Biochemistry

 Volume 35, Number 45 November 12, 1996

New Concepts in Biochemistry

Unraveling the Kinetic Complexity of Interprotein Electron Transfer Reactions†

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Received July 1, 1996; Revised Manuscript Received September 30, 1996

Long range electron transfer (ET)[1] between proteins is a process which is fundamental to respiration, photosynthesis, and redox reactions of intermediary metabolism. Many physiologic long range biologic ET reactions are bimolecular processes involving donor and acceptor proteins. The overall redox reaction may require several steps, including specific binding of proteins, protein rearrangements which optimize the coupling between redox centers, chemical transformations such as proton transfer, and the actual ET step. Furthermore, because the redox centers reside within a protein matrix, the possibility exists that the reorganizational energy (λ) or electronic coupling between donor and acceptor (H_{AB}) may vary with reaction conditions as a result of protein conformational fluctuations. Direct application of ET theory to these processes is problematic because the kinetic complexity of the overall reaction often makes it difficult to identify the true ET rate constant. In model studies of protein ET reactions, it has become standard to analyze the variation in ET rate with the systematic variation of $\Delta G°$ to verify that the ET event is rate-limiting for the observed reaction and to obtain values for λ and H_{AB}. This approach, however, will not be applicable to most physiologic interprotein ET reactions. This paper describes alternative approaches for the analysis of protein ET reactions. Models are presented for the kinetic analysis of interprotein ET reactions and the interpretation of experimentally derived values of λ for protein ET reactions whose rates may be influenced by non-ET events.

†Work from the author's laboratory was supported by National Institutes of Health Grant GM-41574.

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[1] Abbreviations: E_m, midpoint redox potential; ET, electron transfer; H_{AB}, electronic coupling; λ, reorganizational energy.

APPLICATION OF ET THEORY TO PROTEIN ET REACTIONS

ET theory predicts that the rate of an ET reaction will vary predictably with temperature (T), $\Delta G°$, and donor−acceptor distance (r) according to the relationships given in eqs 1 and 2 (Marcus & Sutin, 1985)

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

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

where h is Planck's constant, R is the gas constant (the Boltzmann constant may alternatively be used), k_0 is the characteristic frequency of the nuclei which is usually assigned a value of 10^{13} s^{-1}, and r_0 is the close contact distance usually assigned a value of 3.0 Å. H_{AB} is the electronic coupling between redox centers and describes the degree of wave function overlap between donor and acceptor sites. λ is the reorganizational energy. Detailed discussions of the mathematical and physical meaning of H_{AB} and λ may be found in a number of excellent reviews of ET theory (Marcus & Sutin, 1987; Gray & Winkler, 1996; Moser et al., 1992; McLendon & Hake, 1992; Rees & Farrelly, 1990). The factor β is related to the nature of the intervening medium between redox centers. The experimentally derived value of r will be dependent upon the value of β which is used in the analysis. Values which are typically used range from 0.7 to 1.4 $Å^{-1}$. The most appropriate value for β and the issue of whether r describes the direct donor−acceptor distance or is pathway-dependent have been a matter of much debate (Moser et al., 1992; Onuchic et al., 1992; Langen et al., 1995).

In most published studies of protein ET reactions, values for λ and H_{AB} have been obtained from analysis of the

