Electrostatic effects in electron transfer reactions of [2Fe-2S] ferredoxins with inorganic reagents

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Abstract

The kinetics of electron transfer from the reduced [2Fe-2S] ferredoxins from the cyanobacterium *Anabaena* 7120 and the protozoan *Trichomonas vaginalis* to select cobalt coordination compounds have been studied in order to gain insight into the mechanism of electron transfer and intrinsic reactivity of [2Fe-2S] active sites. With tripositive cobalt complexes, reactions of both proteins displayed saturation kinetics; values of association constants of 12,900 and 1,400 M⁻¹ and limiting rate constants of 7.6 and 3.5 s⁻¹ were found for oxidation of *T. vaginalis* and *Anabaena* ferredoxins, respectively, by Co(NH₃)₆³⁺ at room temperature and *I* = 0.1 M. An activation enthalpy of 12.1 kcal/mol and activation entropy of -14.3 cal/mol K for oxidation of *T. vaginalis* ferredoxin by Co(NH₃)₆³⁺ contrasted with corresponding values of 13.4 kcal/mol and -10.5 cal/mol K for the *Spirulina platensis* protein, which is homologous to *Anabaena* ferredoxin. The dependence of the reaction rates on ionic strength were measured to probe the importance of electrostatics on the reactivity of the proteins. Analysis of the ionic strength dependence of the oxidation of the proteins by Co(NH₃)₆³⁺ by the "parallel plate" model of Watkins et al. (1994, *Protein Sci* 3:2104–2114) afforded values for active site charges of -0.7 and -1.1 and limiting rate constants at infinite ionic strength of 25,800 and 76 M⁻¹s⁻¹ for *T. vaginalis* and *Anabaena* ferredoxins, respectively. These results suggest that the [2Fe-2S] center of the protozoal ferredoxin is more accessible and adjacent to a less highly charged, more compact patch of negative charges than the photosynthetic protein.

Keywords: electron transfer; electrostatics; ferredoxin; kinetics

The [2Fe-2S] ferredoxins comprise a diverse class of iron-sulfur proteins that are widespread in biology (Lovenberg, 1973). These proteins participate in a variety of electron transfer pathways in important biological processes such as nitrogen fixation and photosynthesis. The [2Fe-2S] clusters of these proteins display a wide range of redox potentials as well as substantial diversity in their spectroscopic properties (Johnson, 1994).

[2Fe-2S] Ferredoxins are generally highly charged molecules whose sequences contain significant numbers of acidic residues. For example, the estimated charge of the oxidized plant-type ferredoxin from spinach is -17 at pH 7.0 (Rogers, 1987). During the course of electron shuttling, [2Fe-2S] ferredoxins often form stable precursor complexes with their respective redox partners (Knaff & Hirasawa, 1991). These complexes are stabilized by electrostatic interactions between oppositely charged surface regions of the two

proteins. The formation of such bimolecular structures ensures specificity in biological electron transfer processes.

Studies of redox reactions between small molecular weight inorganic reagents and metalloproteins have afforded insight into patterns of reactivity within various biological electron transfer systems. An advantage of using coordination compounds as redox probes includes the ability for the redox potential, intrinsic reactivity, hydrophobicity, and overall charge of the probe by manipulation of the ligand set. Through the use of inorganic reagents, distinct electron transfer sites have been identified on the surfaces of blue copper proteins (Sykes, 1991a), cytochromes (Wherland & Gray, 1976), and iron-sulfur proteins (Armstrong, 1982). These sites often are used by the proteins for binding to their physiologic redox partners as well.

The electron transfer reactivity of [2Fe-2S] ferredoxins has been investigated by a number of groups. Electron transfer reactions between plant-type [2Fe-2S] ferredoxins and inorganic reagents have been studied extensively by Sykes (1991b). Tollin and co-workers have characterized the kinetics of electron transfer between photosynthetic ferredoxins and their physiologic redox partners, including ferredoxin-NADP⁺ reductase (Hurley et al., 1993) and photosystem I (Hervas et al., 1992). Structure-reactivity analysis of these proteins was aided greatly by the presence of

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; en, ethylenediamine; FPLC, fast protein liquid chromatography; IPTG, isopropyl- β -thiogalactoside; NHE, normal hydrogen electrode; ox, oxalate; RT, room temperature; Tris, tris(hydroxymethyl)aminomethane.

high-resolution crystal structures for five cyanobacterial ferredoxins (Tsukihara et al., 1990; Rypniewski et al., 1991; Jacobsen et al., 1993; Ikemizu et al., 1994; Fukuyama et al., 1995).

