Reactions of Five Spinach Plastocyanin PCu(I) Mutants with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) and Related Studies†

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Rate constants (25 °C) have been determined by stopped-flow spectrophotometry for the oxidation of five spinach plastocyanin (PCu) mutants in the copper(I) form, with inorganic redox partners $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline), I = 0.100 M (NaCl). The mutants investigated, Leu12Asn, Leu12Glu, Asp42Asn, Tyr83His and Tyr83Phe, incorporate changes at the Leu-12 position of the adjacent (to the Cu) hydrophobic site, and at the remote acidic patch residues Tyr-83 and Asp-42. At pH >7.5 the acid-dissociated glutamate (pK_a 6.9) form of Leu12Glu impedes the $[Fe(CN)_6]^{3-}$ oxidant which reacts ≈90% more slowly than with the native protein. A 2.2-fold increase in reactivity is however observed with $[Co(phen)_3]^{3+}$, which is consistent with a switch in reaction from the remote to adjacent site. This was confirmed by competitive inhibition studies with a redox-inactive 6 + tetranuclear cobalt(III) complex. Also for this mutant the active-site His-87 pK_a determined by ^1H NMR studies is shifted to a higher apparent value of 6.1 as compared to 4.9 for native protein, which reflects the combined effect of active site/Glu-12 protonation equilibria. Variations in rate constants with pH are also explored for the Tyr83His mutant, where acid dissociation (pK_a 8.4) affects reaction with both oxidants in a similar manner. At pH 7.5 no significant change in reactivity is observed with the Asp42Asn and Tyr83Phe mutants, while there is a five-fold enhancement in the reaction of the Leu12Asn mutant with $[Fe(CN)_6]^{3-}$. Rate constants were also determined for the $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ reductions of the copper(II) mutant forms at pH 7.5. Reduction potentials PCu(II)/(I) for the five mutants are in the range 360–402 mV, as compared to 375 mV for native protein.

Plastocyanin (M_r ≈ 10 500; ≈99 amino acids) is a single (type 1) blue copper protein located in the thylakoid of the chloroplast and involved in photosynthetic electron transport.[1] Its function is to transfer electrons from the membrane-bound cytochrome f to the P700 centre of photosystem I. The structure of the protein has been determined in both oxidation states.[2] A number of kinetic studies on plastocyanin (PCu) redox reactions have indicated the use of two sites on the surface.[1] One of these, adjacent (to the Cu), is a hydrophobic site which is close to the surface exposed imidazole ring of His-87, and the other is at the remote acidic patch site. The latter is a broad region consisting of a conserved surface Tyr-83 flanked on either side by acidic residues at positions 42–45 and 59–61. The adjacent and remote sites are separated by a ridge which may serve as a division between the two.[2] The expression of the spinach plastocyanin gene in *Escherichia coli* has made it possible to isolate the recombinant (wild-type) protein and a number of site-specific mutant forms.[3] The purpose of the present study is to explore the effects of mutations at the two sites on reactivity. The influence of the two sites on the reactivity of plastocyanin first became apparent from studies with inorganic complexes as redox partners.[1] Subsequently the two sites have been shown to be relevant also in studies with the physiological redox partners cytochrome f and P700.[4–6] Effects of pH are of interest because the inner thylakoid, where plastocyanin functions, has a pH < 5.0. On receiving an electron from cytochrome f redox-inactive trigonally co-ordinated PCu(I) is formed. The mechanism by which the P700 redox activates the PCu(I) in order that electron transfer can occur is not yet understood. The complexes $[Fe(CN)_6]^{3-}$ [reduction potential for 3−/4− couple = 410 mV] and $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) [reduction potential for 3+/2+ couple = 370 mV] were selected as appropriate oxidants for spinach PCu(I) (375 mV at pH 7.5). Single mutations can in some cases have very significant effects. Thus in the case of the Met44Lys mutant of *Pseudomonas aeruginosa* azurin the introduction of a 1+ charge at the adjacent (to the Cu) hydrophobic site results in a 2–3 orders of magnitude smaller self-exchange rate constant at low pH values.[7] A preliminary account of parts of this work has been published.[8]

