The Role of the Cross-Link His-Tyr in the Functional Properties of the Binuclear Center in Cytochrome c Oxidase

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Running title: Properties of the binuclear center of cytochrome c oxidase.

ABBREVIATIONS: CcO= Cytochrome c oxidase; MV= mixed-valence; RR= resonance Raman; FTIR= Fourier transform infrared

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ABSTRACT

Resonance Raman and Fourier transform infrared spectroscopies have been used to study the $aa_3$-type cytochrome $c$ oxidase and the Y280H mutant from Paracoccus denitrificans. The stability of the binuclear center in the absence of the Y280-His276 cross-link is not compromised since heme $a_3$ retains the same proximal environment, spin, and coordination state as in the wild type enzyme in both the oxidized and reduced states. We observe two C-O modes in the Y280H mutant at 1966 and 1975 cm$^{-1}$. The 1975 cm$^{-1}$ mode is assigned to a $\gamma$-form and represents a structure of the active site in which CuB exerts a steric effect on the heme $a_3$-bound CO. Therefore, the role of the cross link is to fix CuB in a certain configuration and distance from heme $a_3$, and not to allow histidine ligands to coordinate to CuB rather than to heme $a_3$, rendering the enzyme inactive, as proposed recently (1998 Biochemistry 37, 14471-14476). The results provide solid evidence that in the Y280H mutant the catalytic site retains its active configuration that allows $O_2$ binding to heme $a_3$. Oxygenated intermediates are formed by mixing oxygen with the CO-bound mixed-valence wild type and Y280H enzymes with similar Soret maxima at 438 nm.
Cytochrome c oxidase (CcO) couples the one-electron oxidation of cytochrome c to the four-electron reduction of molecular oxygen and links these electron transfers to proton translocation across the inner mitochondrial membrane, or the bacterial cytoplasmic membrane, respectively (1-6). The enzyme contains four redox-centers; two hemes \( a \) and three associated copper atoms. Electron injection from cytochrome c to the homo-dinuclear copper center, \( \text{Cu}_A \), is followed by intramolecular electron transfer, via the low-spin heme \( a \), to the binuclear center which contains a high spin heme \( a_3 \) and a \( \text{Cu}_B \) atom. The latter two species serve as the catalytic site where \( \text{O}_2 \) is reduced to \( \text{H}_2\text{O} \). The free energy released in the electron-transfer reactions is conserved as an electrochemical proton gradient across the inner mitochondrial membrane and is used ultimately for ATP synthesis.

The crystal structures of mammalian CcO (7-9) and of the soil bacterium *Paracoccus denitrificans* (10-11) have been determined providing deep insight in the structural properties of the enzyme. The properties of the binuclear center are of particular importance, since the heme \( a_3/\text{Cu}_B \) center is the site where dioxygen reduction takes place and is the most probable site of the proton-electron coupling (2). One of the unique properties of the binuclear center that were determined by the crystal structure is the covalent link of Y280 with one of the three histidine ligands of \( \text{Cu}_B \), namely H276. (If not stated otherwise, we adopt the residue numbering of *P. denitrificans*). This specific tyrosine which is located at the end of the proton K-channel is highly conserved among the heme-copper oxidases and since its discovery it has been proposed to posses an important structural as well as functional role. On the basis of the properties of the
Y→F mutant of *Rh. sphaeroides*, analyzed by resonance Raman spectroscopy (RR), Rousseau and co-workers (12) proposed that the tyrosine-histidine cross-linking stabilizes the binuclear center. They suggested that in the absence of the His-Tyr cross-link one of the histidines normally bound to CuB coordinates to the heme $a_3$, leaving the binuclear center severely disrupted, and rendering the enzyme inactive. Based on the crystal structure of bovine CcO, Yoshikawa and co-workers (9) proposed a proton transfer mechanism from this tyrosine to ferric peroxide to generate a hydroperoxo adduct, and subsequently the electron transfer from CuB$^{1+}$ via the cross-link would cleave the O-O bond of the ferric hydroperoxide. Moreover, other groups have suggested that the tyrosine can serve as a hydrogen atom donor during the cytochrome oxidase/O$_2$ reaction (3-4,6,11,13). Recently, Babcock and co-workers (14) proposed that Tyr280 is the source for both the proton and the electron required in the O-O bond cleavage, and Michel and coworkers (15) proposed that the EPR signal ($g_{iso} \approx 2.0055$) they observed in the cytochrome $aa_3$ H$_2$O$_2$ (*P. denitrificans*) reaction originated from the cross-linked tyrosine.

