Kinetic Studies on 1 : 1 Electron-transfer Reactions Involving Blue Copper Proteins. Part 9.† Effects of Chromium(III) Modification on Reactions of Plastocyanin with $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline), $[Fe(CN)_6]^{3-}$, $[Co(dipic)_2]^-$ (dipic = dipicolinate) (Oxidants) and $[Ru(NH_3)_5(py)]^{2+}$ (py = pyridine) (Reductant) ‡

Stephen K. Chapman, C. Victor Knox, P. Kathirgamanathan, and A. Geoffrey Sykes *
Department of Inorganic Chemistry, The University, Newcastle upon Tyne NE1 7RU

A 1 : 1 chromium(III)-modified form of parsley plastocyanin was obtained by reduction of oxidised protein, here referred to as PCu(II), with $Cr^{2+}(aq)$. At pH 7.5 a 21% decrease in rate constant is observed for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I)·Cr^{III} as compared to native protein. Biphasic kinetics (rate constants k_1 and k_2) are obtained for the same reaction at pH 5.8 suggesting that at this pH the Cr^{III} is attached in two ways. The rate constant k_1 is the same as that observed for native plastocyanin, with k_2 some 72% less. On attachment of a second mol of Cr^{III} by the same procedure to give PCu(I)·$2Cr^{III}$, uniphasic kinetics with a rate constant k_2 are observed. Within experimental error no effect of single modification is observed on rate constants for the $[Fe(CN)_6]^{3-}$ oxidation at pH 7.5 and 5.8. At pH 5.8 the $[Ru(NH_3)_5(py)]^{2+}$ (py = pyridine) reduction of PCu(II)·Cr^{III} gives a net 22% decrease, which increases to 38% for PCu(II)·$2Cr^{III}$. No effect is observed for the $[Co(dipic)_2]^-$ (dipic = dipicolinate) oxidation of PCu(I)·Cr^{III} also at pH 5.8. These observations support the belief that positively charged complexes $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) and $[Ru(NH_3)_5(py)]^{2+}$ react at a broad region on plastocyanin close to Tyr 83 and the negatively charged residues 42—45, whereas $[Fe(CN)_6]^{3-}$ and $[Co(dipic)_2]^-$ react at a site close to His 87. The reduction potential for the PCu(II)–PCu(I) couple (370 mV) is decreased by *ca.* 20 mV on attachment of Cr^{III}.

Plastocyanin (*M* 10 500, 99 amino acids) occurs in all higher plants as well as algae where it has a relatively well defined electron-transport function in photosynthesis.[1] Freeman and co-workers [2] have reported details of the structure of poplar plastocyanin to 1.6 Å resolution. A particularly interesting feature is the matching tetrahedral geometries of the PCu(I) and PCu(II) active sites.[3] From 13 completed amino-acid sequences and 54 partial sequences (>40 residues) of plastocyanin from higher plants it appears that 60 residues are invariant and 7 are conservatively substituted.[2,4] With three algal plastocyanins included there are 39 invariant or conservatively substituted groups. It is believed that the same structural features apply to the whole family, and that highly conserved residues are an indication of functional sites on the protein surface. A particularly striking feature is the distribution of the excess (8−) negative charge on the surface of PCu(II). Freeman [2] has drawn attention to a negative patch, which incorporates four consecutive acid residues at 42—45, as being of possible relevance as a binding site. N.m.r. studies have indicated specificity in the interaction of $[Cr(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) and $[Cr(NH_3)_6]^{3+}$ close to Tyr 83 and this negative patch.[5,6] The negative complex $[Cr(CN)_6]^{3-}$ on the other hand associates at the hydrophobic site close to His 87. From thermolysin proteolysis experiments Farver and Pecht [7] have concluded that the product obtained on reduction of PCu(II) with labelled $[Cr(H_2O)_6]^{2+}$ at pH 7 has Cr^{III} attached to the peptide chain 40—49. Co-ordination of the Cr at one or two carboxylates in the 42—45 patch is favoured.[7]

