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Suicide Inactivation of MauG during Reaction with O₂ or H₂O₂ in the Absence of its Natural Protein Substrate

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Abstract

MauG is a diheme protein that catalyzes the six-electron oxidation of a biosynthetic precursor protein of methylamine dehydrogenase (PreMADH) with partially synthesized tryptophan tryptophylquinone (TTQ) to yield the mature protein with the functional protein-derived TTQ cofactor. The biosynthetic reaction proceeds via a relatively stable high valent *bis*-Fe(IV) intermediate. Oxidizing equivalents ([O]) for this reaction may be provided by either O₂ plus electrons from an external donor, or H₂O₂. The presence or absence of PreMADH has no influence on the reactivity of MauG with [O], however it is demonstrated that MauG is inactivated when supplied with [O] in the absence of PreMADH. The mechanism of inactivation appears to differ depending on the source of [O]. Repeated reaction of diferrous MauG with O₂ leads to loss of activity but not inactivation of heme, as judged by absorption spectroscopy and pyridine hemochrome assay. Repeated reaction of diferric MauG with H₂O₂ leads to loss of activity and inactivation of heme, as well as some covalent cross-linking of MauG molecules. None of these deleterious effects with either source of [O] are observed when PreMADH is present to react with MauG. The radical scavenger hydroxyurea, and small molecule mimics of the monohydroxylated Trp residue of PreMADH also reacted with *bis*-Fe(IV) MauG and afforded protection against inactivation. These results demonstrate that while O₂ and H₂O₂ readily react with MauG in the absence of PreMADH, the presence of this substrate is necessary to prevent suicide inactivation of MauG after formation of the *bis*-Fe(IV) intermediate.

MauG is a 42.3 kDa enzyme containing two *c*-type hemes (1). It exhibits homology to diheme cytochrome *c* peroxidase (CCP) (2,3), but displays significant differences in catalytic and redox behavior (4,5). MauG catalyzes the final steps in the biosynthesis of tryptophan tryptophylquinone (TTQ) (6), the protein-derived cofactor (7) of methylamine dehydrogenase (MADH). MADH exhibits a 119 kDa heterotetrameric $\alpha_2\beta_2$ structure (8) and contained in each β subunit is a TTQ. TTQ is synthesized through posttranslational modification of two endogenous tryptophan residues of MADH. This modification involves crosslinking of β Trp57 and β Trp108, and the incorporation of two oxygens into β Trp57. It has been shown that the incorporation of the second oxygen into β Trp57 and the crosslinking reaction are catalyzed by MauG (1) (Scheme 1). Deletion of *mauG*, a gene in the methylamine utilization (*mau*) gene cluster (9), allowed isolation of a biosynthetic precursor protein of MADH (PreMADH) in which β Trp57 is monohydroxylated at C7 and the cross-link is absent (10,11). MauG catalyzes the six-electron oxidation of PreMADH to yield oxidized MADH with mature TTQ. MauG-dependent TTQ biosynthesis from PreMADH was achieved in vitro using oxidizing equivalents ([O]) provided as either O₂ plus electrons from an external donor, or H₂O₂ (5,

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12). The catalytic high-valent form of MauG is not a ferryl porphyrin cation radical (i.e., Compound I). Instead, MauG utilizes a *bis*-Fe(IV) intermediate with one heme as Fe(IV)=O, and the other as Fe(IV) with two axial ligands from the protein (13). It is believed that an amino acid residue mediates electron transfer between the two hemes during formation of the *bis*-Fe(IV) form and catalysis by MauG (13).

It was previously shown that the order of addition of [O] and PreMADH to MauG is random in the kinetic mechanism (14), in contrast to what is typically observed for other heme-dependent oxygenases and peroxidases (15,16). In this study it is demonstrated that reaction of MauG with [O] in the absence of PreMADH leads to inactivation of MauG. The mechanism of inactivation appears to differ depending on the source of [O]. However, in each case none of these deleterious effects are observed when PreMADH is present to react with MauG. The radical scavenger hydroxyurea, and small molecule mimics of the monohydroxylated Trp residue of PreMADH also reacted with *bis*-Fe(IV) MauG and afforded protection against inactivation. Thus, while O₂ and H₂O₂ readily react with MauG in the absence of PreMADH, the presence of this substrate is necessary to prevent suicide inactivation of MauG after formation of the *bis*-Fe(IV) intermediate.

