Binding and Electron Transfer Reactions between Methanol Dehydrogenase and Its Physiologic Electron Acceptor Cytochrome *c*-551i: A Kinetic and Thermodynamic Analysis†

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ABSTRACT: The quinoprotein methanol dehydrogenase and cytochrome *c*-551i form a physiologic complex in which electrons are transferred from pyrroloquinoline quinone to heme. The reoxidation of methanol dehydrogenase by the cytochrome was studied by stopped-flow spectroscopy. The rate constant for the electron transfer reaction and the dissociation constant for complex formation were each determined at temperatures ranging from 20 to 50 °C. The electron transfer rates varied from 1.4 to 4.6 s^{-1}. Analysis of the electron transfer reaction by Marcus theory yielded values of 1.9 eV for the reorganizational energy and 0.071 cm^{-1} for the electronic coupling and predicted a theoretical distance between redox centers of 15 Å. Kinetically determined dissociation constants correlated well with a K_d of 375 μM which was determined in a direct ultrafiltration binding assay. Thermodynamic analysis of the dissociation constants indicated the importance of the hydrophobic effect in complex formation.

Paracoccus denitrificans provides a powerful and convenient system for studying the mechanisms of long-range intermolecular electron transfer. Regulation of the synthesis of a variety of soluble electron transport proteins is mediated by growth conditions. For example, when methanol or methylamine is used as a sole source of carbon and energy, each is initially oxidized by one of two inducible periplasmic quinoproteins, methanol dehydrogenase (MEDH)[1] (Bamford & Quail, 1979) or methylamine dehydrogenase (MADH) (Husain & Davidson, 1987). The electrons derived from these oxidations are then transferred to a membrane bound cytochrome oxidase by soluble redox proteins, which include *c*-type cytochromes, and a Type I blue copper protein, amicyanin. The periplasmic cytochromes of *P. denitrificans* include a constitutive class I cytochrome *c*-550 and two other *c*-type cytochromes, designated cytochrome *c*-551i and cytochrome *c*-553i, which are induced during growth on methanol or methylamine (Husain & Davidson, 1986). Amicyanin is induced only during growth on methylamine and mediates electron transfer between MADH and cytochrome *c*-551i (Husain & Davidson, 1985; Husain et al., 1986). It has been shown that cytochrome *c*-551i is also the physiological electron acceptor for MEDH (Long & Anthony, 1991). The cytochrome *c*-551i gene is located within the same gene cluster as that for MEDH, and mutants lacking this cytochrome were unable to grow on methanol (van Spanning et al., 1991). It has also been demonstrated *in vitro* that cytochrome *c*-550 mediates electron transfer from cytochrome *c*-551i to the membrane-bound oxidase (Davidson & Kumar, 1989). Except for the membrane-bound oxidase, these periplasmic redox enzymes and proteins are soluble and provide an opportunity to study physiologic electron transfer reactions between proteins in solution.

MEDH possesses an $\alpha_2\beta_2$ structure with subunits of 67 and 9.5 kDa (Nunn et al., 1989; Davidson et al., 1992), noncovalently bound pyrroloquinoline quinone (PQQ) (Salisbury et al., 1979), and tightly bound Ca^{2+}. The steady-state kinetic properties of the reactions of MEDH from *P. denitrificans* with artificial electron acceptors have been characterized (Harris & Davidson, 1993). Steady-state characterization of the interaction of MEDH with cytochrome *c*-551i is complicated by the high *in vitro* pH requirement of MEDH with a pH optimum of 9.0. At this pH cytochrome *c*-551i undergoes autoreduction and, therefore, cannot be used in the assay. MEDH exhibits very little catalytic activity *in vitro* at pH 7.5; however, at this more physiologic pH the cytochrome may be used as an electron acceptor. The low activity is believed to be due to the absence *in vitro* of some natural activator of the substrate oxidation step (Dijkstra et al., 1989), which should not affect the subsequent electron transfer reaction. MEDH is isolated in a stable semiquinone redox state (Frank et al., 1989). In this paper the protein–protein association and the single electron transfer reactions from the semiquinone of MEDH to the oxidized form of cytochrome *c*-551i have been described by stopped-flow kinetic, binding, and thermodynamic studies.