Structural information of the heme-CuB center have been determined from studies of the CO-bound adducts (16-28). In addition to revealing insights concerning the electronic and steric nature of the heme pocket, CO photodissociation studies provided a powerful tool for studying the dynamics and coordination chemistry in the heme-CuB pocket after CO photolysis (29-34). RR spectroscopy is a well-adapted technique in the study of terminal oxidases, as it enables us to selectively enhance the vibrational modes of the hemes without interference from the protein...
matrix and thus identify their oxidation, spin and ligation state by using established marker bands (35-41). Both RR and FTIR spectroscopy have been employed in the study of the carbon monoxy derivatives of cytochrome $aa_3$. The vibrational frequencies of the FeCO unit obtained by the two spectroscopic techniques have been identified in different types of heme-copper oxidases, revealing different conformations of the active site (16-23). The two major conformers found are termed as $\alpha$- and $\beta$-forms and although their functional significance and the origin for the splitting have not been established, it has been demonstrated that in the $\alpha$ form the frequencies of the $\nu$(Fe-CO) and $\nu$(C-O) deviate from the inverse linear curve that exists between the frequencies of these two modes in histidine-coordinated heme proteins, while in the $\beta$ form those frequencies are placed on the curve (17, 22-28). Recently, from the observed pH-dependent conformational changes in the binuclear site it was postulated that the different structures result from a change in the position of the Cu$_B$ atom with respect to the CO due to the presence of one or more ionizable groups (23). Furthermore, it was suggested that the possible candidates are the cross-linked, conserved tyrosine that is adjacent to the oxygen-binding pocket or one of the histidines that coordinate Cu$_B$.

In an effort to gain additional information on the role of Y280 in the catalytic function of CcO we have characterized the wild type and histidine mutant (Y280H) of CcO from $P.\text{denitrificans}$ in the oxidized, reduced and CO-bound forms by optical absorption, RR, and FTIR spectroscopies. Our studies show that the Cu$_B$ modification by the Y280H mutant results in only slight perturbation of the formyl group of heme $a_3$. Therefore, the role of the cross link is not to allow His276 (His240 in bovine, His284 in $Rb.\text{sphaeroides}$) to coordinate to Cu$_B$ instead of the...
heme iron atom, as previously suggested (12), but to hold CuB in a certain configuration and distance from heme $a_3$. Without the cross-linking of Y280 and H276, the heme pocket retains its active configuration that allows O$_2$ binding to heme $a_3$. Upon direct mixing of O$_2$ to the CO-bound mixed-valence wild-type and Y280H enzymes, oxygenated species with similar Soret maxima at 438 nm are formed, which decay to the resting form of the enzymes.

**EXPERIMENTAL PROCEDURES**

Wild type and mutant CcO was purified from *P. denitrificans* according to published procedures (42-43). The activity of wild type and mutant CcO has been reported (43). Mammalian CcO was isolated from beef hearts (44). The samples were concentrated to 100-150 µM in 50 mM Hepes pH 7.4 containing 0.1 % dodecyl β-D-maltoside and stored in liquid nitrogen until use. The fully reduced CO derivative was prepared by flushing CO gas anaerobically to dithionite-reduced enzyme. The mixed-valence CO-bound enzyme was prepared by exposing an anaerobic solution of the resting enzyme to CO for 10 h. RR spectra were obtained from 30-40 µM samples in an anaerobic cylindrical quartz spinning cell. The RR spectra were acquired by using a SPEX 1877 triplemate with an EG&G (model 1530-CUV-1024S) CCD detector. A Coherent Innova K-90 Krypton ion laser was used to provide the excitation wavelength of 413.1 nm. A Coherent 590 dye laser connected with a Coherent Innova 200 argon laser was used to provide the excitation wavelength of 431 nm. The power incident on
the CcO sample was typically 4-6 mW. FTIR spectra were obtained from 200-300 µM samples with a Bruker Equinox 55 FTIR spectrometer equipped with liquid nitrogen cooled MCT detector. The samples were loaded anaerobically into a cell with CaF₂ windows and a 0.025 mm spacer. The spectra were obtained as difference, using the buffer as background, and each spectrum is the average of 1000 scans. The spectral resolution used for the FTIR measurements, was 2 cm⁻¹ for the wild type-CO spectrum and 4 cm⁻¹ for the Y280H mutant, respectively. Optical absorbance spectra were recorded before and after FTIR and Raman measurements in order to assess sample stability with a Perkin-Elmer Lambda 20 UV-visible spectrometer.

