

In memory of S. M. Nelson

Kinetic Studies on 1 : 1 Electron-transfer Reactions involving Blue Copper Proteins. Part 14.† Reactions of Poplar Plastocyanin with Inorganic Complexes‡

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The reactions of poplar plastocyanin, PCu(I), with $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Co}(\text{phen})_3]^{3+}$ (phen = 1,10-phenanthroline) as oxidants, and of PCu(II) with $[\text{Co}(\text{phen})_3]^{2+}$ as reductant, have been studied at 25 °C, $I = 0.10 \text{ M}$ (NaCl). At pH 7.5, 69% of the reaction of $[\text{Co}(\text{phen})_3]^{3+}$ is at the Tyr 83 site, which incorporates the acidic patch, in this case at 42–44. With $[\text{Fe}(\text{CN})_6]^{3-}$ as oxidant the behaviour observed is consistent with reaction at the His 87 (north) site. From the effects of pH (4.5–7.5) on rate constants, active site ($\text{p}K_a$ 4.7) and (acidic patch) binding site ($\text{p}K_a$ 5.25) deprotonation/protonation equilibria are identified. For PCu(II) no active site $\text{p}K_a$ is observed, and the binding site $\text{p}K_a$ is 5.1. Comparisons are made with plastocyanin from parsley, spinach, and French bean. With $[\text{Co}(\text{phen})_3]^{3+}$ as oxidant no limiting kinetic behaviour is observed, and association of PCu(I) with $[\text{Co}(\text{phen})_3]^{3+}$ is at a lower level ($K < 50 \text{ M}^{-1}$). From competitive inhibition studies association with redox inactive $[(\text{NH}_3)_5\text{Co}(\text{NH}_2)\text{Co}(\text{NH}_3)_5]^{5+}$ is also at a lower level ($K = 5650 \text{ M}^{-1}$). The origin of differences in $\text{p}K_a$ for PCu(I) and PCu(II) states is discussed.

The single blue (type 1) copper protein plastocyanin (M 10 500) is a component of the photosynthetic electron-transport chain in the chloroplasts of higher plants and algae. Kinetic and n.m.r. studies have been carried out previously on plastocyanin from parsley, spinach, and French bean.^{1–10} Interpretation of the results has been made with reference to X-ray crystallographic information obtained for plastocyanin from poplar leaves,^{11–14} assuming that the main features of the structure are retained throughout the series. Here, for the first time, kinetic studies on poplar plastocyanin are presented.

Fifteen higher plant plastocyanins, including those referred to above, have been fully sequenced.^{4,15–19} There are 99 amino-acid residues in a single polypeptide chain, parsley (with 97) being the only exception. Of these, 50 residues are invariant and others are highly conserved. The overall charge is also conserved at -9 ± 1 for PCu(I) at pH ~ 7 . Possibly the most significant sequence variation for poplar is the occurrence of serine instead of glutamic acid at position 45, as a result of which the otherwise invariant negative patch of four carboxylates at 42–45 is 3– rather than 4– at the higher pH values (> 7). Previous work⁴ has implicated this negative patch, close to Tyr 83 on the right-hand side of Figure 1, as a binding site for positively charged redox partners. A second electron-transfer site is located at the exposed edge of the imidazole ring of His 87 at the top of the molecule in Figure 1 (the 'north' end). This residue is co-ordinated to the Cu, providing a facile route for electron transfer.

Experimental

Experimental procedures were identical to those of earlier studies^{1–7} unless otherwise stated. Poplar plastocyanin was

