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The Structure of the Hyponitrite Species in a Heme Fe–Cu Binuclear Center**

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The formation of the hyponitrite species from two NO molecules is the key intermediate in the reduction of NO to N_2O in denitrification.[1] The reaction is used by bacteria as an alternative to oxygen-based respiration.[1,2] The bacterial nitric oxide reductase (Nor) and the ba_3-oxidoreductase from *Thermus thermophilus* catalyze the reaction: $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$.[2] The latter enzyme contains a homodinuclear copper complex (Cu_A), one low-spin, six-coordinate heme *b*, and a binuclear center that consists of Cu_B and a high-spin heme a_3 in which the catalytic reactions take place.[3] A full elucidation of the structure and the electronic configuration of various intermediates are of profound importance for understanding the mechanism by which the enzymes form and cleave the N–N and N–O bonds, respectively. The mechanisms of the reduction of NO to N_2O by both Nor and ba_3-oxidoreductase are poorly understood because of the lack of detection of the short-lived intermediate species.[4,5] However, a six-coordinate heme Fe^{3+}–NO species[6] and a five-coordinate heme Fe^{2+}–NO species in Nor[7] have been characterized. In addition, a six-coordinate heme Fe^{2+}–NO species has been detected in ba_3-oxidoreductase and it has been proposed that a hyponitrite ion ($HONNO^-$) bound to the heme Fe^{3+}/Cu_B^+ center is transiently formed under reducing conditions.[8] Recently, density functional theory (DFT) calculations have provided a quantitative accurate atomic-level description of the key steps in the mechanism for the formation of the N–N bond and cleavage of the N–O bond.[9,10] The optimized molecular structure of the heme Fe–Cu_B-bound hyponitrite intermediate computed at the B3LYP/6-31G* theory level is shown in Figure 1. We have studied the reaction of ba_3-oxidoreductase with NO to determine how two NO molecules are activated in the binuclear heme a_3 Fe–Cu_B center of the enzyme. The resonance Raman (RR) data reveal a surprising and unprecedented coordination of the formed hyponitrite species that provides insight into the catalytic mechanism. The initial binding of two NO molecules to the heme a_3 Fe–Cu_B binuclear center is followed by protonation of the heme a_3–NO species and concomitant formation of the N–N bond.

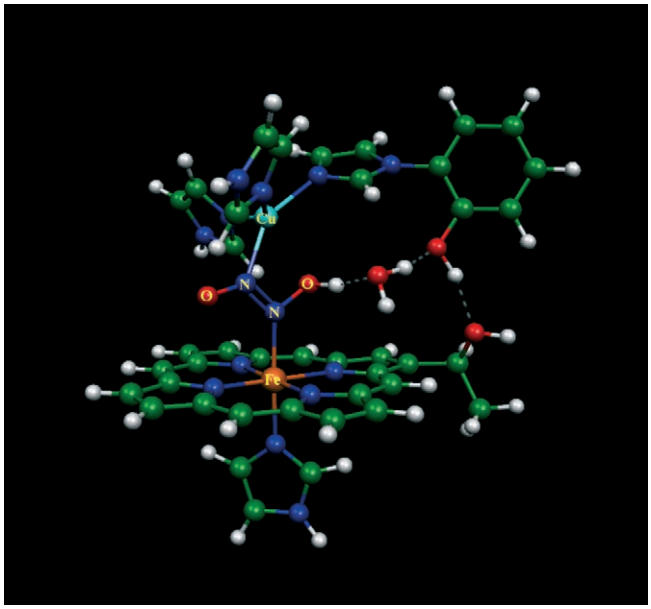

Figure 1. Optimized molecular structure of the heme a_3 Fe–Cu_B bound-hyponitrite intermediate.

