# Free Energy Dependence of the Electron Transfer Reaction between Methylamine Dehydrogenase and Amicyanin

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Methylamine dehydrogenase (MADH), which catalyzes the oxidation of methylamine to formaldehyde and ammonia,[1] possesses the prosthetic group tryptophan tryptophylquinone (TTQ).[2,3] Oxidized MADH is the redox form obtained upon purification. Stable reduced and semiquinone MADH may be generated by titration with dithionite.[4] The physiologic electron acceptor for MADH is a type I copper protein, amicyanin.[5,6] It is also stable in the oxidized and reduced forms. The structure of the protein which separates TTQ and copper is known from crystallographic analyses of a binary complex of MADH and amicyanin[7] and a ternary complex of these proteins in association with cytochrome *c*-551i.[8] In each complex, the two redox centers are separated by approximately 9.4 Å. These and the cytochrome *c*−cytochrome *c* peroxidase complex[9] are the only physiologic complexes of soluble redox proteins for which detailed crystal structures are known. Because TTQ is a two-electron carrier and amicyanin is a one-electron carrier, it is possible to monitor two different one-electron transfer reactions from reduced and semiquinone TTQ to oxidized copper. Furthermore, the driving forces for these reactions are low enough that it is also possible to monitor the reverse electron transfer (ET) reactions from reduced copper to either oxidized or semiquinone TTQ. Thus, it is possible in this system to monitor four separate ET reactions, each of which follows the same pathway and each of which has a different driving force.

Marcus theory (eq 1)[10] may be used to describe ET reactions,

$$k_{ET} = \frac{4\pi^2 H_{AB}^{\ 2}}{h\sqrt{4\pi\lambda RT}} e^{-(\Delta G^\circ+\lambda)^2/4\lambda RT} \quad (1)$$

where $H_{AB}$ is the electronic coupling between redox centers, $\lambda$ is the reorganizational energy, $h$ is Planck's constant, $R$ is the gas constant, $T$ is temperature, and $\Delta G^\circ$ is the standard free energy difference. According to eq 1, it should be possible to experimentally vary $k_{ET}$ as a function of either temperature or $\Delta G^\circ$. Analysis of the $\Delta G^\circ$ dependence of ET rates through proteins by Marcus theory has typically yielded values of $\lambda$ between 0.7 and 1.4 eV. Values reported for protein systems of known structure include the bacterial photosynthetic reaction center, $\lambda$ = 0.7 eV;[11] ruthenated azurins, $\lambda$ = 0.9 eV;[12] ruthenated cytochromes, $\lambda$ = 1.2 eV;[12] ruthenated myoglobin, $\lambda$ = 1.3 eV;[12] the cytochrome *c*−cytochrome $b_5$ complex, $\lambda$ = 0.8 eV;[13] the hemoglobin−cytochrome $b_5$ complex, $\lambda$ = 0.9 eV;[14] and the cytochrome *c*−cytochrom *c* peroxidase complex, $\lambda$ = 1.4 eV.[13] In these systems it was possible to modulate $\Delta G^\circ$ by attaching different ruthenium centers or modifying dissociable metal ions or cofactors. Extracting $\lambda$ from the temperature dependence of a protein ET reaction is complicated, especially in the case of an intermolecular reaction. We previously analyzed the temperature dependence of the ET reaction between reduced MADH and oxidized amicyanin and obtained a value of $H_{AB}$ of 11.6 cm$^{-1}$ and an unusually large $\lambda$ of 2.2 eV.[15] Temperature dependence studies of the physiologic ET reaction between methanol dehydrogenase and cytochrome *c*-551i also yielded a large $\lambda$ value of 1.9 eV.[16] These large $\lambda$ values were attributed to protein rearrangements which were coupled to the ET reaction. The large $\lambda$ values obtained from temperature dependence studies raise the question of whether they may be a consequence of the technique. In this work we examined the $\Delta G^\circ$ dependence of ET reactions between MADH and amicyanin by monitoring reactions between different redox forms of the proteins. This is the first study in which a $\Delta G^\circ$ dependence of an ET rate has been examined using only the unmodified physiologic protein redox partners.

MADH[17] and amicyanin[5] from *Paracoccus denitrificans* were purified as previously described, and protein concentrations were calculated from their known extinction coefficients.[4,5] Semiquinone MADH and reduced amicyanin were prepared by titration with dithionite. An On-Line Instrument Systems (OLIS) sample handling unit coupled to either Durrum or OLIS RSM1000 optics was used for stopped-flow experiments. All reactions were performed at 10 °C in 10 mM potassium phosphate, pH 7.5.

