Kinetic Studies on 1 :1 Electron-transfer Reactions involving Blue Copper Proteins. Part 10.[1] The Assignment of Binding Sites in the Reactions of Plastocyanin (and Azurin) with Non-physiological Protein Redox Partners †

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One-equivalent reductions of plastocyanin, PCu(II) (estimated charge 8−), with cytochrome c(II) (8+), and with high-potential iron–sulphur protein in the reduced form, Hipip(r)(3−), have been studied at I = 0.10 M (NaCl). Three effects have been tested for. First the different responses of the reactions to pH in the range 4.5—8.8 are noted. Secondly competitive inhibition of the reaction cytochrome c(II) + PCu(II), but not Hipip(r) + PCu(II), with redox-inactive $[Pt(NH_3)_6]^{4+}$ has been observed. In the third approach PCu(II) modified by attachment of Cr^{III} at residues 42—45 reacts more slowly with cytochrome c(II), but at the same rate with Hipip(r). All three approaches are consistent with cytochrome c reacting at the previously defined Tyr 83/42—45 site on plastocyanin. The responses with Hipip(r) suggest that this reductant reacts at a different binding site on PCu(II), which may be the same as that used by $[Fe(CN)_6]^{4-}$, namely the His 87 site. Since it has previously been demonstrated that plastocyanin reacts at the exposed haem edge of cytochrome c, it can be concluded that the Cu to Fe separation at the time of electron transfer is *ca.* 20 Å. Effects of pH on the reduction of azurin, ACu(II) (1−), with cytochrome c(II) and Hipip(r) are also considered.

In previous studies on reactions of plastocyanin with inorganic complexes three different approaches have been used to define binding sites for redox partners on the surface of plastocyanin. These involve the effect of chromium(III) modification,[1] response to pH,[2] and the use of $[Pt(NH_3)_6]^{4+}$ as a competitive inhibitor.[2,3] In addition, the n.m.r. line-broadening method has been used to study the interaction of plastocyanin with redox-inactive chromium(III) complexes.[4,5] Inorganic complexes appear to be selective, with the site adopted according to whether the charge on the complex is positive or negative.[2,4,5] The picture which has emerged is that positive complexes react at the Tyr 83 site, which is close to the negative patch incorporating the four consecutive acid residues 42—45, while negative complexes react at a site which is close to His 87. Both these regions have been defined in Freeman's crystal structure of plastocyanin, which has also indicated a striking imbalance in the distribution of charges on plastocyanin with none of the ten conserved acidic residues (which are on the surface and in contact with solvent) in the top third of the molecule.[6,7]

We have now used the three approaches defined to see whether for protein redox partners cytochrome c(II) (8+), cyt c(II), and high-potential iron–sulphur (3−) protein, Hipip(r), binding sites can be tested for in the same way, and indeed whether the same binding sites are effective for these protein reactants. Some of the findings have been referred to in an earlier report.[8] In addition, effects of pH have been examined for the reduction of azurin, ACu(II) (1−), with cytochrome c(II) and Hipip(r). Effects of pH on the reaction of azurin with inorganic complexes have been discussed in an earlier paper.[9]

Experimental

Reactants.—The isolation (and handling) of the single (type 1) Cu proteins plastocyanin (from the chloroplast of parsley leaves) and azurin (from *Pseudomonas aeruginosa*), of high-potential iron–sulphur protein from *Chromatium vinosum* (strain D), and the purification of horse heart cytochrome c (Sigma Chemicals) were all as previously described.[10] The chromium(III) modification of plastocyanin to give PCu(I)·Cr^{III} and PCu(I)·2Cr^{III} with one and two Cr^{III} attached has been described.[1] Hexa-ammineplatinum(IV) chloride $[Pt(NH_3)_6]Cl_4·H_2O$ was prepared by a literature method.[3,11]

Buffers.—For the pH 7—9 range, solutions of 1.0×10^{-2} M NaOH were adjusted to the required pH by addition of a solution containing quantities of tris(hydroxymethyl)aminoethane ('Tris') and maleic acid buffer (both 0.0050 M). In some cases Tris–HCl buffer (1.0×10^{-2} M) was used. For the pH 5—7 range, 2-(*N*-morpholino)ethanesulphonic acid (mes) was added to 1.0×10^{-2} M NaOH to give the required pH. Acetate buffer used for pH <5 was prepared by addition of acetic acid to 10^{-2} M sodium acetate. All these buffers have given satisfactory agreement in overlap regions in previous studies; pH values were checked on a Radiometer (PHM 62) meter fitted with a Russell (CWR/22) combined electrode.

