participate in some way and provide a facile rate for complexation.

The acid dissociation pK_a values obtained from spectrophotometric changes for $CrN_4(H_2O)_2^{3+}$ (2.9), $CrN_4(H_2O)(OH)^{2+}$ (7.8) and $CrN_4(H_2O)(NCS)^{2+}$ (4.6) are acceptable in terms of known Cr(III) chemistry. However, it should be noted that the pK_{a1} value of 2.9 for $CrN_4(H_2O)_2^{3+}$ is small compared to values 5.2, 4.1, 4.8, and 6.0 quoted for the complexes $Cr(NH_3)_5H_2O^{3+}$,[14] *cis*- and *trans*-$Cr(en)_2(H_2O)_2^{3+}$,[15] and $Cr(en)(NCS)_2(H_2O)_2^{+}$,[16] respectively, and that there is an unusually big difference in pK_{a2} and pK_{aT} (4.6) for the 2+-charged complexes. The agreement of pK_{a2} determined kinetically (8.0) and spectrophotometrically (7.8) for $CrN_4(H_2O)(OH)^{2+}$ is encouraging. Information from $[H^+]$ dependences as in (11), and relating to Figure 6, indicate that $CrN_4(H_2O)_2^{3+}$ reacts 10^2 times more slowly than $CrN_4(H_2O)OH^{2+}$ and that $CrN_4(OH)_2^{+}$ makes little or no contribution. The latter is consistent with it being difficult to replace a negative ligand (OH^-) by another negative ligand (NCS^-).[17] We do not however understand fully the behavior of $CrN_4(H_2O)(OH)^{2+}$ and its reactivity with NCS^-. Had there been a similar correspondence of rate constants with spectrophotometric observations to give a pK_{a1} of 2.9 then the rate constant profile (Figure 6) would have looked somewhat different, with no increase in k_{obsd} over the range pH 5-7. Even without buffers (which have presented some difficulties), it has been demonstrated that k_{obsd} from the initial absorbance changes, with the initial pH adjusted to 5.5, is far greater than the values of k_{obsd} determined at pH 3-4. Since buffer effects have been minimized in all the runs reported, it is necessary to account for the variation in k_{obsd} at pHs in the range 5-7.

The view that configurational and/or cis isomers are relevant is supported by the observation that a nonlinear dependence on NCS^- is observed at pH 6.3 and 7.65 (but not at pH <5.3). There are a number of possible explanations for kinetic behavior of this kind. The possibility that ion-pair formation is relevant seems unlikely since the value $K_{IP} = 7.1\ M^{-1}$ required by the kinetics is not in agreement with the spectrophotometrically determined value of $0.7\ M^{-1}$ (which is perfectly reasonable in terms of electrostatic models for a 2+,1- interaction).[18] An interpretation in terms of (9)-(10), where C* represents a more reactive form resulting from an isomerization process ($k_1 = 1.17 \times 10^{-4}\ s^{-1}$), seems more likely. A further possibility, that C* is a 5-coordinate complex, seems unlikely in terms of known Cr(III) chemistry.

The orientation of N-H groups or participation of cis geometric isomers is expected to have an effect on rate constants, since metal-to-ligand bond distances as well as pK_a values will be affected. Of further interest is the observation that at pHs 1.0-3.0, $CrN_4(H_2O)(NCS)^{2+}$ does not appear to react with a second thiocyanate. Indeed, over 1 week, the only changes observed are the formation of crystals of $[Cr(N_4)(H_2O)(NCS)](NCS)_2$; in addition we did not observe aquation of thiocyanate in $CrN_4(NCS)H_2O^{2+}$ and $CrN_4(NCS)OH^+$.

Details of the crystal structure will be published elsewhere.[19]

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Registry No. $CrN_4(H_2O)_2^{3+}$, 70832-95-0; $CrN_4(H_2O)(OH)^{2+}$, 91208-72-9; $CrN_4(H_2O)(NCS)^{2+}$, 91202-09-4; CNS^-, 302-04-5.

