^{-1}/1731 cm^{-1} in ^2H_2O was assigned to the conserved amino acid Glu 286 (*P. denitrificans*: Glu 278). This difference signal was interpreted as a perturbation due to environmental change upon CO photolysis. In the low-temperature photolysis of *P. denitrificans* cytochrome *c* oxidase (Figure 2), no signal of comparable amplitude is observed in this range. The absence of a signal in this spectral range that could be attributed to a protonation/deprotonation process or to the perturbation of a protonated group thus points to a clear difference between the *E. coli* ubiquinol oxidase and the *P. denitrificans* cytochrome *c* oxidase: In the **fully reduced** form, both at cryogenic temperature and at 268 K in the millisecond time domain analyzed here, the impact of CO rebinding on Glu 278 and other Glu/Asp residues in the *P. denitrificans* cytochrome *c* oxidase is negligible.

Mixed Valence Enzyme at 268 and 84 K. The mixed valence form of the cytochrome *c* oxidase reveals a different behavior (cf. Figure 5). In contrast to the fully reduced protein, there is only a single heme a_3-bound CO stretching frequency at 1968.0 cm^{-1} corresponding to only a single conformer. The slightly higher frequency of the CO stretching mode, in comparison to the fully reduced enzyme, is in accordance with investigations on the redox dependence of the heme-bound CO mode for the bovine heart cytochrome *c* oxidase (*29*). The position of the band is independent of pH and temperature.

The preparation of the mixed valence form by addition of $Na_2S_2O_4$ can lead to a minor part of fully reduced protein. This is indicated by a small shoulder at around 1955 cm^{-1} (cf. Figure 5), which corresponds to the CO mode at 1955 cm^{-1} in Figure 1. The possibility that this fraction of fully reduced enzyme might lead to additional bands in the spectra was tested by decomposition of the band structure at 1968 cm^{-1} and the small shoulder at approximately 1955 cm^{-1}. Weighted subtraction of a spectrum of the fully reduced enzyme produces a clean, single 1968 cm^{-1} band. Due to the small amount of fully reduced cytochrome *c* oxidase, however, the difference spectrum of the mixed valence cytochrome *c* oxidase is essentially unaffected in the range from 1200 to 1800 cm^{-1} (data not shown).

Rebinding of CO is much slower as compared with the fully reduced oxidase, but also reveals a biphasic kinetic behavior (cf. Figure 4B). A global analysis of the time course for all bands with sufficient signal/noise ratio reveals identical kinetic parameters for all bands. Rebinding is

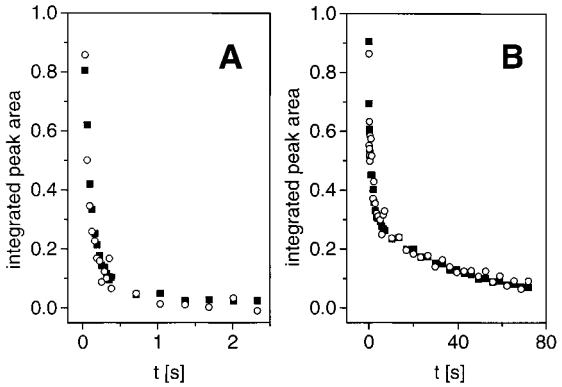

FIGURE 4: Time course for the intensities of a CO mode and corresponding modes in the range between 1200 and 1800 cm^{-1} at 268 K with WT cytochrome *c* oxidase (^1H_2O solution, pH 7). (A) Integrated peak area of the CO mode at 1965.5 cm^{-1} (solid squares) and the mode at 1662.0 cm^{-1} (open circles) from the fully reduced form. (B) Integrated peak area of the CO mode at 1968.0 cm^{-1} (solid squares) and the mode at 1660.0 cm^{-1} (open circles) from the mixed valence form.