The specificity of protein–protein recognition is of fundamental importance to the mechanism of electron transfer between two redox proteins. The magnitude and sign of the thermodynamic parameters $\Delta H°_{app}$, $\Delta S°_{app}$, and $\Delta C_{p,app}$, obtained for the temperature dependence of the kinetically determined binding constant may be used to describe the nature of the forces which contribute to the stability of the protein–protein association between MEDH and cytochrome *c*-551i, such as van der Waals interactions, hydrogen bonding, and electrostatic interactions (Ross & Subramanian, 1981).

The effect of temperature on the rate constants of electron transfer reactions is predicted by Marcus theory (Marcus & Sutin, 1985). The electron transfer rate (k_{ET}) is dependent on the free energy change ($\Delta G°$) and temperature (T), as well as the electronic coupling element (H_{AB}) and the

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[1] Abbreviations: ΔC_p, heat capacity change; ΔH, enthalpy change; ΔS, entropy change; E_m, oxidation–reduction midpoint potential; H_{AB}, electronic coupling; k_{ET}, electron transfer rate constant; λ, reorganizational energy; MADH, methylamine dehydrogenase; MEDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; TFA, trifluoroacetic acid.

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reorganizational energy (λ):

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

H_{AB} describes the probability of electron transfer or the electronic coupling between redox centers and depends upon the distance and nature of the intervening medium. λ relates to the magnitude of any protein conformational changes and solvent rearrangement associated with the electron transfer reaction. R is the gas constant, and h is Planck's constant. Electron transfer rate constants are also predicted to be dependent on the distance (r) between the electron donor and the electron acceptor by use of

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

where the electronic decay factor (β) is often approximated to be 1.4 Å^{-1} for the interior of a protein, r_0 is the van der Waals contact distance (3 Å), and k_0 is the characteristic frequency of the nuclei which is usually assigned a value of 10^{13} s^{-1} (Marcus & Sutin, 1985; Rees & Farrelly, 1990). It can be seen from eqs 1 and 2 that the decrease in H_{AB} with increasing distance depends on β, which is related the nature of the intervening medium.

In this paper we report values for H_{AB}, λ, and the predicted distance for the physiologic electron transfer reaction between MEDH and cytochrome *c*-551i from *P. denitrificans*. These values are discussed in terms of the structural information available from X-ray crystallographic studies of MEDHs (Xia et al., 1992) and cytochrome *c*-551i (Chen et al., 1993).

EXPERIMENTAL PROCEDURES

Purification of MEDH (Davidson et al., 1992) and cytochrome *c*-551i (Husain & Davidson, 1986) from *P. denitrificans* was as described previously. MEDH was isolated as the semiquinone form of the enzyme (Frank et al., 1989). MEDH concentrations were calculated from an $\epsilon_{1mg/mL}$ at 280 nm of 3.70 under native conditions as given by a dry weight determination. Cytochrome *c*-551i concentrations were calculated from the known extinction coefficient (Husain & Davidson, 1986). All reagents were purchased from commercial sources.

Direct binding studies of the interaction between MEDH and cytochrome *c*-551i by ultrafiltration were performed according to Davidson et al. (1993). Separation and quantitation of MEDH and cytochrome *c*-551i by HPLC after ultrafiltration was done using a C4 Vydac reverse-phase column. Solvent A contained 0.06% trifluoroacetic acid (TFA) in water and solvent B contained 0.052% TFA in 60% acetonitrile in water. Proteins were eluted using a convex gradient of 80% A/20% B to 20% A/80% B over 25 min at a flow rate of 0.6 mL/min. The small subunit of MEDH eluted at approximately 12 min and the large subunit at approximately 20 min. The concentration of MEDH was monitored by the absorbance of the small subunit at 277 nm, a maximum in this solvent system. Cytochrome *c*-551i eluted at approximately 22 min and was monitored at 395 nm, also a maximum in this solvent system.

