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THE ELECTRON-TRANSFER KINETICS OF SPINACH FERREDOXIN WITH STRONG REDUCTANTS

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The reduction of spinach ferredoxin by V(II)-EDTA, Eu(II)-DPTA and propylene viologen was monitored electrochemically. The rates of these reactions were found to be $3.0 \cdot 10^4$, $3.2 \cdot 10^5$ and $1.2 \cdot 10^5$ $M^{-1} \cdot s^{-1}$, respectively, by the use of chronoamperometry, pulse polarography, differential pulse polarography and rotating-disk voltammetry. These reaction rates were analyzed by tunneling theory for electron transfers, and the comparisons between the theoretical and experimental values were quite good. The tunneling theory also worked quite well with other ferredoxin electron-transfer rates that are available in the literature. In addition to that, the activation enthalpy and entropy compared well with the tunneling theory.

Introduction

Detailed mechanistic studies of iron-sulfur proteins are still in their infancy when compared to the studies with cytochrome *c*. In the early 1970's, Bennett et al. [1,2] undertook the study of electron transfer to clostridial rubredoxin from hexaammine Ru(II), V^{2+} and Cr^{2+}. It was clear that outer-sphere agents could oxidize or reduce the iron site in rubredoxin quite rapidly, as indicated by the large value of the rate constant (approx. 10^7 $M^{-1} \cdot s^{-1}$) for an only slightly favorable (in a thermodynamic sense) hexammine Ru(II)-rubredoxin reaction [2] ($\Delta G^0 = -1.8$ kcal/mol). Most of the electron-transfer studies of iron-sulfur proteins have centered on the *Chromatium* ferredoxins (Hipip) [3–5], while there have been relatively few studies of the low-potential proteins.

Rawlings et al. [6] have studied the oxidation of spinach ferredoxin with Fe(III)-EDTA, Fe(III)-HEDTA, ferricytochrome *c* and metmyoglobin. In their study, the electrostatically corrected self-exchange rate constant for ferredoxin, based on the Fe(III)-EDTA reaction, was quite small (approx. 10^{-3} $M^{-1} \cdot s^{-1}$), indicating that the redox site was extremely inaccessible. But, recent X-ray studies of a related ferredoxin [7] from *Spirulina platensis* indicate that the cluster is relatively close to the surface of the protein. Armstrong and Sykes [8] have investigated the oxidation of parsley and spinach ferredoxins with various Co(III) complexes. In their work, they observed an equilibrium reaction between the cobalt complexes and ferredoxin prior to electron transfer, especially for the highly charged cationic complexes. Richard et al. [9] studied the reduction of spinach ferredoxin with methyl viologen using spectroelectrochemistry. They found that the reaction rate was rapid, which is quite interesting because of the small driving force for the reaction (approx. 22 mV).

It is the purpose of this work to investigate these diverse results for the electron-transfer be-

Abbreviations: DPTA, 1,2-diaminopropane-*N,N,N',N'*-tetraacetic acid; Hipip, high-potential iron-sulfur protein; HEDTA, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid.

havior of ferredoxin. In order to do this, the tunneling approach of Hopfield [10] and Chien [11,12] will be used. Chien [12] has achieved considerable success in predicting the electron-transfer rates for cytochrome *c* reactions using this approach, and this work will show that the same approaches can be used to study the iron-sulfur protein kinetics.

All of the previous work on the reaction kinetics of small molecule-ferredoxin reactions, except for the work of Richard et al. [9] and Lambeth and Palmer [23], was done by studying the oxidation of the protein. It is quite difficult to generate and maintain the strong reductants that are necessary for studying the reduction kinetics of ferredoxin. These strong reductants are particularly difficult to manipulate in the classical stopped-flow spectrophotometric method. In addition, the difference in extinction coefficients between the oxidized and reduced protein is much smaller for ferredoxin than for cytochrome. The advantage of the electrochemical approach is even more important for those proteins whose redox site is colorless. Recent work [13–15] in this laboratory has shown three different electrochemical methods that can be used for protein-small molecule studies: chronoamperometry [13], pulse polarography [14] and rotating-disk voltammetry [15]. All three of these methods were used in this work.

