Site-Directed Mutagenesis of Phe 97 to Glu in Amicyanin Alters the Electronic Coupling for Interprotein Electron Transfer from Quinol Methylamine Dehydrogenase†

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ABSTRACT: Conversion by site-directed mutagenesis of Phe 97 of amicyanin to Glu significantly decreases the rate constant for the electron-transfer reaction from the quinol form of methylamine dehydrogenase to amicyanin. It is shown that the $\Delta G°$ and reorganizational energy (λ) associated with the electron-transfer reaction are unaffected by the mutation and that the decrease in the electron-transfer rate is attributable completely to a decrease in the electronic coupling matrix element (H_{AB}). Phe 97 is not a part of the predicted pathway for electron-transfer from the tryptophan tryptophylquinone cofactor of MADH to the copper of amicyanin. The most likely explanation for these results is that the mutation of this residue at the protein−protein interface causes an increase in the interprotein distance within the protein complex. The change in distance necessary to cause the observed reduction of H_{AB} is calculated assuming a range of β values, and assuming either solely a direct distance dependence or a pathway dependence, for the long-range electron-transfer reaction. Thermodynamic analysis of the association constants for complex formation reveal that the reaction with the mutant amicyanin exhibits a large positive change in heat capacity whereas this is not observed in the reaction with the wild-type. This may be explained by the replacement of a hydrophobic residue with a polar residue at what is normally a hydrophobic protein−protein interface. The impact of these results on possible explanations for the relatively large reorganizational energy associated with this interprotein electron-transfer reaction is also discussed.

Methylamine dehydrogenase (MADH)[1] and amicyanin from *Paracoccus denitrificans* form one of the best characterized physiologic electron-transfer (ET) complexes of proteins. High-resolution crystal structures are available for the binary complex of MADH and amicyanin (*1*) and for a ternary protein complex (*2*) which also includes the cytochrome *c* which is the electron acceptor for amicyanin. The protein complexes have been shown to be functional in the crystalline state by single-crystal polarized absorption spectroscopy (*3*). The ET reaction between the tryptophan tryptophylquinone (TTQ) (*4*) prosthetic group of MADH and the type I copper center of amicyanin has been studied in solution by stopped-flow spectroscopy. The reactions of several different redox forms of these proteins were characterized. Stable quinol and semiquinone forms of MADH were generated by titration with dithionite, and the forward and reverse ET reactions of these redox forms with amicyanin were studied. It was possible in this manner to examine the $\Delta G°$ dependence of the ET rate (*5*). The temperature dependence of the rate of the ET reaction from quinol MADH to amicyanin was also determined (*6*). Analysis of the $\Delta G°$ and temperature dependencies of the limiting first-order rate constants by Marcus theory (*7*) yielded values for the reorganizational energy (λ) and electronic coupling (H_{AB}) associated with the ET reaction, and predicted an ET distance between redox centers that closely matched the distance seen in the crystal structures of the complexes (*6*). Reduction of MADH by substrate yields an aminoquinol form of MADH in which the substrate-derived amino group remains covalently bound to the reduced TTQ (*8*). Analysis of the ET reaction to amicyanin from this form of MADH revealed that, unlike the ET reaction from quinol MADH, the reaction from the aminoquinol was gated by a proton-transfer reaction step (*9, 10*).

The validity of the protein interface between MADH and amicyanin which is revealed in the crystal structures was proven by site-directed mutagenesis of interfacial amino acid residues of amicyanin (*11*). The K_d for the MADH/amicyanin complex was significantly increased either by conversion of Phe 97 to Glu, which prevents stabilization of the complex by hydrophobic van der Waals contacts with MADH, or by conversion of Arg 99 to Asp or Leu, which prevents stabilization of the complex by a salt bridge with a carboxylate on MADH.

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[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; ET, electron transfer; H_{AB}, electronic coupling; λ, reorganizational energy; E_m, oxidation−reduction midpoint potential.

