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Resonance Raman Characterization of the Ammonia-Generated Oxo Intermediate of Cytochrome *c* Oxidase from *Paracoccus denitrificans*

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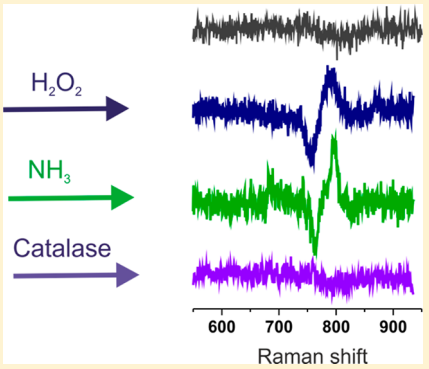
Supporting Information

ABSTRACT: A novel oxo state of cytochrome *c* oxidase from *Paracoccus denitrificans* generated by successive addition of excess H_2O_2 and ammonia was investigated using resonance Raman (RR) spectroscopy. Addition of ammonia to the H_2O_2-generated artificial F state resulted in an upshift of the oxoferryl stretching vibration from 790 to 796 cm^{-1}, indicating that ammonia influences ligation of the heme-bound oxygen in the binuclear center. Concomitantly performed RR measurements in the high-frequency region between 1300 and 1700 cm^{-1} showed a high-spin to low-spin transition of heme a_3 upon generation of the F state that was not altered by addition of ammonia. Removal of H_2O_2 by addition of catalase resulted in the disappearance of the oxoferryl stretching vibration and major back transformation of heme a_3 into the high-spin state. The ratio of high-spin to low-spin states was identical for intermediates created with and without ammonia, leading to the conclusion that ammonia does not interact directly with heme a_3. Only for the ammonia-created state was a band at 612 nm observed in the UV−visible difference spectrum that was shifted to 608 nm after addition of catalase. Our results support the hypothesis by von der Hocht et al. [von der Hocht, I., et al. (2011) *Proc. Natl. Acad. Sci. U.S.A. 108*, 3964−3969] that addition of ammonia creates a novel oxo intermediate state called P_N where ammonia binds to Cu_B once the oxo intermediate F state has been formed.



The membrane-bound cytochrome *c* oxidase (CcO) is a key enzyme in the respiratory chain as it couples the reduction of oxygen to the generation of an electrochemical proton gradient across the mitochondrial inner membrane.[1,2] During the catalytic cycle, several intermediate states with characteristic optical properties are formed;[3] thus, spectroscopic investigations of these intermediate states have a great impact on the understanding of the mechanism of catalytic oxygen reduction and proton pumping in these enzymes. Two transient oxoferryl states, named P and F, are frequently defined by their α-band absorption profile in the UV−vis region between 570 and 620 nm. The P state exhibits a distinct difference absorption peak at 607 nm, while the F state has its maximum near 580 nm. Steady state artificial P and F intermediates can be selectively generated by adding H_2O_2 to the enzyme as a function of pH and H_2O_2 excess ratio.[4−6] Similar artificial P states that have a difference absorption peak around 607 nm could also be created via other routes from the O state; therefore, the H_2O_2-generated P state is named P_H. Recently, von der Hocht et al.[7] discovered a new P-like state that was generated from the H_2O_2-induced F state upon addition of ammonia. This novel, so-called P_N state showed a characteristic α-band difference absorption at 612 nm. CcO in the P_N state showed a higher catalase activity compared to that of the F state, which was proposed to be a result of ammonia binding to Cu_B.[8] A direct proof of ammonia interacting with atoms in the binuclear center, however, could not be provided by the UV−vis measurements. It was pointed out by Wikstrom[3] that ammonia could possibly block the entrance channel for H_2O_2, which would decrease the amount of H_2O_2 reaching the catalytic center. As a consequence, the P_N state would not be formed via the F state but would be generated directly from the O state because of a low effective H_2O_2 concentration within the protein (similar to the P_H state) and without direct interaction of ammonia and the binuclear center of CcO. In this way, the UV−visible (UV−vis) difference spectrum of the P_N state would not be ascribed to a new state but would comprise a mixture of the P_H and F states. This scenario, however, would not explain the higher catalase activity observed for the P_N state.

