Published in final edited form as:

Biochemistry. 2016 October 11; 55(40): 5738–5745. doi:10.1021/acs.biochem.6b00816.

A suicide mutation affecting proton transfers to high-valent hemes causes inactivation of MauG during catalysis

Zhongxin Ma, Heather R. Williamson[#], and Victor L. Davidson^{*}

Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL 32827

Abstract

In the absence of its substrate, the auto-reduction of the high-valent *bis*-Fe^{IV} state of the hemes of MauG to the diferric state proceeds via a Compound I-like and then a Compound II-like intermediate. This process is coupled to oxidative damage to specific methionine residues and inactivation MauG. The auto-reduction of a P107V MauG variant, which is more prone to oxidative damage, proceeds directly from the *bis*-Fe^{IV} to the compound II-like state with no detectable Compound I intermediate. Comparison of the crystal structures of native and P107V MauG reveal that this mutation alters the positions of amino acid residues in the heme site as well as the water network that delivers protons from solvent to the hemes during their reduction. Kinetic, spectroscopic and solvent kinetic isotope effect studies demonstrate that these changes in the heme site affect the protonation state of the ferryl heme and the relative efficiencies of two alternative pathways for proton transfer from solvent to the hemes. These changes enhance the rate of auto-reduction of P107V MauG such that it competes with the catalytic reaction with substrate and causes the enzyme to inactivate itself during the steady-state reaction with H₂O₂ and its substrate. Thus, while this mutation has negligible effects on the initial steady-state kinetic parameters of MauG, it is a fatal mutation as it causes inactivation during catalysis.

TOC image

^{*}Corresponding authors: Victor L. Davidson, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd., Orlando, FL 32827 Tel: 407-266-7111, Fax: 407-266-7002, victor.davidson@ucf.edu.

[#]Present Address: Department of Chemistry, Xavier University of Louisiana, 1 Drexel Dr., New Orleans, LA, 70125

ASSOCIATED CONTENT

None

Author Contributions

The manuscript was written through contributions of all authors. All authors have approved the final version of the manuscript.

The diheme enzyme MauG from *Paracoccus denitrificans*¹ stabilizes a unique high-valent redox state² in which the two hemes are each present as Fe^{IV}. The hemes share charge via an intervening tryptophan residue by a mechanism of charge-resonance (CR) stabilization³. The predominant species in this ensemble of resonance structures is termed *bis*-Fe^{IV}, in which one Fe^{IV} heme has a single axial His ligand and the other is six-coordinate with axial ligands provided by His and Tyr residues^{3–5}. The physiological reaction that requires this high-valent state is the posttranslational modification of its substrate, a precursor protein of methylamine dehydrogenase (preMADH)⁶, to generate the protein-derived tryptophan tryptophylquinone (TTQ) cofactor⁷. The overall reaction is a six-electron oxidation and catalysis requires long-range electron transfer (ET) over several angstroms from the residues of preMADH that are post-translationally modified to the high-valent hemes of MauG^{5, 8, 9}.

In the absence of the preMADH substrate, the *bis*-Fe^{IV} state undergoes auto-reduction to the diferric state which results in inactivation of MauG due to oxidative damage to specific methionine residues^{10, 11}. These Met residues are the source of electrons which reduce the Fe^{IV} hemes via two sequential long-range ET reactions¹². The mechanism by which this process occurs was previously characterized by transient kinetic, spectroscopic and kinetic solvent isotope effect (KSIE) studies^{12, 13} (Figure 1). A priming step precedes the first ET and is required to activate the diheme system for that first ET. That step is a proton transfer (PT) from the solvent to the ferryl heme which converts the CR-stabilized *bis*-Fe^{IV} state to a CR-stabilized Compound I-like state¹³. The first ET is then coupled to a second PT to the hemes from solvent and yields a CR-stabilized Compound II-like state. The subsequent second ET into the diheme system, which yields the diferric state and release of water, is coupled to another PT which occurs at the Met site during the oxidation of the Met to a sulfoxide. This latter reaction does not involve the water network in the heme site.