Stopped-flow experiments were performed with an On-Line Instrument Systems (OLIS, Bogart, GA) stopped-flow sample handing unit coupled to Durram optics. Data were collected and analyzed using OLIS software on an IBM-compatible 486 personal computer. Experiments were carried out by mixing MEDH in 0.1 M potassium phosphate buffer, pH 7.5, containing 0.1 M NaCl, with an equal volume of cytochrome *c*-551i in the same buffer. Reduction of oxidized cytochrome

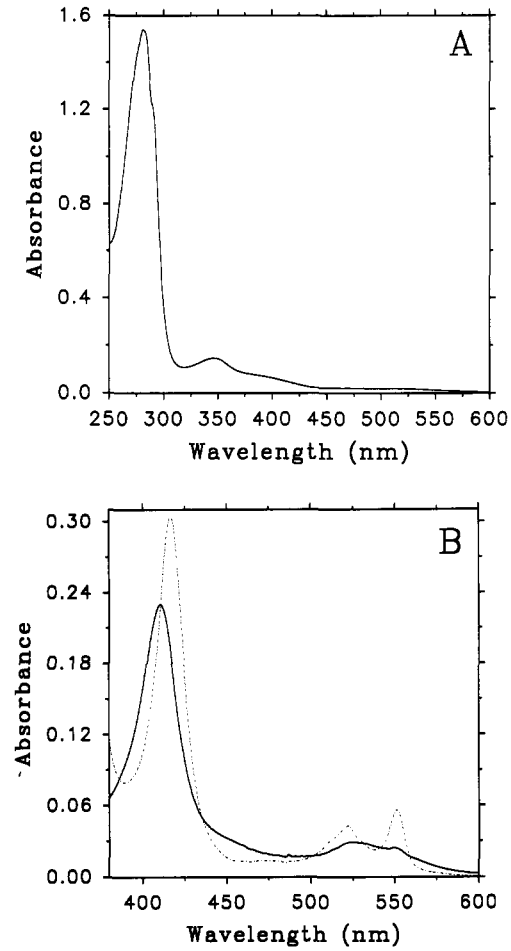

FIGURE 1: (A) Absorption spectrum of MEDH (0.42 mg/mL). (B) Absorption spectra of the oxidized (—) and reduced (- - -) forms of cytochrome *c*-551i (2.2 μM).

c-551i by MEDH was monitored at 551 nm ($\Delta\epsilon$ = 13.3 mM^{-1} cm^{-1}) where absorbance by MEDH is minimal (Figure 1). Under conditions where the varied reactant, $MEDH_{sem}$, was in excess of the concentration of the fixed reactant, cytochrome *c*-551i, the observed rate constants (k_{obs}) at each temperature could be determined from the data fit to the equation for a single-exponential decay

$$\Delta A_{551\ nm} = C(e^{-kt}) + b \quad (3)$$

where C is a constant related to the initial absorbance and b represents an offset value to account for a nonzero basline (Figure 2). All observed absorbance changes were monophasic, indicating that the reduction of cytochrome *c*-551i was pseudo-first-order under these conditions. The k_{obs} for each reaction was determined from the average of at least three measurements. Nonlinear curve fitting of data was performed with the Enzfitter (Elsevier-BIOSOFT, Cambridge) and Sigma Plot 5.0 (Jandel Scientific, San Raphael, CA) computer programs.

RESULTS

Binding Studies. An ultrafiltration binding assay (Davidson et al., 1993) was used as a direct method for determining the equilibrium binding constant between MEDH and cytochrome *c*-551i. A range of concentrations of cytochrome from 30 to 900 μM was mixed with MEDH in the top compartment of a Centricon-100 (Amicon Inc., Beverly, MA) concentrator at room temperature in 0.1 M potassium phosphate buffer with 0.1 M NaCl. To verify that the free cytochrome concentrations were identical on either side of the membrane, different

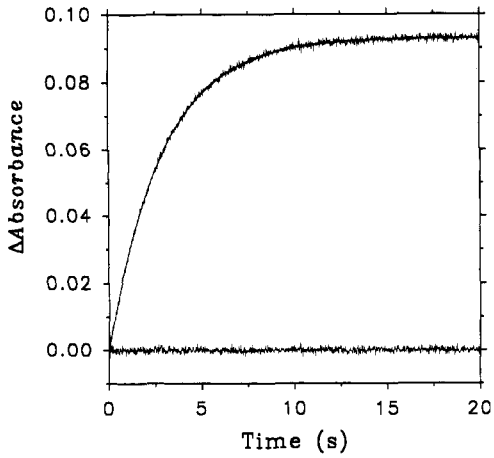

FIGURE 2: Reduction of cytochrome c-551i by MEDH. Oxidized cytochrome c-551i (3.5 μM) was mixed with MEDH (74.2 μM) in 0.1 M potassium phosphate, pH 7.5, with 0.1 M NaCl at 30 °C. The conversion of cytochrome c-551i from oxidized to reduced was monitored at 551 nm with a 2-cm path length. The solid line is a fit of the experimental data to eq 3. The residuals (experimental curve – calculated curve) are plotted near the origin of the y axis.