Structural information about the two heme cofactors of CcO and its direct environment can be extracted from resonance Raman (RR) spectroscopy using Soret excitation.[9] This technique provides information about the immediate coordination sphere of the heme-bound iron and the interaction of the heme-bound ligands with the protein environment.[10] Using RR spectroscopy the vibrational bands at 790 (785) and 804 cm^{-1} were assigned to the Fe(IV)=O vibration of the H_2O_2-

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generated F and P states, respectively.[11,12] The same band positions were observed for the F and P intermediates during turnover using time-resolved RR spectroscopy initiated by CO photolysis,[13−15] indicating that the artificially created F and P states resemble the natural intermediates. The shift in frequency between P and F was attributed to either the different hydrogen bonding of the oxoferryl oxygen to distal amino acids or a change in the trans ligand strength of the proximal histidine.[12,16,17] In this paper, RR spectroscopy will be applied to analyze the structural state of the binuclear center and to characterize the novel P_N state.

MATERIALS AND METHODS

Protein Purification. CcO was purified from *Paracoccus denitrificans* membranes grown in succinate medium.[18] Protein purification was performed as described previously[8,19] by affinity chromatography with the following modifications: The β-dodecyl maltoside concentration was reduced to 0.01% (w/v) for the purification steps. After the final concentration step (Amicon concentrators, 100 kDa cutoff, Millipore, Billerica, MA), the protein was washed three times with 10 mM KP_i [mixture of K_2HPO_4 and KH_2PO_4 (pH 8.0) with 0.2 mM EDTA] to decrease the detergent concentration. To obtain the pure oxidized state of the protein, the sample was oxidized with equimolar amounts of potassium ferricyanide overnight. Directly before the measurement, the protein was washed several times with 10 mM KP_i buffer using a concentrator (Amicon concentrator, 100 kDa cutoff) until the complete oxidizing agent was removed.

Spectroscopy. UV−vis spectra were recorded using an Agilent Technologies Cary 4000 UV−vis spectrophotometer. RR measurements were taken using the 413 nm laser line of a krypton ion laser (Coherent Innova 300 c). The spectra were recorded with a confocal Raman spectrometer (LabRam HR-800, Jobin Yvon). All RR spectra were measured with a total volume of 100 μL at a final concentration of 50 μM CcO [in 100 mM glycine buffer (pH 9.0)]. Ammonia was added from a buffered $NH_3/(NH_4^+)_2SO_4$ solution adjusted to pH 9.0. The pH values were controlled before each experiment. RR spectra at room temperature were recorded with a laser power of 4 mW using a rotating cuvette to ensure a fast exchange of the sample in the focus of the laser beam. RR spectra in the high-frequency region were obtained with an accumulation time of 1 min. $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$ RR spectra in the low-frequency region were accumulated over 10 min. This experiment was repeated four times to achieve a satisfactory signal-to-noise ratio. For each repetition, 100 μL of a fresh solution was prepared.

RR spectra at 77 K were measured using a sample volume of 20 μL and a laser power of 4 mW at 413 nm. The sample was moved constantly during the measurement. The accumulation time per spectrum was 10 min without exchanging the sample. Freezing was accomplished by injecting the liquid sample into the cryostat that was equilibrated to the desired temperature.

RESULTS

Prior to each experiment, CcO was oxidized with ferricyanide, yielding the fully oxidized O state. The steady state F state of CcO was created upon addition of H_2O_2 in a ratio of 1:500. The P_N state was generated in analogy to the procedure described by von der Hocht et al.[7] by addition of ammonia (25 mM) to the F state at high pH. To decompose the present H_2O_2, we added catalase from bovine liver to a final concentration of 80 units/100 μL to yield the degraded P_N (P_{Ndeg}) and degraded F (F_{deg}) states, respectively. UV−vis spectra of the states mentioned above are shown in Figure 1.