The optical absorption spectrum of oxidized ba_3 enzyme displays Soret band maxima at 414 nm (heme b^{3+} and heme $a_3{}^{3+}$), absorption maxima at 558 nm (heme b^{3+}), and a

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weak charge-transfer transition at 660 nm (heme a_3^{3+}; Figure 2, trace A). Flushing NO over the oxidized enzyme shifts the Soret band of heme a_3^{3+} to 434 nm, while the bands corresponding to heme b^{3+} remain, as expected, unchanged

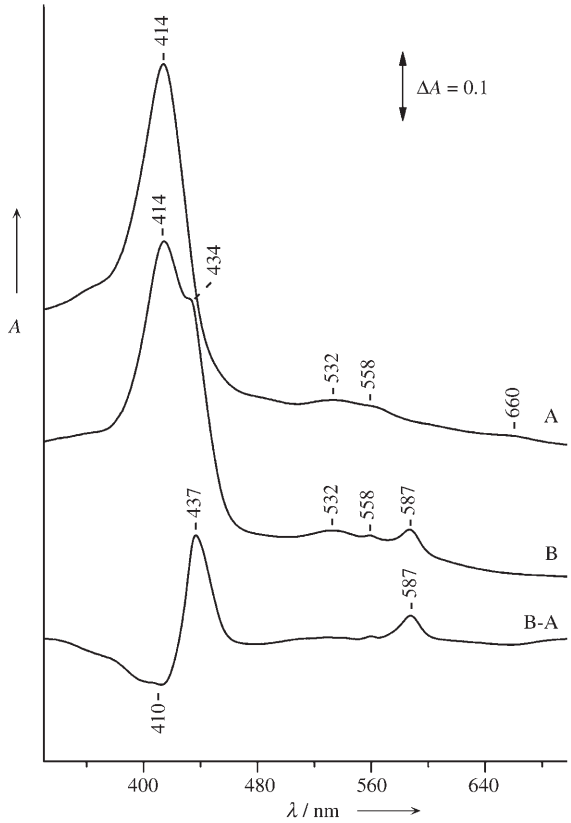


Figure 2. Optical absorption spectra at room temperature of oxidized ba_3-oxidase from *T. thermophilus* (trace A) and upon addition of NO (trace B). B−A is the difference spectrum.

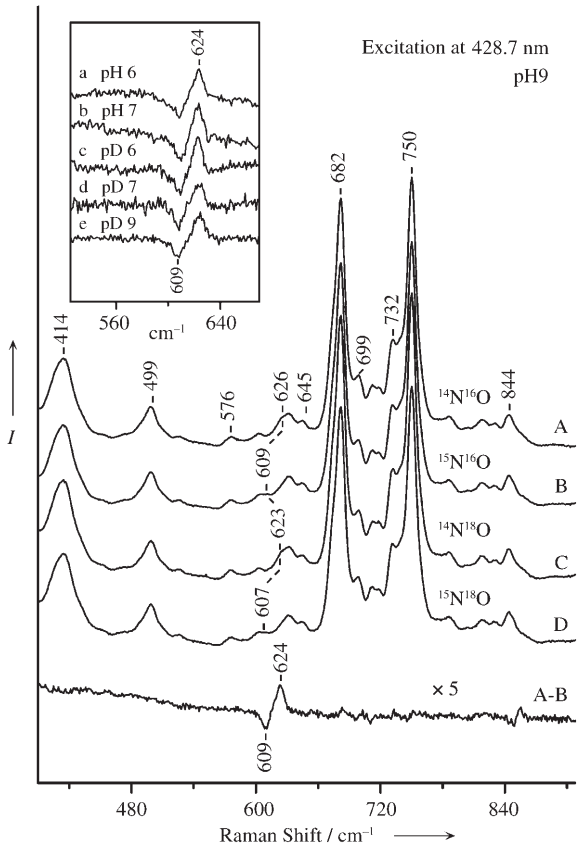


Figure 3. Low-frequency resonance Raman spectra at room temperature of oxidized ba_3-oxidase upon addition of $^{14}N^{16}O$ (trace A), $^{15}N^{16}O$ (trace B), $^{14}N^{18}O$ (trace C), and $^{15}N^{18}O$ (trace D) at pH 9. The difference spectrum A−B is the difference in the spectra of the $^{14}N^{16}O$ and $^{15}N^{16}O$ adducts. Inset: Difference in the spectra of the $^{14}N^{16}O$ and $^{15}N^{16}O$ adducts at pH 6 (trace a), pH 7 (trace b), pD 6 (trace c), pD 7 (trace d), and pD 9 (trace e). The excitation laser wavelength was 428.7 nm and the incident laser power 1 mW. Total accumulation time was 20–30 min for each spectrum.

