Kinetic Studies on 1 :1 Electron-transfer Reactions involving Blue Copper Proteins. Part 6.† Competitive Inhibition of the $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) Oxidation of Parsley Plastocyanin PCu(I) by Redox-inactive Complexes ‡

Stephen K. Chapman, Alan D. Watson, and A. Geoffrey Sykes *
Department of Inorganic Chemistry, The University, Newcastle upon Tyne NE1 7RU

A search has been carried out for redox-inactive inorganic complexes which exhibit competitive inhibition (*i.e.* blocking) of the $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) oxidation of negatively charged parsley plastocyanin, PCu(I), at 25 °C and I = 0.10 M (NaCl). Three complexes have been identified which give greater association (K_B/M^{-1}) with PCu(I) than that previously determined for $[Cr(phen)_3]^{3+}$ (176 M^{-1}). Association constants (M^{-1}) obtained at pH 7.5 are: $[Co(NH_3)_3]^{3+}$, 470; $[Pt(NH_3)_6]^{4+}$, 22 300; and $[(NH_3)_5Co(NH_2)Co(NH_3)_5]^{5+}$, 16 000. Acid dissociation of $[Pt(NH_3)_6]^{4+}$, pK_a 7.1, requires that a lower pH be used for maximum effectiveness of this complex. Partial blocking only is observed in all three cases, and it is concluded that the site for electron transfer involves a broad region of protein surface incorporating Tyr 83 and the negative patch of residues including 42—45. The size of the effect for the 4+ and 5+ complexes makes these complexes most appropriate for further studies in which use of this site is tested for. It has been demonstrated with $[Pt(NH_3)_6]^{4+}$ that association with PCu(I) becomes less effective at pH < 6.6. The protein acid dissociation pK_a of 5.8 which is obtained is coincident with pK_a values reported previously for the inactivation of PCu(I). A possible explanation involving a co-operative effect between the active site and negative patch 42—45 is proposed. No similar competitive inhibition of the $[Fe(CN)_6]^{3-}$ oxidation of PCu(I) by redox-inactive complexes $[Zr(C_2O_4)_4]^{4-}$ and $[Mo(CN)_8]^{4-}$ is observed at pH 7.5.

The single (type 1) copper protein plastocyanin (M 10 500; 99 amino acids) [1-3] is one of a number of metalloproteins involved in electron transport between photosystems II and I in the chloroplast of higher plants.[4] Crystal structure information has been obtained by Freeman and co-workers for poplar leaf plastocyanin [5] in both the PCu(II) and PCu(I) states, with refinement reported to 1.6 Å resolution.[6] An understanding of the way in which plastocyanin functions includes an identification of the site or sites on the protein surface at which electron transfer takes place, and (implied) the distance over which electrons are transferred. Use of inorganic complexes as probes for redox reactivity is of continuing interest in attempting to define such sites.[7,8] The long-term aim is to define approaches which can first be tested using selected protein redox partners for plastocyanin, and which can then be applied to reactions involving the physiological redox partners cytochrome f (as reductant) and P700 (as oxidant).

Results reported here are for the competitive inhibition effects of positively charged redox-inactive complexes on the $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) oxidation of PCu(I). Observations in previous studies that first-order rate constants ($k_{obs.}$) for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) approach a limiting value with increasing concentration of oxidant (present in >10-fold excess), dependence as in equation (1),[7] form the basis of the present study. An explanation in terms of association of the reactants (K) prior to electron transfer (k_{et}) has been proposed.[7] For the $[Fe(CN)_6]^{3-}$ oxidation of PCu(I), K is at a lower level and the denominator in equation (1) is unity.[9] Since K can be determined for $[Co(phen)_3]^{3+}$ a reasonable expectation is that redox-inactive complexes, having similar properties, might also associate strongly. At relatively low $[Co(phen)_3]^{3+}$ concentrations, runs in which varying amounts of redox-inactive complex are introduced can be carried out and effects noted. It has already been shown in this way that redox-inactive $[Cr(phen)_3]^{3+}$ inhibits [8] the reaction with $[Co(phen)_3]^{3+}$. Moreover from ^{1}H n.m.r. line broadening, it has been demonstrated that the (paramagnetic) chromium(III) complex associates preferentially at a site close to Tyr 83.[10,11] However, association of $[Cr(phen)_3]^{3+}$ is not sufficiently extensive (K_B = 176 M^{-1}) to provide a certain means of identifying utilisation of this same site by other redox partners. We therefore decided to explore further the possibility of identifying complexes which might give larger effects.

$$k_{obs.} = \frac{Kk_{et}[\text{oxidant}]}{1 + K[\text{oxidant}]} \qquad (1)$$

Experimental

Preparation of Protein.—Plastocyanin was isolated from parsley leaves according to the method of Plesničar and Bendall.[12] It was purified by column chromatography, stored (*ca.* 2.5 × 10^{-5} M), and prepared for runs by procedures previously described.[7] The oxidised form PCu(II) gave an absorbance peak ratio A_{278}/A_{597} = 1.7 ± 0.1 consistent with previous reports and indicating satisfactory purity. At 597 nm the absorption coefficient for PCu(II) is 4.5 × 10^3 M^{-1} cm^{-1}. To obtain PCu(I) a few crystals of sodium dithionite (BDH, GPR grade), sufficient to remove the blue colour and representing an excess of reductant, were added. Solutions were then dialysed (Sigma, 250-7U sacks) against the appropriate buffer for at least 30 h at *ca.* 2 °C.