Kinetic Studies.—The ionic strength of reactant solutions was adjusted to I = 0.10 M (NaCl). Rate constants were obtained by the stopped-flow method using a Dionex-D110 spectrophotometer. The proteins PCu(II) and ACu(II) were in large (≥10-fold) excess of the reductant. The reaction cyt c(II) + PCu(II) was monitored at 416 nm, $\Delta\varepsilon = 4.0 \times 10^4$ M^{-1} cm^{-1} for cyt c(II) and cyt c(III), Hipip(r) + PCu(II) at 480 nm, which gives the largest difference in absorption for the two Hipip(r) states, cyt c(II) + ACu(II) at 416 nm, and Hipip(r) + ACu(II) at 480 nm. Relevant spectra have been illustrated in previous papers.[12–14] An absorption coefficient ε = 4 800 M^{-1} cm^{-1}, as reported by Tennent and McMillin,[15] has been assumed for the ACu(II) peak at 625 nm. Rate constants were obtained using a Datalab DL901 transient recorder and Commodore PET 2001-16K desktop computer. Plots of absorbance (A) changes $\ln|A_\infty - A_t|$ against time were linear to at least 3—4 half-lives. First-order rate constants, $k_{obs.}$, were obtained from the slopes. Because the reactions with Hipip(r) as reductant are not very favourable thermodynamically, it is important that a sufficiently high oxidant concentration be used to ensure ≥90% completion, see comments in ref. 13. With this proviso, all absorbance changes

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

Table 1. Dependence of first-order rate constants $k_{obs.}$ (25 °C) for the plastocyanin, PCu(II), oxidation of cytochrome c(II) (*ca.* 1×10^{-6} M) on pH, $\lambda = 416$ nm, $I = 0.10$ M (NaCl)

pH	10^5[PCu(II)]/M	$k_{obs.}/s^{-1}$	$10^{-6} k_{exp.}/M^{-1} s^{-1}$	pH	10^5[PCu(II)]/M	$k_{obs.}/s^{-1}$	$10^{-6} k_{exp.}/M^{-1} s^{-1}$
4.60 [a]	1.25	5.8	0.46	5.55 [b]	1.76	21.7	1.23
	2.57	11.2	0.44		3.79	48.4	1.28
4.90 [a]	1.74	13.1	0.75	6.10 [b]	2.23	34.4	1.54
	2.77	20.9	0.75	7.00 [c]	2.10	31.7	1.51
5.17 [b]	1.92	18.6	0.97	7.60 [c]	—	—	1.49 [d]
	3.93	39.0	0.99	8.00 [c]	2.10	32.1	1.53

[a] Acetate. [b] mes. [c] Tris-maleate. [d] Average of 14 independent determinations from ref. 10 with [PCu(II)] in the range (2.0—4.0) $\times 10^{-5}$ M.

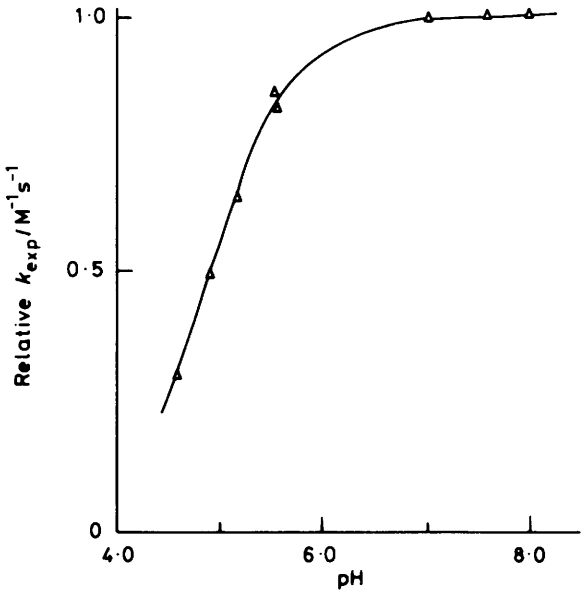

Figure 1. The dependence of second-order rate constants (25 °C) on pH for the cytochrome c(II) reduction of plastocyanin, PCu(II), $I = 0.10$ M (NaCl). (Δ) Value from ref. 10 on an average of 14 independent determinations at pH 7.6 (Tris-maleate)