Experimental Procedures

The methods for homologous expression of MauG in *Paracoccus denitrificans* and its purification were as described previously (1). PreMADH, the biosynthetic precursor of MADH with incompletely synthesized TTQ, which contains monohydroxylated βTrp57 and no crosslink to βTrp108 (11), was heterologously expressed in *Rhodobacter sphaeroides* and purified as described previously (11). Monohydroxylated indole derivatives were obtained from Combi-Blocks, Inc. (San Diego, CA). Hydroxyurea and 5-hydroxy-L-tryptophan were obtained from Sigma-Aldrich.

The effects on MauG of redox cycling in the absence of substrate were studied using either O₂ plus sodium dithionite or H₂O₂ as [O]. Stock solutions of sodium dithionite were freshly prepared anaerobically in 50 mM potassium phosphate buffer, pH 7.5, which had been previously degassed and purged with nitrogen gas. The concentration of dithionite was standardized by anaerobic titration against amicyanin, a copper protein with a known extinction coefficient whose visible absorbance is bleached on reduction (17), and which is used routinely in this laboratory. The reaction mixture contained 3.5 μM of MauG in 3 mL of 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. Aliquots of the solution of sodium dithionite were quantitatively transferred with a gastight syringe and the MauG sample was mixed with a stir bar at the bottom of the sealed cuvette. After each cycle of anaerobic stoichiometric reduction of MauG with dithionite, air was introduced by bubbling into the reaction mixture to reoxidize the diferrous MauG. To remove excess air after each cycle, the MauG sample was again made anaerobic by repeated evacuation and argon replacement. The changes in redox state of MauG were monitored spectrophotometrically (Figure 1A) and the process was repeated for several cycles. In a parallel redox cycling study, MauG was reduced by stoichiometric dithionite and then reoxidized by stoichiometric ferricyanide under anaerobic conditions to specifically ascertain the role of O₂, independent of the simple redox reaction. Stock solutions of ferricyanide were prepared in 50 mM potassium phosphate buffer, pH 7.5. The concentration of ferricyanide was standardized by anaerobic titration with the stock solution of sodium dithionite. For studies with H₂O₂, stoichiometric H₂O₂ was added to 3.5 μM MauG under anaerobic conditions. The reaction with H₂O₂ and the spontaneous return to the diferric state were monitored spectrophotometrically (14) (Figure 1B). When the absorption spectrum indicated that MauG had returned to the diferric state, stoichiometric H₂O₂ was again added to MauG and this process was repeated for several cycles. In parallel studies, this H₂O₂ cycling reaction was performed under aerobic conditions to ascertain the effect if any of the presence

of O₂. The redox cycling studies described above were also performed in the presence of 8 μM PreMADH, 500 μM 6-hydroxyindole, or 100 μM hydroxyurea.

Aliquots of 0.15 mL MauG were removed at different points in the redox cycling experiments and were assessed by absorption spectrum, reactive heme content, steady-state TTQ biosynthesis activity, and SDS-PAGE. Reactive heme content was determined by the pyridine hemochrome method described by Berry and Trumpower (18). Briefly, 0.1 mL of sample was mixed with 0.1 mL of a stock solution containing 200 mM NaOH, 40% (v/v) pyridine, and 3 μL of 0.1 M K₃Fe(CN)₆. The absorption spectrum of this oxidized species was recorded. Solid sodium dithionite was then added and the reduced spectrum was recorded. The concentration of heme was calculated from an extinction coefficient of 24.04 mM⁻¹cm⁻¹ for the difference of A₅₅₀ of the reduced spectrum minus A₅₃₅ of the oxidized spectrum. The enzyme activity of MauG was evaluated by a spectrophotometric assay of MauG-dependent TTQ biosynthesis as described previously (5). In this assay, 0.3 μM MauG samples from the cycling experiments were mixed with 5 μM PreMADH, and 100 μM H₂O₂ was used as the source of [O] during steady-state TTQ biosynthesis. The reaction was monitored by the increase in A₄₄₀ which corresponds to the formation of product, mature MADH with oxidized TTQ.