Experimental

Preparation of Mutants.—Recombinant (wild-type) and mutant forms of spinach plastocyanin were prepared using the system previously described for over-expression of plastocyanin in *E. coli* employing the expression-vector pUG101t_t.[5] The mutant protein was constructed using polymerase chain reaction (PCR) amplification according to the method of Land *et al.*,[9] with the modifications described.[5] Growth and fractionation of *E. coli* cells and purification of the different plastocyanins was carried out as in ref. 3 with some changes. The bacterial strain used was *E. coli* RV 308 (ATCC 31608).[10] A Sepharose HP (26%) (Pharmacia) fast protein liquid chromatography (FPLC) column was employed in the last ion-exchange chromatography, and a Sephacryl S-100 column for

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

the final gel-filtration step. After each of these steps the plastocyanin containing fractions were pooled and concentrated by dialysis against the appropriate buffer. The homogeneity of the mutants was confirmed by SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), and the purity by UV/VIS absorbance (A) peak ratios A_{278}/A_{597} of 1.2. The Tyr83His mutant has a smaller absorbance at 278 nm reflecting a smaller tyrosine content.[5] For the studies described ≈10 mg of each of the mutants Leu12Asn, Asp42Asn and Tyr83Phe were used. For the more extensive studies with Leu12Glu and Tyr83His larger amounts (≈30 mg) were required.

Buffers.—Acetate–acetic acid buffer was appropriate in the range pH 4.25–5.20. For the range pH 5.1–6.9 the buffer 2-morpholinoethanesulfonic acid (mes; Sigma), to which NaOH was added, for pH 7.00–8.8 tris(hydroxymethyl)aminomethane (Tris; Sigma) to which HCl was added, and for pH > 8.9 2-(*N*-cyclohexylamino)ethanesulfonic acid (ches; Sigma) to which NaOH was added, were used.

Inorganic Complexes.—The preparations/sources were as described previously. Characterisation was by UV/VIS absorbance spectra, peak positions λ/nm (ε/M^{-1} cm^{-1}) as follows: $[Co(phen)_3]Cl_3 \cdot 7H_2O$, 330 (4460), 350 (3620), 450 (100),[11] potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, 300 (1600), 420 (1010) and potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \cdot 3H_2O$, 330(330), as supplied (BDH, AnalaR). To minimise effects of air oxidation solutions of $[Fe(CN)_6]^{4-}$ were used within 30 min of preparation. The preparation of the tetranuclear complex $[Co_4(NH_3)_{12}(\mu\text{-}OH)_4(\mu_4\text{-}C_2O_4)][ClO_4]_6 \cdot 4H_2O$, [525 (227) and 380 nm (400 M^{-1} cm^{-1})] has also been described.[12] Solutions of $[Co(phen)_3]^{2+}$ were obtained by addition of 1,10-phenanthroline (in five-fold excess over Co) to a solution of $CoCl_2 \cdot 6H_2O$ (BDH, AnalaR).

Kinetic Studies.—All kinetic runs were monitored at the 597 nm peak for PCu(II) on a Dionex D-100 stopped-flow spectrophotometer at 25 °C, I = 0.100 M (NaCl). The PCu(I) form has no visible absorbance. Investigations of pH effects can consume large amounts of protein and the 'pH-jump' method was therefore used as in previous studies.[1,13] In this procedure the pH of the solution of protein (with small 2 mM concentrations of buffer) has no controlling influence as compared to the solution of inorganic complex (≈40 mM), and the latter determines the final pH. In this way one protein solution, *e.g.* at pH 7.0, can be used for studies in the range pH 6.0–8.0. In some check experiments both solutions were buffered at the same pH.

NMR Studies.—For the acquisition of proton NMR spectra the Leu12Glu mutant was exchanged into 99.9% deuteriated 50 mM phosphate buffer to give a final protein concentration of 1 mM. Immediately prior to NMR measurements, under appropriate air-free conditions, the protein was reduced by the addition of small cooled aliquots of 0.10 M $Na_2S_2O_4$ in 99.9% D_2O containing 0.10 M NaOD. The NMR tube was then flushed with argon to prevent air oxidation of the protein. Spectra were acquired in the range pH 7.8–4.8 using small amounts of 0.10 M solutions of DCl or NaOD to adjust the pH to the required value. The pH of the protein sample in the NMR tube was measured using a narrow Russell CMAWL/3.7/180 pH probe in combination with a Radiometer PHM62 pH meter, which was calibrated using Colour-key buffers in water. The pH values quoted are uncorrected for the deuterium isotope effect.

All NMR spectra were acquired on a Bruker AMX500 spectrometer at 298 K. Typically 512 free induction decays at each pH value were accumulated in 16 K data points and transformed into 32 K data points after zero filling. The residual HDO resonance was suppressed by presaturation at its resonant frequency. All chemical shifts are quoted relative to dioxane (internal) at δ 3.741.

