Neutral complexes as oxidants for the reduced form of parsley (*Petroselinum crispum*) [2Fe–2S] ferredoxin

Evidence for partial blocking by redox-inactive Cr(III) complexes

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The 1:1 reactions of three neutral Co(III) oxidants, $\text{Co}(\text{acac})_3$, $\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3$ and $\text{Co}(\text{acac})_2(\text{NH}_3)(\text{NO}_2)$, with reduced parsley (*Petroselinum crispum*) [2Fe–2S] ferredoxin (which carries a substantial negative charge), have been studied at 25°C, pH 8.0 (Tris/HCl), 10.10 (NaCl). Whereas it has previously been demonstrated that with $\text{Co}(\text{NH}_3)_6^{3+}$ as oxidant the reaction is completely blocked by redox-inactive $\text{Cr}(\text{NH}_3)_6^{3+}$, the neutral oxidants are only partially blocked by this same complex. The effects of three Cr(III) complexes, $\text{Cr}(\text{NH}_3)_6^{3+}$, $\text{Cr}(\text{en})_3^{3+}$ and $(\text{en})_2\text{Cr} \cdot \mu(\text{OH}, \text{O}_2\text{CCH}_3) \cdot \text{Cr}(\text{en})_2^{4+}$ have been investigated. Kinetic data for the response of 3+, neutral, as well as 1– oxidants to the presence of 3+ (and 4+) Cr(III) complexes can now be rationalized in terms of a single functional site on the protein for electron transfer. Electrostatics have a significant influence on association at this site.

The recently reported amino acid sequence for parsley (*Petroselinum crispum*) 2Fe–2S ferredoxin (Nakano *et al.*, 1981) has demonstrated that the protein (M_r 10 500; 96 amino acids; single 2Fe–2S cluster) has, in common with other 2Fe–2S ferredoxins, a high negative charge at pH ~7 (–18 for the reduced form from the amino acid composition). Previous papers in this series have been concerned with the oxidation of the reduced form of the protein by inorganic complexes, and provided evidence for protein–complex association (Armstrong & Sykes, 1978), as well as for a dependence of kinetic behaviour on the oxidant charge type. The effect of redox-inactive complexes $\text{Cr}(\text{NH}_3)_6^{3+}$ [and $\text{Cr}(\text{en})_3^{3+}$] on reactivity has also been investigated (Armstrong *et al.*, 1978, 1979). Interpretation in terms of a number of functional sites for electron transfer on the protein has not proved clear cut, however. Recent studies on the [2[4Fe–4S] protein from *Clostridium pasteurianum* (F. A. Armstrong, R. A. Henderson, H. Ong Wah Kim & A. G. Sykes, unpublished work) have indicated that the response of neutral oxidants to such Cr(III) complexes is particularly crucial to the interpretation. Accordingly, three complexes $\text{Co}(\text{acac})_3$, $\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3$ and $\text{Co}(\text{acac})_2\text{NH}_3\text{NO}_2$, have been selected for further studies, and the effectiveness of different Cr(III) complexes investigated. The Cr(III)

complexes were found to inhibit the oxidation of reduced [2Fe–2S] ferredoxin by the neutral oxidants. Only partial blocking is observed, however, and the reactivity is not decreased to zero. As in previous studies, all the complexes used are inert to ligand substitution, and interactions with the protein are therefore of the outer-sphere kind with retention of original co-ordination spheres.

Methods and materials

The isolation of parsley [2Fe–2S] ferredoxin containing the $\text{Fe}_2\text{S}_2(\text{cysteine})_4$ active site, absorbance peak ratio $A_{422}/A_{277} = 0.61$ and absorption peak at 422 nm ($\epsilon = 9200 \text{ M}^{-1} \cdot \text{cm}^{-1}$), generation of the one-electron reduced protein using dithionite, and adjustment of pH to 8.0 with 10 mM-Tris (with HCl added) were as in previous studies (Armstrong *et al.*, 1978, 1979). The three neutral cobalt(III) inorganic complexes selected were all demonstrated to be suitable oxidants (eqn. 1):

where Fe(II,III) and Fe(III,III) represent respectively the reduced and oxidized forms of the protein. References to the preparations and details of u.v.–visible spectra λ , nm (ϵ , $\text{M}^{-1} \cdot \text{cm}^{-1}$) for the cobalt(III) complexes are as follows: tris(acetylcholine)cobalt(III) (Bryant & Fernelius, 1963) $\text{Co}(\text{acac})_3$, 590(129) and 257(3.4×10^4); tri-