RESULTS AND DISCUSSION

The optical absorption spectra of resting (as isolated) and fully-reduced $aa_3$ from $P.\ denitrificans$ display maxima at 423 and 598 nm in resting form (Fig. 1A, trace a), and at 444 and 605 nm in the reduced form (Fig. 1A, trace b). The difference CO-bound-reduced spectrum displays a positive band at 430 nm and a shoulder at 416 nm with a trough at 449 nm (Fig. 1A, trace c). The optical spectrum of the resting Y280H mutant shown in Fig. 1B (trace a) shows in addition to the 423 nm band a shoulder at 441 nm in the Soret region and an increased absorption of the 604 nm band. This indicates that a sizeable percentage of heme $a$ ($\sim 30\%$) is reduced in the mutant. Addition of dithionite to Y280H (Fig. 1B, trace b) shifts the maxima at 441 and 604 nm, consistent with the maxima of the fully reduced wild type enzyme. The difference spectrum of the reduced CO-bound minus reduced form is characteristic of CO binding to heme $a_3$, as
denoted by the peaks at 432 and 592 nm (Fig. 1 B, trace c).

The high frequency region of the RR spectrum of the resting and reduced wild type, which are shown in Fig. 2A (traces a and b) are in agreement with those previously reported for heme-copper oxidases (35-37, 39-40). In the spectrum of the resting enzyme, the oxidation state marker $\nu_4$ is at 1371, establishing that both hemes are in the ferric (Fe$^{3+}$) state. The modes at 1477 and 1498 cm$^{-1}$ originating from $\nu_3$ of high-spin heme $a_3$ and low spin heme $a$, respectively, indicate that both hemes are six-coordinate. This is also consistent with structural data which indicate that in this form of the enzyme heme $a$ is coordinated by two histidine ligands and that there is a bridging ligand between heme $a_3$ and CuB (10-11). The core expansion region shows two vibrations at 1572 cm$^{-1}$, (high-spin heme $a_3^{3+}$) and 1584 cm$^{-1}$ (low-spin heme $a^{3+}$). The 1612 and 1635 cm$^{-1}$ modes arise from $\nu_{10}$ of heme $a_3^{3+}$ and heme $a^{3+}$, respectively. The 1646 and 1671 cm$^{-1}$ modes have been assigned as the C=O stretching vibrations of the formyl groups (-CHO) of heme $a_3^{3+}$ and heme $a_3^{2+}$, respectively (39-40). The spectrum from the fully reduced enzyme (Fig. 1A, trace b) has the oxidation state marker, $\nu_4$, at 1359 cm$^{-1}$ indicating that both hemes are in the ferrous state. The modes at 1464 and 1490 cm$^{-1}$ originate from the ferrous, five-coordinate high spin (5chs) heme $a_3^{2+}$ and the ferrous six-coordinate low-spin (6cls) heme $a$, respectively. The mode at 1519 originates from $\nu_{19}$ of heme $a_2^{2+}$. The modes at 1569 and 1584 cm$^{-1}$ originate from $\nu_2$ of heme $a_3^{2+}$ and heme $a$, respectively. The 1612 and 1662 have been assigned as the C=O stretching vibrations of the
formyl group (-CHO) of heme $a^{2+}$ and heme $a^{3+}$, respectively (35,40). The 1623 cm$^{-1}$ mode arises from the C=C stretching vibration of heme $a^{2+}$ and heme $a^{3+}$. The spectral perturbations caused by the Y280H mutation are very limited except for the decrease in intensities of the heme $a^{3+}$ modes and the concomitant appearance of modes originating from a fraction of heme $a^{2+}$ (Fig. 2 B, trace a). The reduction of heme $a$ is apparent from the increase in intensities of $\nu_4$ at 1356, $\nu_{11}$ at 1519, $\nu_2$ at 1584, $\nu_{10}$ at 1612 cm$^{-1}$, as well the decrease in intensity of the formyl stretching vibration at 1646 cm$^{-1}$. All the modes associated with the high spin heme $a^{3+}$ are very similar to those of the wild-type enzyme. The only exception is the reduced intensity of the formyl stretching vibration at 1671 cm$^{-1}$. The RR spectrum of the fully-reduced Y280H enzyme (Fig. 2 B, trace b) is very similar to that of the fully-reduced wild-type, with the exception of the formyl vibration at 1662 cm$^{-1}$ which is weaker in the mutant.

