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Converting the *bis*-Fe^{IV} state of the diheme enzyme MauG to Compound I decreases the reorganization energy for electron transfer

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Abstract

The electron transfer (ET) properties of two types of high-valent hemes were studied within the same protein matrix; the *bis*-Fe^{IV} state of MauG and the Compound I state of Y294H MauG. The latter is formed as a consequence of mutation of the Tyr which forms the distal axial ligand of the six-coordinate heme that allows it to stabilize Fe^{IV} in the absence of an external ligand. The rates of the ET reaction of each high-valent species with the type I copper protein, amicyanin, were determined at different temperatures and analyzed by ET theory. The reaction with *bis*-Fe^{IV} WT MauG exhibited a reorganization energy (λ) that was 0.39 eV greater than that for the reaction of Compound I Y295H MauG. It is concluded that the delocalization of charge over the two hemes in the *bis*-Fe^{IV} state is responsible for the larger λ , relative to the Compound I state in which the Fe^V equivalent is isolated on one heme. While the increase in λ decreases the rate of ET, the delocalization of charge decreases the ET distance in its natural substrate protein, thus increasing the ET rate. This describes how proteins can balance different ET properties of complex redox cofactors to optimize each system for its particular ET or catalytic reaction.

Keywords

Charge resonance; cytochrome; electron transfer; ferryl heme; redox protein

INTRODUCTION

High-valent hemes in proteins participate in a variety of important biological processes. They are used in the oxidation of substrates, as in oxygenases including cytochrome P450 enzymes [1, 2] and as intermediates in the breakdown of peroxides in peroxidases [3]. The hemes are usually present as Compound I or Compound ES, in which the ferryl heme iron is Fe^{IV}=O with a cation radical present on the porphyrin ring or a nearby Trp or Tyr residue, respectively [4]. The *bis*-Fe^{IV} state of MauG is an alternative strategy by which to stabilize a

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Author contributions

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high-valent heme iron which utilizes two hemes [5]. In MauG from *Paracoccus denitrificans* this high-valent state has one heme present as $\text{Fe}^{\text{IV}}=\text{O}$ with an axial ligand provided by His35 and the other present as Fe^{IV} with two axial ligands provided by His205 and Tyr294 and no exogenous ligand (Figure 1) [6, 7]. Ultrafast electron transfer (ET) between the hemes in the *bis*- Fe^{IV} state is mediated by hopping through Trp93, which lies between the hemes. This leads to charge resonance stabilization of this high-valent heme species [8]. Trp93 is reversibly oxidized to a radical species during this process. It is believed that as a consequence of the CR stabilization, the high-valent state is actually comprised of an ensemble of resonance structures with the true *bis*- Fe^{IV} state as the dominant species [8, 9]. The function of MauG is unique among other enzymes that use high-valent hemes as reaction intermediates. This di-*c*-type heme enzyme catalyzes the posttranslational modification of a precursor of MADH (preMADH) to generate the protein-derived TTQ cofactor [10]. TTQ is generated by the six-electron oxidation by MauG of two Trp residues in preMADH. The oxidizing intermediate is the *bis*- Fe^{IV} species. In contrast to the mechanism of heme-dependent oxygenases, the hemes of MauG make no direct contact with the substrate. Instead preMADH binds to the surface of MauG with the residues on preMADH that are oxidized at a distance of 40 Å and 19 Å, respectively, from the two heme irons [6]. Due to the long distance, the catalytic reactions require long range ET from the substrate to the hemes.

Hemes are also involved in many non-catalytic physiological ET reactions. Nearly all of these involve the Fe^{II} and Fe^{III} redox states as the electron donor and acceptor. The long range ET reaction from preMADH to the *bis*- Fe^{IV} hemes of MauG, which is required as a part of the catalytic mechanism, has been studied and the ET parameters for this reaction have been determined[11]. Similar long range ET studies have not been performed with other types of high-valent hemes. In this study we present a direct comparison of the ET properties of the hemes in the *bis*- Fe^{IV} and Compound I redox states within the same protein framework of MauG.

