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Heme Iron Nitrosyl Complex of MauG Reveals an Efficient Redox Equilibrium between Hemes with Only One Heme Exclusively Binding Exogenous Ligands†

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ABSTRACT: MauG is a diheme enzyme that oxidizes two protein-bound tryptophan residues to generate a catalytic tryptophan tryptophylquinone cofactor within methylamine dehydrogenase. Upon the two-electron oxidation of bis-ferric MauG, the two *c*-type hemes exist as a spin-uncoupled bis-Fe(IV) species with only one binding oxygen, which is chemically equivalent to a single ferryl heme plus a π porphyrin cation radical (Li, X. et al. (2008) Proc. Natl. Acad. Sci. U.S.A. *105*, 8597−8600). The EPR spectrum of the nitrosyl complex of fully reduced MauG shows a single six-coordinate Fe(II)-NO species, which is characteristic of a histidine-ligated Fe(II)-NO moiety in the heme environment. Exposure of partially reduced MauG to NO reveals a redox equilibrium with facile electron transfer between hemes but with only one binding nitric oxide. Thus, the second heme is able to stabilize all three redox states of iron (Fe(II), Fe(III), and Fe(IV)) in a six-coordinate protein-bound heme without binding exogenous ligands. This is unprecedented behavior for a protein-bound heme for which each of these redox states is relevant to the overall catalytic mechanism. The results also illustrate the electronic communication between the two iron centers, which function as a diheme unit rather than independent heme cofactors.

MauG is a diheme enzyme that catalyzes a six-electron oxidation of protein-bound tryptophan residues to generate a catalytic tryptophan tryptophylquinone (TTQ) cofactor within methylamine dehydrogenase (*1*−*3*). Spectroscopic studies indicate that MauG contains one five-coordinate high-spin ferric heme (Heme 1) and one six-coordinate low-spin ferric heme (Heme 2) (*1*, *4*). The high-valent reactive form of MauG may be generated by the addition of H_2O_2 to the diferric enzyme or O_2 to the diferrous enzyme. When it is oxidized by one equivalent of H_2O_2, MauG produces an unprecedented bis-Fe(IV) intermediate, which consists of two distinct Fe(IV) species that are well-characterized by Mössbauer spectroscopy (*5*). Heme 1 is Fe(IV)═O (δ_1 = 0.06 mm/s and ΔE_{Q1} = 1.70 mm/s), and Heme 2 is an unusual Fe(IV) without an exogenous oxygen ligand (δ_2 = 0.17 mm/s and ΔE_{Q2} = 2.54 mm/s) (*5*).

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Given the novel redox properties of MauG described above, it is of particular interest to investigate the reactivity of the two hemes toward exogenous molecules and metal coordination at the ferrous oxidation state. EPR spectroscopy was used to characterize the coordination and spin states of the hemes of MauG in the diferric state. The bis-Fe(II) state its EPR silent, and thus far, there is no evidence that Heme 2 retains its two axial ligands from protein residues in the ferrous state. Even if this could be shown by structural and spectroscopic studies, it would not exclude the possibility that an axial ligand may be released during the reaction cycle to allow binding of an exogenous axial ligand. For example, in cytochrome cd_1 nitrite reductase, where the six-coordinate heme d_1 is the reaction site, the sixth axial iron ligand does come off to allow substrate binding to heme d_1 (*8*). In this study, nitric oxide (NO) is used to probe the chemistry and reactivity of the hemes of MauG at the diferrous state by EPR spectroscopy.

NO is an ideal O_2 surrogate. It is also a spin probe for the Fe(II) centers in proteins because it has an unpaired electron, which makes the Fe(II)-NO adduct paramagnetic and EPR-active. The present study examines the reactivity of MauG toward NO to determine whether one or both hemes are reactive toward NO and to characterize the spectroscopic properties of the NO-heme adduct(s).

The EPR spectrum of the NO adduct of the fully reduced MauG (Figure 1), which was generated by the addition of two equivalents of NO to the dithionite-reduced MauG, presents a signal in a rhombic pattern with the principal *g* values at $g_x \approx 1.972$, $g_y \approx 2.019$, and $g_z \approx 2.078$. This is characteristic of a low-spin six-coordinate $S = 1/2$ $\{Fe(II)\text{-}NO\}^7$ heme complex following the Enemark and Feltham notation (*9*). An extensive least-squares fit using an EPR program developed by Dr. Frank Neese (*10*) shows that the central g_y component is dominated by triplet hyperfine interactions due to the ^{14}N nuclei (I=1) from the NO ligand (A_{NO} = 32.5 G). Additional superhyperfine features due to the ^{14}N nuclei from the histidine ligand are also present but poorly resolved (Figure 1). Overall, these EPR spectra are similar to data previously reported for histidine-ligated heme Fe(II)-nitrosyl adducts of cytochrome *c* oxidase, cytochrome *c* peroxidase, heme oxygenase, hemoglobin, myoglobin, and horseradish peroxidase (*11*−*13*). Addition of excess NO to the NO−MauG complex does not result in any further spectral change. Spin quantitation indicates, however, that only one Fe is present at the Fe-nitrosyl adduct and that the other Fe remains at the EPR-inactive ferrous state.