The oxidative half-reaction of MADH proceeds through two sequential one-electron reductions as described by the minimal scheme below (eq 2), in which $M_r$, $M_s$, and $M_o$ are respectively reduced, semiquinone, and oxidized MADH. $A_o$ and $A_r$ are

$$M_r + A_o \underset{k_2}{\overset{k_1}{\rightleftharpoons}} M_r{-}A_o \underset{k_4}{\overset{k_3}{\rightleftharpoons}} M_s{-}A_r \underset{k_6}{\overset{k_5}{\rightleftharpoons}} M_s + A_r$$
$$M_s + A_o \underset{k_8}{\overset{k_7}{\rightleftharpoons}} M_s{-}A_o \underset{k_{10}}{\overset{k_9}{\rightleftharpoons}} M_o{-}A_r \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} M_o + A_r \quad (2)$$

respectively oxidized and reduced amicyanin. The forward ($k_3$ and $k_9$) and reverse ($k_4$ and $k_{10}$) ET reactions were studied by mixing appropriate combinations of redox forms of the two proteins. Except for $k_9$, ET rate constants were determined from the concentration dependence of the initial rate of the stopped-flow reaction;[15,18] $k_9$ was determined from the limiting value of $k_{obs}$ (discussed below). It is important to note that these are complex kinetic reactions; however, it is possible to extract the individual $k_{ET}$ values from these data because we are looking at the initial rates which do not reflect the reverse or subsequent reactions.[15]

The reaction described by $k_3$ was studied by mixing reduced MADH with oxidized amicyanin. This reaction was monitored

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at 443 nm, which corresponds to the interconversion of semiquinone and reduced MADH[4] and which is isosbestic for oxidized and semiquinone MADH. The value obtained for $k_3$ was 2.4 ± 0.4 $s^{-1}$ at 10 °C. The reaction described by $k_4$ was studied by mixing oxidized MADH with reduced amicyanin and was also monitored at 443 nm. This rate constant was 0.67 ± 0.07 $s^{-1}$. The $\Delta G°$ for this reversible reaction was calculated from the equilibrium constant for the ET reaction ($K_{ET} = k_3/k_4$) according to eq 3. The experimentally determined rate

$$\Delta G° = -RT \ln K_{ET} \quad (3)$$

constants yielded values of $\Delta G°$ of −3.0 ± 0.6 kJ $mol^{-1}$ for the forward reaction and +3.0 ± 0.6 kJ $mol^{-1}$ for the reverse reaction.

The reaction described by $k_9$ was studied by mixing semiquinone MADH with oxidized amicyanin. This reaction was monitored at 428 nm, which corresponds to the interconversion of semiquinone and oxidized MADH.[4] This reaction was much faster than the ET reaction from reduced MADH and was too fast to allow determination of initial rates. Instead, $k_9$ was determined from the limiting value of $k_{obs}$ at saturating amicyanin concentrations. When the varied reactant is in large excess, $k_{obs}$ will be equal to the sum of the rate constants for the forward and reverse reactions.[19] At 10 °C, this rate was 240 ± 50 $s^{-1}$. As discussed below, the rate of the reverse reaction is insignificant compared to $k_9$, and, therefore, $k_9$ = 240 ± 50 $s^{-1}$. Even though this reaction is essentially irreversible, it was possible to monitor the reverse reaction by mixing a large excess of reduced amicyanin with oxidized MADH. This reaction was slow enough to be analyzed by the initial rate method. Because the concentration of amicyanin required to drive the reaction was well in excess of the $K_d$, the rate was independent of amicyanin concentration. This limiting initial rate ($k_{10}$) was equal to 0.05 ± 0.03 $s^{-1}$. The experimentally determined values of $k_9$ and $k_{10}$ were used to calculate $K_{ET}$ for this reversible reaction and yielded values of $\Delta G°$ of −20.0 ± 1.9 kJ $mol^{-1}$ for the forward reaction and +20.0 ± 1.9 kJ $mol^{-1}$ for the reverse reaction. The calculated $\Delta G°$ values for $k_3/k_4$ and $k_9/k_{10}$ are consistent with the known redox potentials for these proteins.[20]