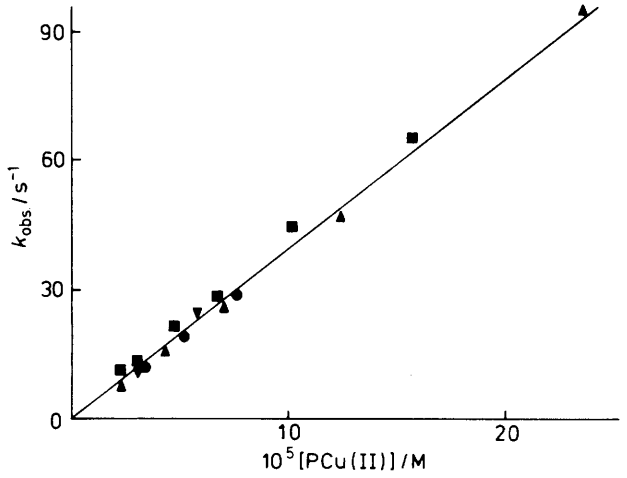

Figure 2. Linear dependence of first-order rate constants (25 °C) for the Hipip(r) reduction of plastocyanin, PCu(II), on concentration of PCu(II) at $I = 0.10$ M (NaCl), and the independence of rate constants on pH: pH 5.0 (●), pH 5.8 (▲), pH 7.5 (■), and pH 8.4 (▼)

were consistent with 1 : 1 stoicheiometries as in equations (1) and (2). First-order rate constants, $k_{obs.}$, can be converted

$$\text{cyt c(II)} + \text{PCu(II)} \longrightarrow \text{cyt c(III)} + \text{PCu(I)} \quad (1)$$

$$\text{Hipip(r)} + \text{ACu(II)} \longrightarrow \text{Hipip(o)} + \text{ACu(I)} \quad (2)$$

into second-order rate constants, $k_{exp.}$, in those cases in which a linear dependence on reductant concentration has been demonstrated.[10]

Treatment of Data.—All computations involving equations (6) and (7) employed an unweighted non-linear least-squares program.

Results

Effects of pH.—These are interpreted in terms of the sequence (3)—(5), where C denotes the copper protein and P

$$H^+ + C \overset{K_H}{\rightleftharpoons} H^+C \quad (3)$$

$$C + P \xrightarrow{k_o} \text{products} \quad (4)$$

$$H^+C + P \xrightarrow{k_H} \text{products} \quad (5)$$

the redox partner cyt c(II) or Hipip(r). Protonation effects are assigned to the copper protein, since it is known that reactions of cytochrome c(II) are not influenced by pH within the range investigated,[16,17] and those of Hipip(r) are generally confined to the (at first) small effects at pH < 6.[14]

Rate constants for the cyt c(II) + PCu(II) reaction are very dependent on pH, Table 1. The variation of second-order rate constants, $k_{exp.}$, with pH is shown in Figure 1. Equation (6) can be derived from (3)—(5). A good fit is obtained with

$$k_{exp.} = \frac{k_o + k_H K_H [H^+]}{1 + K_H [H^+]} \quad (6)$$

$k_o = (1.54 \pm 0.02) \times 10^6$ M^{-1} s^{-1}, $k_H = -(0.18 \pm 0.21) \times 10^6$ M^{-1} s^{-1} (assumed to be zero), and $K_H = (7.9 \pm 1.6) \times 10^4$ M^{-1} which corresponds to a pK_a of 4.9 ± 0.1.

Only random (±7%) effects of pH are observed on rate constants $k_{exp.}$ (Table 2) for the Hipip(r) + PCu(II) reaction (see slopes in Figure 2), and it is concluded that there is no significant pH dependence in the range 5.0—8.4.

Rate constants, $k_{exp.}$, for the cyt c(II) and Hipip(r) reduction of ACu(II), Tables 3 and 4, give pH profiles as shown in Figure 3. Parameters obtained for the cyt c(II) + ACu(II) reaction are $k_o = (4.0 \pm 0.07) \times 10^3$ M^{-1} s^{-1}, $k_H = (6.7 \pm 0.06) \times 10^3$ M^{-1} s^{-1}, $K_H = (7.4 \pm 0.9) \times 10^6$ M^{-1}, which corresponds to a $pK_a = 6.9 \pm 0.1$; and for the Hipip(r) + ACu(II) reaction, $k_o = (1.23 \pm 0.03) \times 10^5$ M^{-1} s^{-1}, $k_H = (2.49 \pm 0.04) \times 10^5$ M^{-1} s^{-1}, $K_H = (2.0 \pm 0.5) \times 10^6$ M^{-1} (pK_a 6.3 ± 0.1).

