Electron Transfer from Copper to Heme within the Methylamine Dehydrogenase−Amicyanin−Cytochrome *c*-551i Complex†

Victor L. Davidson* and Limei Hsu Jones

Department of Biochemistry, The University of Mississippi Medical Center, 2500 North State Street, Jackson, Mississippi 39216-4505

Received December 4, 1995; Revised Manuscript Received April 17, 1996⊗

ABSTRACT: Methylamine dehydrogenase (MADH), amicyanin, and cytochrome *c*-551i are soluble redox proteins that form a complex in solution [Chen, L., Durley, R., Mathews, F. S., & Davidson, V. L. (1994) *Science 264*, 86−90] which is required for the physiologic electron transfer from the tryptophan tryptophylquinone cofactor of MADH to heme via the copper center of amicyanin. The electron transfer reaction from copper to heme within the protein complex has been characterized by transient kinetic and thermodynamic analysis. The rate of this electron transfer reaction is 87 s^{-1} at 30 °C, and it varied with temperature. The reaction exhibited a reorganizational energy (λ) of 1.1 eV and an electronic coupling (H_{AB}) of 0.3 cm^{-1}. The results of these analyses also predict an electron transfer distance, depending upon the value of β which is used, of 13−24 Å. The larger value approximates the direct copper to heme distance observed in the crystal structure of the complex. The most efficient pathways for electron transfer were predicted from the crystal structure using the Greenpath program, and these predictions were correlated with the results of the solution studies of the electron transfer reaction. It is concluded that electron transfer is, in fact, rate limiting for the observed electron transfer reaction in solution and that the two redox centers are strongly coupled, given the distance which separates them.

The electron transfer complex of methylamine dehydrogenase (MADH),[1] amicyanin, and cytochrome *c*-551i from *Paracoccus denitrificans* is the only such physiologic complex of three weakly associating redox proteins for which a detailed crystal structure is available (Chen et al., 1994). Only two other two-protein electron transfer complexes have been structurally characterized, the binary complex of MADH and amicyanin (Chen et al., 1992) and the complex of cytochrome *c* peroxidase and cytochrome *c* (Pelletier & Kraut, 1992). MADH (Davidson, 1993) catalyzes the oxidative deamination of methylamine to formaldehyde plus ammonia and possesses the tryptophan tryptophylquinone (TTQ) (McIntire et al., 1991) prosthetic group which participates in catalysis and electron transfer. The physiologic electron acceptor for most MADHs is amicyanin (Husain & Davidson, 1985), a type I copper protein. In *P. denitrificans*, amicyanin is absolutely required to mediate the transfer of electrons from MADH to the respiratory chain via *c*-type cytochromes. This has been demonstrated in vitro (Husain & Davidson, 1986), and it was also shown that inactivation of amicyanin in vivo by means of gene replacement resulted in complete loss of the ability to grow on methylamine (Van Spanning et al., 1990). In vitro studies have shown that, of the *c*-type cytochromes isolated from *P. denitrificans*, the most efficient electron acceptor for the MADH−amicyanin complex is cytochrome *c*-551i (Husain & Davidson, 1986).

From the crystal structure of the protein complex it is possible to infer the sites of protein−protein interaction and putative electron transfer pathways which connect the redox centers. One would like to correlate this structural information with kinetic data on the electron transfer reactions which occur within the protein complex. Ideally, these kinetic studies would be performed using the unmodified proteins. We have previously shown that amicyanin binding to cytochrome *c*-551i occurs at different sites when amicyanin is free and when it is in complex with MADH (Davidson & Jones, 1995). The electron transfer from free, uncomplexed, amicyanin to cytochrome *c*-551i occurs much more rapidly, but only to a very small extent (Davidson & Jones, 1995), because the reaction is thermodynamically much less favorable when amicyanin is not associated with MADH (Gray et al., 1988). Thus, complex-dependent electron transfer appears to be the physiologically relevant reaction. We have previously characterized the electron transfer reaction from the TTQ of MADH to amicyanin by transient kinetic and thermodynamic analyses (Brooks & Davidson 1994a,b; Bishop & Davidson, 1995) and demonstrated that the electron transfer reaction from amicyanin to cytochrome *c*-551i within the ternary complex may be monitored by stopped-flow spectroscopy (Davidson & Jones, 1995). The steady-state kinetics of the methylamine-dependent reduction of cytochrome *c*-551i by MADH and amicyanin have also been characterized (Davidson & Jones, 1991). In this paper we describe a kinetic and thermodynamic analysis of the electron transfer reaction from amicyanin to cytochrome *c*-551i in the ternary protein complex. These results have allowed us to calculate the reorganizational energy and electronic

† This work was supported by National Institutes of Health Grant GM-41574.