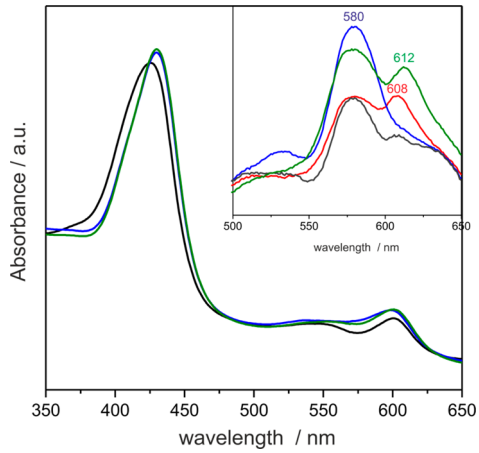

Figure 1. UV−vis spectra of the O (black), F (blue), and P_N (green) states of CcO. The inset shows the UV−vis difference spectra in the region between 500 and 650 nm of the F−O (blue), P_N−O (green), P_{Ndeg}−O (red), and F_{deg}−O (gray) states.

The transition from the O state to the F state caused a red shift of the Soret band absorption from 425 to 429 nm. Further addition of ammonia to create the P_N state did not result in another change of the Soret band maximum. The UV−vis difference spectra of the F−O and P_N−O states in the region between 500 and 650 nm are presented in the inset of Figure 1. Here the characteristic difference absorption maximum of the F state at 580 nm can be seen as well as the occurrence of a new maximum at 612 nm in the P_N state that was first observed by von der Hocht et al. The P_N state can also be obtained when the sequence of the additions of ammonia and catalase is changed: generating the F state and decomposing the free hydrogen peroxide in the medium with catalase directly before ammonia is added to this spectrally unchanged F state also leads to formation of the P_N state (spectra not shown). In the spectra of the degraded F and P_N states, which were recorded 75 min after the addition of catalase, the magnitude of the 580 nm band is decreased, but still present in both states. However, only in the degraded P_N state is an additional band at 608 nm clearly visible.

Figure 2 displays the RR spectra of the O state and the intermediates in the high-frequency region using 413 nm laser excitation. The spectral region between 1300 and 1700 cm^{-1} is of special interest as it provides direct information about the heme geometry.[20,21] In Figure 2, clear differences in the spectra of F and P_N states with respect to the O state can be seen, most prominently in a shift of the ν_4 band from 1370 to 1373 cm^{-1}, the disappearance of the bands at 1569 and 1607 cm^{-1}, and the appearance of a band at 1639 cm^{-1}. In the RR spectra of the degraded P_N and F states that were recorded 75 min after the addition of catalase (Figure 2d,e), the 1569 and 1607 cm^{-1} bands reappear. Thus, an at least partial back reaction to the O state is indicated for these states. It has to be noted that addition of ammonia directly to the O state did not lead to any changes in the UV−vis and RR spectra (data not shown).

The Soret band of the O state in Figure 1 is a superposition of the respective absorption maxima of heme *a* and heme a_3 at 427 and 413 nm, respectively.[9,22] Using 413 nm excitation,

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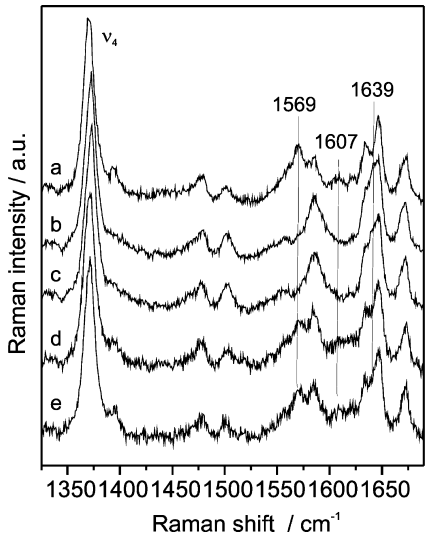

Figure 2. High-frequency spectra of CcO in the (a) O, (b) F, (c) P_N, (d) P_{Ndeg}, and (e) F_{deg} states.

heme a_3 is in resonance with the incoming laser light while heme a is in resonance with the energy of the Raman scattered light for frequencies above 1000 cm^{-1};[9] thus, a similar intensity contribution of both hemes is expected in the high-frequency region presented in Figure 2 (*vide infra*). This feature allows the assignment of the Raman bands of the O state spectrum to the different heme a and a_3 vibrational modes as shown in trace a of Figure 3. The band positions are listed additionally in Table 1 and compared to previous assignments.[9,23] The frequencies of the heme a_3 bands are generally downshifted with respect to the bands of heme a as a result of the larger size of the porphyrin core of the high-spin heme a_3 compared to that of the low-spin heme a.[21,24] With respect to the previous work of Heibel et al., only one noteworthy different assignment

