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Nitric oxide activation by *caa*$_3$ oxidoreductase from *Thermus thermophilus*

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Visible and UV-resonance Raman spectroscopy was employed to investigate the reaction of NO with cytochrome *caa*$_3$ from *Thermus thermophilus*. We show the formation of the hyponitrite (HO-N=N-O)$^-$ bound to heme a_3 species ($\nu_{N=N}$= 1330 cm^{-1}) forming a high spin complex in the oxidized heme a_3 Fe/Cu$_B$ binuclear center of *caa*$_3$-oxidoreductase. In the absence of heme a_3 Fe^{2+}-NO formation, the electron required for the formation of the N=N bond originates from the autoreduction of Cu$_B$ by NO, producing nitrite. With the identification of the hyponitrite intermediate the hypothesis of a common phylogeny of aerobic respiration and bacterial denitrification is fully supported and the mechanism for the 2e$^-$/2H$^+$ reduction of NO to N_2O can be described with more certainty.

Introduction

The formation of the hyponitrite species (HO-N=N-O) and the cleavage of the N-O bonds are the key steps towards our understanding of the mechanism of the reduction of NO to N_2O in denitrification.[1-5] The reaction is used by bacteria as an alternative to oxygen-based respiration.[4] The bacterial Nitric oxide reductase (Nor) from *P. denitrificans* and several heme-coper oxidases including *ba*$_3$ and *caa*$_3$ from *Thermus thermophilus*, *cbb*$_3$ from *P. stutzeri*, and *bo*$_3$ from *E. coli* display NO and O_2 reductase activity, supporting the hypothesis of the two types of enzymes having a common phylogeny.[1,5] On the other hand, despite the similarity in the structure of the reductive heme-Cu$_B$ binuclear site, the *aa*$_3$-type heme-copper oxidase do not display NO reductase activity.[6] The thermophilic Gram-negative eubacterium *Thermus thermophilus* HB8 (ATCC27634) expresses cytochromes *caa*$_3$ and *ba*$_3$ that serve as terminal oxidases for reducing oxygen to water, and also catalyze the reduction of nitric oxide to nitrous oxide under anaerobic conditions.[5,7] These findings support the hypothesis of a common evolutionary origin of bacterial denitrification and aerobic respiration.[5] Cytochrome *caa*$_3$ from *Thermus thermophilus* contains a mixed-valence [Cu$_A^{1.5+}$-Cu$_A^{1.5+}$] homodinuclear copper complex, two low-spin hemes (*a*- and -*c* type), and a binuclear center that consists of Cu$_B$ and heme a_3.[8,9] The *a*-type heme in cytochrome *caa*$_3$ contains a hydrophobic hydroxyethylgeranylgeranyl-group instead of a hydroxyethylfarnesyl chain found in most bacterial and eucaryotic *aa*$_3$ oxidases.[8] Importantly, heme *c* in cytochrome *caa*$_3$ is covalently bound providing an entity that has been found only in a few bacteria. Given that *caa*$_3$ is one of the heme-copper oxidases that show NO reductase activity, it is essential to learn the electronic, ligand coordination structure, and the unique NO-chemistry catalyzed by the enzyme.

Structural information on the heme a_3-Cu$_B$ center of *ba*$_3$ and *caa*$_3$ from *T. thermophilus* has been determined from a great number of resonance Raman studies.[10-16] Recently, the first RR excitation spectra of *aa*$_3$ oxidase in the UV region where strong bands derived from aromatic residues such as tyrosine (Y) and tryptophan (W) were reported.[14] An innovative UV-RR spectroscopic method has been developed for detecting the NO stretching mode in a variety of heme Fe^{3+}-NO proteins.[17] Although the Fe^{3+}-NO stretching and the Fe^{3+}-N-O bending modes of ferric nitrosyl-heme proteins/enzymes have been detected under Soret excitation, the N-O stretching mode in the 1900-1925 cm^{-1} region has been observed only under UV-excitation.[17] It was 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.[17]