No unusual stereochemical influences on heme $a_3$ are apparent in the Y280H mutant that would modify the heme $a_3$ ligand binding site. In addition, no evidence for any histidine coordination from CuB to heme $a_3$ is apparent. Thus, our data do not support the conclusions of Das et al. (12) that, without the cross-linking of Y288 and H284 (R. sphaeroides residue numbering), the heme pocket becomes severely disrupted and one of the histidines bound to CuB coordinates to heme $a_3$, lowering its redox potential.

Fig. 3 shows the low-frequency RR spectra of the fully-reduced wild-type and Y280H mutant, and that of the wild-type CO-bound form. The RR spectrum of the fully reduced
enzyme (trace a) is characterized by the Fe-His stretching mode at 220 cm\(^{-1}\) and porphyrin modes of both hemes \(a\) and \(a_3\). Very similar spectra were obtained for the Y280H mutant as shown in trace b. The Fe-His stretching mode is 6 cm\(^{-1}\) higher in \textit{P. denitrificans}\) than it is in CcO (36). The binding of CO to heme \(a_3^{2+}\) is accompanied by the disappearance of the Fe-His stretching vibration at 220 cm\(^{-1}\) and of the porphyrin mode at 369 cm\(^{-1}\) (trace c). In the 400-600 cm\(^{-1}\) region of the RR spectrum of the CO complex, one frequency for \(\nu(\text{Fe-CO})\) is detected at 517 cm\(^{-1}\). Assignment of this frequency is confirmed by isotope (\(^{13}\)CO) replacement experiment (trace d), where the corresponding line appears at 513 cm\(^{-1}\), close to the value expected for a two-harmonic oscillator between iron and CO. The difference spectrum (trace e) confirms the above assignment. In the \(^{13}\)CO-bound adduct we also detect a line at 559 cm\(^{-1}\). Although the presence of a porphyrin mode at \(\sim\) 580 cm\(^{-1}\) partially obscures band assignment with \(^{12}\)CO in this region, the difference spectrum shows that the 559 cm\(^{-1}\) band shifts to 573 cm\(^{-1}\) when the experiment is repeated with \(^{12}\)CO. We assign the 573 cm\(^{-1}\) mode to the Fe-C-O bending mode \(\delta(\text{Fe-C-O})\). The frequencies of the 517 and 573 cm\(^{-1}\) modes are similar to those that have been reported for the \(aa_3\)-type oxidases from beef heart (16), \textit{R. sphaeroides} (17) and \(aa_3\)-600 from \textit{B. subtilis} (21).

The increased frequency of the Fe-His mode we observe in the \(aa_3\) from \textit{P. denitrificans} can be attributed to the strength of the H-bond of the proximal His411 ligand to Gly387 (41). From the model compound work, the complex with a weaker (or absent) hydrogen bond to the
proximal His is expected to have the weaker Fe-His bond and the lower frequency vibration (36). The observation of the Fe-His at 220 cm$^{-1}$ in conjunction with the high frequency data further supports our conclusion that heme $a_3^{2+}$ in the Y280H mutant is five-coordinated, high-spin, and the proximal environment of heme $a_3^{2+}$ in the mutant behaves in a manner analogous to the wild type. Since the Fe-His411 forms the only covalent linkage between the five-coordinate heme $a_3$ and the protein, protein structural changes should be manifested through this bond. From the absence of such changes in the proximal environment of heme $a_3^{2+}$, any protein structural change due to the mutation is not widespread but is more localized in the CuB environment.