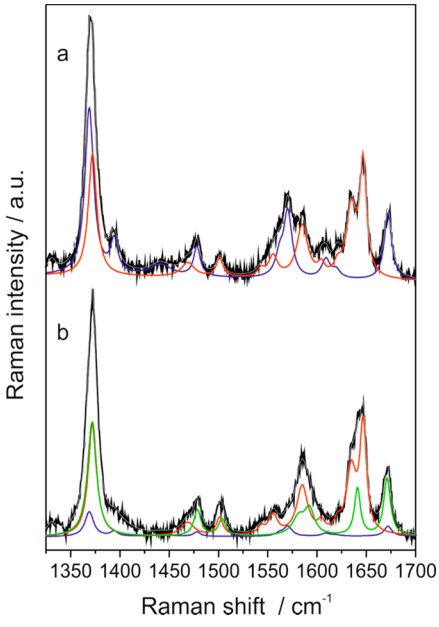

Figure 3. RR spectra of CcO in the O (a) and F (b) states in the high-frequency region that is sensible to the spin state of heme a and heme a_3 with the corresponding component spectra: red for heme a, blue for heme a_3, and green for heme a_3=O.

was made as the position of ν_{38x} for heme a and heme a_3 was exchanged. This change is based on the observation that the band intensity at 1555.3 cm^{-1} was not affected by the addition of H_2O_2 while the band at 1562 cm^{-1} was clearly shifted (*vide infra*).

Addition of H_2O_2 results in the formation of an oxoferryl state that is generally associated with a transformation of heme a_3 to a low-spin state.[12] Assignment of the bands of the oxo heme state, denoted as heme a_3=O,[a] is more difficult as the measured RR spectrum will always be a mixture of at least three species; heme a, deoxygenated heme a_3, and oxygenated heme a_3=O. The F state spectrum, presented in trace b of Figure 3, was used to assign the bands of heme a_3=O. Assignment was conducted under the assumptions that (i) the spectrum of heme a is not altered by the addition of H_2O_2 and (ii) the contribution of heme a_3 is decreased but the relative intensities of the marker bands have not changed. The band assignment of the oxygenated heme a_3=O state derived thusly is also listed in Table 1. The most dominant differences between the vibrational modes of heme a_3 and heme a_3=O correspond to an upshift of porphyrin ring vibrations ν_4, ν_2, and ν_{10}, which is in line with a high-spin (HS) to low-spin (LS) heme a_3 transition. Consequently, vibrational modes assigned to side chain vibrations should not be affected by oxygen binding, which explains why the formyl stretching vibrations of heme a_3 and heme a_3=O are at the same position.

For a more quantitative determination of the HS to LS ratio of heme a_3, the intensity ratio of heme a to heme a_3, determined exemplarily for the respective ν_4 vibration, was taken as a reference. In the O state, corresponding to a molar fraction of 1 for HS heme a_3, the intensity ratio of the ν_4 vibrations of heme a to heme a_3 was determined to be 1.4. In the F intermediate state, this ratio decreased according to the decrease of HS heme a_3 (Figure 3, trace b). From the new intensity ratio of heme a to HS heme a_3, the molar fraction of the latter could be directly derived (Figure 4). The other oxo intermediates were fit as follows. The band positions and relative intensities of the three component spectra were left untouched, and only a factor representing the contribution of each component was varied. With this procedure also the P_N intermediate could be fit nicely, yielding a HS to LS ratio of heme a_3 comparable to that of the F state. Addition of catalase resulted in a continuous increase in the level of HS heme a_3 at the expense of LS heme a_3 (see Figure S1 of the Supporting Information). A good fit to the time-dependent spectra could be achieved for all different HS:LS ratios, indicating that the individual components were assigned correctly. The component spectrum analysis further revealed that HS and LS heme a_3 contribute almost equally to the spectrum.

In the degraded P_N and F states that were recorded 75 min after the addition of catalase, the spectrum is again dominated by the contribution of the high-spin heme a_3. It should be noted that no significant difference in the RR spectra of the degraded P_N and F states could be seen.

