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Resonance Raman Detection of a Ferrous Five-Coordinate Nitrosylheme b_3 Complex in Cytochrome cbb_3 Oxidase from *Pseudomonas stutzeri*

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The chemistry of nitric oxide interactions with the binuclear center of heme-copper oxidases is of profound physiological relevance.[1,2] For example, the inhibition of cytochrome oxidase by NO may play a normal role in controlling mitochondrial O_2 consumption and can have significant consequences for the cell in pathophysiological states.[3,4] Evidence for a common phylogeny of aerobic respiration and bacterial denitrification, which was proposed on the basis of structural similarities between the *Pseudomonas stutzeri* NO reductase and the cbb_3 terminal oxidase, has been reported.[2] The cbb_3 oxidase from *Pseudomonas stutzeri* contains three *c*-type low-spin hemes, one low-spin *b*-type heme, and a heme b_3-Cu_B binuclear center.[5a,b] Stopped-flow studies of cbb_3 revealed strong similarities of NO reductase activity with those found in the ba_3 and caa_3 oxidases from *Thermus thermophilus*.[1,5a] In this study, we have used resonance Raman spectroscopy (RR), a technique that enables iron ligand stretching modes to be identified, to characterize the NO-bound complex of cytochrome cbb_3 oxidase.[6] Although the heme $b_3{}^{2+}-$NO should not be significantly populated in the productive enzymatic cycle, the characterization of its formation is important in establishing the conditions under which the enzyme becomes locked into a dead-end species, due to the very slow dissociation of NO from this adduct. We show simultaneous observation of the NO and $Fe^{2+}-$NO stretching modes at 1679 and 524 cm^{-1}, respectively, in ferrous nitrosyl cbb_3. These frequencies indicate that addition of NO to the fully reduced enzyme causes the cleavage of the His$-Fe^{2+}$ heme b_3 bond producing a five-coordinate heme $b_3{}^{2+}-$NO complex.

In Figure 1 we present the RR spectra of fully reduced cbb_3 from *Pseudomonas stutzeri*, and the NO-bound form in H_2O and D_2O. The principal bands seen in Figure 1A are assigned as analogous to those vibrations found in reduced cbb_3 from *Rhodobacter sphaeroides*.[7] The modes at 1362 (ν_4), 1467 (ν_3), and 1605 (ν_{10}) cm^{-1} indicate the presence of five-coordinate high-spin heme b_3. Also present in this spectrum are modes at 1494 (ν_3) and 1592 (ν_2) cm^{-1} indicating the presence of six-coordinated low-spin hemes b^{2+} and c^{2+}. In the reduced enzyme, NO ligation to high-spin heme $b_3{}^{2+}$ produces the low-spin heme $b_3{}^{2+}-$NO complex. The ligation and spin state change can be inferred by the modes at 1373 (ν_4), 1507 (ν_3), and 1644 (ν_{10}) cm^{-1}. In addition, a new mode appears at 1679 cm^{-1}. The most reasonable assignment of the 1679 cm^{-1} mode is that it arises from a five-coordinate nitrosyl heme $b_3{}^{2+}-$NO complex. Such an assignment is consistent with ν(NO) frequencies observed in other five-coordinate heme $Fe^{2+}-$NO complexes.[9−17a] Figure 1C shows the spectrum of the NO-bound cbb_3 in D_2O. Comparison of spectrum B with spectrum C shows

