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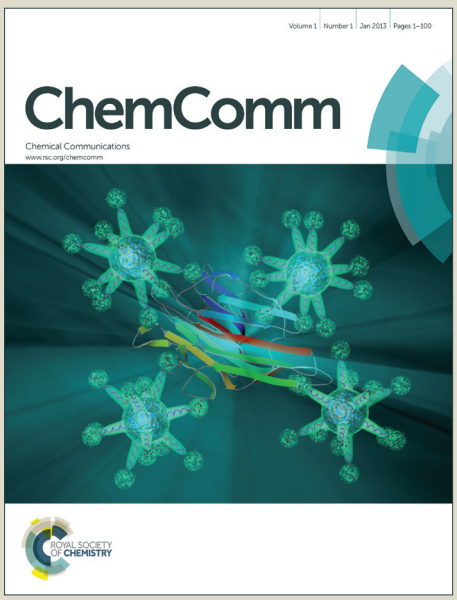

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Probing the nitrite and nitric oxide reductase activity of *cbb*$_3$ oxidase: Resonance Raman detection of a six-coordinate ferrous heme-nitrosyl species in the binuclear b_3/Cu_B center

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Andreas Loullis[a] and Eftychia Pinakoulaki[a]*

In this work we report the first spectroscopic evidence demonstrating that *cbb*$_3$ oxidase catalyzes the reduction of nitrite to nitrous oxide under reducing anaerobic conditions. The reaction proceeds through the formation of a ferrous six-coordinate heme b_3-nitrosyl species that has been characterized by resonance Raman spectroscopy.

Heme-copper oxidases (HCOs) form a family of key enzymes in respiration as they couple the reduction of oxygen to proton pumping across the inner mitochondrial (or bacterial) membrane to generate a transmembrane electrochemical gradient required for ATP synthesis.[1,2] HCOs are diverse in terms of electron donors, subunit composition and the heme type they encompass. However, they share a central subunit that contains a low spin heme (*a* or *b*) and a high spin heme (a_3, o_3 or b_3)/copper (Cu_B) binuclear center, where oxygen is reduced during the catalytic cycle. The molecular mechanisms of oxygen reduction and proton pumping have been extensively investigated and key features have been elucidated.[1-3] During the last years additional catalytic functions have been described for HCOs. Recently, the reduction of nitrite to nitric oxide ($NO_2^- + e^- + 2H^+ \rightarrow NO + H_2O$) by mitochondrial HCO (an A-family HCO) under hypoxic conditions was reported, outlining the potential role of HCO in signaling pathways, as NO is a multitask signaling molecule of great importance in living organisms that regulates various physiological functions.[4,5] Furthermore, bacterial HCOs, including the *caa*$_3$-and *ba*$_3$-cytochrome *c* oxidases from *Thermus thermophilus*, *cbb*$_3$-cytochrome *c* oxidase from *Pseudomonas stutzeri* and *bo*$_3$-quinol oxidase from *Escherichia coli* are able to catalyze the reduction of NO to nitrous oxide ($2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$), in contrast to their mammalian counterpart.[6-8] The reduction of NO_2^- to NO and of NO to N_2O is also part of denitrification. Denitrification is the pathway of the nitrogen biochemical cycle that involves the reduction of nitrate to molecular dinitrogen in four sequential steps ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) and is found in a wide range of organisms, from archaea to eubacteria and even some eukaryotes. In addition, it is interesting to note that bacteria need to respond to various insults, including NO during their lifespans. This is seen in pathogens that are subjected to the mammalian immune response.

The diverse biological roles of NO_2^- and NO underline the importance of elucidating the fundamental chemistry in the reduction of these small molecules. The reduction of these molecules by HCO occurs in the binuclear heme/copper center, however, the role of each metal and the intermediates formed in the catalytic reactions have not been elucidated. Recently, heme/copper model assemblies were synthesized and their reactions with nitrite were investigated.[9] In our previous work, we initiated our effort to investigate the HCO/nitrite chemistry by examining the reaction of *ba*$_3$-oxidase (a B-family HCO) with NO_2^- under reducing conditions. The utilization of resonance Raman (RR) spectroscopy allowed us to describe the first structure of a heme-nitro species in the binuclear heme/copper center of HCO and suggested that bacterial and mammalian HCOs may exhibit different reactivity towards nitrite.[10] Such difference in the reactivity towards a substrate would not be unprecedented since, as described previously, the bacterial (*ba*$_3$, *caa*$_3$, *bo*$_3$) oxidases are able to catalyze the reduction of NO to N_2O in contrast to the mammalian counterpart.[6-8] Noteworthy, the molecular mechanism of NO to N_2O reduction by HCO is also not clarified yet, as diverse species have been observed in the reactions of NO with different members of the HCO family. The formation of a ferric six-coordinate heme b_3-nitrosyl adduct has been reported upon the reaction of NO with the oxidized form of *cbb*$_3$ oxidase, while under reducing conditions the proximal His-Fe_{b3} bond was cleaved and a five-coordinate ferrous heme b_3-nitrosyl species was detected.[11,12] UV and Vis resonance Raman experiments revealed that the reactions of oxidized *bo*$_3$ and *caa*$_3$ oxidases with NO yield the heme a_3-hyponitrite (HO-N=N-O$^-$) species.[13,14] Furthermore, a six-coordinate ferrous heme-nitrosyl adduct was detected under reducing conditions.[15] FTIR photolysis experiments at low temperature

