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Oxidative damage in MauG: implications for control of high-valent iron species and radical propagation pathways

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Abstract

The di-heme enzyme MauG catalyzes the oxidative biosynthesis of a tryptophan tryptophylquinone (TTQ) cofactor on a precursor of the enzyme methylamine dehydrogenase (preMADH). Reaction of H₂O₂ with the diferric form of MauG, or reaction of O₂ with diferrous MauG, forms the catalytic intermediate known as *bis*-Fe(IV), which acts as the key oxidant during turnover. The site of substrate oxidation is over 40 Å from the high-spin heme iron where H₂O₂ initially reacts, and catalysis relies on radical hopping through an interfacial residue, MauG Trp199. In the absence of preMADH, the *bis*-Fe(IV) intermediate is remarkably stable, but repeated exposure to H₂O₂ results in suicide inactivation. Using mass spectrometry, we show that this process involves the oxidation of three Met residues (108, 114 and 116) near the high-spin heme through ancillary electron transfer pathways engaged in the absence of substrate. The mutation of a conserved Pro107 in the distal pocket of the high-spin heme results in a dramatic increase in oxidation of these Met residues. These results illustrate structural mechanisms by which MauG controls reaction with its high-valent heme cofactor and limits uncontrolled oxidation of protein residues and loss of catalytic activity. The conservation of Met residues near the high-spin heme among MauG homologues from different organisms suggests that eventual deactivation of MauG may function in a biological context. That is, methionine oxidation may represent a protective mechanism that prevents the generation of reactive oxygen species by MauG in the absence of preMADH.

Keywords

Heme; methionine oxidation; electron transfer

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ASSOCIATED CONTENT

Supporting Information. Figures S1 and S2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

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Many enzymes utilize metals or metal-containing cofactors capable of forming high-valent species to catalyze difficult oxidative reactions. One well known example of this comes from the cytochromes P450 that use a heme cofactor to activate molecular oxygen, generating an Fe(IV)=O heme with an associated cation radical centered either on the heme macrocycle (compound I) or on a nearby amino acid (compound ES).¹ The utilization of amino acid radicals, which are often generated by a high-valent metallo-cofactor,² is also widespread in biological catalysis. In a process known as hole “hopping”, transient amino acid radicals enable efficient electron transfer across larger distances than are supported by electron tunneling alone.³ In this way, a substrate can be oxidized at a site many angstroms from where the oxidative potential is initially generated. For example, the class Ia ribonucleotide reductases (RNR) utilize a di-iron cofactor to generate a stable radical on a nearby Tyr residue. This radical can then reversibly generate a transient catalytic Cys radical ~35 Å away across a protein interface through a hopping mechanism involving transient Tyr radicals between the two sites.⁴

Although high-valent cofactor intermediates and amino acid radicals are central to many essential cellular processes, they are also potentially dangerous to the cell. By nature, both are highly reactive and must be carefully controlled to prevent the generation of harmful reactive oxygen species (ROS) and oxidation of unintended substrates. Underscoring the importance of such regulation is the observation that elevated ROS and oxidative modifications to proteins are implicated in a wide range of disease states, as well as the natural process of aging.⁵ Thus, it is of interest to determine how proteins are able to stabilize and control high-valent species and amino acid radicals to prevent unwanted oxidation events.

The di-heme *c* enzyme MauG catalyzes the oxidative modification of two tryptophan residues on a precursor of methylamine dehydrogenase (preMADH) to generate the tryptophan tryptophylquinone (TTQ) cofactor^{6, 7} (Fig. 1). The catalytic species is generated by reaction of diferric MauG with H₂O₂ to form a high-valent intermediate termed *bis*-Fe(IV).⁸ In this species, both hemes are oxidized to the Fe(IV) state, but the formerly high-spin heme becomes a Fe(IV)=O (ferryl) species while the low-spin heme retains its Tyr and His ligands (Fig. 1). *Bis*-Fe(IV) MauG provides the oxidizing power required to extract electrons from the protein substrate, preMADH, during TTQ biosynthesis. A conserved Trp93 residue sits between the two MauG hemes, and its rapid, reversible oxidation is responsible for the charge-resonance stabilization of the high-valent intermediate.⁹ Although *bis*-Fe(IV) is the dominant resonance structure, compound ES and compound I-like resonance structures make small contributions to the ensemble of two-electron oxidized species. The electron transfer pathway from the preMADH substrate to the MauG *bis*-Fe(IV) hemes has been characterized and shown to involve the transient oxidation of MauG Trp199 that lies at the interface with preMADH.^{10, 11} Mutation of this residue to Phe, which cannot be oxidized by the *bis*-Fe(IV) hemes, completely abrogates activity toward preMADH. Thus, completion of TTQ biosynthesis by MauG requires the formation of a high-valent intermediate (*bis*-Fe(IV)) involving an amino acid radical (Trp93), as well as an additional tryptophan radical (Trp199) involved in hole hopping.