Abbreviations used: acac, acetylacetone; en, ethylenediamine; μ denotes bridging ligands.

amminetrinitrocobalt(III) (Schlessinger, 1964) $\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3$, 432(303), 340(6390), 250(1.99×10^4); and monoamminemononitrobis(acetylaceton)cobalt(III) (Boucher & Bailar, 1965) $\text{Co}(\text{acac})_2(\text{NH}_3)(\text{NO}_2)$, 534(118), 332(5012), 248(shoulder)(1.59×10^4), 224(3.98×10^4). Similar details for the chromium(III) complexes are as follows: hexa-amminechromium(III) chloride (Oppegard & Bailar, 1965) $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3 \cdot \text{H}_2\text{O}$, 462(40) and 350(33); tris(ethylenediamine)chromium(III) (Gillard & Mitchell, 1972) $[\text{Cr}(\text{en})_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}$, 457(73) and 351(63); and μ -hydroxo- μ -acetatobis[bis(ethylenediamine) chromium(III)] (Springbord & Toftland, 1979) $[(\text{en})_2\text{Cr} \cdot \mu(\text{OH}, \text{O}_2\text{CCH}_3) \cdot \text{Cr}(\text{en})_2(\text{ClO}_4)_4 \cdot 2\text{H}_2\text{O}]$, 505(209), 378(100), 431(46). Because of light-sensitivity, solutions of Cr(III) complexes were prepared just before use and protected by wrapping the container in aluminium foil. Fresh Cr(III) stock solutions were prepared for each run.

Reactions solutions were made up to $I 0.10$ with NaCl. The kinetics (25°C) were monitored at 422 nm (absorbance increase for oxidation of protein, $\Delta\epsilon$, is $4700\text{M}^{-1}\cdot\text{cm}^{-1}$) on a Dionex D-100 stopped-flow spectrophotometer. The inorganic complexes were in >10 -fold excess of the protein. First-order rate constants, k_{obs} , were obtained from the slope of plots of absorbance (A) changes ($\ln \Delta A$) against time (linearity $>90\%$ completion) or in the case of $\text{Co}(\text{acac})_3$, because of the instability of A_∞ , by using the Guggenheim method (Guggenheim, 1926; Frost & Pearson, 1961).

Results

Rate constants, k_{obs} , (Table 1) for all three oxidants gave a linear dependence on $[\text{Co(III)}]$ up to

Table 1. First-order rate constants, k_{obs} , for the $\text{Co}(\text{acac})_3$ (A), $\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3$ (B), and $\text{Co}(\text{acac})_2(\text{NO}_2)(\text{NH}_3)$ (C) oxidations of reduced $[2\text{Fe}-2\text{S}]$ ferredoxin at 25°C , pH 8.0 (Tris/HCl), $I 0.10$ (NaCl)

	$10^4 \times [\text{Co(III)}] \text{ (M)}$	$k_{\text{obs.}} \text{ (s}^{-1}\text{)}$
A	2.5	1.00
	5.0	2.09
	7.5	3.11
	10.0	4.21
B	0.91	0.54
	1.8	1.22
	4.0	2.67
	4.7	3.18
	6.02	4.1
C	1.0	1.06
	4.0	5.1
	6.0	7.7
	10.0	12.8

the highest value ($1.0 \times 10^{-3}\text{M}$) investigated. The rate law is therefore as in eqn. (2):

$$\text{Rate} = k_1[\text{Fe(II,III)}][\text{Co(III)}] \quad (2)$$

then $k_1 = Kk_{\text{eff}}$. For the conditions employed, it can be concluded that K is less than 100M^{-1} . Values of k_1 are summarized in Table 2. At fixed Co(III) concentrations the effect of addition of various amounts of $\text{Cr}(\text{NH}_3)_6^{3+}$ was investigated for $\text{Co}(\text{acac})_3$ (Table 3), $\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3$ (Table 4) and $\text{Co}(\text{acac})_2(\text{NH}_3)(\text{NO}_2)$ (Table 5) (see also, Fig. 1). The effect of Cr(III) complexes $\text{Cr}(\text{en})_3^{3+}$ and