S0006-2960(96)01577-2 CCC: $12.00

dependence of the rate of the reaction on $\Delta G°$. Ideally, $\Delta G°$ is varied over a wide range which spans the Marcus "inverted" region, where $-\Delta G° > \lambda$. Two experimental approaches have primarily been used to modify $\Delta G°$ for protein ET reactions [reviewed in Winkler and Gray (1992), Moser et al. (1992), and McLendon and Hake (1992)]. Intramolecular ET through the protein matrix has been studied by covalently attaching ruthenium complexes to specific sites on the surface of a redox protein. $\Delta G°$ is modulated by use of different ruthenium complexes with varied redox potentials, and intramolecular ET rates between the ruthenium complex and the native redox center are monitored. Alternatively, redox potentials of the donor or acceptor have been modulated by substitution of metal cofactors or modification of organic prosthetic groups. The ability to modify proteins and achieve activationless ET ($-\Delta G° = \lambda$) has led to the development of important hypotheses regarding the dependence of the ET rate constant (k_{ET}) on the distance and the nature of the protein matrix which separates redox centers (Moser et al., 1992; Onuchic et al., 1992). Recently, genetic modifications of amino acid residues near the redox center have been used to modify $\Delta G°$ [e.g., Ortega et al. (1996), Arlt et al. (1996), and Nagarajan et al. (1993)]. Previous studies have also raised questions as to the roles of protein dynamics in controlling, or gating, the rates of ET reactions (Hoffman & Ratner, 1987; Brunschwig & Sutin, 1989; Harris et al., 1994).

A limitation of the $\Delta G°$ dependence approach to the study of physiologic interprotein ET reactions is that it is not always possible to modulate the redox potential of the native redox center without perturbing or destroying the protein matrix. Furthermore, for many physiologic protein ET reactions, $-\Delta G°$ is relatively low compared to λ. This complicates the task of varying $-\Delta G°$ into the Marcus inverted region. It may not be possible to design a system by which to generate a sufficiently large $-\Delta G°$. Given the kinetic complexity of interprotein ET reactions, it is also possible that high artificial values of $-\Delta G°$ that approximate λ may cause k_{ET} to become so fast that some other non-ET event, such as a protein conformational change, will become rate-limiting so that the reaction becomes gated. An example of this is the ET reactions from metal-substituted cytochromes *c* to plastocyanin (Zhou & Kostic, 1993), where the reaction was gated or not gated depending on the magnitude of the imposed $\Delta G°$.

ANALYSIS OF THE TEMPERATURE DEPENDENCE OF PROTEIN ET REACTIONS

According to eqs 1 and 2, k_{ET} will vary predictably as a function of temperature. Given the limitations in varying $\Delta G°$ for many physiologic reactions, experimental variation of k_{ET} by temperature may be the only approach available by which to obtain estimates for H_{AB} and λ. This approach has not been widely used, and there are important caveats to consider when studying the temperature dependence of intermolecular protein ET reactions.

(1) $\Delta G°$ *May Be Dependent upon Temperature*. Even subtle temperature-dependent changes in protein conformation could conceivably perturb the environment of the redox cofactor so as to alter its E_m value. As such, the experimental temperature range should not exceed that over which the protein is known to be stable, and the temperature dependence of E_m values should be measured to verify the implicit assumption that $\Delta G°$ is constant. For most biologic ET reactions, $-\Delta G°$ is small relative to λ. Thus, any change in $\Delta G°$ with temperature, or uncertainty in the precise value of $\Delta G°$, would have to be very large to compromise data analysis by eqs 1 and 2. This is illustrated in Table 1 where variation of $\Delta G°$ on calculated values of H_{AB} and λ is simulated for a hypothetical biologic ET reaction. In this example, an uncertainty of ± 100 mV in ΔE_m has essentially no effect on the calculated values of H_{AB} and r. The uncertainty in $\Delta G°$ leads to an error in the calculated λ about 2-fold greater than the error in the assumed value of $\Delta G°$. If random error is included in the simulation, differences in the calculated λ values become even more difficult to discern.[2] The larger the true λ, the less sensitive the experimentally determined λ will be to uncertainties in $\Delta G°$. Interpretation of λ values tends to be somewhat qualitative. In this example, the negligible effect on the calculated value of H_{AB} and the uncertainty as to whether λ is 1.3 ± 0.2 eV would not significantly impact the mechanistic interpretation of the results. It is important to note that, while variation in $\Delta G°$ will have a substantial effect on k_{ET}, its effect on the temperature dependence of k_{ET} and experimentally derived values of H_{AB} and λ will depend on the relative magnitudes of $\Delta G°$ and λ.