In the low-frequency region (300−1000 cm^{-1}), the Fe(IV)=O oxoferryl stretching vibration was observed in $H_2{}^{16}O_2$ − $H_2{}^{18}O_2$ difference spectra shown in Figure 5. We determined the band position to be at 790 cm^{-1} for the F state at room temperature (trace a), which is in agreement with the literature.[12] However, the oxoferryl stretching band in the P_N state occurred at 796 cm^{-1} (trace c). The band position is upshifted with respect to the F state but also downshifted with respect to the P_H state in which the oxoferryl band is located at

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Table 1. Band Assignments for the High-Frequency Region (1300−1700 cm^{-1}) of Heme *a*, Heme a_3, and Heme a_3=O Compared to Data from refs 9 and 23

	Heibel et al.[23]		Babcock et al.[9] (4 °C)			this work		
mode	heme *a*	heme a_3	heme *a*	heme a_3	heme a_3−CN	heme *a*	heme a_3	heme a_3=O
ν_4			1373	1373	1373	1372.2	1368.9	1371.6
ν_{28}	1467.3		1474		1474	1468.8		
ν_3	1501.9	1478.2	1506	1478	1506	1501.2	1478.1	1478.6
								1503.6
ν_{11}	1541.1	1520.6				1543.2		
ν_{38x}	1563.8	1553.9				1555.3	1562.2	1570.8
ν_2	1585.4	1570.4	1590	1572	1590	1585.0	1570.0	1581.2
ν_{37}	1603.1	1576.2				1604.2	1573.3	
$\nu_{C=C}$	1622.7	1621.5				1620.8	1619.5	not visible
ν_{10}	1634.1	1609.9	1641	1615	1641	1634.7	1608	1641.0
$\nu_{C=O}$	1646.6	1671.6	1650	1676	1676	1646.7	1672.0	1670.9
								(1674)

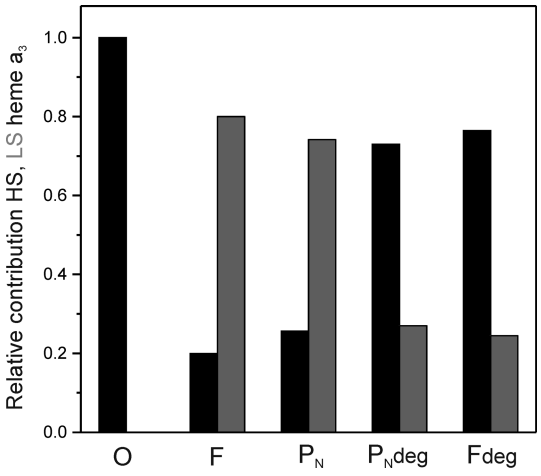

Figure 4. Molar fraction of the high-spin (HS) heme a_3 (black) and low-spin (LS) heme a_3=O (gray) components in the different states.

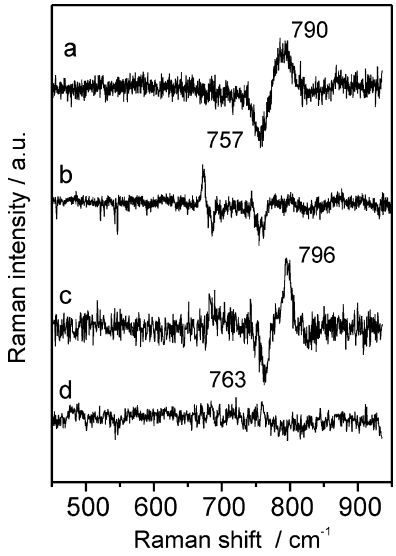

Figure 5. Low-frequency $H_2{}^{16}O_2$ − $H_2{}^{18}O_2$ difference spectra of CcO in the (a) F, (b) F at 77 K, (c) P_N, and (d) P_{Ndeg} states.

804 cm^{-1}. The peak width of the 796 cm^{-1} band in the P_N state is very narrow, and no contributions from an admixture of the F state could be seen. For both states (F and P_N), the intensity of the difference band decreased in the first 30 min (see Figure S2 of the Supporting Information) most likely because of isotope exchange of ^{18}O with bulk water. This decrease in intensity occurred faster for the F state than for the P_N state, of which even after 30 min a sharp difference band could be observed. The position of the band remained constant during the time scale of 30 min for both states. In the degraded P_N state, the oxoferryl vibration could not be detected (trace d). In the $H_2{}^{16}O_2$ − $H_2{}^{18}O_2$ difference spectra also, no other isotope-induced bands could be seen.