[a] University of Cyprus, Department of Chemistry, PO Box 20537, 1678 Nicosia, Cyprus. E-mail: effiep@ucy.ac.cy

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on the ferrous heme-nitrosyl complexes of ba_3 and bo_3 oxidases demonstrated that upon the photodissociation of NO from the heme, a copper-nitrosyl adduct is formed in ba_3 oxidase but not in bo_3.[16,17]

In the work described herein, we investigated the reaction of nitrite with the C-family cbb_3 oxidase from *P. stutzeri* under reducing anaerobic conditions by employing resonance Raman and FTIR spectroscopy. We provide experimental evidence that cbb_3 oxidase catalyzes the reduction of NO_2^- to N_2O through the formation of a six-coordinate ferrous heme-nitrosyl adduct. To the best of our knowledge cbb_3 oxidase is the first member of the HCO family identified as capable of functioning both as nitrite and nitric oxide reductase.

The RR spectra of the oxidized and reduced forms of cbb_3 oxidase (Figure S1, ESI) are similar to those previously reported.[18] Briefly, in the RR spectrum of the cysteine-reduced enzyme the porphyrin π* electron density sensitive mode (ν_4) is observed at 1362 cm^{-1} establishing that all hemes are in the ferrous state. The ν_3 at 1494 cm^{-1} and ν_2 at 1590 cm^{-1} originate from the low spin hemes *c* and *b*, while the corresponding ν_2 for the five coordinate high spin heme b_3 is observed at 1467 cm^{-1}. To investigate the reaction of nitrite with cbb_3 oxidase we have used cysteine, which is a mild reductant, instead of other widely used reductants such as dithionite and ascorbate to avoid the non-enzymatic reduction of NO_2^-, as described in previous work.[10] Figure 1 shows the high frequency RR spectra of the reaction of cysteine-reduced cbb_3 oxidase with $^{14}N^{16}O_2^-$ (trace a) and $^{15}N^{16}O_2^-$ (trace b) at pH 7.5. The most notable changes in the RR spectra of cysteine-reduced cbb_3 oxidase subsequent to nitrite addition are the appearance of a shoulder at 1373 cm^{-1} (ν_4) and the shift of the ν_2 mode of heme b_3 to 1494 cm^{-1}, where it coincides with the corresponding modes of the low spin hemes *c* and *b*, indicating the formation of a six-coordinate low spin heme b_3 species. In addition, increased intensity at 1575 cm^{-1} is observed in trace b (reaction with $^{15}N^{16}O_2^-$) compared to trace a (reaction with $^{14}N^{16}O_2^-$). The difference (trace a-b) RR spectrum shows the presence of a peak/trough feature at 1592/1572 cm^{-1} revealing the presence of mode sensitive to nitrogen isotopic substitution that remains unchanged at pH 9.0 (Fig. 1, inset).

The low frequency RR spectra of the reaction of reduced cbb_3 oxidase with $^{14}N^{16}O_2^-$ (trace a), $^{15}N^{16}O_2^-$ (trace b) and $^{15}N^{18}O_2^-$ (trace c) at pH 7.5 are shown in Figure 2. Trace a displays a mode at 550 cm^{-1} that shifts to ~540 cm^{-1} when $^{15}N^{16}O_2^-$ and $^{15}N^{18}O_2^-$ isotopes are used (traces b and c). The presence of peak/trough features at 555/538 cm^{-1} and 555/536 cm^{-1} in traces a-b and a-c, respectively, confirm the sensitivity of the 550 cm^{-1} mode to nitrogen and oxygen isotopic substitutions. The same frequencies are observed in the corresponding difference RR spectra at pH 9.0 (Fig2, inset).

