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Characterization of the free energy dependence of an interprotein electron transfer reaction by variation of pH and site-directed mutagenesis

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ABSTRACT

The interprotein electron transfer (ET) reactions of the cupredoxin amicyanin, which mediates ET from the tryptophan tryptophylquinone (TTQ) cofactor of methylamine dehydrogenase to cytochrome *c*-551i have been extensively studied. However, it was not possible to perform certain key experiments in that native system. This study examines the ET reaction from reduced amicyanin to an alternative electron acceptor, the diheme protein MauG. It was possible to vary the ΔG° for this ET reaction by simply changing pH to determine the dependence of k_{ET} on ΔG°. A P94A mutation of amicyanin significantly altered its oxidation–reduction midpoint potential value. It was not possible to study the ET from reduced P94A amicyanin to cytochrome *c*-551i in the native system because that reaction was kinetically coupled. However, the reaction from reduced P94A amicyanin to MauG was a true ET reaction and it was possible to determine values of reorganization energy (λ) and electronic coupling for the reactions of this variant as well as native amicyanin. Comparison of the λ values associated with the ET reactions between amicyanin and the TTQ of methylamine dehydrogenase, the diheme center of MauG and the single heme of cytochrome *c*-551i, provides insight into the factors that dictate the λ values for the respective reactions. These results demonstrate how study of ET reactions with alternative redox partner proteins can complement and enhance our understanding of the reactions with the natural redox partners, and further our understanding of mechanisms of protein ET reactions.

1. Introduction

Long range electron transfer (ET) is a fundamental cellular process necessary for respiration, photosynthesis and redox reactions of intermediary metabolism. Although this is a fundamental biochemical process, there is still much to be understood with regard to the different ET mechanisms and how ET is controlled to direct the flow of electrons within the cell. Dysfunctional ET can cause disastrous cellular consequences, including increased production of reactive oxygen species. The rate of an ET reaction (k_{ET}) can be described by ET theory, often termed Marcus theory [1]. In this formalism the parameters that determine k_{ET} are temperature (T), free energy (ΔG°), electronic coupling (H_{AB}), and the reorganization energy (λ). The free energy is determined by the difference in the oxidation–reduction midpoint potential (E_m) values of the donor and acceptor redox sites. H_{AB} describes the extent of overlap of the wave functions of the reactant and product state and is dependent upon the ET distance and the nature of the intervening medium [2–4]. λ reflects the amount of energy needed to optimize the system for ET; i.e., the energy required to bring the reactant and product states to the state in which the ET event occurs. In principle, one could experimentally determine H_{AB} and λ values by examining the dependence of k_{ET} on either ΔG° or temperature. However, application of ET theory to interprotein ET reactions is challenging. The kinetic complexity of these reactions may mask the true k_{ET} [5,6]. Even when it is possible to monitor k_{ET}, in contrast to ET reactions involving small molecules, it is difficult and usually impossible to systematically alter the ΔG° for the reaction. It is possible to examine the temperature dependence of protein ET reactions, although one is limited to studies over a fairly narrow range of temperatures given the relative instability of proteins.

Amicyanin from *Paracoccus denitrificans* [7] is a blue copper protein that mediates ET from the protein-derived tryptophan tryptophylquinone (TTQ) [8] cofactor of methylamine dehydrogenase (MADH) [9] to the heme of cytochrome *c*-551i [10] via its type 1 copper site [11]. The type 1 copper site consists of a single copper ion coordinated by His53, His95, Cys92, and Met98 [12]. The complex of MADH, amicyanin, and cytochrome *c*-551i is one of the best characterized physiological protein

Abbreviations: bis-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; ET, electron transfer; H_{AB}, electronic coupling; λ, reorganization energy; MADH, methylamine dehydrogenase; preMADH, the biosynthetic precursor protein of MADH with incompletely synthesized TTQ; SVD, singular-value decomposition; TTQ, tryptophan tryptophylquinone; WT, wild-type.

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ET systems. The proteins have been structurally characterized by X-ray crystallography of the binary complex of MADH and amicyanin [13] and the ternary protein complex, which includes cytochrome *c*-551i [14]. The protein complexes were shown to be catalytically active and able to perform ET in the crystalline state [15–17]. The ET reactions to [18–21] and from [11] the type I copper center of amicyanin within the ternary protein complex have been studied in solution by stopped-flow spectroscopy, and many of these reactions have been analyzed by ET theory.