(Figure 2, trace B). The difference spectrum of the oxidized NO-bound form minus that of the oxidized form (B−A) is characteristic of NO binding to heme a_3, as denoted by the transitions at 437 and 587 nm. This difference spectrum (B−A) is not due to autoreduction because the heme a_3 Fe^{2+}–NO complex absorbs at 423 and 599 nm.[8]

In all the visible RR experiments we probed the species generating the absorption bands at 437/587 nm that is formed upon the addition of NO to oxidized ba_3-oxidoreductase. The high-frequency (1200–1925 cm^{-1}) RR spectra (see the Supporting Information) indicate that heme a_3 becomes ferric six-coordinate and low-spin upon exposing the oxidized enzyme to NO. In addition, no autoreduction (heme Fe^{2+}–NO, $\tilde{\nu}_{NO}$ = 1620 cm^{-1}) was observed.[8] The difference spectrum of the $^{14}N^{16}O$- and $^{15}N^{18}O$-bound oxidized enzymes, however, shows no evidence of a peak/trough pattern in that frequency range which could be assigned to a heme a_3 Fe^{3+}–NO species.

The low-frequency RR spectra of oxidized ba_3 upon addition of NO are shown in Figure 3. Trace A is from a sample of oxidized enzyme that was exposed to $^{14}N^{16}O$, while traces B, C, and D are from an oxidized enzyme sample that was exposed to $^{15}N^{16}O$, $^{14}N^{18}O$, and $^{15}N^{18}O$, respectively. The RR mode at 626 cm^{-1} (trace A) exhibits nitrogen and oxygen isotopic sensitivity as seem by a shifting of the band to 609 (trace B), 623 cm^{-1} (trace C), and 607 cm^{-1} (trace D), respectively. The difference spectrum (A−B) confirms the 626 cm^{-1} peak as a mode that involves motion of the NO molecule, since it downshifts by 15 cm^{-1} upon isotope substitution. In general, an Fe–ligand (NO) stretching vibration displays a monotonic shift toward lower wavenumbers as the ligand mass is increased. A zigzag shift pattern (decrease-increase-decrease) on isotopic substitution from $^{14}N^{16}O$ to $^{15}N^{16}O$ to $^{14}N^{18}O$ to $^{15}N^{18}O$ is a characteristic behavior of a bending mode. The relative insensitivity of the bending vibration to the mass of the terminal oxygen atom in NO is cited as evidence for its assignment as the bending mode rather than as a stretching mode; thus, the mode observed at 626 cm^{-1}, despite its similarity in frequency with ν(Fe-N(H)O) of myoglobin,[11] has bending character. No other isotope-sensitive modes were identified in the low-frequency RR spectrum of ba_3–NO. The inset shows that the species that was detected is insensitive to pH/pD exchanges over pH/pD 6–9.

