Kinetic studies on reactions of iron–sulphur proteins

Oxidation of the reduced form of *Spirulina platensis* [2Fe-2S] ferredoxin with inorganic complexes

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Kinetic results are presented for the reaction of reduced [2Fe-2S] ferredoxin from the blue–green alga *Spirulina platensis* with $\text{Co}(\text{NH}_3)_6^{3+}$, $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$ as oxidants at pH 8.0 at 1.010 (NaCl). The aim is to compare results obtained with those previously reported for the [2Fe-2S] ferredoxin from parsley, where the two ferredoxins under consideration are in evolutionary terms widely divergent (35% amino acid variations). The three oxidants chosen have different ligand sets and different charges, and are the complexes that in previous studies have given greatest diversity in behaviour. With $\text{Co}(\text{NH}_3)_6^{3+}$ first-order rate constants (oxidant in large excess) tend to a limiting value with increasing concentration of oxidant. With $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$ there is no similar tendency to limiting behaviour and a first-order dependence on oxidant is observed. The temperature-dependence of the $\text{Co}(\text{NH}_3)_6^{3+}$ reaction was investigated, and values were obtained for ΔH° [$19.8 \text{ kJ} \cdot \text{mol}^{-1}$ ($4.7 \text{ kcal} \cdot \text{mol}^{-1}$)] and ΔS° [$129.3 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ($30.9 \text{ cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$)] for the association step that occurs before electron transfer. Whereas redox-inactive $\text{Cr}(\text{NH}_3)_6^{3+}$ displays competitive inhibition in the reaction of $\text{Co}(\text{NH}_3)_6^{3+}$, it accelerates the reaction of $\text{Co}(\text{edta})^-$, and only partially blocks the reaction with $\text{Co}(\text{acac})_3$. Results obtained are similar to those previously reported for parsley (and spinach) ferredoxin. It is concluded that electrostatics play a dominant role and that a negatively charged functional site on the protein common to all three ferredoxins is influential. Conserved negative patches at positions 67–69 and 94–96 within 1.0 nm (10 Å) of an Fe atom of the active site, as well as the exposed S atoms of cysteine residues 41 and 46, which are a part of the $\text{Fe}_2\text{S}_2^*(\text{SR})_4^{3-}$ cluster, are the most likely possibilities. The various effects of $\text{Cr}(\text{NH}_3)_6^{3+}$ provide a means of testing for utilization of the same site in reactions of the ferredoxins with physiological partners.

Iron–sulphur proteins have emerged in recent years as an important class of redox metalloproteins (see review volumes edited by Lovenberg, 1973*a,b*, 1977; Spiro, 1982). Occurrence of the [2Fe-2S] ferredoxins (M_r approx. 10000) is widespread, and these are now known to be present in algae, bacteria and mammalian organs, as well as in higher plants. The [2Fe-2S] ferredoxins are one-electron carriers, reduction potential approx. -0.42 V , utilizing oxidation states Fe(III,III) and Fe(II,III). On the basis of physical–chemical measurements (Beinert, 1973), and analogue studies (Ibers & Holm, 1980), it has been recognized for some time that the binuclear

active site consists of $\text{Fe}_2\text{S}_2^*(\text{SR})_4^{2-}$ (oxidized) and $\text{Fe}_2\text{S}_2^*(\text{SR})_4^{3-}$ (reduced) clusters (where SR^- represents a co-ordinated cysteine residue), and that two tetrahedrally co-ordinated Fe atoms are bridged by sulphides (designated S^*).

Previous kinetic studies on [2Fe-2S] ferredoxins, with the use of inorganic complexes as probes for redox reactivity, have been with the protein isolated from parsley chloroplasts (Armstrong *et al.*, 1979; Adzamli *et al.*, 1982). A brief comparative study with spinach [2Fe-2S] ferredoxin has also been reported (Armstrong & Sykes, 1978). To test more vigorously the effect of amino acid variations on reactivity we now turn to the [2Fe-2S] ferredoxin

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from the blue-green-algal source *Spirulina platensis*. This ferredoxin has been sequenced (Tanaka *et al.*, 1976), and a comparison of the sequence with that of parsley ferredoxin (Tsukihara *et al.*, 1981) indicates that occupancy of as many as 34 out of 98 amino acid positions is different. Comparison of redox-reactivity patterns of two such proteins with a series of inorganic reagents is a possible means therefore of assessing the location of the functional site (or sites) on the protein used for electron transfer. Moreover, an X-ray crystal structure of *S. platensis* ferredoxin (Tsukihara *et al.*, 1981), the only one at present available for a [2Fe-2S] ferredoxin, has been reported, and this enables results obtained to be directly related to structure.

