Site-Directed Mutagenesis of Proline 94 to Alanine in Amicyanin Converts a True Electron Transfer Reaction into One That Is Kinetically Coupled†

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ABSTRACT: Amicyanin is a type I copper protein that mediates electron transfer (ET) from methylamine dehydrogenase (MADH) to cytochrome *c*-551i. Pro^{94} resides in the "ligand loop" of amicyanin, a sequence of amino acids that contains three of the four copper ligands. ET from the reduced *O*-quinol tryptophan tryptophylquinone of MADH to oxidized P94A amicyanin is a true ET reaction that exhibits values of electronic coupling (H_{AB}) and reorganization energy (λ) that are the same as for the reaction of native amicyanin. In contrast, the parameters for the ET reaction from reduced P94A amicyanin to oxidized cytochrome *c*-551i have been significantly altered as a consequence of the mutation. These values of H_{AB} and λ are 8.3 cm^{-1} and 2.3 eV, respectively, compared to values of 0.3 cm^{-1} and 1.2 eV for the reaction of native reduced amicyanin. The crystal structure of reduced P94A amicyanin exhibits two alternate conformations with the positions of the copper 1.4 Å apart [Carrell, C. J., Sun, D., Jiang, S., Davidson, V. L., and Mathews, F. S. (2004) *Biochemistry 43*, 9372−9380]. In one of these, conformation B, a water molecule has replaced Met^{98} as a copper ligand, and the ET distance to the heme of the cytochrome is increased by 1.4 Å. Analysis of these structures suggests that the true k_{ET} for ET from the copper in conformation B to heme would be much less than for ET from conformation A. A novel kinetic mechanism is proposed to explain these data in which the reduction of Cu^{2+} by methylamine dehydrogenase is a true ET reaction while the oxidation of Cu^{1+} by cytochrome *c*-551i is kinetically coupled ET. By comparison of the temperature dependence of the observed rate of the coupled ET reaction from reduced P94A amicyanin to cytochrome *c*-551i with the predicted rates and temperature dependence for the true ET reaction from conformation A, it was possible to determine the K_{eq} and values of $\Delta H°$ and $\Delta S°$ that are associated with the non-ET reaction that modulates the observed ET rate.

Long-range interprotein electron transfer (ET)[1] reactions are fundamental to respiration, photosynthesis, and redox reactions of intermediary metabolism. Small soluble type I blue copper proteins are common mediators of ET between other redox proteins (*1*). Such proteins include azurin and amicyanin in bacteria and plastocyanin in plants. Methylamine dehydrogenase (MADH) (*2*), amicyanin (*3*), and cytochrome *c*-551i (*4*) from *Paracoccus denitrificans* form one of the best characterized physiologic ET complexes of proteins (*5*). Amicyanin mediates electron transfer from the tryptophan tryptophylquinone (TTQ) cofactor (*6*) of MADH to the heme of the cytochrome (Figure 1). Inactivation of the gene for amicyanin results in loss of the ability to grow on methylamine (*7*). A crystal structure of the complex of these three soluble redox proteins has been determined (*5*),

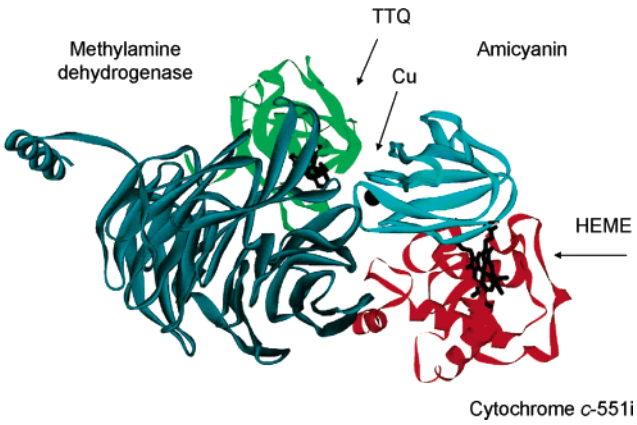

FIGURE 1: Orientation of redox cofactors in the MADH−amicyanin−cytochrome *c*-551i complex. A portion of the crystal structure showing one-half of the symmetrical complex is shown. Coordinates are available in Protein Data Bank entry 2MTA.

and the protein complex has been shown to be functional in the crystalline state (*8*, *9*). While MADH, amicyanin, and cytochrome *c*-551i are isolated as individual soluble proteins, it has been demonstrated that in solution they must form, at least transiently, a ternary complex to catalyze methylamine-dependent cytochrome *c*-551i reduction (*10*, *11*). The ET reactions to (*12*−*15*) and from (*16*) the type I copper center

