# Effects of Engineering Uphill Electron Transfer into the Methylamine Dehydrogenase−Amicyanin−Cytochrome *c*-551i Complex[†]

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ABSTRACT: Within the methylamine dehydrogenase−amicyanin−cytochrome *c*-551i complex, electrons are transferred from tryptophan tryptophylquinone (TTQ) to heme via the type I copper center of amicyanin. Mutation of Pro94 of amicyanin to Phe increases the redox potential of the copper center within the protein complex by approximately 195 mV. This introduces a large energy barrier for the second electron transfer (ET) step in this three-protein ET chain. As a consequence of this mutation, the ET rate from TTQ to copper exhibits about a 6-fold increase and the ET rate from copper to heme exhibits about a 100-fold decrease. These changes in ET rate are consistent with the predictions of Marcus theory. Temperature dependence studies of these reactions indicate that the reorganization energies for the ET to and from the copper center are unchanged by the P94F mutation, despite the large change in redox potential that it causes. Steady-state kinetic studies indicate 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. The turnover number for this steady-state reaction, however, is decreased 50-fold relative to that of the native complex. As a consequence of the P94F mutation, the rate constant for the unfavorable uphill ET reaction from copper to heme has become the rate-limiting step in the overall reaction. The evolutionary implications of the effects of this mutation on the function of this naturally occurring simple ET chain are discussed.

Methylamine dehydrogenase (MADH)[1] (*1*), amicyanin (*2*), and cytochrome *c*-551i (*3*) from *Paracoccus denitrificans* form one of the best characterized physiologic electron transfer (ET) complexes of proteins (*4*). Electrons are transferred from the tryptophan tryptophylquinone (TTQ) cofactor (*5*) of MADH to the type I copper center of amicyanin and then to the heme of the cytochrome (Figure 1). This is the only complex of three soluble redox proteins for which a high-resolution crystal structure is available (*4*), and the protein complex has been shown to be functional in the crystalline state by single-crystal polarized absorption spectroscopy (*6*). In *P. denitrificans*, amicyanin is an obligatory mediator of ET from MADH to soluble *c*-type cytochromes. The amicyanin gene is located immediately downstream of that for MADH, and inactivation of the former by gene replacement resulted in loss of the ability to grow on methylamine (*7*). MADH, amicyanin, and cytochrome *c*-551i are isolated as individual soluble proteins, but it has been demonstrated that in solution they must form, at least transiently, a ternary complex to catalyze methylamine-dependent cytochrome *c*-551i reduction (*8*, *9*). The ET reactions to and from the type I copper center of amicyanin within the protein complex have been studied in solution by stopped-flow spectroscopy (*10*−*14*). ET reactions from different redox forms of TTQ to copper are possible (*15*). It has been shown that the ET reaction from the O-quinol form of TTQ to amicyanin is a true ET reaction (*11*). Analyses by ET theory (*16*) of the temperature dependencies of this ET reaction (*10*), and the ET reaction from reduced copper to heme (*14*), have yielded values for the reorganization energy ($\lambda$) and electronic coupling ($H_{AB}$) that are associated with each of these ET reactions.

It was recently shown that the oxidation−reduction midpoint potential ($E_m$) value of amicyanin may be made significantly more positive by site-directed mutagenesis of Pro94 to Phe (*17*). This residue does not provide a ligand for copper, but it is located in a loop structure that contains three of the four copper ligands (Figure 2) (*18*). The $E_m$ values at pH 7.5 are +415 and +250 mV for P94F amicyanin (*17*) and native amicyanin (*19*), respectively. To examine the consequences of this mutation on the ET properties of amicyanin, the ET reactions of P94F amicyanin as an electron acceptor for MADH and as an electron donor to cytochrome *c*-551i have been studied. ET rates and $\lambda$ and $H_{AB}$ values associated with each ET reaction were determined. Since the P94F amicyanin mutant possesses an $E_m$ value that is significantly more positive than cytochrome *c*-551i, the ET reaction from the intermediate redox center to the terminal electron acceptor in the MADH−P94F amicyanin−cytochrome *c*-551i ET chain is thermodynamically very unfavorable. As such, examination of the consequences of this mutation on the rate and flux of electrons through this relatively simple three-protein ET chain has provided insight into the complex phenomenon of "uphill ET".