Exploration of the electron transfer reactivity of nonphotosynthetic [2Fe-2S] ferredoxins has been less extensive. A drawback to studying these proteins has been the lack of high-resolution X-ray structures. The recent disclosure of an NMR-derived structure for the [2Fe-2S] protein putidaredoxin from *Pseudomonas putida*, however, has aided understanding the reactivity of this protein (Pochapsky et al., 1994). Several groups have probed the electron transfer reactions between putidaredoxin and mitochondrial ferredoxins and their physiologic redox partners (Coghlan & Vickery, 1992; Davies & Silgar, 1992; Uhlmann & Bernhardt, 1995). To date, no reports of reactions between nonphotosynthetic [2Fe-2S] proteins and inorganic reagents have appeared.

This article describes studies directed to the identification of the reactive surface sites and the characterization of the redox reactivity of two distinct [2Fe-2S] proteins. The two proteins, the ferredoxins from vegetative cells of the cyanobacterium *Anabaena* 7120 (Bohme & Schrautemeier, 1987) and from the protozoan *Trichomonas vaginalis* (Gorrell et al., 1984), are spectroscopically distinct and share little sequence homology. These proteins are representative of two broad classes of [2Fe-2S] proteins: the plant-type ferredoxins and the oxidase ferredoxins. Evaluating the kinetics of the redox reactions of these two proteins under conditions of varying ionic strength has provided insight into the intrinsic reactivity and the electrostatic characteristics of the surface sites that these ferredoxins use in electron transfer.

Results

Absorbance spectra of reduced [$\text{Fe}_2\text{S}_2(\text{S}^{\text{Cys}})_4^{3-}$] and oxidized [$\text{Fe}_2\text{S}_2(\text{S}^{\text{Cys}})_4^{2-}$] forms of *T. vaginalis* ferredoxin are shown in Figure 1. Absorbance spectra of reduced and oxidized *Anabaena* ferredoxin were identical to those of other plant-type ferredoxins (Armstrong & Sykes, 1978). Quantitative regeneration of the oxidized proteins was observed upon the reaction of the reduced forms with trivalent cobalt complexes. The rate of reaction between the reduced proteins and oxidants in large excess conformed to Equation 1.

$$d[\text{Fe}_2\text{S}_2(\text{S}^{\text{Cys}})_4^{2-}]/dt = k_{\text{obs}}[\text{Fe}_2\text{S}_2(\text{S}^{\text{Cys}})_4^{3-}] \quad (1)$$

Fig. 1. Absorption spectra of oxidized and reduced *T. vaginalis* ferredoxins.

Table 1. Second-order rate constants of the reaction of reduced ferredoxin with select oxidants

Oxidant	k ($\text{M}^{-1} \text{s}^{-1}$)			
	Parsley ^a	<i>S. platensis</i> ^b	<i>Anabaena</i> ^c	<i>T. vaginalis</i> ^c
$[\text{Co}(\text{NH}_3)_4(\text{ox})]^+$	5.7×10^3	—	—	—
$[\text{Co}(\text{en})_2(\text{ox})]^{3+}$	—	—	3.8×10^1	1.01×10^3
$[\text{Co}(\text{EDTA})]^-$	7.2×10^3	4.05×10^3	2.33×10^3	2.9×10^4

^a Armstrong and Sykes (1978).

^b Adzamli et al. (1983).

^c This work.