Structural information such as the geometry of the bound CO to the heme and its interactions with CuB has been determined from the vibrational modes involving the CO. Although the ν(Fe-CO) and δ(Fe-C-O) we have observed is similar to other $aa_3$-type oxidases the $I_\delta/I_\nu \sim 0.25$ we observe is low compared to those of CcO and $R.sphaeroides aa_3$-type oxidase ($I_\delta/I_\nu = 0.43$) and significantly higher than that of cytochrome $bo_3$ (20) ($I_\delta/I_\nu = 0.1$). It has been argued that a high $I_\delta/I_\nu$ is an indication of a strong interaction between the CO and CuB. Consequently, our data suggest that the Fe&CuB distance is longer in our case. However, despite the increased Fe-CuB distance we have not been able to detect a low-energy Fe-C bond (~490 cm$^{-1}$) corresponding to the high energy C-O bond (~1950 cm$^{-1}$) of the β-conformer. Recently, it was
postulated that the two distinctly different Fe-CO modes observed in the RR of the Rb. sphaeroides spectra result from a change in the position of CuB with respect to the CO due to the presence of one or more ionizable groups in the vicinity of the binuclear center (23). Additional experiments are in progress in our laboratory to address this possibility in the aa3 oxidase from P. denitrificans.

Fig. 4 shows the FTIR spectra of the CO-bound wild-type- and Y280H mutant of aa3 from P. denitrificans at room temperature. For comparison, the fully reduced aa3-CO complex of the mammalian enzyme is included (trace c). Trace a, shows that the C-O stretching modes from the P. denitrificans aa3 enzyme split into three components just as was found in the low-temperature experiments (27). The major component is centered at 1966 cm\(^{-1}\) (\(\alpha\)-form) and two minors are located at 1956 cm\(^{-1}\) (\(\beta\)-form) and 1975 cm\(^{-1}\). The frequency of the major CO stretching mode at 1966 cm\(^{-1}\), which we detect in P. denitrificans is 3 cm\(^{-1}\) higher than the corresponding frequency of the CO-bound heme a3 of CcO (trace c). Trace b, shows that the Y280H mutant has two conformers that have \(\nu(CO)\) at 1966 and 1975 cm\(^{-1}\). Similar results have been obtained in the low-temperature experiments (P. Hellwig, manuscript submitted).

The structural basis for the splitting of the enzyme into the \(\alpha\) and \(\beta\) forms has not been determined and no information regarding the origin of the 1975 cm\(^{-1}\) has been reported. The FWHM is \(~ 5\) cm\(^{-1}\) in the C-O modes of the major conformer in both the mammalian and P. denitrificans enzymes indicating the absence of a wide distribution of allowed CO conformations. Thus, these data confirm the similarity in the properties of the active sites in these
two terminal oxidases. The presence of the 1956 and 1975 cm$^{-1}$ modes in the *P. denitrificans* do indicate, however, that the binuclear site of the bacterial enzyme, while similar, is not identical to its mammalian counterpart. Conversion between the $\alpha$ and $\beta$ forms is pH-dependent and has been attributed to changes in the iron-copper distance (23). It has also been demonstrated from the low-temperature FTIR data that the amplitudes of the bands attributed to the $\alpha$- and $\beta$-forms are temperature and pH dependent (27). Thus the $\alpha$-form represents a constricted pocket that will not allow CO to coordinate to heme iron without strong distal polar interactions between the CO and the copper atom, while in the $\beta$-form the Cu$_B$ atom is moved away from the bound CO. Based on the above interpretation regarding the origin of the $\alpha$ and $\beta$ forms we assign the 1975 cm$^{-1}$ mode to the $\gamma$-conformation in which Cu$_B$ is moved closer to the CO-bound heme $a_3$, thereby the Fe-C-O moiety is further distorted from its preferred symmetry in the $\alpha$-form. Unlike the wild-type enzyme which has a prominent $\alpha$-form, the mutant enzyme exhibits the 1975 cm$^{-1}$ ($\gamma$-form)/1966 cm$^{-1}$($\alpha$-form) in 1.8 ratio indicating that the $\gamma$-confomer is the major conformer in the mutant. Consequently, in the absence of the cross-link Tyr-His the Cu$_B$ atom has moved further closer to the CO-bound heme $a_3$. This is further supported by the absence of the $\beta$-form (1956 cm$^{-1}$) in the mutant enzyme in which the CO is bound without anomalous polar or steric interactions. Both C-O stretches for the Y280H mutant are broad indicating a wide distribution of allowed CO conformations. In the absence of Y280, the heme $a_3$-Cu$_B$ distance has changed and Cu$_B$ is not fixed in a certain position resulting in different Cu$_B$ conformations. The different conformations of Cu$_B$ in the mutant reflect significant differences.
in the heme environment, thereby alter the properties of the CO modes observed in the FTIR spectra. In the CO derivatives, the different conformations of Cuβ could easily cause the change in the C-O frequency and bandwidth since it is well established that ligand frequencies and bandwidths in heme proteins are modulated by the properties of the distal environment.