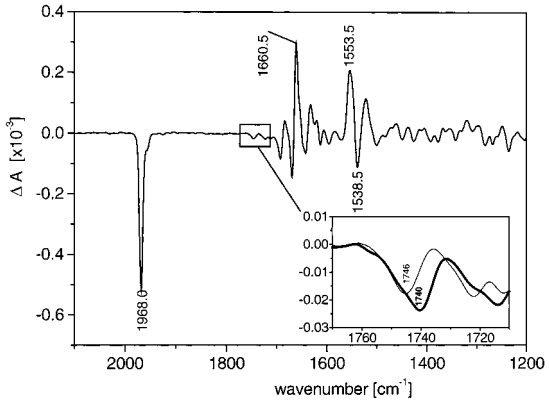

FIGURE 5: Difference spectrum of the mixed valence WT cytochrome *c* oxidase at 268 K (^1H_2O, pH 7) averaged over a time period from 0.3 to 3.6 s. Inset: comparison between the difference spectra of the enzyme in ^1H_2O (thin line) and ^2H_2O (thick line) solution in the range from 1710 to 1770 cm^{-1}.

complete after approximately 90 s at 268 K. For the mixed valence form, no signal is observed above 2000 cm^{-1} which could be assigned to the Cu_B-bound CO in the spectra recorded at 268 K. At this temperature, the lifetime of the Cu_B-CO form is clearly too short to be detected with the millisecond time resolution used here.

The reversible dissociation of CO after photoexcitation leads to a drop of the midpoint potential of heme a_3 and therefore to electron transfer from heme a_3 to heme *a* and Cu_A combined with proton release to the medium (*19*). Rebinding of CO induces the reverse electron transfer and, as a consequence, leads to the uptake of a proton (*21, 22*).

The FT-IR difference spectrum at 268 K obtained as a consequence of this electron transfer upon CO rebinding (Figure 5) is completely different from that of CO rebinding for the fully reduced enzyme (cf. Figure 1) under otherwise identical conditions. While the latter exhibits only one major signal in the wavenumber range from 1600 to 1700 cm^{-1} where amide I and heme modes might dominate, the difference spectrum of the mixed valence enzyme exhibits a multitude of bands in this range as well as in the amide II range (approximately 1520−1560 cm^{-1}). It closely resembles the electrochemically induced FT-IR difference spectra of

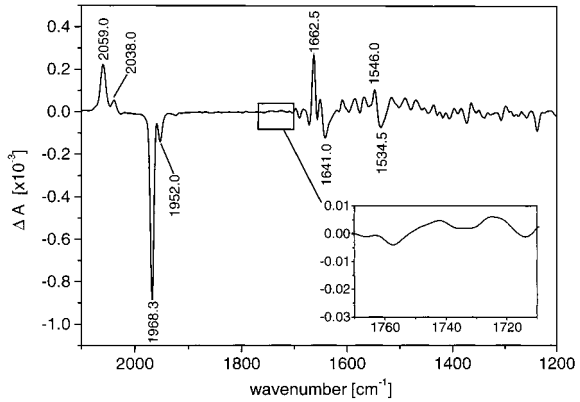

FIGURE 6: Difference spectrum (after flash minus before flash) of the mixed valence WT cytochrome *c* oxidase at 84 K (^1H_2O, pH 7).

the reduction of the *P. denitrificans* cytochrome *c* oxidase recently reported by us (*25, 33, 35*). A detailed analysis of this difference spectrum and a comparison with electrochemically induced difference spectra will be given in a future manuscript.

In the spectral region above 1700 cm^{-1}, where the difference spectra of fully reduced enzyme at 268 and at 84 K were devoid of bands (see insets of Figure 1 and Figure 2), a clear negative signal at 1746 cm^{-1} is observed at 268 K. This signal is shifted in ^2H_2O with almost equal intensity to 1740 cm^{-1} (cf. inset of Figure 5), a shift which is typical for a carboxyl group of an Asp/Glu side chain. In contrast to the electrochemically induced difference spectra (*25*), where difference signals with a positive lobe at 1733 cm^{-1} and a negative lobe at 1745 cm^{-1} are observed for the reduction of the cytochrome *c* oxidase, only a negative difference signal is observed here. The signal observed for low-temperature CO photodissociation of the fully reduced *E. coli* ubiquinol oxidase also consists of a negative and a positive difference signal (*15*). The presence of only a negative signal in the *P. denitrificans* spectra presented here suggests the loss of COOH absorbance, either by significant change of the extinction coefficient of a COOH group or by deprotonation of a side chain group. We thus conclude that upon CO photolysis in the mixed valence enzyme, at least one COOH group responds to the electron-transfer processes following CO photolysis.