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[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; ET, electron transfer; $H_{AB}$, electronic coupling; $\lambda$, reorganization energy; O-quinol, fully reduced TTQ with oxygen at the C6 carbon; N-quinol, fully reduced TTQ with nitrogen bonded to the C6 carbon; $E_m$, oxidation−reduction midpoint potential.

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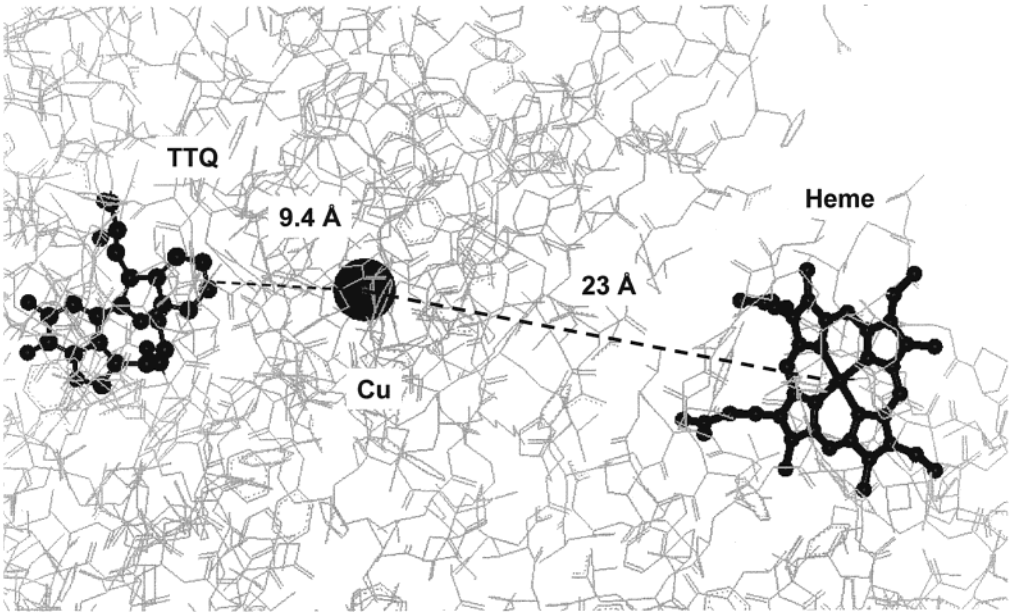


FIGURE 1: Orientation of redox cofactors in the MADH−amicyanin−cytochrome *c*-551i complex. A portion of the crystal structure is shown with the direct distances between the cofactors indicated. Coordinates are available in the Protein Data Bank, entry 2MTA.

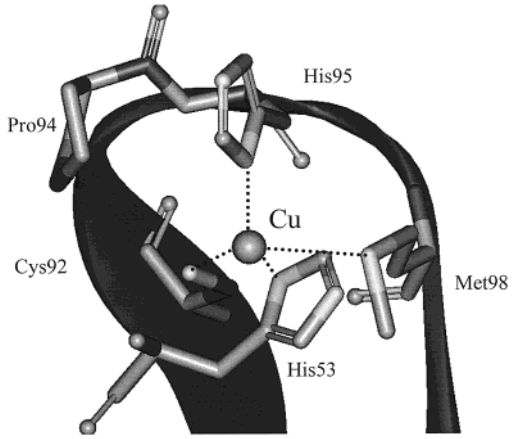


FIGURE 2: Copper center of amicyanin. The amino acids that provide copper ligands and Pro94 are indicated. The loop which contains amino acids Cys92−Met98 is indicated as a ribbon. Coordinates are available in the Protein Data Bank, entry 1AAC.

## EXPERIMENTAL PROCEDURES

Previously described procedures were used to purify MADH (*20*), amicyanin (*2*), and cytochrome *c*-551i (*3*) from *P. denitrificans* (ATCC 13543). P94F amicyanin (*17*) was expressed in *Escherichia coli* and purified from the periplasmic fraction as described previously for other recombinant amicyanin mutants (*21*). The gene for P94F amicyanin was kindly provided by M. C. Machczynski and H. B. Gray (Caltech). O-Quinol MADH and reduced amicyanins were generated by anaerobic titration of the oxidized proteins with stoichiometric amounts of dithionite. N-Quinol MADH (*15*) was prepared by stoichiometric reduction with methylamine. Cytochrome *c*-551i was oxidized by addition of potassium ferricyanide. All reagents were obtained from commercial sources and used without further purification.