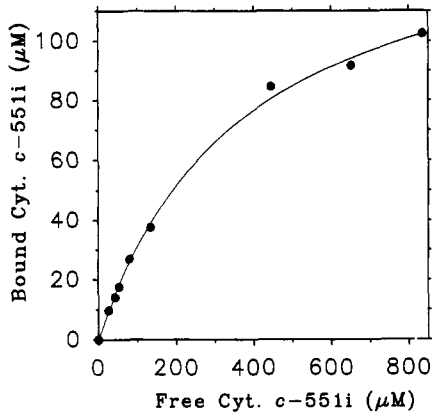

FIGURE 3: Binding of cytochrome c-551i to MEDH. Binding was assayed in 0.1 M potassium phosphate, pH 7.5, with 0.1 M NaCl at room temperature. The solid line represents the fit of the data to eq 4.

concentrations of the cytochrome component covering the range of that used in the assay for binding were centrifuged in Centricon-100s in the absence of MEDH. A plot of the concentration of cytochrome that was retained in the top compartment versus that in the filtrate was linear with a slope of 1.01 ± 0.02 (data not shown). Retention of MEDH in the top compartment was greater than 99% and the final MEDH concentration was 142 μM. Free cytochrome was separated by ultrafiltration and the concentrations of free and bound cytochrome were determined by HPLC analysis as described under Experimental Procedures. These data were best fit to eq 4, which describes ligand binding to a single class of sites (Figure 3):

$$\text{bound cyt } c\text{-551i} = \frac{C[\text{free cyt } c\text{-551i}]}{[\text{free cyt } c\text{-551i}] + K_d} \quad (4)$$

A specific capacity of 1.07 ± 0.08 was calculated by dividing the measured capacity (C) of 149 ± 6 μM by the final MEDH concentration. Analysis of these data yielded a K_d of 375 ± 33 μM. This value correlates very closely with the K_d obtained in kinetic studies (see below).

Stopped-Flow Kinetic Studies. Values of k_{obs} were obtained at different temperatures by varying the MEDH concentration (36–360 μM) with a fixed concentration of cytochrome c-551i (3.5 μM) (Figure 4). It was not possible to obtain data at

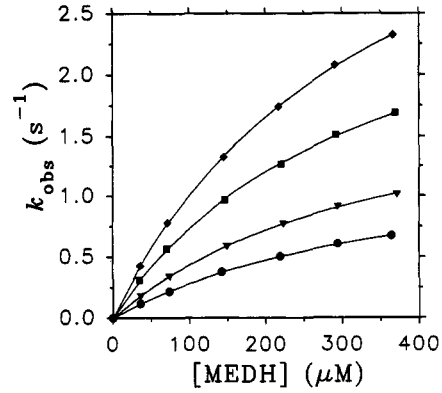

FIGURE 4: Dependence of the observed rate constants (k_{obs}) for the reduction of cytochrome c-551i as a function of MEDH concentration and temperature. Stopped-flow measurements were made as described under Experimental Procedures at 20 °C (●), 30 °C (▲), 40 °C (■), and 50 °C (◆). Solid lines are fits of each data set to eq 6.

more saturating conditions due to the requirement for excessively high concentrations of MEDH (>720 μM before mixing). For all temperatures, it was assumed that the observed reactions obeyed the simple kinetic model (Scheme 1) where oxidized cytochrome c-551i and $MEDH_{sem}$, react to form reduced cytochrome c-551i and $MEDH_{ox}$.