Although *bis*-Fe(IV) MauG reacts rapidly with preMADH, it is remarkably stable in the absence of substrate, decaying back to the di-Fe(III) state over the course of minutes.¹² Despite this stability, MauG has been shown to undergo suicide inactivation with H₂O₂ in the absence of preMADH.¹³ Since only a small amount of heme degradation was observed, it was postulated that the loss of activity was most likely due to the oxidation of a critical amino acid. The subsequent observation of a +16 species by electrospray ionization mass spectrometry (ESI-MS) of intact MauG incubated with H₂O₂ is consistent with that hypothesis.¹⁴ In this study, we further investigate the process of oxidative modification in

MauG using mass spectrometry and UV-visible-near infrared (UV-vis-NIR) absorption spectroscopy. Results from the WT protein compared with several variants of MauG elucidate general mechanisms that prevent uncontrolled reaction of the potent *bis*-Fe(IV) oxidant.

Materials and Methods

Protein Expression and Purification

Recombinant MauG was expressed in *Paracoccus denitrificans* and purified by Ni affinity chromatography as described previously.¹⁵ Mutants of Pro107,¹⁴ Glu113,¹⁶ and Trp199¹¹ were generated by site-directed mutagenesis and expressed and purified from *P. denitrificans* as for the WT enzyme.

UV-visible-near infrared Absorption Spectroscopy

Concentrations of MauG and H₂O₂ were calculated using extinction coefficients $\epsilon_{405} = 208,000 \text{ M}^{-1} \text{ cm}^{-1}$ ¹⁷ and $\epsilon_{240} = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$,¹⁸ respectively. MauG was prepared in 10 mM potassium phosphate pH 7.5 in a quartz cuvette at a concentration of 5.0 μM and equilibrated to 25°C in a temperature-controlled cuvette holder. Stoichiometric H₂O₂ was added from a concentrated stock solution and the initial formation of *bis*-Fe(IV) MauG was confirmed spectroscopically. Spectra monitoring the subsequent decay of the *bis*-Fe(IV) species back to the diferric enzyme were recorded until no further changes were observed (typically ~1 h). This process was repeated three times, at which point no further reaction with H₂O₂ could be detected. All spectra were recorded on a Cary 50 UV-vis spectrophotometer (Varian).

Whole Protein Mass Spectrometry

For reaction with stoichiometric H₂O₂, reaction mixtures were prepared at a final volume of 80 μL and contained 20 μM MauG and 100 μM diethylene triamine pantacetic acid (DTPA) in 10 mM potassium phosphate pH 7.5. Reactions were initiated by the addition of H₂O₂ to 20 μM and incubated for 1 h at room temperature. A 20 μL aliquot was then removed and 250 units of bovine liver catalase (Sigma) added to remove residual H₂O₂. This process was repeated three times. For reaction with excess H₂O₂, reaction mixtures were prepared at a final volume of 20 μL and contained 20 μM MauG and 100 μM diethylene triamine pantacetic acid (DTPA) in 10 mM potassium phosphate pH 7.5. Reactions were initiated by the addition of H₂O₂ to 200 μM and incubated for 1 h at room temperature. Two hundred and fifty units of bovine liver catalase (Sigma) were then added to remove residual H₂O₂. Control samples were prepared under identical conditions in the absence of H₂O₂. Half of the control and reaction mixtures were set aside for trypsin digest and liquid chromatography tandem mass spectrometry (LC/MS/MS) (see below). The remaining samples were desalted and exchanged into 75:25 (v/v) acetonitrile/water with 0.1% formic acid using C4 resin ZipTip pipet tips (Millipore) prior to introduction into the mass spectrometer. Data were acquired on a QSTAR XL (AB Sciex, Framingham, MA) quadrupole time-of-flight (TOF) mass spectrometer with the IonSpray electrospray ionization (ESI) source as described previously.¹⁴