The UV-Raman detection of the N-N stretching frequency at 1338 cm^{-1} in *ba*$_3$ indicated that the N=N is not a single bond

and at least one electron is necessary to form such a strong N=N bond. Since there is a consensus on the mechanism of autoreduction of Cu by NO to produce NO_2^- the required electron must originated from the autoreduction of Cu_B by NO.[6,18] Based on our observations we proposed a reaction scheme for the initial binding of two NO molecules to the oxidized heme a_3-Cu_B binuclear center.[18] In the scheme we suggested that upon addition of NO to the oxidized enzyme a heme a_3 Fe^{3+}-NO Cu_B^{1+}-NO^+ complex is formed and in the presence of a H_2O molecule, NO_2^- and $2H^+$ are released forming a heme a_3 Fe^{3+}-NO Cu_B^{1+} complex. Addition of NO to heme a_3 Fe^{3+}-NO Cu_B^{1+} complex produces heme a_3 Fe^{3+}-NO ON-Cu_B^{1+} and when protonated the steady state hyponitrite species is formed.

In the present work we have explored the reaction of oxidized *caa*$_3$ oxidase with NO with the aim to fully characterize the unique coordination of the NO ligand with heme a_3. The 428.7 nm Soret excitation indicated the formation of a six-coordinated high spin complex without, however, detection of the bound ligand. The UV-excitation data in the 1800-2000 cm^{-1} region showed the absence of the N-O stretching mode. Instead, we observed the formation of the hyponitrite species, as a result of the presence of two NO molecules in the binuclear center, with a characteristic frequency (ν_{N-N}= 1330 cm^{-1}). The 62 cm^{-1} difference between the hyponitrite-bound to heme a_3 (1330 cm^{-1}) and the free hyponitrite ion (1392 cm^{-1}) indicates that the N=N bond has less double character in the bound to the heme a_3 form.[20,21] The $\Delta\nu$=8 cm^{-1} in the frequency of the bound hyponitrite between *ba*$_3$ and *caa*$_3$ indicates that the bound hyponitrite experiences different interactions in their binuclear centers that affects the strength of the N-N bond. The ability of Cu_B^{2+} to produce NO_2 is common in both the *ba*$_3$ and *caa*$_3$ oxidoreductases from *T. thermophiles* and that of *aa*$_3$ cytochrome oxidase.[6]

Experimental

Fermentation of the *Thermus thermophilus* was performed at the Gesellschaft für Biotechnologische Forschung (Braunschweig, Germany). The purification of *caa*$_3$ oxidase and Nitric oxide reductase were according to published procedures.[8,9,21] Mb was purchased from Sigma-Aldrich. The concentration of *caa*$_3$ was determined from reduced-oxidized enzyme using an absorption coefficient ε_{605}=11.7 $mM^{-1}.cm^{-1}$. Oxidized samples were exposed to 1 atm NO in an anaerobic cell and loaded anaerobically into a cell with CaF_2 windows and a 0.025 mm spacer. NO gas was obtained from Messer (Germany) and isotopic NO (^{14}NO) was purchased from Isotec. FTIR spectra were obtained from 400-500 μM samples with a Bruker Equinox 55 FTIR spectrometer equipped with liquid nitrogen cooled mercury cadmium telluride detector. The FTIR 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 static FTIR measurements was 2 cm^{-1}. Optical absorbance spectra were recorded before and after FTIR measurements in order to assess sample stability with a Perkin-Elmer Lamda 20 UV-visible spectrometer. 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 the 428.7 nm excitation and 150 μM for the 244 nm excitation experiments, placed in a 50 mM HEPES buffer, pH 7.5. The resonance Raman spectra were acquired as described elsewhere.[12,13,14] The incident laser power for the 406 and 428.7 nm excitation was 3-4 mW and for the 244 nm experiment was 200 μW. The total accumulation time was 30-40 min for each spectrum for the 406 and 428.7 nm excitations Different samples of *caa*$_3$ were accumulated for 8-10 min each resulting in a total accumulation time of 100-120 min for each spectrum. Optical absorption apectra after 10 min of 244 nm Raman excitation showed reduced intensity at 442 nm.