An On-Line Instruments (OLIS, Bogart, GA) RSM16 stopped-flow rapid scanning spectrophotometer was used for kinetic measurements. To study the ET reaction from MADH to amicyanin, one syringe contained O-quinol MADH and the other contained oxidized amicyanin. All experiments were performed in 0.01 M potassium phosphate, pH 7.5. The experimental details and methods of data analysis have been previously described (*22*). 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. MADH and reduced amicyanin were present in a 1:1 molar ratio. Since ET in this system is from amicyanin in complex, the data are reported in terms of bound amicyanin, which was calculated using eq 1, where $[A]_{bound}$ is the concentration of

$$[A]_{bound} = (2[M]_{total} + K_d - (4[M]_{total}K_d + K_d^2)^{0.5})/2 \quad (1)$$

bound amicyanin and $[M]_{total}$ is the total concentration of MADH or amicyanin. All experiments were performed in 0.01 M potassium phosphate, pH 7.5. The experimental details and methods of data analysis have been previously described (*14*). In each study, the reactions were fit to a simple kinetic model (eq 2) using eq 3.

$$A_{ox} + B_{red} \overset{K_d}{\rightleftharpoons} A_{ox}/B_{red} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} A_{red}/B_{ox} \quad (2)$$

$$k_{obs} = k_3[A]/(K_d + [A]) + k_4 \quad (3)$$

For analysis of the temperature dependence of $k_3$ by ET theory, data were fit to eq 4, where $\lambda$ is the reorganization

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

energy, $H_{AB}$ is the electronic coupling matrix element, $h$ is Planck's constant, $T$ is temperature, and $R$ is the gas constant. Detailed discussions of the mathematical and physical meaning of $H_{AB}$ and $\lambda$ may be found in several reviews of ET theory (*23*−*26*). $\Delta G^\circ$ is determined from the $\Delta E_m$ value for the reaction. It was previously shown that the $E_m$ value of amicyanin in complex with MADH is essentially equal

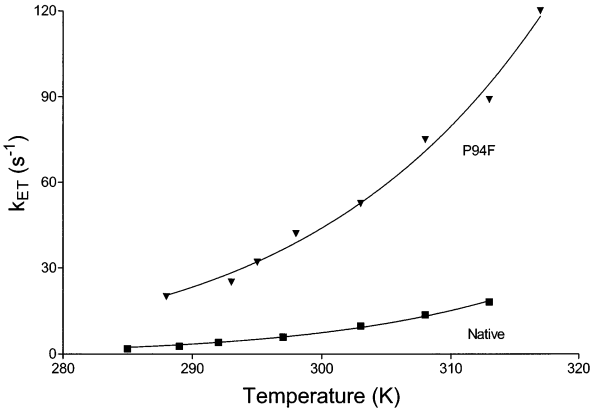


FIGURE 3: Effect of the P94F mutation on the ET reaction from O-quinol MADH to oxidized amicyanin. Solid lines represent fits of the data to eq 4.

to the $E_m$ value of free amicyanin in the pH-independent region above the p$K_a$ for the pH dependence of its $E_m$ value (*19*). These values are +415 mV for P94F amicyanin and +220 mV for native amicyanin. These values, the $E_m$ value of +190 mV for the O-quinol/O-semiquinone couple of MADH (*11*) and the $E_m$ value of +190 mV for the reduced/oxidized cytochrome *c*-551i couple (*27*), were used to determine the $\Delta G°$ values for the ET reactions described in this study.