Proteolysis and Liquid Chromatography Tandem Mass Spectrometry

Aliquots of the control and H₂O₂-treated MauG samples described above were diluted two-fold to 10 μL with 100 mM ammonium bicarbonate pH 8.5. Proteomics grade trypsin (Agilent) was added at a mass ratio of 1:50 to MauG and incubated at 37°C overnight. Reactions were quenched by the addition of formic acid to 0.1%. Samples were desalted and exchanged into 80:20 acetonitrile/water with 0.1% formic acid using a STAGE tip desalting

procedure.¹⁹ Samples were evaporated to dryness and dissolved in 2:98 acetonitrile / water with 0.1 % trifluoroacetic acid for analysis by LC/MS/MS on a LTQ Orbitrap Velos (Thermo Scientific) as described previously.²⁰ ProteinPilot 4.2 (AB Sciex), which uses the Paragon™ scoring algorithm²¹ and the ProGroup™ protein grouping algorithm, was used for tandem mass spectrometry data searching against a *P. denitrificans* database from NCBI Reference Sequence, combined with common contaminants database from <http://www.thegpm.org/crap/>. Search parameters were: trypsin; instrument Orbi MS (1–3ppm) Orbi MS/MS; biological modifications and amino acid substitutions ID focus; thorough search effort; and False Discovery Rate analysis.²² After identification of possible modifications, MS/MS data were manually inspected using Xcalibur software version 2.1.0. for validation of search results and match integrity.

Offline HPLC and Mass Spectrometry of Heme Peptides

20 μL of control and H_2O_2 -treated samples were made up as described above, except with a ratio of 50:500 μM MauG/ H_2O_2 . After 1 h incubation at room temperature, catalase was added and the samples diluted to 50 μL in 50 mM ammonium bicarbonate pH 7.5. Proteomics grade trypsin was added at a final mass ratio of 1:50 to MauG and the samples incubated overnight at 37°C. Formic acid was added to 1% and samples were filtered through 0.22 μm centrifugal spin filters (Millipore) prior to manual injection into a 50 μL sample loop. Samples were loaded onto a C4 resin column (Phenomenex) pre-equilibrated with 5:95 (v/v) acetonitrile/water with 0.1% formic acid at a rate of 1 mL/min. The column was washed with the above mixture for 3 min, at which point a linear gradient was applied from 5–50 % acetonitrile at a flow rate of 1 mL/min over 27 min. Fractions were collected every 30 s and the elution profile monitored by a dual channel detector at 215 and 370 nm. Fractions containing the heme-peptides were evaporated to dryness, reconstituted in 30 μL of 50:50 (v/v) acetonitrile/water with 0.1% formic acid and 10 μL was directly injected into the QSTAR XL (AB Sciex) instrument and analyzed as described above. After peptides of interest were identified, another 10 μL aliquot was injected and the m/z of interest subjected to MS/MS. Time-of-flight (TOF) MS/MS spectra were acquired from 100 – 1100 m/z for approximately 5 min with a 1 s accumulation time. The acquisition software was Analyst™ QS v1.0 (AB Sciex).

Results

Oxidative Modification to MauG Correlates with Loss of Function

It was previously shown that 4 cycles of *bis*-Fe(IV) formation and decay in the absence of substrate were sufficient to completely eliminate MauG catalytic activity.¹³ Here, UV-vis-NIR absorption spectroscopy was used to track this process. Upon addition of stoichiometric H_2O_2 , features characteristic of the *bis*-Fe(IV) intermediate were observed. These include a decrease in absorbance and red shift of the Soret band, a sharpening and increase in absorbance in the 500–600 nm region (the α/β bands)¹² and a broad feature in the NIR at 950 nm.⁹ The characteristic *bis*-Fe(IV) spectrum then slowly decayed to a spectrum similar, but not identical to the initial di-Fe(III) spectrum (Fig. 2A). After the decay process was complete and no further changes were observed (~ 1 h), a second stoichiometric addition of H_2O_2 was made. Features consistent with *bis*-Fe(IV) formation were again observed, but at reduced yield. Quantifying the relative concentration of *bis*-Fe(IV) by absorbance at 950 nm suggests that only $\sim 50\%$ of the intermediate is reformed on the second addition of H_2O_2 . This trend continued until no changes were observed upon the fourth addition of stoichiometric H_2O_2 (Fig. 2B). The loss of the ability of MauG to form the *bis*-Fe(IV) species closely matches the loss in catalytic activity reported previously¹³ suggesting a causal relationship. The di-Fe(III) state to which *bis*-Fe(IV) MauG decayed also continued

to change over this interval (Fig. 2C), indicative of a change in the heme environment for the resting state.