Table 2. The effect of pH on first-order rate constants, $k_{obs.}$, at 25 °C, for the plastocyanin, PCu(II), oxidation of Hipip(r) (*ca.* 4×10^{-6} M), $\lambda = 480$ nm, $I = 0.10$ M (NaCl)

pH	10^5[PCu(II)]/M	$k_{obs.}/s^{-1}$	$10^{-5} k_{exp.}/M^{-1} s^{-1}$	pH	10^5[PCu(II)]/M	$k_{obs.}/s^{-1}$	$10^{-5} k_{exp.}/M^{-1} s^{-1}$
5.0 [a]	3.4	11.5	3.4	7.5 [b]	3.1	12.8	4.1
	5.2	19.1	3.7		4.8	20.9	4.4
	7.6	28.9	3.8		6.7	27.9	4.2
5.8 [c]	4.3	15.5	3.6		10.1	45.0	4.5
	7.0	25.7	3.7		15.8	65.0	4.1
	12.4	49.0	4.0	8.4 [b]	3.1	10.7	3.5
	23.8	95.0	4.0		5.8	24.4	4.2

[a] Acetate. [b] Tris. [c] mes.

Table 3. Dependence of rate constants (25 °C) on pH for the cytochrome c(II) (*ca.* 10^{-6} M) reduction of azurin, ACu(II), $\lambda = 416$ nm, $I = 0.10$ M (NaCl)

pH	10^5[ACu(II)]/M	$10^{-1} k_{obs.}/s^{-1}$	$10^{-3} k_{exp.}/M^{-1} s^{-1}$	pH	10^5[ACu(II)]/M	$10^{-1} k_{obs.}/s^{-1}$	$10^{-3} k_{exp.}/M^{-1} s^{-1}$
5.17 [a]	0.70	0.47	6.7	6.83 [a]	3.30	1.79	5.4
	0.92	0.60	6.5		4.04	2.20	5.5
6.10 [a]	1.57	1.04	6.6	7.30 [b]	2.60	1.24	4.8
	4.60	2.95	6.4		3.36	1.55	4.6
	4.97	3.13	6.3	8.60 [b]	1.81	0.75	4.1
	8.40	5.41	6.4		8.53	3.43	4.0
	24.5	15.3	6.2	8.80 [b]	4.55	1.83	4.0
6.30 [a]	3.44	2.14	6.2				
	4.29	2.66	6.2				

[a] mes. [b] Tris–maleate.

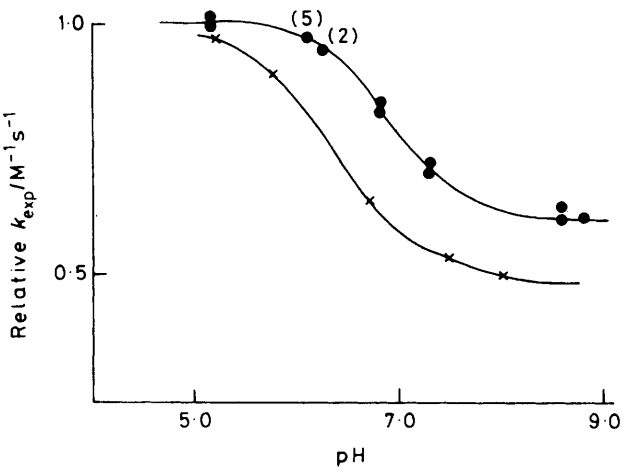

Figure 3. The dependence of rate constants, $k_{exp.}$, on pH for the cytochrome c(II) (●) and Hipip(r) (×) reductions of azurin, ACu(II), $I = 0.10$ M (NaCl). Numbers of points averaged are indicated in parentheses