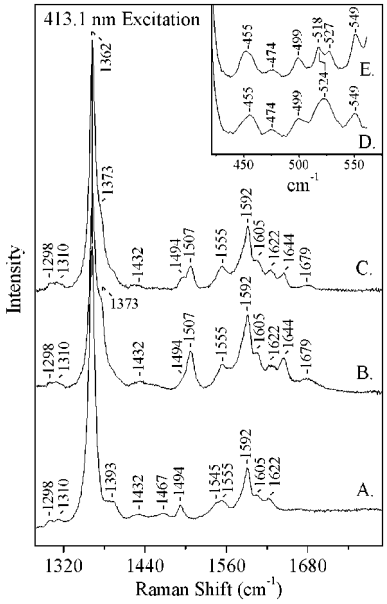

Figure 1. High-frequency resonance Raman spectra of (A) ferrous cbb_3, (B) ferrous cbb_3-NO in H_2O, and (C) ferrous cbb_3-NO in D_2O obtained with 413.1 nm excitation. Ferrous cbb_3 was prepared by injection of a degassed buffer solution containing dithionite into a spinning cell containing ferric enzyme. The NO adduct of ferrous cbb_3 was made by injection of 120 μL of NO into the headspace of the spinning cell (5000 rpm) under Ar atmosphere. The resonance Raman measurements were done as described previously.[6,19] Typically, several 30 s spectra were recorded and averaged. The power incident on the samples was typically 3−5 mW. Inset: Low-frequency resonance Raman spectra of (D) ferrous cbb_3-NO and (E) ferrous $cbb_3-{}^{15}$NO in H_2O.

that similar results are obtained in H_2O and D_2O buffers. This implies that there is no hydrogen bonding of the heme $b_3{}^{2+}-$NO complex at pH 7.4. The high-frequency spectrum of the ^{15}NO-bound heme b_3 (data not shown) shows that the vibration at 1679 cm^{-1} disappears. The $\nu(^{15}NO)$ is expected at 1636 cm^{-1} in a very congested region of the spectrum, preventing us from obtaining an isotope shift to assign $\nu(^{15}NO)$. In the low-frequency spectrum of NO-bound cbb_3 (inset, Figure 1D), we observe a vibration at 524 cm^{-1} that displays nitrogen isotopic sensitivity by shifting to 518 cm^{-1} in the ^{15}NO adduct (Figure 1E). This allows us to assign it as the Fe−NO stretching vibration, as the 6 cm^{-1} shift is in agreement with that expected from the two body harmonic oscillator approximation for $Fe^{2+}-$NO, but more important it is in excellent agreement with $\nu(Fe^{2+}-NO)$ frequencies observed in other five-coordinated model- and protein heme $Fe^{2+}-$NO complexes.[9−16] For an Fe−NO diatomic model, a 6 cm^{-1} frequency shift is predicted for the $^{14}N^{16}O$ to $^{15}N^{16}O$ comparison for a linear structure, whereas for a bent structure, a 16 cm^{-1} shift is predicted. Although the RR

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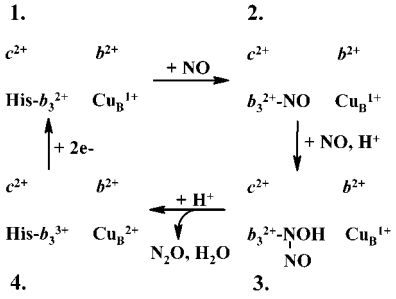
data presented here suggest a linear structure for heme b_3^{+2}−NO, X-ray crystallographic analyses of model porphyrin Fe^{2+}−NO complexes demonstrate a bent geometry.[17b]

The unusual low frequency of the Fe^{2+}−CO stretching frequency found in *cbb*$_3$ was attributed to an open structure lacking the distal interactions from the nearby Cu_B atom, thereby allowing the heme b_3-bound CO to adopt a near-straight and perpendicular conformation with respect to the heme.[18] The results reported here indicate that the distal heme pocket structure of the ferrous nitrosyl complex is similar to that of the CO-bound form of the enzyme. The frequencies for $\nu(Fe^{2+}-NO) = 524\ cm^{-1}$ and $\nu(NO) = 1679\ cm^{-1}$ that we observe are in the same range as those observed for other five-coordinated heme protein NO complexes. The range found for the $\nu(Fe^{2+}-NO)$ frequency is small, 523−526 cm^{-1}, while the ν(NO) covers a range from 1667 to 1681 cm^{-1}. Whereas it remains to be clarified as to what extent the polarity of the distal heme pocket, as is in the case for $\nu(Fe^{2+}-CO)$, influences the properties of the ν(NO), our data demonstrate that the distal heme pocket polarity in *cbb*$_3$ is similar to those of sGC ($\nu_{NO} = 1681\ cm^{-1}$) and FixL ($\nu_{NO} = 1677\ cm^{-1}$).[8,9,16]