The results of proton inventory studies of the reactions involving PT from solvent to the hemes describe a model for two alternative pathways for PT, each involving multiple protons. In both the first and second PTs from solvent to the hemes, the experimentally-determined fractionation factors were consistent with one of the two alternative pathways involving proton tunneling and the other not involving proton tunneling. From the crystal structure of MauG⁵ it was possible to identify these two PT pathways within an ordered water network in the distal pocket of the ferryl heme (Figure 2A). It was proposed that these networks of amino acid side chains and structured waters are used to coordinate the multiple proton and electron transfers that are responsible for the CR stabilization of the high-valent state and control of its reactivity^{12, 13}. The pathway including waters W0–W2 was assigned as the pathway for proton tunneling and the pathway including waters W4–W6 was assigned as the non-tunneling pathway.

The importance of amino acid residues in the heme pocket of MauG was demonstrated by site-directed mutagenesis studies^{14–16}. One of these residues is Pro107¹⁵. It was shown that the kinetic parameters for the reaction of P107V MauG with the preMADH and H₂O₂ substrates were similar to those of wild-type (WT) MauG. However, P107V was more prone to oxidative damage. Inspection of the crystal structure of P107V MauG¹⁵ shows that as a consequence of this mutation the water networks linking the ferryl heme to bulk solvent are perturbed (Figure 2B). As such, it was of interest to investigate the mechanism of the auto-

reduction of the *bis*-Fe^{IV} state of P107V MauG. The results presented in this study demonstrate that subtle changes in the position of residues in the ferryl heme pocket alter the two water networks. This results in dramatic changes in the mechanism and rate of the auto-reduction of the high-valent hemes, and concomitant oxidative damage. Furthermore, whereas WT MauG is protected from self-inactivation during the reaction with its natural substrate, P107V MauG lost activity during the steady-state assay and exhibited oxidative damage. This is because the P107V mutation allows the auto-reduction of the high-valent hemes by the Met residue to compete with the catalytic reaction with substrate. Thus, while this mutation has negligible effects on the initial steady-state kinetic parameters of MauG, it is a fatal mutation as it causes inactivation during catalysis.

EXPERIMENTAL PROCEDURES

Materials

Homologous expression of WT MauG¹ and P107V MauG¹⁵ in *P. denitrificans*, and the methods for the isolation and purification of the proteins were as have been described previously. Expression of preMADH in *Rhodobacter sphaeroides* and its purification were as described previously^{6, 17}. D2O (99.8%) was obtained from Acros Organics.

Spectroscopic and kinetic studies of the auto-reduction of *bis*-Fe^{IV} MauG

Studies were performed in 10 mM potassium phosphate, at pH 9.0, at the indicated temperatures. The studies were performed at pH 9.0 because it was previously shown that the accumulation of reaction intermediates during the reaction with WT MauG were maximal under these conditions¹². Formation of *bis*-Fe^{IV} MauG was achieved by addition of stoichiometric H₂O₂ to the diferric protein 2. The reactions were monitored spectroscopically using a HP8452A diode array spectrophotometer run by the OLIS SpectralWorks/GlobalWorks software. Complete spectra were collected at each time point and the data were globally fit to include the changes in absorbance at each wavelength with time in order to determine rate constants for the reaction steps. Data reduction by factor analysis using the singular-value decomposition algorithm was performed with the fitting routines of OLIS software.

Steady-state kinetic analysis of the reactions with preMADH

The steady-state kinetic activity of MauG-dependent TTQ biosynthesis from preMADH and H₂O₂ was assayed spectrophotometrically as previously described¹⁵. Product formation was monitored by the increase in absorbance centered at 440 nm, which is characteristic of the fully oxidized TTQ. PreMADH lacks absorbance in this area. Studies were performed in 10 mM potassium phosphate, pH 7.5, at 25 °C with 0.5 μM WT or P107V MauG and saturating concentrations of substrates of 5 μM preMADH and 100 μM H₂O₂. In this study, the reaction was initiated by the immediate addition of the H₂O₂ substrate and allowed to go to completion. Then a subsequent reaction was initiated by the addition of another 5 μM preMADH and 100 μM H₂O₂ to the same reaction mixture and this was further monitored.