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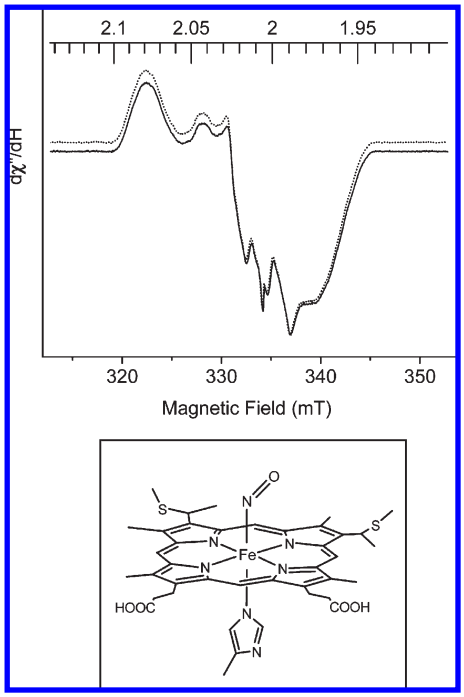

FIGURE 1: EPR spectrum of fully reduced MauG (200 μM) nitrosyl complex (solid line) and partially reduced MauG nitrosyl complex (dotted line) (top panel), and the proposed *c*-type heme structure (bottom panel). The spectrometer parameters were temperature, 10 K; microwave frequency, 9.38 GHz; microwave power, 0.025 mW; modulation, 5 G; and Q-value, 5500.

In a separate set of experiments, MauG was partially reduced by the addition of 1.2 equivalents of dithionite under anaerobic conditions prior to NO exposure. The EPR spectrum of the NO adduct is shown in Figure 2 and juxtaposed to that of the diferric oxidation state MauG and the partially reduced spectrum. The results of these experiments confirmed that the $S = 1/2$ Fe(II)-NO EPR signal is from only one of the hemes.

Analysis of the EPR spectra in Figure 2 also allows precise determination of which of the two hemes is accessible and able to react with NO. The two ferric hemes of MauG exhibit distinct EPR signals. There is no sign of spin interactions between the two hemes, indicating that the two hemes are distantly separated. A minor component at g_z 2.89 and g_y 2.32, values similar to those of diheme cytochrome *c* peroxidase, in the oxidized MauG spectrum has been previously noted to be a minor subpopulation (*1*) that can be reduced by dithionite. Thus, if both hemes were able to bind nitric oxide in the fully reduced state, two distinct Fe(II)-NO species may be observed in the EPR spectrum. This is not the case for MauG, for which only one Fe(II)-NO EPR signal is observable (Figure 2). The EPR signal of the Fe(II)−nitrosyl complex in Figure 2 resembles the NO adduct spectrum of fully reduced MauG (Figure 1), and spin quantitation indicates that it only represents one heme Fe of MauG. Significantly, the EPR spectrum of the partially reduced NO-treated MauG also includes the signal for the ferric low-spin heme.

The results obtained for the experiments with partially reduced MauG are explained by the scheme shown in Figure 3. When only one electron is introduced into the diheme system, equilibrium is established where the electron has a similar probability of being present on either the high- or the low-spin heme. If both hemes were accessible and reactive toward NO, then one should observe a mixture of signals from the NO-heme adducts of both hemes as well as decreased ferric heme EPR signal intensity.

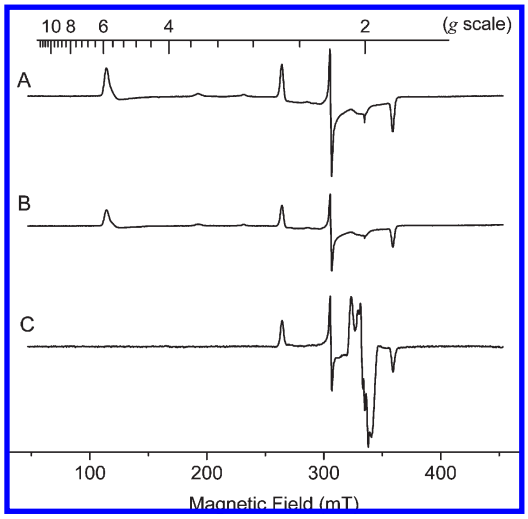

FIGURE 2: EPR spectra of the oxidized diferric MauG (A), partially reduced MauG (B), and the NO adduct with partially reduced MauG from a parallel sample (C). The spectrometer conditions were the same as that of Figure 1 with the exception of microwave power reduced to 0.006 mW.