Mutation of the Tyr294 axial ligand of the 6-coordinate heme of MauG resulted in a heme with His-His axial ligation rather than Tyr-His ligation [12]. The crystal structure of Y294H MauG showed that apart from the ligand substitution, this mutation did not significantly perturb the structure of the diheme region of MauG. Whereas addition of H_2O_2 to MauG results in formation of the *bis*- Fe^{IV} state, addition of H_2O_2 to Y294H MauG resulted in formation of a Compound I species because the six-coordinate heme with His-His ligation could not stabilize Fe^{IV} . The high-valent Compound I species in Y294H MauG was unable to oxidize preMADH and catalyze TTQ biosynthesis. It was concluded that the inability of Compound I in Y294H MauG to oxidize preMADH was because the ET distance from the substrate to this single heme was too great (~40 Å), whereas the delocalization of the oxidizing equivalent over both hemes in the *bis*- Fe^{IV} state of MauG reduced the ET distance to a more manageable distance (~19 Å). Thus, it appears that a major function of the six-coordinate heme of MauG in TTQ biosynthesis is to shorten the ET distance, which exponentially increases the ET rate. Unfortunately, because of the inability of Y294H MauG to react with preMADH, this meant that it was not possible to compare the ET properties of the different high-valent redox states of wild-type (WT) and Y294 MauG.

Recently an inter-protein ET reaction between the cupredoxin amicyanin and MauG was described [13]. Amicyanin is a small beta-barrel protein from *P. denitrificans* with a type 1 copper center that consists of a single copper ion coordinated by His53, His95, Cys92, and Met98 [14]. The ET reaction that was studied was that from Cu^{I} amicyanin to $\text{bis-Fe}^{\text{IV}}$ MauG. A docking model of the MauG-amicyanin complex placed the copper of amicyanin 16.7 Å from the porphyrin ring and 17.9 Å from the heme iron of the five-coordinate heme of MauG [13]. This distance matched that estimated from analysis of the temperature-dependence of the rate of the ET reaction (k_{ET}). The shorter ET distance to the five-coordinate heme in MauG-amicyanin complex than in the preMADH-MauG complex suggested that it might be possible to study an ET reaction of the Compound I redox state of Y294H MauG in complex with amicyanin. In this study the ET reaction between Y294H MauG and amicyanin is analyzed and compared to the reaction with WT MauG. This allows a direct comparison of the ET properties associated with the ET from the $\text{bis-Fe}^{\text{IV}}$ and Compound I redox states. This provides a unique opportunity to study an inter-protein ET reaction involving a Compound I redox center in the same protein matrix as the $\text{bis-Fe}^{\text{IV}}$ redox center, and it allows for direct comparison of the ET parameters, *i.e.* reorganization energy (λ) and electronic coupling (H_{AB}) associated with ET from these two different types of high-valent heme redox states.

MATERIALS AND METHODS

Protein purification

Recombinant amicyanin was expressed in *Escherichia coli* BL21 (DE3) and purified from the periplasmic fraction as described previously [15]. Recombinant Y294H MauG was expressed in and purified from *Paracoccus denitrificans* as described previously [12, 16].

Determination of k_{ET}

The rates of the ET reactions from reduced amicyanin to Compound-I Y294H MauG were determined using an On-Line Instruments (OLIS, Bogart, GA) RSM1000 stopped-flow rapid scanning spectrophotometer. Each reaction was performed in 10 mM potassium phosphate buffer, pH 7.5, at the indicated temperature. One syringe contained the limiting reactant, 2 μM Y294H MauG, and the second syringe contained varying concentrations of reduced amicyanin. Amicyanin was reduced by addition of an equimolar amount of sodium dithionite [17]. The high valent species of Y294H MauG was generated by addition of equimolar H_2O_2 [12]. After rapid mixing the reactions, the absorbance spectrum was monitored over the range from 365 to 435 nm to observe the conversion of Compound-I Y294H MauG to diferric Y294H MauG. Kinetic data were reduced by factor analysis using the singular-value decomposition (SVD) algorithm and then globally fit using the fitting routines of OLIS GlobalWorks software. In each of the reactions that were performed, the observed rate constant (k_{obs}) was best fit to a single-exponential relaxation. The rate constants that were obtained at different concentrations of amicyanin were analyzed by eq 1 to determine the limiting first-order rate constant (k_{ET}) for the reaction and the K_d for complex formation. The errors which are listed in the text are standard errors of the fits.

$$k_{obs} = k_{ET} [Amicaynin(Cu^I)] / ([Amicaynin(Cu^I)] + K_d) + k_{-ET} \quad (1)$$