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

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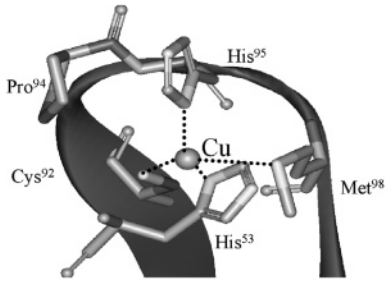


FIGURE 2: Copper center of amicyanin. The amino acids that provide copper ligands and Pro^{94} are indicated. The loop which contains amino acids Cys^{92}−Met^{98} is indicated as a ribbon. Coordinates are available in the Protein Data Bank entry 1AAC.

of amicyanin within the protein complex have been studied in solution by stopped-flow spectroscopy. Analyses by ET theory (*17*) of the temperature dependencies of these ET reactions yielded values for the reorganization energy (λ) and electronic coupling (H_{AB}) that are associated with each of these ET reactions (*12*, *16*). We have previously used site-directed mutagenesis to alter ET rates within this protein complex by selectively altering the values of $\Delta G°$ (*18*), H_{AB} (*19*), and λ (*20*) that are associated with specific ET reactions. In this paper we report the use of site-directed mutagenesis to introduce a novel mechanism of kinetic control of the ET reactions of amicyanin.

Pro^{94} resides in the "ligand loop" of amicyanin, a sequence of amino acids that contains three of the four copper ligands (Figure 2). Mutations of Pro^{94} of amicyanin to Phe or Ala increase its oxidation−reduction midpoint potential (E_m) value at pH 8.0 by 150 and 115 mV, respectively, and shift the pK_a for the pH dependence of the E_m value to more acidic values (*21*). Atomic resolution structures (0.99−1.10 Å) of both P94F and P94A amicyanin were determined in the oxidized and reduced states (*22*). In each mutant a hydrogen bond to the copper-coordinating thiolate sulfur of Cys^{92} is introduced by movement of the amide nitrogens of Phe^{94} and Ala^{94} much closer to the thiolate sulfur than the nitrogen of Pro^{94} in wild-type amicyanin. The S−H bond distances that were estimated from the crystal structures are 2.9 and 3.2 Å for oxidized P94F and P94A amicyanin, respectively. In native amicyanin, this amide nitrogen is 5.5 Å away and possesses no hydrogen since it is a proline. This is the likely explanation for the much more positive E_m values which result from these mutations. In wild-type amicyanin, the His^{95} side chain undergoes a redox and pH-dependent conformational change that accounts for the pH dependence of the E_m value of amicyanin (*23*). The observed decreases in the pK_a value for the pH dependence of the E_m values that are seen in the mutants correlate with steric hindrance to the rotation of the His^{95} copper ligand that is introduced as a consequence of the mutations.

The increased E_m values of P94F and P94A amicyanin within the ET protein complex introduce a large energy barrier for the second ET step in this three-protein ET chain. Previous studies with P94F amicyanin (*18*) have shown that, as a consequence of the changes in ΔE_m, the k_{ET} from TTQ to copper exhibits a 6-fold increase and the k_{ET} from copper to heme exhibits a 100-fold decrease. These changes in k_{ET} with $\Delta G°$ are consistent with the predictions of Marcus theory (*17*). Analysis of the temperature dependence of the reactions with P94F amicyanin indicated that the λ values for ET to and from the copper center were unchanged by the P94F mutation, despite the large change in E_m value. The H_{AB} value for ET to the cytochrome was unaffected, and the H_{AB} for ET from MADH was slightly decreased, probably because of subtle changes in the MADH−amicyanin interface. Steady-state kinetic studies indicated that, despite the large energy barrier for the ET from copper to heme, methylamine-dependent reduction of heme by the three-protein complex with P94F amicyanin goes to completion (*18*).

