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Mechanism of protein oxidative damage that is coupled to long-range electron transfer to high-valent hemes

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 autoreduction of the high-valent *bis*-Fe^{IV} state of the diheme enzyme MauG is coupled to oxidative damage of a methionine residue. Transient kinetic and solvent isotope effect studies reveal that this process occurs via two sequential long-range electron transfer (ET) reactions from methionine to the hemes. The first ET is coupled to proton transfer (PT) to the hemes from solvent via an ordered water network. The second ET is coupled to PT at the methionine site and occurs during the oxidation of the methionine to a sulfoxide. This process proceeds via Compound I- and Compound II-like heme intermediates. It is proposed that the methionine radical is stabilized by a two-center three-electron bond. This provides insight into how oxidative damage to proteins may occur without direct contact with a reactive oxygen species, and how that damage can be propagated through the protein.

Keywords

Charge resonance; cytochrome; electron transfer; ferryl heme; methionine sulfoxide; protein radical

INTRODUCTION

Oxidative damage to proteins has deleterious effects on cell function and is related to many disease states as well as aging [1, 2]. This study describes a mechanism of oxidative damage of amino acids in a protein which does not occur as a result of direct reaction with reactive oxygen species. Instead it is a consequence of the reactivity of the heme cofactors of the protein. Furthermore, the oxidative damage occurs at a site in the protein that is not in close proximity to the hemes.

MauG from *Paracoccus denitrificans* [3] is a diheme enzyme that catalyzes a six-electron oxidation of a precursor protein of methylamine dehydrogenase (preMADH) to generate its protein-derived tryptophan tryptophylquinone (TTQ) cofactor [4]. Catalysis requires long-

*Corresponding author: 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.

Author Contributions

Z.M., H.R.W. and V.L.D. designed research; Z.M. performed research; Z.M., H.R.W. and V.L.D. analyzed data; Z.M., H.R.W. and V.L.D. wrote the paper.

range electron transfer (ET) over several angstroms from the residues of preMADH that are post-translationally modified to the hemes of MauG [5-7]. The high-valent redox form of MauG that accepts electrons is a *bis*-Fe^{IV} redox state in which one heme present as Fe^{IV}=O with an axial ligand provided by His35, and the other present as Fe^{IV} with two axial ligands provided by His205 and Tyr294 with Trp93 residing approximately 4 Å from each heme (Figure 1A) [7, 8]. Amino acid residues [9-11] and structured waters [12] in the distal pocket of the five-coordinate heme (Figure 1B) control the reactivity of this high-valent state. This high-valent state is also stabilized by a charge-resonance (CR) transition phenomenon during which both hemes share charge via ultrafast ET that is mediated by the intervening Trp93 [13, 14]. The conversion of the high-valent state to the diferric state is accompanied by multiple changes in the visible and near infrared (NIR) spectra (Figure 1C). As a consequence of the CR stabilization of the high-valent state of MauG, this redox state is viewed as an ensemble of resonance structures involving the two hemes and Trp93, with the true *bis*-Fe^{IV} state as the predominant resonance species [14, 15] (Figure 2). This redox state is distinct from other well-characterized high-valent redox states of protein-bound hemes, such as Compound I and Compound ES [16-18]. In those states the Fe^V equivalent is centered on a single ferryl heme with a cation radical present on either the porphyrin ring or on a nearby amino acid residue.

Spectroscopic and kinetic solvent isotope effect (KSIE) studies demonstrated that the conversion of the high-valent state to the diferric state in the absence of substrate occurred in at least two phases and involved multiple proton transfer (PT) and ET reactions [12]. The first phase involved protonation of the ferryl heme, without addition of an electron. This altered the distribution of resonance species that support CR stabilization within the ensemble that comprises the high-valent state, such that the proportion of *bis*-Fe^{IV} is decreased and a Compound I-like species becomes more prominent (Figure 2). The subsequent two-electron reduction to the diferric state was also coupled to PT. The source of electrons for this autoreduction of the high-valent state is believed to be a Met residue on MauG. It was previously shown that cycling between the high-valent and diferric states results in inactivation of MauG [19] and that this process is accompanied by the oxidation of three Met residues which are located 7.5-15.2 Å from the ferryl heme iron (Figure 1B) [20]. These Met residues each exhibited increased mass of 16 Da consistent with conversion to methionine sulfoxide, and in some cases increases of 32 Da or 48 Da [20].