Fig. 1. Effect of $\text{Cr}(\text{NH}_3)_6^{3+}$ on first-order rate constants, k_{obs} , (25°C) for the $\text{Co}(\text{acac})_2$ oxidation of reduced parsley $[2\text{Fe}-2\text{S}]$ ferredoxin at three different $\text{Co}(\text{acac})_3$ concentrations, $5.0 \times 10^{-4}\text{M}$ ($\square$), $7.5 \times 10^{-4}\text{M}$ (Δ) and $1.0 \times 10^{-4}\text{M}$ ($\circ$), pH 8.0 (Tris), $I 0.10$ (NaCl)

Table 2. Second-order rate constants k_1 , for the oxidation of reduced $[2\text{Fe}-2\text{S}]$ ferredoxin with neutral Co(III) complexes at 25°C , pH 8.0 (Tris/HCl) $I 0.10$ (NaCl)

Oxidant	$k_1 \text{ (M}^{-1} \cdot \text{s}^{-1}\text{)}$
$\text{Co}(\text{acac})_3$	$4250 \pm 75^*$
$\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3$	6800 ± 70
$\text{Co}(\text{acac})_2(\text{NO}_2)(\text{NH}_3)$	12900 ± 120

* Replaces previous values (Armstrong *et al.*, 1979).

Table 3. First-order rate constants, $k_{\text{obs.}}$, for the $\text{Co}(\text{acac})_3$ oxidation of reduced [2Fe-2S]ferredoxin in the presence of the redox inactive blocking agent $\text{Cr}(\text{NH}_3)_6^{3+}$, $\text{Cr}(\text{en})_3^{3+}$ and $(\text{en})_2\text{Cr} \cdot \mu(\text{OH}, \text{O}_2\text{CCH}_3) \cdot \text{Cr}(\text{en})_2^{4+}$ at 25°C, pH 8.0 (Tris/HCl), 10.10 (NaCl)

Blocking agent	$10^4 \times [\text{Co}(\text{acac})_3]$ (M)	$10^4 \times [\text{Cr}(\text{III})]^*$ (M)	$k_{\text{obs.}}$ (s ⁻¹)
$\text{Cr}(\text{NH}_3)_6^{3+}$	10.0	0	4.21
		2.0	4.03
		5.0	3.74
		7.5	3.45
		10.0	3.31
	7.5	20.0	3.08
		0	3.11
		5.0	2.82
		10.0	2.49
		15.0	2.38
	5.0	20.0	2.31
		0	2.09
		2.0	1.94
		5.0	1.86
		7.5	1.74
$\text{Cr}(\text{en})_3^{3+}$	6.8	10.0	1.70
		20.0	1.54
		0	3.50
		2.5	3.30
		5.0	3.10
$(\text{en})_2\text{Cr} \cdot \mu(\text{OH}, \text{O}_2\text{CCH}_3) \cdot \text{Cr}(\text{en})_2^{4+}$	6.8	10.0	2.85
		15.0	2.65
		20.0	2.55
		0	3.50
		2.5	3.00
		5.0	2.70
		7.5	2.59
		10.0	2.45
		12.5	2.35
		15.0	2.25
		17.5	2.15

* $\text{Cr}(\text{III})_2$ in case of binuclear complex.

$(\text{en})_2\text{Cr} \cdot \mu(\text{OH}, \text{O}_2\text{CCH}_3) \cdot \text{Cr}(\text{en})_2^{4+}$ on the $\text{Co}(\text{acac})_3$ oxidation was also investigated (Table 3). The concentration of $\text{Co}(\text{acac})_3$ used was considerably higher than previously (Armstrong *et al.*, 1979). Blocking by the $\text{Cr}(\text{III})$ reagent was observed in all cases. However, data could not be accounted for by eqns. (3) and (4) alone:

which gives the following dependence (eqn. 5):

$$\frac{k_{\text{obs.}}}{[\text{Co}(\text{III})]} = \frac{k_1}{1 + K_{\text{Cr}}[\text{Cr}(\text{III})]} \quad (5)$$

Table 4. First-order rate constants, $k_{\text{obs.}}$, for the $\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3$ oxidation of reduced [2Fe-2S]ferredoxin in the presence of the redox inactive blocking agent $\text{Cr}(\text{NH}_3)_6^{3+}$ at 25°C, pH 8.0 (Tris/HCl), 10.10 (NaCl)