Also at 77 K (trace b), no positive oxoferryl band could be seen under conditions were the F state is formed at room temperature, although a small negative band appeared at 757 cm^{-1} where the isotopically labeled oxoferryl vibration is expected. We assume that the absence of a clear oxoferryl band is due to the drastic decrease in the diffusion coefficient of H_2O_2 at 77 K. As a result, Fe=O splitting is faster than H_2O_2 supply. The most stable state, which will dominate the RR spectrum, is therefore the deoxygenated state. This interpretation is further supported by high-frequency measurements of CcO at 77 K that show that heme a_3 remains to a great extent in the HS state (Figure S3 of the Supporting Information). At the moment, the possibility that laser radiation promotes a faster splitting of the Fe=O bond cannot be excluded, although we did not observe other typical laser-induced effects such as photoreduction during the measurements.

DISCUSSION

Addition of ammonia to the H_2O_2-generated F state causes a 6 cm^{-1} upshift of the Fe(IV)=O vibrational band. The band at 796 cm^{-1} was not broadened with respect to the F state. This observation clearly indicates that a new state, called P_N, is created upon addition of ammonia and not a mixture of F and P_H states. The broad α-band absorption bands at 580 and 612 nm, seen in the UV−vis spectrum of the P_N state, therefore do not originate from admixtures of the F and P_H intermediates but are characteristic of the P_N state. The shift of the oxoferryl band in F and P_H is generally attributed to either different hydrogen bonding of the oxoferryl oxygen or a change in the trans ligand strength of the proximal histidine.[12,16,17] The strong affinity of nitrogen for Cu ions, however, strongly supports a scenario in which ammonia, if it can access the binuclear center, will bind to Cu_B with additional hydrogen bonding to the Fe-bound oxygen.

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In the degraded P_N state, the oxoferryl vibration could not been detected. The same is expected for the degraded F state.[11] Furthermore, both degraded states, P_{Ndeg} and F_{deg}, show almost identical RR spectra in the high-frequency region with a strong contribution from a high-spin heme a_3. From this observation, we conclude that the axial ligation of heme a_3 is the same for both degraded states.

The disappearance of the oxoferryl isotope difference band under a low level of H_2O_2 has been previously attributed to a rapid exchange of the heme-bound oxygen with oxygen from bulk water.[11] As the spectra of the degenerated F and P_N states are measured more than 75 min after the addition of H_2O_2, oxygen exchange most likely has taken place, and thus, no isotope bands are expected to be seen. However, concomitant measurements of the degraded P_N and F states at high frequencies additionally indicate that the majority of heme a_3 is back in a high-spin state. Thus, for a major fraction of heme a_3, the oxoferryl oxygen is replaced by another ligand, most likely a water or hydroxyl. Why this major ligand replacement has only a minor influence on the UV−vis difference spectra in the α-band region (shown in Figure 1) remains to be elucidated. One has to consider that the position of the α-band is generally a measure of the deviation from the axial symmetry of the central iron with porphyrin plane.[3] Any deviation from this axial symmetry caused by bonding of hydrogen to the axial ligand can lead to a change in the α-band absorption profile. Therefore, it is not necessarily linked to the presence of an oxoferryl, but it is strongly connected to the interactions between the Fe and Cu_B ligands.

In agreement with the measurements of von der Hocht et al.,[7] a band shift from 612 to 608 nm in the UV−vis spectrum is exclusively observed upon the transition from the P_N state to the degraded P_N state. As this band is missing in the F and degraded F states, it has to be attributed to the presence of ammonia. The possibility that the absorption band originates from direct binding of ammonia to the heme iron can be excluded, as this would most likely result in a different high-spin to low-spin ratio in the degraded P_N and F states. Thus, the 608 nm band has to be a result of the interaction of the Fe ligand with other atoms of the binuclear center. In the most probable scenario, it is a result of hydrogen bonding between the Fe ligand and ammonia, which is still bound to Cu_B in the degraded P_N state.