Preparation of Complexes.—These were obtained and purified to known spectra (λ/nm, ε/M^{-1} cm^{-1}) by procedures already described: tris(1,10-phenanthroline)cobalt(III) perchlorate, $[Co(phen)_3][ClO_4]_3$,[13] 330 (4 660), 350 (3 620), and 450 (100); hexa-amminecobalt(III) chloride, $[Co(NH_3)_6]Cl_3$,[14] 339 (47) and 473 (58); hexa-ammineplatinum(IV) chloride, $[Pt(NH_3)_6]Cl_4 \cdot H_2O$,[15] 260 (129); μ-amido-bis[penta-ammine-

---

† Part 5 is ref. 9.

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

cobalt(III)] bromide, $[(NH_3)_5Co(NH_2)Co(NH_3)_5]Br_5$,[16] 360 (705) and 505 (420); potassium tetrakis(oxalato)zirconate(IV), $K_4[Zr(C_2O_4)_4]\cdot5H_2O$, by titration, oxalate 51.5% (calc. 51.1%);[17] and potassium octacyanomolybdate(IV), $K_4[Mo(CN)_8]\cdot2H_2O$,[18] 367 (170). The chloride salt $[Co(phen)_3]Cl_3\cdot7H_2O$ [19] was used when the perchlorate salt was not sufficiently soluble. Commercial grade potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (BDH, AnalaR), λ 300 (ε 1 600) and 420 nm (1 010 M^{-1} cm^{-1}), was used without further purification.

Buffers.—Tris(hydroxymethyl)aminomethane ('Tris') (Sigma Chemicals) in 0.01 M HCl was used to buffer solutions at pH 7.5. Knowing log $K_T = 8.09$ at 25 °C (0.10 M KCl) for its protonation,[20] the required amount of buffer was calculated. For the pH range 5.0—7.0 the buffer used was 2-(*N*-morpholino)ethanesulphonic acid (mes) (Sigma Chemicals), log $K_T = 6.1$ at 25 °C. All pH values were checked using a Radiometer (PHM 62) pH-meter fitted with a combined electrode.

Kinetics.—All runs were at an ionic strength of 0.10 M adjusted with NaCl (BDH, AnalaR) except for one series in which the effect of $I = 0.20$ M (NaCl) was investigated. A Dionex D-110 stopped-flow spectrophotometer was used to monitor absorbance changes at the PCu(II) peak at 597 nm. The reduced form PCu(I) does not absorb in the visible range. A large (>10-fold) excess of the $[Co(phen)_3]^{3+}$ oxidant was used. Redox-inactive complexes could be introduced along with the $[Co(phen)_3]^{3+}$ or PCu(I) reactant (generally the latter) without any difference in behaviour. Both solutions were made up to the required pH prior to mixing. Absorbance changes (output) were either photographed from an oscilloscope or stored digitally on a Datalab DL901 transient recorder, which was interfaced to a Commodore PET 2001-16K desk-top computer. A simple program permitted display of $\ln(A_\infty - A_t)$ against time plots and relevant rate constant information. All absorbance changes were consistent with a 1 : 1 stoicheiometry, equation (2). The $\ln(A_\infty - A_t)$ plots were linear for a least four half-lives. First-order rate constants were converted into second-order values, k_{exp}, by dividing by $[Co(phen)_3{}^{3+}]$. Rate constants listed are the average from at least three determinations. When there is competitive inhibition (giving partial blocking) in the presence of a redox-inactive complex B, the reaction sequence can be expressed as in equations (3)—(6).

$$\mathrm{PCu(I)} + \mathrm{Co^{III}} \longrightarrow \mathrm{PCu(II)} + \mathrm{Co^{II}} \tag{2}$$

$$\mathrm{PCu(I)} + [\mathrm{Co(phen)_3}]^{3+} \overset{K}{\rightleftharpoons} \mathrm{PCu(I)},[\mathrm{Co(phen)_3}]^{3+} \tag{3}$$

$$\mathrm{PCu(I)},[\mathrm{Co(phen)_3}]^{3+} \xrightarrow{k_{et}} \text{products} \tag{4}$$

$$\mathrm{PCu(I)} + \mathrm{B} \xrightarrow{K_B} \mathrm{PCu(I),B} \tag{5}$$

$$\mathrm{PCu(I),B} + [\mathrm{Co(phen)_3}]^{3+} \xrightarrow{k_B} \text{products} \tag{6}$$

$$k_{exp} = \frac{Kk_{et} + K_Bk_B[\mathrm{B}]}{1 + K_B[\mathrm{B}] + K[\mathrm{Co(phen)_3}^{3+}]} \tag{7}$$

Table 1. The effect of the redox-inactive complex $[Co(NH_3)_6]^{3+}$ on second-order rate constants (25 °C) for the $[Co(phen)_3]^{3+}$ (5×10^{-4} M) oxidation of PCu(I) plastocyanin (*ca.* 10^{-5} M), at pH 7.5 (Tris–HCl) and $I = 0.10$ M (NaCl)