Kinetic solvent isotope effect studies

For experiments performed in buffered D2O, the pL of the buffer was determined using Eq 1, where x is the mole fraction of D2O¹⁸. In proton inventory experiments, the spectroscopic and kinetic analyses were performed in buffers containing different mole fractions of deuterium in H2O-D2O mixtures^{13, 19–21}. In these studies the dependence of rate (k_x) on mole fraction of D2O (x) is described by Eq 2 where k_0 is the rate constant at $x = 0$, and ϕ_i^{TS} is the isotopic fractionation factor for the transition state for m PTs. Eq 2 reduces to Eqs 3, 4, or 5 for cases in which one, two, or multiple protons, respectively, are transferred in the transition state. If the proton inventory plot of the data exhibited extreme curvature as was previously the case for WT MauG^{12, 13}, it was fit by Eq 6¹³, which describes a model with two alternative pathways with each involving multiple PTs, where f is the fraction of contribution from first pathway where m PTs occur, and $(1-f)$ is the fraction of contribution from the other pathway in which n PTs occur.

$$pL = pH_{obs} + 0.331x + 0.0766x^2 \quad (1)$$

$$k_x = k_0 \prod_{i=1}^m (1 - x + x\phi_i^{TS}) \quad (2)$$

$$k_x = k_0 (1 - x + x\phi^{TS}) \quad (3)$$

$$k_x = k_0 \left(1 - x + x\sqrt{\phi^{TS}}\right)^2 \quad (4)$$

$$k_x = k_0 (\phi^{TS})^x \quad (5)$$

$$k_x = k_0 \left\{ f \prod_{i=1}^m (1 - x + x\phi_i^{TS_1}) + (1 - f) \prod_{j=1}^n (1 - x + x\phi_j^{TS_2}) \right\} \quad (6)$$

Thermodynamic analysis

In order to determine the activation energy (E_a) of the reaction steps that are described by each rate constant, the dependence of each reaction rate constants on temperature was fit by eq 7.

$$\ln(k) = -\frac{E_a}{RT} + \ln(A) \quad (7)$$

RESULTS

Spectroscopic and kinetic analysis of the effects of the P107V mutation on the auto-reduction of the high-valent hemes of MauG

The high-valent states of WT MauG and P107V MauG were generated by the addition of H₂O₂ and the changes in the resultant visible spectrum with time were recorded (Figure 3). The initial spectra of the high-valent states of WT MauG and P107V MauG (red spectra) are very similar as each initially forms the CR-stabilized *bis*-Fe^{IV} state. The final spectra which describe the diferric state of each (purple spectra) are also very similar. However, whereas the conversion of the CR-stabilized *bis*-Fe^{IV} state to the diferric state in WT MauG proceeds via two clearly detectable spectroscopic intermediates (green and blue spectra), only one intermediate (blue spectrum) is observed with P107V MauG. These species in WT MauG were previously ascribed to be first a CR-stabilized Compound I-like state (green spectrum) and then a CR-stabilized Compound II-like state (blue spectrum)¹². The appearance of the Compound I-like species is evidenced by a shift in the Soret peak maximum with a decrease in intensity and appearance of an absorbance feature centered at 650 nm. The Compound II-like species lacks the absorbance feature at 650 nm. It also exhibits a decrease in intensity of the α band at 559 nm and β band at 526 nm, and an increase in absorbance around 498 nm. This spectrum is consistent with spectra that have been reported for Compound II^{12, 22–25}. In P107V MauG, the Compound I-like intermediate is absent and only the Compound II-like intermediate is observed.