The ET reactions of P94A amicyanin with MADH and cytochrome *c*-551i have now been studied. As described herein, ET from O-quinol MADH to oxidized P94A amicyanin is a true ET reaction that exhibits values of H_{AB} and λ that are the same as for the reactions of native and P94F amicyanin. In contrast, the parameters for the ET reaction from reduced P94A amicyanin to oxidized cytochrome *c*-551i have been significantly altered as a consequence of the mutation, and the observed reaction rate is less than predicted by the change in $\Delta G°$ that is caused by the mutation. Interestingly, the crystal structure of reduced P94A amicyanin revealed an unusual structural feature that is not seen with native amicyanin or P94F amicyanin (*22*). Reduced P94A amicyanin exhibits two alternate conformations with the positions of the copper 1.4 Å apart. In one of these conformations, a water molecule has replaced Met^{98} as a copper ligand. This feature has not been reported for any other native or mutant type I copper protein. Using this structural information, a novel kinetic mechanism is proposed to explain the ET data in which the reduction of Cu^{2+} by methylamine dehydrogenase is a true ET reaction while the oxidation of Cu^{1+} by cytochrome *c*-551i is kinetically coupled ET. It was also possible to determine the K_{eq} and values of $\Delta H°$ and $\Delta S°$ that are associated with this non-ET reaction that modulates the observed ET rate.

EXPERIMENTAL PROCEDURES

Protein Purification. Previously described procedures were used to purify MADH (*24*), amicyanin (*3*), and cytochrome *c*-551i (*4*) from *P. denitrificans* (ATCC 13543). P94F and P94A amicyanins were expressed in *Escherichia coli* and purified from the periplasmic fraction as described previously for other recombinant amicyanin mutants (*25*).

Electron Transfer Reactions of P94A Amicyanin. The rates of the ET reactions from O-quinol MADH to oxidized P94A amicyanin and from reduced P94A amicyanin to oxidized cytochrome *c*-551i were determined as described previously for study of the analogous reactions of P94F amicyanin (*18*). An On-Line Instruments (OLIS, Bogart, GA) RSM16 stopped-flow rapid scanning spectrophotometer was used for kinetic measurements. All experiments were performed in 0.01 M potassium phosphate, pH 7.5. The ET reactions were fit to a simple kinetic model (eq 1) using eq 2.

$$A_{ox} + B_{red} \overset{K_d}{\rightleftharpoons} A_{ox}/B_{red} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} A_{red}/B_{ox} \quad (1)$$

$$k_{obs} = k_3[A]/(K_d + [A]) + k_4 \quad (2)$$

To study the ET reaction from MADH to amicyanin, one sample syringe contained oxidized amicyanin, and the other contained reduced O-quinol MADH. Psuedo-first-order

conditions were maintained with excess oxidized amicyanin. To study the ET reaction from amicyanin to cytochrome *c*-551i in the ternary protein complex, one syringe contained oxidized cytochrome *c*-551i, and the other contained reduced MADH plus reduced amicyanin at concentrations such that essentially all amicyanin is in complex with MADH prior to mixing. Psuedo-first-order conditions were maintained with excess preformed MADH−amicyanin complex. MADH and reduced amicyanin were present in a 1:1 molar ratio. Since ET in this system is from amicyanin in the complex, the data are reported in terms of bound amicyanin which was calculated using the equation

$$[A]_{bound} = (2[M]_{total} + K_d - (4[M]_{total}K_d + K_d^2))^{0.5}/2 \quad (3)$$

where $[A]_{bound}$ is the concentration of bound amicyanin and $[M]_{total}$ is the total concentration of MADH or amicyanin.

Equations 4 and 5 were used for the analysis of the data for the dependence of k_3 on temperature and theoretical calculations of ET rates. In these equations λ is the

$$k_{ET} = \frac{4\pi^2 H_{AB}^{\ 2}}{h\sqrt{4\pi\lambda RT}} e^{-(\Delta G^\circ + \lambda)^2/4\lambda RT} \quad (4)$$

$$k_{ET} = k_0 \exp[-\beta(r - r_0)] \exp[-(\Delta G^\circ + \lambda)^2/4\lambda RT] \quad (5)$$

reorganization energy, H_{AB} is the electronic coupling matrix element, h is Planck's constant, T is temperature, R is the gas constant, and k_0 is the characteristic frequency of the nuclei (10^{13} s^{-1}), which is the maximum ET rate when donor and acceptor are in van der Waals contact and $\lambda = -\Delta G^\circ$. The donor to acceptor distance is r, and r_0 is the close contact distance (3 Å). The parameter β is used to quantitate the nature of the intervening medium with respect to its efficiency to mediate ET. ΔG° is determined from the ΔE_m value for the reaction. The E_m values for P94F amicyanin and native amicyanin when in complex with MADH are +380 and +220 mV, respectively (*21*, *26*). The E_m value for the O-quinol/O-semiquinone couple of MADH is +190 mV (*13*), and the E_m value for the reduced/oxidized cytochrome *c*-551i couple is +190 mV (*27*). Rate constants were determined at several different temperatures as described above, and variation in replicate measurements at each temperature varied by <5%.

