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Nitric oxide reductase activity in heme-nonheme binuclear engineered myoglobins through a one-electron reduction cycle

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Abstract

Fe_BMbs are structural and functional models of native bacterial nitric oxide reductases (NORs) generated through engineering of myoglobin. These biosynthetic models replicate the heme-nonheme diiron site of NORs and allow substitutions of metal centers and heme cofactors. Here, we provide evidence for multiple NOR turnover in monoformyl-heme-containing Fe_BMb1 proteins loaded with Fe^{II}, Co^{II}, or Zn^{II} metal ions at the Fe_B site (Fe^{II}/Co^{II}/Zn^{II}-Fe_BMb1(MF-heme)). FTIR detection of the $\nu(\text{NNO})$ band of N₂O at 2231 cm⁻¹ provides a direct quantitative measurement of the product in solution. A maximum number of turnover is observed with Fe^{II}-Fe_BMb1(MF-heme), but the NOR activity is retained when the Fe_B site is loaded with Zn^{II}. These data support the viability of a one-electron semi-reduced pathway for the reduction of NO at binuclear centers in reducing conditions.

Graphical abstract

Denitrifying nitric oxide reductases (NORs) catalyze the two-electron reduction of nitric oxide (NO) to nitrous oxide (N₂O) (1–3). This process has important significance to human health since the reduction of toxic NO into unreactive N₂O is used by pathogenic bacteria as a defense mechanism against the mammalian immune response (4–6). NORs are members

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ASSOCIATED CONTENT

Supporting Information

Experimental procedures and UV-vis spectra of the IR films used for multi-turnover NO reduction experiments. This material is available free of charge on the ACS Publications website.

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of the heme-copper oxidase superfamily with catalytic subunits that anchor a high-spin heme b_3 and a non-heme Fe_B metal center rather than copper as found in oxidases (7, 8). NORs and some heme-copper oxidases such as ba_3 and cbb_3 oxidases show cross-reactivity depending on the presence of O_2 and NO in the environment (9–12). Despite progress in the structural characterization of NORs (8, 13–16), the mechanism of NO reduction at the binuclear active site is still under debate. A commonly suggested mechanism is the *trans*-pathway where one NO molecule binds at each Fe^{II} centers to form two $\{\text{FeNO}\}^7$ species (17) before formation of a hyponitrite complex through a radical combination reaction (18–20). Alternatively, in the *cis*-heme mechanism, NO binds first to heme Fe^{II} before electrophilic attack by a second NO to form a heme Fe^{III} -hyponitrite dianion radical further reduced by the Fe_B^{II} center (21–23). A similar pathway centered on coordination at the Fe_B site rather than at the heme iron has also been suggested (*cis*- Fe_B mechanism) (24, 25).

There are many roadblocks to mechanistic studies of NORs, including the sub-millisecond rates of NO reaction and the presence of additional redox centers that partly mask spectroscopic signatures for the diiron active site (26–28). Synthetic biomimetic inorganic complexes and engineered proteins are exciting systems to study the mechanism of NORs (29, 30). Previous studies with engineered myoglobins called Fe_BMbs have been particularly fruitful (30–34). E- $\text{Fe}_B\text{Mb1}$ is an engineered myoglobin with an empty Fe_B site composed of three His and one Glu (L29H, F43H, H64 and V68E). The next generation of Fe_BMb construct called $\text{Fe}_B\text{Mb2}$ includes an additional Glu (I107E) at the periphery of the two metal centers; this mimics a fully conserved residue in bacterial NORs (30, 31). The crystal structures of Fe^{II} -loaded reduced $\text{Fe}_B\text{Mb1}$ and $\text{Fe}_B\text{Mb2}$ ($\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb1}$ and $\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb2}$, respectively) confirmed the anticipated Fe_B coordination to the three His and one Glu, and the reproduction of a short distance between the two metal ions. Using time-resolved spectroscopy, we showed that both $\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb1}$ and $\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb2}$ bind one NO per iron(II) to form *trans* iron-nitrosyl dimer intermediate complexes (33). In $\text{Fe}_B\text{Mb2}$, this $[\{\text{FeNO}\}^7]_2$ iron-nitrosyl dimer proceeds to generate N_2O even though a branching reaction with NO that displaces the heme proximal histidine and forms a 5-coordinate low-spin heme dead-end complex limits the extent of N_2O production. In fact, the higher rate of this inhibitory reaction in $\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb1}$ prevents any significant N_2O production in this construct (33). While the exact role of the peripheral Glu remains uncertain (34), these experiments strongly validate the viability of the *trans* mechanism in NORs.