Results and discussion

The optical absorption spectrum of resting (as isolated) *caa*$_3$ from *T. thermophilus* display Soret maxima at 410 (heme c^{3+}) and 424 nm (hemes a^{3+} and a_3^{3+}), and a visible band at 598 nm (Fig. 1, *trace A*). Flushing NO over the oxidized enzyme shifts the Soret to 440 nm (heme a_3^{3+}) while the bands due to hemes c^{3+} and a^{3+} remained, as expected, unchanged (Fig. 2, trace B) . The difference spectrum (B-A) of the oxidized NO-bound *minus* oxidized form is characteristic of NO binding to heme a_3, as denoted by the transitions at 442 and 603 nm. The resulted difference spectrum (B-A) upon addition of NO is not the result of autoreduction because the heme a_3 Fe^{2+}-NO complex absorbs at 423 and 599 nm.[10]

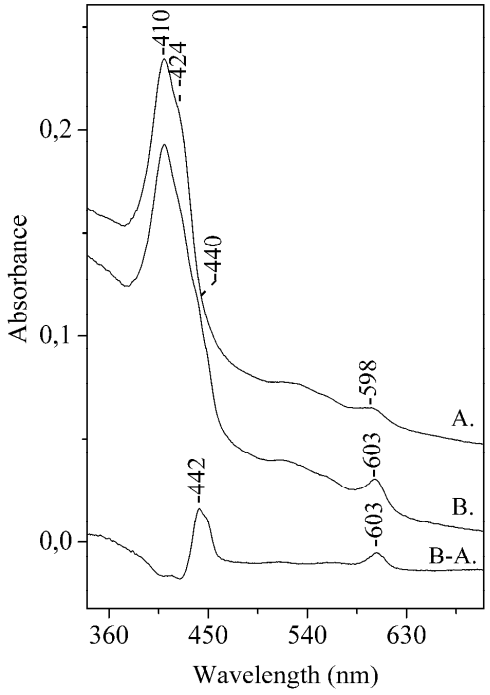

Fig. 1. Optical absorption spectra of cytochrome *caa*$_3$ from *T. thermophilus* at room temperature in the oxidized (*trace A*) and upon the addition of NO *(trace B)* forms. B-A is the difference spectrum.

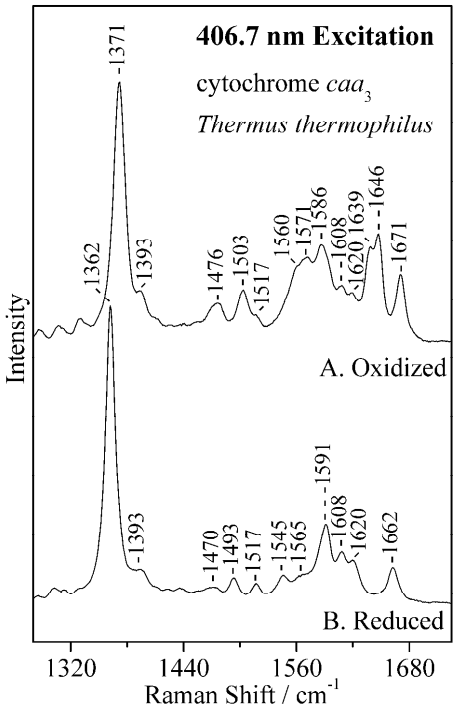

Fig. 2. High-frequency RR spectra of caa_3 in the oxidized (*trace A*) and reduced (*trace B*) forms.The excitation laser wavelength was 406.7 nm. The accumulation time was 10 min for each spectrum.