Second-order rate constants for oxidation of reduced [2Fe-2S] ferredoxins with the monopositive complex $\text{Co}(\text{en})_2(\text{C}_2\text{O}_4)^+$ and the anionic complex $\text{Co}(\text{EDTA})^-$ were obtained from the slopes of the linear plots of k_{obs} versus the concentration of the cobalt reagent (Table 1). Plots of the pseudo-first-order rate constants versus concentration of the tripositively charged cobalt complexes $\text{Co}(\text{NH}_3)_6^{3+}$ and $\text{Co}(\text{en})_3^{3+}$, however, gave nonlinear dependences for both ferredoxins at low ionic strengths (Fig. 2). These dependences were consistent with a mechanism in which rapid association (Equation 2) preceded electron transfer (Equation 3):

The rate law describing this reaction sequence led to an expression (Equation 4) that was used to obtain association constants (K) and electron transfer rates (k_{ET}) from a linear plot of $1/k_{\text{obs}}$ versus $1/[\text{Co}^{\text{III}}]$, where the slope and intercept correspond to $1/Kk_{\text{ET}}$ and $1/k_{\text{ET}}$, respectively. Alternatively, the values of K and k_{ET} could be obtained by nonlinear least-squares fits to Equation 4; values for the parameters for both ferredoxins are presented in Table 2.

$$k_{\text{obs}} = (Kk_{\text{ET}}[\text{Co}^{\text{III}}]) / (1 + K[\text{Co}^{\text{III}}]). \quad (4)$$

Fig. 2. Nonlinear dependence of the pseudo-first-order rate constants k_{obs} on the concentration of oxidant $\text{Co}(\text{NH}_3)_6^{3+}$. The oxidation of reduced *Anabaena* ($\square$) and *T. vaginalis* ($\circ$) ferredoxins were performed at pH 8.0 (Tris-HCl), $I = 0.1 \text{ M}$ (NaCl), at 25 °C. Lines are plots of fits of the data to Equation 4, using the parameters of Table 2.

Table 2. Summary of overall rate constants, k ($= Kk_{ET}$), association constants, K , and electron transfer rate constants, k_{ET} , for oxidation of select ferredoxins with different oxidants, pH 8.0 (Tris-HCl), $I = 0.1$ M (NaCl), 25 °C

Oxidant	Parameter	Parsley ^a	<i>S. platensis</i> ^b	<i>Anabaena</i> ^c	<i>T. vaginalis</i> ^c
[Co(NH ₃) ₆] ³⁺	k (M ⁻¹ s ⁻¹)	1.9×10^4	1.01×10^4	5.0×10^3	9.8×10^4
	K (M ⁻¹)	998	2,070	1,412	12,900
	k_{ET} (s ⁻¹)	19.2	4.9	3.5	7.6
[Co(en) ₃] ³⁺	k (M ⁻¹ s ⁻¹)	1.6×10^3	—	1.2×10^2	5.4×10^4
	K (M ⁻¹)	597	—	—	9,524
	k_{ET} (s ⁻¹)	2.7	—	—	5.6

^a Armstrong and Sykes (1978).^b Adzamli et al. (1983).^c This work.

From the temperature dependence of the value for k_{ET} (Fig. 3), activation parameters for electron transfer were obtained for the reaction of *T. vaginalis* ferredoxin with Co(NH₃)₆³⁺. These values are given Table 3, along with those for the [2Fe-2S] ferredoxin from parsley leaves (Armstrong & Sykes, 1978) and from the cyanobacterium *Spirulina platensis* (Adzamli et al., 1983), which has high homology to the *Anabaena* protein (Alam et al., 1986; Rogers, 1987).

Observed rates were influenced strongly by ionic strength. Figure 4 shows the effect of variation of ionic strength on the apparent second-order rate constant Kk_{ET} for the *Anabaena* and *T. vaginalis* ferredoxin-Co(NH₃)₆³⁺ reactions. Observed rate constants (k_{obs}) and calculated association constants (K) for the reactions with Co(NH₃)₆³⁺ decreased with increasing ionic strength in compliance with presumed electrostatic stabilization of the bimolecular complex. The ionic strength effects were used to probe the electrostatic features of the reactive site on the proteins' surfaces. The dependences of the apparent second-order rate constants k ($= Kk_{ET}$) for reoxidation of the two [2Fe-2S] ferredoxins with Co(NH₃)₆³⁺ on ionic strength were analyzed in terms of a "parallel plate" electrostatic model (Watkins et al., 1994) (Table 4).