The complexes selected for the present study are the oxidants $\text{Co}(\text{NH}_3)_6^{3+}$, $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$. The effect of the redox-inactive $\text{Cr}(\text{NH}_3)_6^{3+}$ on these reactions has also been investigated. A review of oxidation-reduction (and substitution) reactions of iron-sulphur centres is available (Armstrong, 1982).

Experimental

Protein

The [2Fe-2S] ferredoxin was isolated from *Spirulina platensis* cells provided by Mlle. G. Clement, Institute Français du Pétrole, Paris, France, by the same procedure as that used for *Spirulina maxima* ferredoxin (Hall *et al.*, 1972). Purification was by absorption on a calcium phosphate column (5 cm $\times$ 25 cm) equilibrated in 10 mM-sodium phosphate buffer, pH 7. The column was washed with buffer until the washings no longer absorbed at 250 nm. Elution was with more concentrated (20–100 mM) buffer. Those fractions with absorbance peak ratios $A_{420}/A_{280} > 0.4$ were pooled and re-chromatographed on a DEAE-cellulose (Whatman DE-23) column. Fractions with an A_{420}/A_{280} ratio of 0.45 were concentrated on a short DEAE-cellulose column (1 cm $\times$ 5 cm). The peak at 422 nm (ϵ 9200 $\text{M}^{-1} \cdot \text{cm}^{-1}$) was used to estimate protein concentrations. Generation of the one-electron reduced protein with $\text{Na}_2\text{S}_2\text{O}_4$ and adjustment of pH to 8.0 with 10 mM-Tris (with HCl added) were as previously described (Armstrong & Sykes, 1978). The spectrum of the reduced protein is assumed to have an absorption coefficient at 422 nm of 4500 $\text{M}^{-1} \cdot \text{cm}^{-1}$, as with other [2Fe-2S] proteins.

Complexes

The following complexes were obtained by published methods (Armstrong *et al.*, 1979), and characterized by peak positions in the u.v.-visible-absorption spectra, given as wavelength λ in nm (ϵ in $\text{M}^{-1} \cdot \text{cm}^{-1}$): hexa-amminecobalt(III) chloride, $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$, 473 (57.1); sodium ethylene-

diamminetetra-acetatecobaltate(III) tetrahydrate, $\text{Na}[\text{Co}(\text{edta})] \cdot 4\text{H}_2\text{O}$, 535 (320); tris(acetylacetonate)cobalt(III), $\text{Co}(\text{acac})_3$, 257 (3.4×10^4); hexa-amminechromium(III) chloride, $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$, 462 (40).

Stoichiometry

This was assumed to be as previously determined for reactions of parsley [2Fe-2S] ferredoxin (Armstrong & Sykes, 1978). All absorbance changes at 420 nm with oxidant in excess of the 1:1 reactant ratio were consistent with a one-equivalent change as in eqn. (1):

Kinetics

Reactions were monitored with a Dionex stopped-flow spectrophotometer. The oxidant was always in large (at least 10-fold) excess of the protein. Previous studies with parsley [2Fe-2S] ferredoxin have established a kinetic pattern consistent with an association step (K) before electron transfer (k_{et}), as in eqns. (2) and (3):

where such behaviour is detected by observed rate constants tending to a limiting value as a function of increasing concentration of oxidant. For experiments in which the competitive inhibition (or blocking effect) of redox-inactive $\text{Cr}(\text{NH}_3)_6^{3+}$ on the $\text{Co}(\text{NH}_3)_6^{3+}$ reaction was investigated, it was not possible to use a low concentration of $\text{Co}(\text{NH}_3)_6^{3+}$, as in previous studies, such that $K[\text{Co(III)}] \ll 1$ (see below), and a fuller kinetic treatment was required. The ionic strength of reaction solutions was adjusted to 1.0 (NaCl). Rate constants, $k_{\text{obs.}}$, were obtained from the slope of plots of absorbance changes $\ln \Delta A$ against time.

