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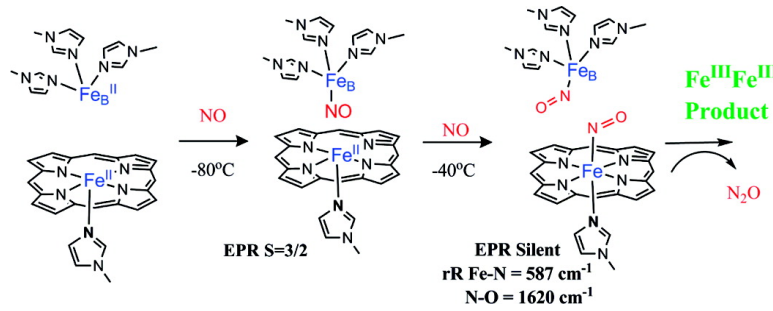
J. Am. Chem. Soc., **2008**, 130 (49), 16498-16499 • DOI: 10.1021/ja807700n • Publication Date (Web): 17 November 2008



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Intermediates Involved in the Two Electron Reduction of NO to N_2O by a Functional Synthetic Model of Heme Containing Bacterial NO Reductase

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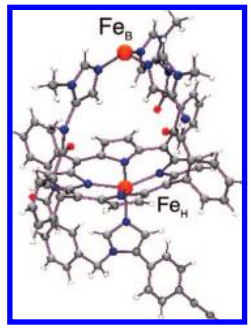
Received September 29, 2008; E-mail: jpc@stanford.edu

Nitric oxide (NO) is a freely diffusing hormone that is an integral component of the physiology of all advanced life forms. It is involved in regulation of metabolism, blood flow, immune responses, and apoptosis.[1] NO is also an intermediate in bacterial denitrification (i.e., reduction of NO_3^- to N_2) and is further reduced to N_2O by the enzyme nitric oxide reductase (NOR).[2] Bacterial NORs are membrane bound enzymes that are members of the heme-copper oxidase superfamily. NORs are proposed to have a bimetallic heme/nonheme catalytic active site with two electron transfer sites quite like the cytochrome c oxidases (CcO).[3] While heme a_3 in NORs is proposed to be similar to that in CcO, the distal metal in NORs is Fe (referred to as Fe_B) instead of Cu_B in CcO. Also the post-translationally cross-linked Tyr244-His ligand in CcO is absent in NORs; rather a few glutamate residues are proposed to play an important role in NORs reactivity.[3]

In the absence of structural data on these enzymes, much of the available insight has been acquired via spectroscopic probes and biochemical studies. Not surprisingly the mechanism of action of NORs is a matter of debate. Spectroscopic evidence suggests that the reduction of NO to N_2O involves a bis-nitrosyl intermediate where each of the reduced (ferrous) irons is bound to a molecule of NO.[4] Then N−N bond formation and further rearrangement lead to the formation of N_2O and a bis-ferric resting active site.[4] This mechanism has not been supported by synthetic model complexes of NOR, such as the one reported by Karlin and co-workers, as such a bis-nitrosyl complex was found to be either inactive or it did not stoichimetrically form N_2O.[5a,b,6] An alternate cis mechanism has been proposed where two NO molecules bind the distal metal and then undergo reductive cleavage forming N_2O.[7]

Scheme 1. A Synthetic Functional Model of NOR[a]



[a] It includes a tris-imidazole distal pocket that binds Fe_B and a porphyrin macrocycle with a covalently attached proximal imidazole that binds Fe_H.

Recently a synthetic functional model of NOR has been reported by Collman et al. where a bis-ferrous heme nonheme model (Fe^{II}_H/Fe_B^{II}) reduces two molecules of NO to generate N_2O (Scheme 1).[8] In the present study we report two nitrosyl intermediates formed along the reaction of this functional NOR model and characterize them using electron paramagnetic resonance (EPR) and resonance Raman (rR) spectroscopy.

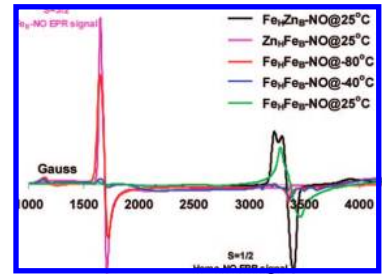


Figure 1. EPR of the intermediates and final product in the reaction of NO with the bis-ferrous model. In the legend H = heme and B = distal pocket. All samples are 1 mM in CH_2Cl_2, $T = 4$ K, and microwave power = 10 mW.