A curious feature of the reactivity of amicyanin in this system is that while these three proteins are isolated from *P. denitrificans* as individual soluble proteins, they must form the ternary protein complex in order to catalyze methylamine-dependent cytochrome *c*-551i reduction [11, 22]. Although it is a thermodynamically favorable reaction, MADH does not directly reduce cytochrome *c*-551i in the absence of amicyanin. Furthermore, reduced free amicyanin does not reduce oxidized cytochrome *c*-551i in the absence of MADH at physiologic pH because the E_m value of free amicyanin is much more positive than that of the cytochrome [23]. The redox properties of amicyanin are altered on complex formation with MADH so as to facilitate the reaction by lowering the E_m value of amicyanin in the complex under physiological conditions [23]. This phenomenon is related to the pH-dependence of the E_m value of free amicyanin which is due to a change in the geometry of the reduced form of the copper site upon deprotonation of the surface exposed His95 copper ligand (Fig. 1). When His95 is deprotonated the imidazole nitrogen points towards the copper ion to form a ligand. When it is protonated, the imidazole side chain rotates 180° out of the coordination sphere of the copper ion. Thus, the Cu(I) is three-coordinate while the Cu(II) is four-coordinate. There is no evidence that within the pH range of this study the Cu(II) ever becomes three-coordinate. As such, since the E_m value of amicyanin is pH-dependent, one would expect the ΔG° for the ET reactions of amicyanin to be pH-dependent. However, when amicyanin is in complex with MADH, this rotation of His95 is sterically hindered by MADH and so the Cu(I) remains four-coordinate and the E_m value of amicyanin in complex is now independent of pH [23]. In the present study, ET is studied from amicyanin to an alternative protein electron acceptor, the diheme enzyme MauG [24]. In the amicyanin–MauG complex, the pH-dependence of the E_m value of amicyanin is retained allowing the characterization of the dependence of k_{ET} on ΔG° for this interprotein ET reaction.

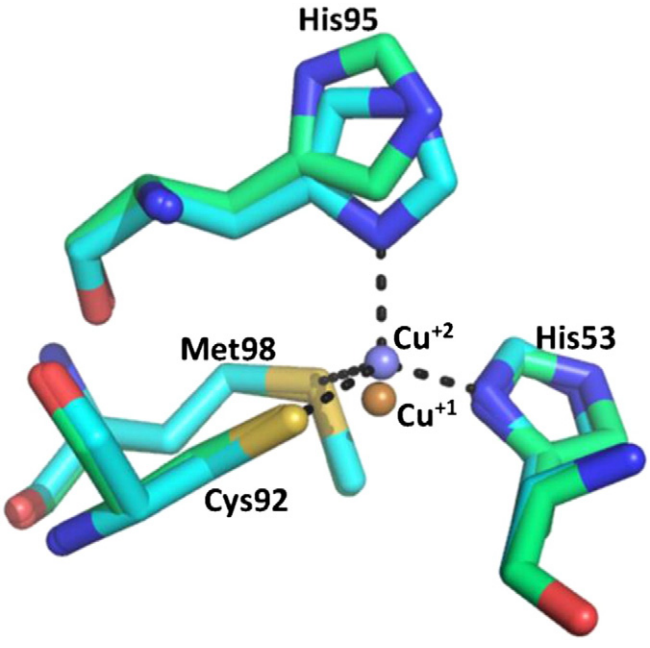

Fig. 1. The type 1 copper site of amicyanin. An overlay is shown of the structures of the oxidized protein (PDB ID: 2OV0) (carbons colored cyan) and the reduced protein (PDB ID: 2RAC) (carbons colored green) below the pK_a for the pH-dependence of the E_m value. The four amino acid residues that provide ligands are shown. The other atoms are colored blue for N, yellow for S and red for O. The positions of the copper ion in the two structures are indicated. The dashed lines indicate the coordination of the copper in the oxidized form.