Treatment of data

For the $\text{Co}(\text{NH}_3)_6^{3+}$ reaction a non-linear least-squares program (no weighting factor) was used to obtain enthalpy and entropy parameters from the temperature-dependence. Standard least-squares treatments were used in all other cases (no weighting).

Results

Cobalt(III) complexes as oxidants

Three complexes, $\text{Co}(\text{NH}_3)_6^{3+}$, $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$, were used. First-order rate constants, $k_{\text{obs.}}$, for the reaction with $\text{Co}(\text{NH}_3)_6^{3+}$ give a

Fig. 1. Variation with temperature of first-order rate constants, k_{obs} , for the $\text{Co}(\text{NH}_3)_6^{3+}$ oxidation of reduced *Spirulina platensis* [2Fe-2S] ferredoxin (approx. $10\ \mu\text{M}$) at pH 8.0 (Tris/HCl) at 1.0 (NaCl). Temperature: ●, 5°C; ■, 15°C; ▲, 25°C.

non-linear dependence on the concentration of the complex (Fig. 1). A good fit to eqn. (4):

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

is obtained (computed curves are illustrated in Fig. 1), consistent with a mechanism involving association (K) before electron transfer (k_{et}), as in eqns. (2) and (3). At 25°C, $K = 2070 \pm 92\ \text{M}^{-1}$ and $k_{\text{et}} = 4.94 \pm 0.07\ \text{s}^{-1}$. From the temperature-dependence the following parameters relating to these constants were obtained: $\Delta H^0 = 19.8 \pm 1.1\ \text{kJ} \cdot \text{mol}^{-1}$ ($4.7 \pm 0.3\ \text{kcal} \cdot \text{mol}^{-1}$), $\Delta S^0 = 129.3 \pm 38.6\ \text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ($30.9 \pm 8.8\ \text{cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$), $\Delta H_{\text{et}}^\ddagger = 56.1 \pm 3.1\ \text{kJ} \cdot \text{mol}^{-1}$ ($13.4 \pm 0.73\ \text{kcal} \cdot \text{mol}^{-1}$), $\Delta S_{\text{et}}^\ddagger = -43.9 \pm 10.5\ \text{J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ($-10.5 \pm 2.5\ \text{cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$).

Limiting kinetics of the above kind are not observed in studies with $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$ as oxidants. Rate constants, k_{obs} (Table 1), exhibit first-order dependences on the concentration of Co(III) , i.e. the condition $1 \gg K[\text{Co(III)}]$ applies in eqn. (2). Second-order rate constants $k (=Kk_{\text{et}})$ at 25°C are $(4.05 \pm 0.07) \times 10^3\ \text{M}^{-1} \cdot \text{s}^{-1}$ for $\text{Co}(\text{edta})^-$ and $(3.88 \pm 0.06) \times 10^3\ \text{M}^{-1} \cdot \text{s}^{-1}$ for $\text{Co}(\text{acac})_3$. Individual values of K and k_{et} cannot be obtained

Table 1. First-order rate constants, k_{obs} , for the $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$ oxidations of reduced *Spirulina platensis* [2FE-2S] ferredoxin (approx. $10\ \mu\text{M}$) at 25°C at pH 8.0 (Tris/HCl) at 1.0 (NaCl)

Oxidant $\text{Co}(\text{edta})^-$							
[Co(III)] (mM)	...	0.23	0.41	0.81	1.60	2.40	
$k_{\text{obs.}}$ (s^{-1})	...	0.90	1.78	3.28	6.7	9.7	
Oxidant $\text{Co}(\text{acac})_3$							
[Co(III)] (mM)	...	0.10	0.126	0.166	0.25	0.50	0.96
$k_{\text{obs.}}$ (s^{-1})	...	0.35	0.56	0.70	0.93	1.93	3.70
							3.93

Table 2. Effect of redox-inactive $\text{Cr}(\text{NH}_3)_6^{3+}$ on rate constants, k_{obs} , for the Co(III) oxidations of reduced *Spirulina platensis* [2Fe-2S] ferredoxin (approx. $10\ \mu\text{M}$) at 25°C at pH 8.0 (Tris/HCl) at 1.0 (NaCl)