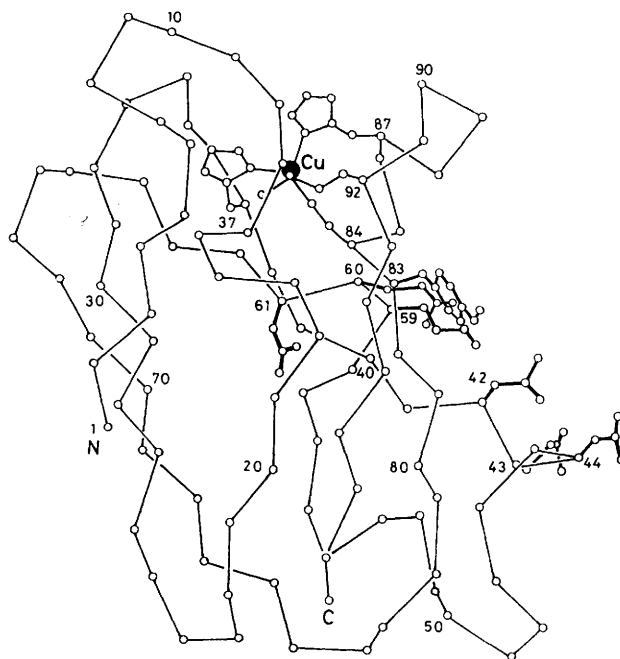


Figure 1. The structure of poplar plastocyanin, PCu(II), showing the α -carbon framework plus the side chains at the Cu site and at the acidic patch, as reported by Guss and Freeman¹¹

extracted and purified as in ref. 11. Final purification was on DEAE-Sephadex A-50 to an absorbance (A) peak ratio, A_{280}/A_{597} , generally in the range 0.8–0.9. Buffers (20 mM) used were acetate (pH < 5.5), mes (pH 5.5–6.8) [mes = 2-(*N*-morpholino)ethanesulphonic acid], and Tris (pH > 7) [Tris = tris(hydroxymethyl)methylamine]. In some cases, including all runs at pH < 4.7 , the pH-jump method (involving mixing of reactant solutions at different values of pH) was employed. Complexes and kinetic procedures were as

† Part 13, J. McGinnis, W. J. Ingledew, and A. G. Sykes, *Inorg. Chem.*, 1986, **25**, 3730.

‡ *Non-S.I. unit employed:* M = mol dm⁻³.

§ The following are included: spinach, French bean, potato, elder, marrow, broad bean, lettuce, dog's mercury, shepherd's purse, solanum, dock, poplar, cucumber, parsley, and white campion.

Table 1. Pseudo-first-order rate constants, $k_{\text{obs.}}$, for the oxidation of poplar plastocyanin PCu(I) with $[\text{Fe}(\text{CN})_6]^{3-}$ at 25 °C, $I = 0.10 \text{ M}$ (NaCl)

pH	$10^4[\text{Fe}(\text{CN})_6^{3-}]/\text{M}$	$k_{\text{obs.}}/\text{s}^{-1}$
4.23	3.82	9.1
	1.90	4.7
4.45	4.86	14.1
	1.79	4.9
4.75	4.62	17.6
	2.10	8.1
5.27	3.61	20.6
	1.49	8.1
5.74	3.47	21.9
	1.69	10.8
5.93	4.00	26.3
	1.41	9.1
6.01	1.73	11.5
6.61	6.24	43.6
7.20	3.49	25.9

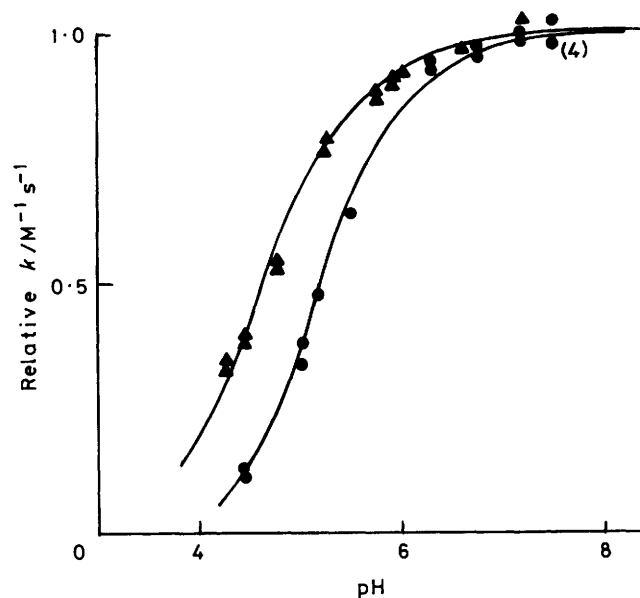


Figure 2. The effect of pH on second-order rate constants (relative scale) for the oxidation of poplar PCu(I) with $[\text{Fe}(\text{CN})_6]^{3-}$ (▲) and $[\text{Co}(\text{phen})_3]^{3+}$ (●), value in parentheses indicates runs averaged

previously described.¹⁻⁷ All studies were at 25 °C, $I = 0.10 \text{ M}$ (NaCl).