Here we aim to investigate the effect of this modification on reactivity with inorganic complexes of different charge using $[Co(phen)_3]^{3+}$, $[Fe(CN)_6]^{3-}$, and $[Co(dipic)_2]^-$ [dipic = dipicolinate (pyridine-2,6-dicarboxylate)] as oxidants for PCu(I), and $[Ru(NH_3)_5(py)]^{2+}$ (py = pyridine) as a reductant for PCu(II). Reactions of all four complexes with plastocyanin have previously been studied.[8–10]

Experimental

Protein.—Plastocyanin was isolated from parsley leaves as previously described.[11] After purification plastocyanin in the oxidised form, PCu(II), gave a u.v.–visible absorbance (*A*) peak ratio A_{278}/A_{597} of 1.7 ± 0.1. Concentrations were based on an absorption coefficient ε = 4 500 M^{-1} cm^{-1} at 597 nm. Protein solutions were dialysed against buffer as required at 4 °C.

Complexes.—Tris(1,10-phenanthroline)cobalt(III) was prepared as the perchlorate salt, $[Co(phen)_3][ClO_4]_3 \cdot 2H_2O$,[12] which was precipitated out of solution by addition of saturated $NaClO_4$, and recrystallised from water with addition of a few drops of 5 M $HClO_4$. The chloride salt $[Co(phen)_3]Cl_3 \cdot 7H_2O$ was also used.[8] Peak positions, λ/nm (ε/M^{-1} cm^{-1}), were at 330 (4 660), 350 (3 620), and 450 (100), in agreement with the literature.[12] Penta-ammine(pyridine)ruthenium(II) perchlorate, $[Ru(NH_3)_5(py)][ClO_4]_2$, peak at 407 nm (7 800 M^{-1} cm^{-1}), was prepared by a literature method starting from $[Ru(NH_3)_6]Cl_3$.[13] Potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (BDH, AnalaR), peaks at 300 (1 600) and 410 nm (1 010 M^{-1} cm^{-1}) was as supplied. The ammonium salt of the bis(pyridine-2,6-dicarboxylato)cobaltate(III) complex, $NH_4[Co(dipic)_2]$, peak at 510 nm (630 M^{-1} cm^{-1}), was prepared by a procedure described.[14]

Chromium Modification.—Solutions of hexa-aquachromium(II) perchlorate in perchloric acid were prepared by electrolytic reduction of hexa-aquachromium(III) perchlorate under N_2. The Cr^{II} was determined spectrophotometrically at 715 nm (ε = 4.82 M^{-1} cm^{-1}), and the $[H^+]$ by titration following replacement of the Cr^{II} by H^+ using ion-exchange

† Part 8 is M. A. Augustin, S. K. Chapman, D. M. Davies, A. D. Watson, and A. G. Sykes, *J. Inorg. Biochem.*, 1984, **20**, 281.

‡ *Non-S.I. unit employed:* M = mol dm^{-3}.

chromatography. Typical concentrations were $[Cr^{2+}]$ = *ca.* 0.2 M and $[H^+]$ = *ca.* 0.6 M. The stock solution was diluted, and an appropriate amount transferred into a buffered reaction solution using a 0.25-cm^3 Hamilton microsyringe. The anaerobic addition of a stoicheiometric amount of Cr^{II} to a deoxygenated sample of protein (5 cm^3 of 10^{-4}—10^{-5} M) at pH 7.5 ('Tris'–HCl) generated a colourless solution indicating reduction of the protein.

Farver and Pecht [7] established a 1 : 1 stoicheiometry for the singly modified protein by monitoring the α activity of ^{51}Cr. In this work the Cr to Cu ratio of the modified protein was determined after dialysis (*ca.* 24 h) and/or column procedures (as below), using a Varian 575 atomic absorption spectrometer. An air–acetylene flame, and hollow chromium and copper cathode lamps were used (bandwidth 0.2 nm). Solutions of $CrCl_3 \cdot 6H_2O$ and $CuCl_2 \cdot 2H_2O$ (both BDH, AnalaR) made up at pH 7.5 (Tris–HCl), I = 0.10 M (NaCl), were used to calibrate the instrument. A 1 : 1 ratio (±10%) consistent with the $PCu(I) \cdot Cr^{III}$ formulation was obtained. The Cr was also checked by chromate analysis. No effect of the chromium(III) modification on the u.v.–visible spectrum of PCu(II) was detected. For studies with inorganic oxidants, the reduced protein was exposed to air for a few minutes (to oxidise excess of Cr^{2+}), and then dialysed against appropriate buffer (0.010 M) to remove any unbound Cr. For studies involving reductants and the addition of a second mol of Cr, the sample of reduced protein was re-oxidised with a crystal of $K_3[Fe(CN)_6]$ before dialysis. Reduction of the protein with a second Cr^{2+} gave the doubly modified protein $PCu(I) \cdot 2Cr^{III}$.