At temperatures below 260 K, at which the protein can be expected to be in a more rigid state, the band at 1746 cm^{-1} disappears, and the difference spectrum of the mixed valence cytochrome *c* oxidase at 84 K (cf. Figure 6) becomes almost identical to that of the fully reduced cytochrome *c* oxidase at the same temperature (cf. Figure 2). We conclude from this that at low temperature not only proton transfer is inhibited, but also essential changes in the protein environment coupled to electron transfer between heme a_3 and heme *a* are suppressed.

In the electrochemically induced FT-IR difference spectra of *P. denitrificans* cytochrome *c* oxidase, Hellwig et al. (*25*) have assigned a difference signal at 1746 and 1734 cm^{-1} as arising predominantly from the C═O stretching mode of Glu 278. The analogous group in the *E. coli* ubiquinol oxidase, Glu 286, was identified by Puustinen et al. as the group which was perturbed upon low-temperature CO photolysis of the fully reduced enzyme.

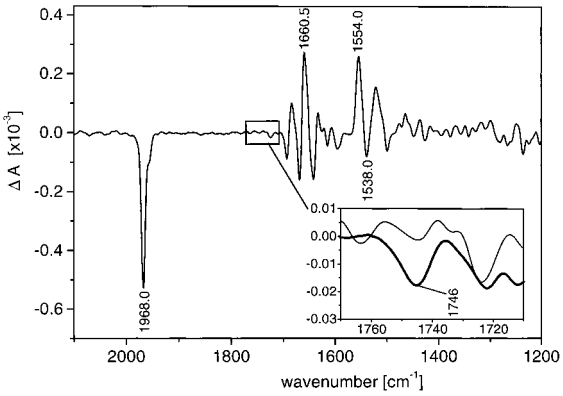

FIGURE 7: Spectrum of the Glu 278 Gln mutant at 268 K (^1H_2O, pH 7) averaged over a time period from 0.3 to 3.6 s. Inset: comparison between the Glu 278 Gln spectrum (thin line) and the corresponding WT spectrum at 286 K (thick line, cf. Figure 5).

The difference spectrum induced by CO photolysis of the mixed valence form of the *P. denitrificans* Glu 278 Gln mutant is shown in Figure 7. The C═O stretching mode peaking at 1968 cm^{-1} is at the same position as in the WT enzyme, with almost identical shoulder structure pointing to a small fraction of fully reduced enzyme (for a discussion, see above). This strongly supports the view that this mutation has no impact on the local conformation of the binuclear site and thus on the CO binding properties. Further support for this view comes from the kinetic analysis of CO rebinding: almost identical parameters are observed for the WT and mutant enzymes (data not shown).

The FT-IR difference spectrum of the mutant enzyme (cf. Figure 7) is essentially identical to that of the WT. Small deviations of band amplitudes originate from the lower signal/noise ratio in the spectrum of the mutant enzyme due to the lower concentration of the samples. The difference spectra were normalized to the same C═O stretching frequency absorption at 1968 cm^{-1}. The inset in Figure 7 shows an enlarged view of the 1710−1770 cm^{-1} range. The dominating peak in the spectrum of the WT enzyme has almost completely disappeared for the mutant enzyme, while the negative signal at 1722 cm^{-1} appears at comparable amplitude, though sharper and at a slightly upshifted frequency.

Ädelroth et al. report the absence of an influence of the Glu 286 Gln mutation on the kinetic properties of the electron transfer from heme a_3 to heme *a* and to the coupled proton release after CO photolysis from the mixed valence *R. sphaeroides* cytochrome *c* oxidase (*26*). Electrostatic calculations also indicate that Glu 278 remains protonated at various redox states (*36*). It would be tempting to assume, on the basis of the data presented here, that Glu 278 is directly or indirectly involved in proton release upon CO photolysis from the mixed valence cytochrome *c* oxidase. However, in view of the data presented by Ädelroth et al. (*26*) and of calculations reported by Kannt et al. (*36*), an alternative interpretation seems more likely for the change of intensity of the signal at 1746 cm^{-1}.