It has been established by Rousseau et al. (12,16-18,20) that the ν(Fe-CO) and ν(CO) frequencies of heme proteins and the correlation between them reflect a) the identity and properties of the proximal ligand because the bound CO competes with the proximal ligand for the same iron d\(_{z^2}\) orbital, and b) indicate the polarity of the distal heme pocket. A highly polar environment favors π-back donation, resulting in an increased ν(Fe-CO) and reduced ν(C-O) due to the increased density in the CO antibonding orbitals. Additional information concerning the properties of the proximal environment in heme-copper oxidases and how it influences the ligand properties on the distal site has been deduced from the unique inverse linear correlation that exists between the frequencies of Fe-His and the Fe-CO stretching modes. Recently, we have shown that in heme-Cuβ oxidases the strength of the proximal histidine H-bonding interaction affects the strength of both the Fe-C and C-O bonds which are further influenced by the Cuβ distal environment (21). We consider both proximal and distal effects on the origin of the ν(Fe-CO) and ν(CO) frequencies we have observed.

The ~4 cm\(^{-1}\) downshift in the ν(Fe-CO) of \textit{P. denitrificans}, when compared to that found in CcO, is brought about by a stronger hydrogen bonding interaction of the proximal histidine, and by distal effects on the heme a\(_3\)-bound CO exerted by Cuβ. This argument is supported by the
observed high frequency of the Fe-His stretching mode at 220 cm\(^{-1}\), as compared to \(aa_3\) and \(bo_3\) type oxidases. A similar conclusion concerning the effect of the proximal ligand to the properties of the distal CO was reached recently by Wang et al. (45) for prostogladin H synthase. On the other hand, Das et al. (23) has argued recently that the pH dependence of the Fe-C-O modes they observed in \(Rb.\ sphaeroides\) cannot be attributed to proximal effects due to the absence of any significant pH-dependent change of the proximal Fe-His stretching frequency.

The proximal protein pocket in heme \(a_3\) appears to be hydrophobic and inaccessible to solvent as indicated by Raman studies in D\(_2\)O (41). Consequently, the invariance of the frequency of the Fe-His does not necessarily rule out a hydrogen bond interaction to the proximal histidine of heme \(a_3^{2+}\). This is further supported from the crystal structure of the \(P.\ denitrificans\) enzyme where it is shown that the proximal His to heme \(a_3\) is indeed hydrogen-bonded to Gly387. The frequencies of the Fe-His and Fe-CO of the enzyme place it on the correlation curve shown in Fig. 5A, and posit that it has the same structure as that of the major \(\alpha\)-form of the mammalian and \(Rb.\ sphaeroides\) enzymes. In the absence of a proximal effect as indicated by the strength of the Fe\(^{2+}\)-His located at 220 cm\(^{-1}\) in the mutant, the anomalously high C-O stretching mode we observed in the CO-bound Y280H mutant is attributed solely to distal effects. Proximal effects should not be taken under consideration since the Fe-His stretching frequency remains unaffected by the mutation, evidence that a global conformational change is unlikely to have taken place.

The properties of the heme \(a_3\)-Cu\(_B\) binuclear center have been determined from the
correlation of $\nu(\text{Fe-CO})$ and $\nu(\text{C-O})$ frequencies. The major component ($\alpha$-form) of the wild type $aa_3$, shown in Fig. 5B, deviates from the inverse linear correlation curve that exists between the frequencies of $\nu(\text{Fe-CO})$ and $\nu(\text{C-O})$ between histidine coordinates proteins but fits to the curve of the rest of the terminal oxidases. Although we were unable to detect the $\nu(\text{Fe-CO})$ in the $\beta$-form, the frequency of the $\nu(\text{C-O})$ in the $\beta$-form is identical to that reported for the $\beta$-form of $aa_3$ from $\text{Rb. sphaeroides}$ suggesting that in $P.\ denitrificans$ the frequencies of the $\beta$-form are placed on the curve of the histidine-coordinated proteins in which CO can bind to the iron without anomalous polar and steric interactions.