An experiment was carried out in which the Cr^{2+}-reduction procedure was repeated five times. This involved addition of amounts of Cr^{2+} (0.06 cm^3, 8.2×10^{-3} M) to PCu(II) (initially 7 cm^3 of 7.2×10^{-5} M), with $[Fe(CN)_6]^{3-}$ oxidation and dialysis (24 h at 0 °C) preceding each successive addition. After the fifth addition the protein was analysed by atomic absorption and gave a Cr : Cu ratio of 5.7 suggesting attachment of 5 mol of Cr^{III}.

The 1 : 1 modified protein $PCu(I) \cdot Cr^{III}$ was prepared at pH 7.5 in Tris–HCl buffer (0.010 M), I = 0.1 M (NaCl), and also in hepes (0.01 M), used by Farver and Pecht.[7] At the outset the modified protein was purified by passing down a gel column. Subsequently dialysis was used to remove an excess of Cr and adjust conditions as required. The modified protein was also prepared by 1 : 1 addition of Cr^{2+} at pH 5.8 in mes buffer. All these different preparations gave satisfactory reproducibility in kinetic runs (at pH 7.5 or 5.8) with $[Co(phen)_3]^{3+}$ as oxidant. Identical results were obtained for protein modified at pH 7.5 and then adjusted to pH 5.8, as for protein modified at pH 5.8. Protein modified by addition of a 4 : 1 excess of Cr^{2+}, followed by air oxidation, gave singly modified protein with the same reactivity.

Buffers—These were from Sigma Chemicals and were made up as follows. Tris(hydroxymethyl)aminomethane ('Tris') was added to 0.010 M HCl, *N*-2-hydroxyethylpiperazine-*N*′-ethane-2-sulphonic acid (hepes) to 0.010 M NaOH, and 2-(*N*-morpholino)ethanesulphonic acid (mes) to 0.010 M NaOH, until required pH values were obtained. All pH values were measured on a Radiometer (PHM62) meter fitted with a Russell (CWR/22) combined electrode.

Kinetic Runs.—A Dionex D-110 stopped-flow spectrophotometer was used to monitor all kinetic runs. Ionic strengths of reactant solutions were made up to I = 0.10 M (NaCl). Except as stated, plots of absorbance changes $\ln(A_t - A_\infty)$ against time were linear for 3—4 half-lives. For other runs a standard concurrent reaction treatment was used.[15] In the case of the $[Ru(NH_3)_5(py)]^{2+}$ reduction of $PCu(II) \cdot Cr^{III}$ at pH 5.8, difficulties were experienced in separating the two stages (which had similar rates), and a single rate constant obtained by standard first-order treatment is reported.

Table 1. Reduction potentials for native plastocyanin and samples of the singly and doubly chromium(III)-modified protein using two inorganic couples as mediators, I = 0.10 M (NaCl)

	Reduction potential (mV)		
	$[Fe(CN)_6]^{3-,4-}$		
Plastocyanin	*a*	*b*	[b] $[Co(phen)_3]^{3+,2+}$
Native	375	368	372
Singly modified	352	348	330
Doubly modified	340	341	318

[a] pH 5.8 (mes). [b] pH 7.5 (Tris–HCl).