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Of the site-directed mutants made thus far, only one has caused the ET rate to change by greater than an order of magnitude. We report here that the Phe 97 to Glu mutation decreases the rate constant for the ET reaction from quinol MADH to amicyanin by approximately 24-fold. It is shown that the $\Delta G°$ and λ associated with the ET reaction are unaffected by the mutation and that the decrease in the ET rate is attributable completely to a decrease in H_{AB}. This may be explained by an increase in the interprotein distance within the protein complex. Comparison of the thermodynamic parameters which describe the associations of MADH with the wild-type and mutant amicyanins provides insight into the nature of the factors which have disrupted the protein−protein interfaces in the respective complexes and caused the decrease in the electronic coupling matrix element.

EXPERIMENTAL PROCEDURES

Purification of MADH (*12*) and amicyanin (*13*) from *P. denitrificans* was as described previously. The methods for the preparation of the F97E mutant of amicyanin, its expression in *Escherichia coli*, and its purification have been previously described (*11*). Protein concentrations were determined from the previously reported extinction coefficients of MADH (*14*) and amicyanin (*13*). Reduced forms of proteins were prepared by titration with sodium dithionite. Fully oxidized forms of proteins were prepared by reaction with potassium ferricyanide. All reagents were obtained from commercial sources.

An On-Line Instrument Systems (OLIS) RSM1000 stopped-flow spectrophotometer was used for transient kinetic experiments. For each set of mixing experiments, the concentration of MADH was fixed at 2 μM and the amicyanin concentration was varied. The amicyanin concentration was always in at least 10-fold excess of MADH to maintain pseudo-first-order reaction conditions. Values of k_{obs} were determined from global fits of the raw data or analysis of the change in absorbance at 443 nm. This is an isosbestic point for the semiquinone and oxidized forms of MADH where MADH exhibits a $\Delta\epsilon$ of 26 200 M^{-1} cm^{-1} on conversion from the reduced to the semiquinone form (*14*). The changes in absorbance with time were best described by a single exponential rise (eq 1). In all reactions, saturation behavior

$$A_{443} = C(1 - e^{-kt}) + b \quad (1)$$

was observed and data were fit according to the model (eq 2) and equation (eq 3, ref 15) given below. Nonlinear curve

$$\mathrm{MADH_{red}} + \mathrm{amicyanin_{ox}} \overset{K_d}{\rightleftharpoons} \mathrm{MADH_{red}/amicyanin_{ox}} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} \mathrm{MADH_{semi}/amicyanin_{red}} \quad (2)$$

$$k_{obs} = \frac{k_3[\mathrm{amicyanin}]}{[\mathrm{amicyanin}] + K_d} + k_4 \quad (3)$$

fitting of data was performed with OLIS software and the Sigma Plot 5.0 and Enzfitter computer programs.

The E_m values of amicyanins were determined by spectrochemical titration. The ambient potential was measured directly with a Corning combination redox electrode which was calibrated using quinhydrone (a 1:1 mixture of hydroquinone and benzoquinone) as a standard with an $E_m$value of +286 mV at pH 7.0 (*16*). The reaction mixtures contained 20−80 μM amicyanin in 10 mM potassium phosphate buffer, pH 7.5, at 25 °C. Ferricyanide (0.2 mM) and quinhydrone (0.4 mM) were present as mediators. The mixture was titrated by addition of incremental amounts of ascorbate, which was used as a reductant. The absorption spectrum of amicyanin was recorded at different potentials, and the concentrations of the oxidized and reduced forms were determined by comparison with the spectra of the completely oxidized and reduced forms (*17*). $E_m$values were obtained from plots of potential versus log(oxidized amicyanin/reduced amicyanin).

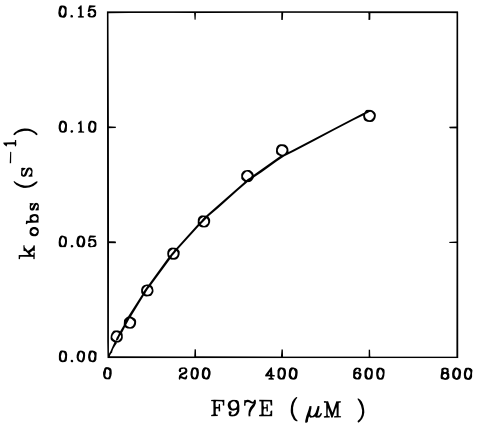

FIGURE 1: Rates of oxidation of quinol MADH by the F97E mutant amicyanin. These experiments were performed at 29 °C in 10 mM potassium phosphate, pH 7.5, as described under Experimental Procedures. The solid line is a fit of the data to eq 3.