Oxidant $[\text{Co}(\text{NH}_3)_6^{3+}] = 0.2\ \text{mM}$								
[Cr(III)] (mM)	...	0	0.5	1.0	1.5	2.0	2.5	3.0
$k_{\text{obs.}}$ (s^{-1})	...	1.43	1.01	0.78	0.66	0.56	0.51	0.43
Oxidant $[\text{Co}(\text{edta})^-] = 0.4\ \text{mM}$								
[Cr(III)] (mM)	...	0	0.5	1.0	1.5	2.0	3.0	
$k_{\text{obs.}}$ (s^{-1})	...	1.62	2.55	3.04	3.41	3.60	3.85	
Oxidant $[\text{Co}(\text{acac})_3] = 0.5\ \text{mM}$								
[Cr(III)] (mM)	...	0	0.2	0.5	1.0	1.5	2.0	
$k_{\text{obs.}}$ (s^{-1})	...	1.94	1.89	1.85	1.65	1.58	1.49	
Oxidant $[\text{Co}(\text{acac})_3] = 0.92\ \text{mM}$								
[Cr(III)] (mM)	...	0	0.2	0.5	1.0	1.2	1.5	1.8
$k_{\text{obs.}}$ (s^{-1})	...	3.70	3.39	3.18	2.90	2.85	2.73	2.67
								2.59

(although they presumably still apply), and k corresponds to reaction as expressed by the overall stoichiometric eqn. (1).

Effect of $\text{Cr}(\text{NH}_3)_6^{3+}$ on $\text{Co}(\text{III})$ oxidations

Rate constants, $k_{\text{obs.}}$, obtained in the presence of redox-inactive $\text{Cr}(\text{NH}_3)_6^{3+}$ are listed in Table 2. The various effects are illustrated in Fig. 2. With $\text{Co}(\text{NH}_3)_6^{3+}$ a satisfactory fit is obtained to eqn. (5):

$$k_{\text{obs.}} = \frac{Kk_{\text{et}}[\text{Co}(\text{III})]}{1 + K[\text{Co}(\text{III})] + K_{\text{Cr}}[\text{Cr}(\text{III})]} \quad (5)$$

which can be derived by consideration of eqns. (2), (3) and (6):

From a plot of $1/k_{\text{obs.}}$ against $[\text{Cr}(\text{III})]$, with $K = 2070 \text{ M}^{-1}$ as determined above, $K_{\text{Cr}} = 1020 \pm 70 \text{ M}^{-1}$ at 25°C . With $\text{Co}(\text{edta})^-$ as oxidant the further path eqn. (7):

is included, when eqn. (8) can be derived:

$$\frac{k_{\text{obs.}}}{[\text{Co}(\text{III})]} (1 + K_{\text{Cr}}[\text{Cr}(\text{III})]) = k + k_{\text{Cr}}K_{\text{Cr}}[\text{Cr}(\text{III})] \quad (8)$$

A plot of the left-hand side of eqn. (8) against $[\text{Cr}(\text{III})]$ is linear with $K_{\text{Cr}} = 1020 \text{ M}^{-1}$ as determined for the $\text{Co}(\text{NH}_3)_6^{3+}$ reaction. This gives $k_{\text{Cr}} = (11.5 \pm 0.8) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 25°C , which is greater than k for the $\text{Co}(\text{edta})^-$ oxidation in the absence of $\text{Cr}(\text{NH}_3)_6^{3+}$. Alternatively from eqns. (1), (6) and (7) the expression (9) can be derived:

$$\frac{[\text{Co}(\text{III})]}{k_{\text{obs.}} - k_0} = \frac{1}{K_{\text{Cr}}(k_{\text{Cr}} - k)} \cdot \frac{1}{[\text{Cr}(\text{III})]} + \frac{1}{(k_{\text{Cr}} - k)} \quad (9)$$

and independent estimates of K_{Cr} (835 M^{-1}) and k_{Cr} ($11.9 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) obtained by plotting the left-hand side of eqn. (9) against $[\text{Cr}(\text{III})]^{-1}$ (k_0 is the rate constant at $[\text{Cr}(\text{III})] = 0$ in Table 2).

The effect of $\text{Cr}(\text{NH}_3)_6^{3+}$ on the $\text{Co}(\text{acac})_3$ oxidation is accounted for by the reaction scheme eqns. (1), (6) and (7). A fit of rate constants $k_{\text{obs.}}$ (Table 2) to eqn. (8), with $K_{\text{Cr}} = 1020 \text{ M}^{-1}$, gives $k_{\text{Cr}} = (2.34 \pm 0.08) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. Thus k_{Cr} is less than k ($3.88 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$) and partial inhibition is observed, a feature that has been established for three neutral oxidants in their reactions with parsley [2Fe-2S] ferredoxin (Adzamli *et al.*, 1982).