Reduction Potentials.—Two mediator couples $[Fe(CN)_6]^{3-}$–$[Fe(CN)_6]^{4-}$ (413 mV) and $[Co(phen)_3]^{3+}$–$[Co(phen)_3]^{2+}$ (372 mV) both *vs.* the normal hydrogen electrode (n.h.e.) were used. In the latter case a three-fold excess of free phenanthroline was present to retain the Co^{II} in the form of the tris(phenanthroline) complex. All solutions were kept under an atmosphere of N_2. The procedure with $[Fe(CN)_6]^{3-}$–$[Fe(CN)_6]^{4-}$ was to dialyse *ca.* 4×10^{-5} M samples of PCu(II), $PCu(I) \cdot Cr^{III}$, and $PCu(I) \cdot 2Cr^{III}$ (2 cm^3 samples) against a solution of $[Fe(CN)_6]^{4-}$ (*ca.* 4×10^{-3} M) and $[Fe(CN)_6]^{3-}$ (*ca.* 4×10^{-4} M) at the required pH, total volume 120 cm^3, for 24 h. During this time the outer solution was changed six times. The concentration of the copper(II) protein was then determined from the absorbance at 597 nm (ε 4 500 M^{-1} cm^{-1}), and the concentration of $[Fe(CN)_6]^{3-}$ checked at 410 nm (ε 1 010 M^{-1} cm^{-1}). The procedure with $[Co(phen)_3]^{3+}$–$[Co(phen)_3]^{2+}$ was to first reduce the protein samples with dithionite, and subsequently dialyse against a solution containing $[Co(phen)_3]^{3+}$ (*ca.* 4×10^{-5} M) and free phenanthroline. The concentration of copper(II) protein was determined as above. Solutions were also prepared by direct addition of reagents to the protein solution. Reduction potentials for the plastocyanin couple, Table 1, were calculated using the Nernst equation. Errors were estimated to be ±2 mV. Differences in $E^\ominus$ values may well originate from the two mediator couples using different binding sites on the protein.

Results

$[Co(phen)_3]^{3+}$ *as Oxidant.*—At pH 7.5 rate constants, $k_{obs.}$, using singly modified protein $PCu(I) \cdot Cr^{III}$, Table 2, are 21 ± 5% less than for the reaction of unmodified protein.* With the same modified protein at pH 5.8 biphasic kinetics are observed. Using a concurrent reaction, first-order rate constants k_1 and k_2 are obtained for the first and second stages, Table 3. The rate constant k_1 is the same as $k_{obs.}$ for oxidation of the unmodified protein, while k_2 (representing a 72 ± 7% decrease in second-order rate constant) is the only process observed when doubly modified $PCu(I) \cdot 2Cr^{III}$ is used, Figure 1.

$[Ru(NH_3)_5(py)]^{2+}$ *as Reductant.*—All rate constants were at pH 5.8, Table 4. Slight biphasic nature was observed, but we were unable to separate the two stages. Comparison is made in Figure 2 of $k_{obs.}$ for the reduction of PCu(II) [9] with the

* Values in this paper have been further checked and supercede those previously reported in ref. 10.

Table 2. First-order rate constants (25 °C) for the $[Co(phen)_3]^{3+}$ oxidation of singly chromium(III)-modified PCu(I) (*ca.* 1×10^{-5} M) at pH 7.5 (Tris–HCl), $I = 0.10$ M (NaCl)

$10^3[Co(phen)_3^{3+}]$/M	$k_{obs.}$/s^{-1}
0.30	0.62
0.47	1.04
0.63	1.38
0.66	1.34
0.81	1.50
0.85	1.82
1.08	2.38
1.41	2.75
1.58	3.3

Table 3. First-order rate constants (25 °C) for the $[Co(phen)_3]^{3+}$ oxidation of plastocyanin, PCu(I) (*ca.* 1×10^{-5} M), singly modified plastocyanin PCu(I)·Cr^{III}, and doubly modified plastocyanin, PCu(I)·$2Cr^{III}$, at pH 5.8 (mes), $I = 0.10$ M (NaCl)

	Rate constant/s^{-1}		
$10^3[Co(phen)_3^{3+}]$/M	PCu(I)	PCu(I)·Cr^{III} [a]	PCu(I)·$2Cr^{III}$
0.74	0.94		0.32
0.91		1.45 and 0.37	
0.97	1.23		
1.30	1.83		
1.35		1.73 and 0.52	
1.40		2.00 and 0.52 [b]	
1.72			0.70

[a] Biphasic kinetics; rate constants obtained by consecutive treatment. [b] PCu(I)·Cr^{III} prepared at pH 7.5 in hepes and dialysed to pH 5.8 (mes).