RESULTS

Effect of the F97E Mutation on the ET Rate. Quinol MADH was mixed with different concentrations of the oxidized wild-type and F97E mutant amicyanins. All reactions exhibited monophasic kinetics. It has been previously established that the ET reaction from quinol MADH is rate-limiting for the overall two-step oxidation reaction of quinol MADH by excess amicyanin. This is because the rate of the second ET reaction from semiquinone MADH to amicyanin is much faster than the first (*18*). Because of the high K_d value for the F97E mutant, it was not possible to perform experiments with high enough concentrations of amicyanin to fully saturate the reaction. It was possible, however, to obtain enough data at subsaturating concentrations to define the hyperbolic dependence of rate on concentration (Figure 1). From the concentration dependence of k_{obs}, it was possible, by fitting these data to eq 3, to determine the limiting first-order rate constant for the ET reaction (Figure 1). At 29 °C, the ET rate constants were 4.6 and 0.19 s^{-1}, respectively, for the wild-type and F97E mutant. The fitted curves for data sets that were obtained at each temperature passed through the origin (Figure 1 and data not shown), indicating that the reaction was irreversible. This will be true either if $k_4 = 0$, or if the dissociation of the $\mathrm{MADH_{semi}/amicyanin_{red}}$ complex is much more rapid than k_4 (see eq 3). As discussed previously (*5*, *6*) the ET rate for this reaction is relatively slow because of its small driving force (ΔE_m= +31 mV) relative to the large λ (2.3 eV) exhibited by this reaction.

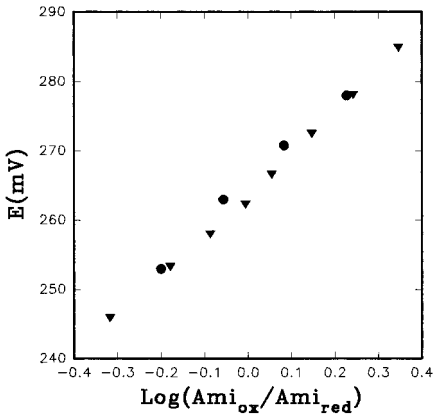

FIGURE 2: Spectrochemical redox titration of wild-type (●) and F97E (▼) amicyanin. These experiments were performed as described under Experimental Procedures.

Redox Potential of Amicyanin is Not Affected by the F97E Mutation. Spectrochemical redox titration of the F97E mutant amicyanin yielded an $E_m$value which was within experimental error (±7 mV) of that obtained from titration of the wild-type amicyanin (Figure 2). We have previously shown that the $E_m$value of amicyanin decreases by 73 mV on complex formation with MADH (*19*). Therefore, a redox titration of the F97E mutant in complex with amicyanin was also performed. Because of the large K_d value for complex formation with the mutant (i.e., 228 μM) it was not possible to completely saturate the system. At MADH concentration in excess of 350 μM, the background absorbance of MADH becomes too high to allow measurement of spectral changes in amicyanin. At subsaturating conditions, one obtains an $E_m$value that is intermediate between those of free and complexed amicyanin. Under conditions where approximately 65% of the amicyanin is in complex with MADH, an $E_m$value of 245 mV was obtained. Correction for incomplete complex formation and extrapolation of the data to 100% complex formation yields an $E_m$value of 228 ± 12 mV. This is essentially the same as the previously determined $E_m$value of 221 ± 7 mV that was obtained for the wild-type amicyanin in complex with MADH (*19*). Thus, it was concluded that the mutation caused no change in the $\Delta G°$ value for the ET reaction from quinol MADH to oxidized amicyanin.