Although the Fe^{3+}–NO stretching and the Fe^{3+}–N–O bending modes of ferric nitrosyl-heme proteins/enzymes have been detected under excitation of the Soret band,[6,11,12,13] the N–O stretching mode in the 1800–1925 cm^{-1} region has been observed, with the exception of P450 Nor,[12] only under excitation with UV light.[14] It has been suggested that there is little orbital conjugation between the NO ligand and the heme, and because such conjugation is not necessary to occur for UV/RR detection, it was proposed that the UV/RR enhancement of the N–O stretching mode is the result of a localized π–π* transition.[14] Resonance Raman excitation in the UV region enhances strong bands derived from aromatic residues such as tyrosine (Y) and tryptophan (W).[15] The RR excitation of oxidized ba_3 and myoglobin (Mb) at 244 nm upon addition of NO is shown in the Supporting Information. The peak/trough pattern at 1921/1883 cm^{-1} confirms the detection of the Mb–NO species under excitation with UV light.[14,16] We probed the species with absorption bands at 437/587 nm that is formed upon addition of NO to oxidized ba_3-oxidoreductase in all the UV/RR experiments. The UV/RR spectra of ba_3 upon addition of NO to the oxidized enzyme, however, show no evidence for the formation of the NO-bound species. If the NO ligand was bound to heme a_3 to form an abnormal Fe^{3+}–nitrosyl species, the N–O stretching vibration should have been observed. However, there is no evidence of any isotope-sensitive NO modes in the entire 1550–2400 cm^{-1} frequency range. Therefore, we conclude that the heme a_3 transition at 437/587 nm that we have probed by excitation with visible and UV light is not due to NO-bound heme a_3^{3+}.

Figure 4 shows the UV/RR spectra in the 1100–1580 cm^{-1} frequency range of the oxidized ba_3-oxidase in the pH/pD 6–9 range upon addition of $^{14}N^{16}O$, $^{15}N^{16}O$, and $^{15}N^{18}O$. The strong bands in the spectra arise from 1553 (W3), 1491 (W4), 1458 (W5), 1440 (W6), 1356 (W7), 1339 (W7), 1235 (W10), 1204 (Y7a), 1173 (Y9a), and 1127 (W13). Spectra C ($^{14}N^{16}O-^{15}N^{16}O$), D ($^{14}N^{16}O-^{15}N^{18}O$), and E ($^{14}N^{16}O-^{15}N^{16}O$) show the presence of peak/trough patterns at 1334/1298, 1338/1300, and 1338/1300 cm^{-1}, respectively, thus indicating a ^{15}N isotope sensitivity of the 1334 cm^{-1} peak. No other distinct features are present in the difference spectra, thus indicating that ligand binding to heme a_3 and H/D exchanges cause no significant structural changes to the Tyr and Trp residues.

Infrared and Raman spectroscopic studies on sodium, silver, and mercury salts of hyponitrous acid have furnished conclusive evidence that the hyponitrite ion ($HO-N=N-O^-$) has a *trans* form.[17,18] The fundamental frequency of the N–N bond at 1392 cm^{-1} for sodium hyponitrite in aqueous solution has been characterized.[17] We identify the mode at 1334 cm^{-1} in ba_3 as the N–N stretching vibration of the hyponitrite species on the basis of the ^{15}NO isotope shift and the similarity of its frequency to that reported for the free hyponitrite ion ($HO-N=N-O^-$).[17] The difference of 58 cm^{-1} between the hyponitrite bound to heme a_3 and the hyponitrite ion indicates that the N–N bond has less double-bond character when bound to heme a_3. Our assignment is further supported by the similarity in the frequency as well as the nitrogen and oxygen isotope shifts to those predicted from density functional theory (DFT) calculations.[9] The computed $\nu_{N\text{-}N}$ frequency at 1252 cm^{-1} exhibits sensitivity to the ^{15}N isotope ($\Delta\nu = 36$ cm^{-1}), while the $^{14}N^{18}O$ isotope does not affect the frequency significantly. Indeed, the N–N vibration, which is detected only by excitation with UV light, is not affected by substitution with the ^{18}O isotope. This observation further supports our assignment and excludes the possible assignment to an N–O stretch. Such an NO stretch ($\tilde{\nu}_{NO} = 1385$ cm^{-1}) with comparable ^{15}N isotope shift has been detected by excitation of the Soret band of Mb–HNO.[11]