Steady-state kinetic experiments with MADH, amicyanin, and cytochrome *c*-551i as the terminal electron acceptor were performed as described previously (*28*). The assay mixture contained 16 nM MADH, 0.5 $\mu$M amicyanin, and 21 $\mu$M cytochrome *c*-551i in 10 mM potassium phosphate, pH 7.5. The reaction was initiated by the addition of 0.1 mM methylamine, and activity was monitored by the change in absorbance caused by the reduction of the cytochrome at 416 nm. Assays were performed at 30 °C, and data were fit to eq 5.

$$v/[E] = k_{cat}[S]/(K_m + [S]) \quad (5)$$

## RESULTS

*ET from O-Quinol MADH to Oxidized Amicyanin.* The dependence of the ET rate from O-quinol MADH to oxidized P94F amicyanin on concentration was determined with MADH as the limiting reactant with its concentration fixed at 2 $\mu$M. The P94F amicyanin concentration was varied from 2 to 100 $\mu$M. As has been observed previously for the reaction with native amicyanin (*10*), the observed rate is already maximal at the lower concentrations so that one can only estimate an upper limit for the $K_d$ value of approximately 5 $\mu$M. It is also known from previous studies that the reaction is irreversible (i.e., $k_4$ is 0 in eq 2). As such, the limiting first-order rate constant is equal to $k_{ET}$ for the forward reaction. Therefore, it was not necessary to examine a complete concentration profile to determine limiting first-order rate constants in temperature dependence studies. Pseudo-first-order conditions were maintained at each temperature with a concentration of P94F amicyanin of 80 $\mu$M, which is much greater than that of MADH and well above the $K_d$ for complex formation. Rates were determined at temperatures from 12 to 45 °C. The temperature dependence of the ET rate and fits of the data to eq 4 are shown in Figure 3 and compared with data for the reaction with native amicyanin. The ET rate for the reaction of P94F amicyanin is approximately 5−6-fold faster than that of native amicyanin. Analysis by ET theory indicates that the increase in rate may be attributed to the greater $-\Delta G°$ for the reaction with the mutant (Table 1). The values for $\lambda$ for each reaction are the same within experimental error. The observed value of $H_{AB}$ for the reaction of P94F amicyanin is about 2-fold smaller. This decrease in $H_{AB}$ will cause the ET rate to decrease by 4-fold (i.e., $k_{ET} \sim H_{AB}^2$). Thus, the actual rate enhancement caused by increasing the redox potential in P94F amicyanin would be 20−24-fold greater than that observed for native amicyanin in the absence of the perturbation of $H_{AB}$.

Table 1: Kinetic and Electron Transfer Parameters for the Reactions of Native and P94F Amicyanin

| | native amicyanin | P94F amicyanin |
|---|---|---|
| ET from O-quinol MADH | | |
| $\Delta G°$ ($J \cdot mol^{-1}$) | −3184 | −21710 |
| $\lambda$ (eV) | 2.3 ± 0.1 | 2.3 ± 0.1 |
| $H_{AB}$ ($cm^{-1}$) | 12 ± 7 | 4.6 ± 1.3 |
| ET to cytochrome *c*-551i | | |
| $\Delta G°$ ($J \cdot mol^{-1}$) | +3184 | +21710 |
| $\lambda$ (eV) | 1.2 ± 0.1 | 1.3 ± 0.1 |
| $H_{AB}$ ($cm^{-1}$) | 0.3 ± 0.1 | 0.3 ± 0.1 |
| steady-state reaction | | |
| $k_{cat}$ at 30 °C ($s^{-1}$) | 18 | 0.4 |

Table 2: Forward and Reverse Rate Constants for the ET Reaction from P94F Amicyanin to Cytochrome *c*-551i

| temp (K) | $k_3$ ($s^{-1}$) | $k_4$ ($s^{-1}$) |
|---|---|---|
| 288 | 0.24 ± 0.04 | 0.027 ± 0.002 |
| 293 | 0.30 ± 0.09 | 0.034 ± 0.005 |
| 298 | 0.46 ± 0.09 | 0.023 ± 0.014 |
| 303 | 0.56 ± 0.08 | 0.045 ± 0.003 |
| 308 | 0.73 ± 0.025 | 0.047 ± 0.015 |
| 313 | 0.99 ± 0.021 | 0.055 ± 0.029 |