Discussion

The kinetics of the electron transfer reactions of reduced *T. vaginalis* and *Anabaena* ferredoxins with cobalt complexes of various

overall charge and ligand structure have been examined. The reactions between the ferredoxins and Co(en)₂(ox)⁺ and Co(EDTA)⁻ conform to simple second-order kinetics without evidence for the formation of an intermediate complex (Table 1). However, saturation behavior was observed with the tripositive cobalt complexes Co(NH₃)₆³⁺ and Co(en)₃³⁺. The values of the association constants K and electron transfer rates k_{ET} were determined by non-linear least-squares fits of the data to Equation 4 (Table 2). The dependence of the value of the association constants on ionic strength indicated that electrostatic interactions played a role in stabilizing the bimolecular complexes.

Despite the fact that both oxidants carried the same overall charge, the association constants involving Co(NH₃)₆³⁺ were larger than those for Co(en)₃³⁺. Although the length of Co(III)-N bond is the same in both complexes (1.96 Å) (Hancock, 1991), the significantly smaller size of the amino group relative to the ethylenediamine ligand would allow more intimate contact of the inorganic reagent with residues of the binding site. In addition, hydrogen bonding from the amino group to the protein surface is likely to contribute to the formation of a more stable complex.

The value of the association constant K for the binding of Co(NH₃)₆³⁺ to reduced *Anabaena* ferredoxin was similar in magnitude to those for Co(NH₃)₆³⁺ binding to other plant-type ferredoxins (Adzamli et al., 1983; Lloyd et al., 1992) (Table 2). The value of K for *T. vaginalis* ferredoxin, however, was almost one order of magnitude larger than that for *Anabaena* ferredoxin (Table 2). Comparison of the amino acid sequence of *T. vaginalis* ferredoxin (Johnson et al., 1990) with those of plant-type ferredoxins (Matsubara & Hase, 1983) shows that the photosynthetic ferredoxins have greater overall negative charge. Thus, results in Table 2 suggest that net charge seems unlikely to be a dominant

Fig. 3. Temperature dependence of the pseudo-first-order rate constant for [Co(NH₃)₆]³⁺ oxidation of reduced *T. vaginalis* ferredoxin, pH 8.0 (Tris-HCl), $I = 0.1$ M (NaCl).**Table 3.** Activation parameters for the oxidation of select ferredoxins with Co(NH₃)₆³⁺, pH 8.0 (Tris-HCl), $I = 0.1$ M (NaCl)

Parameter	<i>S. platensis</i>	Parsley	<i>T. vaginalis</i> ^a
$E^{\circ\prime}$ (mV versus NHE)	-381 ^b	-425 ^b	-360
ΔH° (kcal/mol)	13.4 ^c	8.5 ^c	12.1
ΔS° (cal/mol K)	-10.5 ^c	-24.1 ^c	-14.3

^a This work.^b Cammack et al. (1977).^c Adzamli et al. (1983).

Fig. 4. Ionic strength dependence of the apparent second-order rate constant (Kk_{ET}) for $[\text{Co}(\text{NH}_3)_6]^{3+}$ oxidation of reduced *T. vaginalis* ($\square$) and *Anabaena* ($\circ$) ferredoxins, pH 8.0 (Tris-HCl), 25 °C. Lines are fits to Equation 5 using the parameters given in Table 4.

factor in the formation of electrostatically stabilized, transient complexes. Consequently, a specific patch on the protein surface was responsible for the binding of positively charged oxidants and subsequent electron transfer.

Extracted values for the rates of the electron transfer step (k_{ET}) show typical Marcus behavior, where the reaction with $[\text{Co}(\text{NH}_3)_6]^{3+}$ was faster than the $\text{Co}(\text{en})_3^{3+}$ reaction due to the larger thermodynamic driving force (Table 2) [$E^{0'}$ = 60 and -210 mV versus NHE for $[\text{Co}(\text{NH}_3)_6]^{3+}$ and $\text{Co}(\text{en})_3^{3+}$, respectively (Geselowitz & Taube, 1982)]. The differences between the rates of electron transfer, however, were smaller than expected from the values of the redox potentials; this discrepancy probably reflected the differences in self-exchange rates, and therefore reorganization energies, observed for these inorganic complexes [k = 2.0×10^{-7} and $7.7 \times 10^{-5} \text{ M}^{-1}\text{s}^{-1}$ for $[\text{Co}(\text{NH}_3)_6]^{3+}$ and $\text{Co}(\text{en})_3^{3+}$, respectively (Dwyer & Sargeson, 1961)].

