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## Intermediates Involved in the Two Electron Reduction of NO to N<sub>2</sub>O by a Functional Synthetic Model of Heme Containing Bacterial NO Reductase

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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.<sup>1</sup> NO is also an intermediate in bacterial denitrification (i.e. reduction of NO<sub>3</sub><sup>-</sup> to N<sub>2</sub>) and is further reduced to N<sub>2</sub>O by the enzyme nitric oxide reductase (NOR).<sup>2</sup> Bacterial NOR's are membrane bound enzymes that are members of the heme-copper oxidase super-family. NORs are proposed to have a bimetallic heme/non-heme catalytic active site with two electron transfer sites quite like the cytochrome c oxidases (CcO).<sup>3</sup> While heme a<sub>3</sub> in NORs is proposed to be similar to that in CcO, the distal metal in NORs is Fe (referred to as Fe<sub>B</sub>) instead of Cu<sub>B</sub> in CcO. Also the post-translationally crosslinked Tyr244-His ligand in CcO is absent in NORs; rather a few glutamate residues are proposed to play an important role in NOR's reactivity.<sup>3</sup>

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<sub>2</sub>O involves a bis-nitrosyl intermediate where each of the reduced (ferrous) irons is bound to a molecule of NO.<sup>4</sup> Then N-N bond formation and further rearrangement lead to the formation of N<sub>2</sub>O and a bis-ferrous resting active site.<sup>4</sup> 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 stoichiometrically form N<sub>2</sub>O.<sup>5a,5b,6</sup> An alternate cismechanism has been proposed where two NO molecules bind the distal metal and then undergo reductive cleavage forming N<sub>2</sub>O.<sup>7</sup>

Recently a synthetic functional model of NOR has been reported by Collman *et.al.* where a bis-ferrous heme non-heme model (Fe<sup>II</sup><sub>H</sub>/Fe<sup>II</sup><sub>B</sub>) reduces two molecules of NO to generate N<sub>2</sub>O (Scheme 1).<sup>8</sup> 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.

The low-spin S=1/2 Fe<sup>II</sup><sub>H</sub>-NO has a characteristic EPR signal around 3200 Gauss (g= 2.07, 2.02, 1.96; Fe<sup>II</sup><sub>H</sub>-NO/Zn<sub>B</sub> in Fig.1 black)<sup>9</sup> which is very different from the EPR signal of the S=3/2 Fe<sup>II</sup><sub>B</sub>-NO around 1700 Gauss (g=3.92; Fe<sup>II</sup><sub>H</sub>/Fe<sup>II</sup><sub>B</sub>-NO in Fig.1 pink).<sup>10</sup> The rR spectrum of the Fe<sup>II</sup><sub>H</sub>-NO/Zn<sub>B</sub> show that the Fe<sup>II</sup><sub>H</sub>-NO stretch at 581 cm<sup>-1</sup> shifts to 545 cm<sup>-1</sup> upon <sup>15</sup>NO (Fig. 2) substitution. The 239 cm<sup>-1</sup> mode (Fig. 2) is possibly a Fe<sup>II</sup><sub>H</sub>-N<sub>Imz</sub> vibration as it is lowered from 242 cm<sup>-1</sup> in the Fe<sup>II</sup><sub>H</sub> complex upon NO binding (Fig. S1)

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**Supporting Information Available:** The experimental details, the FTIR data of the N-O stretch and the data for the bis nitrosyl adduct in DMF are available free of charge at (<http://pubs.acs.org>).

forming a six coordinate complex.<sup>9</sup> The N-O vibration is found (FTIR, Fig. S2) to be at  $1635\text{ cm}^{-1}$  and shifts to  $1590\text{ cm}^{-1}$  upon  $^{15}\text{NO}$  substitution. Furthermore the porphyrin  $\nu_4$  band, which is very sensitive to the charge and spin state of the bound heme Fe,<sup>11</sup> is observed at  $1357\text{ cm}^{-1}$  and  $1365\text{ cm}^{-1}$  (Fig. 3A) for  $\text{Fe}_\text{H}^\text{II}$  and  $\text{Fe}_\text{H}^\text{II}\text{-NO}$ , respectively. These characteristic EPR and rR features are used to identify the reaction intermediates.<sup>12</sup>