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Kinetic Studies on Reactions of Iron-Sulfur Proteins. 8. Inner-Sphere Reductions of Parsley [2Fe-2S] and *Clostridium pasteurianum* 2[4Fe-4S] Ferredoxins with a Chromium(II)-Macrocycle Complex

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Stopped-flow kinetic studies have been carried out on the reduction of parsley [2Fe-2S] and *Clostridium pasteurianum* 2[4Fe-4S] ferredoxins with the 1,4,8,12-tetraazacyclopentadecane complex of Cr(II), $Cr(15\text{-ane}N_4)^{2+}$, $I = 0.10$ M (NaCl). The [2Fe-2S] protein is one-electron active, and the two one-electron-active clusters in the 2[4Fe-4S] protein react in an equivalent (statistical) manner. Product analyses have demonstrated that the Cr(III) is attached to the protein and that the reactions fall within the definition of inner-sphere electron transfer. Both reactions exhibit pH effects in the 7.0-8.0 region, the proteins becoming more reactive on protonation. The more detailed study with 2[4Fe-4S] gives a pK_a of 7.46 for the protein in its oxidized form, in accordance with a previously reported value of 7.4 from potentiometric studies. Rate constants (25 °C) and activation parameters at pH 8.0 for 2[Fe-2S] are $k = 1.03 \times 10^3\ M^{-1}\ s^{-1}$, $\Delta H^* = 4.7$ kcal mol^{-1}, and $\Delta S^* = -29.4$ cal K^{-1} mol^{-1} and for 2[4Fe-4S] are $k = 6.9 \times 10^3\ M^{-1}\ s^{-1}$, $\Delta H^* = 3.2$ kcal mol^{-1}, and $\Delta S^* = -31.0$ cal K^{-1} mol^{-1}. When the Cr(III) is bound to the reduced protein, it has little or no effect on rate constants for the $[Co(NH_3)_6]^{3+}$ oxidation. For both proteins, addition of redox-inactive $[Cr(en)_3]^{3+}$ does not give competitive inhibition with $Cr(15\text{-ane}N_4)^{2+}$ as reductant. Instead, an acceleration is observed with the [2Fe-2S] protein and no effect with the 2[4Fe-4S] protein. The implications of these results with regard to binding site(s) on the protein are considered. Electrostatics do not appear to have the same controlling influence in reactions of $Cr(15\text{-ane}N_4)^{2+}$ with the [2Fe-2S] and 2[4Fe-4S] proteins as in previous studies.

Introduction

Inorganic complexes are used extensively as probes for assessing the reactivity of electron-transport metalloproteins. Of interest, but at present little explored, is the possibility that inner-sphere electron transfer can be demonstrated for reactions of this kind. If the inorganic product is substitution inert so that it remains attached to the protein, this can also help define more precisely the lead-in group (or groups) for electron

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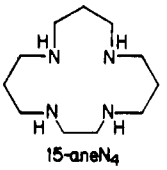
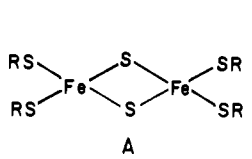
transfer and, hence, the distance over which electrons are transferred. Since Cr(III) is normally inert, the labile reductant Cr(II) meets this requirement. The use of hexaaquachromium(II) has met with some success,[1,2] but there remains some uncertainty regarding the substitution inertness of the aquachromium(III) at pH >5 (due to the presence of conjugate-base forms) and whether the Cr will remain firmly attached to one point on the protein. In this study we have chosen therefore to work with the Cr(II) complex of the saturated macrocyclic ligand 1,4,8,12-tetraazacyclopentadecane



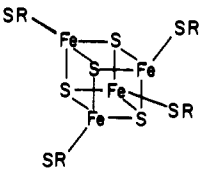
15-aneN$_4$

which is assumed to be present as the diaqua complex $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$. Thiocyanate anation studies on the Cr(III) complex over a wide range of pH 1–9 suggest that the Cr(III) coordination sphere remains inert.[3]

The active sites of the [2Fe-2S] and 2[4Fe-4S] proteins from parsley and *Clostridium pasteurianum*, respectively, are shown in A and B, where SR represents a coordinated cysteine residue. Product analyses[4] have demonstrated that with $Cr(15\text{-}aneN_4)^{2+}$ as reductant the Cr(III) remains attached to both proteins and that an inner-sphere mechanism can be assigned.[5] In the corresponding reductions of high-potential [4Fe-4S] protein from *Chromatium vinosum*, and the single Fe protein rubredoxin from *Clostridium pasteurianum*, the Cr(III) product is not found attached to the protein, and an outer-sphere mechanism seems likely.[6]



A



B

Kinetic results reported here are for the $Cr(15\text{-}aneN_4)^{2+}$ reductions of the [2Fe-2S] protein (1) and the 2[4Fe-4S]

$$Cr^{II} + [2Fe\text{-}2S](o) \rightarrow ([2Fe\text{-}2S](r))\cdot Cr^{III} \quad (1)$$

protein. In the latter there are two [4Fe-4S] clusters, each of which is reduced according to (2). Statistical kinetics are

$$Cr^{II} + [4Fe\text{-}4S](o) \rightarrow ([4Fe\text{-}4S](r))\cdot Cr^{III} \quad (2)$$

relevant.[10] Related studies including the effect on rates of redox-inactive $[Cr(en)_3]^{3+}$ and the effect of attached Cr(III)–macrocycle complex on the reaction with $[Co(NH_3)_6]^{3+}$ are considered.