* Corresponding author: telephone, 601-984-1516; Fax, 601-984-1501; Email, davidson@fiona.umsmed.edu.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: MADH, methylamine dehydrogenase; TTQ, tryptophan tryptophylquinone; H_{AB}, electronic coupling; λ, reorganizational energy.

S0006-2960(95)02854-6 CCC: $12.00

coupling for this reaction and use electron transfer theory to predict the electron transfer distance between redox centers. These data are correlated with the crystal structure of the electron transfer complex and a Pathways (Regan et al., 1993) analysis of potential electron transfer paths from copper to heme within the complex.

EXPERIMENTAL PROCEDURES

Previously described procedures were used to purify MADH (Davidson, 1990), amicyanin (Husain & Davidson, 1985), and cytochrome *c*-551i (Husain & Davidson, 1986) from *P. denitrificans* (ATCC 13543). MADH was reduced by addition of methylamine. Amicyanin was reduced by addition of sodium dithionite. Cytochrome *c*-551i was oxidized by addition of potassium ferricyanide. All reagents were obtained from commercial sources.

An On-Line Instruments (OLIS, Bogart, GA) RSM1000 stopped-flow rapid scanning spectrophotometer was used for kinetic measurements. All experiments were performed in 0.01 M potassium phosphate, pH 7.5, as described previously (Davidson & Jones, 1995). Protein concentrations were determined from the previously reported extinction coefficients for each protein (Husain & Davidson, 1985; 1986; Husain et al., 1987). Nonlinear curve fitting of data was performed using either OLIS software or Sigma Plot 5.0 (Jandel Scientific).

RESULTS AND DISCUSSION

In our experimental design, one mixing syringe contained oxidized cytochrome *c*-551i and the other contained reduced MADH plus reduced amicyanin. The reduced forms of MADH and amicyanin were quite stable against reoxidation by air, and so anaerobic conditions were not necessary. Our aim was to mix fully reduced complex with oxidized cytochrome *c*-551i so that the initial event detectable after mixing would be the reduction of the cytochrome by amicyanin. It was previously demonstrated that no reaction occurred between reduced MADH and oxidized cytochrome *c*-551i in the absence of amicyanin and that no redox reaction between free amicyanin and cytochrome is detectable during the time course of these experiments (Davidson & Jones, 1995). The latter reaction is so fast that it is complete within the dead time for mixing and only occurs to a small extent because it is thermodynamically very unfavorable. To ensure that most of the reduced amicyanin was in complex with MADH, experiments were performed at concentrations of MADH and amicyanin which were well above the K_d for the MADH−amicyanin complex. The K_d value for the MADH−amicyanin complex has been previously determined to be approximately 4 μM (Davidson et al., 1993; Brooks & Davidson, 1994a) under these buffer conditions. The concentration of amicyanin actually in complex with MADH was calculated using this K_d value and the concentrations of MADH and amicyanin present in solution. The amicyanin in complex with MADH will subsequently be referred to as "bound amicyanin". The percent of total amicyanin which was bound varied from approximately 70% at the lowest concentrations of complex to 95% at the highest concentrations.

The kinetic data obtained from the stopped-flow experiments were analyzed assuming the simple model described

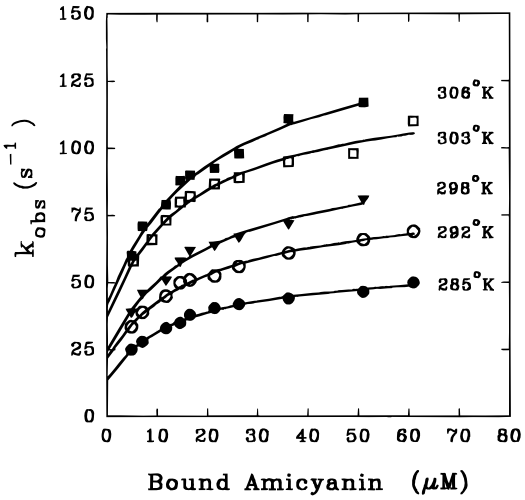

FIGURE 1: Concentration and temperature dependence of k_{obs} for the reactions of cytochrome *c*-551i with the reduced MADH−amicyanin complex. The concentration of the limiting reactant, oxidized cytochrome *c*-551i , was 0.7−3.0 μM. The varied reactant was the reduced MADH−amicyanin complex. Reduced MADH and reduced amicyanin were present in a 1:1 molar ratio with the concentrations of each ranging from 7.5 to 65 μM. The concentration of bound amicyanin was calculated from the K_d value for the complex and indicates the concentration present in complex with MADH, excluding any free amicyanin. The temperature at which each set of experiments was performed is indicated. The solid lines represent the fits of these data to eq 2.