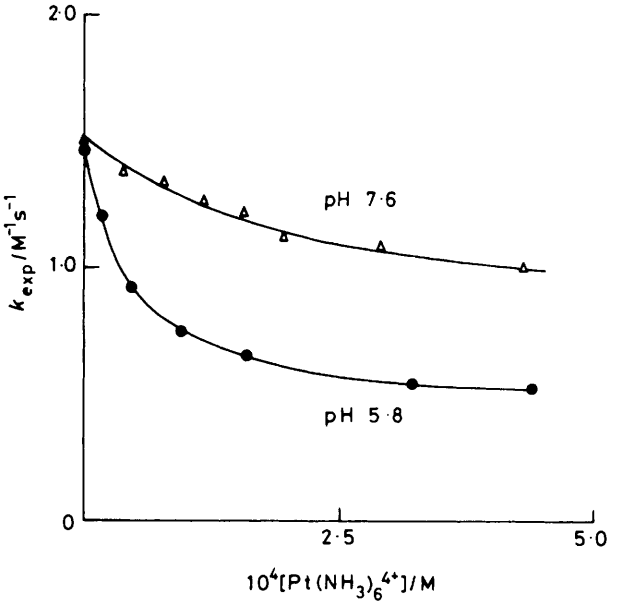

Figure 4. The competitive effect of redox-inactive $[Pt(NH_3)_6]^{4+}$ on rate constants, $k_{exp.}$, at 25 °C for the cytochrome c(II) reduction of plastocyanin at pH 7.6 (Tris–maleate) and 5.8 (mes), $I = 0.10$ M (NaCl)

Effects of $[Pt(NH_3)_6]^{4+}$.—It has been demonstrated previously that redox-inactive $[Pt(NH_3)_6]^{4+}$ produces strong partial competitive inhibition of PCu(II) and PCu(I) reactions with positively charged inorganic redox partners.[2,3] The same effect is observed for the cyt c(II) and PCu(II) reaction, Table 5. Two pH values, one either side of the acid dissociation of $[Pt(NH_3)_6]^{4+}$ (pK_a 7.1) were studied, Figure 4. Data at each pH can be fitted to the expression (7), which is obtained for the sequence (8)—(10). At pH 7.6, $k = (1.52 \pm 0.10) \times$

$$k_{exp.} = \frac{k + k_B K_B [Pt^{IV}]}{1 + K_B [Pt^{IV}]} \quad (7)$$

$$PCu(II) + Pt^{IV} \overset{K_B}{\rightleftharpoons} PCu(II)\cdot Pt^{IV} \quad (8)$$

$$\text{cyt c(II)} + PCu(II) \xrightarrow{k} \text{products} \quad (9)$$

$$\text{cyt c(II)} + PCu(II)\cdot Pt^{IV} \xrightarrow{k_B} \text{products} \quad (10)$$

10^6 M^{-1} s^{-1}, and from the fit $K_B = 4\,300 \pm 800$ M^{-1}, $k_B = (0.58 \pm 0.09) \times 10^6$ M^{-1} s^{-1}. At pH 5.8, $k = (1.50 \pm 0.10) \times 10^6$ M^{-1} s^{-1}, $K_B = 20\,900 \pm 500$ M^{-1}, and $k_B = (0.41 \pm 0.01) \times 10^6$ M^{-1} s^{-1}. The 3+ conjugate-base form $[Pt(NH_3)_5(NH_2)]^{3+}$ binds less strongly than $[Pt(NH_3)_6]^{4+}$.

Table 4. The dependence of rate constants (25 °C) on pH for the Hipip(r) (*ca.* 2 × 10^{-6} M) reduction of azurin, ACu(II), λ = 450 nm, I = 0.10 M (NaCl)

pH	10^5[ACu(II)]/M	$k_{obs.}/s^{-1}$	$10^{-5}k_{exp.}/M^{-1}\ s^{-1}$	pH	10^5[ACu(II)]/M	$k_{obs.}/s^{-1}$	$10^{-5}k_{exp.}/M^{-1}\ s^{-1}$
5.2 [a]	3.67	8.8	2.40	6.78 [b]	2.59	3.9	1.52
					2.76	4.1	1.49
5.8 [a]	3.86	8.4	2.18		10.3	15.7	1.52
	4.2	9.6	2.29				
	4.5	10.0	2.22	7.5 [b]	3.8	5.1	1.34
	4.8	10.4	2.17				
	7.0	15.3	2.19	8.0 [b]	3.3	4.1	1.24
6.73 [b]	2.67	4.9	1.84				
	4.2	7.0	1.67				
	6.6	10.7	1.62				
	15.6	23.2	1.49				
	21.6	34.3	1.59				

[a] mes. [b] Tris.