Experimental Section

Isolation of Ferredoxins. Parsley [2Fe-2S] ferredoxin was isolated from fresh leaves as previously described by the method of Plesničar and Bendall.[11] The final column for purification was made up from DE52 resin (20 cm by 1 cm in diameter) instead of DE23. Protein of absorbance peak ratios $A_{422}/A_{277} \geq 0.60$ was used for kinetic experiments. The 2[4Fe-4S] ferredoxin was isolated from *Clostridium pasteurianum* (Microbiological Products) as previously described by using the procedure of Thompson et al.[13] Minor modifications included introduction of a N_2 atmosphere whenever possible during the preparation, and use of a Whatman DE52 anion-exchange resin instead of DE23 in the final stages of purification. Protein with an absorbance peak ratio A_{390}/A_{285} equal to or greater than 0.79 was used for kinetic runs. UV–visible spectra of oxidized and reduced forms of both proteins have been illustrated.[10,12] Samples of rubredoxin from *Clostridium pasteurianum* and high-potential Fe/S protein from *Chromatium* were obtained as previously described.[4]

Complexes. A sample of chromium(II) chloride, $CrCl_2\cdot 4H_2O$, was prepared anaerobically from chromium metal (Johnson and Matthey, Specpure) by a literature method.[14] Required amounts of the saturated macrocycle ligand 1,4,8,12-tetraazacyclopentadecane (Strem Chemicals), 15-aneN$_4$, were added to $CrCl_2\cdot 4H_2O$ in Tris/HCl buffer,[15] at an ionic strength I = 0.10 M (NaCl). Over a wide range of pHs the complex $[Cr^{II}(15\text{-}aneN_4)(H_2O)_2]^{2+}$, hereafter $Cr(15\text{-}aneN_4)^{2+}$ (reduction potential for the III/II couple –0.58 V),[15] gives a UV–visible absorbance peak at λ = 540 nm (ϵ = 36.5 M^{-1} cm^{-1}), determined by chromate(VI) analysis (a previous value of 28.7 M^{-1} cm^{-1} was in 1:1 aqueous *tert*-butyl alcohol[15]). The higher ϵ was confirmed by air oxidation (pH adjusted to 1.5) to the diaquachromium(III)–macrocycle complex, the spectrum of which gives peaks at 377 (88) and 454 nm (87) as previously reported.[15] Rigorous air-free conditions were required in handling all Cr(II) solutions. Samples of tris(ethylenediamine)chromium(III), $[Cr(en)_3]Cl_3\cdot 3H_2O$,[16] peak positions ($\lambda$/nm ($\epsilon$/$M^{-1}$ cm^{-1})) at 351 (63) and 457 (73), and hexaamminecobalt(III), $[Co(NH_3)_6]Cl_3$,[12] 339 (46.4) and 473 (57.1), were prepared by procedures indicated.

Buffers. Solutions of tris(hydroxymethyl)methylamine (Trizma), here referred to as Tris (Sigma Chemicals), in 0.010 M HCl were used as buffer over the range pH 7–9. As already indicated[12] the protonation constant for Tris varies significantly with temperature, and the influence on pH has to be taken into account as the temperature is varied. A Radiometer PHM62 pH meter was used to check prepared pHs. The ionic strength of all solutions was adjusted to I = 0.100 ± 0.003 M with NaCl.

Reaction Product. Details of product analysis including extrusion and EPR experiments have been indicated in an earlier communication.[4] Protein held at the top of chromatographic columns is believed to be denatured product.[4]

Kinetics. The reductant was in large >10-fold excess of the protein (~2 × 10^{-5} M). On reduction, the [2Fe-2S] cluster changes from the Fe^{III}_2 to the $Fe^{II}Fe^{III}$ state (–0.42 V), which can be formulated alternatively as a change $[Fe_2S_2(SR)_4]^{2-}$ to $[Fe_2S_2(SR)_4]^{3-}$. Each [4Fe-4S] cluster (ca. –0.40 V) likewise undergoes a one-electron change from an average Fe oxidation state of 2.5 to 2.25, which can be formulated as the change $[Fe_4S_4(SR)_4]^{2-}$ to $[Fe_4S_4(SR)_4]^{3-}$. Absorbance changes were monitored on a Dionex D-110 stopped-flow spectrophotometer equipped with logarithmic amplifier, and the output was stored digitally on a Datalab DL901 transient recorder. Traces were displayed on a oscilloscope using the digital analogue facility of the transient recorder. A Commodore PET 2001-16K computer was interfaced to the recorder, and a simple program was used to display absorbance changes, calculated as ln ($A_\infty - A_t$) against time graphs. Such plots were linear to 3–4 half-lives. First-order rate constants k_{obsd} were obtained from the slopes. For runs using [2Fe-2S]