Scheme 1

$$MEDH_{sem} + \text{cyt } c\text{-551i}_{ox} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [MEDH_{sem}\cdot\text{cyt } c\text{-551i}_{ox}] \underset{k_4}{\overset{k_3}{\rightleftharpoons}} MEDH_{ox}\cdot\text{cyt } c\text{-551i}_{red}$$

In this scheme, k_3 is the forward rate constant of the electron transfer reaction and k_4 is the rate of the reverse reaction if any. The ratio of the rate constants, k_1/k_2, describes the equilibrium binding constant for the protein–protein complex association. Thus, k_3 may be considered k_{ET} and K_d is equal to k_2/k_1.

For all temperatures, k_{obs} showed a hyperbolic concentration dependence (Figure 4), indicating a two-step process in which an initial binding step equilibrates much faster than the following electron transfer step. Furthermore, no lag was observed in the initial portions of k_{obs} (Figure 2), indicating that steady-state conditions do not apply. With the rapid equilibrium assumption where the first step equilibrates much faster than the second step, the data were analyzed by the method of Strickland et al. (1975) according to

$$k_{obs} = \frac{k_3[MEDH]}{[MEDH] + K_d} + k_4 \quad (5)$$

This will be true for many cases where complex formation of the reacting proteins occurs in a fast diffusion-controlled process followed by a slow rate-determining electron transfer step.

At each temperature, the y-intercepts for plots of k_{obs} versus the concentration of MEDH passed through the origin (Figure 4), indicating that k_4 is essentially zero (Strickland et al., 1975). The finding that this electron transfer reaction is irreversible is consistent with redox potentials of the reactants. The oxidation–reduction midpoint potential (E_m) value of cytochrome c-551i is +190 mV (Gray et al., 1986). Although the redox potential of the $MEDH_{sem}/MEDH_{ox}$ couple is unknown, a good approximation can be inferred from the known E_m value of free PQQ. The one-electron redox potential

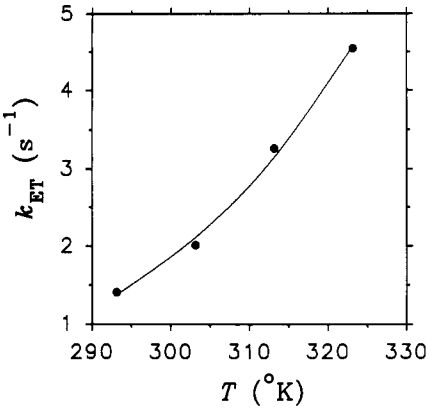

FIGURE 5: Dependence of electron transfer rate (k_{ET}) on temperature. The solid line represents the fits of the values of k_{ET} to classical Marcus equations 1 and 2. Fits to the two equations are superimposable.

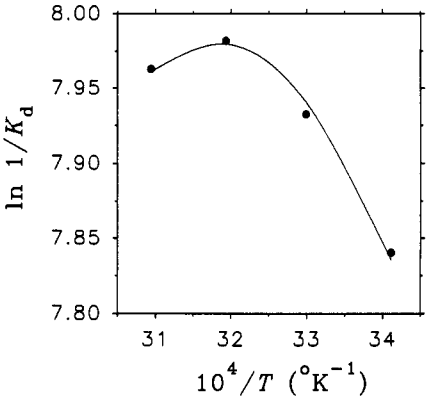

FIGURE 6: Nonlinear van't Hoff plot for the binding of MEDH and cytochrome *c*-551i. The solid line represents the fit of the values of the association constant, $1/K_d$, obtained in kinetic studies to eq 7.

of the semiquinone/quinone couple for free PQQ at pH 7 has been shown to be −100 mV (Ohsiro & Itoh, 1993). This suggests that the approximate ΔE_m for this reaction is +290 mV, which is consistent with an essentially irreversible reaction. When the reverse reaction for the electron transfer is negligible (*i.e.*, $k_4 = 0$), eq 5 is reduced to eq 6, which was used to fit the data shown in Figure 4.

$$k_{obs} = \frac{k_3[\text{MEDH}]}{[\text{MEDH}] + K_d} \quad (6)$$

Nonlinear regression analysis yielded a range of values for K_d between 342 and 394 μM and for k_{ET} (k_3) from 1.41 to 4.55 s^{-1} for temperatures 20–50 °C. The standard errors in K_d were less than 4%, and those for k_{ET} were less than 3%. The rapid equilibrium assumption for this mechanism is supported by the close correlation between the kinetically determined K_d and that independently obtained in the direct binding assay (Bernasconi, 1976).