To determine whether oxidative modifications to MauG correlated with the observed loss of H₂O₂ reactivity, aliquots from a sample of WT MauG were removed 1 h after each addition of stoichiometric H₂O₂, quenched with catalase and analyzed by whole protein ESI-MS (Fig. 3A). After the first addition of H₂O₂ a significant proportion of a +16 adduct appears, which increases after the second addition. The third and fourth cycles result in an increasing proportion of a +32 adduct, the appearance of a +48 adduct, and the near disappearance of the native protein. These data show that oxidative modification accompanies the loss of H₂O₂ reactivity and catalytic activity in MauG.

In order to localize the observed modifications to H₂O₂-treated MauG, the above samples were digested with trypsin and subjected to LC/MS/MS. Sequence coverage was 72% for all samples at a confidence of 99% (a Paragon scoring parameter). The MS/MS data were searched using ProteinPilot (AB Sciex) with parameters set to identify biological modifications. Among the peptide modifications identified were multiple oxidations to the peptide Q102-R119 as a result of H₂O₂ treatment, which was validated by manual inspection of the data (Fig. 3B). The increasing oxidation with increasing H₂O₂ addition at the peptide level correlates with the whole protein data, which collectively support appearance of a +16 adduct followed by a +32 adduct and a small amount of a +48 adduct. Tandem mass spectra of the oxidized species demonstrate that the initial oxidation preferentially occurs at Met108, followed by Met114 and Met116 (Table 1).

Oxidation of a second peptide, N322-R338 (Fig. 3C), was also identified at Met333 in samples treated with multiple sequential additions of stoichiometric H₂O₂. This modification was interesting in that Met333 is adjacent to Trp199 in the crystal structure of MauG-preMADH with the nearest approach between residues being 3.1 Å.²³ Thus, oxidation of Met333 may be due to radical hopping through Trp199 to the *bis*-Fe(IV) MauG hemes. To test the radical hopping hypothesis, a W199F variant of MauG was subjected to identical H₂O₂ treatment and analyzed by MS. The results were very similar to the results for the WT protein (Fig. 3D-F). Most importantly, Met333 is oxidized upon repeated exposure to stoichiometric H₂O₂ as it was in the WT protein, which suggests that oxidation of this residue does not require an intact radical hopping pathway through Trp199. Rather, oxidation may be a result of direct reaction with H₂O₂ since, based on the crystal structure,²³ this residue would be surface exposed in the absence of preMADH.

Oxidation of MauG variants of high-spin heme distal residues

Oxidation of the E113Q variant of MauG was analyzed to confirm that oxidation of Met108, 114 and 116 requires efficient formation of *bis*-Fe(IV), while oxidation of Met333 is the result of direct, nonspecific reaction with H₂O₂. Glu113 is proposed to act as a general base in the high-spin heme active site, promoting O-O bond cleavage and the formation of the *bis*-Fe(IV) intermediate.²⁴ The E113Q mutation does not alter the structure of MauG, but it decreases the rate of reaction with H₂O₂ and alters the distribution of high-valent resonance structures such that *bis*-Fe(IV) is no longer the predominant species and activity toward preMADH is lost.¹⁶ Upon incubation of E113Q MauG with a 10-fold excess of H₂O₂, no significant oxidation was detected by whole protein ESI-MS (Fig. S1D), nor was any increased oxidation of the Q102-R119 peptide observed (Fig S2A). Conversely, LC/MS/MS data showed increased oxidation of Met333 in the presence of H₂O₂ (Fig S2B), demonstrating that oxidation of MauG Met108, 114 and 116 requires the efficient formation of *bis*-Fe(IV) and is not a simply a result of direct oxidation by H₂O₂.