Temperature Dependence and Marcus Analysis of ET Rates. Analysis of the concentration dependence of k_{obs} was performed at several temperatures ranging from 20 to 40 °C. The variation with temperature of the limiting first-order rate constant for the ET reaction from quinol MADH to amicyanin (k_{ET}) was analyzed by ET theory (eq 4) (*7*). λ is the

$$k_{ET} = \frac{4\pi^2 H_{AB}{}^2}{h\sqrt{4\pi\lambda RT}} \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \qquad (4)$$

reorganizational energy, H_{AB} is the electronic coupling matrix element, h is Planck's constant, and R is the gas constant. As discussed earlier, the F97E mutation caused no change in the $\Delta G°$ value for the ET reaction. A value of −3.0 kJ mol^{-1} (ΔE_m= +31 mV) was used for each reaction. This value was previously shown to be the driving force for the one-electron oxidation by amicyanin of quinol MADH to the semiquinone (*5*). From fits to eq 4 (Figure 3) of the

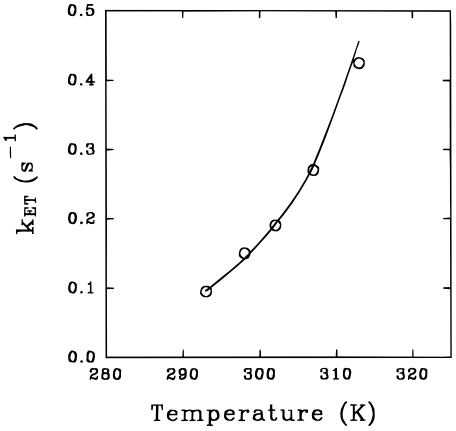

FIGURE 3: Analysis of the temperature dependence of the ET rate constant for the reaction of quinol MADH with the F97E amicyanin mutant. Values of k_{ET} are the values of k_3 which were obtained from fits of the concentration dependence of the observed rate to eq 3. The solid line is a fit of the data to eq 4.

Table 1: Thermodynamic Analysis of the Electron-Transfer Reactions from Quinol MADH to Amicyanins

activation and Marcus parameters	amicyanin	
	wild-type[a]	F97E[b]
λ (eV)	2.3 ± 0.1	2.3 ± 0.1
H_{AB} (cm^{-1})	12 ± 4	2.9 ± 0.8
ΔH* (kJ mol^{-1})	59 ± 4	53 ± 2
ΔS* (J mol^{-1} K^{-1})	−28 ± 13	−82 ± 1

[a] Values were taken from ref 6. [b] Values were obtained from fits of the data in Figure 3 to eqs 4−6. Identical values of λ were obtained from fits using eqs 4 and 6.

dependence of the ET rate constant on temperature, it was possible to obtain values for λ and H_{AB}. These data were also fit to the Eyring eq (eq 5) to obtain values for the

$$\ln(k_{ET}h/k_BT) = -\Delta H*/RT + \Delta S*/R \qquad (5)$$

activation parameters that are associated with the ET reaction. The values obtained for the reaction with the F97E mutant were compared with those previously obtained for the reaction with the wild-type amicyanin (Table 1). For a nonadiabatic ET reaction, ΔH* and ΔS* values are difficult to interpret and such a reaction is more appropriately described by eq 4 (*7*). It is clear from the analysis of the temperature dependence of these ET reactions by eq 4 that the decrease in rate observed with the F97E mutant is attributable completely to a decrease in H_{AB}. The implications of the decreased coupling between redox centers in the mutant complex relative to the wild-type complex are discussed below.

Correlation of Changes in H_{AB} with Structure. An alternative form of eq 4 is given in eq 6, in which H_{AB} is

$$k_{ET} = k_0 \exp[-\beta(r - r_0)] \exp[-(\Delta G° + \lambda)^2/4\lambda RT] \qquad (6)$$

factored into terms which define the ET distance between redox centers (r is the center-to-center distance and r_0 is the close contact distance which is taken to be 3 Å) and describe the nature of the medium which separates the redox centers (β). The other parameter (k_0) is the characteristic frequency of the nuclei which is set at 10^{-13} s^{-1}.