by eq 1, where M is MADH, A is amicyanin, and C is

$$M_{red}-A_{red} + C_{ox} \overset{K_d}{\rightleftharpoons} M_{red}-A_{red}-C_{ox} \overset{k_3}{\underset{k_4}{\rightleftharpoons}} M_{red}-A_{ox}-C_{red} \quad (1)$$

cytochrome *c*-551i. The subscripts indicate whether the redox state is reduced or oxidized . After oxidized cytochrome *c*-551i was mixed with the reduced MADH−amicyanin complex, spectral changes occurred which were indicative of reduction of the cytochrome (Davidson & Jones, 1995). For kinetic analyses, the observed rate constant (k_{obs}) was determined from the absorbance change with time at either 552 or 419 nm. Under these experimental pseudo-first-order conditions, all absorbance changes could be fit to a single exponential (data not shown). This is consistent with our previous observation (Davidson & Jones, 1995) that the kinetic components associated with any reaction of free amicyanin occur within the dead time of the mixing and do not have any influence on the observed complex-dependent kinetics. At the wavelengths being monitored, all steps beyond k_3/k_4 in eq 1 are spectroscopically invisible. For the reactions of the reduced MADH−amicyanin complex with oxidized cytochrome *c*-551i, data for the concentration dependence of k_{obs} were fit to eq 2 (Figure 1), which is

$$k_{obs} = \frac{k_3[\text{amicyanin}]}{[\text{amicyanin}] + K_d} + k_4 \quad (2)$$

derived (Strickland et al., 1975) for the model given in eq 1. Since the amicyanin which is bound to MADH is the relevant species, data were analyzed in terms of the concentration of bound amicyanin rather than total amicyanin present. In each case, the data were described by a simple hyperbolic dependence of k_{obs} on concentration (Figure 1). As such, it was not possible to determine unique values for

Table 1: Kinetic Parameters for the Electron Transfer Reaction from the MADH−Amicyanin Complex to Cytochrome *c*-551i[a]

temp (K)	K_d (μM)	k_3 (s^{-1})	k_4 (s^{-1})
285	14.2 ± 3.1	43.3 ± 1.8	13.7 ± 2.6
292	19.1 ± 3.9	60.5 ± 2.1	22.0 ± 2.8
298	19.5 ± 6.2	76.1 ± 4.3	24.5 ± 4.9
303	16.9 ± 6.1	87.2 ± 5.4	37.2 ± 8.3
306	20.6 ± 6.5	105.6 ± 6.3	41.6 ± 6.4

[a] The parameters are obtained from the fits of the data sets shown in Figure 1 to eq 2.

k_1 and k_2. Only a unique value for the ratio k_2/k_1 (K_d) could be determined in addition to values for k_3 and k_4. Sets of experiments were performed at temperatures ranging from 12 to 33 °C. The values obtained for each kinetic parameter at each temperature are given in Table 1. The oxidation−reduction midpoint potential values for amicyanin in complex with MADH (Gray et al., 1988) and cytochrome *c*-551i (Gray et al., 1986) are +221 and +190 mV, respectively. Thus, this reaction is slightly unfavorable, and from the difference in redox potentials of −31 mV, the equilibrium constant for this reaction is predicted to be 0.3. One would, therefore, expect k_4 to be larger than k_3. Finite values of k_4 were observed; however, these values were less than the fitted values of k_3 (Table 1). For the kinetic model in eq 1, the fitted value of k_4 obtained from eq 2 will only be a true k_4 if the $M_{red}-A_{ox}-C_{red}$ complex accumulates (i.e., dissociation of reduced cytochrome *c*-551i is much slower than k_4). That the observed value for k_4 is smaller than predicted indicates that the dissociation of reduced cytochrome *c*-551i from the ternary complex is faster than k_4. Therefore, the fitted value for k_4 is really an apparent rate constant, which also contains contributions from the dissociation constant for the release of reduced cytochrome *c*-551i from the ternary complex.

The limiting first-order rate constant (k_3) strictly describes the redox change of cytochrome *c*-551i from the oxidized to reduced state. This rate should be considered an apparent electron transfer rate constant (k_{ET}) because in this kinetic model any spectroscopically invisible reaction steps which may occur subsequent to binding and before electron transfer may be reflected in k_3. The k_3 will only be a true k_{ET} if no such additional reaction steps occur or if electron transfer is rate limiting in a multistep mechanism where all other steps are energetically favorable (Hoffman & Ratner, 1988; Brunschwig & Sutin, 1989; Harris et al., 1994). Whether or not k_3 describes an electron transfer event or some adiabatic process which is a prerequisite for electron transfer may be inferred from the results of the analysis of the temperature dependence of k_3 (Bishop & Davidson, 1995).