The kinetic plots, which depict the global fits of the overall spectral changes, with time illustrate the rates of formation and decay of each intermediate are shown in Figure 4 and the results of the kinetic and KSIE studies are shown in Table 1. For the reaction of WT MauG, the data are best fit by a three-exponential process with rate constants $k_1 = (2.5 \pm 0.3) \times 10^{-2} \text{ s}^{-1}$, $k_2 = (1.19 \pm 0.03) \times 10^{-2} \text{ s}^{-1}$ and $k_3 = (8.8 \pm 0.7) \times 10^{-4} \text{ s}^{-1}$. These rate constants correspond to the conversion of the formation of the CR-stabilized Compound I-like state via PT (k_1), the formation of the CR-stabilized Compound II-like state via proton-coupled ET (PCET) (k_2), and the formation of the diferric state via another PCET (k_3) (see Figure 1). For P107V MauG, the data are best fit by a two-exponential process with rate constants of $(1.41 \pm 0.03) \times 10^{-2} \text{ s}^{-1}$ and $(1.24 \pm 0.05) \times 10^{-3} \text{ s}^{-1}$. This is because the initial CR-stabilized *bis*-Fe^{IV} state is directly converted to the CR-stabilized Compound II-like state. As such, the rate constant that describes the direct formation of that state will also be referred to as k_2 (see Figure 1). The slower rate constant corresponds to k_3 for WT MauG and also describes the reduction of the CR-stabilized Compound II-like state to the diferric state. This reaction, which does not involve PT from the water network in the heme site, is unaffected by the P107V mutation. It is significant that the initial ET to the CR-stabilized *bis*-Fe^{IV} state in P107V MauG occurs immediately after formation of the high-valent species. The lag time for ET observed in auto-reduction of WT MauG is absent since it is not required to first form the CR-stabilized Compound I-like state. Thus, the ET reaction from Met begins immediately, and the

formation of the Compound-II-like intermediate (blue in Figure 4) is completed faster in P107V MauG than in WT MauG. This reaction at the heme site is accompanied by the one-electron oxidation of Met to generate a Met radical. As such, the process of oxidative damage is initiated more quickly in P107V MauG than in WT MauG.

Kinetic solvent isotope effects studies

Given the previous results with WT MauG that k_1 , k_2 , and k_3 each exhibited a KSIE, the conversion of the CR-stabilized *bis*-Fe^{IV} state to the diferric state in P107V MauG was examined in buffered D₂O. A KSIE for P107V MauG was obtained for k_2 of 1.8 ± 0.1 and for k_3 of 1.5 ± 0.1 . Each of these values is smaller than the corresponding KSIE values that were obtained for WT MauG of 2.6 ± 0.5 and 1.8 ± 0.4 , respectively (Table 1). In order to further explore the basis for the differences in the KSIE values that were caused by the P107V mutation, proton inventory studies were performed (Figure 5). For WT MauG the plot of a proton inventory of k_2 exhibited hyper-curvature, whereas the plot of k_3 did not¹². Analogous studies were performed with P107V MauG. The plot for the rate constant k_2 that describes the formation of the CR-stabilized Compound II-like state in P107V MauG also exhibited hyper-curvature, but not as extreme as that for the k_2 for WT MauG. A fit of the data to Eq 6 revealed that k_2 does indeed describe a reaction in which two alternative pathways involving multiple PTs is operative in the transition state. The basis for the difference in the curvatures of the plots for WT MauG and P107V MauG may be inferred from the fitted parameters obtained from the analysis by Eq 6. The values of the fractionation factors for the two pathways in P107V MauG were similar to those for WT MauG. However, the contribution to the reaction from the tunneling pathway in P107V MauG was much smaller. This is what accounts for the lesser degree of curvature of the proton inventory plot. The fit of the data by Eq 6 indicates that one pathway contributes $3 \pm 9\%$ with $\phi^{\text{TS1}} < 0.01$ and the other pathway contributes $97 \pm 9\%$ with $\phi^{\text{TS2}} = 0.57 \pm 0.07$. For the WT MauG, one pathway contributes $27 \pm 11\%$ with $\phi^{\text{TS1}} < 0.01$ and the other pathway contributes $73 \pm 11\%$ with $\phi^{\text{TS2}} = 0.52 \pm 0.10$. The fractionation factors with values < 0.01 are consistent with proton tunneling. Thus, an effect of the P107V mutation was to decrease the contribution of the tunneling pathway to this PCET reaction step. The proton inventory plots for k_3 in both WT and P107V MauG which describe the conversion of the CR-stabilized Compound II-like state to the diferric state did not exhibit hyper-curvature. This reaction occurs concomitantly with the oxidation of Met and is consistent with the PT occurring at the Met site with no involvement of the water network in the heme site.