Kinetic studies of the reaction of small molecules with *bis*-Fe(IV) MauG were performed using an On-Line Instrument Systems (OLIS, Bogart GA) RSM stopped-flow spectrophotometer. Kinetic data collected in the rapid-scanning mode were reduced by factor analysis using the singular value decomposition algorithm and then globally fit using the fitting routines of the OLIS Global Fit software. All reactions were performed in 10 mM potassium phosphate buffer, pH 7.5, at 25 °C. The methods were as described previously for the reaction of *bis*-Fe(IV) MauG with preMADH (14). One syringe contained a fixed concentration of *bis*-Fe(IV) MauG (4 μM) that was generated by stoichiometric addition of H₂O₂, and the other contained the small molecule, the concentration of which was varied. Reactions were monitored between 360 and 440 nm. The *K_d* value and limiting first-order rate constant (*k₃*) for the conversion of *bis*-Fe(IV) to diferric of MauG was determined from the concentration dependence of the observed rate using Eqs 1 and 2, where [S] is the concentration of the varied reactant, E is *bis*-Fe(IV) MauG and E' is diferric MauG.

$$k_{\text{obs}} = k_3 [S] / ([S] + K_d) + k_4 \quad (2)$$

Results

The effects of redox cycling of MauG on absorption spectrum

MauG catalyzes the maturation of MADH using O₂ with electrons provided by a variety of donors (5). It was previously observed that diferrous MauG was reoxidized by O₂ (1) and readily reacted with the O₂ analog, CO (14). To investigate the consequences, if any, of the reaction with O₂ in the absence of substrate, MauG was subjected to repeated redox cycling during which it was reduced anaerobically by addition of stoichiometric (two equivalents) sodium dithionite, and then reoxidized by air in the absence of the PreMADH substrate. The absorption spectra of the initial oxidized and reduced MauG are shown in Figure 1A. These spectra were monitored during 12 cycles of reduction by dithionite and reaction with O₂. No significant change in intensity of the Soret peak was observed after 12 cycles (Figure 2A).

H₂O₂ can also serve as the source of [O] for MauG-dependent TTQ biosynthesis (5) and previous studies showed that the addition of one equivalent of H₂O₂ to diferric MauG generated a di-heme *bis*-Fe(IV) species (i.e., Fe(IV)=O/Fe(IV)) (12). This species spontaneously returned to the diferric state at a rate of $2 \times 10^{-4} \text{ s}^{-1}$ in the absence of PreMADH (14). On conversion to the *bis*-Fe(IV) state, one observes a decrease in the intensity of the Soret peak and a shift in its maximum from 405 to 407 nm (14) (Figure 1B). The absorption spectrum was monitored during 12 cycles of H₂O₂ addition and spontaneous return to the diferric state. An irreversible decrease in intensity of the Soret peak, as well as a change in its shape was observed after 12 cycles (Figure 2B).

The effects of redox cycling of MauG on reactive heme content

Given the results described in the previous section, it was of interest to determine whether the redox cycling in the absence of PreMADH, particularly the H₂O₂-driven cycling, had any direct effect on heme reactivity. In each of the two sets of cycling experiments aliquots were removed after reaction cycles and the reactive heme concentration was determined using the pyridine hemochrome assay (18) (Figure 3A). Essentially no change in heme content was observed during the redox cycling with dithionite plus O₂, consistent with the lack of change in the absorption spectrum. In contrast, after 12 cycles of reaction with H₂O₂ approximately 25% of the covalent *c*-type hemes were inactivated. This correlates with the approximately 25% decrease in the overall size of the Soret peak (i.e., area under the peak) observed in Figure 2B.

The effects of redox cycling of MauG on its catalytic activity

For each of the cycling experiments that are described above, aliquots were removed after reaction cycles and the enzymatic activity of MauG was assessed using a spectroscopic steady-state assay of TTQ biosynthesis. Significant loss of enzymatic activity was observed during the redox cycling studies with both O₂ and H₂O₂ (Figure 3B). During the redox cycling with dithionite plus O₂ full activity was retained after 4 cycles but then decreased to approximately 40% after 12 cycles. During the redox cycling with H₂O₂, activity substantially decreased after one cycle and was completely lost after 4 cycles. Thus, in each cycling experiment significant loss of activity was observed, even in samples which still exhibited 100% reactive heme (Figure 3A and B).

To be certain that the observed loss of activity during redox cycling with dithionite plus O₂ was indeed a consequence of decay of the *bis*-Fe(IV) species formed from reaction of O₂ with diferric MauG, and not a consequence of damage from dithionite reduction or simply the cycling between the diferric and diferrous states, simple redox cycling was also performed under anaerobic conditions. In this parallel study, MauG was subjected to 12 cycles of reduction by dithionite followed by oxidation by ferricyanide in the absence of O₂. At the end of this experiment MauG retained 100% of its activity as shown in Figure 3A and 3B confirming that the observed loss of activity in Figure 3A was an O₂-dependent phenomenon.