$10^3[Co(NH_3)_6{}^{3+}]$/M	$10^{-3}k_{exp}$/M^{-1} s^{-1}
0	2.67
0.41	2.21
0.61	2.06
0.82	1.97
1.02	1.80
1.38	1.64

Equation (7) can be derived from this sequence, where K and k_{et} are as determined previously.[8] At pH < 6.6, K is small and, for the range of $[Co(phen)_3{}^{3+}]$ adopted, contributions from $K[Co(phen)_3{}^{3+}]$ can be ignored. Equation (7) can be re-arranged to give (8), with $k = Kk_{et}$. At pH 6.6 and 7.5, K is close to 92 M^{-1} (determination at pH 6.5)[8] and 167 M^{-1} respectively, and contributions from $K[Co(phen)_3{}^{3+}]$ become important and have to be taken into account.

$$\frac{1}{(k - k_{exp})} = \frac{1}{(k - k_B)} + \frac{1}{(k - k_B)K_B[\mathrm{B}]} \tag{8}$$

Treatment of Data.—An unweighted non-linear least-squares program was used to determine all relevent parameters, *e.g.* those relating to equations (7) and (10).

Results

Effect of $[Co(NH_3)_6]^{3+}$.—Second-order rate constants, Table 1, decrease with increasing $[Co(NH_3)_6{}^{3+}]$. This complex produces a stronger inhibition than $[Cr(phen)_3]^{3+}$, but one which remains relatively mild in the context of the present studies. Since partial competitive inhibition has not been firmly established, as also with $[Cr(phen)_3]^{3+}$,[8] k_B has been assumed

Table 2. The effect of the redox-inactive complex $[Pt(NH_3)_6]^{4+}$ on second-order rate constants (25 °C) for the $[Co(phen)_3]^{3+}$ (*ca.* 1.3×10^{-4} M) oxidation of PCu(I) plastocyanin (*ca.* 10^{-5} M), at different pH values; mes buffer and $I = 0.10$ M (NaCl) except as stated

pH	$10^4[Pt^{IV}]$/ M	$10^{-3}k_{exp}$/ M^{-1} s^{-1}	pH	$10^4[Pt^{IV}]$/ M	$10^{-3}k_{exp}$/ M^{-1} s^{-1}
7.5[a]	0	2.40	5.8[b]	0	0.64
	0.23	2.04		0.84	0.59
	0.57	1.77		1.26	0.58
	1.14	1.58		1.66	0.57
	2.29	1.44		2.49	0.55
	2.29	1.40		3.30	0.54
6.6	0	2.00	5.4	0	0.73
	0.15	1.67		0.30	0.65
	0.37	1.49		0.60	0.60
	0.74	1.34		0.86	0.57
	1.49	1.19		1.59	0.52
	2.98	1.12		2.45	0.49
6.2	0	1.85	5.00	0	0.36
	0.20	1.54		0.48	0.34
	0.35	1.39		0.96	0.33
	0.70	1.25		1.60	0.31
	1.40	1.13		2.40	0.30
	2.42	1.04			
5.8	0	1.40			
	0.35	1.17			
	0.46	1.09			
	0.75	1.04			
	1.12	0.96			
	1.30	0.92			
	1.66	0.90			
	2.43	0.87			

[a] Tris–HCl. [b] $I = 0.20$ M (NaCl).

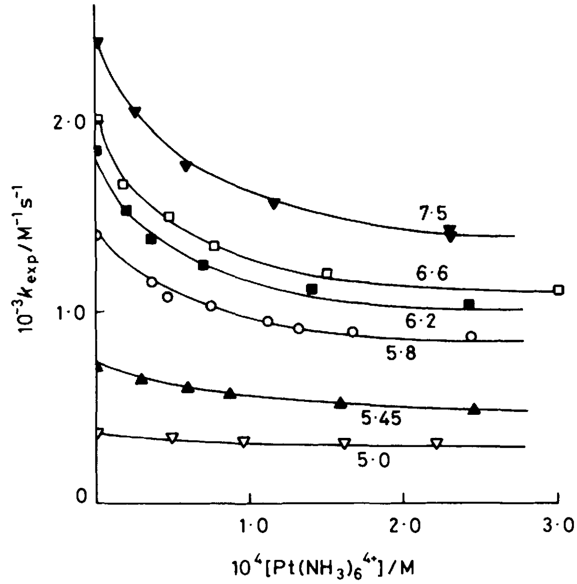

Figure 1. Variation of second-order rate constants, k_{exp} (25 °C), for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) with concentration of inhibitor $[Pt(NH_3)_6]^{4+}$, and pH (Tris–HCl for 7.5, otherwise mes) and $I = 0.10$ M (NaCl)