The complexity of biological ET reactions must also be considered when attempting to apply ET theory to protein ET reactions. ET reactions may be either gated [25–27] or coupled [5]. In these cases, the observed rate of the ET reaction is not a true $k_{ET,}$ and therefore the true λ and H_{AB} associated with the ET event cannot be determined by ET theory. A P94A mutation of amicyanin increased its E_m value [28,29]. In addition to altering the E_m value, the P94A mutation also converted the ET from the reduced copper site to cytochrome *c*-551i from a true ET reaction to one which was kinetically coupled [30]. It is shown herein that the ET reaction of reduced P94A amicyanin to MauG is not coupled and so the effect of the mutation on the ET parameters of P94A amicyanin can now be characterized in the amicyanin–MauG system.

MauG is a di-*c*-type heme enzyme responsible for the posttranslational modification of a precursor of MADH (preMADH) to generate the protein-derived TTQ cofactor [31–33]. These oxidative biosynthetic reactions require the formation of a high-valent *bis*-Fe(IV) redox state of MauG in which one heme is present as Fe(IV)═O with an axial ligand provided by a His and the other is present as Fe(IV) with His-Tyr axial ligation and no exogenous ligand [31,34]. This *bis*-Fe(IV) form of MauG is used as the electron acceptor for reduced amicyanin in the present study. The studies of this ET reaction with amicyanin also provide an opportunity to further examine and gain insight into the ET properties of this unique high-valent heme species.

2. Materials and methods

2.1. Protein purification

Recombinant amicyanin was expressed in *Escherichia coli* BL21 (DE3) and purified from the periplasmic fraction as described previously for WT [35] and P94A [30] amicyanins. Recombinant MauG was expressed in and purified from *Paracoccus denitrificans* as described previously [24].

2.2. Determination of k_{ET}

The rates of the ET reactions from reduced amicyanin to *bis*-Fe(IV) 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 (unless otherwise specified), at the indicated temperature. One syringe contained the limiting reactant, 1–2 μM *bis*-Fe(IV) MauG, and the second syringe contained varying concentrations of reduced amicyanin. The concentration of amicyanin was always in at least 10-fold excess. Amicyanins were reduced by addition of a stoichiometric amount of sodium dithionite [36]. The *bis*-Fe(IV) MauG was generated by addition of equimolar hydrogen peroxide [34]. After rapid mixing the reactions were monitored over the range from 350 to 450 nm to observe the conversion of *bis*-Fe(IV) MauG to diferric 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 Global Fit.

Kinetic data were analyzed using the model described in Eq. (1) where A and B are amicyanin and MauG, respectively. In each of the single-turnover kinetic experiments, the observed rate constant (k_{obs}) was best fit to a single-exponential relaxation. The limiting first-order rate constant for each reaction was determined from the concentration dependence of k_{obs} using Eq. (2).

$$A(CuI) + B(FeIV) \overset{K_d}{\rightleftharpoons} A(CuI)-B(FeIV) \underset{k_{-ET}}{\overset{k_{ET}}{\rightleftharpoons}} A(CuII) + B(FeIII) \quad (1)$$

$$k_{obs} = k_{ET}[A(CuI)]/([A(CuI)] + K_d) + k_{-ET}. \quad (2)$$

2.3. Analysis of k_{ET} by ET theory

Data for the temperature-dependence of k_{ET} and the ΔG°-dependence of k_{ET} were each analyzed using Eq. (3). The other terms in this equation are Planck's constant (h) and the gas constant (R). An alternative equation (Eq. (4)) was also used which takes into account the exponential decrease in k_{ET} over distance. This equation can be used to predict the ET distance between the donor and acceptor redox sites [2–4]. The parameter β is used to quantitate the nature of the intervening medium with respect to its efficiency to mediate ET. The donor to acceptor distance is r, and r_0 is the close contact distance (3 Å). k_0 is the characteristic frequency of nuclei ($k_0 = 10^{13}\ s^{-1}$) which is the maximum ET rate when donor and acceptor are in van der Waals' contact and $\lambda = -\Delta G°$.

$$k_{ET} = \left[4\pi^2 H_{AB}{}^2/h(4\pi\lambda RT)^{0.5}\right] \exp\left[-\left(\frac{\text{Å}}{\Delta G + \lambda}\right)^2/4\lambda RT\right] \quad (3)$$

$$k_{ET} \approx k_0 \exp[-\beta(r-r_0)] \exp\left[-\left(\frac{\text{Å}}{\Delta G + \lambda}\right)^2/4\lambda RT\right]. \quad (4)$$

2.4. In silico docking of WT amicyanin and MauG

A docking model of WT amicyanin with MauG was constructed by using the ZDOCK utility version 3.02 and server (http://zdock.umassmed.edu) [37]. The PDB files of WT amicyanin (PDB ID: 2OV0) and MauG (PDB ID: 3L4M, chain A) were used in model building. All residues of both chains were used to explicitly search the rotational space, and the translational space was searched using fast Fourier transform. No residues were blocked from the binding site during docking, and no specific residues were selected for filtering binding site predictions.