In contrast to the E113Q mutant, mutation of high-spin heme distal residue Pro107 to Val or Ser results in dramatically increased susceptibility to oxidation upon incubation with a 10-fold excess of H₂O₂ as demonstrated by whole protein ESI-MS¹⁴ (Fig S1). Given their hyperoxidation phenotype, it was of interest to determine the sites of oxidation in the Pro107 variants. Therefore, control and H₂O₂-treated WT, P107V and P107S MauG were trypsin digested and subjected to LC/MS/MS as above. Sequence coverage for the mutant proteins was similar to the coverage obtained for the WT experiments. Like the WT, oxidative modifications of the Q102-R119 peptide were identified in P107S and P107V MauG upon treatment with H₂O₂ (Fig. 4). However, the degree of oxidation was significantly increased in these mutants, consistent with the whole protein ESI-MS (Fig. S1). Namely, some of the unmodified peptide is retained in H₂O₂-treated P107V MauG, but there is also a series of multiply oxidized species formed. In H₂O₂-treated P107S MauG, the unmodified peptide is completely absent and a series of even greater oxidized species is observed. The precise positions of oxidative modifications on this peptide were determined by acquiring MS/MS spectra for each species. The results are summarized in Table 1 and show that all modifications can be localized to the same three Met residues oxidized in the WT protein. For peptides where more than 3 oxidation events are indicated, these Met residues are di-oxidized as demonstrated in the MS/MS spectrum of the +96 adduct peptide in P107S, where all three Met residues contain an additional two oxygen atoms (Fig. 5).

Assessment of Oxidation to Heme-Containing Peptides

Although the above results suggest that essentially all of the observed oxidative damage to WT MauG upon reaction with H₂O₂ is localized to Met108, Met114 and Met116, sequence coverage in this experiment was not complete. Importantly, the peptides containing the covalently attached *c*-hemes were not observed under conditions used for LC/MS/MS. Therefore, the visible absorbance of the heme peptides was used to detect and isolate them by offline HPLC or control and H₂O₂-treated samples (Fig. 6A). Two major peaks, albeit at significantly different absorbance intensities, were observed in the chromatogram of trypsin-digested WT MauG monitored at 370 nm, consistent with the presence of two heme-containing peptides. ESI-MS of the first peak yielded a strong signal with a mass consistent with the tryptic peptide bearing the high-spin heme (N26-R39, 2089.8 Da). MS/MS of this species confirmed this assignment and further showed that the heme cofactor is liberated upon fragmentation, yielding a peak for the +1 ion at 617.2 (Fig. 6B). There were no mass changes observed for this peptide or its fragments in the sample treated with excess H₂O₂.

Analysis of the second heme peak is problematic, as a trypsin cleavage site exists within the low-spin *c*-heme binding motif CRLCH. Thus, several masses are possible depending upon whether trypsin can cleave at this site and whether the *c*-heme remains covalently bound bridging the resulting peptides. In our analysis, no masses were identified that could be unambiguously assigned to the low-spin heme containing peptide. Unfortunately, this peptide also contains Trp199, a key residue in the catalytic radical hopping pathway and a potential site for oxidation. However, the elution profiles for both peaks were completely unchanged in protein samples treated with H₂O₂ (Fig. 6A). Oxidation would be expected to alter the elution time relative to the unmodified form. Thus, these results suggest that the heme-containing peptides are not sites of significant oxidative modification during reaction of MauG with H₂O₂ in the absence of substrate.

Discussion

The results presented here show that of the seven Met residues in the MauG sequence (Fig 7A), those at positions 108, 114 and 116 are preferentially oxidized during cycles of *bis*-Fe(IV) formation and decay in the absence of substrate. These oxidation events correlate

with a loss in the ability to form the *bis*-Fe(IV) intermediate and, consequently, catalytic activity. Mutations to high-spin heme distal residues Glu113 and Pro107 had opposing effects on the susceptibility of MauG to oxidation. Specifically, while we observed no significant oxidation to E113Q MauG treated with excess H₂O₂, P107V and P107S MauG exhibited a hyperoxidation phenotype in which Met108, Met114 and Met116 are all oxidized to a greater degree than in the WT protein. Since the peptide containing Trp199 was not observed by mass spectrometry, we cannot conclusively rule out oxidation of this residue during H₂O₂-induced deactivation of WT MauG. However, several pieces of indirect evidence suggest that it is not significantly oxidized in H₂O₂ treated samples: 1) The extent of oxidation in whole protein mass spectra of WT MauG correlates closely with the extent of oxidation in its Q102-R119 peptide; 2) WT and W199F are oxidized to equivalent degrees, both at the whole protein and Q102-R119 peptide levels; 3) The elution profiles of the heme-containing peptides in WT MauG are completely unchanged by treatment with H₂O₂. Therefore, since the only other oxidative modification identified was minor, nonspecific oxidation of Met333, it is concluded that the large majority of oxidative damage to WT, W199F, P107S and P107V MauG occurs at Met 108, 114 and 116.