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(6) Examples of Cr(III) complexes having labile ligands are known, e.g. the Cr(III)–aldehyde linkage generated in the Cr^{2+} reduction of the (*p*-formylbenzoato)pentaamminecobalt(III) complex[7] and the H_2O in $[Cr(edta)(H_2O)]^-$ (edta = ethylenediaminetetraacetate).[8,9] Although an inner-sphere mechanism is unlikely in these two instances, it cannot be ruled out entirely.
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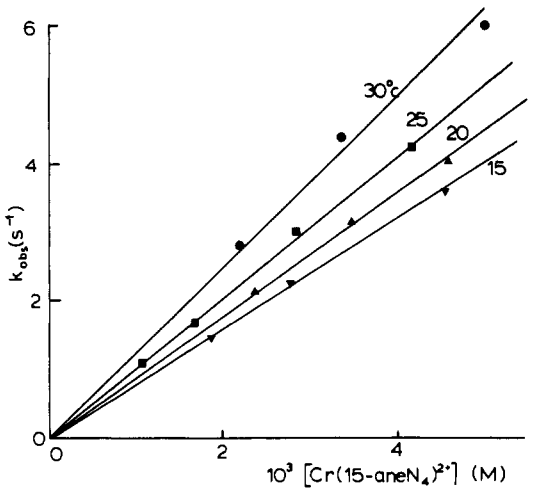

Figure 1. Linear dependence of first-order rate constants, k_{obsd}, on concentration of reductant for the Cr(15-aneN$_4$)$^{2+}$ reduction of parsley [2Fe-2S] ferredoxin (2×10^{-5} M; I = 0.10 M (NaCl)).

Table I. Effect of pH (Tris/HCl) on Rate Constants, k (25 °C), for the Cr(15-aneN$_4$)$^{2+}$ Reduction of [2Fe-2S] Ferredoxin, I = 0.10 M (NaCl)

pH	$10^3 \times$ [Cr(II)], M	$10^{-3}k$, M^{-1} s^{-1}	pH	$10^3 \times$ [Cr(II)], M	$10^{-3}k$, M^{-1} s^{-1}
7.2	1.37	1.33	8.0	1.06	1.04
	2.04	1.42		1.67	1.02
	3.03	1.26		2.8	1.07
	5.11	1.17		4.2	1.02

Table II. Dependence of First-Order Rate Constants, k_{obsd}, for the Cr(15-aneN$_4$)$^{2+}$ Reduction of [2Fe-2S] Ferredoxin on Temperature at pH 8.0 (Tris/HCl), I = 0.10 M (NaCl)

T, °C	$10^3 \times$ [Cr(II)], M	k_{obsd}, s^{-1}	T, °C	$10^3 \times$ [Cr(II)], M	k_{obsd}, s^{-1}
15.0	1.87	1.48	25.0	1.06	1.10
	2.7	2.3		1.67	1.70
	4.6	3.6		2.8	3.0
20.0	2.4	2.14		4.2	4.3
	3.5	3.2	30.0	2.2	2.8
	4.6	4.1		3.38	4.4
				5.0	6.0

with the Cr(III)-macrocycle complex attached, the [2Fe-2S] protein was first reduced with a 1.5-fold excess of Cr(15-aneN$_4$)$^{2+}$. The product was air oxidized and separated on a DE52 column. It was then reduced with dithionite as in previous studies.[12] The ratio Cr:Fe was confirmed as 1:1 by atomic absorption spectroscopy. Attachment of Cr produced no significant effect on the protein spectrum.

Treatment of Data. Nonlinear and linear least-squares programs (unit weighting) were used as appropriate. Rate constants for the 2[4Fe-4S] protein correspond to the second stage of reduction in a statistical kinetic situation.[10] These have to be multiplied by 2 to obtain rate constants for the first stage.