Electron transfer coupling analysis was performed using the HARLEM computer program (*28*) by the Pathways approach of Beratan, Onuchic, and co-workers (*29*) and the direct distance approach of Dutton and co-workers (*30*).

RESULTS AND DISCUSSION

Previous studies of the ET reactions of native (*16*) and P94F (*18*) amicyanin with MADH and cytochrome *c*-551i established the validity of this ternary protein complex as a system for analysis of ET reaction rates by ET theory (eqs 4 and 5). It was demonstrated that the changes in ΔG° caused by the P94F mutation yielded predictable changes in k_{ET} values that were consistent with true nonadiabatic ET reactions to and from the copper center. Furthermore, the P94F mutation did not cause any significant changes in the other parameters (λ and H_{AB}) for these ET reactions. While the P94A mutation seems to have had little effect on the ET reaction from reduced MADH to oxidized P94A amicyanin other than changing ΔG°, the values of λ and H_{AB} for the reaction of reduced P94A amicyanin to oxidized cytochrome *c*-551i are significantly altered relative to those of native and P94F amicyanin.

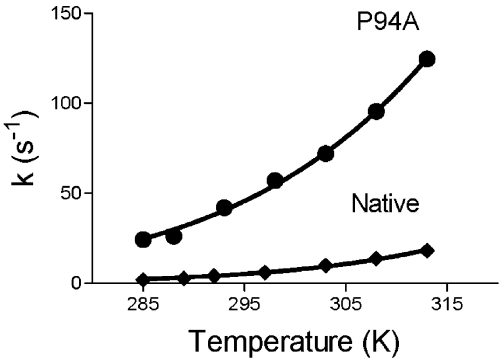


FIGURE 3: Effect of the P94A mutation on the ET reaction from reduced O-quinol MADH to oxidized amicyanin. The data for native and P94A amicyanin are indicated. Solid lines represent fits of the data to eq 4.

Table 1: Electron Transfer Parameters for Reactions of Native and Mutant Amicyanins with MADH and Cytochrome *c*-551i

	native amicyanin[a]	P94A amicyanin	P94F amicyanin[b]
	ET from O-quinol MADH		
ΔG° (J·mol^{-1})	−3184	−18875	−21710
λ (eV)	2.3 ± 0.1	2.2 ± 0.1	2.3 ± 0.1
H_{AB} (cm^{-1})	12 ± 7	3.8 ± 1.2	4.6 ± 1.3
	ET to cytochrome *c*-551i		
ΔG° (J·mol^{-1})	+3184	+18875	+21710
λ (eV)	1.2 ± 0.1	2.3 ± 0.2	1.3 ± 0.1
H_{AB} (cm^{-1})	0.3 ± 0.1	8.3 ± 5.5	0.3 ± 0.1

[a] Taken from refs *12* and *16*. [b] Taken from ref *18*.

ET from MADH to Amicyanin. The k_3 value (see eq 1) for the ET reaction from O-quinol MADH to oxidized P94A amicyanin was determined at temperatures from 15 to 45 °C. As shown in Figure 3, the reaction rates with P94A amicyanin are much greater than those of native amicyanin at all temperatures (approximately 10−20-fold depending on temperature). The results of the analysis of these data by eqs 4 and 5 are shown in Table 1 and compared with previously obtained values for the reactions of O-quinol MADH with native and P94F amicyanin.

The reactions of P94A and P94F amicyanin with MADH are each significantly faster than those of native amicyanin because the reactions with each mutant amicyanin have much more favorable ΔG° values. The H_{AB} values for the reactions of the P94A and P94F amicyanin are a little less than those for the reaction of native amicyanin. This is likely because mutation of residue Pro[94], which lies at the MADH−amicyanin interface, causes a small disruption in the ET pathway. Such a phenomenon was previously characterized for the ET reaction of F97E amicyanin with MADH (*19*). The λ values for the reaction of P94A amicyanin are also similar to those for the reactions of native and P94F amicyanin. These results are consistent with true ET reactions with ET rates that exhibit a predictable dependence on ΔG°, as predicted by ET theory (eq 4).