Values for the electron transfer rate constants, k_{ET} , within the complex do show trends in reactivity for different ferredoxins with the same oxidant. For example, parsley ferredoxin is more reactive (~2.5 times), whereas *Anabaena* ferredoxin and *S. platensis* ferredoxin are less reactive (~2 times and ~1.5 times, respectively) than the protozoal ferredoxin with $[\text{Co}(\text{NH}_3)_6]^{3+}$ (Table 2). The larger values of electron transfer rates for the higher plant ferredoxins can be rationalized in terms of corresponding more negative redox potentials, and therefore larger driving forces, relative

to that of *T. vaginalis* ferredoxin (Hutson et al., 1978; Vidakovic et al., 1996). The slower rate for the *S. platensis* protein could be explained by a correspondingly smaller driving force (Hutson et al., 1978). The lower electron transfer rates observed for *Anabaena* ferredoxin could not be solely due to thermodynamic factors, because the midpoint potential of the *Anabaena* protein is lower than that of the protozoal protein (Vidakovic et al., 1995). In addition to the redox potential differences, other factors, such as steric and electronic properties of the protein active site, and nuclear and solvent rearrangements within the transient complex, could contribute to the observation.

Analysis of the temperature dependence of the intramolecular electron transfer rate constant, k_{ET} , for the reaction of *T. vaginalis* ferredoxin with $[\text{Co}(\text{NH}_3)_6]^{3+}$ allowed extraction of activation parameters for this process (Table 3). The values of $\Delta H^\ddagger$ and $\Delta S^\ddagger$ fell between those for the reaction of the *S. platensis* and parsley proteins with the same reagent (Adzamli et al., 1983) (Table 3). A general trend of decreasing activation parameters with increasing thermodynamic driving force was found.

The kinetic behavior of *T. vaginalis* and *Anabaena* ferredoxins with positively charged electron acceptors suggested that a significant negative charge was located in the proximity of the proposed electron-transfer active sites. To clarify the role of electrostatics in the reactions with the tripositive cobalt reagents, the rates of electron transfer between *T. vaginalis* and *Anabaena* ferredoxins with $[\text{Co}(\text{NH}_3)_6]^{3+}$ under varying ionic strength conditions were determined. The values of the apparent second-order rate constants were then analyzed in terms of a "parallel plate" model for the electrostatic potential energy (Tollin et al., 1984; Watkins et al., 1994). This model emphasizes local electrostatic interactions between the charged moieties, and gives a more realistic description of the reaction than does an earlier model of Wherland and Gray (1976), which considers the entire charge of the protein.

In the form of the "parallel plate" model in which ion-dipole and dipole-dipole interactions are neglected, the rate constant at a particular ionic strength [$k(I)$] is given by Equation 5:

$$\ln k(I) = \ln k_i - (\alpha Z_1 Z_2 r_{12} / D_{\text{eff}} \rho^2) \exp(-\kappa \rho) / (1 + \kappa \rho). \quad (5)$$

In this equation, k_i is the limiting rate constant at infinite ionic strength, $\kappa = 0.3295 I^{1/2} \text{ \AA}^{-1}$, Z_1 and Z_2 are the charges on the reactants, α is a constant, r_{12} is the distance between the surface electron-transfer groups within the reactive complex, D_{eff} is the effective dielectric constant, and ρ is the surface radius of the active site. Values for the parameters were obtained by nonlinear least-squares fits of the data to the theoretical model, while fixing the values for r_{12} to 3.5 Å, and Z_2 to +3 (Table 4). Uncertainties in the values for ρ are less than 1 Å, and less than 10% for the other parameters, according to the originators of the analytical solution to the model (Watkins et al., 1994).