Experimental Procedure

Chemicals. Spinach ferredoxin was isolated and purified by the method of Petering and Palmer [16]. Some of the ferredoxin was obtained from Sigma Chemical Co. and was purified as needed. The purity of all the ferredoxin used was checked by the ratio of the absorbance at 420 nm to that at 275 nm, which was over 0.47. V(III)-EDTA was prepared by the electrochemical reduction of V_2O_5 (Alfa Division, Ventron Corp.) in the presence of EDTA at pH 5. The V(III)-EDTA was stored under nitrogen. The Eu(III)-DPTA solution was obtained by dissolving Eu_2O_3 (Sigma Chemical Co.) in 2 ml of 1:1 $HClO_4$, followed by the addition of 25 ml of water. A 20% excess of DPTA (Fluka AG) was added and the solution stirred until the DPTA dissolved. The pH of the solution was adjusted to the desired value (pH 7.3 in most cases) with a small volume of HCl or NaOH, and finally Tris-HCl buffer was added to make the volume up to 100 ml. The dibromide salt of propylene viologen was prepared by the method of Steckhan and Kuwana [17]. The physical and electrochemical properties of the viologen were consistent with the literature values [18].

Equipment. The electrochemical apparatus has been described previously [13–15]. A microcell of 4 ml volume was used in this work in order to use as little protein as possible. The microcell was a Radiometer Copenhagen cell (type V521) with a thermostat jacket (type 526). The thermostat jacket has one inlet and one outlet for water flow to control the cell temperature. All solutions were degassed with prepurified nitrogen which was passed through an alkaline pyrogallol scrubbing solution. The electrode systems were the same as described previously [13–15]. Pulse polarographic waves were obtained with a Princeton Applied Research Corp. Model 174A polarographic analyzer. The scan rate was 2 mV/s.

Procedures. The experimental and analysis procedures have been described in detail in previous work [13–15].

Results

The first difficulty in trying to study the reduction kinetics of spinach ferredoxin was to obtain suitable redox agents because of the negative potentials that are necessary. After examining several possibilities, the following reductants were found to be suitable: V(II)-EDTA, Eu(II)-DPTA and the propylene violgen radical cation. V(III)-EDTA is reduced at a mercury electrode to V(II)-EDTA (reaction 1) with an $E_{1/2}$ value of -1.27 V as compared to a standard calomel electrode. Chronoamperometry and cyclic voltammetry of V(II)-EDTA indicated a simple Cotrell-type behavior without any additional chemical reactions, as shown in Table I.

$$\text{V(III)-EDTA}^- + e^- \rightleftharpoons \text{V(II)-EDTA}^{2-} \quad (1)$$

$$\text{V(II)-EDTA}^{2-} + \text{Fd}_{ox} \xrightarrow{k_f} \text{V(III)-EDTA}^- + \text{Fd}_{red} \quad (2)$$

If ferredoxin in the oxidized state (Fd_{ox}) is pre-

TABLE I

CHRONOAMPEROMETRY OF V(III)-EDTA IN THE ABSENCE AND PRESENCE OF SPINACH FERREDOXIN

$C_{V(III)\text{-}EDTA} = 0.10$ mM; $C_{Fd} = 0.20$ mM; 0.010 M acetate buffer, pH 5. Average value of $k_f = 3.2 \cdot 10^4$ $M^{-1} \cdot s^{-1}$.

t (s)	I_d (μA)	$I_d t^{1/2}/C$ ($\times 10^3$)	I_k (μA)	n_{app}	k_f ($\times 10^{-4}$) ($M^{-1} \cdot s^{-1}$)
0.10	0.930	2.94	1.52	1.63	4.2
0.20	0.600	2.68	1.06	1.76	2.8
0.30	0.481	2.63	0.95	1.98	2.8
0.50	0.370	2.62	0.82	2.22	2.8
0.70	0.313	2.62	0.76	2.45	3.4
1.0	0.240	2.40	0.646	2.69	-

