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Active-Site Models of Bacterial Nitric Oxide Reductase Featuring Tris-Histidyl and Glutamic Acid Mimics: Influence of Carboxylate Ligand on Fe_B Binding and Heme Fe/Fe_B Redox Potential

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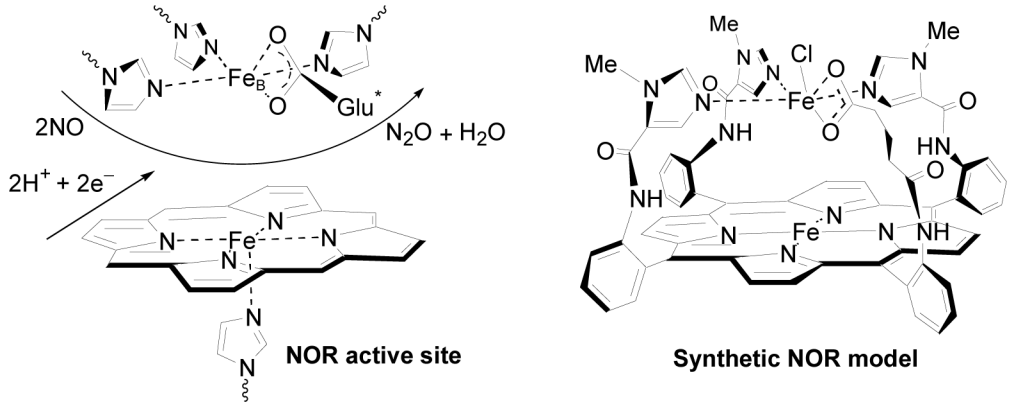
Abstract



Active-site models of bacterial nitric oxide reductase (NOR) featuring a heme iron and a trisimidazole and a glutaric acid-bound non-heme iron (Fe_B) have been synthesized. These models closely replicate the proposed active site of native NORs. Examination of these models shows that the glutamic acid mimic is required for both Fe_B retention in the distal binding site and proper modulation of the redox potentials of both the heme and non-heme irons.

Biological denitrification is a four-step process that reduces nitrate to dinitrogen (eq 1). This process is not only important for bacteria in anaerobic energy generation but also represents a major pathway by which vast amounts of fixed nitrogen is returned to the atmosphere.[1-3] Bacterial nitric oxide reductase (NOR) is a membrane-bound enzyme that catalyzes the third step of denitrification: the two-electron reduction of nitric oxide to nitrous oxide (eq 2 and Figure 1). NORs are members of the heme-copper oxidase (HCO) superfamily and are believed to be ancestral relatives of cytochrome *c* oxidase (C*c*O).

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \quad (1)$$

$$2NO+2e^-+2H^+ \rightarrow N_2O+H_2O \quad (2)$$

The active sites of both C*c*O and NOR are bimetallic with a proximal imidazole ligated heme iron and a distal tris-histidine coordinated metal ion. In C*c*O, the distal ion is copper (Cu_B),

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Supporting Information **Available:** ^{1}H NMR and ^{13}C NMR spectra of **1a**-**1c**, high resolution mass spectra (HRMS) of **1a**—**1c**, **2a**—**2c**, and **3a**—**3c**, cyclic volammetry of **2a**—**2c** and **3a**—**3c**. This material is available free of charge via the internet at http://pubs.acs.org.

while in NOR, it is a non-heme iron (Fe_B) (Figure 1).[1-3] While the distal metal binding sites of C*c*O and NOR are very similar, there are a few differences between them that likely account for the selectivity between Cu and Fe, and thus the preferred reactivity for O_2 and NO respectively. In C*c*O, a redox active phenol group from a Tyr residue is post-translationally coupled to one of the Cu_B ligating imidazoles. In NOR, this phenol group is absent, but a conserved glutamic acid residue is located near the active site and is reported to be essential for normal levels of NOR activity.[4] It has been suggested that this glutamic acid provides an additional ligand for Fe_B, which prefers octahedral coordination. It has been proposed that this glutamic acid residue increases the selective binding of the distal non-heme Fe_B (over that of Cu), regulates the charge at the active site, and possibly mediates the uptake of protons during catalytic turnover of NO.[4,5]