In the current study, the pH-dependence of the conversion of the high-valent state of MauG to the diferric state is examined. At pH 9.0 the kinetics of the overall reaction are altered such that it was possible to kinetically distinguish the two one-electron reductions of the high-valent hemes to the diferric state. Evidence is presented that the reaction proceeds via a Compound II-like intermediate (Figure 2) and that each reduction is a proton-coupled ET (PCET) reaction. A mechanism is proposed for the overall conversion of the high-valent hemes to diferric that is linked to the oxidation of a Met residue. The first step is a PT without ET and the subsequent two steps are PCET reactions. The rate constants for these steps will be referred to as k_1 , k_2 and k_3 in this paper (Figure 3). These results have implications not only for understanding how proteins control the reactivity of high-valent hemes, but also for describing a mechanism by which oxidative damage to proteins may

occur without contact with reactive oxygen species. This expands our view of the potential causes of and possible interventions for oxidative damage to proteins.

MATERIALS AND METHODS

Protein purification

Recombinant MauG was expressed in *Paracoccus denitrificans* and purified as described previously [3].

Kinetic studies

Studies were performed in 10 mM potassium phosphate, at the indicated pH. Formation of *bis*-Fe^{IV} MauG was achieved by addition of stoichiometric H₂O₂ [13]. Reactions were monitored spectroscopically in the NIR range using a Beckman Coulter DU 800 spectrophotometer and in the visible range using an HP8452A diode array spectrophotometer run by the OLIS SpectralWorks/GlobalWorks software. Kinetic data collected in the scanning mode were globally fit to include the changes in absorbance at each wavelength with time. Data were reduced by factor analysis using the singular-value decomposition (SVD) algorithm and then globally fit using the fitting routines of OLIS software.

Kinetic solvent isotope effect studies

For experiments performed in D₂O the pL of the buffer was determined using eq 1, where x is the mole fraction of D₂O [21]. In proton inventory experiments the reaction rate was determined in buffers containing different atomic fractions of deuterium in H₂O-D₂O mixtures [22-24]. This relationship is described by eq 2 where k_x is the rate constant at mole fraction of D₂O equal to x , k_0 is the rate constant at $x = 0$, and ϕ_i^{TS} and ϕ_j^R are the isotopic fractionation factors for the transition and reactant states, respectively, for m or n PTs. In the case of a single proton transferred in the transition state there will be a linear relationship (eq 3). If two protons are transferred in the transition state this will result in a quadratic relationship (eq 4). If multiple PTs contribute to the rate the relationship reduces to eq 5.

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

$$k_x = k_0 \left\{ \prod_{i=1}^m (1 - x + x\phi_i^{TS}) / \prod_{j=1}^n (1 - x + x\phi_j^R) \right\} \quad (2)$$

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

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

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

As described previously [12], a plot of the data exhibiting extreme curvature was fit by eq 6 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.

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

RESULTS

Dependence on pH of the kinetic mechanism of the conversion of the high-valent state of MauG to the diferric state

Addition of H_2O_2 to diferric MauG generates a high-valent state, the formation of which is accompanied by changes in the absorbance spectrum. One observes a decrease in intensity and shift of the Soret peak from 406 nm to 408 nm, the appearance of minor peaks at 526 and 559 nm, and appearance of an absorbance feature at 950 nm in the NIR region (Figure 1C) [13, 14, 25]. These spectral changes occur very quickly [25] and in the absence of the preMADH substrate return over several minutes to the spectrum of the diferric MauG. It was previously shown that this process occurred in at least two phases, an initial PT followed by PCET for the two-electron reduction of the high-valent hemes [12]. The dependence of the rate constants for the two reaction phases were examined over a range of pH values from 6.5 to 9.0 (Figure 4). MauG was not stable outside of this range. The rate of each reaction phase increased with increasing pH. However, the rate of the first phase increased more rapidly than the rate of the second phase with increasing pH.

In the previous study performed at pH 7.5 the global fit of the kinetic data was well-described by two exponentials [12]. However, at pH 9.0 the data were better described by a three-exponential fit (Figure 5A). The global fit of the data in the visible absorbance range from 300-750 nm yielded rate constants for $k_1 = 0.019 \pm 0.004 \text{ s}^{-1}$, $k_2 = 0.0086 \pm 0.0007 \text{ s}^{-1}$ and $k_3 = 0.0013 \pm 0.0002 \text{ s}^{-1}$. The altered kinetics at pH 9.0 allowed characterization of an additional reaction intermediate. The changes in the visible spectrum during the overall reaction are shown in Figures 5B and 5C. After reaction of MauG with H_2O_2 , the formation of the *bis*- Fe^{IV} predominant high-valent species exhibits a Soret peak maximum at 408 nm, a β band at 526 nm and α band at 559 nm. The loss of the *bis*- Fe^{IV} predominant species (red in Figure 5) and appearance of the Compound I-like predominant high-valent state (green in