Results

All first-order rate constants, k_{obsd}, conformed to (3), with

$$k_{obsd} = k[\text{Cr(15-aneN}_4)^{2+}] \quad (3)$$

Cr(II) in large excess and as high as 5.1×10^{-3} M for the [2Fe-2S] reaction (Figure 1) and 3.9×10^{-3} M for the 2-[4Fe-4S] reaction. From studies on [2Fe-2S] at two pHs (Table I) it can be concluded that second-order rate constants k exhibit a small dependence on pH. Because of the smallness of the effect, and the need to change buffers to investigate at lower pHs, this was not explored further. From the temperature dependence at pH 8.0 (Table II), $k = 1.03 \times 10^3$ M^{-1} s^{-1} (25 °C), $\Delta H^{\ddagger} = 4.7 \pm 0.3$ kcal mol^{-1}, and $\Delta S^{\ddagger} = -29.0 \pm 1.1$ cal K^{-1} mol^{-1}. The linear dependence of k_{obsd} on [Cr(II)] for the 2[4Fe-4S] reaction at pH 8.0 (Table III) is illustrated

Table III. Effect of pH (Tris/HCl) on First-Order Rate Constants, k_{obsd} (25 °C), for the Cr(15-aneN$_4$)$^{2+}$ Reduction of 2[4Fe-4S] Ferredoxin, I = 0.10 M (NaCl)

pH	$10^3 \times$ [Cr(II)], M	k_{obsd},[a] s^{-1}	pH	$10^3 \times$ [Cr(II)], M	k_{obsd},[a] s^{-1}
7.0	1.59	15.4	8.2	1.19	7.7
	2.9	27.6		3.9	22.9
7.5	1.38	11.6	8.9	1.61	9.4
	3.3	28.3		0.62	3.7
7.8	0.80	5.6		1.23	6.7
	2.20	16.7		1.16	7.0
8.0	0.64	4.6		1.57	9.6
	3.9	25.2		2.8	15.6

[a] Rate constants listed are for the second stage of reduction in a situation where statistical kinetics apply. Multiply by 2 to obtain rate constants for the first stage.

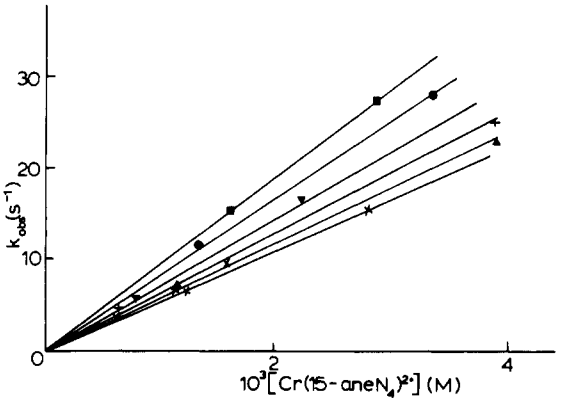

Figure 2. Linear dependence of first-order rate constants, k_{obsd} (25 °C), on concentration of reductant for the Cr(15-aneN$_4$)$^{2+}$ reduction of *Clostridium pasteurianum* 2[4Fe-4S] ferredoxin (2×10^{-5} M; I = 0.10 M (NaCl)): pH 7.0 (—); 7.5 (●); 7.8 (▼); 8.0 (+); 8.2 (▲); 9.0 (×).

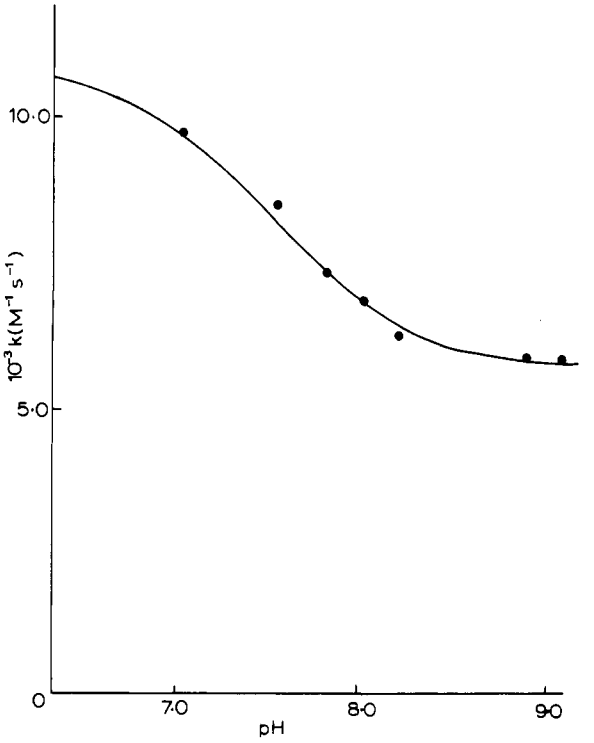

Figure 3. Variation of second-order rate constants, k (25 °C), with pH for the Cr(15-aneN$_4$)$^{2+}$ reduction of *Clostridium pasteurianum* 2[4Fe-4S] ferredoxin (I = 0.10 M (NaCl)).