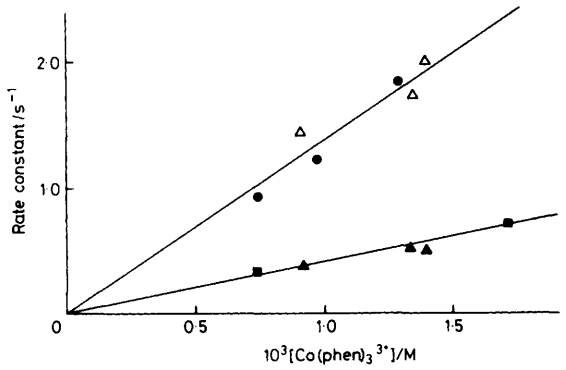

Figure 1. A comparison of first-order rate constants $k_{obs.}$ for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) (●) with rate constants k_1 (△) and k_2 (▲) for the biphasic oxidation of singly modified plastocyanin PCu(I)·Cr^{III} and rate constants k_2 (■) for the oxidation of doubly modified plastocyanin, PCu(I)·$2Cr^{III}$, at 25 °C, pH 5.8 (mes), $I = 0.10$ M (NaCl)

apparent rate constant k_{12} for the reduction of PCu(II)·Cr^{III} and k_2 for the reduction of PCu(II)·$2Cr^{III}$. Second-order rate constants from the slopes in Figure 2 indicate 22 ± 7% and 38 ± 5% decreases respectively in rate constants for singly and doubly modified protein.

$[Fe(CN)_6]^{3-}$ *as Oxidant.*—At pH 7.5 and 5.8 first-order rate constants $k_{obs.}$ for the oxidation of PCu(I) and PCu(I)·Cr^{III} are the same within experimental error (<5% differences), Table 5. The decrease in rate constants with pH, Figure 3, is as reported previously.[8,10]

Table 4. First-order rate constants (25 °C) for the reduction of plastocyanin PCu(II) (*ca.* 5×10^{-6} M), PCu(II)·Cr^{III}, and PCu(II)·$2Cr^{III}$, with $[Ru(NH_3)_5(py)]^{2+}$ at pH 5.8 (mes), $I = 0.10$ M (NaCl)

	Rate constant/s^{-1}		
$10^4[Ru(NH_3)_5(py)^{2+}]$/M	PCu(II)	PCu(II)·Cr^{III} *	PCu(II)·$2Cr^{III}$
0.44		11.6	
0.51	17.3		8.1
0.74	26.0	19.2	
0.96		24.0	
1.00	31.4		19.8

* Rate constants for a biphasic process are too close to be separated. An apparent rate constant from a standard $\ln\Delta A$ against time plot is reported here.

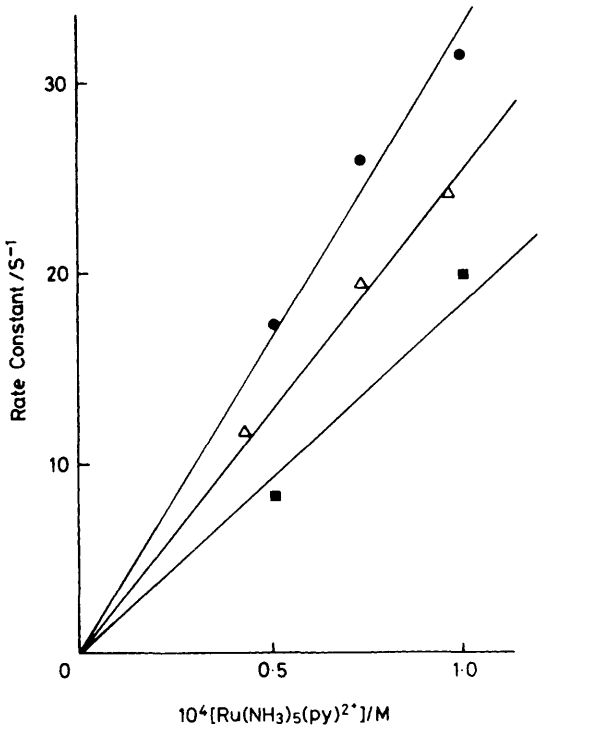

Figure 2. A comparison of first-order rate constants, $k_{obs.}$, for the $[Ru(NH_3)_5(py)]^{2+}$ reduction of PCu(II) (●) with the rate constant k_{12} (△) for the reduction of PCu(II)·Cr^{III}, and k_2 (■) for the reduction of PCu(II)·$2Cr^{III}$, at 25 °C, pH 5.8 (mes), $I = 0.10$ M (NaCl)

$[Co(dipic)_2]^-$ *as Oxidant.*—Rate constants, $k_{obs.}$, at pH 5.8 show little or no effect (<3%) of modification of PCu(I) to PCu(I)·Cr^{III}, Table 6.