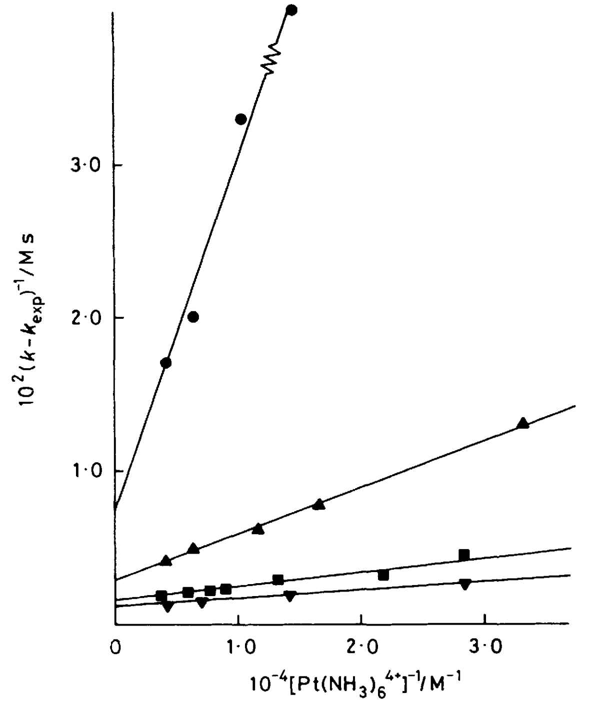

Figure 2. The dependence of second-order rate constants, k_{exp} (25 °C), for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) on inhibitor $[Pt(NH_3)_6]^{4+}$, at pH 5.0 (●), 5.4 (▲), 5.8 (■), and 6.2 (▼); $I = 0.10$ M (NaCl)

to be zero in equation (7), when $K_B = 470 \pm 20\ M^{-1}$. With the inclusion of k_B, values obtained are $K_B = 580 \pm 120\ M^{-1}$ and $k_B = 540 \pm 100\ M^{-1}\ s^{-1}$.

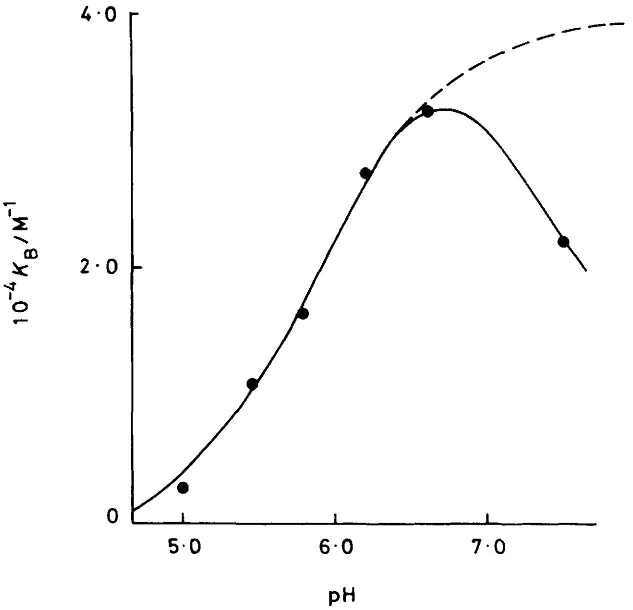

Figure 3. The variation of K_B for association of inhibitor $[Pt(NH_3)_6]^{4+}$ and PCu(I) with pH at 25 °C and $I = 0.10$ M (NaCl). The broken line is from a computer fit, and indicates the behaviour which would be observed if acid dissociation of $[Pt(NH_3)_6]^{4+}$ did not occur

Effect of $[Pt(NH_3)_6]^{4+}$.—Second-order rate constants, k_{exp}, are listed in Table 2. The effect of $[Pt(NH_3)_6]^{4+}$ at different pH values is indicated in Figure 1. An acid dissociation pK_a has been determined for $[Pt(NH_3)_6]^{4+}$, equation (9),

$$[Pt(NH_3)_6]^{4+} \rightleftharpoons [Pt(NH_3)_5(NH_2)]^{3+} + H^+ \quad (9)$$

from the effects of pH (mes buffer) on the absorbance at 275 nm. At 25 °C and $I = 0.10$ M (NaCl), pK_a = 7.1 in good agreement with the literature value of 7.2 at $I = 0.02$ M.[21] At

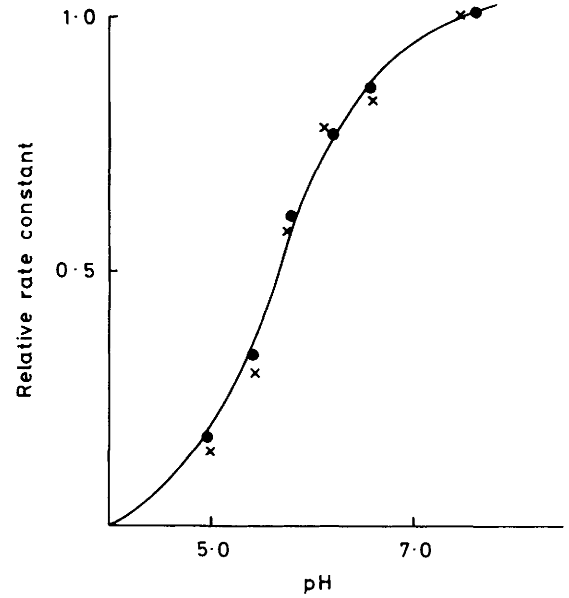

Figure 4. A comparison of the effect of pH on second-order rate constants (relative scale), k (×), for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) with those, k_B (●), for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I),$[Pt(NH_3)_6]^{4+}$ at 25 °C and $I = 0.10$ M (NaCl)