The high-frequency RR spectra of caa_3 in the oxidized (*trace A*) and reduced (*trace B*), forms are depicted in Fig. 2. With our excitation wavelength (406.7 nm) all hemes contribute to the intensity of the Raman scattering. The high-frequency (1300-1700 cm^{-1}) RR data contain several porphyrin modes termed as the oxidation state (ν_4) and the ligation state (ν_3, ν_2, and ν_{10}) modes. The modes of the oxidized enzyme (*trace A*) at 1371 (ν_4), 1475 (ν_3), 1571 (ν_2) and 1608 (ν_{10}) cm^{-1} indicate the presence of a six-coordinate high spin (6C/HS) heme a_3 as observed previously.[16] Also present in this spectrum are modes at 1503 (ν_3), 1586 (ν_2) and 1639 (ν_{10}) cm^{-1}, indicating the presence of six-coordinate low-spin (6C/LS) heme *c* and heme *b*. The modes at 1646 and 1671 cm^{-1} originate from the formyl (C=O) mode of hemes *a* and a_3, respectively. In the spectrum of the reduced enzyme (*trace B*), the ν_4 mode is located at 1362 cm^{-1} demonstrating that all hemes are in the ferrous state. The ν_3 at 1470 cm^{-1}, ν_2 at 1565 cm^{-1}, and ν_{10} at 1608 cm^{-1} establish the presence of a ferrous, five-coordinate high spin heme a_3. The presence of low-spin hemes *c* and *b* is shown by ν_3 intensity at 1493 cm^{-1}. The mode at 1591 cm^{-1} is assigned to ν_2 of the low spin hemes *a* and *c*. The 1620 cm^{-1} mode originates from the vinyl (C=C) group of all hemes and the 1662 cm^{-1} from the formyl group (C=O) of heme a_3.

Addition of NO to oxidized caa_3 causes noticeable changes in the RR spectra (Fig. 3 *traces A* and *B*). Ligand binding to heme a_3 is apparent from the concomitant observation of ν_4 at 1360 and ν_3 at 1468 cm^{-1}. The ferric nitrosyl adducts of His-heme proteins exhibit the Fe^{3+}-NO vibrations in the 1900 cm^{-1} region.[17] The absence of a trough/peak in the difference spectrum (*trace A-trace B*) clearly demonstrates that none of the changes we observe in the high-frequency spectra are due to nitrosyl ligation to heme a_3. It should be noted, however, that the NO stretching vibration, with the exception of PN450 Nor, has not been observed under Soret excitation.[22] In the FTIR spectrum (inset) of oxidized caa_3 upon addition of NO (440 nm species), as opposed to other His-heme-NO proteins such as Mb trace a (ν_{NO} =1921 cm^{-1}) and Nitric oxide reductase (Nor, ν_{NO} =1904 cm^{-1}), no characteristic NO stretching frequency is present. Consequently, the ligation to heme a_3 observed under Soret excitation is not due to nitrosyl ligation to heme a_3 or to the formation of the heme a_3 Fe^{2+}-NO species (ν_{NO} =1620 cm^{-1}).[10]