A particularly interesting aspect of Fe_BMbs is that they can be loaded with other metal ions than Fe^{II} (30–32, 35). In contrast, attempts to exchange Cu_B/Fe_B in heme-copper oxidases and NORs led to disrupted protein structure and loss of activity (36, 37). Studies with different non-heme metals in E- Fe_BMbs showed that divalent metals result in an increase in nitroxyl-like character of the heme iron-nitrosyl complex through electrostatic interactions (32), but that this polarization of $\{\text{FeNO}\}^7$ species is not sufficient to allow electrophilic attack by a second NO as proposed in the *cis*-heme mechanism (33). Besides the incorporation of different metals at the Fe_B site, substitutions of the heme cofactor have allowed tuning of the redox potential of the heme iron in Fe_BMbs (38). Specifically, replacing the heme b in $\text{Fe}_B\text{Mb1}$ with mono-formyl or diformyl heme derivatives (Scheme 1) increases the heme redox potential by +53 mV and +148 mV, respectively (38). This same study also showed that only $\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb1}(\text{MF-heme})$ (i.e., out of $\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb1}$, Fe^{II} -

Fe_BMb1(MF-heme), and Fe^{II}-Fe_BMb1(DF-heme)) efficiently consumes excess NO in the presence of excess reducing agent, strongly suggesting that this construct can perform multiple NOR turnovers – a first for these engineered myoglobin systems (38). Here, we further demonstrate that the product of these multi-turnovers is N₂O and we compare the efficacy of Fe_BMb1(MF-heme) with different non-heme metal centers at the Fe_B site, including the non-redox active divalent ion Zn^{II}. A major implication of this work is that raising the reduction potential of the heme iron permits N₂O production through a one-electron reductive pathway.

Loading of Fe^{II}, Co^{II}, and Zn^{II} in the Fe_B site of E-Fe_BMb1(MF-heme) was monitored by a 4-nm red-shift of the Soret absorption feature from the ferrous mono-formyl-heme cofactor (Figure 1).

Quantitative measurements of N₂O production were performed using transmittance FTIR spectroscopy on aqueous protein films exposed to NO released by the diethylamine-NONOate (DEA-NONOate) inside the IR cell (see Supporting Information for details). Monitoring the 2231 cm⁻¹ band of N₂O provides an estimation of the yield of a single turnover reaction when 1-mM solutions of Fe^{II}/Co^{II}/Zn^{II}-Fe_BMb1(MF-heme) are exposed to 4-equiv of NO generated in the course of the NONOate decay. Comparison with buffer solutions equilibrated with different N₂O partial pressures indicates that 0.25 to 0.3 equiv of N₂O are produced per reduced Fe^{II}/Co^{II}-Fe_BMb1(MF-heme) and as little as 0.10 equiv of N₂O per Zn^{II}-Fe_BMb1(MF-heme) (Figure 2).

FTIR spectra of 100 μM solutions of Fe^{II}-Fe_BMb1(MF-heme) exposed to 10 mM DEA-NONOate in presence of 50 mM ascorbate show intense ν(NNO) bands consistent with 1.2 mM N₂O production (Figure 3). Control experiments with enzyme free ascorbate/NONOate solution or supplemented with 100 μM E-Fe_BMb1(MF-heme) or Fe^{II}-Fe_BMb2, show only ~10% of this N₂O signal, confirming that N₂O is produced by multiple NOR catalytic turnovers from Fe^{II}-Fe_BMb1(MF-heme). Although the DEA-NONOate concentration used in these experiments produces a large excess of NO, Fe^{II}-Fe_BMb1(MF-heme) only performs 12 turnovers before losing activity. UV-vis spectra collected on the IR films show that this loss of activity is due to the formation of 5-coordinate low-spin heme (Figure S1), as previously identified as a dead-end complex in Fe^{II}-Fe_BMb1 (33).

Equivalent FTIR measurements with Co^{II}-Fe_BMb1(MF-heme) in multi-turnover conditions show a slight decrease in numbers of turnover achieved, from 12 to 9 relative to Fe^{II}-Fe_BMb1(MF-heme), and UV-vis spectra consistent with the same inhibition process. Most strikingly, experiments with Zn^{II}-Fe_BMb1(MF-heme) show that it can perform 7 consecutive turnovers despite the lack of redox activity from the Zn^{II} ion (Figure 3).

While time-resolved resonance Raman experiments have shown that both iron(II) centers in Fe^{II}-Fe_BMb2 bind one NO molecule before generating N₂O (33), the multiple turnovers exhibited by Zn^{II}-Fe_BMb1(MF-heme) highlight that NO binding to the non-heme metal center is not required for activity in these constructs. Vibrational analyses of heme {FeNO}⁷ species in Fe_BMbs (32) and Fe_BMb1(MF-heme) (38) have shown how interactions of divalent metals at the Fe_B site with the heme-bound NO group result in large downshifts of

the $\nu(\text{NO})$ indicative of an increase in NO^- nitroxyl-like character. Since the $\text{E-Fe}_\text{B}\text{Mb1(MF-heme)}$ does not turn over, our current data suggest that this increased electron density on the NO moiety facilitates a reductive catalytic route where the heme $\{\text{FeNO}\}^7$ species is further reduced to an $\{\text{FeNO}\}^8$ species prone to electrophilic attack by a second NO molecule and N-N bond formation (Scheme 2). Experiments with $\text{Fe}_\text{B}\text{Mb}$ proteins with Cu(I) bound at the Fe_B site support these conclusions since the monovalent ion fails to increase the NO^- nitroxyl-like character of the heme $\{\text{FeNO}\}^7$ (32) and does not permit multi-turnover in $\text{Cu}^\text{I}\text{-Fe}_\text{B}\text{Mb1(MF-heme)}$ (see Supporting Information for details).