The low-spin $S = {}^1/_2$ Fe_H^{II}−NO has a characteristic EPR signal of ∼3200 G (g = 2.07, 2.02, 1.96; Fe_H^{II}−NO/Zn_B in Figure 1 black)[9] which is very different from the EPR signal of the $S = {}^3/_2$ Fe_B−NO ∼1700 G (g = 3.92; Fe_H^{II}/Fe_B−NO in Figure 1 pink).[10] The rR spectrum of the Fe_H^{II}−NO/Zn_B shows that the Fe_H^{II}−NO stretch at 581 cm^{-1} shifts to 545 cm^{-1} upon ^{15}NO (Figure 2) substitution. The 239 cm^{-1} mode (Figure 2) is possibly an Fe_H^{II}−N_{Imz} vibration as it is lowered from 242 cm^{-1} in the Fe_H^{II} complex upon NO binding (Figure S1) forming a six-coordinate complex.[9] The N−O vibration is found (FTIR, Figure S2) to be at 1635 cm^{-1} and shifts to 1590 cm^{-1} upon ^{15}NO substitution. Furthermore the porphyrin υ_4 band, which is very sensitive to the charge and spin state of the bound heme Fe,[11] is observed at 1357 and 1365 cm^{-1} (Figure 3A) for Fe_H^{II} and Fe_H^{II}−NO, repectively. These characteristic EPR and rR features are used to identify the reaction intermediates.[12]

Figure 1 shows the EPR spectrum obtained by adding NO to the bis-ferrous complex in dichloromethane solution at different temperatures. At −80 °C (intermediate A) the spectrum has a signal at 1700 G (g = 3.92, Figure 1, red) which is identical to the Zn_H/Fe_B−NO complex in both energy and intensity (Figure 1, pink), implying that this is a mononitrosyl Fe_B−NO species. The rR data of intermediate A in the high-energy region (Figure 3A, red) shows a υ_4 band at 1356.5 cm^{-1} characteristic of an Fe_H^{II} (1357 cm^{-1})

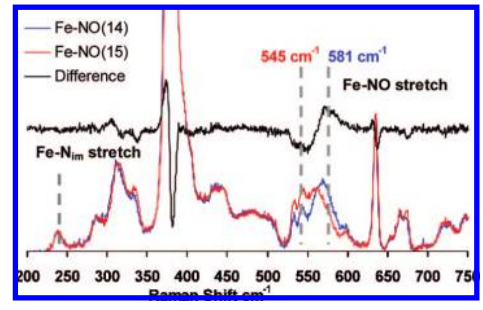


Figure 2. rR data for the heme Fe−NO species ^{14}NO (blue) and ^{15}NO (red).

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indicating that there is no reaction at the Fe_H^{II} in this intermediate. No vibrational data on the Fe_B−NO could be obtained due to its weaker enhancement relative to heme vibrations. When the reaction is warmed to −40 °C (intermediate B), no signal is observed in the EPR spectrum. The same EPR data can also be obtained when the reaction is rapidly frozen at room temperature.

Intermediate B shows a υ_4 band at 1366.1 cm^{-1} (Figure 3A, blue), ~8 cm^{-1} higher than intermediate A showing that NO is now bound to Fe_H. There is also a band at 1620 cm^{-1} that shifts to 1579 cm^{-1} when ^{15}NO was used (Figure S3). In the low energy region a band is observed (Figure 3 B) at 587 cm^{-1} (blue) that shifts to 559 cm^{-1} with ^{15}NO (red) while another band is observed at 460 cm^{-1} which shifts to 432 cm^{-1} with ^{15}NO substitution. The 587 cm^{-1} band is assigned as an Fe_H^{II}−NO stretching vibration based on data obtained with the Fe_H^{II}−NO complex (Figure 2). The band at 460 cm^{-1} is consistent with an Fe−N−O bending mode (an Fe_B−N vibration can not be ruled out). These data indicate the presence of an Fe_H^{II}−NO center in intermediate B.