In previous work, we reported the first RR detection of a ferrous heme-nitro species in the binuclear heme/copper center of HCO in the ba_3 oxidase/NO_2^- reaction. The species was characterized by the ν(Fe-NO_2) and δ(NO_2) modes at 568 cm^{-1} and 786 cm^{-1}, respectively.[10] Although the 555 cm^{-1} mode we observe in the cbb_3/NO_2^- reaction appears at a frequency close to that of the ν(Fe-NO_2) of the ferrous heme-nitro adduct

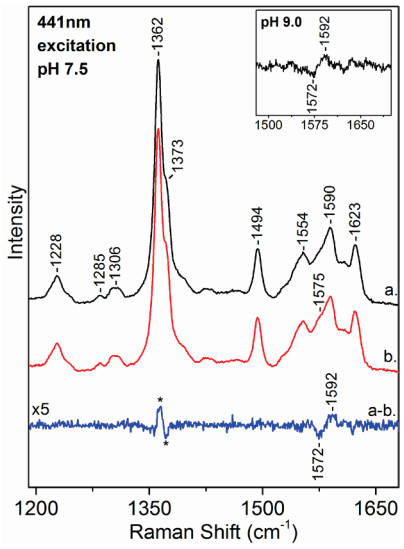

Figure 1. High frequency resonance Raman spectra of the cbb_3 oxidase adducts formed after addition of $^{14}N^{16}O_2^-$ (trace a) and $^{15}N^{16}O_2^-$ (trace b) to cysteine-reduced enzyme at pH 7.5. The difference a-b resonance Raman spectrum multiplied by a factor of 5 is included. The inset shows the corresponding difference resonance Raman spectrum at pH 9.0. The excitation wavelength wa 441 nm and the power incident on the sample was 4 mW.

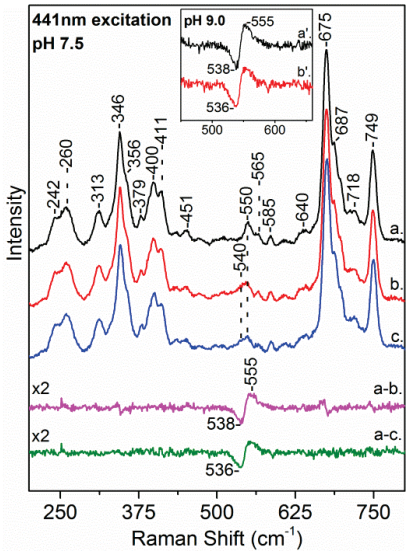

Figure 2. Low frequency resonance Raman spectra of the cbb_3 oxidase adducts forme after addition of $^{14}N^{16}O_2^-$ (trace a), $^{15}N^{16}O_2^-$ (trace b) and $^{15}N^{18}O_2^-$ (trace c) to cysteine-reduced enzyme at pH 7.5. The difference a-b and a-c resonance Raman spectra multiplied by a factor of 2 are included. The inset shows the corresponding difference resonance Raman spectra at pH 9.0. The excitation wavelength was 441 nm and the power incident on the sample was 4 mW.

of ba_3 oxidase, it is also within the range of frequen ies reported for ferrous heme-nitrosyl adducts. Indicatively, th ν(Fe-NO) for the six-coordinate ferrous heme-nitros complexes of ba_3 and bo_3 oxidases have been reported at 53 cm^{-1} and 534 cm^{-1}, respectively,[15,17] and for the correspondi myoglobin (Mb) adduct at 551 cm^{-1}.[19] At this point it should b noted that recent nuclear resonance vibrational spectrosco (NRVS) studies of the myoglobin ferrous heme-nitrosyl adduc and six-coordinate model heme-nitrosyl complexes suppo that the mode at ~550 cm^{-1} (higher frequency mode), more