Thermodynamic analysis

In order to obtain additional evidence to support the conclusion that k_2 and k_3 in P107V MauG each describe the same reaction step in WT MauG, the temperature dependence of each rate constant for each protein was examined under the same reaction conditions. The reactions were monitored at temperatures ranging from 15–30 °C and k_2 and k_3 were determined at each temperature. These data sets were fit by Eq 7 to determine the activation energy (E_a) for each reaction (Figure 6). For P107V MauG, values of E_a for k_2 and k_3 were 17.8 ± 0.4 and 6.3 ± 0.1 , respectively. These values are essentially the same as were obtained for WT MauG of 17.5 ± 0.5 and 6.5 ± 0.1 , respectively. These findings are consistent with k_2 and k_3 each describing the same reaction steps in WT and P107V MauG.

Effect of the P107V mutation on the steady-state reaction of MauG with preMADH

It was previously shown that analysis of the initial rates of the steady-state kinetic reactions with WT MauG and P107 MauG yielded similar k_{cat} values of $0.16 \pm 0.02 \text{ s}^{-1}$ and $0.10 \pm 0.01 \text{ s}^{-1}$, respectively¹⁵. However, when the steady-state reaction is allowed to proceed to completion distinct differences are seen (Figure 7). The rate of product formation by P107V MauG slows and levels off much earlier than for WT MauG, and the total amount of product formed by P107V MauG after the reaction is less than for WT MauG. This suggests that P107V MauG has inactivated prior to the depletion of substrate. To test this hypothesis, identical amounts of preMADH and H₂O₂ were added to each reaction mixture at this point and the steady-state reaction was monitored further. For WT MauG, the initial reaction rate was similar to that observed in the initial steady-state reaction and product formation continued. In contrast, addition of more substrates to P107V MauG led to no further product formation, confirming that the enzyme had been inactivated during the initial steady-state reaction.

DISCUSSION

A notable difference in the conversion of the CR-stabilized *bis*-Fe^{IV} state to the diferric state in P107V MauG is the absence of the CR-stabilized Compound I intermediate. In WT MauG it is formed by PT from the water network to the ferryl heme. The lack of this intermediate in P107V MauG can be explained either by a change in the relative rates of the first two steps that are observed in WT MauG (k_1 and k_2) or a change in mechanism of reduction of the diheme system. A kinetic effect is unlikely. For P107V MauG, k_2 is faster than the combined k_1 and k_2 for WT MauG. Thus, the mutation could not have caused either k_1 (if truly present in P107V MauG) or k_2 to become slower. If it caused k_1 to become faster in P107V MauG, then the accumulation of the Compound I-like intermediate would have been greater rather than absent. If it caused k_2 to become faster, the overall process would still be rate-limited by k_1 and one would not expect the E_a values for the “observed” k_2 of P107V to be identical to those for the true k_2 of WT MauG.