The overall similarity between the frequencies and relative enhancements for the vibrational resonances of oxidized heme $a_3$ in the wild-type and Y280H mutant indicates that the protein milieu surrounding the heme $a_3$ is the same in these two forms. A comparison of the high-frequency resonance Raman spectra of the mutant enzyme with that of the wild type, upon reduction, shows no significant differences. However, a conformational change is likely to occur in the binuclear center as indicated by the differences in the intensity and bandwidth of the formyl line of $a_3$ in both oxidation states of the heme $a_3$ in the Y280H mutant compared to the wild type enzyme. This, nevertheless, cannot be attributed to a different oxidation, spin or ligation change since we have established that no such changes occur as a result of the mutation. Therefore, other possibilities should be considered.

In the absence of a conformational change in heme $a_3$ the only possibility to account for the reduced intensity of the heme $a_3$ formyl group is the histidine ligands coordinated to Cu$_B$. His 276 is the histidine that forms the cross-link with Y280 in the wild-type and is not close to the
formyl group, thus it is unlikely that it can interact with the formyl group. His325 and His326 are on the same helix and are close to the formyl group. The closest residue to the formyl oxygen atom of heme $a_3$ is His325 (His290 in bovine). It is noteworthy that the absence of Tyr280 in the heme pocket results in the loss of the hydrogen-bonding interaction between the hydroxy group on the farnesyl chain of heme $a_3$ and the hydroxy group on the tyrosine. If the observed changes were due to a change in the hydrogen bonding state of the formyl group then, a frequency shift would have been expected (46). We postulate that changes in the Fe-Cu$_B$ distance could modulate the position of His325 with respect to the formyl group. Since no frequency shift is observed, we postulate that the intensity difference is due to a change in the geometry of the formyl group that does not allow the electronic coupling between the formyl group and the porphyrin core to be as effective as in heme $a_3$ of the wild type enzyme. The complete absence of the C=O stretching vibration of the formyl group (-CHO), reported in the reduced form of the Y280F mutant from *Rb. sphaeroides*, was attributed to an altered heme $a_3$ conformation. The question arising is whether the change in the orientation of the formyl, which is more evident in the Y280F mutant from *Rb. sphaeroides* than the Y280H $aa_3$ from *P. denitrificans*, could have an effect in the catalytic function of the enzyme. Recently, Das *et al.* (46) reported a redox-link deprotonation event at the binuclear site of the quinol oxidase from *Acidianus ambivalens*, in which the changes in heme $a_3$ formyl C-O stretching mode upon heme reduction were attributed to a change in H-bonding to the formyl group. As a possible candidate for the pH-dependent changes they observed suggested His290 (bovine sequence numbering) and discussed the implications for proton translocation. However, since this is a single observation and no similar
observation has been made for any other $aa_3$ CcO we cannot consider that the small conformational change we detect in the formyl group of heme $a_3$ in the Y280H mutant will influence the functional properties of the binuclear center.

Oxygenated intermediate(s) that occur after the decay of the oxy-intermediate (Im) in the MV/O$_2$ reaction may be generated by direct mixing of oxygen with the CO-bound MV oxidase. These species have been observed by others to have relatively long lifetimes, and thus, they can be studied in a conventional absorption spectrometer. The Soret region optical absorption spectra of oxygenated species formed this way are shown in Fig. 6. The difference spectrum (MV-CO minus oxidized) of the wild type enzyme (panel A, inset), with maxima and minima at 432 and 411 nm, agrees with that of the CO-bound MV Y280H enzyme (panel B, inset). In both the wild type and Y280H MV enzymes, mixing with O$_2$ shifts the Soret maximum to 438 nm (1-18 min). This indicates that in both the wild-type and mutant enzymes O$_2$ spontaneously replaces CO. The progression of changes in the Soret indicates that the decay of the oxygenated 438 nm species to the pulsed and subsequently to the resting form occurs on a time scale of tens of seconds. These results suggest that Y280 is not involved in the formation and decay of the 438 nm species because similar observations were obtained in the wild type and mutant enzymes.

Similar observations in the decay of II$_m$ to the resting form have been reported in the bovine MV CcO/O$_2$ reaction by Rousseau and co-workers (47). It was also demonstrated by the same authors that intermediates formed by mixing O$_2$ with CO-bound MV CcO and allowing O$_2$ to spontaneously replace CO are the same as those of II$_m$ formed in the flow-flash-probe
experiments (47).