The values of the second-order rate constants extrapolated to infinite ionic strength (k_i) provided a measure of the intrinsic reactivity of the two proteins to the oxidant (van Leeuwen et al., 1981). The k_i values obtained in this way revealed *T. vaginalis* ferredoxin to be more than two orders of magnitude more reactive than *Anabaena* ferredoxin (Table 4). The origin of this large difference could be attributed to differences in reorganization parameters as well as in the steric and electronic properties of the active site. The larger value of the second-order rate constant for the reaction of *T. vaginalis* ferredoxin with the negatively charged complex, $\text{Co}(\text{EDTA})^-$ relative to plant-type ferredoxins (Table 1)

Table 4. Values for parameters from fits to the "parallel plate" model of ionic strength dependences of pseudo-second-order rates for reaction of reduced ferredoxins with $[\text{Co}(\text{NH}_3)_6]^{3+}$, pH 8.0 (Tris-HCl), 25 °C

Parameter	<i>T. vaginalis</i>	<i>Anabaena</i>
$k_i (\text{M}^{-1} \text{s}^{-1})$	25,800	76
$Z_1 Z_2$	-2.2	-3.2
$r_{12} (\text{\AA})$	3.5	3.5
D_{eff}	47.0	14.0
$\rho (\text{\AA})$	3.0	4.5

was also consistent with the greater intrinsic reactivity of the protozoal protein with $\text{Co}(\text{NH}_3)_6^{3+}$ determined by evaluation of ionic strength effects.

The "parallel plate" model also afforded an estimate of the size and the amount of charge at the proteins' reactive sites. The best fit gave an active site charge of -1.1 and radius of $4.5 \text{ \AA}$ for *Anabaena* ferredoxin. These values are similar to those found by Tollin and co-workers for the reaction of spinach ferredoxin with a positively charged viologen analog ($Z_2 = -0.9$, $\rho = 4.5 \text{ \AA}$), suggesting that both reagents use the same reactive site on the protein surface (Navarro et al., 1989). For the *T. vaginalis* protein, an active site charge of -0.7 and radius of $3.0 \text{ \AA}$ were found, indicating the presence of a smaller, less highly charged reactive site on the surface of this protein.

To complement the kinetic studies, the electrostatic properties of the $[2\text{Fe-2S}]$ ferredoxins were also investigated by computer modeling. A high-resolution X-ray crystal structure of oxidized *Anabaena* ferredoxin was available to aid the modeling of this protein (Rypniewski et al., 1991); the absence of a three-dimensional structure of *T. vaginalis* ferredoxin, however, hampered similar analysis of the protozoal protein. An estimate for the structure of *T. vaginalis* ferredoxin was obtained by homology modeling using the NMR-derived structure of putidaredoxin (Pochapsky et al., 1994), which is homologous with the *T. vaginalis* protein, as the template.

The electrostatic potential maps of the two proteins were calculated and are shown in Figure 5. A large region of negative potential, covering approximately $40 \text{ \AA}^2$, is present on the surface of the *Anabaena* protein in the vicinity of the residues Glu 24, Asp 28, and Glu 32. A smaller negative patch ($30 \text{ \AA}^2$) is seen on the surface of the protozoal ferredoxin in the vicinity of Asp 35 and Asp 36. The sizes and charges of the patches are in relative agreement with the values found by analysis of the kinetic data with the parallel plate model, which proposed patch areas of $60 \text{ \AA}^2$ and $30 \text{ \AA}^2$ on the surfaces of *Anabaena* and *T. vaginalis* ferredoxins, respectively.