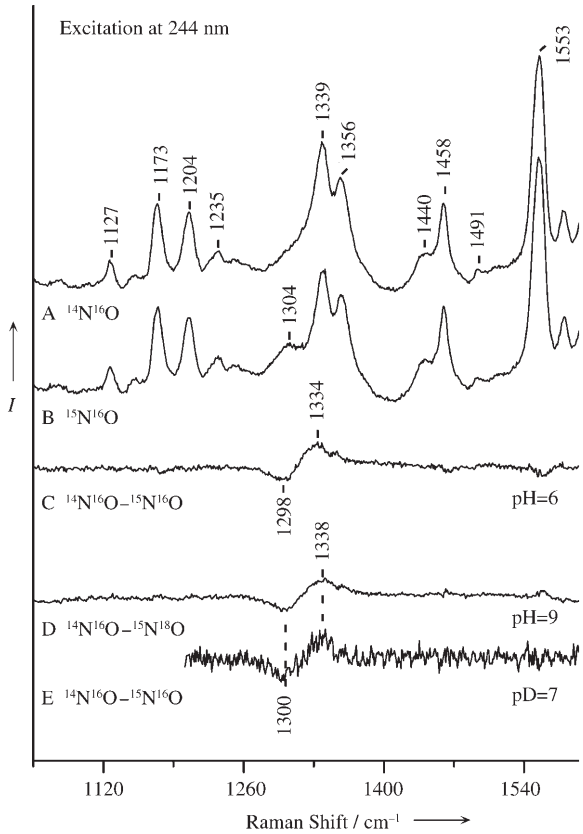
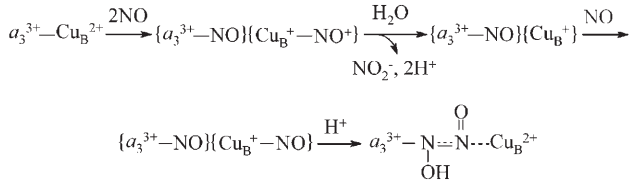


Figure 4. UV resonance Raman spectra at room temperature of oxidized ba_3 upon addition of $^{14}N^{16}O$ (trace A) and $^{15}N^{16}O$ (trace B) at pH 6. The difference in the spectra of the $^{14}N^{16}O$ and $^{15}N^{16}O$ adducts is shown in trace C. The difference in the spectra of the $^{14}N^{16}O$ and $^{15}N^{18}O$ adducts at pH 9 is shown in trace D, and the difference in the spectra of the $^{14}N^{16}O$ and $^{15}N^{16}O$ adducts at pD 7 is shown in trace E. The excitation laser wavelength was 244 nm, the incident laser power 200 μW, and the total accumulation time was 30–40 min for each spectrum.

The difference spectra D (pH 9) and E (pD 7) show that the 1334/1298 cm^{-1} pattern is shifted to 1338/1300 cm^{-1}. If the normal coordinate of a vibrational mode includes a proton contribution, the increase in mass due to deuterium substitution is expected to lead to a decrease in frequency. We detect a small but clear increase (4 cm^{-1}) in frequency upon deuteration (pD 7) and under alkaline conditions (pH 9). This observation indicates that if the heme a_3 Fe-N-O(H) unit of the bound hyponitrite is protonated, the bound proton is not exchangeable. Although $HO-N=N-O^- \xrightleftharpoons{pK_a=11.5} {}^-O-N=N-O^-$, it is known that the pK_a value of substrates bound to proteins deviate from those in aqueous solution.[19]

The N–N stretching frequency we have detected is only slightly perturbed over pH 6–9. Therefore, the bound hyponitrite retains the same protonation state over this pH range.