Discussion

The present study (summary Table 7) has demonstrated that single chromium(III) modification of plastocyanin discriminates between positively and negatively charged redox partners. Thus with $[Co(phen)_3]^{3+}$ and $[Ru(NH_3)_5(py)]^{2+}$ rate constants are decreased by the modification, whereas no effect is observed with $[Fe(CN)_6]^{3-}$ and $[Co(dipic)_2]^-$. The discrimination is believed to originate from the positively charged complexes using a binding site on plastocyanin close to the chromium modification, whereas negatively charged complexes use an alternative site which is unaffected by

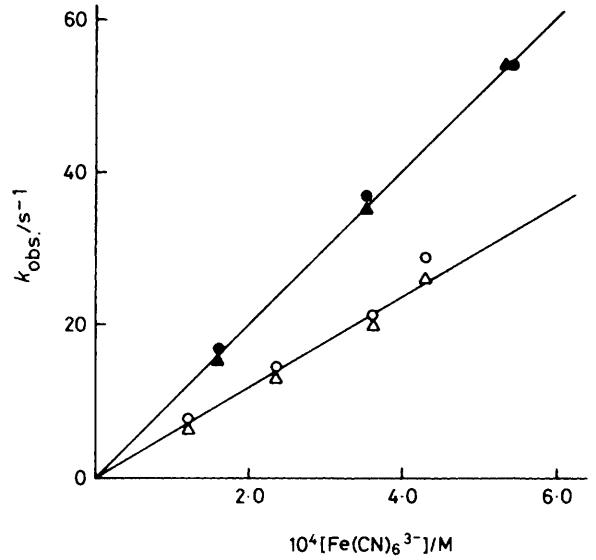

Figure 3. A comparison of first-order rate constants, $k_{obs.}$ (25 °C), for the $[Fe(CN)_6]^{3-}$ oxidation of PCu(I) (●, ○) and PCu(I)·Cr^{III} (▲, △), showing little or no effect of the modification at pH 7.5 (closed symbols) and 5.8 (open symbols), I = 0.10 M (NaCl)

Table 5. First-order rate constants (25 °C) for the $[Fe(CN)_6]^{3-}$ oxidation of plastocyanin PCu(I) (*ca.* 1 × 10^{-5} M), and modified plastocyanin PCu(I)·Cr^{III}, at pH 7.5 (Tris–HCl) and pH 5.8 (mes), I = 0.10 M (NaCl)

	Rate constant/s^{-1}	
$10^4[Fe(CN)_6^{3-}]/M$	PCu(I)	PCu(I)·Cr^{III}
At pH 7.5		
1.67	16.5	16.0
3.5	37	35
5.4	54	54
At pH 5.8		
1.23	7.7	7.4
2.35	14.2	13.6
3.62	21.3	20.2
4.30	28.8	26.0

Table 6. First-order rate constants (25 °C) for the $[Co(dipic)_2]^-$ oxidation of plastocyanin PCu(I) (*ca.* 1 × 10^{-5} M), and modified plastocyanin PCu(I)·Cr^{III}, at pH 5.8 (mes), I = 0.10 M (NaCl)

	Rate constant/s^{-1}	
$10^4[Co(dipic)_2^-]/M$	PCu(I)	PCu(I)·Cr^{III}
2.5	0.080	0.075
3.6	0.110	0.111
4.3	0.124	0.124
5.8	0.168	0.158

Table 7. Summary of effects of chromium(III) modifications on rate constants (25 °C) for reactions of plastocyanin with inorganic redox partners, I = 0.10 M (NaCl)