Table 1: Effects of Uncertainty in ΔE_m ($\Delta G°$) on the Analysis of the Temperature Dependence of k_{ET}[a]

ΔE_m (V)	$\Delta G°$ (kJ mol^{-1})[b]	H_{AB} (cm^{-1})	λ (kJ mol^{-1})	r (Å)[c]
0	0	0.096	106 (1.10 eV)	18.8
0.1	−9.65	0.100	125 (1.30 eV)	18.8
0.2	−19.3	0.103	142 (1.47 eV)	18.8

[a] A data set was simulated using eq 1 and $\Delta E_m = 0.1$ V (which corresponds to $\Delta G° = -9.65$ kJ mol^{-1}), $H_{AB} = 0.100$ cm^{-1}, and $\lambda = 125$ kJ mol^{-1} (which corresponds to 1.3 eV). These data were then fit to eqs 1 and 2 with different values input for $\Delta G°$, and the fitted values for H_{AB}, λ, and r were calculated. No noise was included in this simulation, so there are no reported error values for the calculated values. [b] $\Delta G°$ is calculated from the redox potential difference between the donor and acceptor according to $-\Delta G° = nF\Delta E_m$, where F is the Faraday constant (23 063 cal V^{-1} equiv^{-1}) and n is the number of electrons transferred per mole. [c] Values of r were calculated with eq 2 using a β value of 1.0 Å^{-1}.

(2) A Change in Temperature May Alter the Rate-Limiting Step for the ET Reaction. If a temperature-dependent conformational change causes a change in the rate-limiting step for the overall ET reaction so that the ET event is no longer rate-limiting, this would result in a transition where the limiting first-order rate constant switches from that of the ET event to that of a non-ET reaction step. Different temperature dependencies of the apparent k_{ET} would be observed on either side of the transition temperature because different reaction steps are being described. When such a transition is observed for an ET reaction, this provides the opportunity to analyze the two processes observed on either side of the transition temperature. One may obtain Marcus parameters (i.e., λ and H_{AB}) which describe the ET event and activation parameters (i.e., $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) that describe

[2] The simulation in Table 1 was repeated with random noise included in the data. Random error assuming a mean of 0 ± 1 standard deviation with 10% noise was introduced into the perfect data set. In an example of such a simulation with 10% noise, fits of these data sets assuming ΔE_m values of 0, 0.1, and 0.2 V yielded fitted values of λ of 117 ± 3, 124 ± 11, and 141 ± 12 kJ mol^{-1}, respectively.

the non-ET event which precedes ET. A change in the rate-limiting step could also complicate $\Delta G°$ dependence studies of protein ET reactions. As k_{ET} increases with increasing $-\Delta G°$, some prerequisite non-ET reaction step which is insensitive to $\Delta G°$ may become rate-limiting. For kinetically complex protein ET reactions, the extent to which k_{ET} contributes to the observed rate may depend on either varied parameter, temperature, or $\Delta G°$.