To account for the possible oxidation and ligand-binding states upon addition of NO to the fully reduced *cbb*$_3$, we propose the mechanism shown in Scheme 1. Addition of NO to fully reduced enzyme causes the rupture of the proximal His−Fe heme b_3 bond producing a five-coordinate heme b_3^{2+}−NO species **2**. A second NO molecule attacks the N atom of the ferrous-NO species, and protonation occurs to transiently yield hyponitrite ($HONNO^-$), and thus the N−N bond formation. This form of the enzyme is unstable to oxidation and with the addition of a H^+ is leading to a mixed valence form **4** and the concomitant production of N_2O and H_2O. In the presence of dithionite, this form will return to the fully reduced form **1**. In our experiments on the fully reduced-NO enzyme, we find no evidence for any oxidized components, suggesting that sufficient dithionite is present in our samples to prevent formation of any oxidized species. Therefore, the formation of hyponitrite **3** from heme b_3^{2+}−NO **2**, which depends on excess NO, should be inhibited under our conditions, and the fully reduced heme b_3^{2+}−NO enzyme becomes the dominant species under our reducing conditions. This mechanism describing the generation of the nitrosyl species under the conditions used here can be postulated to serve as a working model for future experiments.

Scheme 1. Schematic Structures of the Redox Centers of Fully Reduced *cbb*$_3$ upon Addition of Nitric Oxide



The heme b_3 in *cbb*$_3$ is only partially reduced by ascorbate, and readily reoxidized by NO.[5] Full reduction of the enzyme requires the use of dithionite, as it is required for full reduction of nitric oxide reductase (Nor) from *Paracoccus denitrificans* and *cbb*$_3$ from *Rhodothermus marinus*.[19,20] This behavior seems indicative of an unusually low redox potential of heme b_3. A low midpoint redox potential for heme b_3 ($E_m = 60$ mV) in Nor has been reported, and it was suggested that full reduction of the dinuclear center is thermodynamically unfavorable.[21] Similar observations have been reported for *cbb*$_3$ from *Rhodothermus marinus*.[20] The nitrosyl-bound species we have detected requires dithionite and thus a fully reduced binuclear center. On this line, it has been recently proposed that NO activation in Nor occurs with a fully reduced dinuclear heme b_3/non-heme Fe center, in which the binding of NO to the ferrous heme b_3 results in the dissociation of the proximal histidine.[22]

The data reported here clearly demonstrate the existence of a five-coordinate heme b_3 Fe^{2+}−NO complex. The detection of the nitrosyl species is surprising and was not expected a priori. Such a species has been postulated in the past in many different NO reduction schemes.[22] However, no spectroscopic characterization of this species or even confirmation of its existence has been possible owing to a presumed short lifetime under turnover conditions. Future studies should allow other properties of the nitrosyl complex to be examined so that its complete role in the enzymatic function of nitric oxide reductase, cytochrome *ba*$_3$ from *T. thermophilus*, and cytochrome *cbb*$_3$ under physiological conditions may be determined. Moreover, the comparison of the five-coordinated to six-coordinated heme-bound NO complexes will allow us to investigate structural parameters that may facilitate the cleavage of the Fe−His bond by NO. These experiments are in progress in our laboratory.

References

(1) Giuffrè, A.; Stubauer, G.; Sart, P.; Brunori, M.; Zumft, W. G.; Buse, G.; Soulimane, T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14718−14723.

(2) Zumft, W. G. *Microbiol. Mol. Biol. Rev.* **1997**, *61*, 533−616.

(3) Brown, G. C. *FEBS Lett*. **1995**, *369*, 136−139.