Table IV. Temperature Dependence of First-Order Rate Constants for the $Cr(15\text{-}aneN_4)^{2+}$ Reduction of 2[4Fe-4S] Ferredoxin at pH 8.0 (Tris/HCl), $I = 0.10$ M (NaCl)

T, °C	$10^3 \times$ [Cr(II)], M	k_{obsd},[a] s^{-1}	T, °C	$10^3 \times$ [Cr(II)], M	k_{obsd},[a] s^{-1}
7.0	0.45	0.81	25.0	0.38	1.59
	0.85	2.60		0.78	4.1
	1.70	5.1		1.72	7.5
	3.25	9.6		1.95	9.3
15.0	0.95	3.4		2.73	12.2
	1.70	6.1		3.80	17.9
	2.50	9.2			
	3.60	13.1			

[a] Rate constants listed are for the second stage of a reduction in a situation where statistical kinetics apply. Multiply by 2 to obtain rate constants for the first stage.

Table V. Effect of Redox-Inactive $[Cr(en)_3]^{3+}$ on First-Order Rate Constants at 25 °C for the $Cr(15\text{-}aneN_4)^{2+}$ (2.8×10^{-3} M) Reduction of [2Fe-2S] Ferredoxin at pH 8.0 (Tris/HCl), $I = 0.10$ M (NaCl)

$10^3 \times$ $[Cr(en)_3^{3+}]$, M	k_{obsd}, s^{-1}	$10^3 \times$ $[Cr(en)_3^{3+}]$, M	k_{obsd}, s^{-1}
0	2.28	0.61	2.73
0.35	2.53	1.03	2.87
0.52	2.70	1.53	3.07

in Figure 2, and the dependence of k on pH, in Figure 3. Equation 4 can be derived for the reaction sequence (5)–(7),

$$k = k_1 + (k_2 - k_1)K_a/(K_a + [H^+]) \quad (4)$$

$$H^+P \overset{K_a}{\rightleftharpoons} H^+ + P \quad (5)$$

$$H^+P + Cr(II) \xrightarrow{k_1} \quad (6)$$

$$P + Cr(II) \xrightarrow{k_2} \quad (7)$$

where P designates the protein. A pK_a value of 7.46 ± 0.1 is obtained with $k_1 = (11.2 \pm 0.6) \times 10^3$ M^{-1} s^{-1} and $k_2 = (5.6 \pm 0.2) \times 10^3$ M^{-1} s^{-1} at 25 °C. The rate constant k_2 applies at pH 8.0. From the temperature dependence (Table IV), activation parameters for k_2 are $\Delta H^{\ddagger} = 3.2 \pm 0.2$ kcal mol^{-1} and $\Delta S^{\ddagger} = -31.0 \pm 0.7$ cal K^{-1} mol^{-1}.

Rate constants for the outer-sphere $Cr(15\text{-}aneN_4)^{2+}$ reductions of rubredoxin and Hipip were too fast to monitor ($k > 10^5$ M^{-1} s^{-1} at 25 °C, pH 7.5).

Rate constants (Table V) for the $Cr(15\text{-}aneN_4)^{2+}$ reduction of the [2Fe-2S] protein increase with increasing amounts of redox-inactive $[Cr(en)_3]^{3+}$ (Figure 4). No effect is observed for the 2[4Fe-4S] protein. For the reaction sequence (8)–(10),

$$P + Cr(en)_3^{3+} \overset{K}{\rightleftharpoons} P, [Cr(en)_3]^{3+} \quad (8)$$

$$P + Cr(15\text{-}aneN_4)^{2+} \xrightarrow{k_2} \quad (9)$$

$$P, Cr(en)_3^{3+} + Cr(15\text{-}aneN_4)^{2+} \xrightarrow{k_{Cr}} \quad (10)$$

an expression (11) is obtained. Experimental k values give

$$k = (k_2 + k_{Cr}K[Cr(en)_3^{3+}])/(1 + K[Cr(en)_3^{3+}]) \quad (11)$$

a satisfactory fit with $K = 610 \pm 165$ M^{-1} and $k_{Cr} = (1.39 \pm 0.10) \times 10^3$ M^{-1} s^{-1}. A value $K = 590$ M^{-1} has been reported previously.[17]

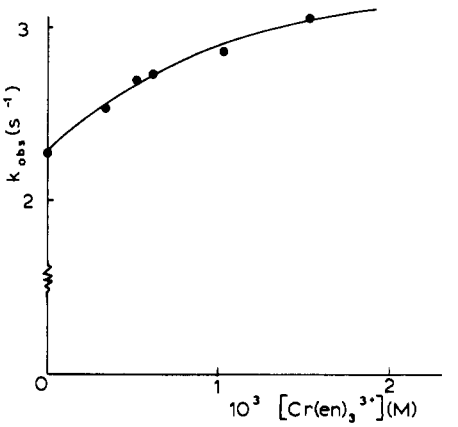

Figure 4. Effect of redox-inactive $[Cr(en)_3]^{3+}$ on first-order rate constants (25 °C) for the $Cr(15\text{-}aneN_4)^{2+}$ (2.8×10^{-3} M) reduction of parsley 2[Fe-2S] ferredoxin (pH 8.0; $I = 0.10$ M (NaCl)).