(3) H_{AB} or λ May Be Dependent on Temperature. If the protein matrix is not perturbed by a change in temperature, then H_{AB} should not be directly dependent on temperature. H_{AB} may vary with nuclear configuration. However, in the physiologic temperature range, the contribution of nuclear tunneling to the reaction rate will be negligible since thermal energy exceeds the characteristic vibrational energies of the system (De Vault & Chance, 1966; Peterson-Kennedy et al., 1984). It is still possible that temperature-dependent protein conformational changes may occur which modulate H_{AB} or λ and in doing so affect k_{ET} in a temperature-dependent manner. An extreme example of this was discussed above in point (2), but such conformational perturbations need not cause a change in the rate-limiting step. It was recently shown (Ortega et al., 1996) that the λ for charge recombination from the primary quinone to bacteriochlorophyll in the photosynthetic reaction center varied with temperature. It is also conceivable that H_{AB} could be altered by a subtle temperature-dependent conformational change that could alter the donor−acceptor distance or the relative efficiencies of alternative ET pathways, especially ones that require through-space jumps. The extent to which indirect temperature-dependent changes in H_{AB} compromise data analysis of ET by eqs 1 and 2 will depend on how severely the value of H_{AB} is affected. Protein modifications required to vary $\Delta G°$ may also potentially alter H_{AB} or λ by altering protein conformation. In practice, temperature dependence studies of most interprotein ET reactions can realistically be performed only over a relatively narrow temperature range to avoid freezing or thermal denaturation. This may actually be advantageous as it minimizes the possibility of significant protein conformational changes over the experimental range. Temperature-dependent changes in protein conformation or protein dynamics may cause H_{AB} or λ to be different at temperatures outside of this experimental range. However, biologic protein ET reactions will likely be studied in the physiologic temperature range, and the parameters obtained at these temperatures will be the most relevant to physiologic function.

IMPLICATIONS OF KINETIC COMPLEXITY OF PROTEIN ET REACTIONS

The definition of λ includes contributions from inner shell (λ_i) and outer shell (λ_o) reorganization (Marcus & Sutin, 1985). For a protein ET reaction, λ_o will include contributions not only from solvent reorganization but also from any nuclear displacements in the protein matrix that are associated with ET. More importantly, λ_{obs} may also contain contributions from non-ET reaction steps due to kinetic complexity. One must consider the implications of the kinetic complexity of an interprotein ET reaction on experimentally derived values of λ.

Scheme 1

simple kinetic model

$$A_{ox} + B_{red} \overset{K_d}{\rightleftharpoons} A_{ox}-B_{red} \underset{-k_{lim}}{\overset{k_{lim}}{\rightleftharpoons}} A_{red}-B_{ox}$$

kinetic complexity

$$A_{ox} + B_{red} \overset{K_d}{\rightleftharpoons} A_{ox}-B_{red} \underset{-k_x}{\overset{k_x}{\rightleftharpoons}} [A_{ox}-B_{red}]^* \underset{-k_{ET}}{\overset{k_{ET}}{\rightleftharpoons}} A_{red}-B_{ox}$$

true ET

$k_{ET} \ll k_x \quad K_x(k_x/k_{-x}) \gg 1 \quad k_{lim} = k_{ET} \quad \lambda_{obs} = \lambda_{ET}$

gated ET

$k_x \ll k_{ET} \quad k_{lim} = k_x \quad \lambda_{obs} = f(\Delta G^{\ddagger}_x)$

coupled ET

$k_{ET} \ll k_x \quad K_x(k_x/k_{-x}) \ll 1 \quad k_{lim} = K_x k_{ET} \quad \lambda_{obs} = f(\lambda_{ET}, \lambda_x)$

For redox reactions involving proteins, the actual meaning of the kinetically determined limiting first-order rate constant for the ET reaction (k_{lim} in Scheme 1)[3] must be interpreted with caution. It may not be a true ET rate constant (Hoffman & Ratner, 1987; Brunschwig & Sutin, 1989; Harris et al., 1994). Protein dynamics (i.e., transient formation of unstable conformational intermediates) or catalytic events (e.g., protonation/deprotonation) may contribute to the observed rate. In kinetic models that are used to analyze these data, any spectroscopically invisible, or otherwise undetectable, events subsequent to binding and preceding the spectral change associated with the redox reaction will be reflected in this rate constant. These events could include substrate-induced, product-induced, or redox-linked protein conformational changes. Three situations are considered below in which the rate of the ET event (k_{ET}) is preceded by some reversible prerequisite adiabatic event which has a rate constant k_x and an equilibrium constant K_x.

(1) True ET ($k_{lim} = k_{ET}$). λ_{obs} will include no contributions from non-ET reaction steps. It will be a true value that will reflect nuclear displacements in the redox centers, protein matrix, and solvent.