3. Results

3.1. Determination of k_{ET} for the reaction of bis-Fe(IV) MauG with reduced amicyanin

The reaction of *bis*-Fe(IV) MauG with varied concentrations of reduced amicyanin at pH 7.5 at 25 °C exhibited saturation behavior (Fig. 2). The fit of this data to Eq. (2) yielded a limiting first-order rate constant (k_{ET}) of 22 ± 2 s^{-1} and a K_d of 165 ± 50 μM. It should be noted that reduction of *bis*-Fe(IV) MauG to diferric MauG requires two electrons and that 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. This means that after the first Cu(I) amicyanin reacts it dissociates and then a second Cu(I) amicyanin binds and reacts. The fact that a single exponential relaxation is observed means that the dissociation/association steps are very rapid relative to k_{ET} and since excess Cu(I) amicyanin is present, rebinding of the Cu(II) amicyanin after the single turnover is not a factor.

3.2. Temperature dependence of k_{ET} for the reaction of bis-Fe(IV) MauG with reduced amicyanin

The k_{ET} for the ET reaction from reduced amicyanin to *bis*-Fe(IV) MauG was determined at temperatures from 10 °C to 30 °C (Fig. 3). The *bis*-Fe(IV) MauG was unstable at higher temperatures and so this limited the range that could be studied. The E_m value for the *bis*-Fe(IV)/diferric couple of MauG is unknown. E_m values for Fe(IV)/Fe(III) couples in heme-dependent peroxidases have been determined

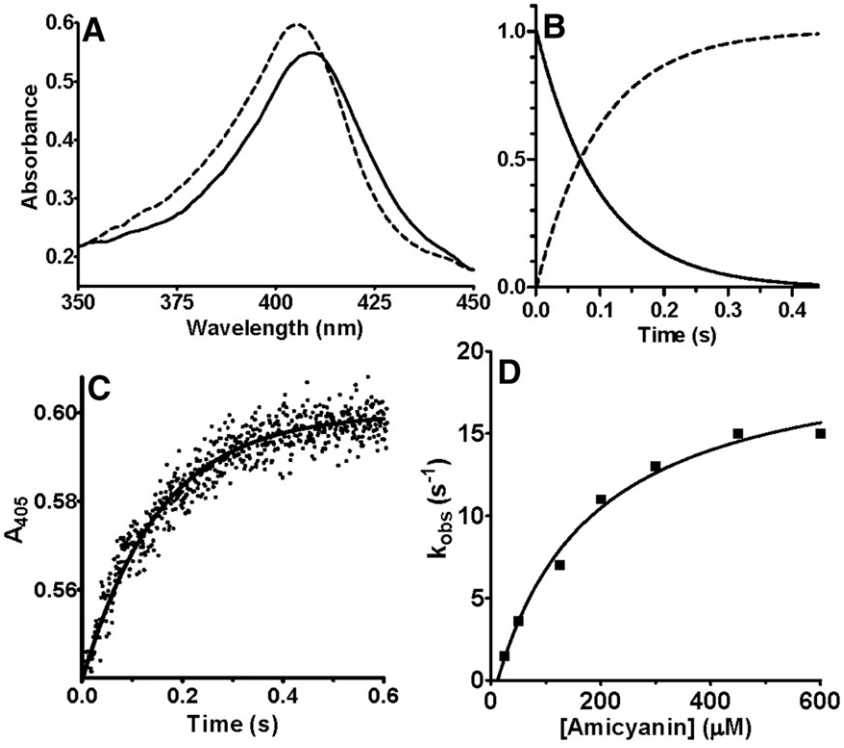

Fig. 2. The reduction of *bis*-Fe(IV) MauG by reduced amicyanin. A. Spectral changes associated with the ET reaction. The visible spectrum of the Soret region of the hemes of *bis*-Fe(IV) before (solid line) and after (dashed line) reaction with reduced amicyanin. B. The kinetic plots depict global fits 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, each of which were fit by a single exponential transition. C. The time course for the change in absorbance at 405 nm after formation of the *bis*-Fe(IV) state. The solid line is the fit of the data by a single exponential transition. D. The concentration dependence of observed rate of ET from reduced amicyanin to *bis*-Fe(IV). The line is a fit of the data by Eq. (2).