One might ask whether the observed changes in the RR and UV−vis spectra could also be observed if ammonia is not able to access the binuclear center. In such a scenario, ammonia would block one of the entrance channels that are used for H_2O_2 or proton supply. H_2O_2 blocking by ammonia can be ruled out, as this contradicts the measured higher catalase activity of the P_N state.[8] Furthermore, the generation of the P_N state directly after degradation of all free H_2O_2 in solution by catalase would be impossible. Additionally, a poor H_2O_2 supply would not lead to the observed pure and stable RR difference spectra of the oxoferryl vibrations. An indirect influence of ammonia on proton uptake could in principle lead to a stable shifted oxoferryl band. However, in such a case, ammonia would have to bind with high affinity and stably to a residue at the entrance of one of the proton channels. We cannot imagine a mechanism by which this occurs. One has to keep in mind that hydrogen bonds formed by ammonia are considerably weaker than those formed by water or -OH groups. Moreover, it is highly unlikely that this binding occurs only for ammonia and not for the ammonium ion as observed in previous pH-dependent measurements.[7] Furthermore, if ammonia is not present in the binuclear center, no differences between the UV−vis spectra of the degraded F and P_N states should be observed. Therefore, we conclude that ammonia accesses the binuclear center, even after prior removal of excess H_2O_2.

The question of why the degraded P_N state cannot be directly formed upon addition of ammonia to CcO in the O state remains. In the resting O state, a stable bridging ligand, potentially a peroxide,[25,26] is located between the heme iron and Cu_B. The strong binding of this bridging peroxide might be too strong for ammonia to replace the Cu_B ligand. Binding of oxygen to Fe in the F state distinctly weakens the stability of the initial Cu_B ligand such that it now can be replaced by ammonia. One has to take into account the fact that the H_2O_2-generated oxoferryl is just transiently formed. It may very well be that ammonia is able to bind to Cu_B only when the oxoferryl is present, but once it has been bound, it will remain ligated to Cu_B after the Fe ligand has been exchanged. In such a scenario, CcO could not go back to the initial, resting, O state once H_2O_2 is consumed as the formation of a peroxide bridge between Fe and Cu_B is prevented by the presence of ammonia. A very slow regeneration of the resting O state might also be the reason for the observation that the 580 nm band can still be seen very clearly in the degraded F state.

CONCLUSIONS

In this paper, the H_2O_2 (and ammonia)-generated F and P_N states were investigated using resonance Raman spectroscopy. A shift of the oxoferryl stretching vibration from 790 cm^{-1}, seen in the F state, to 796 cm^{-1} in the P_N state was observed upon addition of ammonia. This observation proves that the presence of ammonia has a direct influence on the interaction of the heme-bound oxygen with nearby ligands in the binuclear center. This conclusion is further supported by the occurrence of a band at 608 nm in the UV−vis spectrum of the P_N state after addition of catalase, which is missing in the absence of ammonia.

Component analysis of CcO in the RR high-frequency region showed a dominant high-spin to low-spin heme a_3 transition in the F and P_N states followed by a major back transition to its high-spin state after addition of catalase. The high-spin to low-spin ratio was equal for the F and P_N states with and without catalase. From this result, we conclude that ammonia does not interact directly with the heme iron. In summary, these observations strongly support the hypothesis that ammonia in the P_N state, possible also in the degraded P_N state, is bound to the Cu_B as a fourth ligand and interacts via hydrogen bonds with the heme-bound oxygen.

ASSOCIATED CONTENT

Supporting Information

Kinetics of the high-spin to low-spin conversion of heme a_3 after addition of catalase, low-frequency RR $H_2{}^{16}O - H_2{}^{18}O$ difference spectra of the F and P_N states as a function of time, and RR spectra of CcO from *P. denitrificans* at 77 K. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

O, fully oxidized state; F, oxoferryl state after addition of a 1:500 excess of H_2O_2; P_N, oxoferryl state after addition of a 1:500 excess of H_2O_2 and 25 mM ammonia; F_{deg}, degraded F state resulting from F, after excess H_2O_2 had been removed with catalase; P_{Ndeg}, degraded P_N state resulting from P_N, after excess H_2O_2 had been removed with catalase.

■ ADDITIONAL NOTE

[a]Heme a_3=O denotes the new state that is created after the addition of H_2O_2. Whether this corresponds fully to an oxoferryl state cannot be unambiguously determined.

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