Results

$[\text{Fe}(\text{CN})_6]^{3-}$ as Oxidant.—First-order rate constants $k_{\text{obs.}}$ (Table 1) exhibit a linear dependence on $[\text{Fe}(\text{CN})_6^{3-}]$, $(1.4-6.2) \times 10^{-4} \text{ M}$, enabling second-order rate constants k to be determined. The variation of k with pH is illustrated in Figure 2. Rate constants were fitted with equation (1), which was

$$k = \frac{k_0 K_a + k_H [\text{H}^+]}{K_a + [\text{H}^+]} \quad (1)$$

obtained from the reaction sequence (2)–(4), where P is the

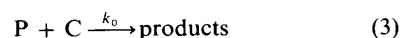
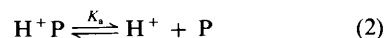


Table 2. Pseudo-first-order rate constants, $k_{\text{obs.}}$, for the oxidation of poplar plastocyanin PCu(I) with $[\text{Co}(\text{phen})_3]^{3+}$ at 25 °C, $I = 0.10 \text{ M}$ (NaCl)

pH	$10^4[\text{Co}(\text{phen})_3^{3+}]/\text{M}$	$k_{\text{obs.}}/\text{s}^{-1}$
4.45	4.96	0.163
	2.33	0.086
5.00	4.52	0.49
	1.73	0.170
5.16	3.67	0.50
5.51	4.27	0.78
6.30	4.06	1.09
	1.66	0.45
6.77	3.20	0.88
	1.86	0.52
7.18	4.90	1.43
	2.14	0.61
7.50	5.76	1.71
	10.6	3.0
	11.0	3.1
	12.7	3.6
	22.0	6.2

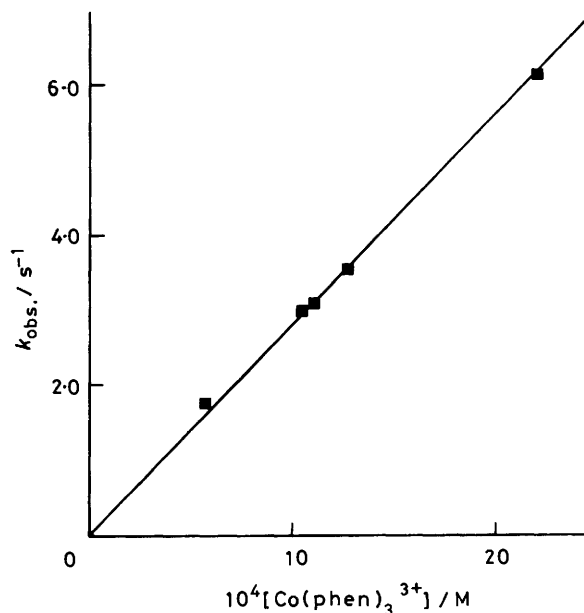
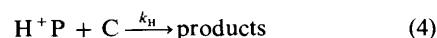


Figure 3. Linear dependence of first-order rate constants $k_{\text{obs.}}$ (25 °C) on oxidant concentration for the $[\text{Co}(\text{phen})_3]^{3+}$ oxidation of poplar PCu(I) at pH 7.5, $I = 0.10 \text{ M}$ (NaCl)



protein and C the inorganic complex. The form $\text{H}^+ \text{P}$ in reaction (2) represents the protein with His 87 protonated and dissociated from the Cu. In previous studies^{1,5} this form has been found to be redox inactive, and in our present computations k_H has been fixed at zero by inclusion of a weighting factor $k_H = 0$ (five points) at pH 2. The curve drawn in Figure 2 is in satisfactory agreement with experimental points, and gives $\text{p}K_a = 4.68 \pm 0.05$, $k_0 = (6.94 \pm 0.09) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, and $k_H = (0.09 \pm 0.12) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ consistent with a value of zero.