While the above results confirmed that inactivation was O₂-dependent during the cycling experiment with dithionite, inactivation occurred more rapidly during cycling with H₂O₂. That the latter reactions were performed anaerobically raises the question of whether O₂ has any effect on the H₂O₂-induced inactivation. To examine this possibility, the cycling reaction of H₂O₂ with diferric MauG was also performed under aerobic conditions. The results which were obtained were essentially identical to those obtained under anaerobic conditions (Figure 3).

Assessment of the effects of redox cycling of MauG by SDS-PAGE

For each of the cycling experiments, aliquots were removed after reaction cycles and analyzed by SDS-PAGE. SDS-PAGE of MauG which had undergone redox cycling with dithionite plus O₂ revealed no evidence of significant degradation or change in migration of the protein (Figure

4A). In contrast, SDS-PAGE of MauG at different points during the H₂O₂ cycling revealed that repeated reaction of H₂O₂ to diferric MauG addition caused some accumulation of covalent higher molecular weight MauG aggregates (Figure 4B). The primary species that was formed exhibited a mass on SDS-PAGE of approximately twice the mass of MauG (Figure 4C).

Protective effect of the PreMADH substrate

When each of the redox cycling experiments described above was repeated in the presence of PreMADH, no change in MauG was observed after 12 reaction cycles. The absorption spectrum, heme content and enzymatic activity were unchanged and there was no evidence of formation of higher molecular weight crosslinked MauG species (data not shown). In this experiment, 3.5 μ M MauG was incubated with 8 μ M PreMADH. MauG requires three reaction cycles per TTQ synthesized. Since each PreMADH contains two TTQ sites, 7 μ M PreMADH will be consumed after 12 cycles. It is noteworthy that the presence of even this slight excess of PreMADH is sufficient to afford complete protection against inactivation. This protective effect of substrate against inactivation of MauG by [O] is also consistent with the knowledge that MauG is able to catalyze multiple turnovers during the steady-state TTQ biosynthesis reactions using either source of [O] with no apparent time-dependent loss of activity (5).

Protective effect of Hydroxyurea on MauG inactivation

It was previously shown that the radical scavenger, hydroxyurea, either quenched or blocked formation of the PreMADH-based radical which is formed on addition of PreMADH to *bis*-Fe(IV) MauG (13). As such, the effect of hydroxyurea on the H₂O₂-induced inactivation of MauG was examined. When the H₂O₂ cycling experiments were repeated in the presence 100 μ M hydroxyurea, protection against inactivation was observed, as judged by the onset and extent of inactivation of heme and loss of enzyme activity (Figure 5). However, it did not protect as well as PreMADH did at much lower concentration (8 μ M). These experiments were not performed with higher concentrations of hydroxyurea because at higher concentrations hydroxyurea-dependent damage to MauG was observed under these incubation conditions (data not shown). Nevertheless these data indicate that hydroxyurea does attenuate H₂O₂-induced damage to MauG, consistent with the inactivation being a free radical-mediated process.

Kinetic analysis of the reaction of small molecule substrate analogs of PreMADH with *bis*-Fe(IV) MauG

Given the ability of the PreMADH substrate to protect MauG against suicide inactivation at relatively low concentration, it was of interest to determine if small molecules with similar structural features could do the same. PreMADH contains monohydroxylated β Trp57 and no crosslink to β Trp108. As a first step towards identifying small molecules that might protect against inactivation, several commercially available tryptophan and indole derivatives which are monohydroxylated on the indole ring were screened for reactivity with MauG. Unfortunately many of these compounds were not sufficiently soluble to include in the study, but interesting results were obtained with those that were. As shown in Table 1, 5-hydroxyindole, 6-hydroxyindole, and 5-hydroxy-L-tryptophan each readily reacted with *bis*-Fe(IV) MauG to yield diferric MauG as does PreMADH. The limiting first-order rate constant for the reaction of the tryptophan derivative was similar to that with PreMADH, and those for the indole derivatives were greater than that with PreMADH. The K_d values for these compounds, however, were greater than three orders larger than the K_d value for the natural protein substrate. The reaction with 7-hydroxyindole, which is structurally the most similar to PreMADH with respect to the position of the hydroxyl group, could not be studied because of its poor solubility.