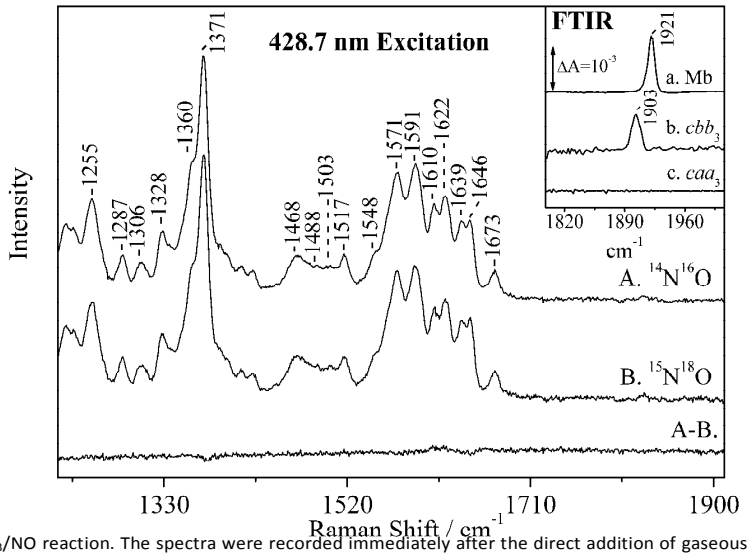

Fig. 3. RR spectra of the oxidized caa_3/NO reaction. The spectra were recorded immediately after the direct addition of gaseous $^{14}N^{16}O$ (*trace A*) and $^{15}N^{18}O$ (*trace B*) to oxidized enzyme. A-B is the difference spectrum. The excitation laser wavelength was 428.7 nm. The accumulation time was 30 min for each spectrum. The inset shows the FTIR spectra of NO bound to Mb (*trace* a), Nitric oxide reductase (*trace* b) and caa_3 (*trace* c).

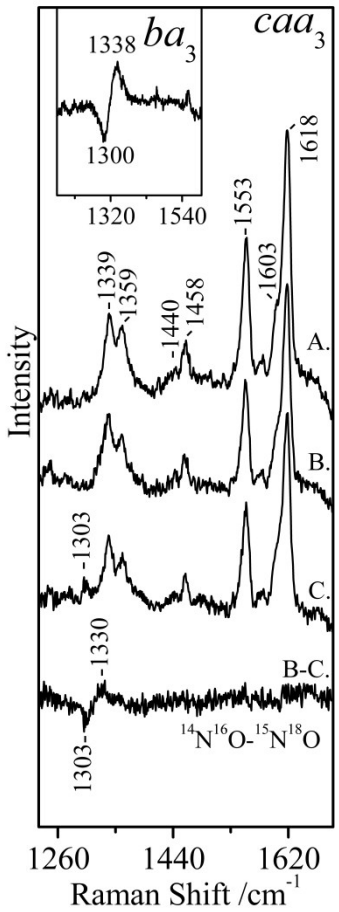

Fig. 4. UV-resonance Raman spectra of oxidized *caa₃ (trace* A), upon addition of $^{14}N^{16}O$ (*trace* B) and $^{15}N^{18}O$ (*trace C*), at room temperature. Trace B – trace C is the difference spectrum. The inset shows, the difference spectrum of the $^{14}N^{16}O$ *minus* $^{15}N^{18}O$ oxidized *ba*$_3$ adduct. The excitation laser wavelength was 244 nm, the incident laser power 150 μW, and the total accumulation time 120 min for each spectrum.

In all UV-RR experiments we probed the 440 nm species that is formed upon addition of NO to oxidized *caa*$_3$ at pH 8. The UV-RR spectra of *caa*$_3$ upon addition of NO to the oxidized enzyme in the 1700-1900 cm^{-1} spectral region, however, show no evidence, in agreement with the FTIR results, for the formation of the NO-bound species. The UV-RR spectra in the 1250-1680 cm^{-1} frequency range of the oxidized *caa*$_3$ (trace A) upon addition of $^{14}N^{16}O$ (trace B) and $^{15}N^{18}O$ (trace C) are shown in Fig 4. Trace B-C is the difference trace B-trace C spectrum. The strong bands in the spectra arise from 1618 (Y8a, W1), 1553 (W3), 1458 (W5), 1359 (W7) and 1339 (W7). A relative decrease in intensity and bandwidth of the 1339 cm^{-1} mode and the appearance of a new mode at 1303 cm^{-1} is observed in trace C. The difference spectrum B-C ($^{14}N^{16}O$-$^{15}N^{18}O$) shows the presence of a peak/trough pattern at 1330/1303 cm^{-1}, indicating ^{15}N isotope sensitivity of the 1330 cm^{-1} peak. No other distinct features are present in the difference spectra indicating that ligand binding to heme a_3 exchanges cause little structural changes to Tyr and Trp residues. For comparison we have included, in the inset, the difference spectrum of the oxidized *ba*$_3$ upon addition of $^{14}N^{16}O$ and $^{15}N^{18}O$.[14] Based on the previous assignment of the hyponitrite species in *ba*$_3$ oxidoreductases we assign the 1330 cm^{-1} to the hyponitrite species in the binuclear center of *caa*$_3$ oxidoreductase.[14] The 8 cm^{-1} difference between the hyponitrite-bound to heme a_3 of *caa*$_3$ and the hyponitrite bound to heme a_3 of *ba*$_3$ indicates that the N-N bond has less double bond character, when bound to heme a_3 of *caa*$_3$. Our assignment is further supported by the similarity in the frequency, and the nitrogen and oxygen isotope shifts to those predicted from density functional theory (DFT) calculations.[23] It is suggested that upon addition of NO to the oxidized enzyme the heme a_3 Fe^{3+}-NO Cu_B^{1+}-NO^+ complex is formed and in the presence of a H_2O molecule, NO_2^- and $2H^+$ are released forming a heme a_3 Fe^{3+}-NO Cu_B^{1+} complex (Scheme 1). 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 N-N bond formation must originate from the autoreduction of Cu_B by NO. The mechanism for the reduction of Cu (II) by NO has been described in the case of *ba*$_3$ oxidoreductase and also reported in other Cu compounds and in *aa*$_3$ oxidase.[6,18] Addition of NO to heme a_3 Fe^{3+}-NO Cu_B^{1+} complex produces heme a_3 Fe^{3+}-NO ON-Cu_B^{1+} and when protonated the steady state hyponitrite species is formed (Scheme 1).