sent, the V(II)-EDTA that is formed by reaction 1 will react with the ferredoxin to regenerate V(III)-EDTA and form the reduced form of ferredoxin (Fd_{red}) (reaction 2). The regenerated V(III)-EDTA can be reduced again at the electrode surface, giving an increase in the observed current. The current that flows when no ferredoxin is present is called the diffusion current, I_d, while the current that is observed with ferredoxin present is called the kinetic current, I_k. The ratio of the kinetic current to the diffusion current is called the apparent n value, n_{app}. From the n_{app} value, one can calculate the value of the rate constant, k_f, as shown in detail in Ref. 13. The values of n_{app}, k_f and the average rate contant are shown in Table I. There was no appreciable reduction of ferredoxin observed which is consistent with previous workers using various electrodes [19,20]. Similar results were also obtained for 0.10 and 0.50 mM ferredoxin (3.0 and $3.1 \cdot 10^4$ $M^{-1} \cdot s^{-1}$, respectively), indicating the pseudo-first-order nature of the process. The temperature dependence of the V(II)-EDTA-ferredoxin reaction was also studied. The results are shown in Table II. An activation enthalpy of 5.6 kcal/mol and an activation entropy of -21 cal/K per mol were obtained.

The second complex that was studied was Eu(III)-DPTA. The $E_{1/2}$ value for this complex was -1.10 V at pH 7. A typical set of chronoamperometric data for Eu(III)-DPTA is shown in Table III, along with the analysis as described above for the V(III) complex. This reaction rate was significantly faster than the V(II)-ferredoxin reaction, even though the driving force was smaller. The rate of the reduction of ferredoxin by Eu(II)-DPTA was also measured by the use of pulse and differential pulse polarography. By using these

TABLE II

VARIATION OF THE RATE CONSTANT FOR THE REDUCTION OF SPINACH FERREDOXIN AS A FUNCTION OF TEMPERATURE

V(II)-EDTA-Ferredoxin		Eu(II)-DPTA-Ferrredoxin	
t (°C)	k_f ($M^{-1} \cdot s^{-1}$) ($\times 10^{-4}$)	t (°C)	k_f ($M^{-1} \cdot s^{-1}$) ($\times 10^{-5}$)
2	1.3	1	0.72
16	2.2	15	2.0
25	3.0	24	3.2
32	4.5	35	4.5

TABLE III

CHRONOAMPEROMETRY OF Eu(III)-DPTA IN THE ABSENCE AND PRESENCE OF SPINACH FERREDOXIN

$C_{Eu(III)\text{-}DPTA} = 0.010$ mM; $C_{Fd} = 0.050$ mM; 0.050 M Tris-HCl, pH 7.3. Average k_f value $= 2.0 \cdot 10^5$ $M^{-1} \cdot s^{-1}$

t (s)	I_d (μA)	I_k (μA)	n_{app}	k_f ($M^{-1} \cdot s^{-1}$) ($\times 10^{-5}$)
0.10	0.102	0.210	2.06	2.8
0.20	0.0722	0.190	2.63	2.8
0.30	0.0589	0.168	2.85	2.3
0.50	0.0457	0.140	3.06	1.7
0.75	0.0373	0.123	3.30	1.5
1.0	0.0323	0.113	3.50	1.4

TABLE IV

PULSE AND DIFFERENTIAL PULSE POLAROGRAPHY OF Eu(II)-DPTA IN THE ABSENCE AND PRESENCE OF SPINACH FERREDOXIN

$C_{Eu(II)\text{-}DPTA} = 1\ \mu M$; $C_{Fd} = 25\ \mu M$; 0.04 M Tris-HCl, pH 7.3.

t (s)	I_d (nA)	I_k (nA)	n_{app}	k_f ($M^{-1} \cdot s^{-1}$) ($\times 10^{-5}$)
Pulse polarography				
0.5	7.8	17	2.18	10.0
1.0	20	29.7	1.49	3.2
2.0	28	40	1.42	2.8
5.0	50	72.5	1.45	3.1
Differential pulse polarography				
0.5	1.7	2.1	1.24	1.8
1.0	3.1	4.9	1.57	5.2
2.0	4.4	6.3	1.42	3.6
5.0	7.2	10.0	1.38	3.3

methods it was possible to measure the reaction rate under pseudo-first-order conditions, which made the analysis much simpler. In addition, it is possible to verify that ferredoxin was not reduced because the whole potential range can be scanned. The results using this approach are shown in Table IV. The results are somewhat higher but are generally consistent with each other. Finally, the temperature dependence of the Eu(II)-ferredoxin reaction was also studied and the results are given in Table II. An activation enthalpy of 8.4 kcal/mol and an activation entropy of -7.0 cal/K per mol were obtained.