Redox partner	pH	Protein	Rate constant/M^{-1} s^{-1}
$[Co(phen)_3]^{3+}$	7.5	PCu(I)	(2.8 ± 0.6) × 10^3 [a]
		PCu(I)·Cr^{III}	(2.2 ± 0.2) × 10^3
	5.8	PCu(I)	(1.38 ± 0.25) × 10^3
		PCu(I)·Cr^{III}	(1.36 ± 0.43) × 10^3
			(0.39 ± 0.04) × 10^3
		PCu(I)·$2Cr^{III}$	(0.39 ± 0.04) × 10^3
$[Ru(NH_3)_5(py)]^{2+}$	5.8	PCu(II)	(3.2 ± 0.6) × 10^5 [b]
		PCu(II)·Cr^{III}	(2.51 ± 0.17) × 10^5
		PCu(II)·$2Cr^{III}$	(1.98 ± 0.20) × 10^5
$[Fe(CN)_6]^{3-}$	7.5	PCu(I)	(1.00 ± 0.05) × 10^5 [c]
		PCu(I)·Cr^{III}	(1.00 ± 0.03) × 10^5
	5.8	PCu(I)	(6.4 ± 0.9) × 10^4
		PCu(I)·Cr^{III}	(5.9 ± 0.5) × 10^4
$[Co(dipic)_2]^-$	5.8	PCu(I)	(2.9 ± 0.2) × 10^2
		PCu(I)·Cr^{III}	(2.8 ± 0.3) × 10^2

[a] A rate constant (3.0 ± 0.3) × 10^3 M^{-1} s^{-1} was reported in Part 1.[8] [b] A rate constant (3.8 ± 0.1) × 10^5 M^{-1} s^{-1} was reported in Part 7.[9] [c] The rate constant 0.94 × 10^5 M^{-1} s^{-1} reported in ref. 8 was in cacodylate (dimethylarsinate) buffer.

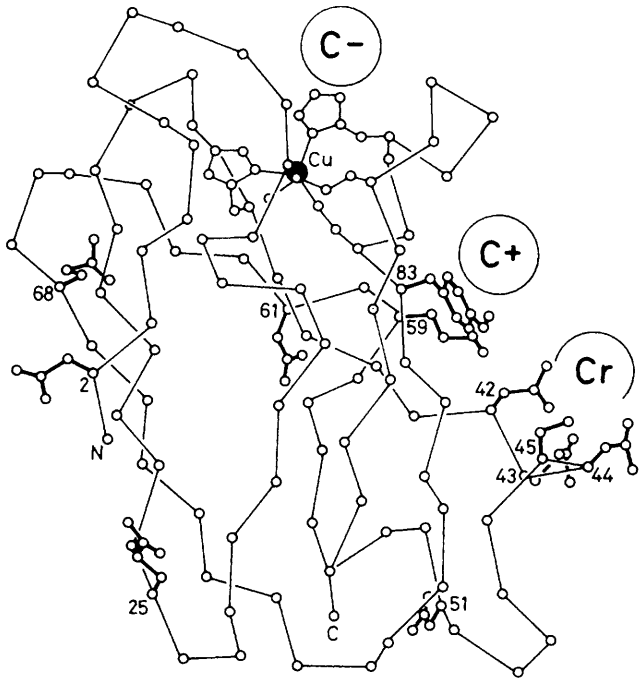

Figure 4. The structure of plastocyanin as reported by Freeman,[2] showing attachment of the Cr^{III} at the 42—45 patch as proposed by Farver and Pecht.[7] The sites for reaction with positively (C^+) and negatively (C^-) charged inorganic redox partners are indicated

modification. Farver and Pecht [7] have concluded that the single chromium modification is at the 42—45 site (Figure 4), and the results obtained are therefore consistent with previous observations that plastocyanin exhibits specificity for positively charged complexes (C^+) at a site close to Tyr 83, which is adjacent to the negative patch incorporating residues 42—45. From n.m.r. experiments [5,6] it has been concluded that negatively charged complexes such as $[Fe(CN)_6]^{3-}$ (C^-) react at the hydrophobic site close to His 87, which represents the closest approach possible to the copper active site.