Figure 5) is described by k_1 . One sees the Soret peak maximum shifted to 407 nm with a small decrease in intensity and appearance of an absorbance feature centered at 650 nm. The one-electron reduction of this state to the Compound II-like state (blue in Figure 5) is described by k_2 . In this step the Soret peak increases in intensity, the absorbance at 650 nm disappears, the α band at 559 nm and β band at 526 nm decrease in intensity, and the absorbance at 498 nm increases. The resulting spectrum is consistent with spectra that have been reported for Compound II [26-29]. Lastly, the one-electron reduction of the Compound II-like intermediate to the diferric state (black in Figure 5) with loss of the absorbance at 498 nm and a slight decrease in the Soret peak is described by k_3 . This spectrum is essentially identical to that of the diferric MauG prior to addition of H_2O_2 . The overall reaction may also be monitored by absorbance changes in the near IR (Figure 5D). The high valent-state of MauG exhibits an absorbance feature centered at 950 nm which is a consequence of the CR stabilization [14]. This absorbance is unchanged during the first reaction phase (k_1) and then decays. The rate of decay was best fit by two exponentials yielding rate constants of $0.0083 \pm 0.0002 \text{ s}^{-1}$ and $0.0018 \pm 0.0001 \text{ s}^{-1}$. These values closely match those of k_2 and k_3 that were obtained from the global fit of the data for the visible absorbance changes. This indicates the CR stabilization is diminished by the reaction described by k_2 , but not completely lost until after the reaction described by k_3 .

As a consequence of the differential effects of pH on the rates, not only was it possible at pH 9.0 to identify the previously undetected Compound II-like intermediate, but also the Compound I-like intermediate formed after the first reaction (see Figure 2) accumulated to a much greater extent than it did previously in the study at pH 7.5. The presence of the Compound I-like species is evidenced by a characteristic feature in the absorbance spectrum that is centered around 650 nm (green in Figure 5). This feature is much more prominent at pH 9.0 than at lower pH (Figure 6). This is because the rate of formation of this intermediate is faster and the rate of its decrease is relatively slower allowing greater accumulation.

Kinetic solvent isotope effect studies of the three reaction steps in the reduction of the high-valent hemes by a Met residue

It was previously shown at pH 7.5 that the two phases of the overall reaction which were kinetically distinguishable exhibited KSIE values of 2.5 ± 0.1 and 2.3 ± 0.2 , respectively. The proton inventory plot of each of these two reactions exhibited extreme curvature and was fit to a model in which there are two alternative pathways for PT, each involving multiple PTs [12]. For comparison, these experiments were repeated for each of the three reaction phases that were identified for the overall reaction at pH 9.0. For the initial reaction, k_1 exhibited a KSIE of 2.5 ± 0.7 and extreme curvature of the proton inventory plot (Figure 7A). The fit of this data to the equation which describes two alternative pathways for PT yielded one pathway contributing $38 \pm 7\%$ with a $\phi^{\text{TS}1} < 0.01$ and the other contributing $61 \pm 7\%$ with a $\phi^{\text{TS}2} = 0.66 \pm 0.10$, where ϕ^{TS} is the isotopic fractionation factor for the transition state in each alternative pathway. The two alternative pathways may be inferred from the ordered water network in the pocket of the high-spin heme (Figure 1B). These results are similar to those obtained for the first reaction phase at pH 7.5 [12]. At pH 9.0, k_2 exhibited a KSIE of 2.6 ± 0.5 and extreme curvature of the proton inventory plot (Figure 7B). The fit of the proton inventory data to the same equation yielded one pathway

contributing $27 \pm 11\%$ with a $\phi^{\text{TS1}} < 0.01$ and the other contributing $73 \pm 11\%$ with a $\phi^{\text{TS2}} = 0.52 \pm 0.10$. The similarity of these fitted parameters to those for the initial PT reaction indicates that the first ET to the high-valent hemes from a Met residue on MauG is linked to a PT that occurs via the same mechanism as the PT that occurs in the absence of ET in the first reaction phase. For k_2 it should be noted that the source of the proton, which is solvent, is different from the source of the electron which is a Met residue. For the final reaction step, k_3 exhibited a KSIE of 1.8 ± 0.4 . In contrast to k_1 and k_2 , the proton inventory plot for k_3 did not show the extreme curvature. From the data it is not possible to accurately distinguish whether this involves the transfer of one or more protons, but it clearly does not involve multiple pathways (Figure 7C). An explanation for this result is that in the final reaction, the proton that is transferred is not being delivered from solvent to the hemes as is the first two reactions. Instead it is a PT that is associated with the conversion of the Met residue which is the source of electrons to methionine sulfoxide. Since this step exhibits a KSIE, it follows the long range ET to the hemes is gated [30] by the PT.