Figure 1 shows the EPR spectrum obtained by adding NO to the bis-ferrous complex in dichloromethane solution at different temperatures. At  $-80^\circ\text{C}$  (intermediate A) the spectrum has a signal at 1700 Gauss ( $g=3.92$ , Fig.1, red) which is identical to the  $\text{Zn}_\text{H}/\text{Fe}_\text{B}\text{-NO}$  complex both in energy and intensity (Fig.1, pink), implying that this is a mono-nitrosyl  $\text{Fe}_\text{B}\text{-NO}$  species. The rR data of intermediate A in the high-energy region (Fig. 3 A, red) shows  $\nu_4$  band at  $1356.5\text{ cm}^{-1}$  characteristic of a  $\text{Fe}_\text{H}^\text{II}$  ( $1357\text{ cm}^{-1}$ ) indicating that there is no reaction at the  $\text{Fe}_\text{H}^\text{II}$  in this intermediate. No vibrational data on the  $\text{Fe}_\text{B}\text{-NO}$  could be obtained due to its weaker enhancement relative to heme vibrations. When the reaction is warmed to  $-40^\circ\text{C}$  (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  $\nu_4$  band at  $1366.1\text{ cm}^{-1}$  (Fig. 3A, blue),  $\sim 8\text{ cm}^{-1}$  higher than intermediate A showing that NO is now bound to  $\text{Fe}_\text{H}$ . There's also a band at  $1620\text{ cm}^{-1}$  that shifts to  $1579\text{ cm}^{-1}$  when  $^{15}\text{NO}$  was used (Fig. S3). In the low energy region a band is observed (Fig. 3 B) at  $585\text{ cm}^{-1}$  (blue) that shifts to  $559\text{ cm}^{-1}$  with  $^{15}\text{NO}$  (red) while another band is observed at  $460\text{ cm}^{-1}$  which shifts to  $432\text{ cm}^{-1}$  with  $^{15}\text{NO}$  substitution. The  $585\text{ cm}^{-1}$  band is assigned as  $\text{Fe}_\text{H}^\text{II}\text{-NO}$  stretching vibration based on data obtained with the  $\text{Fe}_\text{H}^\text{II}\text{-NO}$  complex (Fig. 2). The band at  $432\text{ cm}^{-1}$  is consistent with a Fe-N-O bending mode (an  $\text{Fe}_\text{B}\text{-N}$  vibration can not be ruled out). These data indicate the presence of a  $\text{Fe}_\text{H}^\text{II}\text{-NO}$  center in intermediate B.

Warming the reaction mixture up to room temperature results in a heme- $\text{Fe}^\text{III}\text{-NO}/\text{Fe}_\text{H}^\text{III}\text{-OH}$  product (EPR in Fig.1, light green).<sup>8</sup> Intermediate A is a  $\text{Fe}_\text{H}^\text{II}/\text{Fe}_\text{B}\text{-NO}$  complex as indicated by the  $\nu_4$  band of the  $\text{Fe}_\text{H}^\text{II}$  and the  $S=3/2$  EPR signal of the  $\text{Fe}_\text{B}\text{-NO}$ . Intermediate B has a  $\text{Fe}_\text{H}^\text{II}\text{-NO}$  center in it as indicated by the Fe-N vibration (Fig. 3B) and N-O vibration (Fig. S3). Note that both of these vibrations are different from that of an isolated  $\text{Fe}_\text{H}^\text{II}\text{-NO}/\text{Zn}_\text{B}^\text{II}$  complex. The Fe-N vibration is higher ( $589\text{ cm}^{-1}$  in B,  $581\text{ cm}^{-1}$  in  $\text{Fe}_\text{H}^\text{II}\text{NO}$ ) and the N-O vibration is lower ( $1620\text{ cm}^{-1}$  in B  $1635\text{ cm}^{-1}$  in  $\text{Fe}_\text{H}^\text{II}\text{NO}$ ). Thus intermediate B could either be 1) a mononitrosyl derivative where the NO dissociates from the  $\text{Fe}_\text{B}$  and binds to heme-Fe at higher temperatures or 2) a bis-nitrosyl derivative.<sup>13</sup> The reaction of the same bis-ferrous complex in N,N-dimethylformamide (DMF) results in a bis-nitrosyl species where both  $S=3/2$  ( $\text{Fe}_\text{B}\text{-NO}$ ) and  $S=1/2$  ( $\text{Fe}_\text{H}^\text{II}\text{-NO}$ ) EPR signals (similar to those reported by Shiro *et.al.*)<sup>4</sup> and a heme-Fe-N vibration at  $572\text{ cm}^{-1}$  (unperturbed relative to that for the  $\text{Fe}_\text{H}\text{-NO}/\text{Zn}_\text{B}$  complex, Fig. 2) are observed (Fig. S4, S5). However, this reaction does not yield  $\text{N}_2\text{O}$ . Also the  $\text{Zn}_\text{H}^\text{II}/\text{Fe}_\text{B}\text{-NO}$  species shows no NO dissociation at room temperature. Thus formation of an EPR active but functionally inactive bis-nitrosyl in DMF, the stability of  $\text{Fe}_\text{B}\text{-NO}$ , 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 are directly interact. A very similar situation was reported for the heme non-heme bis carbonyl complex for the active site of NOR.<sup>14</sup>