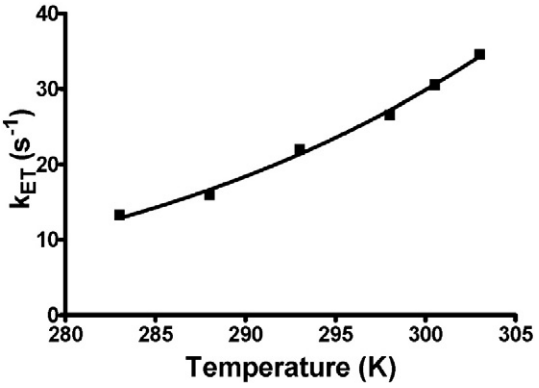

Fig. 3. The temperature dependence of k_{ET} from reduced amicyanin to *bis*-Fe(IV). The line is a fit of the data by Eqs. (3) and (4). Those two fits are superimposable.

and these values range from 724–1160 mV [38]. The results of previous studies of the ET reaction of *bis*-Fe(IV) MauG with preMADH determined that the reduction of the *bis*-Fe(IV) center during hopping-mediated ET via a Trp radical was endergonic by ~200–300 mV [39]. E_m values for the Trp radical/Trp redox couple have been determined to be in the range of 890–1080 mV [40,41]. This suggested that the E_m value for the Fe(IV)/Fe(III) couple in MauG is at the low end of what has been reported for similar systems. As such, for the analysis of the temperature-dependence of the ET rates the E_m value of 724 mV for MauG and the known E_m value of amicyanin at pH 7.5 of 265 mV [23] were used to determine the ΔG° for the reaction to input into Eq. (3). The fit of the data to Eq. (3) yielded values of $\lambda = 2.3 \pm 0.1$ eV and $H_{AB} = 0.6 \pm 0.1$ cm^{-1}. Using Eq. (4) it is also possible to obtain an experimentally-determined estimate of the ET distance. In analyzing protein ET reactions by Eq. (4), average β values of 0.7–1.4 $Å^{-1}$ have been used to describe the nature of the intervening protein medium between the redox centers [2–4]. Analysis of these data inputting β values in this range yielded a range of ET distances of 12–21 Å.

In order to gain insight into the relative orientations of the redox centers in the amicyanin–MauG complex, a protein docking model was constructed using the ZDOCK program [37] from the crystal structures of amicyanin and MauG (Fig. 4). This model places the amicyanin copper approximately 16.7 Å from the porphyrin ring edge of the ferryl heme and 17.9 Å from the ferryl heme iron. These distances correlate well with the experimentally-determined range of ET distance obtained from analysis of the temperature-dependence of the ET rate using Eq. (4).

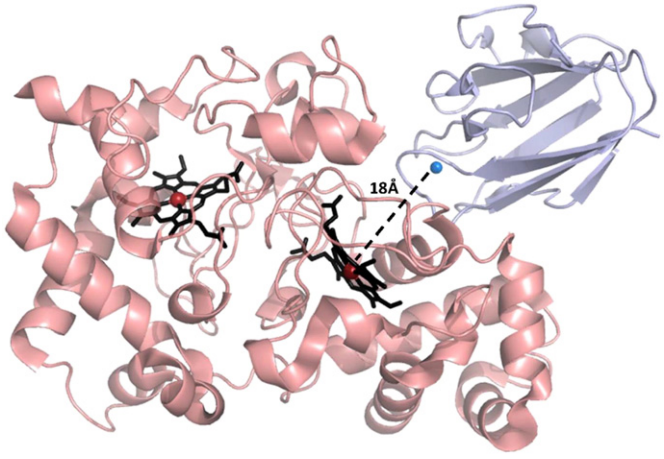

Fig. 4. Docking model of the amicyanin–MauG complex. MauG is colored pink with the porphyrin rings black and irons red. Amicyanin is colored purple with the copper blue. The distance from the copper to the high-spin heme iron is indicated.