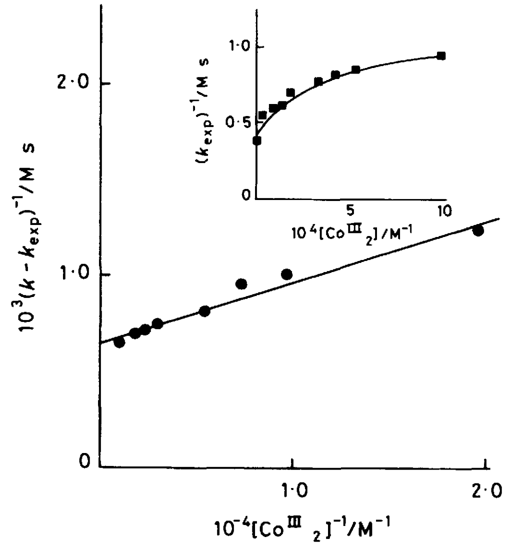

Figure 5. The dependence of second-order rate constants, k_{exp} (25 °C), for the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) on inhibitor $[(NH_3)_5Co(NH_2)Co(NH_3)_5]^{5+}$ at pH 7.5 (Tris-HCl) and I = 0.10 M (NaCl). The insert shows the non-linearity of the $(k_{exp})^{-1}$ against $[Co^{III}{}_2]$ plot and the need to include k_B as in equation (8)

any one pH, equation (8) gives a good fit of k_{exp}, Figure 2, with inclusion of k_B an essential requirement. An increase in K_B is observed as the pH is decreased from 7.5 to 6.6, Table 3, consistent with greater competitive inhibition of $[Pt(NH_3)_6]^{4+}$ as compared to the 3+ conjugate base. Not enough information has been obtained at pH > 6.6 to give an accurate K_B for the conjugate-base form. Below pH 6.6, K_B decreases to zero (Figure 3, a small negative number is obtained) and equation (10) gives a satisfactory fit,[7] where K'_B here is for the

$$K_B = K'_BK_a/(K_a + [H^+]) \qquad (10)$$

association of $[Pt(NH_3)_6]^{4+}$ at high pH. A protein acid dissociation, $pK_a = 5.8 \pm 0.1$, is obtained. The latter is in agreement with values obtained previously from rate constant *vs.* pH profiles (pK_a 5.7—6.1), and assigned to proton inactivation of the copper(I) active site.[6] Rate constants k_B decrease in a similar manner with decreasing pH, Figure 4. An increase in ionic strength from 0.10 to 0.20 M decreases the effectiveness of $[Pt(NH_3)_6]^{4+}$ as expected for an association of positively and negatively charged reactants.

Effect of $[(NH_3)_5Co(NH_2)Co(NH_3)_5]^{5+}$.—Rate constants, Table 4, again indicate a strong inhibition of the PCu(I) + $[Co(phen)_3]^{3+}$ reaction. A linear plot of $(k - k_{exp})^{-1}$ against $[Co^{III}{}_2]^{-1}$, where $Co^{III}{}_2$ represents the binuclear 5+ complex, is obtained, Figure 5. The inset illustrates the inadequacy of the treatment excluding contributions from k_B. From a fit of equation (7) to the data at pH 7.5, $K_B = (1.6 \pm 0.2) \times 10^4$ M^{-1} and $k_B = (1.03 \pm 0.04) \times 10^3$ M^{-1} s^{-1}.

Other Results.—The possibility that redox-inactive complexes $[Mo(CN)_8]^{4-}$ (2×10^{-3} M) and $[Zr(C_2O_4)_4]^{4-}$ (2×10^{-3} M) produce a blocking effect on the $[Fe(CN)_6]^{3-}$ oxidation of PCu(I) was investigated at 25 °C, pH 7.5 (Tris-HCl), and I = 0.10 M (NaCl). No variation in rate constants was obtained on addition of these complexes. Redox-inactive $[Pt(NH_3)_6]^{4+}$ accelerates the $[Fe(CN)_6]^{3-}$ oxidation of PCu(I), Table 5. No simple rate law fit has been possible.

Table 3. Competitive inhibition of the $[Co(phen)_3]^{3+}$ oxidation of PCu(I) by $[Pt(NH_3)_6]^{4+}$ at 25 °C, mes buffer and I = 0.10 M (NaCl) (except as stated), and the effect of pH on K_B and k_B

pH	$10^{-3}K_B$/M^{-1}	$10^{-3}k_B$/M^{-1} s^{-1}
7.5 [a]	22.3 ± 1.6	1.20 ± 0.03
6.6	34.6 ± 2.7	1.03 ± 0.02
6.2	26.9 ± 1.6	0.92 ± 0.02
5.8	16.4 ± 2.0	0.72 ± 0.03
5.8 [b]	4.5 ± 0.4	0.48 ± 0.01
5.45	10.7 ± 0.2	0.40 ± 0.02
5.0	2.7 ± 0.4	0.21 ± 0.01

[a] Tris-HCl. [b] I = 0.20 M (NaCl).