For this system, ET rates have been determined for four different reactions which correspond to four experimentally determined values of $\Delta G°$ (Figure 1). The solid line in Figure 1 represents the predicted dependence of $k_{ET}$ on $\Delta G°$ using the values of $H_{AB}$ (11.6 $cm^{-1}$) and $\lambda$ (2.2 eV) which were previously determined from temperature dependence studies of $k_3$.[15] The good correlation between the data obtained in this study and the Marcus parameters which were determined in the temperature dependence study support the validity of the values of $H_{AB}$ and $\lambda$. This correlation and the clear $\Delta G°$ dependence of the reaction confirm that the unusually large $\lambda$ indeed describes an ET reaction and not a purely gated process.[22] The large $\lambda$ most likely does arise from a change in orientation of the protein complex after binding, from the ground state to an intermediate

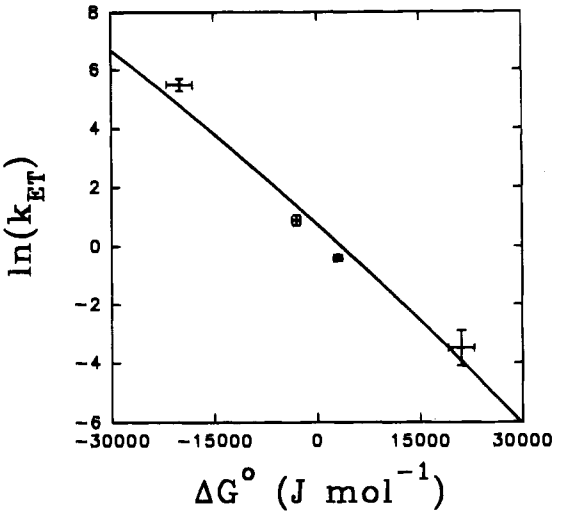


**Figure 1.** Free energy dependence of ET reactions in the MADH–amicyanin complex. Values of $k_{ET}$ and $\Delta G°$ are those which were obtained in the present work as described in the text. Error bars are shown in each direction because $\Delta G°$ values were experimentally determined. The solid line describes the relationship between $k_{ET}$ and $\Delta G°$ that is predicted by eq 1 using the values $\lambda$ = 218 kJ $mol^{-1}$ and $H_{AB}$ = 11.6 $cm^{-1}$, which were determined from thermodynamic analysis of the ET reaction from reduced MADH to oxidized amicyanin with a driving force of 31 mV.[15]

state with an optimum configuration for ET. If the rate of the reorientation were slower than the rate of the actual ET reaction, then the reaction would be gated. If, however, the rate of reorientation is faster than the ET rate, then the reaction will be coupled to this change but not gated.[15,23,24] This means that the experimentally measured $k_{ET}$ is actually an apparent rate which is equal to the product of the true ET rate and the equilibrium constant for the rearrangement process. Furthermore, the experimentally determined $\lambda$ will be an apparent $\lambda$ which reflects the energies required for the ET reaction and the reorientation.[15,23,24] These results highlight the importance of protein dynamics in modulating biological ET reactions, especially intermolecular ET reactions between proteins. The importance of protein dynamics in modulating ET reactions was demonstrated by covalent cross-linking of either cytochrome *c* or cytochrome *f* to plastocyanin, which dramatically lowered the rate of ET between these proteins.[25,26] The requirement for dynamic interactions between proteins has also been inferred from studies of the ET reaction from cytochrome *c* to cytochrome *c* peroxidase.[27] Relatively few studies of physiologic ET reactions between proteins have been reported. This is the first in which a $\Delta G°$ dependence of ET rate has been examined using only the unmodified physiologic redox partners. We have demonstrated that valid Marcus parameters may be obtained from either temperature dependence or $\Delta G°$ dependence studies for this native ET complex. Ultimately it is hoped that studies such as these will allow the formulation of general rules regarding the process of long-range ET between proteins.

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(20) The $\Delta E°$ values for the reactions described by $k_3$ and $k_9$ are calculated from these data to be +31 and +207 mV, respectively. The $E_m$ value for amicyanin in complex with MADH is +221 mV.[21] From this one may calculate $E_m$ values for MADH of +190 (semiquinone/reduced), +14 (oxidized/semiquinone), and +102 mV (oxidized/reduced). The latter value correlates well with the value of +100 mV which are determined by electrochemical titration.[4]

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