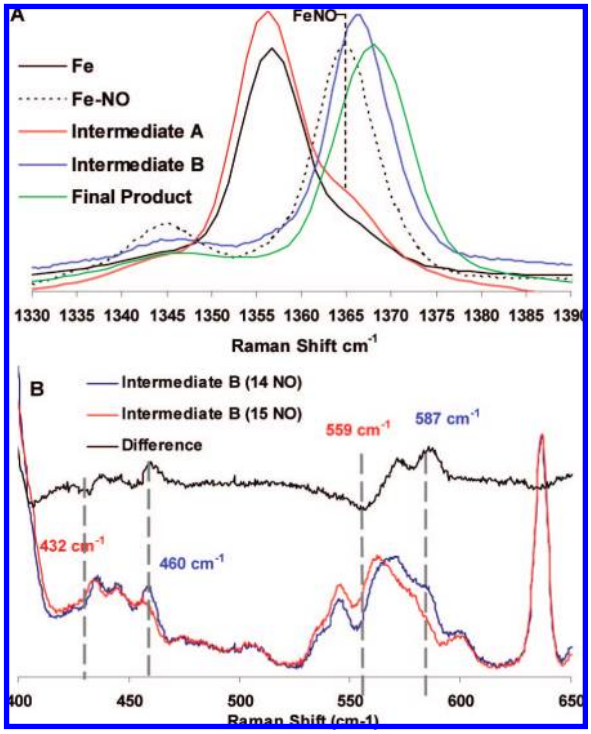
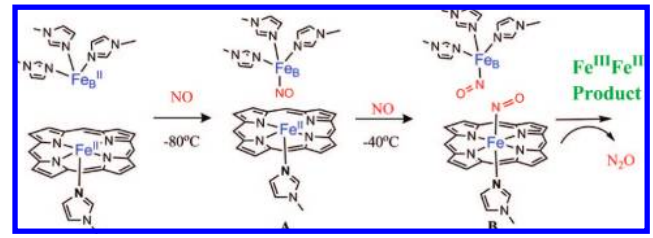


Figure 3. rR data of the starting bis-ferrous complex, intermediates, and end product (A) high-energy region (includes heme-Fe^{II}−NO data for reference) and (B) low-energy region.

Warming the reaction mixture up to room temperature results in a heme-Fe^{III}−NO/Fe_B^{III}−OH product (EPR in Figure 1, light green).[8] Intermediate A is an Fe_H^{II}/Fe_B−NO complex as indicated by the υ_4 band of the Fe_H^{II} and the $S = {}^3/_2$ EPR signal of the Fe_B−NO. Intermediate B has an Fe_H^{II}−NO center in it as indicated by the Fe−N vibration (Figure 3B) and N−O vibration (Figure S3). Note that both of these vibrations are different from that of an isolated Fe_H^{II}−NO/Zn_B^{II} complex. The Fe−N vibration is higher (587 cm^{-1} in B, 581 cm^{-1} in Fe_H^{II}NO), and the N−O vibration is lower (1620 cm^{-1} in B 1635 cm^{-1} in Fe_H^{II}NO). Thus intermediate B could be either (1) a mononitrosyl derivative where the NO dissociates from the Fe_B and binds to heme-Fe at higher temperatures or (2) a bis-nitrosyl derivative.[13] The reaction of the same bis-ferrous complex in *N*,*N*-dimethylformamide (DMF) results in a bis-nitrosyl species where both $S = {}^3/_2$ (Fe_B−NO) and $S = {}^1/_2$ (Fe_H^{II}−NO) EPR signals (similar to those reported by Shiro et al.)[4] and a heme-Fe−N vibration at 579 cm^{-1} (unperturbed relative to that for the Fe_H−NO/Zn_B complex, Figure 2) are observed (Figures S4, S5). However, this reaction does not yield N_2O. Also the Zn_H^{II}/Fe_B−NO species shows no NO dissociation at room temperature. Thus formation of an EPR active but functionally inactive bis-nitrosyl in DMF, the stability of Fe_B−NO, perturbation of the heme-nitrosyl vibrational features, and the lack of an EPR signal indicate that the intermediate B is a bis-nitrosyl complex where the two nitrosyls are close enough to either spin-dipolar couple or directly interact. A very similar situation was reported for the heme nonheme bis-carbonyl complex for the active site of NOR.[14]

Scheme 2. Proposed Mechanism of N_2O Formation from NO



These results support a trans mechanism of N_2O formation by this synthetic functional model of NOR (Scheme 2). The first NO binds to Fe_B forming a high-spin Fe_B−NO species (A), followed by another NO binding to heme Fe^{II} resulting in the formation of an Fe_H^{II}−NO/Fe_B−NO species (B) which then forms N_2O and a bis-ferric product. This study provides the first direct evidence for a trans bis-nitrosyl intermediate as a viable pathway for the two electron reduction of NO to N_2O by the bis-ferrous active site of NOR.

Acknowledgment. This research is funded by NIH GM-69568 (J.P.C.) and NSF DMB-0342808 (E.I.S.). Dr. Somdatta Ghosh is acknowledged for her help in acquiring liquid He EPR data.

Supporting Information Available: The experimental details, the FTIR data of the N−O stretch and the data for the bis nitrosyl adduct in DMF. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA807700N