Fig. 2. Effect of $\text{Cr}(\text{NH}_3)_6^{3+}$ on first-order rate constants, $k_{\text{obs.}}$, for the $\text{Co}(\text{NH}_3)_6^{3+}$ (0.2 mM) (●), $\text{Co}(\text{edta})^-$ (0.4 mM) (■) and $\text{Co}(\text{acac})_3$ (0.5 mM) (▲) oxidation of reduced *Spirulina platensis* [2Fe-2S] ferredoxin (approx. $10 \mu\text{M}$) at 25°C at pH 8.0 (Tris/HCl) at 10.10 (NaCl)

Discussion

The reactivity of [2Fe-2S] ferredoxin isolated from *S. platensis* with three $\text{Co}(\text{III})$ oxidants, $\text{Co}(\text{NH}_3)_6^{3+}$, $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$, has been investigated. Limiting kinetics are observed with the tervalent complex, which is consistent with a mechanism in which association (K) occurs before electron transfer (k_{et}), as in eqns. (2) and (3). This is the simplest possible interpretation, which is supported by earlier studies in which the effect of ionic strength was noted (Armstrong & Sykes, 1978). Thermodynamic parameters for the association constant K (2070 M^{-1} at 25°C) were determined: ΔH° [$19.8 \text{ kJ} \cdot \text{mol}^{-1}$ ($4.73 \text{ kcal} \cdot \text{mol}^{-1}$)] and ΔS° [$129.3 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ($30.9 \text{ cal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$)]. These are somewhat less than values (Armstrong & Sykes, 1978) for the reaction of parsley ferredoxin with $\text{Co}(\text{NH}_3)_6^{3+}$: ΔH° [$42.7 \text{ kJ} \cdot \text{mol}^{-1}$ ($10.2 \text{ kcal} \cdot \text{mol}^{-1}$)] and ΔS° [$200.4 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$ ($47.9 \text{ kcal} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$)]. If desolvation consequent upon charge neutralization is the main contributing factor (Miralles *et al.*, 1977), as has been suggested, then the charge involvement is less for *S. platensis* ferredoxin. Limiting kinetics are also observed in a brief comparative study at 25°C with spinach [2Fe-2S] ferredoxin and $\text{Co}(\text{NH}_3)_6^{3+}$ as oxidant at pH 8.0 at 10.10 (NaCl) (Armstrong & Sykes, 1978). Values of K (933 M^{-1}) and k_{et} (15.9 s^{-1}) are very similar to those for parsley ferredoxin in Table 3. Limiting kinetics are not observed with $\text{Co}(\text{edta})^-$ and $\text{Co}(\text{acac})_3$ as oxidants

Table 3. Comparison of kinetic and thermodynamic parameters obtained for the Co(III) oxidations of reduced *Spirulina platensis* and parsley [2Fe-2S] ferredoxins at 25°C at pH 8.0 (Tris/HCl) at 1.0 (NaCl)

Complex	Parameter	<i>S. platensis</i> ferredoxin (present work)	Parsley ferredoxin
Co(NH ₃) ₆ ³⁺ (oxidant)	<i>k</i> (M ⁻¹ ·s ⁻¹)	1.01 × 10 ⁴	1.9 × 10 ⁴
	<i>K</i> (M ⁻¹)	2070	998
	Δ <i>H</i> ⁰ [kJ·mol ⁻¹ (kcal·mol ⁻¹)]	19.8 (4.7)	42.7 (10.2)
	Δ <i>S</i> ⁰ [J·K ⁻¹ ·mol ⁻¹ (cal·K ⁻¹ ·mol ⁻¹)]	129.3 (30.9)	200.4 (47.9)
	<i>k</i> _{et} (s ⁻¹)	4.9	19.2
	Δ <i>H</i> [kJ·mol ⁻¹ (kcal·mol ⁻¹)]	56.1 (13.4)	35.6 (8.5)
	Δ <i>S</i> [J·K ⁻¹ ·mol ⁻¹ (cal·K ⁻¹ ·mol ⁻¹)]	-43.9 (-10.5)	-100.8 (-24.1)
	<i>k</i> (M ⁻¹ ·s ⁻¹)	4.05 × 10 ³	7.2 × 10 ³
	<i>k</i> (M ⁻¹ ·s ⁻¹)	3.88 × 10 ³	4.1 × 10 ³
	<i>K</i> _{Cr} (M ⁻¹)	1020	468*
Co(edta) ⁻ (oxidant)			
Co(acac) ₃ (oxidant)			
Cr(NH ₃) ₆ ³⁺ (redox-inactive)			