The temperature dependence of k_3 may be analyzed by transition state theory, which provides information about the formation of the activated complex which leads to the conversion of reactants into products in an adiabatic reaction. These data were fit to the Eyring equation (eq 3), in which

$$\ln (k_3 h/k_B T) = -\Delta H^*/RT + \Delta S^*/R \qquad (3)$$

h is Planck's constant, R is the gas constant, T is temperature, k_B is Boltzman's constant, ΔH^* is activation enthalpy, and ΔS^* is activation entropy. The data fit well to a straight line (data not shown), and this fit yielded values for ΔH^* of 27 ± 2 kJ mol^{-1} and for ΔS^* of −117 ± 5 J mol^{-1} K^{-1} for the reaction that is described by k_3. How one interprets the meaning of these thermodynamic parameters depends on whether or not k_3 describes an adiabatic reaction.

An adiabatic process is a reaction which proceeds to completion every time the transition state energy is achieved and is most appropriately described by transition state theory. Conversely, in a nonadiabatic process achievement of the transition state energy does not always lead to product formation (Marcus & Sutin, 1985; McLendon, 1988). Long-range electron transfer reactions are nonadiabatic. Electron transfer reactions within an electron transfer complex are collisionless and do not involve the making or breaking of bonds. The reaction coordinates and energy barriers associated with nonadiabatic electron transfer reactions are described better by electron transfer theory than by classical transition state theory, and for such reactions ΔH^* and ΔS^* are better described in terms of reorganizational energy (λ) and electronic coupling (H_{AB}).

Electron transfer theory (Marcus & Sutin, 1985) predicts that the rate of an electron transfer reaction will vary predictably with temperature and $\Delta G°$ according to eq 4. λ

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

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

may be viewed as the energy needed to move the electron from donor to acceptor without allowing nuclear motion and may reflect nuclear displacements in the chromophores, protein medium, and solvent. H_{AB} describes the degree of wave function overlap occurring between donor and acceptor sites and is related to the distance between redox centers and the intervening medium which separates them. This is seen in eq 4b, where H_{AB} is factored into terms which define the electron transfer distance (r) and nature of the medium (β). The other parameters in these equations are r_o, the close contact distance which is set at 3.0 Å, h, Planck's constant, R, the gas constant, and k_o, the characteristic frequency of the nuclei which is set at 10^{-13} s^{-1}. The $\Delta G°$ for this reaction is calculated from the known redox potentials of the redox centers to be +3.0 kcal/mol (−31 mV). The temperature dependence of k_3 was analyzed by eq 4a (Figure 2). Fitted values were obtained for λ of 1.1 ± 0.1 eV and H_{AB} of 0.3 ± 0.1 cm^{-1}. The temperature dependence of k_4 was not analyzed because it appears to be a composite rate constant influenced by the rates of subsequent steps (discussed earlier).

For a nonadiabatic electron transfer reaction, the probability that the achievement of the activation energy leads to the formation of product is less than unity and approaches zero as H_{AB} approaches zero. If the actual electron transfer step is truly rate limiting for the experimentally determined k_{ET}, then H_{AB} should be less than 80 cm^{-1}. This nonadiabatic limit is calculated from the dynamic relaxation rate of water and occurs when the reorganization of water, as the bulk solvent, becomes rate limiting (Winkler & Gray, 1992). The experimentally determined value of H_{AB} of 0.3 cm^{-1} is, therefore, consistent with k_3 describing an electron transfer reaction. It is also within the range of H_{AB} values which have been obtained from model studies with ruthenated redox proteins, although at the high end of H_{AB} values reported for electron transfer reactions over comparable distances (Onuchic et al., 1992). This suggests that the donor and

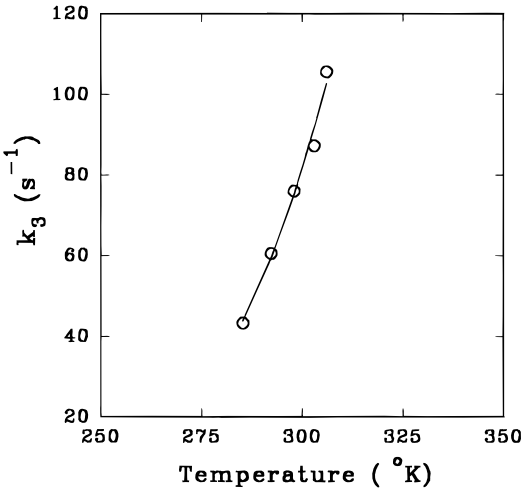

FIGURE 2: Analysis of the temperature dependence of k_3 for the reactions of cytochrome *c*-551i with the reduced MADH−amicyanin complex. Values of k_3 were determined from the data shown in Figure 1, which were fit to eq 2. These values were analyzed according to eqs 4a and 4b. The solid line represents the fits of these data to those equations. The two fits to the data are superimposable.

acceptor are reasonably well coupled given the distance that separates them.