(2) Gated ET ($k_{lim} = k_x$). Some reaction step that precedes ET is completely rate-limiting so that the observed rate is actually that of a non-ET event. Therefore, k_{lim} will not exhibit a predictable dependence on $\Delta G°$ since this reaction step is not being driven by the redox potential difference between the reactants. The reaction will still vary with temperature, but if the temperature dependence data are analyzed by ET theory, the values which are obtained for λ and H_{AB} will be unrelated to the ET event. They may, however, be of diagnostic use (discussed below).

Semiclassical ET theory assumes that ET reactions are nonadiabatic (Marcus, 1964). H_{AB} is related to the degree of wave function overlap between donor and acceptor sites. As H_{AB} approaches 0, the probability that reactants will go to products at the transition state configuration also ap-

[3] For the simple kinetic model given in Scheme 1, if the redox reaction is reversible, the maximum k_{obs} at saturation conditions will be equal to the sum of k_{lim} plus k_{-lim}. For reversible reactions, one must be sure that values of k_{lim} are correctly extracted from the kinetic data and used in the subsequent analysis.

proaches 0. Conversely, if H_{AB} is large, the probability that reactants will go to products at the transition state approaches unity. The latter reactions are adiabatic and not appropriately described by Marcus theory (eqs 1 and 2). One would expect the experimentally derived value for H_{AB} for a gated reaction to exceed the nonadiabatic limit for an ET reaction. As a general rule, one may consider that limit to be approximately 80 cm^{-1}. This value 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). Reorganization of the protein matrix complicates this argument because it will occur on a different time scale than that of water and the nonadiabatic limit may be different. However, if one obtains a value of H_{AB} well in excess of 80 cm^{-1}, that would clearly be suggestive of a gated reaction in which k_{lim} is actually a rate constant which describes a relatively slow non-ET event. For example, analysis of the temperature dependence of the ET reaction from aminoquinol methylamine dehydrogenase to amicyanin, which is gated by a proton transfer, yielded an H_{AB} of 23 000 cm^{-1} (Bishop & Davidson, 1995). When the rate-limiting reaction step is adiabatic, it is not appropriately described by Marcus theory. However, it may be analyzed by transition state theory to obtain activation parameters, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, for the adiabatic reaction step which is gating ET.

(3) Coupled ET ($k_{lim} = k_{ET}K_x$). If a reaction step which precedes ET is rapid relative to ET but very unfavorable (i.e., $\ll 1$), k_{lim} will be influenced by the equilibrium constant for that non-ET process (K_x). Although k_{ET} is rate-limiting, k_{lim} will be equal to the product of k_{ET} and K_x (Harris et al., 1994). It follows that the experimentally derived λ_{obs} will contain contributions from both the ET event and the preceding reaction step [i.e., $\lambda_{obs} = f(\lambda_{ET}, \lambda_x)$]. For example, λ_{obs} for interprotein ET reactions may reflect contributions from an intracomplex rearrangement of proteins after binding to achieve an optimum orientation for ET.