ET from Amicyanin to Cytochrome c-551i. The k_3 value for the ET reaction from reduced P94A amicyanin to

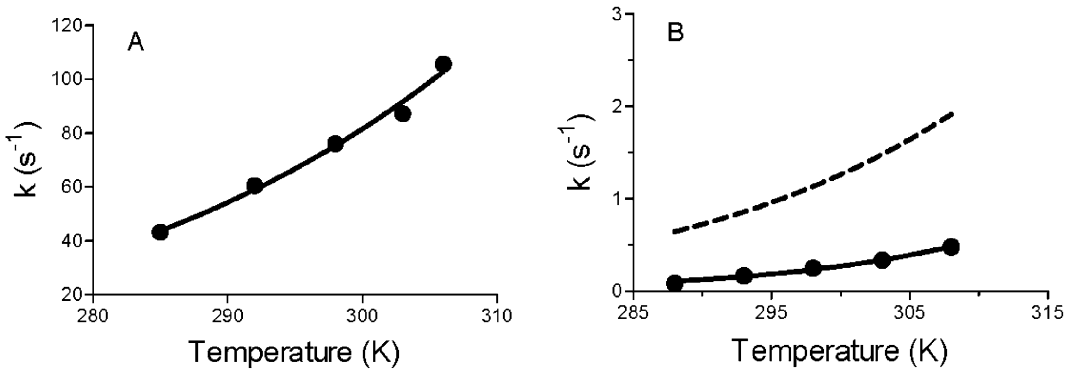


FIGURE 4: Effect of the P94A mutation on the ET reaction from reduced amicyanin to oxidized cytochrome *c*-551i. (A) Reaction of reduced native amicyanin. (B) Reaction of reduced P94A amicyanin. The solid lines are fits of the data to eq 4. The dashed line in (B) is simulated using eq 4 and inputting values of $\Delta G° = +18875$ J·mol^{-1}, $\lambda = 1.2$ eV, and $H_{AB} = 0.3$ cm^{-1}.

oxidized cytochrome *c*-551i within the protein complex was determined at temperatures from 15 to 40 °C. As shown in Figure 4, the reaction rates with P94A amicyanin are much less than those of native amicyanin at all temperatures (approximately 200−500-fold depending on temperature). The results of the analysis of these data by eqs 4 and 5 are shown in Table 1 and compared with previously obtained values for the reactions of reduced P94F amicyanin to oxidized cytochrome *c*-551i.

For the ET reactions from copper to cytochrome *c*-551i, the values of H_{AB} and λ for the reaction with P94A amicyanin are substantially different than those values which were obtained for the reactions with native and P94F amicyanins. Although the H_{AB} value for the reaction of P94A amicyanin may still lie within the so-called nonadiabatic limit [$H_{AB} <$ 80 cm^{-1} (*31*)], it is difficult to imagine how the P94A mutation could cause a 10-fold increase in electronic coupling compared to native and P94F amicyanin. If the reaction with the mutant protein is a true ET reaction, then this would require a significant decrease in the ET distance or introduction of a much more efficient ET pathway, or both. This does not seem reasonable since there are no significant changes in the overall crystal structure of P94A amicyanin compared to native and P94F amicyanin beyond those changes at the metal binding site (*22*). The corresponding increase in λ of 0.7 eV for the reaction of P94A amicyanin is also difficult to rationalize. To illustrate the effects of this mutation on parameters other than $\Delta G°$, the k_{ET} at different temperatures that is predicted using eq 4 was simulated by using the values for H_{AB} and λ that were determined for the true ET reaction and the $\Delta G°$ for P94A amicyanin. These simulated values are compared with the experimental data in Figure 4B. Clearly, the difference is not simply due to changes in $\Delta G°$. The most likely explanation for these drastic changes in the values for the ET parameters is that, as a consequence of the P94A mutation, the ET from copper to cytochrome *c*-551i is no longer a true nonadiabatic ET reaction but instead an ET reaction that is gated or kinetically coupled.

Kinetic Complexity. Correct interpretation of the basis of the change in a rate constant for an ET reaction that results from site-directed mutagenesis depends on whether the observed reaction is true, gated, or coupled ET (Scheme 1) (*32*). We have previously described approaches to distinguish between these possibilities by analysis of the dependence of the observed ET rate constant on temperature and $\Delta G°$ (*32*−*34*), as well as use of other complementary biochemical techniques such as kinetic solvent isotope effect studies (*35*). From the results obtained for the ET reactions from amicyanins to cytochrome *c*-551i (Table 1), it appears that the reactions from native and P94F amicyanin are true ET, but the reaction from P94A amicyanin is not. Thus, the P94A mutation appears to have caused the ET reaction from copper to heme to become either gated or coupled (Scheme 1). There are at least two mechanisms by which this could occur. (i) It is possible that a mutation may slow a preceding non-ET step to cause a true ET reaction to become gated. (ii) The mutation may introduce a new reaction step which is required to optimize the system for ET and which is slower than the ET step (gated ET) or rapid but unfavorable (coupled ET). A possible mechanism for the introduction of such a new reaction step that precedes the ET reaction may be inferred from the crystal structures of the oxidized and reduced forms of P94A amicyanin.