The value of λ of 1.1 eV falls within a range of reported values which have been experimentally determined for electron transfer reactions through proteins: 0.7 eV for the photosynthetic reaction center (Moser et al., 1992); 0.9−1.3 eV for ruthenated azurins, cytochromes, and myoglobin (Winkler & Gray, 1992); 0.8 eV for the cytochrome *c*−cytochrome b_5 complex (McLendon & Hake, 1992); and 1.4 eV for the cytochrome *c*−cytochrome *c* peroxidase complex (Conklin & McLendon, 1988). If the fitted values of either λ or H_{AB} or both would have been unreasonably large, then this would have been diagnostic of a gated reaction (Hoffman & Ratner, 1987) in which k_3 actually described the rate of an adiabatic process, such as a conformational change or catalytic step, which is a prerequisite for electron transfer. For example, analysis of the temperature dependence of the apparent electron transfer rate constant for the reaction of substrate-reduced aminoquinol MADH with amicyanin yielded values of λ of 3.5 eV and H_{AB} of 23 000 cm^{-1}, and this reaction was shown to be gated by a proton transfer (Bishop & Davidson, 1995), unlike the reaction of dithionite-reduced quinol MADH with amicyanin which is not gated (Brooks & Davidson, 1994b). The values of λ and H_{AB} which were obtained from the temperature dependence of k_3 are at least consistent with the notion that the electron transfer step is rate limiting for the observed reaction between amicyanin and cytochrome *c*-551i in the ternary protein complex and that we are not simply measuring the rate of an adiabatic process such as a protein conformational change.

A potential complication which must be considered when examining the temperature dependence of electron transfer rate constants is that $\Delta G°$ may be temperature dependent. This could arise from temperature dependent changes in the conformations of amicyanin or cytochrome *c*-551i which affect the redox potential. We have previously examined the temperature dependence of the electron transfer reaction from the dithionite-reduced quinol MADH to amicyanin (Brooks & Davidson, 1994a). The $\Delta G°$ dependence of this reaction was also characterized by monitoring the forward and reverse redox reactions between quinol and semiquinone MADH and amicyanin (Brooks & Davidson, 1994b). These temperature- and $\Delta G°$-dependence studies yielded comparable values for H_{AB} and λ. Good correlation has also been reported for values of λ which were obtained from temperature- and $\Delta G°$-dependence studies of the electron transfer reaction between cytochrome *c* and cytochrome *c* peroxidase (Conklin & McLendon, 1988). Given that λ is relatively large (1.1 eV) compared to $\Delta G°$ (−31 mV), any changes in $\Delta G°$ with temperature would have to be substantial to compromise our analysis (see eq 4). Fortunately, MADH, amicyanin, and cytochrome *c*-551i are each quite thermally stable toward denaturation. Furthermore, the K_d values which were obtained in this study did not vary significantly over the range of temperatures used in this study (Table 1), suggesting no gross temperature-dependent perturbations of structure. Thus, the likelihood of significant changes in $\Delta G°$ over the range of temperatures used in this study is very small.

We have previously reported relatively large λ values for two intermolecular protein electron transfer reactions. The electron transfer reaction from the dithionite-reduced quinol form of MADH and oxidized amicyanin exhibited a λ of 2.3 eV (Brooks & Davidson, 1994a), and the physiologic electron transfer reaction between methanol dehydrogenase and cytochrome *c*-551i yielded a λ of 1.9 eV (Harris & Davidson, 1993). Despite the large λ values, each of these reactions exhibited H_{AB} values within the nonadiabatic limit, and thermodynamic analysis of k_{ET} predicted electron transfer distances which were consistent with the known crystal structures of these proteins. It was suggested that those large λ values could reflect a contribution from protein rearrangements within the electron transfer complex which were coupled to the electron transfer reaction (Harris et al., 1994). The importance of protein dynamics in modulating electron transfer reactions was demonstrated by covalent cross-linking of either cytochrome *c* or *f* to plastocyanin, which dramatically lowered the rate of electron transfer between these proteins (Peerey et al., 1991; Qin & Kostic, 1993). The requirement for dynamic interactions between proteins has also been inferred from studies of the electron transfer reaction from cytochrome *c* to cytochrome *c* peroxidase which exhibited a λ of 1.4 eV (Conklin & McLendon, 1988). The λ of 1.1 eV reported for the electron transfer reaction from amicyanin to cytochrome *c*-551i within the ternary protein complex is more typical of values reported for intramolecular electron transfer reactions in ruthenated proteins (Winkler & Gray, 1992; Onuchic et al., 1992). This may mean that relatively little rearrangement of proteins within the ternary complex is required to achieve the optimum orientation for the electron transfer reaction from amicyanin to cytochrome *c*-551i. In other words, the optimum orientation of the proteins for binding of cytochrome *c*-551i to the MADH−amicyanin complex is very similar to the optimum orientation of the proteins required to optimize coupling for the electron transfer reaction.