DISCUSSION

Oxidation of Met residues in proteins may be a consequence of reaction with a variety of oxidants and oxygen sources. During reaction with certain reactive oxygen species (*e.g.*, H_2O_2 , O_2^- , HOCl), that species is both the two-electron oxidant and the oxygen source. In contrast, some reactive radicals (*e.g.*, $\text{HO}^\bullet$, $\text{RO}^\bullet$) and metals, act as a one-electron oxidant with varying oxygen sources (*e.g.*, H_2O , OH^-). In the case of Met oxidation within MauG the oxidant is the high-valent state of the diheme center and the oxygen source is likely hydroxide. It is proposed that the product of the first ET from Met is a methionine radical cation. This radical species is very stable (*i.e.*, $k_3 = 0.0013 \text{ s}^{-1}$). A mechanism that has been postulated for stabilization of a Met-based radical is the formation of a two-center three-electron (2c3e) bond. This may occur in a protein through interaction of the Met radical with a peptide amide nitrogen, a peptide amide oxygen or an aromatic residue side-chain [31-36] (Figure 8A). Amino acid residues that could stabilize such a radical intermediate are present in MauG in proximity of the Met residues which are known to be oxidized by the high-valent hemes.

The KSIE of 1.8 that is obtained for the reaction of the Met radical species (k_3) indicates that the source of oxygen that reacts with the Met radical to form methionine sulfoxide is from water or hydroxide. This reaction is proposed to be “base catalyzed” (Figure 8B), which correlates with pH dependence of the reaction rate. During the final one-electron reduction (k_3), the loss of an electron from the 2c3e bond is coupled to PT that is centered at the methionine site. Consistent with this model are the results of the proton inventory for this step, which unlike k_1 and k_2 does not exhibit hyper-curvature. In this final reaction step, PT does not proceed via the dual-pathway water network in the heme pocket but via a traditional single pathway of PT at the Met site in the transition state.

The results of the present study allow expansion of the previously proposed model [12] of conversion of the high-valent hemes to the diferric state and the role of CR stabilization in this process. Evidence is presented that the first one-electron reduction of the high-valent hemes results in the formation of a di-protonated Compound II-like species (Figure 2). It

was noted that some of the 950 nm absorbance feature which is indicative of CR stabilization was retained after formation of the Compound II-like state. Thus, there is still CR stabilization of the Compound II-like state. However, the nature of this CR must be different from that of the high-valent *bis*-Fe^{IV} predominant and the Compound I-like predominant states. Those two states can each be described as a type II CR (Π^{+*})₂ complex, or if one includes the intervening Trp, a type III CR complex [14]. These designations would indicate that the two Π systems (*i.e.*, the hemes) share two holes. In contrast, the Compound II-like state would be considered a type I CR, (Π)₂⁺ complex where the Π systems have a single hole distributed over both sites [37].

In conclusion, the process of the reduction of the high-valent diheme system to the diferric state which is coupled to the oxidation of a Met residue exhibits at least three noteworthy features. (i) The newly characterized Compound II-like intermediate state is a type I CR which has not previously been described in a protein. (ii) While Met cation radicals stabilized by 2c3e bonds have been previously examined theoretically and experimentally in small molecule models and peptides, the mechanism of Met oxidation in MauG involves stabilization of a 2c3e bond in a globular protein. (iii) A mechanism is described by which oxidative damage to proteins may occur without direct reaction with reactive oxygen species. These results not only advance our understanding of how proteins control the reactivity of high-valent hemes, but also expands our view of the potential causes of oxidative damage to proteins.

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ABBREVIATIONS

<i>bis</i> -Fe ^{IV} MauG	redox state of MauG with one heme as Fe ^{IV} =O and the other as Fe ^{IV}
E _m	oxidation-reduction midpoint potential
CR	charge resonance
ET	electron transfer
KSIE	kinetic solvent isotope effect
φ	isotopic fractionation factor
preMADH	the biosynthetic precursor protein of methylamine dehydrogenase with incompletely synthesized TTQ
PCET	proton coupled electron transfer

PT	proton transfer
TTQ	tryptophan tryptophylquinone
2c3e	two-center three-electron

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Summary

This study describes how oxidative damage to a protein may occur without direct reaction with a reactive oxygen species, and how that radical-mediated damage can be propagated through the protein. This process is coupled to the reactivity of high-valent hemes within the same protein.