$10^4 \times [\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3]$ (M)	$10^4 \times [\text{Cr}(\text{NH}_3)_6^{3+}]$ (M)	$k_{\text{obs.}}$ (s ⁻¹)
3.0	0	2.04*
	5.0	1.86
	10.0	1.70
	15.0	1.60
	20.0	1.55
4.0	0	2.67
	2.5	2.54
	5.0	2.47
	7.5	2.39
	10.0	2.25
4.7	12.5	2.16
	0	3.18
	2.5	3.00
	5.0	2.84
	7.5	2.76
6.0	10.0	2.62
	0	4.10
	4.95	3.67
	9.84	3.37
	15.9	3.21
10.2	21.2	3.12
	26.3	3.03
	31.4	2.95
	0	7.2
	5.0	6.0
	10.0	5.7
	15.0	5.3
	25.0	5.0

* From data in Table 2.

Table 5. First-order rate constants, $k_{\text{obs.}}$, for the $\text{Co}(\text{acac})_2(\text{NO}_2)(\text{NH}_3)$ oxidation of reduced [2Fe-2S]ferredoxin in the presence of the redox-inactive blocking agent $\text{Cr}(\text{NH}_3)_6^{3+}$ at 25°C, pH 8.0 (Tris/HCl), 10.10 (NaCl)

$10^4 \times [\text{Co}(\text{acac})_2(\text{NO}_2)(\text{NH}_3)]$ (M)	$10^4 \times [\text{Cr}(\text{NH}_3)_6^{3+}]$ (M)	$k_{\text{obs.}}$ (s ⁻¹)
10.0	0	12.8
	5.0	11.2
	10.0	10.1
	15.0	9.6
	0	7.7
6.0	5.0	6.6
	10.0	6.1
	15.0	5.7
	20.0	5.7
	0	5.1
4.0	5.0	4.4
	10.0	4.1
	15.0	3.8
	20.0	3.6
	25.0	3.4

Table 6. Summary of data for the Cr(III) blocking of the oxidation of reduced [2Fe-2S]ferredoxin with neutral Co(III) complexes

Values of K_{Cr} are as determined previously.

Oxidant	Cr(III)	K_{Cr} (M^{-1})	k_1 ($M^{-1} \cdot s^{-1}$)	k_2 ($M^{-1} \cdot s^{-1}$)
Co(acac) ₃	Cr(NH ₃) ₆ ³⁺	468	4130‡	1780
	Cr(en) ₃ ³⁺	590	4250‡	2080
	Cr(III) ₂ [*]	1100†	4250‡	1800
Co(NH ₃) ₃ (NO ₂) ₃	Cr(NH ₃) ₆ ³⁺	468	6700§	3400
Co(acac) ₂ (NO ₂)(NH ₃)	Cr(NH ₃) ₆ ³⁺	468	12 600	5070

* (en)₂Cr·μ(OH, O₂CCH₃)·Cr(en)₂⁴⁺.† Determined for the Cr(III)₂ blocking of the Co(NH₃)₆³⁺ oxidation of parsley Fe(II,III) ferredoxin under identical conditions; R. A. Henderson & A. G. Sykes, unpublished work).‡ 4250 M⁻¹·s⁻¹ in Table 2.§ 6800 M⁻¹·s⁻¹ in Table 2.|| 12 900 M⁻¹·s⁻¹ in Table 2. Fig. 2. Dependence of first-order rate constants k_{obs} (25°C) on $[Cr(III)]$ for the Co(acac)₃ oxidation of reduced parsley [2Fe-2S] ferredoxin at pH 8.0 (Tris, 10.10 (NaCl)

The effects of three different Cr(III) complexes, Cr(NH₃)₆³⁺ (■), Cr(en)₃³⁺ (Δ) and (en)₂Cr·(OH, O₂CCH₃)·Cr(en)₂⁴⁺ (O) are indicated.

pected value (Armstrong *et al.*, 1979). By including the additional step (6):

an expression (eqn. 7) is obtained.

$$\frac{k_{obs.}}{[Co(III)]} = \frac{k_1 + k_2 K_{Cr}[Cr(III)]}{1 + K_{Cr}[Cr(III)]} \quad (7)$$