commonly assigned to the ν(Fe-NO) in RR studies, has significant contribution from the Fe-N-O bending and a lower frequency mode at ~450 cm^{-1} should be assigned to the Fe-NO stretch.[20,21] Based on the NRVS studies and since the isotope shifts we observed for the ^{15}NO and $^{15}N^{18}O$ complexes are almost identical, we assign the 555 cm^{-1} mode as the δ(Fe-N-O), acknowledging that it contains contribution from the Fe-NO stretching vibration. If the 555 cm^{-1} mode was predominately due to the Fe-NO stretching mode then a much larger isotope shift would be expected for the $^{15}N^{18}O$ complex compared to that of the ^{15}NO adduct.[20] To elucidate the structure of the species that we have detected we should take into account the second isotope-sensitive band, which appears at 1592 cm^{-1}. We assign the 1592 cm^{-1} band to the N-O stretch of the six-coordinate heme b_3^{2+}-NO adduct, since the ν(NO) appears in the 1590-1640 cm^{-1} range for six-coordinate heme-nitrosyl complexes and distinctly higher (1660-1680 cm^{-1}) for five-coordinate NO adducts,[19,22] while the symmetric and antisymmetric stretching vibrations of metal-bound NO_2^- are expected in the 1300-1500 cm^{-1} range for the nitro binding mode.[23] Our assignment of the 555 cm^{-1} and 1592 cm^{-1} modes to the δ(FeNO) and ν(NO) of the heme b_3-nitrosyl species is further supported by the RR detection of the same species in the reaction of cbb_3 oxidase with NO under steady-state anaerobic reducing conditions (Figure S2, ESI).

After the characterization of the six-coordinate ferrous heme b_3-nitrosyl adduct formed in the cbb_3/NO_2^- reaction, we employed FTIR spectroscopy to examine whether N_2O is formed as a final product of the reaction, and thus to confirm that cbb_3 oxidase retains its ability to function as a nitric oxide reductase under our experimental conditions. The FTIR spectrum of the reaction of cysteine-reduced cbb_3 oxidase with $^{14}NO_2^-$ (Figure S3, ESI) displays the characteristic antisymmetric N-N-O stretch of N_2O at 2230 cm^{-1},[16] shifting to 2161 cm^{-1} when $^{15}NO_2^-$ is used (inset in Figure S3, ESI). In the control FTIR experiment (incubation of NO_2^- with cysteine) N_2O formation was not observed. Thus, our findings demonstrate that cbb_3 oxidase is able to function as a nitrite and nitric oxide reductase under reducing anaerobic conditions.

Properties of the six-coordinate ferrous heme-nitrosyl species of cbb_3 oxidase. Previous studies have demonstrated that NO acts as a σ donor/π-acceptor in ferrous heme complexes.[20-22] The variation of the strength of the σ bond leads to a direct correlation of the Fe-NO and N-O stretches, whereas a change in the π backbonding produces an inverse correlation.[20-22] Therefore, depending on which of these bonding interactions is perturbed by the interaction of the heme-NO complex with the protein environment, a different response of the vibrations of the Fe-N-O unit occurs. The reported frequencies for the ferrous heme-nitrosyl adducts of ba_3 and bo_3 oxidases are 539/1620 cm^{-1} and 534/1616 cm^{-1}, respectively.[15,16] The species we have detected exhibits quite distinct structure as the δ(Fe-N-O) at 555 cm^{-1} is higher compared to the corresponding frequencies of ba_3 and bo_3 oxidases, while the ν(N-O) at 1592 cm^{-1} is significantly lower. Interestingly, the frequencies of the cbb_3 nitrosyl complex fall closer to those of the six-coordinate ferrous heme-nitrosyl adduct of wild type Mb (551/1612 cm^{-1}) and especially to its V68N mutant (551/1595 cm^{-1}).[19] The RR data on wild type and several distal site mutants in Mb along with DFT calculations by Spiro and co-workers have indicated that the heme-bound NO is hydrogen bonded through the N atom to the distal HisE7 residue. In the V68N mutant it has been suggested that asparagine introduces a stronger H-bond, further lowering the ν(N-O) frequency.[19,22] The DFT calculations have shown that both Fe-N and N-O bonds weaken when the H-bond acceptor is N, while Fe-N strengthens and N-O weakens when the acceptor is O.[22] In ba_3 oxidase the differences in the frequencies of the Fe-N-O unit compared to Mb have been attributed to the proximal to heme a_3 His384 protein environment that has been proposed to control the strength of the Fe-His384 bond and determine the structure of the heme a_3-nitrosyl complex.[15]

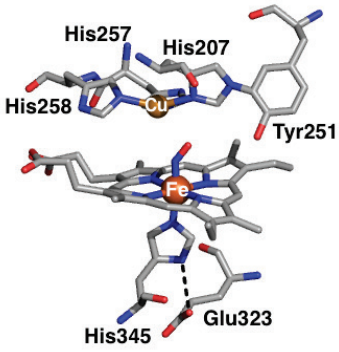

Figure 3. Schematic representation of the heme b_3-nitrosyl adduct of cbb_3 oxidase. The structural features of the b_3/Cu_B center are based on PDB 3KM7.[3]