Protective effect of 6-hydroxyindole on MauG inactivation

Given the demonstrated ability of 6-hydroxyindole to react with *bis*-Fe(IV) MauG, the effect of 6-hydroxyindole on the H₂O₂-induced inactivation of MauG was examined. When the H₂O₂ cycling experiments were repeated in the presence 500 μ M 6-hydroxyindole protection against inactivation was observed, similar to that seen with PreMADH (Figure 5). While 500 μ M is well below the K_d value for 6-hydroxyindole (Table 1), at this concentration the observed rate of the reaction with the *bis*-Fe(IV) MauG of 0.75 s⁻¹ is similar to the rate of reaction with PreMADH at the concentrations used in this study (data not shown). These results indicate that monohydroxylated tryptophan and indole derivatives do react with *bis*-Fe(IV) MauG and protect MauG against H₂O₂-induced damage, however at concentrations much greater than PreMADH.

Discussion

MauG requires three two-electron oxidizing equivalents in the form of reductant plus O₂ or H₂O₂ to catalyze the six-electron oxidation of PreMADH to produce oxidized MADH with the mature TTQ cofactor. It was previously shown that the order of addition of the oxidizing equivalent and substrate to MauG is random in the kinetic mechanism (14). The reactivity towards [O] is neither stimulated nor impeded when MauG binds PreMADH. These results demonstrate that while O₂ and H₂O₂ each readily react with MauG in the absence of PreMADH, the presence of this substrate is necessary to prevent suicide inactivation of MauG after reaction with [O]. It is appropriate to term this suicide inactivation since it requires the reaction of [O] with heme rather than direct modification by [O] of amino acid residues of the protein.

Several heme-dependent enzymes such as cytochrome P450 (19), cytochrome *c* (20), horseradish peroxidase (21), prostaglandin H synthase (22), lignin peroxidase (23), manganese peroxidase (24), and chloroperoxidase (25) have been shown to undergo inactivation on reaction with H₂O₂ without reducing substrates present. These studies have not typically examined the consequences of the reaction of the ferrous heme with O₂ in the absence of substrate. Furthermore, these studies have typically examined the consequences of addition of excess H₂O₂ and have not monitored the effects of sequential stoichiometric additions of [O] to heme proteins, as has been done in this study of MauG.

For heme and nonheme iron enzymes it is generally believed that the O₂-dependent and H₂O₂-dependent oxygenation mechanisms each proceed via a ferric hydroperoxy intermediate (15,26) which may then lose water to yield a high-valent Fe(IV)=O species. Ferryl heme species with a π -porphyrin ring/axial ligand or amino acid radical (known as Compound I) have been observed (15,27). In the absence of substrate, or when exposed to high concentrations of H₂O₂, heme-dependent enzymes undergo suicide inactivation, in which H₂O₂ is the suicide substrate that converts Compound I into a highly reactive Fe(III)-OOH porphyrin free-radical called Compound III (28). Compound III may then react with itself resulting in cleavage of the porphyrin ring or catalyze the oxidation of an amino acid residue of the surrounding protein (29-31).

The mechanism of suicide inactivation of MauG by [O] is not necessarily analogous to that of other heme proteins. The O₂-dependent and H₂O₂-dependent oxygenation mechanisms of MauG are also believed to proceed via a ferric hydroperoxy intermediate (5). However, the reactive high-valent intermediate is not Compound I but an unprecedented *bis*-Fe(IV) species that is an electronic equivalent of Compound I, with the second oxidizing equivalent stored at the second heme iron rather than as a porphyrin or amino acid-based radical (13). In the absence of PreMADH, the *bis*-Fe(IV) species generated by H₂O₂ slowly decays to diferric MauG, as judged by EPR spectroscopy (13) but with an absorption spectrum that exhibits a change in the appearance of the Soret band in the region from 350-400 nm relative to that of the original

diferrous MauG (Figure 1B, compare black and blue spectra). This feature has been observed in other heme proteins and attributed to a change in coordination of the ferric high-spin heme from five- to six-coordinate after reaction with H_2O_2 and return to the ferric (32,33), or to ferric heme in the presence of an amino acid radical (34). This suggests that ferric high-spin heme of MauG might still bind oxygen maintaining a six-coordination.