Table 4. The effect of the redox-inactive complex $[(NH_3)_5Co(NH_2)Co(NH_3)_5]^{5+}$ on second-order rate constants (25 °C) for the $[Co(phen)_3]^{3+}$ (5×10^{-4} M) oxidation of PCu(I) plastocyanin (*ca.* 10^{-5} M) at pH 7.5 (Tris-HCl) and I = 0.10 M (NaCl)

$10^4[Co^{III}{}_2]$/M	$10^{-3}\,k_{exp}$/M^{-1} s^{-1}
0	2.67
0.51	1.86
1.04	1.68
1.39	1.62
1.86	1.43
3.40	1.31
4.09	1.26
5.16	1.22
9.72	1.08

Discussion

Charge is clearly important in determining the magnitude of competitive inhibition on the $[Co(phen)_3]^{3+}$ oxidation of PCu(I), Table 6.* The high charge density of $[Pt(NH_3)_6]^{4+}$ makes it the most powerful inhibitor so far studied. Proton n.m.r. line-broadening experiments[10,11] have demonstrated specificity of both the redox-inactive complexes $[Cr(phen)_3]^{3+}$ and $[Cr(NH_3)_6]^{3+}$ for association with PCu(I) close to Tyr 83, and other positively charged complexes with these same ligands are expected to exhibit the same specificity.

Only partial inhibition is observed with $[Pt(NH_3)_6]^{4+}$ and $[(NH_3)_5Co(NH_2)Co(NH_3)_5]^{5+}$ where the trend of rate constants with increasing concentration of inhibitor gives k_B, or on a relative scale k_B/k (Table 6). The inhibition observed with $[Co(NH_3)_6]^{3+}$, and (previously) with $[Cr(phen)_3]^{3+}$, is too small for a decision to be reached as to whether k_B participates or not. We suspect that k_B will in fact contribute, but note in the $[Co(NH_3)_6]^{3+}$ case that K_B only changes from 470 to 580 M^{-1} with inclusion of this path. From the observation that different k_B/k ratios apply for different inhibitors, it is unlikely that there are two quite separate sites for electron transfer (one of which is completely blocked). Instead, we favour an explanation in terms of a single (dominant) region of protein capable of associating with two positively charged complexes, the presence of one impairing but not preventing associations of the other. The n.m.r. experiments have indicated that this region is near to Tyr 83. Two groups of acidic residues 42—45 and 59—61 have their negatively charged side-chains directed into the solvent, and form an elongated acidic patch with Tyr 83 close to the centre. The existence of this patch is such a striking feature of the structure that a functional significance seems likely. Figure 6 illustrates

---

* A preliminary report on this work is to be found in ref. 22. Results in the present paper have been further refined and, where different, supersede those previously given.

Table 5. The effect of redox-inactive $[\mathrm{Pt(NH_3)_6}]^{4+}$ on rate constants for oxidation of PCu(I) (*ca.* 1×10^{-5} M) by $[\mathrm{Fe(CN)_6}]^{3-}$ (*ca.* 5×10^{-4} M) at pH 7.50 (Tris-HCl) and $I = 0.10$ M (NaCl)

$10^5[\mathrm{Pt(NH_3)_6}^{4+}]/\mathrm{M}$	$10^{-5}k_{\mathrm{exp}}/\mathrm{M^{-1}\,s^{-1}}$
0	1.00
2.08	1.12
4.14	1.25
8.20	1.44
12.2	1.60
16.1	1.84
31.0	2.56

these features and one of a number of possible situations in which both redox-active (C^+) and inhibitor complex (B) are associated with the protein.

The n.m.r. experiments[10,11] have also indicated a high degree of specificity in the association of negatively charged $[\mathrm{Cr(CN)_6}]^{3-}$ close to His 87. The tip of the co-ordinated pseudo-aromatic imidazole ring of His 87 is exposed to solvent and is at the centre of a distinctive hydrophobic patch. The Cu is closest to the protein surface (6 Å) at this point, which has attractive features as a binding site for electron transfer. It has been suggested that the oxidant $[\mathrm{Fe(CN)_6}]^{3-}$ (C^- in Figure 6) uses this locality as a binding site.[10] However, a rate equation of the form (1) in which the term $K[\mathrm{Fe(CN)_6}^{3-}]$ is effective in the denominator giving limiting kinetics is not observed.[9] Consistent with this observation, the two redox-inactive 4− complexes $[\mathrm{Mo(CN)_8}]^{4-}$ and $[\mathrm{Zr(C_2O_4)_4}]^{4-}$ do not give rise to competitive inhibition. We are not able therefore to extend studies using these negatively charged complexes as reagents which specify use of this His 87 site for electron transfer. The observation that $[\mathrm{Pt(NH_3)_6}]^{4+}$ accelerates the $[\mathrm{Fe(CN)_6}]^{3-}$ oxidation of PCu(I) is open to a number of possible interpretations involving more reactive oxidant–$[\mathrm{Pt(NH_3)_6}]^{4+}$ and/or protein–$[\mathrm{Pt(NH_3)_6}]^{4+}$ adducts which, because of the complexity, we have not attempted to quantify.