A possible change in mechanism to account for the fact that the CR-stabilized Compound I-like state is not observed in P107V MauG is as follows. The CR-stabilized *bis*-Fe^{IV} species in P107V MauG is modified by changes in the environment of the ferryl heme such that the initial ET from Met does not require an initial protonation of the ferryl heme to activate the system for ET. A possible explanation for this may be inferred from comparing the structures of WT and P107 MauG (see Figure 2B). Residue Gln103 has been implicated in the stabilization of the *bis*-Fe^{IV} state by donating a hydrogen bond to the ferryl-oxo^{16, 26}. This was strongly supported by quantum chemical computational modeling based on the parameters obtained from the Mössbauer spectrum of the *bis*-Fe^{IV} state of MauG, and the crystal structure. It was noted that the quadrupole splitting value for the ferryl heme of MauG was between reported values for Fe^{IV}=O and Fe^{IV}-OH hemes²⁶. A consequence of the P107 mutation is that Gln103 moves approximately 0.7 Å closer to the Fe^{IV}=O¹⁰. This is a consequence of a shift in position of an α -helix containing residues 100–110. Furthermore, the amide backbone of Val107 forms a hydrogen bond with the carbonyl backbone of the Gln103, also shifting the residue towards the ferryl heme iron¹⁵. This closer proximity

results in a stronger hydrogen bond than present in WT MauG. This could conceivably activate the CR-stabilized *bis*-Fe^{IV} state by allowing it to achieve a lower energy state comparable to the Compound I-like intermediate in WT MauG, without requiring the formal donation of a proton to the ferryl heme. It should be noted that the conversion of the CR-stabilized *bis*-Fe^{IV} state to the CR-stabilized Compound II-like state requires addition of two protons and one electron. This is a two-step process in WT MauG and a one-step process in P107V MauG (see Figure 1). The initial PT step in P107V MauG is not kinetically distinguishable from the first ET. Once the initial PCET step (k_2) occurs in P107V MauG, the donation of a proton to the heme-bound oxygen would weaken the hydrogen bond to Gln103 allowing the space for the second proton to rapidly add to generate the diprotonated Compound II-like state that was reported for WT MauG¹².

Another notable effect of the P107V mutation is the perturbation of the water network in the heme site that changes the relative contributions of the two alternative PT pathways that deliver protons to the ferryl heme. These changes may be attributed to small shifts in the positions of residues Gln106 and Glu113. In WT MauG Gln106 is hydrogen bonded to W2, the entry point for the tunneling pathway. The shift in position of this residue allows an additional water to be present in the tunneling pathway between W2 and the carbonyl oxygen of Gln 106. As the tunneling mechanism of PT through this pathway in WT MauG is optimized for three waters, the presence of the additional water may decrease the efficiency of the proton tunneling pathway in P107V MauG. The fractionation factor for this pathway $\phi^{\text{TS1}} < 0.01$ is still consistent with tunneling, however the flux through the pathway is now minimal, 3% compared to 27% for WT MauG. Residue Glu113 was previously demonstrated to stabilize the *bis*-Fe^{IV} species in WT MauG¹⁴. The shift in position of Glu113, which is hydrogen bonded to Asn110 in WT MauG, also results in the presence of an additional water in the non-tunneling pathway between Gln113 and Asn110. That water can hydrogen bond to those two residues, as well as W4, W1 and W0. This is significant because in WT MauG direct PT from W4 to W0 is not possible. This pathway in WT MauG requires that the side-chain of Asn110 mediate PT from W4 to W1, with subsequent PT to W0. In P107V MauG the additional water replaces Asn110 as a PT mediator and likely functions as a more efficient PT mediator than the amide nitrogen of Asn110. The fraction factor this pathway of $\phi^{\text{TS2}} = 0.57 \pm 0.07$ is similar to that for the corresponding pathway in WT MauG consistent with a similar PT mechanism that does not involve proton tunneling. However, now the relative flux through this pathway increases from 73% to 97%. Therefore, it may be concluded that the shift in preference for the non-tunneling pathway for PT from solvent to the hemes in P107V MauG may be attributed to either greater efficiency of the non-tunneling pathway or decreased efficiency of the tunneling pathway, or both. Each of these possibilities is consistent with the results that were obtained. The observation that the KSIE for k_2 for P107V MauG is less (1.8) than that for WT MauG (2.6) is also consistent with a reduced contribution of proton tunneling to the reaction.