$[\text{Co}(\text{phen})_3]^{3+}$ as Oxidant.—First-order rate constants $k_{\text{obs.}}$ (Table 2) give a linear dependence on $[\text{Co}(\text{phen})_3^{3+}]$ (phen = 1,10-phenanthroline) over the range up to $2.2 \times 10^{-3} \text{ M}$

Table 3. Second-order rate constants (25 °C) for the oxidation of poplar plastocyanin, PCu(I), with $[\text{Co}(\text{phen})_3]^{3+}$ (C), $(0.28\text{--}0.53) \times 10^{-3}$ M, inhibited by addition of $[(\text{NH}_3)_5\text{Co}(\text{NH}_2)\text{Co}(\text{NH}_3)_5]^{5+}$ complex (B), at pH 7.45, $I = 0.10$ M (NaCl)

$10^3[\text{B}]/\text{M}$	$k_{\text{obs.}}/[\text{C}]$ ($\text{M}^{-1} \text{s}^{-1}$)
0	2 845*
0.019	2 600
0.26	1 660
0.41	1 530
0.69	1 280
1.31	1 090

* Mean of 5 values at pH 7.5, see Table 2.

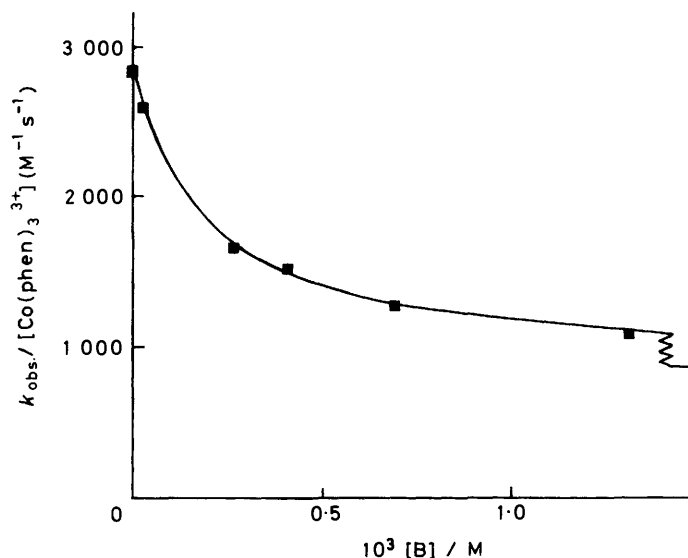


Figure 4. Effect of $[(\text{NH}_3)_5\text{Co}(\text{NH}_2)\text{Co}(\text{NH}_3)_5]^{5+}$, B, on rate constants for the $[\text{Co}(\text{phen})_3]^{3+}$ oxidation of poplar PCu(I) at pH 7.5, $I = 0.10$ M (NaCl)

investigated (Figure 3). There is no evidence for curvature. The effect of pH on second-order rate constants is illustrated in Figure 2. On fitting the data with equation (1) (without a weighting factor) an *apparent* $\text{p}K_a$ of 5.21 ± 0.04 is obtained. The discrepancy in $\text{p}K_a$ values for $[\text{Fe}(\text{CN})_6]^{3-}$ and $[\text{Co}(\text{phen})_3]^{3+}$ is in accord with previous observations on other plastocyanins, and implies that a binding-site protonation is contributing in the $[\text{Co}(\text{phen})_3]^{3+}$ case, in addition to the active-site protonation. The binding-site protonation is denoted $\text{p}K_a'$ and was determined by the same procedure as before,^{3,5} except that the active site $\text{p}K_a$ used was that determined by the $[\text{Fe}(\text{CN})_6]^{3-}$ kinetics (4.68) as there is no n.m.r. value. The value of $\text{p}K_a'$ determined was 5.25 ± 0.05 and the rate constant $k_0 = 2880 \pm 20 \text{ M}^{-1} \text{ s}^{-1}$, at $\text{pH} > 7.5$, was found to decrease to $850 \pm 100 \text{ M}^{-1} \text{ s}^{-1}$ from protonation at the binding site. The latter value decreases to zero as a result of active-site protonation.