The 21% effect of chromium(III) modification on the reaction of $[Co(phen)_3]^{3+}$ at pH 7.5 is small. It is however of similar magnitude to the *ca.* 30% (maximum) inhibition of the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) by redox-inactive $[Co(NH_3)_6]^{3+}$,[10,16] which is believed to originate from the association of $[Co(NH_3)_6]^{3+}$ at the Tyr 83/42—45 site. On decreasing the pH from 7.5 to 5.8 the behaviour of the chromium(III)-modified protein changes and biphasic kinetics

are observed. Analyses have confirmed a 1 : 1 ratio of Cr : Cu and we have no evidence that Cr^{III} has become detached from the protein. Therefore the change in pH would seem to emphasise a difference between (at least) two different chromium(III)-modified forms already present, or alternatively results in two different modified forms being generated. The latter seems most likely from our results, since adjusting the pH of modified protein from 5.8 to 7.5, two kinetic stages are retained. Of the two forms present at pH 5.8 one appears to have a non-influential Cr^{III} attached, and the second a Cr^{III} which produces a 72% retardation of the $[Co(phen)_3]^{3+}$ oxidation. On attachment of a second mol of Cr^{III} uniphasic kinetics are observed, and the rate constant k_2 is the same as for the slower stage in the biphasic reaction of $PCu(I)\cdot Cr^{III}$. In other words chromium(III) saturation of the influential site has been achieved.

With $[Ru(NH_3)_5(py)]^{2+}$ as reductant for $PCu(II)\cdot Cr^{III}$ at pH 5.8 it was not possible to separate two kinetic stages. However, the behaviour observed appears comparable to that with $[Co(phen)_3]^{3+}$ as redox partner, and the effect with $PCu(II)\cdot 2Cr^{III}$ further substantiates this. A brief study was made of the reaction of $[Fe(CN)_6]^{3-}$ with doubly modified PCu(I) when an increase in rate constants compared to those for native protein was noted.

We have no information as to what changes occur on adjustment of the pH to 5.8 to give the modified form with non-influential Cr^{III}. At pH 7.5 it is possible that the Cr^{III} is attached to the protein through one to three co-ordination positions. On changing the pH to 5.8, protonation could result in significant changes affecting the attachment and degree of hydrolysis of the Cr^{III}. Other experiments indicate that on repeating the preparation procedure as many as five Cr^{III} can be attached to the protein, and clearly the Cr^{2+} is capable of binding to more than one site, although not presumably with the same affinity for each site. The results of Farver and Pecht indicate a preference for the 42—45 residues.

The trend in reduction potentials in Table 1 is the same for both mediators. Of particular interest is the decrease observed compared to the increase of 40 mV reported by Burkey and Gross [17] for ethylenediamine modification at the 42—45 site. Kinetic results can be examined assuming that second-order rate constants are the product of two components, K/M^{-1} for association of the two reactants prior to k_{et}/s^{-1} for electron transfer. The decrease in reduction potential would be expected to give an increase in k_{et} for the $[Fe(CN)_6]^{3-}$ and $[Co(dipic)_2]^-$ reactions. That none is observed is surprising and suggests that the effect is balanced by a decrease in K. Changes in k_{et} would also be expected for the $[Co(phen)_3]^{3+}$ and $[Ru(NH_3)_5(py)]^{2+}$ reactions. Here, however, the dominant effect will be the much more pronounced decrease in K due to the proximity of the Cr^{III}.

In further experiments we have noted that the $[CrL(H_2O)_2]^{2+}$ complex, where L is the N_4 tetradentate saturated macrocyclic ligand 1,4,8,12-tetra-azacyclopentadecane, reduces PCu(II) without becoming attached to the protein. This is in contrast to similar experiments on the $[CrL(H_2O)_2]^{2+}$ reduction of [2Fe–2S] and 2[4Fe–4S] ferredoxins,[18] when Cr^{III} is attached to the product. With plastocyanin the different affinities of $Cr^{2+}(aq)$ and $[CrL(H_2O)_2]^{2+}$ for inner- and outer-sphere reaction paths are noted.

In conclusion, the effects of chromium(III) modification provide a means of differentiating between the two binding sites on plastocyanin which have previously been defined. Experiments described support the belief that positive complexes react at a broad region on the protein surface which incorporates Tyr 83 and residues 42—45.

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