There is debate over what the most appropriate value of β is for a protein ET reaction, and whether it is even appropriate to use a single β value for a protein ET reaction or whether β varies with segments of specific pathways through the protein matrix (reviewed in refs 20−22). We have fit our data to eq 6 using β values ranging from 1.4 to 0.7 Å^{-1}. For the wild-type amicyanin, such an analysis yielded values of r that ranged from 7.9 to 12.4 Å, and which compare closely with the distance of 9.4 Å that is observed in the crystal structures (*1*, *2*). For the F97E mutant amicyanin, this analysis yields values of r that range from 9.8 to 16.5 Å. Thus, if one believes that a single β value is appropriate for describing this interprotein ET reaction, then this analysis indicates that the F97E mutation has increased the ET distance between redox centers in the complex by 1.9−4.1 Å, depending on the choice of β.

An alternative interpretation of the decrease in H_{AB} may be considered in the context of a Pathways (or Greenpaths) analysis (*23*) of the complex structure. This algorithm calculates the relative efficiency of ET pathways according to eq 7 where i ranges over the pathway steps and ϵ_i is a

$$H_{AB} \propto \Pi\epsilon_i \qquad (7)$$

wave function decay factor for step i. For the complex with the wild-type amicyanin, the most efficient predicted pathway (i.e., at least an order of magnitude more efficient than alternative pathways) involves a through-space jump of 2.6 Å from a hydrogen on the surface-exposed Trp 108 indole ring of TTQ to the lone pair orbital of the carbonyl oxygen of Pro 94 of amicyanin (Figure 4).[2] The pathway then follows six covalent bonds via the His 95 copper ligand to the copper atom. Phe 97 is not part of the predicted pathway. It is, however, positioned on the surface of amicyanin close enough to make van der Waals contact with residues on MADH. Carbon atoms on the phenyl ring of Phe 97 are within 3.5 Å of Pro 100 on the MADH light subunit and within 3.4 Å of Arg 184 on the MADH heavy subunit. If one assumes that the replacement of Phe 97 with Glu has disrupted the MADH/amicyanin interface, but not the through-bond segment of the pathway from the carbonyl oxygen of Pro 94 to copper, then the observed decrease in H_{AB} would be due to an increase in the length of the through-space jump from MADH to amicyanin. According to the Pathways algorithm, the wave function decay factor for the through-space jump is defined as equal to $0.6e^{-\beta(r-1.4)}$, with β for this process being equal to 1.7 Å^{-1} (*23*). To account for the observed decrease in H_{AB} by this analysis, the distance for the through-space jump in the complex with the F97E mutant must have increased by 0.9 Å. It may also be argued that, for the through-space portion of the pathway, one may use a β of 2.8 Å^{-1}, instead of 1.7 Å^{-1}. The former is believed to describe ET through a vacuum (*20*). If that β value is used in this calculation, then the distance for the through-space jump between proteins is predicted to have increased by only 0.5 Å.