Plots of $k_{obs.}(1 + K_{Cr}[Cr(III)]/[Co(III)])$ against $[Cr(III)]$ are linear, assuming $K_{Cr} = 470 M^{-1}$ (average of previously determined values) for Cr(NH₃)₆³⁺ (Armstrong *et al.*, 1979), $K_{Cr} = 590 M^{-1}$ for Cr(en)₃³⁺ (Armstrong *et al.*, 1979) and $K_{Cr} = 1100 M^{-1}$ for the Cr(III)₂ complex (Fig. 2) [determined for the Cr(III)₂ blocking of the Co(NH₃)₆³⁺ oxidation of parsley Fe(II,III)ferredoxin under identical conditions; R. A. Henderson & A.G. Sykes, unpublished work]. Values of k_2 (±5%) obtained are listed in Table 6. At high Cr(III) concentrations, second-order rate constants, $k_{obs.}/[Co(III)]$, approach k_2 .

Discussion

All three neutral Co(III) oxidants, Co(acac)₃, Co(NH₃)₃(NO₂)₃ and Co(acac)₂(NH₃)(NO₂) give similar kinetic behaviour in their reactions with reduced [2Fe-2S] ferredoxin (eqn. 1), with no evidence for limiting kinetics and only partial blocking of the reaction by redox-inactive Cr(NH₃)₆³⁺. In the case of Co(acac)₃, three Cr(III) complexes were studied, and all three gave similar results in terms of partial blocking. The reaction sequence of eqns. (3), (4) and (6) is an adequate description of the kinetic data, where in eqn. (6) the protein associated with Cr(III) retains some reactivity (k_2).

since plots of $[Co(III)]/k_{obs.}$ against $[Cr(III)]$ were not linear. Moreover, slopes that could be drawn gave a K_{Cr} value approximately one-half the ex-

It was previously demonstrated that $\text{Cr}(\text{NH}_3)_6^{3+}$ blocks completely the oxidation of reduced [2Fe-2S] ferredoxin with $\text{Co}(\text{NH}_3)_6^{3+}$, as well as other positively charged oxidants, e.g. $\text{Co}(\text{NH}_3)_5(\text{C}_2\text{O}_4)^+$, $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$ and $(\text{NH}_3)_5\text{Co}\cdot\text{NH}_2\cdot\text{Co}(\text{NH}_3)_5^{5+}$ (Armstrong *et al.*, 1979). However, it accelerates the reaction with $\text{Co}(\text{EDTA})^-$ and, in the light of the present results, an attractive possibility is that all these oxidants use the same functional site on the ferredoxin, with the influence of $\text{Cr}(\text{NH}_3)_6^{3+}$ according to charge. Thus whereas $\text{Cr}(\text{NH}_3)_6^{3+}$ is able to block completely the reaction with positively charged oxidants, the inhibition with neutral oxidants is only partial, and it actually enhances the rate with negatively charged reactants. This interpretation is supported by the fact that a single value of K_{Cr} 470M^{-1} , is compatible with all the $\text{Cr}(\text{NH}_3)_6^{3+}$ effects. A previous suggestion (Armstrong *et al.*, 1979), that one of three sites on the protein is selected according to ligand types and/or charge on the complex, therefore no longer applies.

The above interpretation in terms of a single reaction site on the protein is the simplest interpretation consistent with available data. There are, however, at least two other interpretations of the sequence of eqns. (3), (4) and (6) that should be mentioned. These centre around the possibility that k_1 and k_2 correspond to reactions at two different sites on the protein. The first requires that only one of the sites is blocked by $\text{Cr}(\text{NH}_3)_6^{3+}$. Such an explanation has to exclude reaction of $\text{Co}(\text{NH}_3)_6^{3+}$ at the second site and permit a faster reaction of $\text{Co}(\text{EDTA})^-$ at the same site. The second possibility is that k_2 represents reaction at the second site and that this reactivity is initiated by $\text{Cr}(\text{NH}_3)_6^{3+}$ associating at the first site (i.e. there is a conformational change). The latter also discriminates against $\text{Co}(\text{NH}_3)_6^{3+}$, which again does not react at

the second site. The complexity of these alternatives, and versatility required of the protein, suggests that they should not at this time be considered as strong possibilities. The interpretation now given agrees with that proposed for the corresponding reactions of the 2[4Fe-4S] protein (F. A. Armstrong, R. A. Henderson, H. Ong Wah Kim & A. G. Sykes, unpublished work).

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