ASCERTAINING THE VALIDITY OF EXPERIMENTALLY DERIVED ET REACTION PARAMETERS

The kinetic complexity of interprotein ET reactions raises concerns about the validity of values of λ, H_{AB}, and r that are derived from temperature dependence studies. Two criteria have been used to argue for the validity of such values: correlation with values obtained from $\Delta G°$ dependence studies and correlation of r with ET distances predicted from protein crystal structures. In at least two cases, correlation has been observed between λ values obtained from temperature and $\Delta G°$ dependence studies. For the ET reaction from cytochrome *c* to cytochrome *c* peroxidase, similar λ values were obtained from temperature and $\Delta G°$ dependence studies (Conklin & McLendon, 1988). For the ET reaction from the dithionite-reduced quinol form of methylamine dehydrogenase to amicyanin, similar λ and H_{AB} values were obtained from temperature (Brooks & Davidson, 1994a) and $\Delta G°$ (Brooks & Davidson, 1994b) dependence studies. For the latter (Brooks & Davidson, 1994a) and the ET reaction from amicyanin to cytochrome *c*-551i (Davidson & Jones, 1996), values of r obtained from temperature dependence studies correlated reasonably well with ET distances revealed by the crystal structure of the complex of methylamine dehydrogenase, amicyanin, and cytochrome *c*-551i (Chen et al., 1994). For the ET reaction from methanol dehydrogenase to cytochrome *c*-551i, the r value obtained from temperature dependence studies correlated well with the minimum ET distance predicted from the crystal structures of the individual proteins (Harris & Davidson, 1994). A temperature dependence study of the ET reaction between cytochrome *a* and cytochrome a_3 of cytochrome oxidase yielded an r value which correlated with the distance predicted from structural models (Adelroth et al., 1995). In one case, calculation of an unreasonable ET distance ($r < 0$) provided evidence that the ET reaction was gated (Bishop & Davidson, 1995).

Analysis of the temperature dependence of ET reaction rates alone will not be sufficient to say whether one is monitoring a true ET reaction or one which is coupled or gated. These data can, however, be useful in providing an initial diagnosis of whether the ET event is the rate-limiting step for the overall ET reaction. Unreasonably large values of H_{AB} or λ, or unrealistic estimates of r, would suggest that the ET reaction is gated by a non-ET reaction step. Other experimental approaches may then be employed to obtain further evidence for gating or coupling and to provide information on the nature of the non-ET process which is controlling the rate of the ET reaction.

For gated and coupled ET reactions, solvent conditions such as viscosity, ionic strength, and pH can alter the observed ET rate by affecting the dynamic conformational fluctuations or catalytic events (k_x or K_x in Scheme 1) that precede ET. Kinetic isotope effect studies may also be employed to determine whether and to what extent the observed rate reflects the true k_{ET} or some non-ET event. Such studies can provide important information to complement temperature and $\Delta G°$ dependence studies of interprotein ET reactions. Viscosity effects on ET reactions from zinc- and tin-substituted cytochrome *c* to plastocyanin demonstrated that these reactions were gated under certain conditions (Zhou & Kostic, 1993). For the ET reaction from methanol dehydrogenase to cytochrome *c*-551i, ionic strength effects were cited as evidence that the reaction was conformationally coupled (Harris et al., 1994). Deuterium kinetic solvent isotope effect studies were used to confirm that the ET reaction from substrate-modified aminoquinol methylamine dehydrogenase to amicyanin was gated by proton transfer while that from the unmodified quinol was not (Bishop & Davidson, 1995).

CONCLUSION

The difficulty or impossibility of examining the rate dependence on $\Delta G°$ for most physiologic interprotein ET reactions should not exclude them from study. Using the approaches outlined above, one may study an interprotein ET reaction without requiring any modification of the proteins or exposure to potentially denaturing conditions. Analysis by ET theory of the temperature dependence of rates of biologic ET reactions may provide valid estimates for λ, H_{AB}, and ET distance, providing that the ET event is rate-limiting for the observed reaction. It may also be useful for identifying gated ET reactions when unrealistic values for these parameters are obtained. When ET reactions are believed to be gated or coupled, they may be further characterized by examining the effects on ET rates of varying solution conditions and performing kinetic isotope effect studies. The results of these studies can provide useful

information on the nature of the reaction step which gates or attenuates the observed rate of the ET reaction. When a sufficient number of reactions have been examined, the results of such combined studies may be used to establish criteria for classifying long range intermolecular ET reactions between proteins, discerning the extent of adiabaticity of such reactions, and elucidating the nature of non-ET processes which influence the observed rates of ET reactions.

ACKNOWLEDGMENT

The author thanks Reid Bishop for many helpful and stimulating discussions on this topic.

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BI961577P