The last mediator that was studied was propylene viologen, which has an $E_{1/2}$ value of -0.80 V as compared to a standard calomel electrode. This molecule is not well behaved at the mercury surface because of adsorption problems. Fortunately, its electrochemistry at the gold surface is quite good. Therefore, rotating-disk voltammetry at a gold surface was used for this work. The two major advantages of rotating-disk voltammetry are the increase in current due to the convection caused by the rotation of the electrode and the elimination of the charging current because the potential is scanned very slowly. The pseudo-first-order rate constant is related to n_{app} by:

$$\chi k' = \left(n_{app}^3 - 1\right)/n_{app} \tag{3a}$$

TABLE V

ROTATING-DISK VOLTAMMETRY OF PROPYLENE VIOLOGEN IN THE ABSENCE AND PRESENCE OF SPINACH FERRODOXIN

$C_{PV} = 10.4\ \mu M$; $C_{Fd} = 0.104$ mM; 0.050 M Tris-HCl, pH 7.3. Average $k_f = 1.2 \cdot 10^5\ M^{-1} \cdot s^{-1}$. PV, propylene viologen.

ω (s^{-1})	I_d (μA)	I_k (μA)	n_{app}	k_f ($M^{-1} \cdot s^{-1}$) ($\times 10^{-5}$)
25.9	1.10	2.90	2.61	0.74
63.5	2.30	5.35	2.33	1.43
102	3.20	5.68	1.78	1.19
140	4.10	6.40	1.56	1.13
181	4.90	7.0	1.43	1.09
219	5.10	7.8	1.53	1.66

where

$$\chi = 1.61 \nu^{1/3} / D^{1/3} \omega \tag{3b}$$

and

$$k' = C_{Fd} k_f \tag{3c}$$

The term n_{app} has been defined before; ν is the kinematic viscosity ($9.11 \cdot 10^{-3}$ P at 24°C), D the diffusion coefficient of the viologen ($3.9 \cdot 10^{-6}$ cm^2/s) and ω the rotation rate in rad/s. The data are shown in Table V for some typical experiments and an average rate constant of $1.2 \cdot 10^5\ M^{-1} \cdot s^{-1}$ was calculated.

Discussion

Small molecule-protein electron-transfer rates can be analyzed using the Marcus theory, as shown in Eqn. 4:

$$k_f = (k_{11} k_{22} K f)^{1/2} \tag{4a}$$

where

$$\ln f = (\ln K)^2 / 4 \ln\left(k_{11} k_{22} / Z^2\right) \tag{4b}$$

and, k_{11} and k_{22} are the self-exchange rates for the protein and the small molecule, respectively, K is the equilibrium constant for reaction 2, and Z the

collision frequency. In general, the electron-transfer rates for protein-small molecule reactions have not correlated very well with the known self-exchange rates using the Marcus theory. Usually, the observed rates are much slower than expected. For example, synthetic iron-sulfur clusters have been found by NMR to have electron self-exchange rates of $10^6\ M^{-1}\cdot s^{-1}$ [21], while the self-exchange rates for iron-sulfur proteins have been calculated by Marcus theory to be 10^{-2}–$10^{-3}\ M^{-1}\cdot s^{-1}$ [3,6]. Thus, from Eqn. 4a, k_{11} is about 10^4-times slower than would be predicted from model studies. Finally, there has been little consistency in calculated k_{11} values.