3.3. ΔG°-dependence of k_{ET} from reduced amicyanin to bis-Fe(IV) MauG

As stated earlier, the E_m value of type I copper site of free amicyanin is pH-dependent due to the fact that the His95 ligand is lost when it is protonated (Fig. 5). The pK_a for this phenomenon is 7.5 [23]. This pH-dependence was not a factor in the ET reaction between MADH and amicyanin because when amicyanin is in complex with MADH the rotation of the His95 ligand out of the copper coordination sphere is sterically prevented. Thus, the E_m value of amicyanin in complex with MADH is pH-independent [23]. Inspection of the docking model for the amicyanin–MauG complex suggested that movement of His95 was not likely to be hindered in this complex, meaning that the E_m value of amicyanin in this complex should be the same as free amicyanin. To examine this further the ET reaction from reduced amicyanin to *bis*-Fe(IV) MauG was examined over a range of pH from 5.6 to 7.9. The range could not be extended further due to instability of the proteins. As can be seen in Fig. 5, k_{ET} increased with increasing pH, consistent with the decrease in E_m value of amicyanin with increasing pH, and consequently an increasingly less negative ΔG° for the ET reaction.

In order to assess whether the dependence of k_{ET} on pH was truly reflecting the ΔG°-dependence of k_{ET}, and not some other effect of pH on the system, these data were analyzed using Eq. (3). A curve simulating the predicted ΔG°-dependence of k_{ET} was constructed using the values of λ and H_{AB} that were obtained from the fit of the data in Fig. 3 at a fixed temperature of 25 °C at which the pH-dependence studies were performed. The values of ΔG° for the reactions at different values of pH were calculated using the known E_m values for free amicyanin at each pH and the E_m value of 724 mv for MauG. As can be seen in Fig. 6, the data points fall nearly exactly on the predicted curve for the ΔG°-dependence of k_{ET}.

As stated above, the E_m value for MauG used in this analysis is an estimation. As per Eq. (3), uncertainty in this value could influence the fitted value of λ. The E_m value that was used is at the low end of literature values for similar systems. If the E_m value of MauG were more positive, then the corresponding fitted value of λ would be proportionately greater. This seems unlikely since the fitted value of λ of 2.3 eV is at the high end of what would be considered a λ value for a true ET reaction. The data in Fig. 6 provide further evidence that this λ value describes a true ET reaction. These data also provide evidence that the E_m value of MauG and λ for the reaction do not vary with pH since the change in ΔG° values determined solely from the pH-dependence of amicyanin fit so well to the curve that is described by pH-independent values for those parameters.

3.4. ET from reduced P94A amicyanin to bis-Fe(IV) MauG

To further explore the ΔG°-dependence of k_{ET} from reduced amicyanin to *bis*-Fe(IV) MauG, studies were performed with the P94A

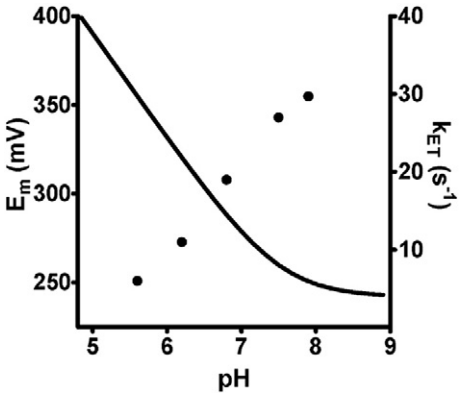

Fig. 5. Dependence on pH of the E_m value of amicyanin and k_{ET} from reduced amicyanin to *bis*-Fe(IV). The solid line describes the pH dependence of the E_m value of free amicyanin [23]. The circles are the k_{ET} values at the indicated pH.