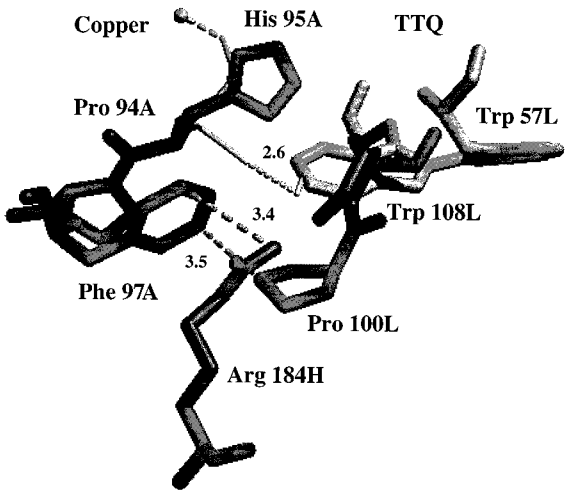

FIGURE 4: Binding site and ET pathway for the reaction of MADH with wild-type amicyanin. Selected residues at the MADH/amicyanin interface are displayed. Residues of amicyanin are indicated by A, residues of the light subunit of MADH are indicated by L, and residues of the heavy subunit of MADH are indicated by H. The copper of amicyanin and the indole rings of TTQ derived from Trp 108L and Trp 57L are also labeled. The most efficient predicted pathway for ET from TTQ to copper is indicated with through-space jumps from the His ligand to copper and from TTQ to Pro 94A displayed as dashed lines. van der Waals contacts between Phe 97A and Arg 184H and Pro 100L which are inferred from the crystal structure are indicated as dashed lines.

One must also consider the possibility that the mutation has caused a pathway different from that shown in Figure 4 to become the most favored. This is unlikely, however, since the analysis of the wild-type complex structure indicates that the pathway shown in Figure 4 is at least 10-fold more favored than any alternative pathways. Since $k_{ET} \propto H_{AB}^2$ (see eq 4), ET transfer via alternative pathways would be expected to be at least 100-fold slower than wild-type values. Thus, the approximate 24-fold decrease in rate which is observed for the F97E mutant is most likely due to an increase in the interprotein distance for the pathway shown in Figure 4, as discussed above.

Thermodynamic Analysis of Complex Association Constants. As yet it has not been possible to obtain crystals of a complex of MADH with the F97E mutant amicyanin. The K_d value for the complex of the F97E mutant is at least 100-fold higher than that for the wild type (*11*). However, significant structural changes in amicyanin near the copper site which is at the protein−protein interface seem unlikely. The spectral properties of the mutant and its redox potential are indistinguishable from that of the wild type. In the absence of structural information for the mutant complex, it is still possible from a thermodynamic analysis of binding constants to gain insight into the nature of the differences at the MADH/amicyanin interface in complexes with wild-type and mutant amicyanin.

Whereas the association constant ($K_a = 1/K_d$) for complex formation between MADH and wild-type amicyanin varied very little with temperature (*6*), the binding constant for complex formation with the F97E mutant exhibited considerable variation with temperature. The temperature depen-

[2] Greenpath v0.97 provides an option of placing hydrogens and lone pair orbitals in a structure which lacks explicit hydrogen placement. We have used this program to predict pathways in the protein complex structure with and without inclusion of the hydrogens and lone pairs. The amino acid residues which participate in electron-transfer pathways are the same in either case. The length of through-space jumps, however, will be shorter and relative coupling greater when hydrogens and lone pairs are included. The discussion regarding the length of through-space jumps refers to predicted pathways for the structure which include hydrogens and lone pairs.

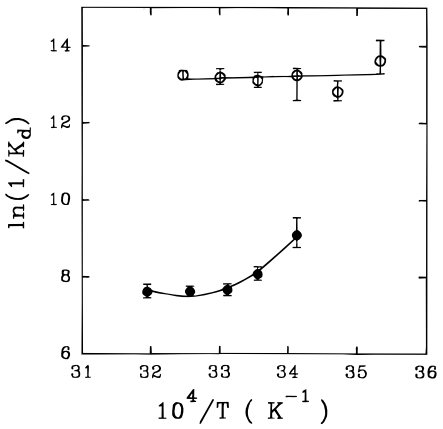

FIGURE 5: Thermodynamic analysis of the binding constant for the reaction of MADH with amicyanin. The solid circles represent data for the binding of the F97E mutant to MADH. The solid line is the fit of these data to eq 9. The open circles represent data for the binding of the wild-type amicyanin to MADH. These data were taken from ref 6. The solid line is the fit of these data to eq 8.

dence of the binding constant with wild-type amicyanin was essentially linear and could be fit to the simple form of the van't Hoff equation (eq 8). In contrast, the variation with

$$\ln(1/K_d) = -\Delta H°/RT + \Delta S°/R \qquad (8)$$

temperature of the binding constant for the F97E mutant was clearly nonlinear (Figure 5).