With MauG it was clearly shown that loss of enzymatic activity can occur without concomitant heme inactivation, and that loss of activity precedes heme inactivation during both O_2 and H_2O_2 -dependent suicide inactivation. This suggests that the initial loss of enzymatic activity involves oxidative damage to a critical amino acid residue rather than to heme, and that the hemes retain their redox reactivity after the initial loss of enzymatic activity. There are at least two possible scenarios by which this could happen. The EPR spectrum of the *bis*-Fe(IV) form of MauG includes a minor signal attributed to an amino acid-based radical (13). This signal was proposed to arise from a residue which mediated electron/radical transfer between the hemes (13). Furthermore, following the addition of PreMADH to *bis*-Fe(IV) MauG, EPR analysis revealed that when the two hemes returned to the ferric state a new PreMADH-based radical signal was observed (13). This new radical species was very stable and exhibited an EPR spectrum similar to that of an aromatic protein-based radical (*i.e.*, Trp or Tyr). Thus, both formation of the *bis*-Fe(IV) state and its reaction with PreMADH appear to require long range electron/radical transfer. It is conceivable that in the absence of PreMADH, defective electron transfer or radical transfer may result in non-specific oxidative damage of amino acid residues of MauG after reaction with [O]. These could be residues normally involved in a specific electron transfer pathway or susceptible amino acids which are oxidized after propagation of the long-lived radical through the protein in the absence of PreMADH. After the protein damage that results in the loss of enzymatic reactivity, subsequent reactions of [O] with heme still occur and ultimately lead to inactivation of heme.

For MauG in the absence of PreMADH, differences in the nature of the inactivation process are observed depending on the source of [O]. Loss of activity occurs much more quickly (*i.e.*, fewer reaction cycles) with H_2O_2 than with O_2 . Inactivation of heme was not observed with H_2O_2 until after total loss of enzymatic activity (Figure 3). Another difference is that some covalent aggregation, primarily dimerization, was observed only during H_2O_2 -driven inactivation. It should be noted that there is no evidence for dimerization of unmodified MauG in solution. It was previously shown that MauG elutes as a monomer when subjected to high-resolution size-exclusion chromatography (35). Some MauG aggregation was observed even after the first reaction cycle with H_2O_2 and the extent increased with subsequent reaction cycles. The extent of protein aggregation, however, did not show any direct correlation with either extent of loss of activity or heme inactivation. While the mechanism by which this occurs is not known, cross-linking of MauG molecules in the absence of PreMADH would be consistent with radical propagation to susceptible surface residues which then could crosslink the protein molecules via radical coupling. While the process of inactivation of MauG appears to differ depending on the source of [O], in neither case is inactivation observed when PreMADH is present and able to react with the high-valent MauG species. Hydroxyurea and monohydroxylated tryptophan and indole derivatives are also able to react with the *bis*-Fe(IV) MauG reaction intermediate and afford protection against suicide inactivation. Considering the much higher K_d values for the interactions of these small molecules with MauG relative to that of PreMADH (Table 1), it is unlikely that their ability to protect against inactivation is of any physiological relevance. However, the protection by the monohydroxylated tryptophan and indole derivatives is consistent with the view that it is the chemical reaction with substrate, rather than substrate binding or some other factor that affords the protection against inactivation. The protection by hydroxyurea is consistent with possibility of a free radical mediated mechanism of inactivation.

In contrast to MauG, heme-dependent monooxygenases are typically not reactive toward oxygen in the absence of substrate. In those enzymes, binding of substrate triggers a conformational change that allows the high-spin heme to bind and activate oxygen (16). The reactivity of MauG towards H₂O₂, O₂ and CO is neither stimulated nor impeded by prior complex formation with PreMADH (1,14). The random-binding kinetic mechanism exhibited by MauG may be necessitated by its unusual natural substrate, specific amino acid side-chains within a 119 kDa tetrameric precursor protein, and the fact that it catalyzes multiple reactions on this substrate. MauG is an inducible enzyme which is only synthesized when the host bacterium is provided with methylamine as a sole source of carbon (9). It is encoded by *mauG* which is a part of the methylamine utilization (*mau*) gene cluster that includes the structural genes for MADH (9). When the induction of these genes is no longer required PreMADH will no longer be available for MauG. Suicide inactivation of MauG in the absence of PreMADH provides a mechanism to remove MauG when that substrate is no longer available, so that MauG will be unable to generate reactive oxygen and radical species which could otherwise damage cell components in the absence of PreMADH. Thus, there may be some physiological relevance to the fact that PreMADH prevents suicide inactivation of MauG but is not required to induce an O₂-binding conformation as in other heme-dependent oxygenases.