Analysis of these data by eq 4b yielded a range of fitted values for the distance between redox centers of 13−23 Å, depending upon the β value that was used. This range was obtained using β values ranging from 1.4 to 0.7 $Å^{-1}$ (Moser et al., 1992; Onuchic et al., 1992), and the standard error of the fitted values of *r* was approximately ±5%. The β value

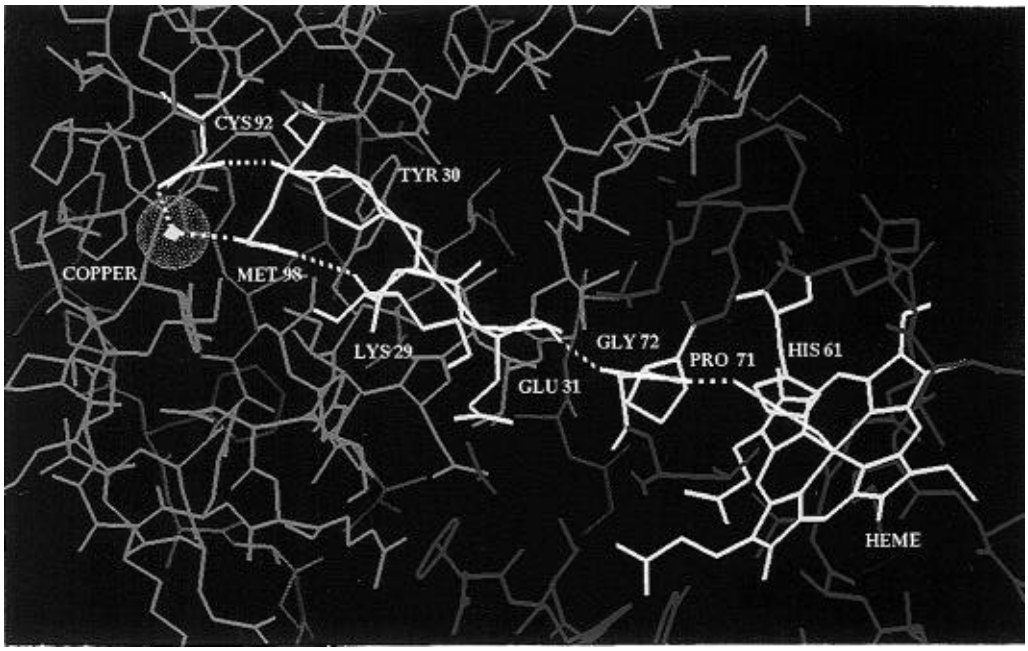

FIGURE 3: Predicted pathways of electron transfer between copper and iron within the MADH−amicyanin−cytochrome *c*-551i complex. A portion of the crystal structure of the ternary protein complex is displayed. Amicyanin is in blue and cytochrome *c*-551i is in red. The heme, copper, and amino acid residues which participate in the two sets of predicted dominant electron transfer pathways are in yellow. Portions of the pathways through covalent bonds are shown as solid white lines, and through space jumps are shown as dashed white lines. The two pathways overlap from Glu^{31} to heme. The predicted relative efficiencies of these pathways are discussed in the text.

of 0.7 is thought to be appropriate for electron tunneling through σ bonds in a covalently coupled system. The value of 1.4 has previously been applied for electron transfer through a protein matrix and is a value between that of a covalently linked systems and a vacuum (Moser et al., 1992). Langen et al. (1995) recently calculated a β value of 1.1 $Å^{-1}$ for electron tunneling through β strands in ruthenated azurin. This range of predicted distance, determined using this range of β values, may be evaluated in the context of the known structure of the protein complex.