An alternate approach that has been used lately is the tunneling theory of Hopfield [10], Jortner [22] and Chien [11,12]. The derivations of the equations are given in Refs. 10–12 and only the conclusions will be given here. The tunneling equations are:

$$k_f = 6.023\cdot 10^{-4}\left[k^{\mu}\left(2\pi\lambda^3 r/R_p\right)\right] \tag{5a}$$

where

$$k^{\mu} = (4\pi/h)|T_{ab}(r)|^2(4\pi k_B T\Delta)^{-1/2} \times \exp\left[-(E_a - E_b - \Delta)^2/4kT\Delta\right] \tag{5b}$$

and

$$|T_{ab(r)}| = 2.7(N_a N_b)^{-1/2}\exp(-0.72r) \tag{5c}$$

and, λ is the characteristic decay constant, equal to 0.7 A, r the distance of closest approach of the redox centers, R_p the radius of the protein, h Planck's constant, k_B Boltzmann's constant, T the absolute temperature, Δ the vibronic coupling parameter, E_a and E_b the standard reduction potentials of the protein and the mediator, respectively, N_a and N_b the number of active sites over which the initial and final wave functions are spread, and T_{ab} the tunneling or electron-exchange matrix element. If we combine the constants in Eqns. 5a and 5b we obtain:

$$k_f = 3.77\cdot 10^{14}(r/R_p)|T_{ab(r)}|^2(T\Delta)^{-1/2} \times \exp\left[-(E_a - E_b - \Delta)^2/3.44\cdot 10^{-4}T\Delta\right] \tag{6}$$

Chien [12] has applied this approach extensively to the cytochrome *c* literature but there has been no application of this approach to other proteins. We are somewhat hampered in this work by the lack of data on ferredoxin and the type of mediators that can reduce it. Chien and Dickinson [11] have used 0.5 eV for the Δ value for iron complexes and cytochrome *c*, and 1.0 eV for cobalt complexes. The Δ value for spinach ferredoxin may be different from the values for the other iron complexes because the irons in spinach ferredoxin are tetrahedrally coordinated, rather than the usual octahedral iron complexes. The E_a and E_b values are experimentally available and the r value can be estimated from the closest approach of the reactants. Δ for propylene viologen will be assumed to be the same as those of methyl and benzyl viologen [15], which have been calculated previously from the viologen-cytochrome *c* reactions. In this case Δ was 0.3.

Using this approach, the values of the rate constants were calculated using the tunneling model, and are shown in Table VI. The results compare well with the experimental values. In calculating these values, the best results were obtained if the Δ value for the europium reaction was 0.5 eV (like iron complexes) and for the vanadium reaction the Δ value was 1.0 eV (like the cobalt complexes). In Table VI, the experimental results from the literature for the oxidation of spinach ferredoxin with two iron complexes [6], cytochrome *c* [6], Co(II)-EDTA [8] and dithionite [23] are given along with the theoretical value calculated from Eqn. 5. These results also compare quite well, indicating that the higher value of Δ for spinach ferredoxin appears to be quite consistent with the available data.

It is recognized that the original derivation of the preexponential factor and the constant in the exponential in Eqn. 5c depends upon the value of the resonance integral, β, between two aromatic π-electron systems. The basic derivation of Eqn. 5c, though, does not assume a given electronic configuration but involves essentially the difference between the barrier height, V_0 ($V_0 \approx 2$ eV) and the energy of the electron (approx. β), which was taken to be about 1 eV. For any given system, V_0 and β will vary but the results depend most strongly on their difference. The consistency of the

TABLE VI

CALCULATION OF THE THEORETICAL RATE CONSTANT FOR THE REDUCTION OR OXIDATION OF SPINACH FERREDOXIN BY THE USE OF THE TUNNELING THEORY [a]

Mediator	k_f (exp.) ($M^{-1} \cdot s^{-1}$)	k_f [d] (theor.) ($M^{-1} \cdot s^{-1}$)	Δ_{med} (eV)	$\Delta H^{\ddagger}$ (exp.) (kcal/mol)	$\Delta H^{\ddagger}$ (theor.) (kcal/mol)	$\Delta S^{\ddagger}$ (exp.) (cal/mol per K)	$\Delta S^{\ddagger}$ (theor.) (cal/mol per K)	Reference
Eu-DPTA	$3.2 \cdot 10^5$	$1.5 \pm 0.7 \cdot 10^5$	0.5	8.4	2.5	−7.0	−26	this work
V-EDTA	$3.0 \cdot 10^4$	$3.0 \pm 1.3 \cdot 10^4$	1.0	5.6	3.8	−21	−25	this work
Propylene viologen [b]	$1.2 \cdot 10^5$	$2.8 \pm 1.4 \cdot 10^4$	0.3	–	–	–	–	this work
Fe-EDTA	$2.9 \cdot 10^5$	$4.6 \cdot 10^5$	0.5	0.7	1.9	−31	−26	6
Fe-HEDTA	$2.5 \cdot 10^4$	$2.1 \cdot 10^5$	0.5	0.3	2.4	−37	−26	6
Cytochrome c [c]	$8.1 \cdot 10^4$	$1.8 \cdot 10^5$	0.5	9.6	0.8	−4	−32	6
Co-EDTA [c]	$7.2 \cdot 10^3$	$5.6 \cdot 10^4$	1.0	5.2	2.3	−23.4	−32	8
SO_2^- [b]	$2.3 \cdot 10^5$	$3.2 \cdot 10^3$	0.5	–	–	–	–	23