The reductive pathway described in Scheme 2 differs from the *cis*-heme mechanism favored by Blomberg (22) or the *trans* mechanism (19, 24, 25), since these two mechanisms involve redox cycling of the diiron site between diferrous and diferric forms. Nevertheless, the NOR activity of $\text{Zn}^\text{II}\text{-Fe}_\text{B}\text{Mb1(MF-heme)}$ is more in line with the *cis*-heme pathway favored by Blomberg (22) since it rules out the formation of a *trans* metal-nitrosyl dimer. A recent study by Richter-Addo and coworkers that showed how Lewis acid coordination to nitrosyl O -atom in porphyrin $\{\text{FeNO}\}^7$ models can promote NO coupling reactions also brings support to the *cis*-heme mechanism (39).

A recent study of a nonheme diiron complex that mimics the flavodiiron nitric oxide reductase (FNORs) showed how a one-electron reduction of a stable $[\{\text{FeNO}\}^7]_2$ synthetic model complex was sufficient to produce N_2O (40). This novel semi-reduced mechanism is distinct from the super-reduction mechanism proposed earlier by Kurtz where an $[\{\text{FeNO}\}^7]_2$ requires a 2-electron reduction for N-N coupling and N_2O production (41). Although flavin-free FNORs has been shown to produce N_2O (42), Lehnert and coworkers suggest that the reaction can proceed more efficiently with the flavin cofactor acting as a single electron donor (40). Our current study with $\text{Fe}_\text{B}\text{Mbs}$ supports an equivalent semi-reduced pathway with a heme-only redox active binuclear NO reduction in $\text{Fe}_\text{B}\text{Mb1(MF-heme)}$ and as a possible catalytic route for NORs.

Quantitative analysis of the multiple NO reduction turnovers showed 12, 9 and 7 turnovers with $\text{Fe}^\text{II}/\text{Co}^\text{II}/\text{Zn}^\text{II}\text{-Fe}_\text{B}\text{Mb1(MF-heme)}$ complexes, respectively. These results clearly show that the presence of a redox active metal center is not required for multiple NO reductase turnovers in metal-loaded $\text{Fe}_\text{B}\text{Mb1(MF-heme)}$. The higher reduction potential of MF-heme facilitates the one-electron reduction of its $\{\text{FeNO}\}^7$ complex and electrophilic addition of a second NO to produce N_2O through a semi-reduced pathway. The divalent metal ion at the Fe_B site is required for electrostatic activation. A similar semi-reduced pathway should be considered in investigations of cytochrome-c-dependent NOR activity in reducing conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

UV-vis absorption spectra of reduced E-Fe_BMb1(MF-heme) where E stands for “empty” (black traces), Fe^{II}-Fe_BMb1(MF-heme) (A, red), Co^{II}-Fe_BMb1(MF-heme) (B, red) and Zn^{II}-Fe_BMb1(MF-heme) (C, red).

Figure 2.

FTIR detection of N_2O produced by 1 mM $\text{Fe}^{\text{II}}\text{-Fe}_B\text{Mb1(MF-heme)}$ (A), $\text{Co}^{\text{II}}\text{-Fe}_B\text{Mb1(MF-heme)}$ (B) and $\text{Zn}^{\text{II}}\text{-Fe}_B\text{Mb1(MF-heme)}$ (C) following exposure to 3 mM DEA-NONOate (see Supporting Information for details). The FTIR spectrum of a 0.3 mM N_2O standard solution is also shown for intensity comparison (black traces).

Figure 3.

FTIR detection of N₂O produced by multiple turnovers of 100 μM Fe^{II}-Fe_BMb1(MF-heme) (red), Co^{II}-Fe_BMb1(MF-heme) (blue) and Zn^{II}-Fe_BMb1(MF-heme) (green) in the presence of 50 mM ascorbate and 10 mM DEA-NONOate. Negative controls with 50 mM ascorbate and 10 mM DEA-NONOate (gray), + E-Fe_BMb1(MF-heme) (yellow) and + Fe^{II}-Fe_BMb2 (purple).

Scheme 1. Structure of heme b (A), monoformyl heme (MF-heme) (B) and diformyl heme (DF-heme) (C).

Scheme 2.

Proposed one-electron reductive cycle for the multiple turnovers in Fe^{II}/Co^{II}/Zn^{II}-Fe_BMb1(MF-heme).