Thermodynamic Analysis of k_{ET}. Analysis of the temperature dependence of k_{ET} by traditional transition-state theory yielded a linear Eyring plot of ln k_{ET} vs $1/T$ and values of $\Delta H^* = 29$ kJ/mol and $\Delta S^* = -140$ J/(mol K). The interpretation of these parameters for an electron transfer reaction, however, is not straightforward, as this reaction does not involve the formation or the breakage of bonds.

Alternatively, the temperature dependence of k_{ET} was analyzed according to the classical Marcus equation 1 (Figure 5). From the estimated value for the redox potential of the $MEDH_{sem}/MEDH_{ox}$ couple and the known redox potential for cytochrome *c*-551i, as described above, $\Delta G°$ for the electron transfer reactions was assumed to be −28.0 kJ/mol. The fit of the data to eq 1 yielded values of 179 ± 6 kJ/mol (1.86 eV) for λ and 0.071 ± 0.022 cm^{-1} for H_{AB}. The distance between redox centers in an electron transfer reaction was predicted with eq 2. When a β value of 1.4 $Å^{-1}$ for a protein electron transfer reaction (Beratan et al., 1991; Onuchic et al., 1992; Moser et al., 1992) was assumed, the direct distance between donor and acceptor was calculated to be 14.9 ± 0.4 Å. The β value of 1.4 $Å^{-1}$ is intermediate between that found for covalent systems and that of a vacuum. Alternatively, with a β value of 0.7 $Å^{-1}$, which corresponds to electron transfer between synthetically coupled redox centers (Beratan et al., 1992; Moser et al., 1992), the distance was calculated by eq 2 to be 26 Å. The latter distance would theoretically correspond to the distance of the electron transfer pathway if the electrons traveled primarily through bonds.

It is important to note that the $\Delta G°$ value for this reaction was estimated using the E_m value for the free PQQ cofactor (described earlier). It has not been possible to directly determine the E_m value for MEDH by spectroelectrochemical techniques because its absorption spectrum does not change significantly on addition of oxidants, reductants, or substrate. For this reaction, however, $\Delta G°$ is relatively small compared to λ. Given the nature of the Marcus equations, the experimentally obtained values of H_{AB} and distance are essentially independent of the value of $\Delta G°$ used to fit the data. If our estimate of $\Delta G°$ is incorrect, it would lead to an approximately equivalent error in the calculated value of λ. It is unlikely that $\Delta G°$ is significantly smaller than our estimate because the reaction is completely irreversible (discussed earlier). If $\Delta G°$ were significantly larger than our estimate, then λ would be even larger than the 1.9 eV reported. Thus, any error in the estimation of $\Delta G°$ will not affect the major results of this study regarding the values of H_{AB} and distance and the relatively large magnitude of λ (discussed later).

An additional 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 conformation of MEDH which affect its redox potential. As indicated above, given the large λ, any changes in $\Delta G°$ with temperature would have to be substantial to compromise our analysis. Fortunately, the likelihood of this is small. MEDH is quite thermally stable toward denaturation (Davidson et al., 1992). Furthermore, the temperature dependence of K_d for MEDH and cytochrome *c*-551i binding is relatively weak (see Figure 6).

Thermodynamic Analysis of Binding Constants. The simplest binding equilibrium describes that between the two isolated proteins and the bound complex in solution. The dependence of the equilibrium constant K_{eq} ($1/K_d$) on temperature is characterized by changes in the thermodynamic functions of state $\Delta X°$ ($X = G, H, S, C_p, V, \ldots$). The free energy change, $\Delta G°$, characterizes the total system in terms of a single equilibrium constant $1/K_d$. The differentials of ln $1/K_d$ with respect to temperature are related to the standard enthalpy, $\Delta H°$. The simplest approach is to assume that $\Delta H°$ is independent of temperature so that ln $1/K_d$ is a linear function of $1/T$. Many times this assumption is valid; often it is not but is made anyway. Often $\Delta H°$ varies with temperature; this will happen, for example whenever there is a difference in heat capacity, $\Delta C_p°$, between reactants and products. Analysis of the change of $\Delta H°$ with respect to temperature yields the value of $\Delta C_p°$.