The physiological relevance of these findings is evidenced by the fact that P107V MauG inactivates itself during the steady-state reaction with preMADH and H₂O₂. WT MauG does not and is only inactivated when it reacts with H₂O₂ in the absence of preMADH. This may be explained by the changes in the kinetics of the auto-reduction of the high-valent hemes by Met, which are linked to oxidation of the Met to a sulfoxide. For WT MauG it was

previously shown that k_1 , which precedes the first ET from Met, is 0.025 s^{-1} ¹³ and the k_{cat} for the reaction of the CR-stabilized *bis*-Fe^{IV} state with preMADH is 0.16 s^{-1} ¹⁵. Thus the catalytic reaction is complete before any significant auto-reduction of the hemes and oxidation of Met can occur. For P107V MauG the k_{cat} for the reaction of the CR-stabilized *bis*-Fe^{IV} state with preMADH is 0.1 s^{-1} ¹⁵. Since there is no k_1 the ET from the Met to the hemes begins immediately with a k_2 of 0.014 s^{-1} . Thus the catalytic rate is only about seven-fold greater than the initial reaction of the inactivation process. The closeness of the rates means that the inactivation does compete with catalysis. Thus, over time during the steady-state reaction oxidative damage will accumulate and result in inactivation even when the substrate is present (see Figure 7).

Conclusion

The results of this study provide mechanistic information regarding protein control of structured water and long-range PT transfer, and its consequent effects on high-valent heme stability and reactivity. They also describe an unusual effect of a point mutation. Typically, a detrimental mutation will affect an enzyme by decreasing k_{cat} , increasing K_m for a substrate or causing the protein to mis-fold and thus become inactive or rapidly degraded. The P107V mutation of MauG has little effect on the steady-state kinetic parameters for the physiological reaction of MauG with preMADH and H₂O₂ which are determined from the initial rate of reaction before significant self-inactivation occurs. The protein is also properly assembled and quite stable in the absence of an oxidant that generates the high-valent diheme state. However, the P107V mutation results in complete inactivation of MauG during catalysis because it enhances the rate of a detrimental side-reaction that causes oxidative damage. This may be a more widely occurring phenomenon than is realized, particularly for oxidoreductases which must stabilize reactive intermediates at least transiently, and control the reactivity of reactive oxygen species and radical intermediates during catalysis. This type of effect should be considered when a naturally occurring or engineered mutation alters the phenotype of an organism without exhibiting an obvious effect on the activity of the variant protein.

Acknowledgments

The authors thank Yu Tang for technical assistance.

Funding Sources

This research was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R37GM41574 (V.L.D.).

ABBREVIATIONS

CR	charge resonance
ET	electron transfer
KSIE	kinetic solvent isotope effect
preMADH	the biosynthetic precursor protein of methylamine dehydrogenase with incompletely synthesized TTQ

PT	proton transfer
TTQ	tryptophan tryptophylquinone
WT	wild-type

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Figure 1.

Reaction steps and intermediates in the auto-reduction of the CR-stabilized *bis*-Fe^{IV} state to the diferric state in WT MauG and P107V MauG. The *bis*-Fe^{IV}, Compound I-like and Compound II-like structures that are shown are each likely the predominate species in ensembles of CR-stabilized structures which were characterized for WT MauG¹².

Figure 2.

Key residues and the ordered water networks in the distal pocket of the five-coordinate heme of WT MauG and P107V MauG. The waters that comprise the proton tunneling pathway are colored red. The waters which comprise the non-tunneling pathway that intersects with W1 are colored blue. The two additional waters that are present in the structure of P107V MauG are colored green. These representations are adapted from the crystal structures of WT MauG (PDB ID: 3L4M)⁵ and P107V MauG (PDB ID: 3SVW, chain A)¹⁵.

Figure 3.