Earlier efforts to determine the location of the binding site for positively charged reagents on the surface of plant-type ferredoxins used the redox-inactive $\text{Cr}(\text{NH}_3)_6^{3+}$ complex (Armstrong et al., 1979). Using various concentrations of $\text{Cr}(\text{NH}_3)_6^{3+}$, the selective broadening of resonances assigned to Tyr 82, Tyr 25, and Glu 24 in the NMR spectrum of *Anabaena variabilis* ferredoxin was observed, whereas the negatively charged complex $\text{Cr}(\text{CN})_6^{3-}$ did not exhibit a similar effect, even at higher concentrations (Chen et al., 1983). X-ray structure analysis (Rypniewski et al., 1991) revealed that an array of evolutionarily conserved, negatively charged residues, including Glu 24, Asp 28, and Glu 32, was located in the proximity of the tyrosine residues. On the other hand, Lloyd et al. (1992) argued that this site was not the first choice for these complexes, because the broadening of the neighboring Arg 42 resonances occurred only at higher concentrations of $\text{Cr}(\text{NH}_3)_6^{3+}$. In an effort to identify the residues primarily responsible for the electrostatic interaction with positively charged inorganic complexes, investigation of the mechanism of electron transfer for this system based on the tunneling pathway model (Beratan et al., 1990) was undertaken (Lloyd et al., 1992). Although this approach did not provide a clear picture of the most favorable path for the electron transfer, it pointed out the importance of the whole region surrounding Tyr 82 and Tyr 25 to the reaction, in agreement with the results of this study.

Structure reactivity studies involving nonphotosynthetic $[2\text{Fe-2S}]$ ferredoxins are less abundant. Coghlan and Vickery (1992)

Fig. 5. Calculated electrostatic potential maps of *Anabaena* and *T. vaginalis* ferredoxins. Calculations were made at an ionic strength of 0.02 M . Blue color indicates positive potential, and red indicates negative potential. Isopotential contours at $\pm 6 \text{ kT/e}$, which indicate the minimum energies.

found that a specific evolutionarily conserved glutamate residue analogous to Glu 63 of *T. vaginalis* ferredoxin was critical for the function of a mitochondrial ferredoxin with its physiologic redox partners. This residue, however, was located rather distantly from the protein's metal center. The negative patch in the vicinity of Asp 35 and Asp 36 of the protozoal protein, however, was located very close to the iron-sulfur cluster, adjacent to the ligand residue Cys 38 (Fig. 5). This negative patch is unique to the protozoal protein among the nonphotosynthetic ferredoxins and may play a role in its function of electron transfer to hydrogenase. The close proximity of this patch to the metal center ($4.1 \text{ \AA}$ between Asp 36 O_δ and Cys 38 S_γ), relative to the long distance between the negative patch of *Anabaena* ferredoxin and its closest iron ligand ($7.8 \text{ \AA}$ from Asp 28 O_δ and Cys 79 S_γ), was also consistent with the

greater intrinsic reactivity of the protozoal ferredoxin with $\text{Co}(\text{NH}_3)_6^{3+}$.

In summary, examination of ionic strength dependences of electron transfer reactions of [2Fe-2S] ferredoxins with cobalt oxidants in conjunction with the "parallel plate" model for electrostatic energy provides considerable insight into differences in reactivity patterns of distinct [2Fe-2S] ferredoxin classes. Studies to clarify the reactive surface sites of the ferredoxins is currently underway.

Materials and methods

T. vaginalis ferredoxin was overexpressed in *Escherichia coli* as described elsewhere (Vidakovic et al., 1996). Briefly, the *E. coli* strain HMS174(DE3) was transformed with a recombinant plasmid containing the gene for *Trichomonas vaginalis* ferredoxin. A culture of bacteria harboring the vector was grown in LB medium supplemented with 100 mg ampicillin/L followed by IPTG induction for ferredoxin expression. After cell disruption using a French press, purification of the protein product was achieved through anion-exchange chromatography on a Q-Sepharose column using a linear gradient from 100 to 300 mM Tris-HCl, pH 7.6, followed by FPLC gel filtration (Superdex 75). The target protein was greater than 95% pure by PAGE and isoelectric focusing. Recombinant *Anabaena* 7120 ferredoxin was overexpressed and purified according to the procedure published previously (Vidakovic et al., 1995).

The following complexes were obtained by published methods and characterized by peak position in the ultraviolet-vis absorption spectra: hexa-amminecobalt(III) chloride $[\text{Co}(\text{NH}_3)_6\text{Cl}_3]$ (Bjerrum & McReynolds, 1946), tris(ethylenediamine)cobalt(III) chloride trihydrate $[\text{Co}(\text{en})_3]\text{Cl}_3 \times 3\text{H}_2\text{O}$ (Work, 1946), sodium ethylenediaminetetraacetatocobaltate(III) tetrahydrate $\text{Na}[\text{Co}(\text{EDTA})] \times 4\text{H}_2\text{O}$ (Schwarzenbach, 1949), oxalato-bis(ethylenediamine) cobalt(III) chloride $[\text{Co}(\text{en})_2(\text{ox})]\text{Cl}$ (Jordan & Froebe, 1978). Extinction coefficients were in good agreement with existing literature values.