NO as a radical (ν=1876 cm^{-1}) has one electron in its π^* antibonding orbitals.[17] Addition of an electron weakens the NO bond, forming NO^- (ν=1284 cm^{-1}).[17] On the other hand, removal of an electron strengthens the N-O bond producing NO^+ (ν=2345 cm^{-1}).[17] The electronic states of all NO-bound adducts of ferric His-heme-proteins including Hb and Mb reported so far, are close to that of $NO^{\bullet}$, and as inferred from the frequency range of NO (1900-1922 cm^{-1}) electron deficient.[17] If the NO ligand was bound to heme a_3 yielding an abnormal Fe^{3+}-nitrosyl, the N-O stretching vibration should have been observed. However, there is no evidence of any NO isotope-sensitive modes in the entire 1550-2400 cm^{-1} frequency range. Therefore, we conclude that the heme a_3 transition at 440/603 nm that we have probed under visible and UV excitations is not due to NO-bound heme a_3^{3+}. If two NO molecules would couple in the binuclear center of *caa*$_3$ oxidoreductase without support of an additional electron then a single N-N bond will form because only two electrons from the N-O π^* are available for bonding. The observed frequency at 1330 cm^{-1} indicates that it originates from a double bond. It is proposed that 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 Fe-NO unit and concomitant formation of the N=N bond. Our data offer direct evidence for the formation of the hyponitrite intermediate and provide the isolated marker mode at 1330 ($\nu_{N=N}$) cm^{-1} which should be useful for kinetic experiments.

Scheme 1. Proposed mechanism for the formation of the hyponitrite species in the oxidized heme a_3-Cu_B binuclear center of *caa*$_3$-oxidoreductase from *Thermus thermophilus*. (See text). The full catalytic cycle is presented in [14].

Conclusions

The molecular mechanisms of the *caa*$_3$ oxidoreductase is expected to be similar to its counterpart *ba*$_3$ oxidase and to other distantly related heme-copper oxidases with respect to the oxygen, carbon monoxide and nitric oxide chemistry since the central features of the active site are similar and the catalyzed chemical reactions identical.[24] The implications of these results with respect to the ability of Cu_B^{2+} to produce NO_2^- upon reaction with NO and the common reaction mechanism for the formation of the hyponitrite species in the heme a_3 Fe/Cu_B binuclear centers of both the *ba*$_3$ and *caa*$_3$ from *T. thermophilus* indicates unique evolution pathways in respiration. Based on our observations we propose that the co-respiration of nitric oxide by *T. thermophilus* and *P. denitrificans* to produce N_2O, strongly indicates the existence of a common co-evolution pathway of bacterial respiration and denitrification.[5]

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Notes and references

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