Analysis of k_{ET} by ET theory

Data for the temperature-dependence of k_{ET} was analyzed using ET theory (eq 2) [18]. The terms in the equation are temperature (T), free energy (ΔG°), electronic coupling (H_{AB}), the reorganization energy (λ), Planck's constant (h) and the gas constant (R). ΔG° is calculated by eq 3, where n is equal to the number of electrons transferred, F is Faraday's constant, and E_m is the difference in the oxidation-reduction midpoint potential value of the electron donor and acceptor sites. Data were also analyzed by eq 4 where E_a is the activation energy and A is the pre-exponential factor.

$$k_{ET} = [4\pi^2 H_{AB}^2 / h(4\pi\lambda RT)^{0.5}] \exp [-(\Delta G^\circ + \lambda)^2 / 4\lambda RT] \quad (2)$$

$$\Delta G^\circ = -nF \Delta E_m \quad (3)$$

$$\ln(k) = (-E_a / RT) + \ln(A) \quad (4)$$

In Silico Docking of amicyanin and Y294MauG

A docking model of amicyanin with Y294H MauG was constructed by using the ZDOCK utility and server (<http://zdock.umassmed.edu>) [19]. The PDB files of amicyanin (PDB ID: 2OV0) and Y294H MauG (PDB ID: 3ORV, chain A) were used in model building.

RESULTS

Determination of k_{ET} for the reaction of Y294H MauG with reduced amicyanin

The formation of the high-valent species in Y294H MauG is accompanied by a decrease in intensity of the Soret peak, which occurs immediately after addition of H_2O_2 . Subsequent addition of reduced amicyanin results in an increase in intensity of the Soret peak and return of the absorbance spectrum to that of the diferric state (Figure 2A). The kinetics of this reaction fit well to a single exponential transition. The kinetic plot derived from the global fit of the SVD-reduced three-dimensional data set is shown in Figure 2B. Analysis of the time course of the reaction at a single wavelength (404 nm) also fit well to a single exponential (Figure 2C). The reaction of the high-valent state of Y294H MauG with varied concentrations of reduced amicyanin at pH 7.5 at 25° C exhibited saturation behavior (Figure 2D). The fit of this data to eq 1 yielded a limiting first-order rate constant (k_{ET}) of $33 \pm 4 \text{ s}^{-1}$ and a K_d of $300 \pm 128 \text{ }\mu\text{M}$. For comparison, the reaction of *bis*-Fe^{IV} WT MauG with reduced amicyanin fit exhibited a k_{ET} of 22 s^{-1} and a K_d of $165 \text{ }\mu\text{M}$ [13]. Similar monophasic kinetics was observed for the reaction of *bis*-Fe^{IV} WT MauG with reduced amicyanin. It should be noted that the reduction of either high-valent MauG species, *bis*-Fe^{IV} or Compound I, requires two electrons whereas the oxidation of Cu^I amicyanin requires one electron. Thus, two molecules of Cu^I amicyanin must be oxidized to observe the

complete reaction. In each reaction, the change in absorbance fit best to a single exponential. The monophasic kinetics means that the reaction of the first Cu^I amicyanin is followed by dissociation of the complex and reaction with a second Cu^I amicyanin. If the dissociation/association steps are very rapid relative to k_{ET} and excess Cu^I amicyanin is present, rebinding of the Cu^{II} amicyanin after the single turnover is not a factor and monophasic kinetics is observed.

Temperature-dependence of k_{ET} for the reaction of Y294H MauG with reduced amicyanin

The k_{ET} for the ET reaction from reduced amicyanin to the Compound I Y294H MauG was determined at temperatures from 10° C to 27 °C (Figure 3A). The high-valent state of Y294H MauG was unstable at higher temperatures, and so this limited the range that could be studied. The E_m value for the Compound I/diferric couple of Y294H MauG is unknown. E_m values have been determined for the Compound I/Fe^{III} couple in soybean ascorbate peroxidase [20], *Arthromyces ramosus* peroxidase [21], yeast cytochrome *c* peroxidase [22], and horseradish peroxidase type A2[23] and type C[23–26]. These E_m values range from 717 to 954 mV, with an average of 887 mV. As such, for the analysis of the temperature-dependence of the ET rates by eqs 2 the E_m value of 887 mV was used for Y294H MauG. That value and the known E_m value of amicyanin at pH 7.5 of 265 mV[27] were used to determine the G° for the ET reaction from the E_m . The fit of the data to eq 2 yielded values of $\lambda = 1.95 \pm 0.08$ eV and $H_{AB} = 0.03 \pm 0.01$ cm⁻¹. For comparison, the reaction of *bis*-Fe^{IV} WT MauG with reduced amicyanin exhibited values of $\lambda = 2.34 \pm 0.16$ eV and $H_{AB} = 0.6 \pm 0.1$ cm⁻¹ [13]. Thus, the mutation decreased the magnitudes of both λ and H_{AB} . These data were also analyzed by eq 3 (Figure 3B). This yielded values of E_a of 8.2 ± 0.3 kcal/mol for the reaction with WT MauG and 4.6 ± 0.3 kcal/mol for the reaction with Y294H MauG.