Figure 1.

A. The crystal structure of MauG. The overall structure (PDB 3L4M) [7] is shown in gray in the background with the two hemes, intervening Trp93, and Met residues that were previously shown [20] to undergo oxidative damage displayed in stick. The segment colored pink in the box is the portion of the surface of MauG which interacts with the preMADH substrate protein. B. The distal pocket of the five-coordinate heme. Residues in proximity to the ferryl heme and ordered water molecule are displayed. C. The absorbance spectra of diferric and *bis*-Fe^{IV} MauG in the Soret region. D. The absorbance spectra of diferric and *bis*-Fe^{IV} MauG in the far visible and NIR regions. Spectra were recorded before (black) and after (red) addition of H₂O₂.

Figure 2.

Ensembles of charge-resonance-stabilized structures of the high-valent state of MauG and intermediate states formed during the reduction to the diferric state. The structures in the initial two states are those proposed previously [14, 15].

Reduction of Fe^{IV} hemes Oxidation of Methionine

Figure 3.

Reactions at the diheme site and methionine site that are associated with the three kinetically distinguishable steps in the two-electron reduction of the high-valent hemes by a Met residue. The rate constants for each step are designated k_1 , k_2 and k_3 .

Figure 4.

Dependence on pH of the kinetic mechanism of the conversion of the high-valent state of MauG to the diferric state. The rate constants for the initial PT to the high-valent hemes (k_1 in Figure 2) are shown as circles. The rate constants for the subsequent two-electron reduction to the diferric state are shown as squares. At pH values below 9.0, the latter process is described by a single rate constant as k_2 , because k_2 and k_3 cannot be kinetically distinguished under those conditions. For comparison with the other pH values, the data point at pH 9.0 is that for a single exponential fit of the remainder of the overall reaction. As it was not possible to obtain data points beyond pH 9.0, it was not possible to properly fit the data to an equation to determine a pK_a for this transition. The lines in this figure are fits to a sigmoidal function which allows an estimation of a pK_a from these data, which in each case is 8.1 ± 0.1 .

Figure 5.

A. Kinetic analysis of changes in the absorbance spectrum that are correlated with the conversion of the high-valent state of MauG to the diferric state. Red is the time course for the disappearance of the starting spectrum of the *bis*-Fe^{IV} predominant state. Green represents the formation and loss of the first intermediate, the Compound I-like predominant state. Blue represents the formation and decay of the second intermediate, the Compound II-like state. Black represents and the formation of the diferric state. The kinetic plots depict global fits of the most statistically significant eigenvector of the SVD reduced three-dimensional data. B. The spectral changes in the Soret region of the spectrum associated with the starting, intermediate, and final states described by the kinetic analysis. C. The spectral changes in the far visible region of the spectrum associated with the starting, intermediate and final states described by the kinetic analysis. D. The spectral changes in the NIR region of the spectrum associated with the starting, intermediate and final states described by the kinetic analysis. No change in the NIR spectrum is observed in first phase of the overall reaction and so the initial spectrum shown is that of the Compound I-like predominant state (green). After formation of this state, blue spectra were recorded every 3 min and then black spectra were subsequently recorded every 5 min.

Figure 6.

A. Time course for the formation and decay of the of the first reaction intermediate, the Compound I-like predominate state. The reaction was monitored at 650 nm, which is the wavelength maximum for this species (green spectrum in Figure 5C). Reactions were monitored at pH 9.0 (green) and pH 7.5 (black). B. Difference spectrum of the first reaction intermediate at its maximum accumulation. The absorbance spectrum at time equal zero was subtracted from the spectrum recorded at the time when A_{650} was maximum. The difference spectra were obtained at pH 9.0 (green) and pH 7.5 (black).

Figure 7.

Proton inventories of the KSIE on k_1 (A), k_2 (B), and k_3 (C). In A and B, the data are fit by eq 6 for the model of a transition state with two alternative pathways, each involving multiple PTs (purple). 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. In C, the fit to a single PT (eq 3) is shown. No improvement to the fit of the data were obtained on fitting the data to the more complex equations.

Figure 8.

Proposed mechanism for methionine oxidation that is coupled to reduction of the high-valent state in MauG. A. Alternative possibilities by which the proposed Met radical may be stabilized by formation of a two-center three-electron bond with either an amide nitrogen or oxygen or an aromatic side-chain. B. Possible mechanism for PCET reaction that converts the Met radical to a sulfoxide. X represents one of the species shown in A that stabilizes the radical. B is a species which abstracts a proton. The half arrow describes the one-electron transfer from the radical.