On the basis of our observations we propose a reaction scheme for the initial binding of two NO molecules to the oxidized heme a_3–Cu_B binuclear center (Scheme 1). We



Scheme 1. Proposed mechanism for the formation of the hyponitrite species in the oxidized heme a_3-Cu_B binuclear center of ba_3-oxidoreductase from *Thermus thermophilus* (see text). The full catalytic cycle is presented in Ref. [8].

suggest that upon addition of NO to the oxidized enzyme a {heme a_3 Fe^{3+}–NO}{Cu_B^{+}-NO^{+}} complex is formed, and in the presence of an H_2O molecule, NO_2^- and $2H^+$ are released to form a {heme a_3 Fe^{3+}–NO}{Cu_B^{+}} complex. The formation of the N–N bond in the oxidized binuclear center requires an electron. Since a heme a_3 Fe^{2+}–NO species was not observed in our experiments, the electron required for the formation of the N–N bond must originate from the autoreduction of Cu_B by NO as described above. The mechanism for the reduction of Cu^{II} by NO has been described previously.[20] Importantly, the autoreduction of Cu_B by NO to produce nitrite in aa_3 cytochrome oxidase was demonstrated recently.[21] The addition of NO to the {heme a_3 Fe^{3+}–NO}{Cu_B^{+}} complex produces {heme a_3 Fe^{3+}-NO}{Cu_B^{+}-NO}, and when protonated the steady-state hyponitrite species is formed. Thus, Fe is in the +3 oxidation state, hyponitrite is neutral (or −1 if deprotonated) and Cu_B is in the +2 state. It should be noted that Cu_B is in the reduced form (+1) and Fe is in the +3 oxidation state when the hyponitrite species is formed in the reaction of fully reduced ba_3-oxidoreductase with NO.[8]

The transient binding of two NO molecules to the heme a_3 Fe–Cu_B binuclear center is accompanied by proton uptake. Evidence for the presence of an H_2O molecule near the bound hyponitrite is provided by the pH- and pD-sensitivity of the N–N vibration. This behavior reflects the proton control aspects of the enzyme and indicates that the proton-uptake reaction and the electron transfer from Cu_B^{+} are the rate-limiting steps in the formation of the N–N bond. The detection of the hyponitrite intermediate presented here underscores the complexity of the NO reduction process. The environment of Cu_B, and thus, the vibrational modes involving the Cu_B-N-O unit should be monitored to obtain a quantitative understanding of the NO reduction mechanism. A quantitative model of the initial electron transfer steps will lead to a better understanding of the structure of the catalytic intermediates and a framework on which to build concepts for the concomitant formation of the N–N bond and cleavage of the N–OH bond to produce laughing gas.

In summary, we have presented resonance Raman spectroscopic evidence for the formation of the hyponitrite species upon the addition of NO to oxidized ba_3-oxidoreductase. The hyponitrite species is characterized from the observation of the heme a_3 Fe-N-OH bending vibration at 626 cm^{-1} and the N–N stretching vibration at 1334 cm^{-1}. The experiments presented here represent our initial efforts to understand the complex interplay between the proton and electron transfers that are necessary to construct and control the formation of the N–N bond and the subsequent cleavage of the N–OH bond to produce laughing gas. Taken together, the detection of the hyponitrite intermediate in the heme a_3 Fe–Cu_B binuclear center is particularly important in view of its key role in the hypothesis of common evolutionary origin of aerobic respiration and bacterial denitrification.

Experimental Section

Oxidized ba_3 enzyme was isolated from *T. thermophilus* HB8 cells according to previously published procedures.[3] Oxidized samples were exposed to 1 atm NO in an anaerobic rotating quartz cell for the Raman measurements. The samples used for the RR measurements had an enzyme concentration of 50 μM for excitation at 428.7 nm and 150 μM for excitation at 244 nm, and were placed in a desired buffer (pH 5.5–6.5, β-morpholinoethanesulfonic acid (MES); pH 7.5, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES); pH 8.5–9.5, 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES)). The resonance Raman spectra were acquired as described elsewhere.[14,15,22–24] The incident laser power of the 428.7-nm excitation frequency was 1 mW and the total accumulation time was 20–30 min for each spectrum. The incident laser power for the experiment with excitation at 244 nm was 200 μW and the total accumulation time was 30–40 min for each spectrum.

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