* Average value.

for *S. platensis* ferredoxin, and second-order rate constants *k* show little variation (within a factor of 2) as compared with corresponding values for parsley ferredoxin (Table 3).

On addition of Cr(NH₃)₆³⁺ an inhibitory effect is observed with Co(NH₃)₆³⁺ as oxidant, and an acceleration with the univalent negative oxidant Co(edta)⁻. Partial inhibition only is observed with the neutral oxidant Co(acac)₃. These effects are identical with those previously reported (Armstrong *et al.*, 1979; Adzamli *et al.*, 1982) for parsley ferredoxin, where the simplest (possibly the only) interpretation is in terms of electrostatic effects resulting from the presence of associated Cr(NH₃)₆³⁺ at a single functional electron-transfer site on the protein. This argument has been presented in a recent paper (Adzamli *et al.*, 1982) and supersedes an earlier suggestion that a number of sites might be involved.

Previously the kinetics for the oxidation of parsley ferredoxin with some ten oxidants having charges from quinquevalent positive to tervalent negative have been presented. With the quinquevalent, quadrivalent, tervalent and (marginally) bivalent positive oxidants various degrees of limiting kinetics are observed, where Δ*H*⁰, Δ*S*⁰ and *K* for association exhibit systematic trends with increasing positive charge. Large overall negative charges are a feature of all the [2Fe-2S] ferredoxins (see below). It appears therefore that association of positively charged redox partners is favoured and that electrostatic considerations are a dominant contributing factor.

The case that has so far been argued does not distinguish between involvement of overall negative

charge on the protein and the charge of the functional site. When the two reactants are separated by a large distance, the overall charge is expected to be dominant. On closer approach to a contact distance, local charge effects are expected to take over and be more influential. The dependence of *K* on oxidant charge strongly suggests that the functional site carries a significant negative charge.

The amino acid sequences of some 27 [2Fe-2S] ferredoxins, all from higher plants with the exception that from *S. platensis*, have been compared (Matsubara *et al.*, 1980). There are 98 amino acid residues with two deletions possible at positions remote from the active site (10 and 17 for parsley ferredoxin). A comparison of the *S. platensis* ferredoxin and parsley ferredoxin sequences is given in Fig. 3. These two ferredoxins give 34 variations, as compared with 25 for the parsley and spinach ferredoxins. When all 27 known chloroplast ferredoxin sequences are considered, variations occur at 76 positions. Overall negative charge is strongly conserved, and from a count of amino acid residues that at neutral pH can be assigned negative (aspartate and glutamate) and positive (lysine, arginine and histidine) charges, and including the charge on the cluster, estimates of the charge are -19 for *S. platensis* ferredoxin, -18 for parsley ferredoxin and -17 for spinach ferredoxin. These should be regarded as maximum values, since such estimates may be modified by internal compensatory effects such as hydrogen-bonding. Surprisingly, only one of the negatively charged amino acid residues, the glutamate at position 94, is totally conserved throughout all 27 ferredoxins. Negative charge (glutamate or aspartate) is, however, con-