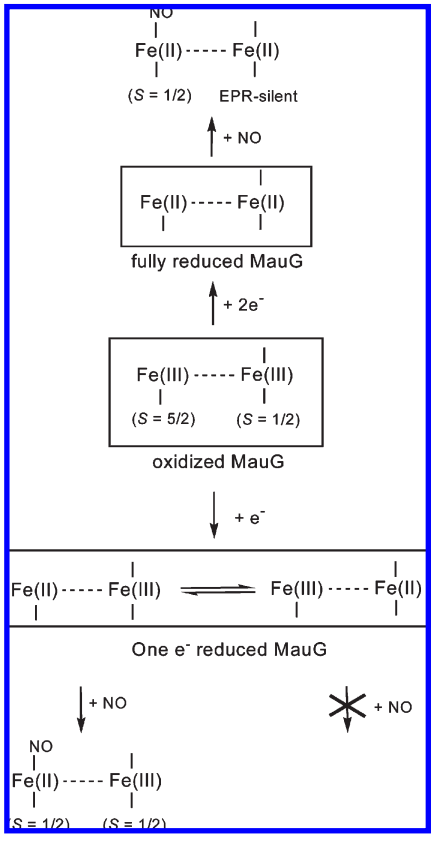

FIGURE 3: Proposed model for the formation of the NO adduct complex with fully and partially reduced MauG.

This was not observed. Instead, only signals from the NO adduct and ferric low-spin heme are observed. An explanation consistent with this finding is that NO reacts selectively at Heme 1 with the ferrous ion, thus removing it from the redox equilibrium. Each time a heme-NO is formed, another ferrous ion will be generated to maintain the equilibrium. When the reaction with NO is complete, all that will remain is MauG with the ferric low-spin Fe at Heme 2 and the NO adduct of Heme 1.

Previous studies show that the two *c*-type hemes of MauG act in concert as a 2-electron redox cofactor (*4*). They exhibit cooperative redox behavior during conversion between the diferric and diferrous redox states. MauG exhibits two distinct E_m values of −159 mV and −244 mV, yet the two hemes are reduced and oxidized simultaneously (*4*). This suggests that these E_m values correspond to the addition of the first and second electron to the diheme system (*4*−*7*). The bis-Fe(IV) intermediate is catalytically competent (*5*); it returns to the diferric state in a slow spontaneous reaction or in a more rapid reaction with its substrate (*5*). Again, during the conversion between the bis-Fe(IV) and diferric states, the hemes appear to oxidize and reduce simultaneously.

It has been proposed that after the addition of H_2O_2 to MauG a compound I type of intermediate, i.e., Fe(IV)═O and a π porphyrin cation radical, is transiently formed, presumably at the five-coordinate Heme 1 site (*7*). In contrast to what is seen with other heme-dependent oxygenases such as cytochrome P-450, this postulated compound I-type species does not accumulate and react with its substrate. Instead, it reacts further with an aromatic acid residue that resides between the hemes, giving rise to a compound ES type of intermediate composed of an oxyferryl heme close to a protein based radical. The transient amino acid radical then oxidizes the second heme, producing a second Fe(IV) at Heme 2 that is stabilized by the porphyrin ring and the axial ligands provided by amino acid residues of the protein (*5*). The present work demonstrates that only the five-coordinate Heme 1 Fe(II) ion is reactive toward NO and is the heme which presumably reacts with O_2. Although the two hemes can share electrons, Heme 2 does not directly react with exogenous compounds and maintains its six-coordinate geometry during the entire reaction cycle.

These results, along with previous studies (*14*), indicate that the hemes of MauG function as a coordinated diheme unit that binds exogenous molecules, including O_2, H_2O_2, CO, and NO, selectively at the Fe-center of the high-spin five-coordinate heme. The results also clearly illustrate the redox equilibrium, previously termed redox cooperativity, and facile electron transfer between hemes. The two hemes are shown here to maintain efficient redox equilibrium. Previous spectroscopic data indicate that Heme 2 Fe ion is six-coordinate with both ligands provided by protein amino acid residues at both the Fe(III) and Fe(IV) states (*5*). The results presented here demonstrate that both protein-derived metal ligands are retained in the Fe(II) state of Heme 2. Thus, it is intriguing that MauG stabilizes three redox states of iron in a six-coordinate protein-bound heme wherein each redox state is relevant to the overall catalytic mechanism. The results also illustrate the electronic communication between the two iron centers which function as a diheme unit rather than independent heme cofactors.

SUPPORTING INFORMATION AVAILABLE

Detailed experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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