The development of biomimetic models to investigate the structural-functional relationships of native metalloenzymes has proven to be a successful strategy.[6-11] Simulation and variation of synthetic models provide insight into the coordination environments, spectroscopic properties, and catalytic mechanisms of metalloenzymes. Such detailed information and systematic variations are difficult to obtain from wild type enzymes or their mutants due to their restricted availability and difficulty in mutagenesis. To date, only a few of synthetic models have been reported that imitate the active site of NOR; none of these contain a mimic for the conserved glutamic acid moiety.[1,12,13] In order to draw accurate conclusions about structural-functional relationships from biomimetic models, it is important that these synthetic models reproduce all of the key structural features of the native enzyme.

In this paper, we report a new synthetic NOR active-site model (**2a**) featuring a heme iron and a trisimidazole- and glutaric acid-bound non-heme iron. This model most closely replicates the active site of native NOR. Examination of this model shows that the glutamic acid mimic is required for both Fe_B retention in the distal binding site and proper modulation of the redox potentials of both the heme and non-heme irons.

NOR model ligands **1** (**1a**—**1c**) bearing trisimidazole pickets (Scheme 1) were prepared following a scheme recently developed in our laboratory.[14] Variation at the R group from glutaric acid (**1a**) to glutaric ester (**1b**) and a hexyl group (**1c**) provides opportunities to investigate the impact of the glutaric acid residue on non-heme Fe binding and the electrochemical properties of the di-Fe active site. **1a**—**1c** were fully characterized by ^{1}H NMR, ^{13}C NMR, and high resolution mass analysis (HRMS).[14,15] While this generation of NOR models does not contain a proximal imidazole for coordination to the heme Fe, it has been reported that in the native enzyme, the imidazole disassociates from the heme Fe after binding of NO.[16,17]

Reaction of **1a**—**1c** under a N_2 atmosphere with excess $FeCl_2$ and K_2CO_3 in THF provide di-Fe models **2a**—**2c**, respectively (Scheme 2).[15] The identities of the paramagnetic di-Fe complexes **2a**—**2c** were confirmed by mass analysis (LRMS and HRMS).[15] LRMS and HRMS data for **2a** reveal that only a single chloride ion is present in the structure and with the glutaric carboxylate accounting for the charge balance of the non-heme Fe in the distal site. In contrast, LRMS and HRMS data reveal two chloride ions are present in **2b** and **2c**.[15]

The coordination of the non-heme Fe by the glutaric carboxylate is consistent with following observations: when a CH_2Cl_2 solution of **2a**, **2b**, or **2c** is washed thoroughly with deoxygenated water under N_2, **2b** and **2c** lost their non-heme Fe to give the corresponding mono heme Fe complex (**3b** and **3c**). In contrast, the non-heme Fe in **2a** survives such aqueous treatment. Stirring a CH_2Cl_2 solution of **2a** with an excess amount of saturated Na_2 EDTA (a strong Fe^{2+} chelator) in water for 5h under N_2 successfully removs the non-heme Fe of **2a** forming **3a**. The identities of **3a**—**3c** were confirmed by LRMS and HRMS and further supported by

electrochemical analysis (Figure 2).[15] Compared with that of **2a**—**2c**, the redox couples corresponding to the non-heme Fe disappeared on the cyclic voltammograms of compounds **3a**—**3c**. These results suggest that the non-heme Fe is only weakly bound by the tris-imidazole pocket of **2b** and **2c**, while coordination of the non-heme Fe by the three imidazoles and the glutaric carboxylate significantly increases the stability of **2a** in aqueous media. Thus, the conserved glutamic acid moiety present at the NOR active site may be required for retention of Fe_B, as native NOR needs to function in an aqueous biological system.