Table 5. Effect of the redox-inactive complex $[Pt(NH_3)_6]^{4+}$ on rate constants (25 °C) for the plastocyanin PCu(II) (*ca.* 2 × 10^{-5} M) oxidation of cytochrome c(II) (*ca.* 1 × 10^{-6} M) at two pH values, I = 0.10 M (NaCl)

pH	10^4[Pt^{IV}]/M	$k_{exp.}/M^{-1}\ s^{-1}$
7.6 [a]	0	1.52
	0.40	1.39
	0.79	1.35
	1.18	1.27
	1.57	1.23
	1.96	1.12
	2.89	1.09
	4.35	1.00
5.8 [b]	0	1.50
	0.16	1.20
	0.47	0.93
	0.94	0.76
	1.57	0.66
	3.15	0.56
	4.4	0.52

[a] Tris–maleate. [b] mes.

Table 6. The increase in rate constant $k_{obs.}$ (25 °C) for the Hipip(r), (3—4) × 10^{-6} M, reduction of PCu(II), (3—5) × 10^{-5} M, brought about by the addition of $[Pt(NH_3)_6]^{4+}$, I = 0.10 M (NaCl)

pH	$10^5[Pt(NH_3)_6^{4+}]$/M	$10^{-5}\ k_{exp.}/M^{-1}\ s^{-1}$
7.5 [a]	0	4.0
	6.0	5.7
	11.9	6.0
	23.2	6.8
5.8 [b]	0	3.6
	6.5	4.5
	12.7	5.6
	24.6	6.1
	35.8	6.4

[a] Tris–HCl. [b] mes.

An acceleration effect of $[Pt(NH_3)_6]^{4+}$ on the Hipip(r) + PCu(II) reaction is observed, Table 6. As in the case of the $[Fe(CN)_6]^{3-}$ + PCu(I) reaction, this is difficult to quantify in a meaningful way. Possible interactions to be considered include that of $[Fe(CN)_6]^{3-}$ with $[Pt(NH_3)_6]^{4+}$.

Effects of Chromium(III) *Modification.*—These effects were studied for the two PCu(II) reactions only. It is possible to attach 1 and 2 mol of Cr^{III} to give PCu(II)·Cr^{III} and PCu(II)·2Cr^{III} as previously indicated.[1]

Table 7. Rate constants (25 °C) indicating the effect of chromium(III) modification of plastocyanin on the reaction with cytochrome c(II) (*ca.* 1 × 10^{-6} M), pH 5.8 (mes), I = 0.10 M (NaCl)

	10^5[PCu(II)]/M	$10^{-1}\ k_{obs.}/s^{-1}$
Native PCu(II)	1.19	1.70
	1.83	2.66
	2.44	3.87
	3.5	5.3
PCu(II)·Cr^{III}	1.03	1.00
	1.57	1.52
	2.29	2.68
PCu(II)·2Cr^{III}	1.82	1.13
	2.50	1.71
	3.43	2.30

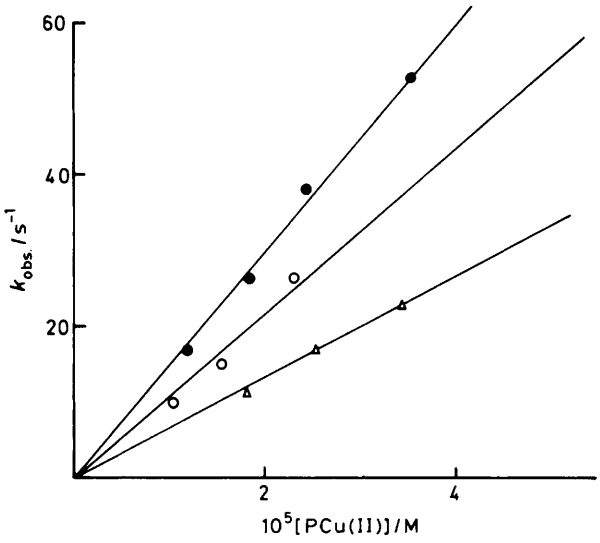

Figure 5. The dependence of first-order rate constants, $k_{obs.}$ (25 °C), on plastocyanin (reactant in large excess) for the cytochrome c(II) reduction of native plastocyanin PCu(II) (●) and singly (○) and doubly (△) chromium(III)-modified PCu(II) at pH 5.8 (mes), I = 0.10 M (NaCl)