This nonlinearity indicates that the latter binding reaction exhibits a nonzero value for the difference in heat capacity ($\Delta C_p°$) between reactants and products. Consequently, this data set was fit to a truncated form of the integrated van't Hoff equation[3] (eq 9) (*24*). The values for the thermo-

$$\ln(1/K_d) = a + b(1/T) + c \ln T \qquad (9)$$

$$\Delta H° = R(cT - b)$$

$$\Delta S° = (\Delta H° - \Delta G°)/T$$

$$\Delta C_p° = Rc$$

dynamic parameters which describe the complex association between MADH and the F97E amicyanin mutant are given in Table 2. These values may be compared with those previously reported for the binding of wild-type amicyanin (*6*). Under these reaction conditions, the previously reported values for the reaction with wild-type amicyanin were $\Delta H°$ = −18 kJ mol^{-1} and $\Delta S°$ = 47 J mol^{-1} K^{-1}. The van't Hoff plot for the wild type is essentially linear suggesting that $\Delta C_p°$ is approximately zero. When the wild type data are fit to eq 9 rather than eq 8, no significant improvement in the fit is obtained and the fitted parameters yield large standard errors of approximately 75%.

Table 2: Thermodynamic Analysis of the Binding Reaction of Quinol MADH with F97E Amicyanin[a]

temperature (°C)	K_d (μM)	$\Delta H°$ (kJ mol^{-1})[b]	$\Delta S°$ (J mol^{-1} K^{-1})[b]	$\Delta C_p°$ (kJ mol^{-1} K^{-1})[b,c]
20	114 ± 41	−155	−452	10.3
25	313 ± 54	−102	−274	10.3
29	469 ± 80	−60	−133	10.3
34	491 ± 66	−7	41	10.3
40	491 ± 86	56	244	10.3

[a] Values were obtained from fits of the data in Figure 4 to eq 9. [b] Errors in the fitted parameters *a*, *b*, and *c* in eq 9, which were used to calculate $\Delta H°$, $\Delta S°$, and $\Delta C_p°$, were ±18%. [c] Equation 9 assumes that $\Delta C_p°$ is independent of temperature.[3]

DISCUSSION

The data presented here indicate that mutation of Phe 97 of amicyanin to Glu affects complex formation with MADH such that it alters the distance or length of the pathway from TTQ to copper. This causes a decrease in the rate of ET from the quinol form of MADH to oxidized amicyanin. These results do not resolve the debate as to whether specific pathways are required for protein ET reactions. Analyses which assume solely a distance dependence for ET, or a specific pathway, each indicate that the observed decrease in H_{AB} is attributable to an increase in the distance which the electron must traverse. Depending on the method of analysis and the choice of β value, the increase in distance may be as little as 0.5 Å or as large as 4.1 Å. At the shorter end, that distance approaches the limit of resolution of X-ray crystallography. Because this is an interprotein ET reaction, it seems reasonable that the interprotein distance should be considered separately from intraprotein ET pathways and distances. This would mean that the more appropriate analyses are those which predict the shorter calculated increases in distance (0.5−0.9 Å) and which are obtained by assuming that only the coupling associated with the interprotein through-space jump is affected by the mutation.

These results also relate to our understanding of the factors which contribute to the reorganizational energy exhibited by the ET reaction within this protein complex. A question which is as yet unresolved in this system is why these reactions for which ET is rate-limiting exhibit such a large λ. As discussed earlier, ET from the aminoquinol form of MADH is gated, but ET from the quinol form is not (*9*, *10*). As indicated in this and previous (*5*, *6*) studies, analysis of the reaction of quinol MADH by ET theory yields values for H_{AB} which are within the nonadiabatic limit and predicts an ET distance which correlates well with the crystal structures. This analysis also yields a value of λ of approximately 2.3 eV. It is possible that the large value for λ may be an apparent value that reflects the kinetic complexity of the experimental system (*25*).