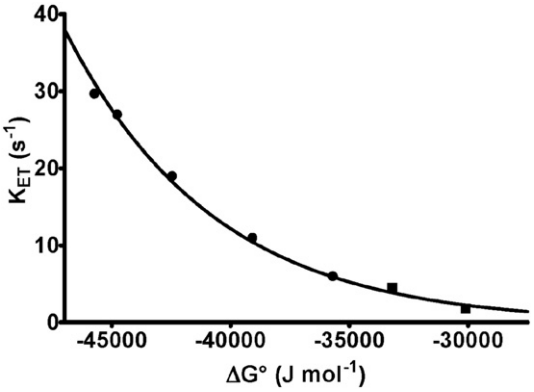

Fig. 6. Free energy dependence of k_{ET} from reduced amicyanin to *bis*-Fe(IV). The line which was generated using Eq. (3) uses the experimentally-determined values of H_{AB} and λ that were determined from the data in Fig. 3 to describe the predicted dependence of k_{ET} on ΔG° at 25 °C. The experimentally determined k_{ET} values are shown for the reactions WT amicyanin (circles) and P94A amicyanin (squares) which were determined at different pH values to allow variation of ΔG°.

amicyanin variant [28]. The mutation of Pro94 to Ala increased its E_m value and shifted the pK_a for the pH dependence of the E_m value to more acidic values [29]. The reaction of *bis*-Fe(IV) MauG with varied concentrations of reduced P94A amicyanin at pH 7.5 exhibited saturation behavior. The fit of this data to Eq. (2) yielded a k_{ET} of 7.8 ± 0.6 s^{-1} and a K_d of 240 ± 82 μM. The k_{ET} for the reaction of P94A amicyanin with *bis*-Fe(IV) MauG was also determined at pH 6.0. At pH 6.0 and 7.5 the E_m values of P94A amicyanin are 412 and 380 mV, respectively [29]. The ΔG° values for these reactions are less than any of those for the reactions of WT amicyanin so that the range of the ΔG°-dependence of the reaction could be expanded. As seen in Fig. 6 these data points also fall on the predicted curve for the ΔG°-dependence of k_{ET}.

The ET reaction from reduced P94A amicyanin to cytochrome *c*-551i could not be analyzed by ET theory because the mutation converted this true ET reaction to a coupled ET reaction [30]. It is noteworthy that the k_{ET} values for the reactions reduced P94A amicyanin are consistent with those for a true ET reaction with λ and H_{AB} values identical to those of the reaction with WT amicyanin (discussed later).

4. Discussion

There are relatively few examples of studies of protein ET reactions in which it was possible to examine the ΔG°-dependence of k_{ET}. Gunner and Dutton [42] were able to study the ΔG° dependence of the ET from bacteriochlorophyll to ubiquinone in the photosynthetic reaction center from *Rhodobacter sphaeroides* by substituting the native Q_A with quinones with different E_m values. Scott et al. [43] studied intramolecular ET in cytochrome b_5 labeled with Ruthenium(II) polypyridine complexes with different E_m values. Farver et al. [44] recently studied the intramolecular ET reactions between the copper of azurin a disulfide radical anion with several site-directed mutants that spanned a wide range of E_m values. The ΔG°-dependence of the ET reaction between MADH and amicyanin had previously been examined by looking at the rates of the forward and reverse reactions of amicyanin with different redox forms of MADH [19,21]. The use of the pH-dependent variation of ΔG° that is described herein is a novel approach for analysis of an interprotein ET reaction by ET theory. This approach could be applied to other systems in which the electron donor or acceptor site has a pH-dependent E_m value. As in this study, it could also be possible to use amicyanin or a another protein with a pH-dependent E_m value to study the ET reactions with a non-physiologic redox protein of interest and gain information on the ET parameters associated with ET to or from its redox center.

In the present study, it is interesting to compare the value of λ obtained in this study of 2.3 eV with the λ values previously obtained for the reactions of amicyanin with MADH of 2.3 eV [19,21] and with cytochrome *c*-551i of 1.1 eV [11]. While the ET reaction with the cytochrome may seem more similar to the reaction with MauG, in that the redox center is a heme, the λ values are quite different and more similar to the reaction with the TTQ cofactor of MADH. However, it should be noted that the redox center of MauG is not just a single heme, but two hemes with an intervening Trp residue that share spin and charge [45]. In this sense, the diheme redox center of MauG is more similar to TTQ. TTQ is comprised of two covalently-linked modified Trp residues which share charge. Another similarity is that while the heme iron of cytochrome *c*-551i is shielded from solvent, the high-spin heme of MauG and the quinone moiety of TTQ are each exposed to solvent. The fact that the reorganization energies associated with the diheme site of MauG and the TTQ of MADH could require changes in lengths and angles of multiple bonds, as well as contributions from reorganization of solvent is likely reflected in the similar and relatively large values of λ for their respective reactions with amicyanin. The magnitude of these λ values is consistent with the contribution of these different processes that poise the system for the ET event and are kinetically indistinguishable from that event.