We consider both proximal and distal effects that could contribute to the distinct structure of the nitrosyl cbb_3 adduct (schematically represented in Figure 3) compared to the corresponding species in ba_3 and bo_3 oxidases. However, the complex orbital structure of the NO adducts and mixed nature of the Fe-NO stretching and Fe-N-O bending coordinates, as well as standing controversies between the NRVS and RR studies for six-coordinate ferrous heme-nitrosyl complexes make it challenging to assess the contribution of each factor.[15,22] In cbb_3 oxidase heme b_3 is ligated by His345, which is hydrogen bonded to Glu323 rather than the Gly residues in other HCOs.[3] This increases the basicity of the proximal His and strengthens the $Fe\text{-}N_{His}$ bond of the ferrous heme b_3. A stronger $Fe\text{-}N_{His}$ bond in cbb_3 oxidase compared to the other oxidases would be expected to weaken the Fe-NO and N-O bonds since σ-donor orbitals of NO and His "compete" for the Fe d_{z^2} orbitals (*trans* effect).[21] Vice versa, the strong *trans* effect of NO may significantly weaken the $Fe\text{-}N_{His}$ bond upon NO binding to heme b_3.[21] This interpretation is consistent with the detection of a five-coordinate heme b_3-nitrosyl species in previous RR experiments (see discussion below).[12] In the distal heme b_3 site, the covalent linkage between the Cu_B His207 ligand and Tyr251 is conserved in cbb_3 oxidase as in all members of the HCO family.[3] However, the tyrosine side chain protrudes from helix a7 rather than helix a8, ~4 Å away from the substrate binding site, in a position that could enable H-

bonding interaction with the heme-bound NO. The interaction of the heme-bound NO with Tyr251 can rationalize the frequencies we observe for the heme b_3-NO complex, based on the similarities with the corresponding NO adduct of the Mb V68N mutant.[19,22]

Nitrite and nitric oxide reductase activities of cbb$_3$ oxidase. The reaction of nitrite with *cbb*$_3$ oxidase in the presence of reductant leads to the formation of a six-coordinate ferrous heme b_3-nitroslyl complex under steady state conditions. Although we do not have evidence for the formation of a nitro- (binding of NO_2^- through the N atom) or nitrito- (binding of NO_2^- through the O atom) heme b_3 species, presumably because this first intermediate is short-lived, we suggest that upon NO_2^- binding to heme b_3 electron transfer from the heme to the substrate occurs and along with its double protonation reduction to NO and release of a H_2O molecule takes place. We speculate that heme b_3 is concomitantly re-reduced by electron transfer from either Cu_B or heme *b*, which in contrast to the other HCO families is in van der Waals contact with heme b_3 implying increased electron transfer rate between the hemes.[3] This way, the ferrous six-coordinate heme b_3-nitrosyl species we detect is formed, trapping NO for the subsequent catalytic reduction to N_2O. It should be noted that in previous RR investigation of NO reduction by *cbb*$_3$ oxidase a five-coordinate ferrous heme b_3-nitrosyl adduct ($\nu_{Fe\text{-}NO}$=524 cm^{-1} and ν_{NO}=1679 cm^{-1}) was detected under 413 nm excitation.[12] In the present work a six-coordinate heme b_3-nitrosyl species was detected under 441 nm excitation in the reactions of *cbb*$_3$ with NO_2^- and NO. The two species (five- and six-coordinate heme-nitrosyls) can therefore exist in equilibrium in the *cbb*$_3$/NO reaction and are detected under different excitations, their ratio depending on experimental conditions. The role of each species in the catalytic cycle requires further investigation. The reduction of NO proceeds with the attack of the heme-bound NO by a second NO molecule; protonation occurs to transiently yield the hyponitrite species ($HONNO^-$) that been detected in reactions of NO with *ba*$_3$, *caa*$_3$ and bacterial nitric oxide reductase enzymes, and thus the N-N bond is formed.[13,14,24] Addition of another H^+ and cleavage of the N-O bond leads to N_2O and H_2O release.

In conclusion, the present report provides spectroscopic evidence for the ability of *cbb*$_3$ oxidase to function as a nitrite and nitric oxide reductase. In contrast, our recent work on the *ba*$_3$ oxidase that also demonstrates nitric oxide reductase activity, revealed the formation of a stable ferrous heme-nitro species upon the *ba*$_3$/NO_2^- reaction and production of NO or N_2O was not observed.[10] Our findings on *cbb*$_3$ oxidase outline a potential role beyond oxygen respiration in bacteria for these multifunctional enzymes, comparable to the emerging role of mammalian HCO in NO signaling.

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