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1 Abbreviations

MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; PreMADH, the biosynthetic precursor protein of MADH with incompletely synthesized TTQ; CCP, cytochrome *c* peroxidase; [O], oxidizing equivalents.

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Scheme 1.

Figure 1.

Changes in the absorption spectrum of MauG on reaction with O_2 or H_2O_2 in the absence of PreMADH. A. Spectra were recorded of diferric MauG (black line), diferrous MauG formed by reaction with sodium dithionite (red line), and diferric MauG after reoxidation by air (blue line). B. Spectra were recorded of diferric MauG (black line), *bis*-Fe(IV) MauG formed by reaction with H_2O_2 (red line), and spontaneous return to diferric MauG (blue line).

Figure 2. Changes in the absorption spectrum of MauG after redox cycling in the absence of PreMADH. A. Spectra of diferric MauG before (black) and after (red) 12 cycles of reduction by stoichiometric dithionite followed by reoxidation by O₂. B. Spectra of diferric MauG before (black) and after (red) 12 cycles of reaction with stoichiometric H₂O₂ followed by spontaneous decay to the diferric state.

Figure 3.

Changes in reactive heme concentration and enzymatic activity of MauG after redox cycling in the absence of PreMADH. Four sets of experiments are shown in which: MauG was cycled between reduction by dithionite followed by oxidation by ferricyanide under anaerobic conditions (black, X); MauG was cycled between reduction by dithionite followed by reoxidation by O₂ (blue, □); MauG was cycled between reaction with stoichiometric H₂O₂ followed by spontaneous decay to the diferric state under anaerobic conditions (red, ○); and MauG was cycled between reaction with stoichiometric H₂O₂ followed by spontaneous decay to the diferric state under aerobic conditions (green, ●). All data are normalized as % relative to the starting sample of diferric MauG. A. The heme concentration of MauG was determined

by the pyridine hemochrome assay. B. The catalytic activity of MauG was determined by the spectrophotometric TTQ biosynthesis assay.

Figure 4.

Analysis of MauG by SDS-PAGE after redox cycling in the absence of PreMADH A. MauG samples were removed during cycling between reduction by dithionite followed by reoxidation by O_2 and were subjected to SDS-PAGE using 12.5% gel. B. MauG samples were removed during cycling between reactions with stoichiometric H_2O_2 followed by spontaneous decay to the diferric state and were subjected to SDS PAGE using 12.5% gel. C. MauG samples from (B) were subjected to SDS PAGE using a 4%-20% gradient gel for more accurate estimation of molecular mass of MauG aggregates. Molecular weights corresponding to each band of Markers are shown in left side of each gel. Lane labels are M for molecular weight markers,

C for the unreacted MauG control, and numbers indicating how many reaction cycles the sample experienced.

Figure 5.

The effects of 6-hydroxyindole and hydroxyurea on changes in reactive heme concentration and enzymatic activity of MauG after redox cycling with H_2O_2 in the absence of PreMADH. Three sets of experiments are shown in which MauG was cycled between reaction with stoichiometric H_2O_2 followed by spontaneous decay in the presence of 500 μ M 6-hydroxyindole (black, Δ), in the presence of 100 μ M hydroxyurea (blue, $\Diamond$), and without any other additions (red, $\circ$). A. The heme concentration of MauG was determined by the pyridine hemochrome assay. B. The catalytic activity of MauG was determined by the spectrophotometric TTQ biosynthesis assay.

Table 1Kinetic parameters for the reactions of PreMADH and small molecules with *bis*-Fe(IV) MauG^a

	K_d (μM)	k_3 (s^{-1})
PreMADH ^b	1.3 $\pm$ 0.2	0.80 $\pm$ 0.02
6-hydroxyindole	2470 $\pm$ 280	4.87 $\pm$ 0.20
5-hydroxyindole	3986 $\pm$ 1143	2.66 $\pm$ 0.33
5-hydroxy-L-Tryptophan	6801 $\pm$ 1656	0.39 $\pm$ 0.05

^aThe kinetic values were determined from the analysis of the variation in k_{obs} with substrate concentration using Eqs. 1 and 2, as described under Experimental Procedures.

^bTaken from ref. (14).