The data reported here have shown that the cross-link His-Tyr generates a unique environment around CuB and holds it in a certain distance and position from heme $a_3$. In the absence of the cross-linking, CuB moves further towards the heme $a_3$ in the CO bound form of the enzyme, affecting the properties of the bound ligands to heme $a_3$. Thus, in the Y280H mutant, heme $a_3$ retains its proximal His ligand and can be oxidized by oxygen. The oxidation of heme $a_3$ in the Y280H mutant and in the wild-type proceeds through oxygenated species with Soret maxima at 438 nm. The nature of the bound oxygen intermediates following the oxy species remains to be determined. The characterization of the functional/structural implications to the heme $a_3$-CuB center by the Y280H mutation reported here, and the determination of the initial electron transfer steps in the Y280H/O$_2$ reaction will lead to a better understanding of the oxidative phase of cytochrome $c$ oxidase. These experiments are in progress in our laboratory.

**Acknowledgment.** This work was supported by Alexander von Humboldt-Stiftung (to C.V and B. L), PENED 99 (to C.V) and DFG (SFB 472; to B.L.) We thank Werner Müller for excellent technical assistance.
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Figure Legends

Fig. 1: Optical absorption spectra of the wild type cytochrome *aa3* from *P. denitrificans* (panel A) and the Y280H mutant (panel B) in the “as isolated” (trace *a*, solid line) and the dithionite reduced (trace *b*, dot line) forms. In both panels the difference spectrum (trace *c*, dashed line) of the reduced CO-bound form minus the reduced indicates the binding of CO to heme *aa*. The concentration of the enzyme was 10 µM and the path length of the cell was 0.5 cm.

Fig. 2: High frequency resonance Raman spectra of the wild type cytochrome *aa3* from *P. denitrificans* (panel A) and the Y280H mutant (panel B) in the resting (trace *a*) and the dithionite reduced (trace *b*) forms. The excitation laser wavelength was 413.1 nm. The accumulation time was 15 min for each spectrum.

Fig. 3: Resonance Raman spectra of the reduced wild type cytochrome *aa3* from *P. denitrificans* (trace *a*) and the Y280H mutant (trace *b*) in the low frequency region. The reduced wild type complex with $^{12}$CO and $^{13}$CO are shown in traces *c* and *d*, respectively. The difference spectrum $^{12}$CO - $^{13}$CO is shown in trace *e*. The excitation laser wavelength was 431 nm. The accumulation time was 20 min for each spectrum.

Fig. 4: FTIR spectra of the fully reduced CO derivative of wild type cytochrome *aa3* from *P. denitrificans* (trace *a*) and of the Y280H mutant (trace *b*). For comparison the spectrum of the CO
derivative of mammalian *aa3* is depicted in trace *c*.

**Fig. 5:** Panel A: Correlation between frequencies of the Fe-His versus C-O stretching modes. (filled circle) cytochrome *bo3* from *E. coli*; (filled left pointed arrow) the *α* form of cytochrome *aa3* from *R. sphaeroides*; (filled square) mammalian cytochrome *c* oxidase and cytochrome *aa3* from *B. subtilis*; (filled downward pointed arrow) cytochrome *aa3* from *P. denitrificans*; (filled upward pointed arrow) cytochrome *cbb3* from *R. capsulatus*. Panel B: Correlation between frequencies of the Fe-CO versus C-O stretching modes. Same as in panel A and (filled right pointed arrow) the *β* form of cytochrome *aa3* from *R. sphaeroides*; (open rhombus) myoglobins and hemoglobins.

**Fig. 6:** Optical absorption difference spectra showing reaction products minus the resting form of wild-type (panel A) and Y280H (panel B) enzymes at the indicated times. The reaction was initiated by mixing O₂ with CO-MV- wild-type and -Y280H enzymes. The *insets* show the CO-MV- wild-type and -Y280 enzymes minus their resting forms. The concentration of the enzymes was 10 µM, and the path length of the cell was 0.5 cm.
Figure 1. Pinakoulaki et al.
Figure 2. Pinakoulaki et al.
Figure 3. Pinakoulaki et al.
Figure 4. Pinakoulaki et al.
Figure 5. Pinakoulaki et al.
The role of the cross-link his-Tyr in the functional properties of the binuclear center in cytochrome c oxidase
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*J. Biol. Chem. published online February 1, 2002*

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