## Supplementary Material

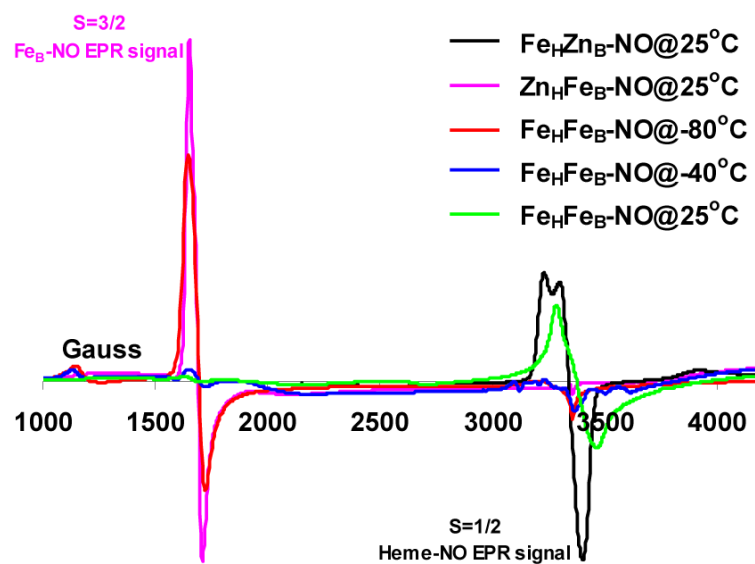
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## Acknowledgments

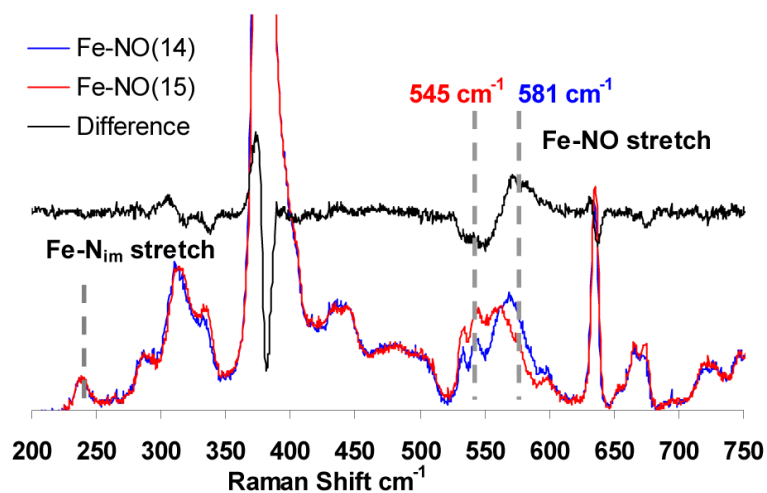
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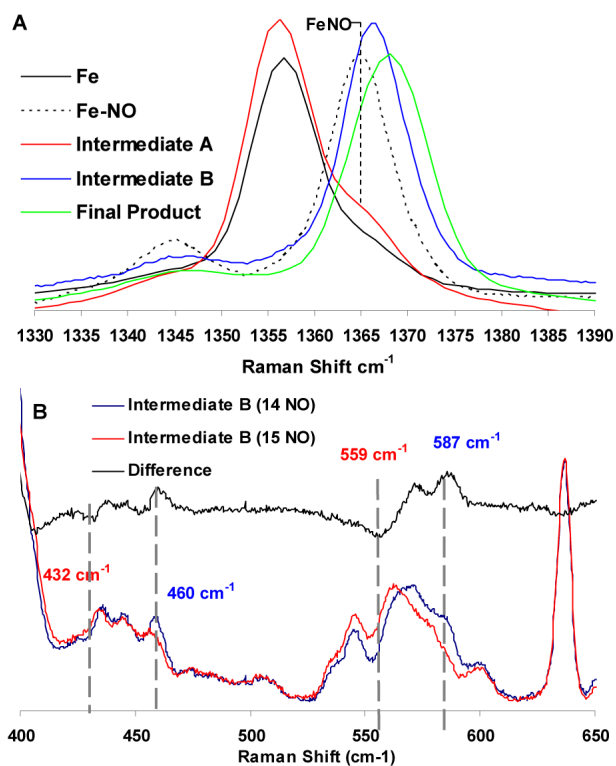
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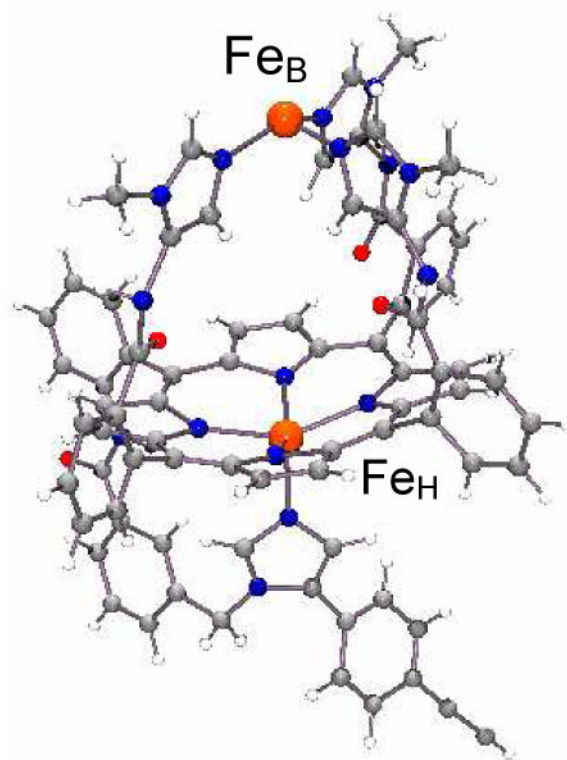
**Figure 1.** EPR of the intermediates and final product in the reaction of NO with the bis-ferrous model. In the legend H=heme and B=distal pocket. All samples are 1mM in  $\text{CH}_2\text{Cl}_2$ ,  $T=4\text{K}$  and microwave power = 10mW.