The direct center to center distance from the copper of amicyanin to the iron of cytochrome *c*-551i in the crystal structures of the electron transfer complex is 24.7 Å. Alternatively, the shortest distance from copper to the nearest edge of the heme porphyrin ring is 22.7 Å. A Pathways analysis (Regan et al., 1993) was performed using the Greenpath v0.97 computer program. Pathways from copper to iron and to the porphyrin ring were calculated. Several pathways were identified with relative couplings which were down to 50% of that of the best pathway identified. Two dominant sets of pathways were apparent (Figure 3). In each set of pathways, the point of intermolecular electron transfer was from Glu^{31} of amicyanin to either Pro^{71} or Gly^{72} of cytochrome *c*-551i, and the entry of electrons to iron occurred either via the porphyrin ring or the His^{61} ligand. In one set of pathways the exit of electrons from copper occurred via the Cys^{92} copper ligand, and Tyr^{30} was an intermediate between Cys^{92} to Glu^{31}. In the other set of pathways the exit of electrons from copper occurred via the Met^{98} copper ligand, and Lys^{29} was an intermediate between Met^{98} to Glu^{31}. These pathways contained no through space jumps which were greater than 3 Å. The absence of long, relatively inefficient, through space jumps in the predicted pathways[2] seems consistent with the experimentally determined H_{AB} values which suggested that copper and iron were well coupled given the distance by which they are separated.

It should be noted that the results of the Greenpath analysis of the ternary complex described above and in Figure 3 were obtained without taking into consideration differences in metal−ligand binding between copper and the amino acid residues which provide ligands. This analysis indicates a similar degree of copper−iron coupling for the two pathways shown in Figure 3. Ullmann and Kostic (1995) recently reported a Pathways analysis for possible electron transfer pathways between iron(II) and copper(II) sites in docked complexes of cytochrome *c* and plastocyanin. Consideration of the anisotropy of metal−ligand coupling, which was based on the electronic structure of the copper(II) site, was incorporated into their analysis. Quantum chemical calculations and biophysical studies (Penfield et al., 1985; Solomon & Lowry, 1993) on which these considerations are based suggested that the Met ligand of the type 1 copper is not likely to be involved in electron transfer into the oxidized center and that the coupling between copper and Met is essentially zero (Ullmann & Kostic, 1995). If similar corrections to account for copper−ligand anisotropy are incorporated into the pathways analysis of the ternary complex, then pathways which utilize Met^{98} would be highly disfavored relative to those which utilize Cys^{92}.

Our ultimate goals are to use this protein complex to determine precisely what factors regulate the observed rates of physiologic biological electron transfer reactions and to test whether or not specific amino acid residues are critical

[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 for the protein 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.

components of electron transfer pathways. For this experimental system to be of any use it must be established that in solution one is truly measuring rates of electron transfer and not some nonelectron-transfer process which is gating the observed reaction. If the reaction were gated, then experimentally induced variations in the true electron transfer rate might not be detected because electron transfer is not rate limiting. It is also important to establish that the crystal structure which one is using as a context in which to interpret data is at least a reasonable approximation of the structure of the complex in solution. It was previously established that the observed redox reactions studied here require formation of the ternary complex in solution (Gray et al., 1986, 1988; Davidson & Jones, 1991, 1995). Recent single crystal microspectrophotometry studies (Merli et al., 1996) have further shown that the crystals of the ternary protein complex are capable of oxidizing methylamine and transferring electrons from TTQ to heme via copper. Thus, the proteins, in the orientation displayed in the crystal structure, are competent for both catalysis and electron transfer. The results of the kinetic and thermodynamic studies reported in this paper provide good evidence that electron transfer is indeed rate limiting for the observed redox reactions in solution. The results also suggest that the crystal structure provides at least a reasonable approximation of the orientation of these proteins in the electron transfer complex in solution. We have previously demonstrated good correlation between results of kinetic and thermodynamic studies of the electron transfer reaction from TTQ to copper and the orientation of the proteins displayed in the crystal structure of this complex (Brooks & Davidson, 1994a). This system should, therefore, serve very well as a paradigm for characterizing the factors which determine the rates of long-range intermolecular electron transfer reactions between proteins.

ACKNOWLEDGMENT

The authors thank Harold Brooks for his contributions to the early stages of this study, Reid Bishop for helpful discussions, Rex Williams for technical assistance, and Jeffrey J. Regan for providing the Greenpath v0.97 program.