(4) Shiva, S.; Brookes, P. S.; Patel, R. P.; Anderson, P. G.; Darley-Usmar, V. M. *Proc. Natl. Acda. Sci. U.S.A.* **2001**, *98*, 7212−7217.

(5) (a) Forte, E.; Urbani, A.; Saraste, M.; Sarti, P.; Brunori, M.; Giuffre, A. *Eur. J. Biochem*. **2001**, *268*, 1−6. (b) Stavrakis, S.; Koutsoupakis, K.; Pinakoulaki, E.; Urbani, A.; Saraste, M.; Varotsis, C. *J. Am. Chem. Soc*. **2002**, *124*, 3814−3815.

(6) Pinakoulaki, E.; Pfitzner, U.; Ludwig, B.; Varotsis, C. *J. Biol. Chem.* **2002**, *277*, 13563−13568.

(7) Varotsis, C.; Babcock, G. T.; Garcia-Horsman, A.; Gennis, R. B. *J. Phys. Chem.* **1995**, *99*, 16817−16820.

(8) Deinum, G.; Stone, J. R.; Babcock, G. T.; Marletta, M. A. *Biochemistry* **1996**, *35*, 1540−1547.

(9) Lukat-Rodgers, G. S.; Rodgers, K. R. *Biochemistry* **1997**, *36*, 4178−4187.

(10) Renolds, M. F.; Parks, R. B.; Burstyn, J. N.; Shelver, D.; Thorsteinsson, M. V.; Kerby, R. L.; Roberts, G. P.; Vogel, K. M.; Spiro, T. G. *Biochemistry* **2000**, *39*, 388−396.

(11) Choi, I. K.; Spiro, T. G.; Langry, K. C.; Smith, K. M. *Inorg. Chem*. **1991**, *30*, 1832−1839.

(12) Vogel, K. M.; Kozlowski, P. M.; Zgierski, M. Z.; Spiro, T. G. *J. Am. Chem. Soc*. **1999**, *121*, 9915−9921.

(13) Tomita, T.; Hirota, S.; Ogura, T.; Olson, J. S.; Kitagawa, T. *J. Phys. Chem. B* **1999**, *103*, 7044−7054.

(14) Schelvis, J. P. M.; Seibold, S. A.; Cerda, J. F.; Garavito, R. M.; Babcock, G. T. *J. Phys. Chem. B* **2000**, *104*, 10844−10850.

(15) Schelvis, J. P. M.; Zhao, Y.; Marletta, M. A.; Babcock, G. T. *Biochemistry* **1998**, *37*, 16289−16297.

(16) Tomita, T.; Ogura, T.; Tsuyama, S.; Imai, Y.; Kitagawa, T. *Biochemistry* **1997**, *36*, 10155−10160.

(17) (a) Nagai, K.; Welborn, C.; Dolphin, D.; Kitagawa, T. *Biochemistry* **1980**, *19*, 4755−4761. (b) Ellison, M. K.; Schheidt, W. R. *J. Am. Chem. Soc*. **1997**, *119*, 7404.

(18) Wang, J.; Gray, K. A.; Daldal, F.; Rousseau, D. L. *J. Am. Chem. Soc*. **1995**, *117*, 9363−9364.

(19) Pinakoulaki, E.; Gemeinhardt, S.; Saraste, M.; Varotsis, C. *J. Biol. Chem*. **2002**, *277*, 23407−23413.

(20) Pereira, M. M.; Carita, J. N.; Anglin, R.; Saraste, M.; Texeira, M. *J. Bioenerg. Biomembr*. **2000**, *32*, 143−152.

(21) Grönberg, K. L. C.; Roldan, M. D.; Prior, L.; Butland, G.; Cheesman, M. R.; Richardson, D. J.; Spiro, S.; Thomson, A. J.; Watmough, N. J. *Biochemistry* **1999**, *38*, 13780−13786.

(22) Moënne-Loccoz, P.; de Vries, S. *J. Am. Chem. Soc.* **1998**, *120*, 5147−5152.

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