Docking model of the complex of Y294H MauG and Amicyanin

A protein docking model was constructed using the the ZDOCK server and utility [19] from the crystal structures of amicyanin and Y294H MauG (Figure 4). The relative orientations of the redox centers in this complex were similar to those in the docking model of the WT MauG-amicyanin [13].

DISCUSSION

The value of λ reflects the amount of energy needed to optimize the system for ET, or in other words the energy required to bring the reactant and product states to the state in which the ET event occurs. The value of λ is composed of an inner sphere λ_{in} , which derives from optimization bond lengths and bond angles of the inner shell of atoms of the redox center for the ET event, and an outer sphere λ_{out} , which reflects reorientation of solvent molecules that that is associated with the ET event. In small molecules, these two parameters are fairly well distinguishable. However, in protein ET reactions, the line between these two terms can become blurred as electron density is distributed asymmetrically about the redox cofactor or metal-ligating residues. Computational methods have been used to predict λ for protein ET reactions which tend to focus on the contributions of the protein medium [28, 29]. λ values have also been determined for ET reactions between native redox cofactors and redox-active

active tags such as Ruthenium complexes [30, 31]. ET reactions between the copper of azurin a disulfide radical anion were used to determine λ [32]. The proteins used in the present study are among the few for which λ values have been experimentally determined for the reactions with their natural redox partners; the reactions of amicyanin with MADH [33] and cytochrome *c*-551i [34], and the reactions between MauG and preMADH and MADH [11]. λ values have also been determined for some other ET reactions between quinoprotein dehydrogenases redox protein partners [35]. However, in general there have been relatively few studies in which λ values have been experimentally determined for ET reactions between two protein-bound redox cofactors.

The structures of WT and Y294H MauG suggest that there should be little difference in the value of λ_{out} during ET into the high-valent hemes since the only solvent accessible portion of the diheme site is the $\text{Fe}^{\text{IV}}=\text{O}$ of the five-coordinate heme [6, 36]. The six-coordinate heme is shielded from solvent by the protein. Therefore, the 0.39 eV decrease in the experimentally-determined λ that was caused by the Y294H mutation is most likely a consequence of a decrease in λ_{in} . It was previously suggested that the relatively large λ values associated the ET reactions of WT *bis*- Fe^{IV} MauG with preMADH [11] and amicyanin [13] could be attributed to the fact that λ_{in} associated with the *bis*- Fe^{IV} state could require alterations in lengths and angles of multiple bonds within the two hemes and intervening Trp93. In Y294H MauG, the ET reaction associated with the high-valent Compound I redox state would only require such changes in bond lengths and angles of the five-coordinate heme, as there is no change in the redox state of the six-coordinate heme. This would explain the decrease in λ that was caused by the Y294H mutation.

The Y294H mutation also caused a decrease in H_{AB} . Comparison of the docking models of the complexes of amicyanin with WT MauG and Y294MauG does not provide a clear explanation for this. H_{AB} is dependent upon the ET distance and the nature of the intervening medium. ET through amino acid bonds in proteins will exhibit a larger H_{AB} than for ET through empty space [37, 38]. For interprotein ET reactions, a through-space segment of the ET pathway is required to get from one protein to the other. As such, the nature of the protein-protein interface in the ET protein complex can be a critical determinant of the overall H_{AB} . An increase of less than an angstrom in the length of the through-space jump can significantly decrease the overall H_{AB} [39]. Such distances cannot be reliably ascertained from docking models. It should be noted that while the structures of the diheme sites of WT amicyanin and Y294H amicyanin are indistinguishable, there were some other changes in structure of the protein. In order for His294 to replace Tyr294 as a heme axial ligand, residues 291-314 in the C-terminal region of the Y294H MauG are displaced relative to WT MauG [12]. While this region is not at the interface with amicyanin, it does leave open the possibility that the mutation may have also caused subtle changes in structure at the protein surface that affect the interface. The fact that the Y294H mutation increased the K_d for complex formation with amicyanin from 165 μM to 265 μM supports the notion that subtle changes have occurred at the protein-protein interface. These could account for the decrease in H_{AB} .