The more extensive studies carried out on the competitive inhibition by $[\mathrm{Pt(NH_3)_6}]^{4+}$ have demonstrated that the association constant K_{B} decreases at pH < 6.6, Figure 3, giving a protein pK_{a} of 5.8. This complete switch-off in association is an unexpected result, and one which it seems appropriate to think of as originating from protonation at the negative patch. The pK_{a} is in the same range as values (5.7—6.1) reported from rate constants for the $[\mathrm{Co(phen)_3}]^{3+}$ and $[\mathrm{Fe(CN)_6}]^{3-}$ oxidations of PCu(I) (no inhibitor present). It has previously been suggested that these pK_{a} values are the result of dissociation and protonation of His 87 at the PCu(I) active site giving a redox-active three-co-ordinate product,[6] a view which we have found acceptable.[22] This suggestion may now require modification, with protonation at the negative patch somehow involved. Previous results (recently confirmed) have indicated that it is K, rather than k_{et}, for association of $[\mathrm{Co(phen)_3}]^{3+}$ to PCu(I) which is responsible for the protein inactivation with decreasing pH. Therefore both the $[\mathrm{Co(phen)_3}]^{3+}$ and $[\mathrm{Pt(NH_3)_6}]^{4+}$ pK_{a} values originate from effects of pH on association constants.

Freeman[6] has indicated an increase (2.3 to 3.4 Å) of the average Cu–N (His 87) bond length on decreasing the pH from 7 to 4, an increase which is sufficient to accommodate a proton. The variation observed gives a pK_{a} of 5.7 ± 0.04 (our calculation), it being assumed that the effective pH in the crystal phase is the same as that of the medium. This gives rise to an inconsistency in that Markley *et al.*[23] from n.m.r. studies have observed proton-induced dissociation of the two co-ordinated histidines at pK_{a} 4.9 and <4.5 respectively. It is possible to accommodate these different observations if indeed the Cu–N (His 87) bond length increased but proton uptake does not occur until, as Markley observes, pH *ca.* 4.9. Since the Cu is known to be in a hydrophobic site,[24] which $\mathrm{H_2O}$ and presumably $\mathrm{H_3O^+}$ do not have access to at the higher pH, this possibility would certainly seem to merit consideration.

Table 6. Summary of association constants (25 °C) for redox-inactive complexes with parsley plastocyanin, PCu(I), from competitive inhibition studies at pH 7.5 (except as indicated) and $I = 0.10$ M (NaCl)

Complex	$K_{\mathrm{B}}/\mathrm{M^{-1}}$	k_{B}/k
$[\mathrm{Cr(phen)_3}]^{3+}$	176[a]	*b*
$[\mathrm{Co(NH_3)_6}]^{3+}$	470	*b*
$[\mathrm{Pt(NH_3)_6}]^{4+}$	4.2×10^4 [c]	0.51
$[\mathrm{Co_2(NH_3)_{10}(NH_2)}]^{5+}$	1.6×10^4	0.39

[a] To be compared with $K = 167\ \mathrm{M^{-1}}$ for the $[\mathrm{Co(phen)_3}]^{3+}$ oxidation of PCu(I) (no redox-inactive complex added).[7] [b] Fitted to equation without inclusion of k_{B}, see comments in text. [c] Computed value, see broken line in Figure 3. Value determined experimentally at pH 7.5, without allowance for $[\mathrm{Pt(NH_3)_2(NH_2)}]^{3+}$, is $2.23 \times 10^4\ \mathrm{M^{-1}}$.

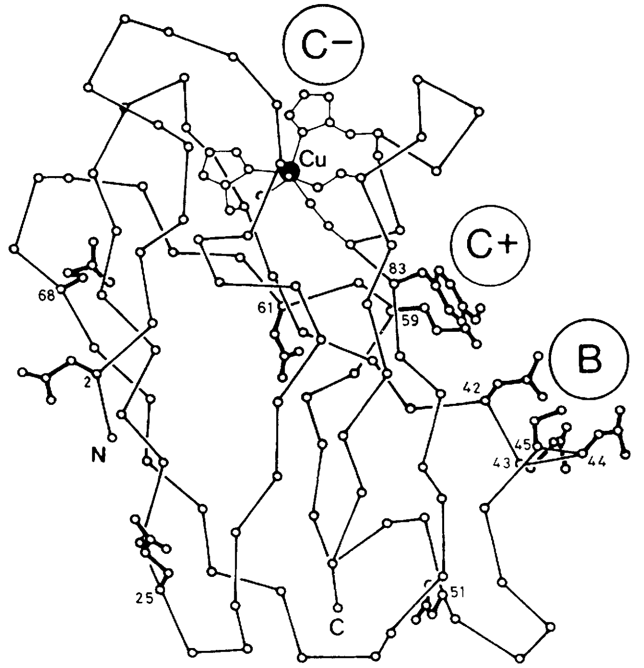

Figure 6. The structure of plastocyanin as reported recently by Freeman,[6] with orientations of acidic residues, Tyr 83, and active site residues indicated. Possible sites for reaction with positively (C^+) and negatively (C^-) charged redox partners are also illustrated. One of a number of ways in which C^+ and a positively charged inhibitor (B) might interact with the region incorporating Tyr 83 and the negative patch (42—45) is indicated