Absorbance spectra of intermediates in the conversion of the *bis*-Fe^{IV} state to the diferric state in WT MauG (A) and P107V MauG (B). The starting spectrum of the *bis*-Fe^{IV} state is red. The Compound I-like state which is observed in WT MauG but not in P107V MauG is green. The Compound II-like state is blue and the diferric state is purple. Samples contained 3 μ M protein and the reactions were initiated by addition of a stoichiometric amount of H₂O₂.

Figure 4.

Kinetic analysis of changes in the absorbance spectrum with time that are correlated with the conversion of the *bis*-Fe^{IV} state to the diferric state in WT MauG (A) and P107V MauG (B). These traces correspond to the disappearance of the starting spectrum of the *bis*-Fe^{IV} state (red), the formation and decay of the spectrum of the Compound I-like state (green) which is only observed in WT MauG, the formation and decay of the spectrum of the Compound II-like state (blue) and the formation of the spectrum of the diferric state (purple). The reference spectra for these redox states are those shown in Figure 3. The kinetic plots depict global fits of the three-dimensional data (time, wavelength and absorbance) which are each derived from 750 spectra. Spectra were recorded every 2 s. A plot of the residuals for each global fit is shown above each plot.

Figure 5.

Proton inventories of the KSIE for reaction steps in the auto-reduction of the high-valent states of P107V MauG. (A) The experimentally-determined values of k_2 for the reaction of P107V MauG are shown as circles. The data are best fit by Eq. for the model of a transition state with two alternative pathways, each involving multiple PTs (purple line). The other lines are simulations of the expected plots for a PT in the transition state of a single pathway involving the transfer of one (red, Eq. 3), two (green, Eq. 4) or multiple (blue, Eq. 5) protons. (B) The values for k_3 for P107V MauG and the fit to a single PT (Eq. 3). No improvement to the fit of the data was obtained on fitting the data to the more complex equations. (C) Comparison of the fits of the proton inventory data for k_2 for P107V MauG (open circles) and WT MauG (closed circles) with each fit to Eq. 6. The simulation of the expected plot for a single PT in the transition state is shown in red for reference. (D) Comparison of the fits of the proton inventory data for k_3 for P107V MauG (open circles) and WT MauG (closed circles) with each fit to Eq. 3.

Figure 6. Dependence on temperature of reaction steps in the auto-reduction of the high-valent states of P107V and WT MauG. The values for k_2 for P107V MauG (open circles) and WT MauG (closed circles), and the values for k_3 for P107V MauG (open squares) and WT MauG (closed squares) are each fit to Eq. 7.

Figure 7. Steady-state kinetics of the reactions catalyzed by WT MauG (blue circles) and P107V MauG (red squares). The reaction was monitored by the increase in absorbance at 450 nm which is due to formation of the TTQ product. Reaction conditions are described under Experimental Procedures. Arrows indicate the time at which additional substrates were added to the reaction mixtures.

Table 1

Reaction parameters for the auto-reduction of the high-valent hemes in WT MauG and P107V MauG

	WT MauG	P107V MauG
k_1 (s ⁻¹)	$(2.5 \pm 0.3) \times 10^{-2}$	–
KSIE for k_1	2.5 ± 0.7	–
k_2 (s ⁻¹)	$(1.19 \pm 0.03) \times 10^{-2}$	$(1.41 \pm 0.03) \times 10^{-2}$
KSIE for k_2	2.6 ± 0.5	1.8 ± 0.1
ϕ^{TS1} (% contribution)	<0.01 (27%)	<0.01 (3%)
ϕ^{TS2} (% contribution)	0.52 ± 0.10 (73%)	0.57 ± 0.00 (97%)
E_a (kcal/mol)	17.5 ± 0.5	17.8 ± 0.4
k_3 (s ⁻¹)	$(8.8 \pm 0.7) \times 10^{-4}$	$(1.24 \pm 0.05) \times 10^{-3}$
KSIE for k_3	1.8 ± 0.4	1.5 ± 0.1
E_a (kcal/mol)	6.5 ± 1.0	6.3 ± 0.9