Analysis of the temperature-dependence of k_{ET} by eq 4 (Figure 3B) also clearly illustrates the effect of the Y294H mutation on λ and H_{AB} as both the slope and y-intercept of the lines

are altered. The change in slope reflects the decrease in E_a for the ET reaction. Since this is an ET reaction, the term in eq 2 which corresponds to E_a in eq 4 is $(G^\circ + \lambda)^2/4\lambda$. Thus, using the experimentally-determined values of λ for the two reactions, it is calculated that the E_a caused by the mutation of -3.6 kcal/mol corresponds to -0.156 eV. This describes the overall effect on the reaction of the 0.39 eV decrease in λ that was caused by the mutation. The change in the y-intercept caused by the mutation reflects the change in H_{AB} , which is present in the pre-exponential term in eq 2.

In addition to providing some insights into the factors which contribute to the magnitude of λ for ET reactions involving hemes, as well as other cofactors, these results provide some insight into why nature uses alternative mechanisms by which to stabilize and use utilize high-valent Fe^{V} equivalent heme species. These results clearly indicate that Compound I is a more efficient electron acceptor in redox reactions than *bis*- Fe^{IV} by virtue of the lower λ associated with the ET reaction. Single-heme enzymes which function as oxygenases utilize the Compound I species catalyze enzymatic reactions requiring a strong oxidant in which the substrate is in close proximity to the heme. In this case, it makes sense to not delocalize the oxidizing potential but to keep it centered on the heme. The results presented herein further suggest that this also minimizes the energy barrier for the short range ET that is part of the catalytic mechanism in the active site. In contrast, the diheme redox center of MauG catalyzes a reaction at the surface of the protein that is distant from the heme site. The ability to delocalize the oxidizing potential over two hemes which are themselves separated by several angstroms significantly reduces the distance for the long range ET required for catalysis. While this delocalization results in a significant increase in the λ associated with the reaction, that deficiency is more than made up for by the decrease in ET distance by approximately $20 \text{ \AA}$. The importance of this consideration is evidenced by that fact that Y294H MauG, which forms the Compound I state instead of the *bis*- Fe^{IV} , was unable to oxidize the natural substrate preMADH over the longer distance [12].

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ABBREVIATIONS

<i>bis</i> - Fe^{IV} MauG	redox state of MauG with one heme as $\text{Fe}^{\text{IV}}=\text{O}$ and the other as Fe^{IV}
E_m	oxidation-reduction midpoint potential
ET	electron transfer
H_{AB}	electron coupling
λ	reorganization energy
MADH	methylamine dehydrogenase

preMADH	the biosynthetic precursor protein of MADH with incompletely synthesized TTQ
TTQ	tryptophan tryptophylquinone

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Figure 1.

The *bis*-Fe^{IV} redox state of WT MauG and the Compound I redox state of Y294H MauG are illustrated. The porphyrin rings are represented as parallelograms with the iron and oxygen atoms, amino acid ligands indicated.

Figure 2.

The reduction of Compound I Y294H MauG by reduced amicyanin. A. Spectral changes associated with the ET reaction. The visible spectrum of the Soret region of Y294H MauG is shown immediately after formation of the Compound I state (solid line) and after (dashed line) reaction with reduced amicyanin. B. The kinetic plots depict global fits of the most statistically significant eigenvector of the SVD reduced three-dimensional data. The time courses for the disappearance of the initial species (solid line) and appearance of the final species (dashed line) are displayed. C. The time course for the change in absorbance at 404 nm after formation of the Compound I state. The solid line is the fit of the data by a single exponential transition. D. The dependence on amicyanin concentration of observed rate of ET from reduced amicyanin to Compound I. The line is a fit of the data by eq. 1. Each data point is the average of at least three measurements which vary by less than 10%.

Figure 3.

The temperature dependence of k_{ET} from reduced amicyanin to *bis*-Fe^{IV} WT MauG and Compound I Y294H MauG. A. The lines are a fit of the data by eq 2. *bis*-Fe^{IV} WT MauG is shown in circles, and Compound-I Y294H MauG is shown in squares. B. The lines are a fit of the data by eq 4. The reactions with *bis*-Fe^{IV} WT MauG are shown as circles with solid lines and the reactions with Compound I Y294H MauG are shown as squares with dashed lines.

Figure 4.
Docking model of amicyanin-Y294H MauG complex. MauG is colored pink with the heme shown as sticks and amicyanin is colored purple with the copper shown as a sphere.