Oxidative modification and loss of H₂O₂ reactivity also correlated with a change in the UV-vis spectrum of the di-Fe(III) state of MauG after suicide inactivation. A similar spectroscopic change was observed after H₂O₂ treatment and loss of reactivity in a myoglobin mutant engineered as a model of cytochrome P450.²⁵ In this case, compound I formed at the heme oxidized a Trp residue engineered into the distal pocket. The oxidized Trp was proposed to form strong hydrogen bond interactions with a heme-coordinated water or hydroxide, yielding a 6-coordinate inhibited state. This could be occurring in WT MauG as well, where oxidized methionine residues could be playing a similar role or potentially even coordinating the heme iron directly. Methionine oxidation is known to cause significant conformational changes,²⁶ and oxidized Cys was observed to coordinate the heme in P107C MauG.¹⁴

In the presence of substrate, *bis*-Fe(IV) MauG rapidly acquires electrons from preMADH during biosynthesis of the TTQ cofactor, which occurs via a hole-hopping mechanism of electron transfer *via* Trp199.¹¹ However, in the absence of substrate, electrons must be acquired from another source. Under these conditions, the current results show that susceptible Met residues can slowly reduce *bis*-Fe(IV) MauG, becoming oxidized themselves in the process. A third source of electrons may be exogenous reductants, such as buffer components or excess H₂O₂, *via* catalase activity. The latter possibility is supported by the observation that sequential exposure to stoichiometric H₂O₂ was significantly more damaging than exposure to a 10-fold excess. No significant catalase activity could be observed by O₂ evolution under these conditions, but it is possible that the activity is sufficiently slow to avoid detection by this method.

Mapping the oxidation-susceptible Met residues onto the structure of WT MauG in complex with preMADH²³ shows that they are clustered in a loop above the distal side of the high-spin heme (Fig. 7B). Oxidation could occur by direct O-atom insertion from the high-valent intermediate as is observed for thioether sulfoxidation mediated by cytochrome P450 compound I.²⁷ However, the sulfur atom of Met108, the most sensitive residue to oxidation, is nearly 11 Å from the heme iron in the crystal structure, making this mechanism unlikely unless the loop is significantly disordered in solution. Perhaps a more likely alternative is that ancillary electron transfer routes terminating in the oxidation of susceptible Met residues may be used when the biosynthetic pathway to preMADH is not available (i.e. in the absence of substrate). In this case, the oxygen atoms introduced in oxidized Met derivatives would most likely derive from solvent water. Thus, we can consider the properties of WT, P107S and P107V MauG in terms of three possible pathways for

reduction of the *bis*-Fe(IV) intermediate: 1) The biosynthetic electron transfer pathway from preMADH; 2) the ancillary electron transfer pathway(s) from Met108, 114 and 116; 3) the direct reduction pathway by exogenous electron donors. Mutations of Pro107 seem to enhance pathway 2 relative to the others, resulting in increased oxidation in the absence of preMADH and decreased catalytic efficiency in its presence. Specifically, P107V MauG exhibits a relatively moderate increase in oxidation sensitivity and a ~ 40% reduction in catalytic activity. P107S MauG exhibits a severe increase in oxidation sensitivity and has no measurable activity toward preMADH. P107C MauG similarly exhibits no catalytic activity and oxidation of the introduced Cys to Cys sulfinic acid was observed even without exogenous H₂O₂ treatment, oxidation apparently having occurred during protein expression or purification.¹⁴