Competitive Inhibition of $[\text{Co}(\text{phen})_3]^{3+}$ Oxidation.—Inhibition of the $[\text{Co}(\text{phen})_3]^{3+}$ (C) oxidation by redox inactive $[(\text{NH}_3)_5\text{Co}(\text{NH}_2)\text{Co}(\text{NH}_3)_5]^{5+}$ (B) was investigated at pH 7.5. Second-order rate constants $k_{\text{obs.}}/[\text{C}]$ (Table 3) decrease with increasing concentration of the 5+ complex to a plateau value indicating maximum 69% inhibition of the $[\text{Co}(\text{phen})_3]^{3+}$ oxidation (Figure 4). This is in accord with the reaction sequence (5)–(7), which gives rise to equation (8). A fit of the

Table 4. Pseudo-first-order rate constants, $k_{\text{obs.}}$, for the reduction of poplar plastocyanin PCu(II) with $[\text{Co}(\text{phen})_3]^{2+}$, at 25 °C, $I = 0.10$ M (NaCl)

pH	$10^4[\text{Co}(\text{phen})_3^{2+}]/\text{M}$	$k_{\text{obs.}}/\text{s}^{-1}$
4.50	1.33	0.284
	2.65	0.56
4.72	2.65	0.59
4.98	1.33	0.32
	2.65	0.65
5.23	2.65	0.68
5.50	2.65	0.76
5.54	1.33	0.39
5.90	1.28	0.38
6.12	1.33	0.41
	2.65	0.81
6.58	1.28	0.41
7.02	2.56	0.83

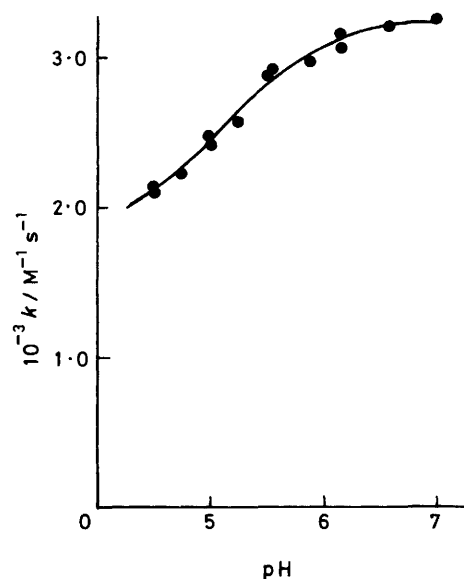
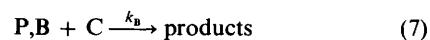
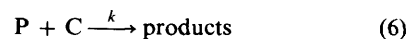


Figure 5. Effect of pH on second-order rate constants (25 °C) for the reduction of poplar PCu(II) with $[\text{Co}(\text{phen})_3]^{2+}$, $I = 0.10$ M (NaCl)



$$\frac{k_{\text{obs.}}}{[\text{C}]} = \frac{k + K_B k_B [\text{B}]}{1 + K_B [\text{B}]} \quad (8)$$

data in Table 3 with equation (8) gives $K_B = 5650 \pm 800 \text{ M}^{-1}$ and $k_B = 880 \pm 75 \text{ M}^{-1} \text{ s}^{-1}$.

$[\text{Co}(\text{phen})_3]^{2+}$ as Reductant.—This complex was prepared using a six-fold molar excess of phenanthroline ligand.⁷ Reaction of the free ligand with the protein was observed to be an order of magnitude slower than the electron-transfer process and could be ignored. First-order rate constants $k_{\text{obs.}}$ (Table 4) exhibit a linear dependence on $[\text{Co}(\text{phen})_3^{2+}]$ in the range investigated. The variation of second-order rate constants k with pH is illustrated in Figure 5. A fit of the data with equation (1) gives $\text{p}K_a = 5.1 \pm 0.1$, $k_0 = 3240 \pm 60 \text{ M}^{-1} \text{ s}^{-1}$, and $k_H = 1800 \pm 90 \text{ M}^{-1} \text{ s}^{-1}$.