The exact molecular mechanism of NO reduction by NOR is still the subject of much debate. To date, two general schemes have been proposed; the "trans" mechanism involves the binding of a molecule of NO to both heme b_3 and Fe_B, while the "cis" method suggests that two molecules of NO bind to Fe_B solely. Spectroscopic examination of single-turnover steps, using time-resolved Raman, EPR, and optical absorption spectroscopies typically start with the fully reduced enzyme (heme b_3 Fe^{II} / Fe_B^{II}). Alternatively, it has been suggested that the mixed-valence state of the active site (heme b_3 Fe^{III} / Fe_B^{II}) may represent the active form of the enzyme.[5,18] Mediated redox potentiometry experiments on NOR isolated from *P. denitrificans* reveal that the midpoint potential of the heme b_3 (E_m = +60 mV vs NHE) is unexpectedly lower than that of C*c*O.[5] In addition, the midpoint potential of Fe_B is approximately 260 mV positive of that of heme b_3. The large potential difference between heme b_3 and Fe_B, combined with the apparent low potential of heme b_3, suggests that the enzyme may not achieve the fully reduced state (heme b_3 Fe^{II} / Fe^{II}_B) under physiological conditions. This would avoid the formation of a stable heme Fe^{II}-NO complex, a potential thermodynamic trap in the catalytic cycle.

The energetic cost of placing a charged carboxylate residue in a lipid layer supports the proposal that the glutamic acid residue ligates Fe_B, regulates the charge and mediates the redox potential of the di-Fe center active site.[4,5] Indeed, replacement of the glutamic acid residue closest to the active site with alanine results in a −120 mV negative shift in the Fe_B midpoint potential from to +200 mV vs NHE in conjunction with a decrease in NO reductase activity.[4]

Figure 2 shows the cyclic voltammograms (CVs) of **2a**/**3a** and **2b**/**3b** adsorbed on an edge plane graphite electrode in deoxygenated pH 7 buffer.[19] All of the di-Fe complexes show a single reduction wave, corresponding to the simultaneous reduction of both the heme Fe and Fe_B. **2b** and **2c**, without a carboxylate available to ligate Fe_B, show the same peak oxidation potentials (E_{pa}) for the nonheme Fe_B and heme Fe at +115 mV vs NHE (Figure 2, panel b). In contrast, the CV for **2a** shows two distinct oxidation waves at +40 mV and –80 mV vs NHE, corresponding to the non-heme Fe and heme Fe respectively (Figure 2, panel a).

The pH dependence of the $Fe^{III}Fe_B^{III}$/$Fe^{II}Fe_B^{II}$ reduction potential ($E^{o'}$) for **2a** was also examined (Figure 3) and found to be –58 mV/pH in the pH range of 6 to 10.[20] This represents a two-electron, two-proton reaction[21,22] for the reduction of **2a** and is consistent with the reduction of a μ-oxo-bridged active site (heme Fe^{III}-O-Fe_B^{III}). The heme Fe^{III}-O-Fe_B^{III} state (or protonated form, heme Fe^{III}-O(H)-Fe_B^{III}) is believed to be the resting (oxidized) state of NOR and possibly the final step in the catalytic cycle of NOR.[1,17,18,23-28]

In summary, we have developed several models that closely replicate the bimetallic active site of bacterial NOR. These complexes reproduce the key structural features of the catalytic di-Fe center of native NOR and represent the best available synthetic NOR active-site models examined to date. The presence of a glutamic acid mimic significantly increases the stability of Fe_B binding in the distal site while modulating the redox potentials of both the heme Fe and Fe_B centers. Further investigation of the single turn-over reactions of NO and O_2 with these models, as well as steady-state electrocatalytic studies of NO reduction are currently in