**Figure 2.**  
rR data for the heme Fe-NO species  $^{14}\text{NO}$  (blue) and  $^{15}\text{NO}$  (red)

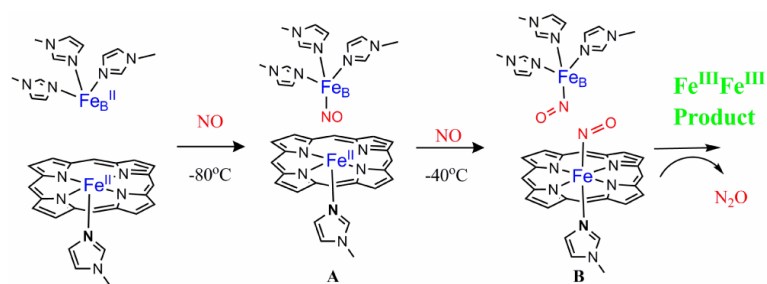


**Figure 3.**  
rR data of the starting bis-ferrous complex, intermediates and end product A) high-energy region (includes heme-Fe<sup>II</sup>-NO data for reference) and B) low-energy region.

**Scheme 1.**

A synthetic functional model of NOR. It includes a tris-imidazole distal pocket that binds  $\text{Fe}_\text{B}$  and a porphyrin macrocycle with a covalently attached proximal imidazole that binds  $\text{Fe}_\text{H}$ .



**Scheme 2.**

Proposed Mechanism of  $\text{N}_2\text{O}$  Formation from  $\text{NO}$ . These results support a trans-mechanism of  $\text{N}_2\text{O}$  formation by this synthetic functional model of NOR (scheme 2). The first  $\text{NO}$  binds to  $\text{Fe}_\text{B}$  forming a high-spin  $\text{Fe}_\text{B}\text{-NO}$  species (A), followed by another  $\text{NO}$  binding to heme  $\text{Fe}^{\text{II}}$  resulting in the formation of a  $\text{Fe}_\text{H}^{\text{II}}\text{-NO}/\text{Fe}_\text{B}\text{-NO}$  species (B) which then forms  $\text{N}_2\text{O}$  and a bis ferric product. This study provides first direct evidence for a trans bis nitrosyl intermediate as a viable pathway for the two electron reduction of  $\text{NO}$  to  $\text{N}_2\text{O}$  by the bis ferrous active site of NOR.