We also wish to note here that a pK_{a} of 5.0 has been observed[25] for the $[\mathrm{Ru(NH_3)_5(py)}]^{2+}$ (py = pyridine) reduction of PCu(II) (no His 87 dissociation is observed in this case[6]), and that this can also be assigned to protonation at the negative patch. If, more specifically, residues 42—45 at *ca.* 18 Å from the active site are involved, a change in oxidation state of Cu from I to II would not be expected to change the pK_{a} from 5.8 to 5.0, unless some additional effect were contributing. A possible explanation is that for PCu(I) there is a co-operative effect between the negative patch and the active site, and we note that there is, for example, conservation of the peptide 37—42 chain. Consistent with this suggestion it has recently been reported that ethylenediamine chemical modification at 42—45 affects the PCu(II)–PCu(I) reduction potential by 40 mV,[26] but that similar modifications at other acid residues have much less effect on the potential. If co-operative effects exist with a special relationship between the negative patch and active site, then the switch-off in reactivity observed for the $[Fe(CN)_6]^{3-}$ oxidation of PCu(I) presumably originates from the k_{et} rather than K component of the second-order rate constant.

Acknowledgements

We thank the S.E.R.C. for a postdoctoral research assistantship (to A. D. W.) and a postgraduate studentship (to S. K. C.).

References

1 A. G. Lappin, ' Metal Ions in Biological Systems,' ed. H. Sigel, Marcel Dekker, New York, 1981, vol. 13, pp. 15—71.
2 O. Farver and I. Pecht, ' Copper Proteins,' ed. T. G. Spiro, Wiley, New York, 1981, pp. 151—193.
3 J. J. Fee, *Struct. Bonding (Berlin)*, 1975, **23**, 1.
4 D. O. Hall and K. K. Rao, ' Studies in Biology No. 37, Photosynthesis,' 3rd edn., Arnold, 1981, pp. 1—84.
5 P. M. Coleman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, and M. P. Venkatappa, *Nature (London)*, 1978, **272**, 319.
6 H. C. Freeman, in ' Coordination Chemistry—21,' ed. J. P. Laurent, Pergamon, Oxford, 1981, p. 29.
7 M. G. Segal and A. G. Sykes, *J. Am. Chem. Soc.*, 1978, **100**, 4585.
8 A. G. Lappin, M. G. Segal, D. C. Weatherburn, and A. G. Sykes, *J. Am. Chem. Soc.*, 1979, **101**, 2297.
9 S. K. Chapman, I. Sanemasa, A. D. Watson, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1983, 1949.
10 D. J. Cookson, M. T. Hayes, and P. E. Wright, *Nature (London)*, 1980, **283**, 682; *Biochim. Biophys. Acta*, 1980, **591**, 162.
11 P. M. Handford, H. A. O. Hill, R. W.-K. Lee, R. A. Henderson, and A. G. Sykes, *J. Inorg. Biochem.*, 1980, **13**, 83.
12 M. Plesničar and D. S. Bendall, *Biochim. Biophys. Acta*, 1970, **216**, 192.
13 T. J. Pryzystas and N. Sutin, *J. Am. Chem. Soc.*, 1973, **95**, 5545.
14 J. Bjerrum and J. P. Reynolds, *Inorg. Synth.*, 1946, **2**, 216.
15 L. N. Essen, *Inorg. Synth.*, 1973, **15**, 93.
16 R. Davies, M. Mori, A. G. Sykes, and J. A. Weil, *Inorg. Synth.*, 1970, **12**, 212.
17 F. A. Johnson and E. M. Larsen, *Inorg. Synth.*, 1966, **8**, 40.
18 Van der Poil and H. M. Newmann, *Inorg. Synth.*, 1968, **11**, 53.
19 P. Pfeiffer and B. Werdelmann, *Z. Anorg. Allg. Chem.*, 1950, **263**, 31.
20 R. M. Smith and A. E. Martell, ' Critical Stability Constants,' Plenum, New York, 1975, vol. 2, p. 20.
21 R. C. Johnson, F. Basolo, and R. G. Pearson, *J. Inorg. Nucl. Chem.*, 1962, **24**, 59.
22 S. K. Chapman, D. M. Davies, A. D. Watson, and A. G. Sykes, ' Inorganic Chemistry into the 21st Century,' ed. M. H. Chisholm, American Chemical Society, Bloomington, 1983, pp. 177—197.
23 J. L. Markley, E. L. Ulrich, S. P. Berg, and D. W. Krogman, *Biochemistry*, 1975, **14**, 4429.
24 N. Boden, M. C. Holmes, and P. F. Knowles, *Biochem. Biophys. Res. Commun.*, 1974, **57**, 845; L. Avigliano, A. Finazzi-Agro, and B. Mondori, *FEBS Lett.*, 1974, **38**, 205; A. Finazzi-Agro, G. Rotilio, L. Avigliano, P. Guerrieri, V. Boffi, and B. Mondori, *Biochemistry*, 1970, **9**, 2009.
25 S. K. Chapman, I. Sanemasa, and A. G. Sykes, following paper.
26 K. O. Burkey and E. L. Gross, *Biochemistry*, 1982, **21**, 5886.

Received 26th January 1983; *Paper* 3/114