progress. Such investigation should provide meaningful information regarding the mechanism of NO reduction to N_2O by bacterial NOR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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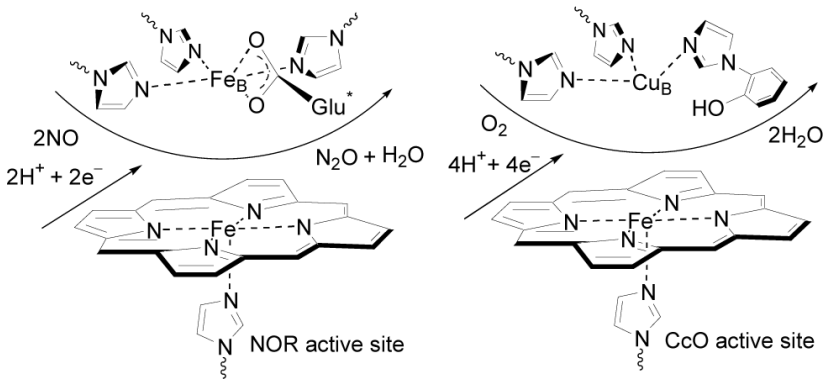
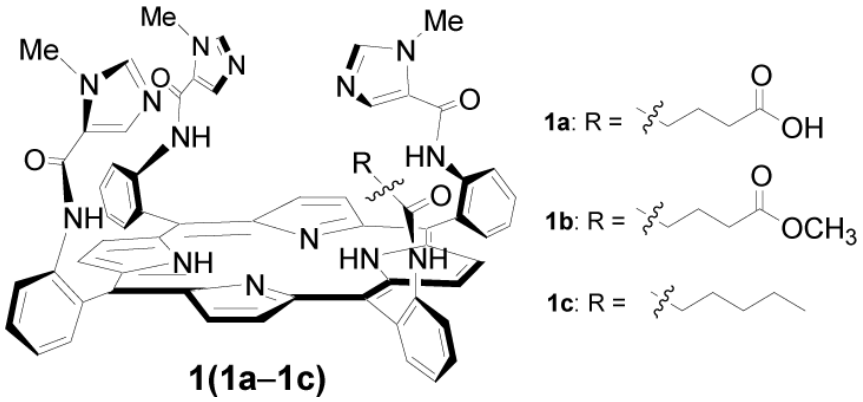
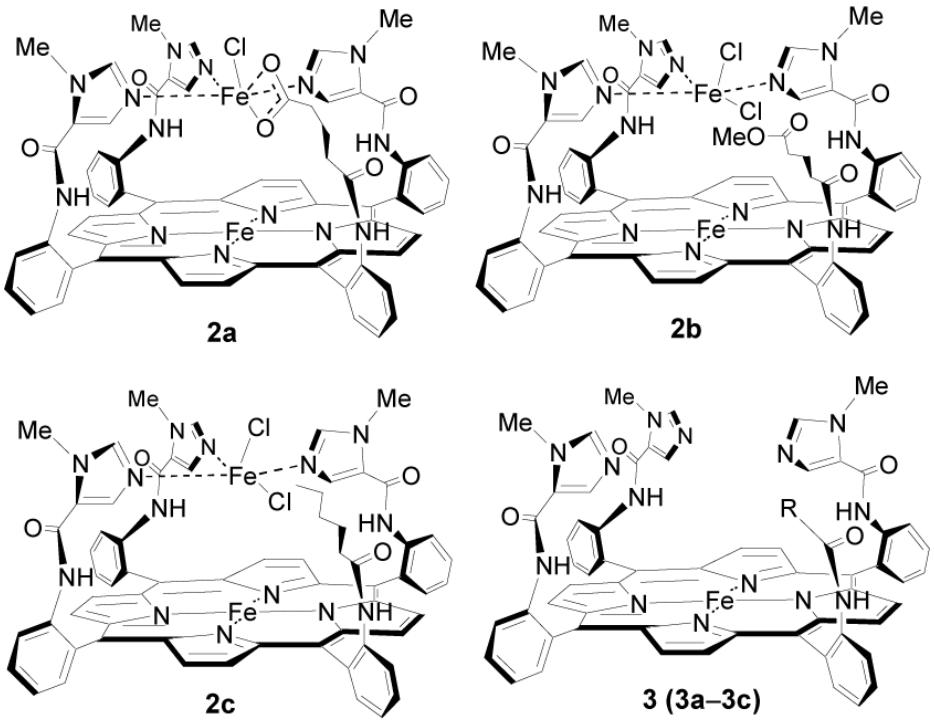


Figure 1.
Schematic representation of the bimetallic active sites of bacterial nitric oxide reductase (NOR) and cytochrome c oxidase (CcO).