Scheme 1

$$A_{ox}\text{-}B_{red} \underset{k_{-X}}{\overset{k_X}{\rightleftharpoons}} [A_{ox}\text{-}B_{red}]^* \underset{k_{-ET}}{\overset{k_{ET}}{\rightleftharpoons}} A_{red}\text{-}B_{ox}$$

true ET:	$k_{ET} \ll k_X$	$K_X(k_X/k_{-X}) \gg 1$	$k_{obs} = k_{ET}$
gated ET:	$k_X \ll k_{ET}$		$k_{obs} = k_X$
coupled ET:	$k_{ET} \ll k_X$	$K_X(k_X/k_{-X}) \ll 1$	$k_{obs} = K_X * k_{ET}$

Possible Effects of Structural Perturbations of P94A on True ET Reactions. High-resolution crystal structures are available for the oxidized and reduced forms of P94A and P94F amicyanins (*22*), as well as native amicyanin (*36*). There is little difference in the overall structures of the three proteins, but for P94A amicyanin, a significant change is seen in the metal binding site of the reduced form. Reduced P94A amicyanin exhibits two alternative conformations with the positions of the copper 1.4 Å apart. One is very similar to that of native reduced amicyanin (conformation A). In the other conformation, Met^{98} is too far from the copper to provide a ligand, and a water molecule has been recruited as a ligand (conformation B) (*22*). Since reduced P94A amicyanin can adopt two alternative conformations, it is of interest to determine how the particular conformation affects the ET distance or pathways and, consequently, H_{AB} and the ET rate constant. We have considered the possible ET reactions from each of the two conformations.

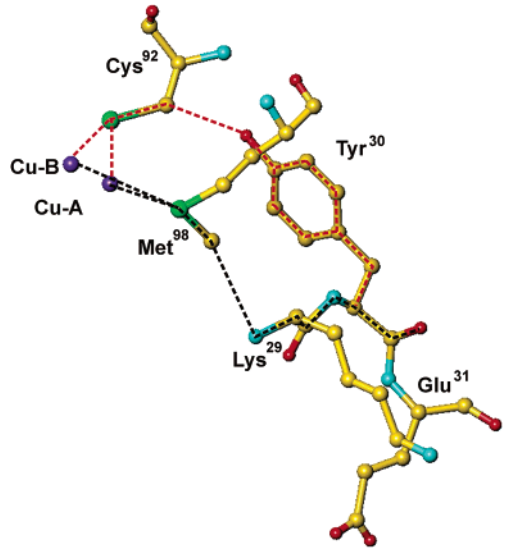


FIGURE 5: Predicted pathways for ET from copper through amicyanin in alternative conformations of reduced P94A amicyanin. The positions of copper in conformations A and B are indicated. The pathways begin at copper and end at the O of Glu[31], which is the point from which ET to cytochrome *c*-551i occurs (*16*). Amino acid residues that form the predicted pathways are shown. Pathways via Cys[92] (red) and Met[98] (black) are indicated as dashed lines which converge at Glu[31]. Coordinates are available in Protein Data Bank entry 1SF3 for reduced P94A amicyanin.

A Pathways analysis (*29*) of the ET reaction from reduced native amicyanin predicted two alternative sets of pathways from copper to heme that were of relatively equivalent efficiency and at least 10-fold more efficient than other possible pathways (*16*, *37*). One set of pathways required the electron exit copper via Cys[92] and the other via Met[98]. Analysis of the ET reactions from each of the two reduced P94A conformations (Figure 5) indicates that the pathways via Cys[92] would not be affected because the Cu−S distance for the Cys ligand is essentially the same in each conformation. The pathways via Met[98] are different for the two conformations. Since the Cu−S distance is 3.01 Å in conformation A and 3.94 Å in conformation B (*22*), the pathway via Met[98] is much more efficient in conformation A than in conformation B. In previous studies, mutation of Tyr[30] to Ile, which should have disrupted the pathways via the Cys[92] ligand, did not affect ET from copper to heme, suggesting that the pathways via Met[98] are important (*37*).