It is significant that whereas the mutation of Pro94 to Ala in amicyanin converts the true ET reaction from reduced amicyanin to cytochrome *c*-551i to a kinetically coupled ET reaction, the reaction of reduced P94A amicyanin with *bis*-Fe(IV) MauG remains a true ET reaction. This is evident from the results shown in Fig. 6. The fact that the rates of reaction of P94A amicyanin with MauG lie on the ΔG°-dependence curve indicate that the λ and H_{AB} for the reaction with reduced amicyanin are not affected by the P94A mutation, despite the fact that the E_m value is significantly altered by the mutation. The basis for the change in the E_m value of the type 1 copper site of P94A amicyanin was that a consequence of the mutation was the introduction of a hydrogen-bond to the Cys thiolate ligand and alteration of the position of the Cu^+ ion [28]. It is noteworthy that the λ associated with its ET reaction from the copper site was not altered in the reaction with MauG. This is information that could not be obtained from the physiologically relevant and well-studied MADH-amicyanin-cytochrome *c*-551i complex because the mutation altered the kinetic mechanism of that reaction such that it was coupled [30].

In true ET reactions, the ET event is the rate limiting step. Thus, k_{obs} is k_{ET}. However, gated and coupled ET reactions cannot be properly analyzed by ET theory. In a gated ET reaction a kinetically indistinguishable reaction step precedes and is required for ET, and the rate of that step (k_x) is slower than k_{ET}. Thus, k_{obs} is k_X rather than k_{ET} [26,27]. In a coupled ET reaction, k_x is faster than k_{ET} but thermodynamically unfavorable such that the equilibrium constant for that step (K_x) is <1. In this case k_{obs} is the product of K_X and k_{ET} [46]. The basis for the coupled reaction mechanism of ET from reduced P94A amicyanin to cytochrome *c*-551i in the ternary protein complex with MADH is as follows. The copper site in reduced P94A amicyanin was found to exist in an equilibrium between two different conformations. In one of these conformations, the Met98 copper ligand is replaced by a water molecule and the copper atom is displaced by 1.4 Å and disfavored for ET to the cytochrome. As such the observed rate of ET is influenced by the equilibrium constant for the interconversion of these two conformations [30]. This is evidently not the case for the reaction with *bis*-Fe(IV) MauG. This may be due to differences in the protein–protein interactions at the protein interface in the respective ET complexes. As discussed earlier, the movement of the His95 copper ligand is constrained at the amicyanin-MADH interface but it is not constrained when in complex with *bis*-Fe(IV) MauG. Residue 94 is also in this interface. The P94A mutation increased the K_d for complex formation with MADH [30] and with *bis*-Fe(IV) MauG (discussed earlier). It is possible that whereas the protein–protein interactions at the amicyanin-MADH interface shift the equilibrium to the conformation that disfavors ET, the protein–protein interactions at the P94A amicyanin–MauG interface shift the equilibrium strongly to the conformation that favors ET, resulting in a true ET reaction.

This study includes the first description of the use of variation of pH as a means to examine the free energy dependence of an interprotein ET reaction. The amicyanin–MauG system allowed study of the ET properties of amicyanin in this manner, which could not be studied this way in the native system with MADH and cytochrome *c*-551i. This study includes the first description of how an alternative redox protein partner can be used to study true ET from a redox cofactor which was obscured due to the kinetic complexity of its reaction with its native electron acceptor. This study also provides an opportunity to examine an interprotein ET reaction to the *bis*-Fe(IV) redox state of MauG. These results demonstrate how ET reactions with alternative redox partner proteins can complement and enhance our understanding of the reactions with the natural redox partners, and further our understanding of mechanisms of protein ET reactions.

Transparency document

The Transparency document associated with this article can be found, in online version.

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