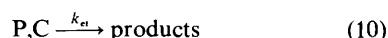
Table 5. Comparison of relevant parameters for plastocyanin from four higher plant sources

	pK_a n.m.r.	pK_a from kinetics with [Fe(CN) ₆] ³⁻	pK_a from kinetics with [Co(phen) ₃] ³⁺ ^a	pK_a' for PCu(I) ^b	pK_a' for PCu(II)	$k_{Fe}/M^{-1} s^{-1} c$	$k_{Co}/M^{-1} s^{-1} c$	k_{Fe}/k_{Co}	K_B/M^{-1}	Blocking %
Parsley	5.7	5.5	6.1	5.8	5.0	94 000	3 000	31	16.0×10^3	61
Spinach	4.9	4.8	5.6	5.6	5.3	85 000	2 500	34	6.8×10^3	75
French bean ^{3,9}	4.8	4.6	5.4	5.5	—	58 000	4 700	12		
Poplar		4.7	5.2	5.25	5.1	69 000	2 900	25	5.7×10^3	69

^a Apparent value. ^b Assuming that two processes, pK_a (determined by n.m.r. or with [Fe(CN)₆]³⁻ as oxidant) and pK_a' , contribute with [Co(phen)₃]³⁺ as oxidant. ^c pH 7.5.

Discussion

One interesting difference between the kinetic behaviour of plastocyanin from poplar and that of other higher plant plastocyanins is the absence of detectable rate saturation (*i.e.* limiting kinetics) in the oxidation with [Co(phen)₃]³⁺. For parsley,¹ spinach,⁵ and French bean⁹ plastocyanins, a plot of k_{obs} against [Co(phen)₃]³⁺ begins to curve at oxidant concentrations above 5×10^{-4} M. This is explained in terms of prior association of the 3+ oxidant at the (4-) negative patch, reactions (9)–(10). Values of K and k_{et} have been determined



based on the assumption that [Co(phen)₃]³⁺ reacts exclusively at the negative patch.¹ Recent results^{6,9,20} have indicated that this is not so, and that some 20–40% of the reaction is at some other site or sites, possibly the His 87 site because of its close proximity to the Cu active site. A revised treatment based on the new model has been carried out for parsley,⁶ giving a value for K of $340 M^{-1}$. In the present case, the plot of k_{obs} against [Co(phen)₃]³⁺ (Figure 3) is linear up to 2.2×10^{-3} M [Co(phen)₃]³⁺, though we note from individual values in Table 2 that there may be a small decrease in apparent second-order rate constant at the higher oxidant concentrations. This is within experimental error and is in any case too small to allow a meaningful treatment based on the above scheme. While it is difficult to give a reliable upper limit for the value of K in the poplar case, it must be less than for parsley and is probably $< 50 M^{-1}$. Such behaviour is in accord with the sequence variation at position 45 which makes the 42–45 negative patch 3- instead of 4-.

Evidence that the reaction of [Co(phen)₃]³⁺ with parsley plastocyanin involves at least two sites is obtained from competitive inhibition studies.⁶ With poplar plastocyanin, the effect of the [(NH₃)₅Co(NH₂)Co(NH₃)₅]⁵⁺ inhibitor is similar to other plastocyanins. The extent of inhibition at pH 7.5, corresponding to the proportion of reaction occurring at the negative patch, is $69 \pm 3\%$ for poplar, which is comparable to $61 \pm 2\%$ for parsley⁶ and $75 \pm 2\%$ for spinach.⁵ The value of the association constant for poplar, $K_B = 5 650 M^{-1}$, is the lowest so far observed among higher plant plastocyanins, but this may well be affected by factors other than the negative patch charge. There is, for example, a significant difference between the K_B values for parsley and spinach (Table 5) which has not so far been explained.

The variation of rate constants with pH for the oxidation of PCu(I) with [Co(phen)₃]³⁺ and [Fe(CN)₆]³⁻ (Figure 2) follows the previous pattern, and the ratio k_{Fe}/k_{Co} lies in the range previously observed (Table 5). This is the case even though the estimated value of K , which is a component part of k_{Co} ($k_{Co} = Kk_{et}$), is less than the effective value for parsley and

spinach by a factor of 2–3. Protonation at the binding site reduces the rate by approximately the same amount as does competitive inhibition, suggesting that protonation at the negative patch results in a complete switch-off in reactivity at this site, as in the case of parsley and spinach plastocyanins.⁴ We note that pK_a' for poplar PCu(I) is smaller than for plastocyanin from other plant sources (Table 5), again in accord with the lower charge on the negative patch.