Table VI. Summary of Rate Constants at 25 °C and Activation Parameters for the Reduction of Fe/S Proteins, $I = 0.10$ M (NaCl)

protein[a]	reductant	k, M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal K^{-1} mol^{-1}	ref
[2Fe-2S]	$Cr(15\text{-}aneN_4)^{2+}$ [b]	1.03×10^3	4.7	−29.0	this work
2[4Fe-4S]	$Cr(15\text{-}aneN_4)^{2+}$ [b]	6.9×10^3	3.2	−31.1	this work
rubredoxin	$[Ru(NH_3)_6]^{2+}$ [c]	9.5×10^4	1.4	~−31	26
rubredoxin	$[V(H_2O)_6]^{2+}$ [d]	1.6×10^4	0.1	−40	26
rubredoxin	$[Cr(H_2O)_6]^{2+}$ [e]	1.2×10^3	~0	~−44	26

[a] Protein in oxidized form. [b] pH 8.0. [c] pH 6.3–7.0. [d] pH 3.5–4.5. [e] pH 3.5–4.0.

Finally, rate constants for the $[Co(NH_3)_6]^{3+}$ oxidation of the [2Fe-2S] protein with the Cr(III)–macrocycle complex attached have been determined. For a range of $[Co(NH_3)_6]^{3+}$ concentrations up to 3.7×10^{-3} M, first-order rate constants were 4–13% less than values for the [2Fe-2S] protein with no Cr(III) attached. This is not regarded as a significant difference bearing in mind that the protein has been recycled.

Discussion

It has been demonstrated that the $Cr(15\text{-}aneN_4)^{2+}$ reduction of [2Fe-2S] and 2[4Fe-4S] ferredoxins gives products in which the Cr(III) is attached to the protein.[4] Since a mixture of unattached Cr(III) complex and protein can be separated by using column chromatography, it is concluded that the Cr(III) becomes attached at the time of electron transfer. In the case of the [2Fe-2S] ferredoxin the Fe:Cr ratio has been shown[4] to be close to the 2:1 ratio required by (1).

Rate constants and activation parameters for the $Cr(15\text{-}aneN_4)_2^+$ reduction of the [2Fe-2S] and 2[4Fe-4S] proteins (Table VI) are of similar magnitude. The thermodynamic driving force is virtually the same (E° values ~ −0.4 V) for both proteins. Fast outer-sphere $Cr(15\text{-}aneN_4)^{2+}$ reduction of rubredoxin ($E^{\circ} = -0.06$ V)[20] and Hipip ($E^{\circ} = 0.35$ V)[21] is observed. Both reactions have a bigger thermodynamic driving force. However, there are in addition structural features, namely the hydrophobic nature of part of the surface of rubredoxin and the buried nature of the Hipip cluster, that may preclude inner-sphere electron transfer. Previously Jacks et al.[22] have concluded that the $[Cr(H_2O)_6]^{2+}$, $[V(H_2O)_6]^{2+}$,

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and $[Ru(NH_3)_6]^{2+}$ reductions of rubredoxin are outer sphere.

Active sites of both proteins, A and B respectively, are near to the surface, with cysteinyl sulfurs exposed to solvent in each case.[18,19] Extrusion experiments[4] have indicated that the Cr(III) of the chromatographed product is not attached to the sulfides of the [2Fe-2S] core. Also, from EPR it is unlikely that the Cr is bound to the cysteinyl sulfurs. The ~13% of [2Fe-2S] and ~40% of 2[4Fe-4S] protein product that is held at the top of chromatograph columns is believed to be denatured protein. Formation of this product can be accounted for by the protein, with Cr(III) attached, being more sensitive to damage on air oxidation, or by the Cr being attached in a different manner (possibly at the protein active site).