Scheme 1.



Scheme 2.

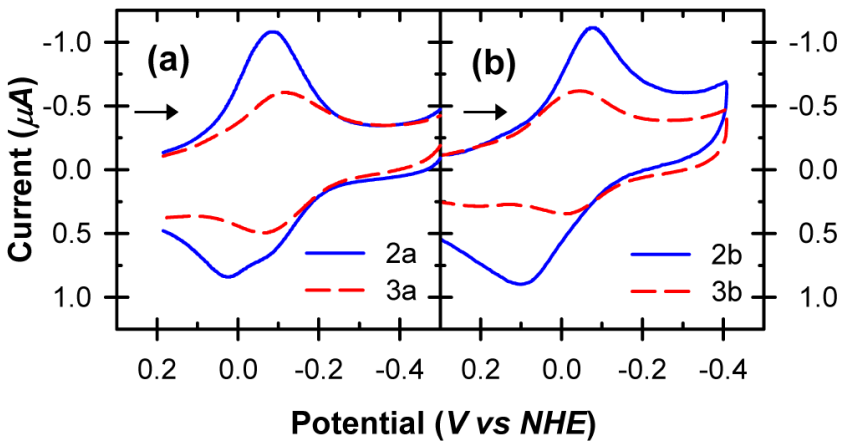


Figure 2.

Cyclic voltammograms of **2a** / **2b** (panel a) and **3a** / **3b** (panel b) adsorbed on an edge plane graphite (EPG) electrode at a coverage of 2.5 nmol / cm^2. Scans were taken in deoxygenated phosphate buffer (pH=7.0) at a scan rate of 10 mV / sec.

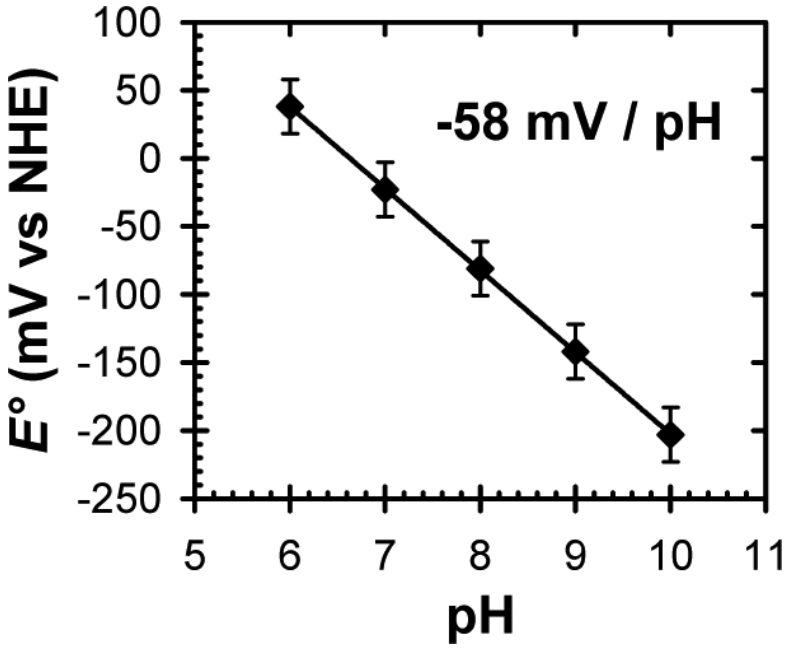


Figure 3.
pH dependence of the $Fe^{III}Fe_B^{III}/Fe^{II}Fe_B^{II}$ potential (E^o) for **2a** at an EPG electrode. Scan rate, 10 mV s^{-1}.