Finally, it is of interest that the pH profile for the reduction of PCu(II) with [Co(phen)₃]²⁺ leads to a pK_a' value of 5.1 ± 0.1 . The kinetic determination is not in this case complicated by contributions from active-site protonation. Comparison with the pK_a' of 5.25 ± 0.05 for PCu(I) suggests a dependence on the oxidation state of the Cu. This has been observed previously for parsley (5.0 and 5.8)⁷ and for spinach (5.3 and 5.6),⁵ the higher value being for PCu(I) in each case. The difference obtained for poplar is smaller, and is almost within experimental error, but is we believe a real effect. There are two plausible explanations for it. First, the effect may have a structural origin. In oxidised plastocyanin the geometry of the Cu site is invariant with pH,¹¹ whereas reduced plastocyanin exists in a high-pH form PCu(I) and a low-pH form HPCu(I) with dramatically different Cu site geometries.¹⁴ At intermediate pH values the two forms are in equilibrium. It is clear from the crystal structures of PCu(I) and HPCu(I) that the change in Cu site geometry is transmitted to the acidic patch, since there are small but significant positional changes at residues 42–44 and 59–61.¹⁴ Even if the pK_a' of the high-pH form PCu(I) is the same as that of PCu(II), the pK_a' of the low-pH form HPCu(I) is likely to be different. The fact that reduced plastocyanin has a slightly higher pK_a' than oxidised plastocyanin can then be ascribed to the presence of HPCu(I). Alternatively, the cause of the difference between the ' pK_a' ' values in oxidised and reduced plastocyanin may be electrostatic in origin. It can for example be argued that a change of -1 in the formal charge of the Cu atom accounts for a pK_a' change of $+0.25$ – 0.40 at a distance of *ca.* 16 Å, if the intervening material has a dielectric constant of *ca.* 40. [The argument is similar to that used by Rees,²¹ to estimate the electrostatic effect of the change in charge at a remote position on the reduction potential of a metal centre.] In reduced plastocyanin at low pH, the electrostatic effect of the -1 change in the charge of the Cu atom must be partly balanced by the effect of the protonation at His 87 (which is more distant from the acidic patch).

The preceding explanations are not mutually exclusive. They have the virtue that a conformational change at the acidic patch is invoked only in the case where a large geometrical change occurs at the Cu site. Previously the difference between the pK_a' values of oxidised and reduced plastocyanin has been explained in terms of a conformational change occurring at the acidic patch when the oxidation state of the Cu atom changes. Evidence for such a conformational change has been reported

from fluorescence measurements,²² but has been criticised,²³ and is not supported by the crystallographic evidence for poplar PCu(I) and PCu(II).¹⁴ It has been suggested²² that the conformational change is masked by the high concentration (2.7 M) of ammonium sulphate from which the crystals are grown. While this suggestion remains to be tested experimentally, we note that ammonium sulphate does not prevent the crystallographic detection of significant changes in side-chain conformations as a function of pH.

The pK_a' for poplar PCu(II) (5.1 ± 0.1) is lower than for spinach (5.33 ± 0.06) in accord with the lower negative patch charge, but is higher than for parsley (5.05 ± 0.03). The differences observed are, however, small. The effect of the PCu(II) pK_a' is to reduce the rate of the reaction with $[\text{Co}(\text{phen})_3]^{2+}$ by 45%, which is similar to the effect observed for the corresponding reactions of PCu(II) from parsley (54%),⁷ and spinach (46%).⁵

In summary, we find that in these kinetic studies poplar behaves similarly to the typical higher plant plastocyanins from spinach and French bean. There are some differences in detail, most notably the absence of detectable association with $[\text{Co}(\text{phen})_3]^{3+}$, which can be rationalised in terms of the sequence variation at residue 45 (Ser replacing Glu). A detailed comparison with parsley plastocyanin is less helpful as parsley possesses other significant sequence variations,¹⁶ which make it atypical among the higher plant sources.

Acknowledgements

We thank the S.E.R.C. for post-doctoral support (to J. McG.) and Johnson-Matthey Co. Ltd. for an S.E.R.C./CASE studentship (to M. P. J.), and the Australian Research Grants Scheme for grant C80/15377 (to H. C. F.). The work was also supported by the British Council under its Academic Links scheme.

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Received 29th December 1986; Paper 6/2483