One possible explanation for the large λ exhibited by this ET reaction is that the reaction is conformationally coupled (*25*, *26*). In coupled ET, some non-ET reaction step occurs after the initial binding reaction and is required to optimize the system for ET. If this preceding non-ET reaction step is much faster than the rate-limiting ET, but very unfavorable (i.e., $K_{eq} \ll 1$), then k_{ET} will be an apparent value equal to the product of K_{eq} for the non-ET reaction step and the true ET rate constant (*25*). Because of this, the experimentally determined value of λ will also be an apparent value which will contain contributions from the prerequisite non-ET reaction step as well as the true ET reaction (*25*, *26*). In the case of a bimolecular protein ET reaction, it is possible that

[3] Equation 9 assumes that $\Delta C_p°$ is independent of temperature. This is statistically justified because determination of the change in $\Delta C_p°$ is described by a third derivative which lacks the precision necessary to yield satisfactory standard errors in $\Delta C_p°$ (*24*).

the optimum orientations for binding and ET transfer may not be the same. Thus, some reorientation within the complex after initial docking may be required to poise the system for ET. Such a conformational rearrangement has been invoked to explain the basis for gated ET from metal-substituted cytochromes *c* to plastocyanin (*27*). The results presented here argue against such a rearrangement of proteins contributing to the large λ for the MADH/amicyanin ET reaction. For an ET reaction that is gated by or coupled to a conformational rearrangement of proteins at the protein−protein interface, one would expect that an interfacial mutation that affected binding and altered the ET distance would also affect that rearrangement process. If it did, then this should be reflected in a change in the experimentally determined λ value, and this was not observed. Previous studies have demonstrated that the ET reaction under study is not gated (*5*, *6*). The present results do not rule out the other possibility that this is coupled ET, but suggest that the putative prerequisite non-ET reaction step involves something other than a reorientation of proteins with respect to each other at the protein−protein interface.

An alternative explanation for the large λ value exhibited by this ET reaction may lie in the nature of the TTQ prosthetic group. TTQ is an unusual redox cofactor in two respects. It contains no metal and it is comprised of two nonplanar ring systems (*28*, *29*) capable of rotation with respect to each other. This raises two important questions. In the absence of a metal center, how is the electron density distributed throughout the cofactor? Since the two rings are not fused, does the orientation of the rings with respect to each other change during the ET reaction? If a rapid but unfavorable reorientation of the two TTQ rings were required to activate the system for ET, then this could be the basis for coupled ET and the large λ value. One would not expect this type of reorientation within MADH to be affected by mutations of amicyanin. The need for such a perturbation of the angle between TTQ rings for ET reactions has also been suggested on the basis of studies with TTQ model compounds (*30*).

The results of the thermodynamic analysis of the association reaction of MADH with the F97E mutant are unusual and interesting. A positive ΔC_p° was obtained for the binding reaction with the F97E mutant amicyanin, whereas the reaction with the wild type exhibited no difference in heat capacity between the individual proteins and the protein complex. Of the protein association reactions that have been subjected to such a thermodynamic analysis and exhibit a nonzero ΔC_p°, that value is usually negative (*31*). A well-studied example of this is the process of tubulin polymerization (*32*). The $-\Delta C_p^\circ$ is typically attributed to the release of water upon burial of hydrophobic residues which are no longer accessible to solvent after the protein−protein association. Positive values of ΔC_p° are associated with protein denaturation and protein unfolding processes (*31*). A $+\Delta C_p^\circ$ is typically attributed to the increase in exposure of hydrophobic groups to solvent. This leads to orientation of water molecules in a hydrogen bonding structure, the disruption of which on heating leads to an increase in heat absorption relative to bulk water. The positioning of a Glu, rather than a Phe, at the hydrophobic protein−protein interface in the MADH/amicyanin complex may have the same effect as exposing a hydrophobic group to water. Whereas the native complex is stabilized by van der Waals interactions involving Phe 97, Glu may instead be forming hydrogen bonding interactions when forced into this position.

It is somewhat remarkable that the ET rate in the mutant complex decreases as little as it does given the large effect it has on the binding reaction. This suggests that the two proteins are not able to adopt alternative sites of interaction that lead to a productive ET reaction and argues strongly for the absolute specificity for this site for protein−protein interaction.

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