The biphasic kinetics observed for the reaction of PCu(II)·Cr^{III} with $[Co(phen)_3]^{3+}$ at pH 5.8 suggest [1,11] that the Cr is bound in two ways. For the cyt c(II) + PCu(II)·Cr^{III} reaction, it was not possible to identify two consecutive stages, if indeed

Table 8. Rate constants (25 °C) indicating the effect of chromium(III) modifications of plastocyanin on the reaction with Hipip(r) (*ca.* 2 × 10^{-6} M), pH 5.8 (mes), I = 0.10 M (NaCl)

	10^5[PCu(II)]/M	$k_{obs.}/s^{-1}$
Native PCu(II)	2.46	6.0
	2.86	6.9
	5.1	12.5
PCu(II)·Cr^{III}	2.05	5.3
	2.69	6.4
	3.33	8.0
PCu(II)·$2Cr^{III}$	2.41	4.0
	2.72	4.8
	3.90	6.4

two exist. Accordingly, single-stage first-order rate constants which give $k_{1Cr} = (1.10 \pm 0.08) \times 10^6$ M^{-1} s^{-1} are reported, Table 7. Similarly, with PCu(II)·$2Cr^{III}$ a second-order rate constant $k_{2Cr} = (0.67 \pm 0.02) \times 10^6$ M^{-1} s^{-1} is obtained from $k_{obs.}$ values in Table 7. The modifications give respectively 29% (k_{1Cr}) and 55% (k_{2Cr}) retardation effects, see Figure 5.

With Hipip(r) as reductant, Table 8, no effect on rate constants is obtained with singly modified protein PCu(II)·Cr^{III}, but a 35% decrease is observed for PCu(II)·$2Cr^{III}$.

Discussion

The different effects of pH on rate constants for the cytochrome c(II) and Hipip(r) reductions of PCu(II) are consistent with utilisation of different binding sites on PCu(II). With cytochrome c(II) a significant pH dependence is observed (pK_a 4.9) which is very similar to that for the $[Ru(NH_3)_5(py)]^{2+}$ (py = pyridine) reduction of PCu(II) (pK_a 5.0).[2] In the latter case a variety of evidence is consistent with electron transfer and protonation taking place at the negative patch on PCu(II) which incorporates residues 42—45 and is near to Tyr 83. Since the two pK_a values are very similar, it is concluded that they are likely to be for the same acid dissociation and that cytochrome c(II) reacts also at the Tyr 83/42—45 binding site. Understandably, in view of its negative charge, the site appears to have specificity for positively charged redox partners, whether these are inorganic complexes or in the present case a protein. At the lower pH, rate constants for the cytochrome c(II) reaction are small, and the reaction appears to be more sensitive to the protonation than $[Ru(NH_3)_5(py)]^{2+}$.

The observation that pH (5.0—8.4) has no effect (±7%) on rate constants for the reaction of Hipip(r) and PCu(II) is in sharp contrast, indicating that a different site on PCu(II) is used. With inorganic complexes as redox partners for Hipip(r), effects on reactivity (at first small) originating from the protein have been noted at pH < 6, and we do not rule out the possibility of two (small) opposing trends cancelling. The results obtained are similar to those for the reaction of $[Fe(CN)_6]^{4-}$ with PCu(II) at pH 4.5—7.5, when only a small upward trend in rate constants was observed with decreasing pH.[2,8] Therefore the implications would seem to be that negatively charged Hipip(r) is behaving like $[Fe(CN)_6]^{4-}$, and using the same reaction site on PCu(II). Line-broadening n.m.r. studies have suggested that this is close to His 87.[4,5]

The competitive inhibition effect of $[Pt(NH_3)_6]^{4+}$ on the cytochrome c(II) reduction of PCu(II), where $[Pt(NH_3)_6]^{4+}$ is known to have a specificity for the Tyr 83/42—45 site, again implicates this binding site on PCu(II). Partial inhibition only is observed and, as with inorganic redox partners, a fairly large area of protein surface appears to be relevant. A case against the proposal that this behaviour is consistent with reaction at two different binding sites has been made.[8] The acceleration effect of $[Pt(NH_3)_6]^{4+}$ on the Hipip(r) reduction of PCu(II), which is similar to that observed for the $[Fe(CN)_6]^{4-}$ reduction of PCu(II), is ambiguous,[2] and has not been quantified. One possible explanation is that association of $[Pt(NH_3)_6]^{4+}$ at the Tyr 83/42—45 site makes the latter electrostatically accessible to the negatively charged Hipip(r) and $[Fe(CN)_6]^{4-}$.