<i>S. platensis</i>	1 *	5	10	15	20
Parsley	Ala-Thr-Tyr-Lys-Val-Thr-Leu-Ile-Asn-Glu-Ala-Glu-Gly-Ile-Asn-Glu-Thr-Ile-Asp-Cys-*				
	Ala-Thr-Tyr-Asn-Val-Lys-Leu-Ile-Thr	—	Pro-Asp-Gly-Glu-Val-Glu	—	Phe-Lys-Cys-
<i>S. platensis</i>	25	30	35	40	*
Parsley	Asp-Asp-Asp-Thr-Tyr-Ile-Leu-Asp-Ala-Ala-Glu-Glu-Ala-Gly-Leu-Asp-Leu-Pro-Tyr-Ser-*				
	Asp-Asp-Val-Tyr-Val-Leu-Asp-Gln-Ala-Glu-Glu-Gly-Ile-Asp-Ile-Pro-Tyr-Ser-				
<i>S. platensis</i>	45	50	55	60	
Parsley	Cys-Arg-Ala-Gly-Ala-Cys-Ser-Thr-Cys-Ala-Gly-Thr-Ile-Thr-Ser-Gly-Thr-Ile-Asp-Gln-*				
	Cys-Arg-Ala-Gly-Ser-Cys-Ser-Ser-Cys-Ala-Gly-Lys-Val-Val-Ser-Gly-Ser-Ile-Asp-Gln- Δ	Δ			
<i>S. platensis</i>	65	70	75	80	
Parsley	Ser-Asp-Gln-Ser-Phe-Leu-Asp-Asp-Gln-Ile-Glu-Ala-Gly-Tyr-Val-Leu-Thr-Cys-Val-*				
	Ser-Asp-Gln-Ser-Phe-Leu-Asp-Asp-Glu-Gln-Met-Asp-Ala-Gly-Tyr-Val-Leu-Thr-Cys-His- Δ				
<i>S. platensis</i>	85	90	95		
Parsley	Ala-Tyr-Pro-Thr-Ser-Asp-Cys-Thr-Ile-Lys-Thr-His-Gln-Glu-Gly-Leu-Tyr*				
	Ala-Tyr-Pro-Thr-Ser-Asp-Val-Val-Ile-Glu-Thr-His-Lys-Glu-Glu-Ile-Val				

Fig. 3. Comparison of amino acid sequences for *Spirulina platensis* and parsley [2Fe-2S]ferredoxins (Matsubara *et al.*, 1980; Nakano *et al.*, 1981). Invariant amino acid residues for the 27 sequenced ferredoxins are indicated (*). Co-ordinated cluster cysteine amino acids are also indicated (Δ).

Fig. 4. Structure of *Spirulina platensis* ferredoxin as determined by Matsubara *et al.* (1980)

Fe atoms (●), S^{2-} (○) and cysteinyl S atoms (◐) are indicated. Amino acid 94 is a totally conserved glutamate residue (negative charge). In addition, negative charge (glutamate or aspartate) is conserved at positions 67 and 68, and partially conserved at positions 69, 95 and 96.

served at positions 32 (which is remote from the active-site cluster), 67 and 68. It is also fairly extensively conserved at positions 69 and 95 (conservation is observed in the three ferredoxins relating to this discussion), and for parsley and spinach ferredoxins at position 96. Insofar as conservation is expected to be related to specific functionality, the two negative patches at positions 67–69 and 94–96 may be singled out as being at (or close to) possible functional sites, with the negative charge serving as a recognition site that is influential in the association process preceding electron transfer. The positions of relevant amino acid residues are indicated in Fig. 4, where both sites are within approx. 1.0 nm (10 Å) range of an Fe atom of the $[2Fe-2S]$ active site. In addition, the sulphur atoms of cysteine-41 and cysteine-46, which are co-ordinated to the $[2Fe-2S]$ core, are exposed to solvent (Tsukihara *et al.*, 1981). Since the cluster carries an overall negative charge (which is conserved), this represents a third possible functional site for electron transfer.

Questions that remain are how to distinguish between these three different possibilities and how to establish whether experiments with inorganic complexes (although interesting in themselves) have any bearing on the electron-transfer process *in vivo* involving physiological redox partners. Although we remain cautious as to the full meaning of ΔH^0 and ΔS^0 parameters corresponding to the association step K , it is of interest to compare the significantly different values for *S. platensis* and parsley ferredoxins in Table 3. These differences could be an indication that amino acid residues 94–96 constitute the functional site, since the charges arising from these amino acid residues are 2– and 3– respectively (Fig. 3). This follows, since desolvation attendant on charge neutralization is expected to be more extensive and ΔH^0 and ΔS^0 are expected to be bigger in the latter case. The present study confirms that redox-inactive $Cr(NH_3)_6^{3+}$ can be used to monitor utilization of a specific electron-transfer site used by Co(III) inorganic oxidants. From reactivity patterns observed with and without addition of

$\text{Cr}(\text{NH}_3)_6^{3+}$, it should be possible to comment whether physiological partners of [2Fe-2S] ferredoxins use this same site.

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