Recently, it has been suggested that Glu 278, whose COOH group is nearly equidistant to the heme *a* and heme a_3 irons, might sense the charge difference between the heme *a* iron and the binuclear site (*37*). Upon electron back-transfer from heme a_3 to heme *a* following CO photolysis of the mixed valence cytochrome *c* oxidase, the binuclear site loses one negative charge, and the heme *a* iron gains one. Since a COOH group, in contrast to a COO^- group, must possess an electric dipole moment along the axis connecting both carboxylate oxygen atoms, one might expect that the COOH group reorients itself in the altered electric field in a way that the proton is preferentially positioned closer to the heme *a* iron after electron back-transfer. This would be most easily accomplished by a rotation around the $C_\gamma-C_\delta$ bond of the Glu 278 side chain, even without further conformational changes. Rotations around the $C_\gamma-C_\delta$ bond also could lead to a repositioning of the proton position toward an energetically more favorable position. Such changes of the preferred proton position would not be expected upon CO photolysis of the fully reduced enzyme, since no electron redistribution occurs, and not at the very low temperatures, since these rotations would be frozen. This explanation is therefore in full agreement with the experimental observations. Residual R−COOH signals in the difference spectrum of the mutant enzyme can be clearly identified; however, we refrain from discussing these signals at the present stage of our analysis.

CONCLUSIONS

Investigation of CO photolysis and rebinding for the fully reduced and for the mixed valence cytochrome *c* oxidase allows the analysis of two essentially different processes: In the case of the fully reduced cytochrome *c* oxidase, CO moves within the binding site and is released from the enzyme at higher temperature. Both processes, CO rebinding at 268 K and at cryogenic temperature, result in FT-IR difference spectra that are similar with regard to the amide I, amide II, and heme modes, but essentially differ as to indicate the motional constraints of the CO molecule at low temperature. Neither at low nor at ambient temperature is participation of Asp or Glu side chains indicated.

In the case of the mixed valence cytochrome *c* oxidase, photodissociation of CO initiates "reverse" electron transfer; proton release to the medium is coupled to this reaction (*21*, *22*). Our data clearly indicate that this reverse electron transfer causes changes at a Glu or Asp residue. This is illustrated by a difference signal at 1746 cm^{-1}, shifted to 1740 cm^{-1} in ^2H_2O. The mutant enzyme Glu 278 Gln clearly shows the absence of this signal and thus allows identification of this residue as being responsible for this signal. Taking into account the data of the kinetics of proton release (*22*) and the electrostatic calculations (*36*), the balance of evidence suggests as a possibility a repositioning of the carboxylate proton in response to changes of the local electric fields rather than proton release, because proton release is still observed in a related Glu to Gln mutant enzyme.

At low temperature, rotations leading to a proton repositioning are most likely frozen, resulting in the absence of the signal assigned to Glu 278. At cryogenic temperature, the difference spectra of mixed valence cytochrome *c* oxidase resemble those of the fully reduced cytochrome *c* oxidase. Further investigations on mutants and at various temperatures are in progress to determine the mechanism and the kinetic and thermodynamic parameters of this electron/proton coupling.