The observation that Met108 is the most susceptible residue to oxidation is intriguing in that it is not the closest Met residue to the heme (Fig. 7B). In fact, Met114 is significantly closer. However, Met108 is sequentially adjacent to Pro107, which is very close to the high-spin heme iron with a Fe-C γ distance of 4.3 Å. This suggests that a through-bond electron transfer pathway may exist from Met108 through Pro107 to the heme that is more efficient than the shorter, through-space pathway from Met114 or the longer route from Met116. As such, the identity and reactivity of the residue at position 107 will have a significant impact on the efficiency of electron transfer through these ancillary pathways. In addition, mutation of the rigid Pro107 seems to increase the flexibility of the distal heme environment, resulting in coordination of the heme Fe by Glu113 in the P107S mutant, and increased disorder of nearby loops in both P107S and P107V structures.¹⁴ This increased flexibility may allow susceptible residues to more closely approach the heme, enhancing the rate of their oxidation such that it effectively competes with the biosynthetic electron transfer pathway. It may also change the relative susceptibility of each residue to oxidation, as is observed in the Pro107 mutants where Met116 is oxidized prior to Met114 in contrast to the WT enzyme.

Although the above explanation adequately describes the effect of Pro107 mutations, it is almost certainly an oversimplification. Mutations to residues in the distal pocket alter the electronic as well as structural properties of the heme environment. For example, the E113Q mutation disrupts redox communication between hemes and alters the distribution of high-valent resonance-structures in the H₂O₂-oxidized state¹⁶. These changes resulted in a mutant that was unable to oxidize either preMADH or its own Met residues. Similarly, Pro107 mutations may alter the nature of the MauG high-valent intermediate in addition to the preferred pathway for electron transfer to that intermediate. Thus, a complete description of these mutants needs to take into consideration the rates of formation and decay of their high-valent species.

The observed charge resonance of *bis*-Fe(IV) MauG involving both hemes and the intervening Trp93 provides an elegant electronic mechanism of stabilization for this species.⁹ However, this alone cannot account for its remarkable stability, as there must be structural elements that prevent the unwanted reaction of the high-valent species with protein residues. This study demonstrates that the rigid proline residue at position 107 near the highly reactive Fe(IV)=O moiety of *bis*-Fe(IV) MauG limits the oxidation of distal Met residues through ancillary electron transfer routes, which only become significant when the biosynthetic pathway is unavailable or Pro107 is mutated. This structural property, in conjunction with charge resonance stabilization between MauG hemes, provides an efficient means of controlling the potent oxidizing potential of *bis*-Fe(IV) MauG and helps to explain its remarkable stability in both electronic and structural terms.

Finally, as Met residues are highly susceptible to oxidative modification, it is perhaps counterintuitive that evolution would place three of them in close proximity to the reactive

heme of MauG. It has been suggested that methionine may play a protective role in some enzymes by being selectively oxidized by ROS, sparing catalytic activity.^{28, 29} Clearly, the roles of Met108, 114 and 116 are not protective in MauG as activity is lost upon their oxidation. However, this process may protect other proteins and cellular structures by preventing the formation of additional ROS by MauG in the absence of preMADH. The potential biological importance of the deactivation process for MauG is highlighted by the fact that confirmed MauG homologues are rich in Met residues in the distal heme region (residues 106–118, *P. denitrificans* numbering) and Met114 in particular appears to be absolutely conserved. Thus, oxidation of MauG Met residues in the absence of preMADH may provide a convenient means to eliminate this potential source of ROS when MauG activity is not required for TTQ biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MADH	methylamine dehydrogenase
TTQ	tryptophan tryptophylquinone
preMADH	a precursor of MADH with incompletely synthesized TTQ
bis-Fe(IV) MauG	redox state of MauG with one heme as Fe(IV)=O and the other as Fe(IV)
WT	wild-type
ROS	reactive oxygen species
ESI-MS	electrospray ionization mass spectrometry
LC/MS/MS	liquid chromatography tandem mass spectrometry
NIR	near-infrared

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Figure 1.
Schematic representation of the reaction of MauG with H_2O_2 to generate the *bis*-Fe (IV) intermediate and its reaction with preMADH to yield the TTQ cofactor.

Figure 2.