REFERENCES

Bishop, G. R., & Davidson, V. L. (1995) *Biochemistry 34*, 12082−12086.
Brooks, H. B., & Davidson, V. L. (1994a) *Biochemistry 33*, 5696−5701.
Brooks, H. B., & Davidson, V. L. (1994b) *J. Am. Chem. Soc. 116*, 11201−11202.
Brunschwig, B. S., & Sutin, N. (1989) *J. Am. Chem. Soc. 111*, 7454−7465.
Chen, L., Durley, R., Poloks, B. J., Hamada, K., Chen, Z., Mathews, F. S., Davidson, V. L., Satow, Y., Huizinga, E., Vellieux, F. M. D., & Hol, W. G. J. (1992) *Biochemistry 31*, 4959−4964.
Chen, L., Durley, R., Mathews, F. S., & Davidson, V. L. (1994) *Science 264*, 86−90.
Conklin, K. T., & McLendon, G. L. (1988) *J. Am. Chem. Soc. 110*, 3345−3350.
Davidson, V. L. (1990) *Methods Enzymol. 188*, 241−246.
Davidson, V. L. (1993) in *Principles and Applications of Quinoproteins* (Davidson, V. L., Ed.) pp 73−95, Marcel Dekker, New York.
Davidson, V. L., & Jones, L. H. (1991) *Anal. Chim. Acta 249*, 235−240.
Davidson, V. L., & Jones, L. H. (1995) *J. Biol. Chem. 270*, 23941−23943.
Davidson, V. L., Graichen, M. E., & Jones, L. H. (1993) *Biochim. Biophys. Acta 1144*, 39−45.
Gray, K. A., Knaff, D. B., Husain, M., & Davidson, V. L. (1986) *FEBS Lett. 207*, 239−242.
Gray, K. A., Davidson, V. L., & Knaff, D. B. (1988) *J. Biol. Chem. 263*, 13987−13990.
Harris, T. K., & Davidson, V. L. (1993) *Biochemistry 32*, 14145−14150.
Harris, T. K., Davidson, V. L., Chen, L., Mathews, F. S., & Xia, Z. (1994) *Biochemistry 33*, 12600−12608.
Hoffman, B. M., & Ratner, M. A. (1987) *J. Am. Chem. Soc. 109*, 6237−6243.
Husain, M., & Davidson, V. L. (1985) *J. Biol. Chem. 260*, 14626−14629.
Husain, M., & Davidson, V. L. (1986) *J. Biol. Chem. 261*, 8577−8580.
Husain, M., Davidson, V. L., Gray, K. A., & Knaff, D. B. (1987) *Biochemistry 26*, 4139−4143.
Langen, R., Chang, I.-J., Germanas, J. P., Richards, J. H., Winkler, J. R., & Gray, H. B. (1995) *Science 268*, 1733−1735.
Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265−322.
McIntire, W. S., Wemmer, D. E., Christoserdov, A. Y., & Lidstrom, M. E. (1991) *Science 252*, 817−824.
McLendon, G. (1988) *Acc. Chem. Res. 21*, 160−167.
McLendon, G., & Hake, R. (1992) *Chem. Rev. 92*, 481−490.
Merli, A., Brodersen, D. E., Morini, B., Chen, Z., Durley, R. C. E., Mathews, F. S., Davidson, V. L., & Rossi, G. L. (1996) *J. Biol. Chem. 271*, 9177−9180.
Moser, C. C., Keske, J. M., Warncke, K., Farid, R. S., & Dutton, P. L. (1992) *Nature 355*, 796−802.
Onuchic, J. N., Beratan, D. N., Wrinkler, J. R., & Gray, H. B. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 349−377.
Peerey, L. M., Brothers, H. M., Hazzard, J. T., Tollin, G., & Kostic, N. M. (1991) *Biochemistry 30*, 9297−9304.
Pelletier, H., & Kraut, J. (1992) *Science 258*, 1748−1755.
Penfield, K. W., Gerwirth, A. A., & Solomon, E. I. (1985) *J. Am. Chem. Soc. 107*, 4519−4529.
Regan, J. J., Risser, S. M., Beratan, D. N., & Onuchic, J. N. (1993) *J. Chem. Phys. 97*, 13083−13088.
Qin, L., & Kostic, N. M. (1992) *Biochemistry 31*, 5145−5150.
Solomon, E. I., & Lowery, M. D. (1993) *Science 259*, 1575−1581.
Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048−4052.
Ullmann, G. M., & Kostic, N. M. (1995) *J. Am. Chem. Soc. 117*, 4766−4774.
van Spanning, R. J. M., Wansell, C. W., Reijnders, W. N. M., Oltmann, L. F., & Stouthamer A. H. (1990) *FEBS Lett. 275*, 217−220.
Winkler, J. R., & Gray, H. B. (1992) *Chem. Rev. 92*, 369−379.

BI952854F