Kinetic measurements were made using an OLIS stopped-flow spectrophotometer. All reactions were performed under pseudo-first-order conditions with oxidant in at least eightfold excess over protein. Ultra-high purity argon was bubbled through all reactant solutions for 15–30 min. Protein solutions were prepared in an anaerobic chamber by adding aliquots of concentrated ferredoxin samples (~2 mM), degassed by blowing humidified argon over the surface for 15 min to deoxygenated buffer. Sodium dithionite solution (0.1 M) was prepared by adding 5 mL degassed 10 mM Tris-HCl, pH 8.0, containing 0.15 mL 3 M NaOH, to 87 mg fresh sodium dithionite. The tube containing solid dithionite was evacuated repeatedly and filled with argon before the buffer solution was added. The reduced protein was generated by adding 2–3 equivalents sodium dithionite to the ferredoxin solution under anaerobic conditions. Solutions of oxidants and ferredoxins were made up at the required pH and ionic strength was adjusted by addition of 5 M NaCl solution.

Reoxidations of reduced *T. vaginalis* ferredoxin and *Anabaena* ferredoxin by inorganic oxidants were monitored at 458 nm and 422 nm, respectively. At least five kinetic traces were collected at the appropriate wavelengths for each oxidant concentration; first-order rate constants (k_{obs}) were calculated by computer fitting to a single-exponential rate equation using the SI-FIT program (OLIS, Inc.).

The theoretical model of the dependence of the rate constants for oxidation of reduced *T. vaginalis* and *Anabaena* ferredoxins

with the tripositively charged cobalt complex $\text{Co}(\text{NH}_3)_6^{3+}$ on ionic strength was based on a "parallel plate" electrostatic model developed by Tollin and coworkers (Watkins et al., 1994). Values for parameters were obtained by nonlinear least-squares fits of the data to the theoretical model, while fixing the values for r_{12} to 3.5 Å, and Z_2 to +3.

Energy minimization and conformational searches of the [2Fe-2S] ferredoxins were conducted with the help of the program QUANTA 4.1 on an SGI work station. Structural coordinates for the *Anabaena* protein (Rypniewski et al., 1991) were obtained from the Brookhaven Protein Data Bank. A structural model for the *T. vaginalis* protein was generated from the NMR-derived structure of *P. putida* ferredoxin (Pochapsky et al., 1994) as follows. All ionizable amino acid residues of the *T. vaginalis* ferredoxin sequence were substituted for the homologous residues of the *P. putida* sequence, and all charged residues of *P. putida* ferredoxin were substituted with the corresponding residues of the protozoal ferredoxin to result in the sequence GVVYVSKGGTKKQLKVED DVSLMQAAVENGIIYSIDDTCCGSGKSCAKCHVYVN KAFTGKVPAAEEDEKEMLECVTADLPNSRLCC QIFRTPELDGIVVEV. The substituted protein was subjected to manual adjustment of side-chain torsion angles to remove unfavorable steric interactions.

Electrostatic potential maps of *Anabaena* and *T. vaginalis* ferredoxins were calculated, visualized with the program GRASP (Nicholls, 1992), which uses a simplified version of the program DELPHI (Gilson & Honig, 1987) for calculation of electrostatic potentials according to the Poisson–Boltzmann equation. Values for charges, in units of electrons, for the iron–sulfur center were added to the charge file as follows: Fe (+1.5), S (−1.1), and cysteinyl S (−0.7) (Tsukihara et al., 1986). The parameters used for the Poisson–Boltzmann calculation were as follows: ionic strength of 0.02 M, water probe radius of 1.4 Å, ion radius of 2.0 Å, and interior and exterior dielectrics of 2.0 and 80.0, respectively. Protein surface areas within specific isopotential contours were determined by manually scribing the surface region and calculating the enscribed area.

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