UV-vis-NIR spectra of (A) the reaction of di-Fe(III) WT MauG (black) with one equivalent of H_2O_2 to form *bis*-Fe(IV) (red) followed by its decay (grayscale) to a final resting state (blue). (B) Decreasing yields of *bis*-Fe(IV) immediately after addition of 1 (red), 2 (green), 3 (blue) and 4 (black) equivalents of H_2O_2 in cycles of stoichiometric additions of H_2O_2 and *bis*-Fe(IV) decay. (C) Comparison of the initial di-Fe(III) MauG (gray) to the decay products formed after 1 (red), 2 (green), 3 (blue) and 4 (black) cycles of stoichiometric additions of H_2O_2 and *bis*-Fe(IV) decay.

Figure 3.

H₂O₂-induced oxidation of WT (A-C) and W199F (D-F) MauG. (A, D) Whole protein mass spectra highlighting the +50 charge peak of MauG after 0–4 sequential cycles of addition of stoichiometric H₂O₂ and *bis*-Fe(IV) decay (increasing additions going downward). (B, E) LC/MS data showing the 2+ charge state of peptide Q102-R119 after 0–4 sequential cycles of addition of stoichiometric H₂O₂ and *bis*-Fe(IV) decay (increasing additions going downward). (C, F) LC/MS data showing the 2+ charge state of peptide N322-R338 after 0 (top) and 4 (bottom) sequential additions of stoichiometric H₂O₂ and *bis*-Fe(IV) decay. The numbers represent the increase in total mass of the peptide relative to the unmodified peptide.

Figure 4. LC/MS data showing the 2+ charge state of peptide Q102-R119 of WT (A), P107V (B), and P107S (C) before (top trace) and after (bottom trace) incubation with a 10-fold excess of H_2O_2 . The numbers represent the increase in total mass of the peptide relative to the unmodified peptide.

Figure 5.

MS/MS spectrum of the 2+ charge state of the +96 mass of peptide Q102-R119 obtained by incubation of P107S MauG with a 10-fold excess of H₂O₂. The monoisotopic $[M + 2H]^{+2}$ observed precursor m/z value was 1050.44. The y- and b-ions identified are mapped onto the primary sequence and demonstrate di-oxidation of all Met residues.

Figure 6.

(A) HPLC chromatogram of trypsin digests of WT MauG before (blue) and after incubation with a 10-fold excess of H₂O₂ (red) monitored at 370 nm. (B) MS/MS spectrum of the 2+ charge state of the high-spin heme-containing peptide. The monoisotopic [M + 2H]²⁺ observed precursor m/z value was 1045.9. The y- and b-ions identified are mapped onto the primary sequence.

Figure 7.
 (A) Structure of WT MauG in the crystal structure of WT MauG-preMADH (PDB ID: 3L4M) ²³ showing the positions of Met residues (spheres), catalytically important Trp residues and hemes (sticks). (B) The high-spin heme environment of WT MauG showing distances between Met sulfur atoms and the heme Fe. Figure was generated in PyMOL, <http://www.pymol.org>.

Table 1

Masses and sites of oxidative modifications to the tryptic peptide Q102-R119 in WT, P107V and P107S MauG upon incubation with a 10-fold excess of H₂O₂.

Peptide	Calc. Mono ^a [M+2H] ⁺ 2	Obs. Mono ^a [M+2H] ⁺ 2	Modification
WT Q102-R119 +16	1014.97	1014.97	OxMet108
WT Q102-R119 +32	1022.96	1022.97	OxMet108, OxMet114
WT Q102-R119 +48	1030.97	1030.96	OxMet108, OxMet114, OxMet116
P107V Q102-R119 +16	1015.97	1015.98	OxMet108
P107V Q102-R119 +32	1023.97	1023.98	OxMet108, OxMet114
P107V Q102-R119 +48	1031.97	1031.97	OxMet108, OxMet114, OxMet116
P107V Q102-R119 +64	1039.97	1039.97	DiOxMet108, OxMet114, OxMet116
P107V Q102-R119 +80	1047.96	1047.97	DiOxMet108, OxMet114, DiOxMet116
P107S Q102-R119 +48	1025.95	1025.94	OxMet108, OxMet114, OxMet116
P107S Q102-R119 +64	1033.95	1033.95	DiOxMet108, OxMet114, OxMet116
P107S Q102-R119 +80	1041.94	1041.95	DiOxMet108, OxMet114, DiOxMet116
P107S Q102-R119 +96	1049.93	1049.95	DiOxMet108, DiOxMet114, DiOxMet116

^a Observed (Obs) and calculated (Calc) monoisotopic (Mono) *m/z* values are for the 2+ charge states.