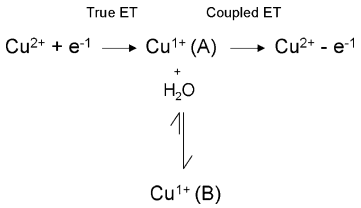
An alternative to the Pathways approach for assessing relative electronic coupling probabilities has been proposed by Dutton and co-workers (*30*). This considers the total distance and the packing density (ρ) of the atoms in the protein that separates the two redox centers. High-resolution structures are only available for the free forms of P94A amicyanin and not those in complex. Therefore, when using this approach, we analyzed the distance and intervening protein from each alternative copper position to the point on the surface of amicyanin from which ET to the cytochrome occurs, the backbone oxygen of residue Glu[31] (*16*) (see Figure 5). The subsequent ET from the surface of amicyanin to the heme of cytochrome *c*-551i will not be affected by the mutation of amicyanin. In this analysis it is significant that the actual distance from copper is 1.4 Å greater in conformation B than in conformation A (Table 2). Furthermore, the average packing density of the atoms

Table 2: Calculated Parameters for Electron Transfer Reactions through Native and Mutant Amicyanins

	native amicyanin	P94F amicyanin	P94A-A[a] amicyanin	P94A-B amicyanin
distance (Cu to Glu[31]O) (Å)	13.8	14.2	14.0	15.3
packing density (ρ)[b]	0.85	0.88	0.85	0.79
average β ($Å^{-1}$)[c]	1.18	1.13	1.18	1.29
max ET rate (s^{-1})[d]	8.5×10^6	1.1×10^7	7.2×10^6	2.5×10^5

[a] P94A-A and P94A-B refer to the two alternative conformations of reduced P94A amicyanin that were observed in the crystal structure. [b] ρ is defined in ref *30*. [c] β is defined in eq 5. These values are not experimentally determined but calculated from the ρ value. [d] The free energy optimized rate of electron transfer when $\Delta G° = -\lambda$.

Scheme 2



separating copper and Glu[31]O is less in conformation B than in conformation A. This is largely a consequence of the increased space between copper and Met[98]. The decrease in ρ from 0.85 to 0.79 is the equivalent of an increase in the average β value in eq 5 from 1.18 to 1.29 Å. On the basis of this analysis the maximum ET rate for conformation B would be approximately 30-fold less than for conformation A.

In addition to distance and pathways considerations, it may also be significant that water is present in the metal binding site and providing a copper ligand in conformation B. This could increase the λ value for the true ET reaction from this conformation because of additional solvent reorganization that is likely to be associated with the ET reaction from reduced P94A amicyanin conformation B, relative to conformation A and native reduced amicyanin. The increased λ value would be expected to substantially decrease the k_{ET} from conformer B. We have previously shown that the presence or absence of a water near the protein-bound redox center can have a large influence on the λ value (*20*).

If we accept that the true k_{ET} from conformation B is much less than from conformation A because of changes in H_{AB} and/or λ associated with the reaction, then it may be necessary to convert from conformation B to conformation A in order to activate the system for efficient ET. The following kinetic model is proposed to explain the results.

A Model for ET from P94A Amicyanin to Cytochrome c-551i. The results for the ET reaction from MADH to oxidized P94A amicyanin suggest that it is a true ET reaction with ET parameters that have not been significantly affected by the mutation. This is consistent with the similarity in active site structures, with respect to the position of Cu^{2+} and its ligands, in the crystal structures of oxidized native, P94F, and P94A amicyanins (*22*). A kinetic model is proposed in Scheme 2 to explain the difference in ET parameters for the ET reaction from reduced P94A amicyanin to cytochrome *c*-551i. As discussed above, it is known from the crystal structure of reduced P94A amicyanin that two conformations are possible and that conformation A may be

much more favorable than conformation B for the ET reaction to cytochrome *c*-551i. It is proposed that, after reduction by MADH, reduced P94A amicyanin adopts a rapid equilibrium between the two conformations which favors conformation B. The rapid unfavorable equilibrium will effectively reduce the concentration of conformation A, resulting in a slower observed rate of ET to heme. This is an example of kinetically coupled ET (*32*, *38*), as described in Scheme 1. In this model, the apparent ET rate will be the product of the true k_{ET} for the reaction from conformation A and the unfavorable K_{eq} for the formation of conformation A.