[a] $r = 7$ Å, $R_p = 16$ Å, $\Delta_{Fd} = 0.8$.
[b] $r = 6$ Å.
[c] $r = 8$ Å.
[d] Uncertainty derived from Eqn. 7 for d $\Delta = \pm 0.05$.

result indicates that the V_0-β term is fairly constant. Attempts to refine the theory in terms of these energies are probably not justified in comparison with the assumptions inherent in the derivation. It should, therefore, be kept in mind that the variations observed in r may really be variations in V_0-β. Thus, we must not interpret r too rigorously. In spite of this, the overall success of this approach indicates that the tunneling theory is quite useful in rationalizing the reaction rates between small molecules and proteins. If we compare this approach with the Marcus theory approach, we find that it is much easier to arrive at an estimate of k_f using reasonable values for the initial parameter. In these terms, the tunneling approach is most useful.

The sensitivity of k_f to variations in Δ can be estimated from the derivative of Eqn. 6, which is shown in Eqn. 7.

$$\frac{dk_f}{d\Delta} = -k_f\left[\frac{1}{2\Delta} - \frac{2\Delta(E_a - E_b - \Delta) + (E_a - E_b - \Delta)^2}{4kT\Delta^2}\right] \quad (7)$$

The value of k_f depends quite strongly on the value of Δ. The results are shown in Table VI. Generally, variations in Δ of ± 0.05 eV lead to variations in k_f of 50%. Thus, one does not have much leeway in the choice of Δ. This consistency between the experimental and theoretical values of k_f, as seen in the last five mediators in Table VI for which the Δ values are already known, supports the validity of the tunneling approach to electron-transfer kinetics.

Finally, the $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values can be estimated from the following equations [12]:

$$\Delta H^{\ddagger} = (E_a - E_b - \Delta)^2/4\Delta - 3RT/2 \quad (8a)$$

$$\Delta S^{\ddagger} = R \ln\left[\frac{2.38 \cdot 10^{-2}}{kT}\left(\frac{2\pi\lambda 3r}{R_p}\right)\right.$$
$$\left.\times\left(\frac{1}{4\pi kT\Delta}\right)^{1/2}|T_{ab}(r)|^2\right] - \frac{3R}{2} \quad (8b)$$

These results are also shown in Table VI. The experimental results compare fairly well with the theoretical values, especially in predicting the low values of $\Delta H^{\ddagger}$ for the iron-EDTA-ferredoxin reaction. There are some significant deviations, as also seen by Chien [12] for cytochrome *c*. But, it is remarkable that the agreement is so good in a large number of cases.

Conclusions

The results of this work show the utility of electrochemical methods in the study of the reduction kinetics of low-potential proteins. In addition, this work has shown the applicability of the tunneling theory to iron-sulfur proteins. This approach should be more useful than the Marcus theory for investigating anamolies in electron-transfer kinetics. In particular, the Marcus theory underestimates the rate constant for reactions with a large driving force, where nonadiabaticity effects would be the largest. As a result, the previous use of Marcus theory predicted an unusually slow electron-transfer reaction, which was interpreted in terms of an 'inaccessible' redox site [6]. This is in contrast to recent X-ray data which indicate that the redox site is near the surface [7]. More data are necessary though in order to assure its predictive power for electron-transfer reactions. Further applications of this are in progress in order to extend the tunneling concept to multi-electron reactions.

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