From studies with positively charged ammine oxidants of charge up to and including 5+, which react by an outer-sphere mechanism, it has been concluded that negative patches at 67–69 and 94–96 constitute possible binding sites.[23] From the magnitude of association constants, a binding site of charge 3– is indicated.[24] Markley and colleagues[25] have examined NMR line-broadening effects of redox-inactive $[Cr(NH_3)_6]^{3+}$ and concluded that association is at a more distant site incorporating Asp22, Asp23, Glu24, and Asp63 (numbering as in ref 25). These three sites remain in contention for reaction with the ammine complexes. In the present studies we had hoped to ascertain the point of attachment of the Cr. This has proved difficult by using amino acid sequencing methods and, in the carboxymethylation and cyanogen bromide procedures, reagents used tend to liberate the Cr. Also it has recently been demonstrated[26] that the [2Fe-2S] protein isolation procedure does not discriminate between ferredoxins I and II. No modified procedure is at present available to separate these two components. The two forms, while conserving important features (such as the charged centers indicated above), do have 25 out of 97 residues different,[26] making determination of the point of attachment of Cr by sequencing methods more difficult. Other approaches including NMR are also correspondingly more complicated. For the present therefore we have no means of determining precisely where the Cr is bound.

Whichever binding site is relevant with $[Co(NH_3)_6]^{3+}$ as redox partner,[12,17] it was of interest to investigate whether the same site was involved with $Cr(15\text{-}aneN_4)^{2+}$. Redox-inactive $[Cr(en)_3]^{3+}$ (K = 590 M^{-1}) gives competitive inhibition in the $[Co(NH_3)_6]^{3+}$ oxidation of the reduced [2Fe-2S] protein[17] but accelerates the reaction with $[Co(edta)]^-$. Effects observed are consistent with electrostatics being dominant, and interpretation in terms of a single binding site is possible.[27] With $Cr(15\text{-}aneN_4)^{2+}$ as reductant, $[Cr(en)_3]^{3+}$ brings about an acceleration that is not compatible with charge considerations. However, no change in the UV–visible spectrum of $Cr(15\text{-}aneN_4)^{2+}$ is observed at pHs in the range 7–9, and it is unlikely that a conjugate-base form of the complex is relevant. Experiments in which the reduced [2Fe-2S] protein with Cr(III) attached is oxidized with $[Co(NH_3)_6]^{3+}$ indicate little or no effect of the Cr(III). We note also that $[Cr(en)_3]^{3+}$ (4×10^{-3} M) does not inhibit the $Cr(15\text{-}aneN_4)^{2+}$ reduction of the 2-[4Fe-4S] protein. If the site at which $[Cr(en)_3]^{3+}$ associates (K = 318 M^{-1}) was being used,[27] a 55% inhibition would have been expected. Therefore, we conclude that $Cr(15\text{-}aneN_4)^{2+}$ reacts with both proteins at a different site to that used by the ammine oxidants. In the case of the [2Fe-2S] protein, the presence of $[Cr(en)_3]^{3+}$ increases the redox reactivity of this site.

The effect of H^+ on the kinetics of the reduction of the 2[4Fe-4S] protein has been examined in this study, and a pK_a of 7.46, obtained. This is in excellent agreement with the value of 7.4 reported by Sweeney and colleagues[28] for the oxidized protein from potentiometric measurements. From a less extensive study on the [2Fe-2S] protein at pHs 7.2 and 8.0 a similar sort of dependence appears to exist here also. Porras and Palmer[29] have concluded from potentiometric studies on xanthine oxidase that the [2Fe-2S] ferredoxin I and II components in the oxidized form have pK_a's of 6.4. These observations are further supported by the pH dependence of potentiometric measurements that Fee et al. have reported for parsley ferredoxin.[30]

The observations that rate constants for the reduction of the [2Fe-2S] and 2[4Fe-4S] proteins increase on decreasing the pH below 8.0 is not consistent with the simple electrostatic effect which protonation of a negatively charged protein would be expected to have on the reaction with a 2+ complex. Protonation at the protein binding site is therefore unlikely. This leaves protonation at the active site, or an H^+-induced conformation change effective at the active site, as possible explanations. Sweeney and colleagues[28] could find no evidence for a pH-dependent conformational change for the 2[4Fe-4S] ferredoxin from NMR, EPR, and CD spectroscopic studies. Job and Bruice[31] have moreover reported a pK_a of 7.4 for the Fe/S core of the water-soluble analogue complex $[Fe_4S_4(SCH_2CH_2CO_2)_4]^{6-}$. However, no NMR or EPR evidence has been obtained in support of protonation of the sulfides of the Fe_2S_2 and Fe_4S_4 cores.[28] In the present work, we have not been able to detect any change in the UV–visible spectrum of the oxidized 2[4Fe-4S] protein over the pH range 7.0–9.0. Definitive experiments are still required therefore to identify the site of protonation.

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Registry No. $[Cr(15\text{-}aneN_4)(H_2O)_2]^{2+}$, 70833-04-4; $[Cr(en)_3]^{3+}$, 15276-13-8.

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