A slight curvature was observed in the van't Hoff plot of ln $1/K_d$ versus $1/T$ (Figure 6), indicating that there is a change in $\Delta H°$ with temperature and therefore $\Delta C_p°$ is nonzero. When

Table 1: Thermodynamic Parameters for the Binding of MEDH and Cytochrome c-551i[a]

T (°C)	$\Delta G°_{app}$ (kcal/mol)	$\Delta H°_{app}$ (cal/mol)	$\Delta S°_{app}$ (eu)
20.0	−4.6	2400	24
30.0	−4.8	1300	20
40.0	−5.0	170	16
50.0	−5.1	−950	13

[a] $\Delta C_{p,app}$ = −110 cal/(mol deg).

the plot of ln $1/K_d$ against $1/T$ passes through a maximum or minimum, the data can be successfully fit by use of the Valentiner equation (eq 7), which is a truncated form of the integrated van't Hoff equation which assumes that $\Delta C_p°$ is independent of temperature being equal to Rc (eq 8) (Blandamer et al., 1982).

$$\ln\frac{1}{K_d} = a + b\left(\frac{1}{T}\right) + c \ln T \tag{7}$$

The values of the free energy, $\Delta G°$, the enthalpy, $\Delta H°$, the entropy, $\Delta S°$, and the heat capacity, ΔC_p, changes in the reaction, summarized in Table 1, are given by

$$\Delta G° = -RT \ln \frac{1}{K_d}$$

$$\Delta H° = R(cT - b)$$

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

$$\Delta C_p = Rc \tag{8}$$

Use of eq 7, rather than the complete van't Hoff equation which also considers changes in $\Delta C_p°$ with temperature, is statistically justified because determination of the change of $\Delta C_p°$ with temperature is described by a third derivative which lacks precision necessary to yield satisfactory standard errors in $\Delta C_p°$ (Blandamer et al., 1982). If the protein–protein association reaction is indeed a simple one-stage reaction, then it has been suggested that a negative $\Delta C_p°$ means that diminishing solvent structure at higher temperatures results in the elimination of positive $\Delta S°$ due to hydrophobic interaction which is a general property of the solvent, water (Ross & Subramanian, 1988; Sturtevant, 1977). An alternative explanation for a nonlinear van't Hoff plot is that there are multiple equilibria dependent on temperature. This possibility cannot be excluded. However, we believe that the very good fits of the data in Figures 5 and 6 to equations which assume the simple two-state model support our analysis and interpretation. The thermodynamic driving force for the binding of MEDH and oxidized cytochrome c-551i is primarily entropic between 20 and 40 °C (Table 1). The enthalpy and entropy for the protein association reaction become less positive at high temperatures as a consequence of the negative change in heat capacity. These values suggest the loss of ordered water upon contact between these two proteins (Kornblatt et al., 1993).

DISCUSSION

Values of λ and H_{AB} for long-range protein electron transfer reactions have been obtained primarily from model systems including multisite metalloproteins and the photosynthetic reaction center. Covalent attachment of ruthenium ions to proteins with an intrinsic redox group has been a useful approach for studying intramolecular electron transfer in proteins. Typical values for λ in these ruthenated proteins are in the range of 0.8–1.3 eV. A λ of 0.7 eV has been reported for the bacterial photosynthetic reaction center (Moser et al., 1992). Measurements of λ and H_{AB} for protein–protein electron transfer reactions are less well characterized. A λ of 0.8 eV for the nonphysiologic cytochrome c–cytochrome b_5 complex and a λ of 0.9 eV for the physiologic hemoglobin–cytochrome b_5 complex were determined by measurement of driving force effects on electron transfer rate constants (McLendon, 1988; Simmons et al., 1993). Both temperature dependence measurements and driving force perturbation methods estimated a λ value of 1.4 eV for the physiologic cytochrome c–cytochrome c peroxidase electron transfer reaction (Conklin & McLendon, 1988). The λ value of 1.9 eV reported in this paper is relatively large and may be a consequence of the required complex formation between MEDH and cytochrome c-551i. This must precede the intermolecular electron transfer reaction. Conformational changes after binding or some rearrangement of the two proteins may have to occur to provide the optimal orientation necessary for electron transfer (Peerey et al., 1991; Qin & Kostic, 1993).