*ET from Reduced Amicyanin to Oxidized Cytochrome c-551i.* To obtain a limiting first-order rate constant for this reaction, it is necessary to analyze the concentration dependence of the reaction. There are two reasons for this. (i) Higher concentrations are required to fully saturate the reaction. (ii) A finite reverse reaction ($k_4$ in eq 2) is observed (*14*). Thus, a fit of the hyperbolic concentration dependence is required to get the true forward rate constant. Experiments were performed at temperatures over the range from 15 to 40 °C. At each temperature, the concentration of cytochrome *c*-551i was limiting and fixed at 0.75 $\mu$M. The varied reactant was the reduced MADH−amicyanin complex as discussed earlier under Experimental Procedures. As was observed with native amicyanin (*14*), the $k_4$ value is finite but much smaller than expected (Table 2). Since the $\Delta G°$ for the reaction is unfavorable, the reverse reaction is actually expected to be much faster than the forward. The observation that $k_3 > k_4$, despite the unfavorable $\Delta G°$, can be explained by relatively rapid dissociation of the cytochrome from the ternary complex after ET that prevents the reverse reaction going to completion in the complex.

The temperature dependence of the ET rate and fits of the data to eq 4 are shown in Figure 4 and compared with data for the reaction with native amicyanin. The ET rate for the reaction of P94F amicyanin is approximately 100-fold

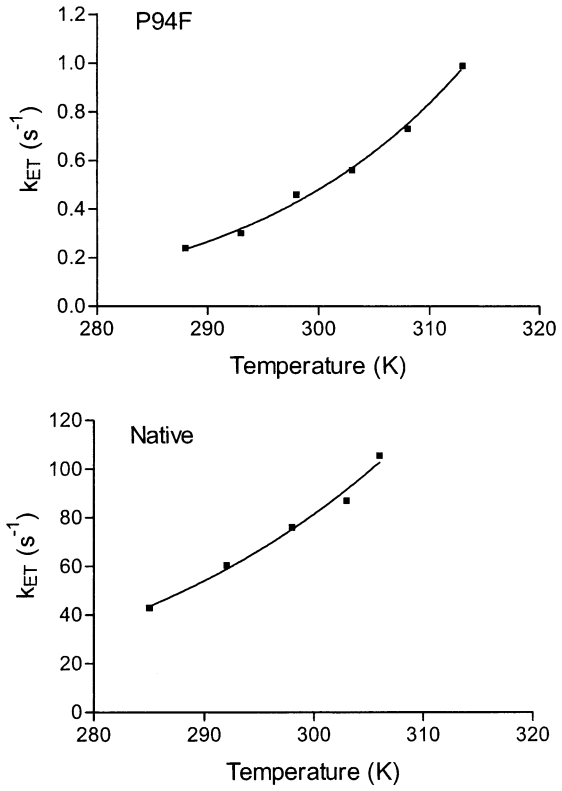


FIGURE 4: Effect of the P94F mutation on the ET reaction from reduced amicyanin to oxidized cytochrome *c*-551i. Solid lines represent fits of the data to eq 4.

smaller than that of native amicyanin. The analysis by ET theory indicates that the decrease in rate may be attributed to the altered $\Delta G°$ for the reaction with the mutant (Table 1). The values of $H_{AB}$ and $\lambda$ for the reaction of P94F amicyanin are the same within the range of experimental uncertainty.

*Steady-State Kinetics*. The steady-state kinetics of methylamine-dependent cytochrome *c*-551i reduction within the ternary protein complex were examined to determine the effect of the P94F mutation on turnover number and extent of flux through the MADH−amicyanin−cytochrome *c*-551i ET chain. In 10 mM potassium phosphate, pH 7.5, the $k_{cat}$ for the reaction with native amicyanin is 18 $s^{-1}$ (*28*). In this assay, the rate-limiting step in the overall steady-state reaction is either the rate of release of the aldehyde product from MADH (*29*) or the rate of the gated ET reaction from the substrate-derived N-quinol MADH to amicyanin (*13*), depending on reaction conditions. When the assay is performed with P94F amicyanin, steady-state activity is observed and the reaction goes to completion. However, the rate of reaction is much slower, exhibiting a $k_{cat}$ value of 0.4 $s^{-1}$. This value is approximately that which is observed for $k_{ET}$ from P94F amicyanin to cytochrome *c*-551 (Table 1). Thus, the P94F mutation has caused the unfavorable ET reaction from copper to heme to become the rate-limiting step in the steady-state reaction.