The approach using chromium(III)-modified PCu(II) is potentially the most powerful, although the background is slightly complicated.[1] Farver and Pecht [18] have concluded that the Cr^{III} is covalently attached at the 42—45 site, and this we accept. However, from studies with inorganic redox partners at pH 5.8 it would appear that *ca.* 40% of the Cr is attached in a more influential manner than the rest.[1] The single chromium(III) modification inhibits reaction with cytochrome c(II) consistent with residues 42—45 being a part of the binding site. Interestingly, this reaction does not appear to be sensitive as to how the Cr^{III} is bound, and only a single rate constant (k_{1Cr}) is detected at pH 5.8. Presumably this is a consequence of the bigger size of the cytochrome c. The singly modified plastocyanin does not affect the reduction with Hipip(r), which supports the belief that binding is at an alternative site. Attachment to two Cr^{III} results in further inhibition of the reaction with cytochrome c(II), which is consistent with effects previously noted.[1] It also produces an inhibition of the Hipip(r) reduction which we have difficulty in explaining.

To summarise, with plastocyanin the effects of pH, competitive inhibition, and chromium(III) modification give meaningful and self-consistent assignments as to binding sites adopted using protein redox partners. The binding sites appear to be the same as those used by inorganic complexes, suggesting that for proteins which are not natural partners, electrostatics play a dominant role. In the case of the cytochrome c(II) reduction of PCu(II) it is of further interest that the approximate location of binding sites on both proteins has now been defined.[19] The distance from the Tyr 83/42—45 site to the Cu of PCu(II) is *ca.* 15 Å. A fairly large protein surface area appears relevant making it difficult to be more specific. It has been demonstrated that the haem edge of the cytochrome c is involved,[19] and a Cu to Fe separation of *ca.* 20 Å is implied for electron transfer.

Studies with azurin are at present less detailed, with no consideration of the effects of competitive inhibition or chromium(III) modification. From the effects of pH on rate constants different acid-dissociation constants have been obtained for the reduction of ACu(II) with cytochrome c(II) (pK_a 6.9) and Hipip(r) (pK_a 6.3). Inspection of Figure 3 makes clear the different responses. For Hipip(r) the variation is in the direction expected from a consideration of simple electrostatics, where association of the two reactants prior to electron transfer is expected to benefit from the presence of the proton. However, with cytochrome c(II) a similar explanation cannot hold and some other factor, possibly a conformational change, is implicated. Previously reported pK_a values for ACu(II) are from n.m.r. studies (pK_a 7.35),[20] and the kinetics of the $[Fe(CN)_6]^{4-}$ reduction (pK_a 6.12).[9] Two different pK_a values have been obtained for ACu(I) from kinetic studies on the reduction of $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) (pK_a 7.6) and $[Fe(CN)_6]^{3-}$ (7.1).[9] Equilibrium studies on the cytochrome c_{551} reaction with azurin have given a pK_a of *ca.* 7.0 for ACu(I).[21] For pK_a values of this magnitude, uncoordinated imidazoles, of which ACu(II) form *Pseudomonas aeruginosa* has two, are most likely to be involved. We note that the oxidation state of the Cu appears to be influential as far as both pK_a values are concerned. As in previous studies with inorganic redox partners, it is possible to assign the pK_a of 6.9 with cytochrome c(II) (8+) to acid dissociation

at His 83 and the pK_a of 6.3 with Hipip(r) (3−) to His 35. If this approach is correct then the binding site for Hipip(r) may be close to His 35, but that for cytochrome c(II) need not necessarily be adjacent to His 83 if a conformational change is involved. The His 83 is in any case probably too far removed from the copper active site to constitute a binding site. Two different binding sites selected according to charge of the redox partners seems to be implied. However, ACu(II) has a small (1−) overall charge at pH *ca.* 7 and from present structural information [22] does not appear to have localities of high charge as a number of other metalloproteins including plastocyanin have.[23] Interpretation for azurin remains less certain for the present therefore.

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