We cannot absolutely rule out the possibility that the reaction is gated rather than coupled. For the reaction to be gated, the interconversion between conformations A and B would have to be much slower than the ET from conformation A (see Scheme 2). Since the ET parameters for the reaction between O-quinol MADH and oxidized P94A amicyanin are essentially the same as for the reaction with native amicyanin, the immediate product of the reaction with MADH is most likely conformation A of reduced P94A amicyanin, which is structurally very similar to that of reduced native amicyanin. If the interconversion of conformations was slower than ET, then ET from the initially formed conformation A would occur before any conversion to conformation B. In that case, the observed k_{ET} from conformation A would be a true ET reaction, which it is not. For this reason the model of coupled ET is a more likely explanation than a gated ET model.

Kinetic and Thermodynamic Characterization of K_{eq} for the Non-ET Reaction in the Coupled ET Model. In the model for coupled ET from P94A amicyanin to cytochrome *c*-551i (Scheme 2), the value of k_3 (see eqs 1 and 2) that is determined from the kinetic analysis is actually equal to $K_{eq}k_{ET}$ (Scheme 1). We have previously determined the values of H_{AB} and λ for the true ET reactions to cytochrome *c*-551i from native and P94F amicyanins. We have also determined the $\Delta G°$ value for the reaction of P94A with cytochrome *c*-551i. Using these values it is possible to predict the true k_{ET} for this reaction and its temperature dependence. This is shown in Figure 4B and compared with the experimentally determined values for the coupled reaction. The temperature dependence of the observed rate of a coupled ET reaction will be influenced by the $\Delta H°$ and $\Delta S°$ values associated with the preceding non-ET reaction step (*38*). Since $k_{obs} = K_{eq}k_{ET}$ for this reaction, it is possible to determine K_{eq} for the non-ET reaction by simply dividing the experimentally determined rate constant by the predicted value of k_{ET}. This can be done over the temperature range that was studied by comparing the simulated curve for the true reaction in Figure 4B and the fitted curve for the experimentally determined values. When this is done, one obtains K_{eq} values that range from 0.13 at 288 K to 0.25 at 308 K. It should be noted that the distribution of the two conformations in the crystal structure of reduce P94A amicyanin is approximately 58% A and 42% B (*22*). The results discussed above indicate that the K_{eq} for the interconversion of conformations is from 0.13 to 0.25. The differences in the equilibrium concentrations of each conformation in the crystal versus in solution may be a consequence of the conditions used to obtain crystals, which include higher pH and much higher salt concentration than are present during the solution studies.

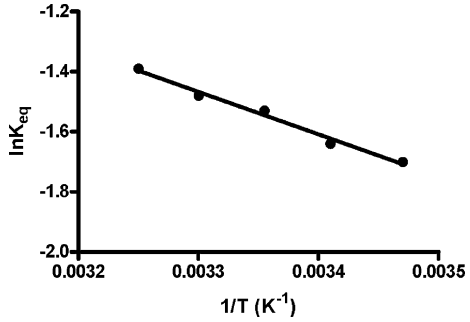


FIGURE 6: van't Hoff analysis of the putative reaction step that precedes ET from reduced P94A amicyanin to cytochrome *c*-551i. Values of K_{eq} were determined by dividing experimentally determined values shown in Figure 4 by simulated values of k_{ET} shown as the dashed line in Figure 4 (i.e., $k_{obs} = K_{eq}k_{ET}$). The data were plotted using eq 6.

Analysis of the data shown in Figure 4B by eq 6 yields a linear plot with values of $\Delta H° = +11.8 \pm 0.8$ kJ·mol^{-1} and

$$\ln K_{eq} = -\Delta H°/RT + \Delta S°/R \qquad (6)$$

$\Delta S° = +26.6 \pm 2.6$ J·mol^{-1}·K^{-1}. The positive value of $\Delta S°$ is consistent with the nature of the proposed coupling reaction (Scheme 2). The conversion of conformation B to conformation A (the forward reaction in this equilibrium) involves the loss of water. This would be expected to increase the entropy of the system since the lost water will be more disordered. The reaction is still unfavorable since the positive enthalpy change more than compensates for the positive entropy change.

Summary. A consequence of site-directed mutagenesis to create P94A amicyanin is that two alternate conformations of an electron donor for a biological ET reaction are generated. The ET parameters for one conformation are such that the ET reaction is prohibitively unfavorable. Thus, the equilibrium between the two conformations becomes important to the overall regulation of the rate of ET. This equilibrium introduces a new reaction step that alters the kinetic mechanism for the ET reaction such that a non-ET reaction controls the availability of a reactive intermediate from which ET occurs. We believe that this is the first example of conversion of a true ET reaction to a coupled ET reaction by site-directed mutagenesis.

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