## DISCUSSION

By analyzing the ET reactions from O-quinol MADH to P94F amicyanin and from P94F amicyanin to cytochrome *c*-551i, it was possible to determine the effects on the ET rates and ET parameters associated with both reactions. With the native proteins, very different $\lambda$ values have been obtained for the ET reactions from O-quinol MADH to amicyanin and from amicyanin to cytochrome *c*-551i (Table 1). The current results indicate that, for each of these ET reactions, the P94F mutant has no significant effect on the $\lambda$ associated with the ET reactions of amicyanin. The value of $H_{AB}$ that is associated with the reaction of the P94F amicyanin with cytochrome *c*-551i does not change, indicating that the distance and/or pathway between copper and heme are (is) not affected by the mutation. A small change in $H_{AB}$ for the reaction with MADH is observed. The predicted pathway of ET from TTQ to copper in the native complex includes a through-space jump from the Trp108 portion of TTQ to the backbone carbonyl oxygen of Pro94 (*14*). A subtle change in the orientation of this carbonyl in P94F could account for the observed difference in $H_{AB}$. Overall, the similarities in the $H_{AB}$ values for the two reactions are consistent with the lack of any significant change in the structure or the orientation of P94F amicyanin in the ternary protein complex with respect to MADH and cytochrome *c*-551i. The observed increase in $k_{ET}$ for the reaction of P94F amicyanin with O-quinol MADH and the observed decrease in $k_{ET}$ for the reaction of P94F amicyanin with cytochrome *c*-551i are as predicted by Marcus theory (eq 4, ref *16*). These rates of ET vary with $\Delta G°$ as expected for true ET reactions.

It is usually assumed that biological ET chains should follow an energetically "downhill" thermodynamic path from redox centers with more negative $E_m$ values to redox centers with more positive $E_m$ values. However, it is possible to find several examples where this is not the case. For example, significantly "uphill" reaction steps occur in the sequential ET reactions that are catalyzed by hydrogenase (*30*), nitrate reductase (*31*), the *Rhodopseudomonas viridis* tetraheme cytochrome (*32*), and membrane-bound respiratory chains (*33*). By examining the effects of the P94F mutation on the rate and extent of ET through the MADH−amicyanin−cytochrome *c*-551i complex, it was possible to experimentally determine the consequences of engineering a significantly uphill ET step in a naturally occurring ET chain.

It has been proposed (*34*) that the relatively close proximity of redox centers alone is sufficient to allow tunneling of electrons to occur at rates far faster than the substrate redox reactions and that this renders naturally occurring ET chains tolerant to endergonic electron tunneling steps. This is likely true for some systems, but only if the distances between redox centers are relatively close and if the $\lambda$ values for the ET reactions are not too large. The latter point is somewhat problematic since $\lambda$ values have been experimentally determined for a relatively small number of interprotein redox reactions. In the MADH−amicyanin−cytochrome *c*-551i chain, ET rates are relatively slow and in the range of the $k_{cat}$ for the overall process of methylamine-dependent reduction of the cytochrome by the three-protein complex. This is because the reaction from TTQ to copper exhibits a relatively large $\lambda$ value and because the ET from copper to heme occurs over a very long distance (i.e., exhibits a relatively small $H_{AB}$). The P94F amicyanin mutant exhibits an $E_m$ value that is 225 mV more positive than that of cytochrome *c*-551i, the terminal electron acceptor in this chain. Since the $E_m$ values of MADH and cytochrome *c*-551i are unchanged, the overall thermodynamic driving force for ET through the three-protein ET chain is unchanged. The

P94F mutation, however, has introduced a very large thermodynamic energy barrier for an intermediate step in the ET chain. Despite this significant energy barrier for a segment of the ET chain, the reaction through the chain does go to completion. However, the rate of ET through the chain is decreased by approximately 50-fold. Comparison of the microscopic rate constants with the steady-state kinetic parameters for methylamine-dependent reduction of cytochrome *c*-551i by the ternary protein complex reveals that the rate of the engineered unfavorable ET reaction from P94F amicyanin to the cytochrome becomes the rate-limiting step in the overall reaction. Thus, while maintaining flux through the ET chain, site-directed mutagenesis of a single residue caused an electron tunneling step to become sufficiently endergonic to become rate limiting relative to the other catalytic and redox reactions. The evolutionary implications of this are that not all ET chains are necessarily tolerant of mutations that vary $E_m$ values of intermediate electron carriers.

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