The value of H_{AB} of 0.07 cm^{-1} is within the range of values observed in model systems (Onuchic et al., 1992). Most model systems have examined activationless electron transfer reactions, those in which $\Delta G°$ is approximately equal to λ. In such a reaction k_{ET} will be primarily dependent on H_{AB}. The reaction between MEDH and cytochrome c-551i is, however, not activationless. It exhibits a large λ and a $\Delta G°$ which is small relative to λ. Thus, the electron transfer rate constants reported here are significantly less than typically reported for model systems with comparable H_{AB} values. These relatively slow rate constants are a consequence of the large λ and relatively low driving force. This will very likely be true for most physiologic electron transfer reactions between soluble proteins.

The crystal structures of two MEDHs have been determined (Xia et al., 1992) and the crystal structure of cytochrome c-551i from *P. denitrificans* is being resolved in a ternary protein complex with MADH and amicyanin (Chen et al., 1993). Although the structure of the complex of MEDH and cytochrome c-551i is not known, from their individual structures one may estimate the minimum possible distance between redox centers and compare this with the electron transfer distance predicted by Marcus theory. In the crystallized MEDH, the nearest surface side chains are located approximately 10 Å from PQQ and are located in an indentation near the active site. Because of steric hindrance with the groove of the indention, the surface of a globular protein such as the cytochrome would then be positioned approximately 15 Å from PQQ.[2] Thus, the value of 14.9 Å predicted from eq 2 for the direct distance between redox centers in the electron transfer complex is a reasonable estimate.

The directly measured binding constant for the interaction of MEDH with cytochrome c-551i correlated very well with the binding constant obtained in transient kinetic studies. This is important because this correlation lends support to the validity of the kinetically determined K_ds and the analysis of the thermodynamic parameters for the temperature dependence of the binding constant obtained in the kinetic studies. The K_d for the MEDH–cytochrome c-551i complex of approximately 0.38 mM seems high for a physiologic reaction. However, when grown on methanol as a carbon source, methylotrophic bacteria synthesize high levels of these periplasmic proteins. The concentrations of MEDH and

[2] F. S. Mathews, personal communication.

cytochrome *c*-551i in the periplasm have each been estimated to be about 0.5 mM (Anthony, 1993). As such, the K_d value determined here is reasonable.

The positive enthalpy and entropy changes for the protein–protein association (Table 1) may be attributed to a hydrophobic process in which hydrophobic amino acid side chains, which were previously accessible to solvent in the isolated subunits, become buried upon complex formation and cause a partial disordering of the more highly organized water molecules that were formerly surrounding these groups. This is true because the increase in ΔS due to hydrophobic association is greatest at low temperatures and can more than compensate the decrease in ΔS due to the loss of translation and rotation in the hydrophobically associated species (Chothia & Janin, 1975). The negative heat capacity change at higher temperatures also suggests the importance of the hydrophobic effect for complex formation. The positive contribution to $\Delta S°$ is *reduced* at higher temperatures, where more randomized solvent structure is less enhanced by the repulsion of water by nonpolar groups, and the enthalpy term begins to contribute to the stability of the complex (Ross & Subramanian, 1988; Sturtevant, 1977).

Detailed crystal structures have been determined for only two naturally occurring complexes of soluble electron transfer proteins, methylamine dehydrogenase and amicyanin (Chen et al., 1992) and cytochrome *c* and cytochrome *c* peroxidase (Pelletier & Kraut, 1992). Attempts to cocrystallize MEDH and cytochrome *c*-551i as a complex are in progress, as are attempts to model a putative structure of this complex from the known crystal structures of the individual proteins. There have been relatively few physiologic intermolecular protein electron transfer reactions for which λ and H_{AB} have been determined. There is also very little information available on the thermodynamic parameters which govern the associations between redox proteins. The acquisition of data such as those reported in this paper is critical to the eventual understanding of the mechanism of a biological long-range electron transfer reaction. Only when such data have been obtained for several naturally occurring systems, and correlated with known structures, will it be possible to begin to draw general conclusions as to the nature of biological long-range electron transport processes.

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