REFERENCES

1. Iwata, S., Ostermeier, C., Ludwig, B., and Michel, H. (1995) *Nature 376*, 660−669.
2. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itho, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1995) *Science 269*, 1069−1074.
3. Antholine, W. E., Kastrau, D. H., Steffens, G. C., Buse, G., Zumft, W. G., and Kroneck, P. M. (1992) *Eur. J. Biochem. 209*(3), 875−881.
4. Steffens, G. C., Soulimane, T., Wolff, G., and Buse, G. (1993) *Eur. J. Biochem. 213*(3), 1149−57.
5. Babcock, G. T., and Wikström, M. (1992) *Nature 356*, 301−309.
6. Ferguson-Miller, S., and Babcock, G. T. (1996) *Chem. Rev. 96*, 2889−2907.
7. Michel, H., Behr, J., Harrenga, A., and Kannt, A. (1998) *Annu. Rev. Biophys. Biol. Struct. 27*, 329−356.
8. Tsubaki, M., and Yoshikawa, S. (1993) *Biochemistry 32*, 164−173.
9. Moody, J., von Germar, F., Mäntele, W., and Rich, P. R. (1995) *Biochem. Soc. Trans*., *23*, 521−524.
10. Einarsdottir, Ó., Dyer, R. B., Lemon, D. D., Killough, P. M., Hubig, S. M., Atherton, S. J., López-Garriga, J. J., Palmer, G., and Woodruff, W. H. (1993) *Biochemistry 32*, 12013−12024.
11. Georgiadis, K. E., Jhon, N.-I., and Einarsdottir, Ó. (1994) *Biochemistry 33*, 9245−9256.
12. Alben, J. O., Moh, P. P., Fiamingo, F. G., and Altschuld, R. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 234−237.
13. Mitchell, D. M., Müller, J. D., Gennis, R. B., and Nienhaus, U. G. (1996) *Biochemistry 35*, 16782−16788.
14. Park, S., Pan, L.-P., Chan, S. I., and Alben, J. O. (1996) *Biophys. J. 71*, 1036−1047.
15. Puustinen, A., Bailey, J. A., Dyer, R. B., Mecklenburg, S. L., Wikström, M., and Woodruff, W. H. (1997) *Biochemistry 36*, 13195−13200.
16. Fiamingo, F. G., Altschuld, R. A., and Alben, J. O. (1986) *J. Biol. Chem. 261*, 12976−12987.
17. Fiamingo, F. G., Altschuld, R. A., Moh, P. P., and Alben, J. O. (1982) *J. Biol. Chem. 257*, 1639−1650.
18. Oliveberg, M., and Malmström, B. G. (1992) *Biochemistry 31*, 3560−3563
19. Hallen, S., Brzezinski, P., and Malmström, B. G. (1994) *Biochemistry 33*, 1467−1472.
20. Ädelroth, P., Brzezinski, P., and Malmström, B. G. (1995) *Biochemistry 34*, 2844−2849.
21. Mitchell, R., and Rich, P. R. (1994) *Biochim. Biophys. Acta 1186*, 19−26.
22. Ädelroth, P., Sigurdson, H., Hallen, S., and Brzezinski, P. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 12292−12297.
23. Tsukihara, T., Aoyama, H., Yamashita, E., Tomizaki, T., Yamaguchi, H., Shinzawa-Itho, K., Nakashima, R., Yaono, R., and Yoshikawa, S. (1996) *Science 272*, 1136−1144.
24. Pfitzner, U., Odenwald, A., Ostermann, T., Weingard, L., Ludwig, B., and Richter, O.-M. H. (1998) *J. Bioenerg. Biomembr. 30*, 89−97.
25. Hellwig, P., Behr, J., Ostermeier, C., Richter, O.-M. H., Pfitzner, U., Odenwald, A., Ludwig, B., Michel, H., and Mäntele, W. (1998) *Biochemistry 37*, 7390−7399.
26. Ädelroth, P., Svensson Ek, M., Mitchell, D. M., Gennis, R. B., and Brzezinski, P. (1997) *Biochemistry 36*, 13824−13829.
27. Kleymann, G., Ostermeier, C., Ludwig, B., Skerra, A., and Michel, H. (1995) *Bio/Technology 13*, 155−160.
28. Brzezinski, P., and Malmström, B. G. (1985) *FEBS Lett. 187*, 111−114.
29. Dodson, E. D., Zhao, X.-J., Caughey, W. S., and Elliott, C. M. (1996) *Biochemistry 35*, 444−452.
30. Wang, J., Takahashi, S., Hosler, J. P., Mitchell, D. M., Ferguson-Miller, S., Gennis, R. B., and Rousseau, D. L. (1995) *Biochemistry 34*, 9819−9825.
31. Mitchell, D. M., Shapleigh, J. P., Archer, A. M., Alben, J. O., and Gennis, R. B. (1996) *Biochemistry 35*, 9446−9450.
32. Dyer, R. B., Einarsdottir, Ó., Killough, P. M., López-Garriga, J. J., and Woodruff, W. H. (1989) *J. Am. Chem. Soc. 111*, 7657−7659.
33. Hellwig, P., Rost, B., Kaiser, U., Ostermeier, C., Michel, H., and Mäntele, W. (1996) *FEBS Lett. 385*, 53−57.
34. Lübben, M., and Gerwert, K. (1996) *FEBS Lett. 397*, 303−307.
35. Hellwig, P., Grzybek, S., Behr, J., Ludwig, B., Michel, H., and Mäntele, W. (1999) *Biochemistry 38*, 1685−1694.
36. Kannt, A., Lancaster, C. R. D., and Michel, H. (1998) *Biophys. J. 74*, 708−721.
37. Michel, H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 12819−12824.

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