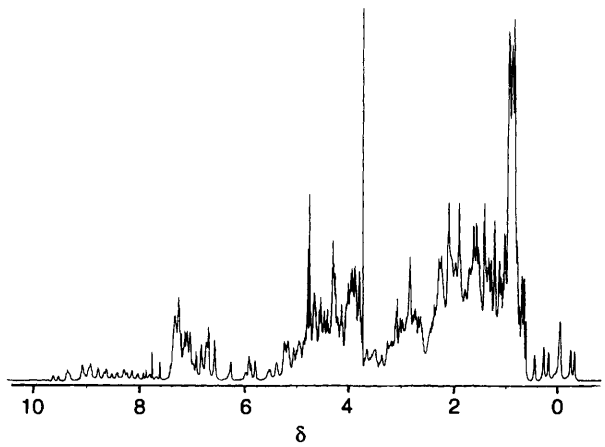

Fig. 1 Proton NMR spectrum of the spinach plastocyanin mutant Leu12Glu PCu(I) at pH 7.4

The quality of NMR spectra is illustrated by that for Leu12Glu PCu(I) at pH 7.4 in Fig. 1. We comment in the Discussion section on the retention of structure in mutant forms.

Treatment of Data.—Experimental second-order rate constants k for the oxidation of Leu12Glu- and Tyr83His-PCu(I) were fitted by equation (1), which can be derived from reaction

$$k = k_H + \frac{(k_0 - k_H)K_{aA}K_{aH} + (k_1 - k_H)K_{aA}[H^+] - k_H K_{aH}[H^+]}{K_{aA}K_{aH} + K_{aH}[H^+] + K_{aA}[H^+] + [H^+]^2} \quad (1)$$

scheme (2), where P represents the plastocyanin. This involves

$$\begin{array}{ccc} H^+ + HP_A^+ & \overset{K_{aA}}{\rightleftharpoons} & 2H^+ + P \\ \Big\uparrow\Big\downarrow K_{aH} & & K_{aH}\Big\uparrow\Big\downarrow \\ H_2P_{AH}^{2+} & \overset{K_{aA}}{\rightleftharpoons} & H^+ + HP_H^+ \end{array} \quad (2)$$

active-site (subscript A) and one other (subscript H) acid dissociation processes K_a. It is assumed that the acid dissociation constants (one at each site) do not change when the other is protonated. This assumption, and the absence of any specific effect from the oxidant, is supported by the agreement of pK_a values obtained from kinetic data and from ^1H NMR studies. Relevant kinetic steps are as defined in equations (3)–(5). The constant K_{aH} is assigned in turn to the

$$P + \text{oxidant} \xrightarrow{k_0} \text{products} \quad (3)$$

$$HP_H^+ + \text{oxidant} \xrightarrow{k_1} \text{products} \quad (4)$$

$$H_2P_{AH}^{2+} + \text{oxidant} \xrightarrow{k_H} \text{products} \quad (5)$$

glutamic acid of the Leu12Glu mutant, and to the His-83 of Tyr83His. Experimental rate constants k at the different $[H^+]$ values were fitted by equation (1) using an iterative program with no weighting factor.

Results

Oxidation of PCu(I) *Mutants at* pH 7.5.—Linear plots of first-order rate constants, k_{obs} (s^{-1}), against oxidant concentra-

Table 1 Summary of rate constants (M^{-1} s^{-1}), k_{Fe} and k_{Co}, for the $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ oxidation of PCu(I), and k_{Fe}' and k_{Co}' for the $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ reduction of PCu(II), using different mutant forms at 25 °C and calculated reduction potentials $E°_f$, pH 7.5, I = 0.100 M(NaCl)

Protein	$10^{-5}k_{Fe}$	$10^{-3}k_{Co}$	k_{Fe}/k_{Co}	$10^{-4}k_{Fe}'$	$10^{-3}k_{Co}'$	$E°_f$*/mV
Native	0.78*	2.38*	33.4	1.80	2.80	372
Wild-type	0.71	2.24	31.7	1.75	2.68	374
Leu12Asn	3.7	2.50	148	6.00	2.10	365
Leu12Glu	0.12	4.5	2.3	0.17	3.00	360
Asp42Asn	0.85	2.24	37.5	2.10	2.60	374
Tyr83His	0.37	0.90	41.1	2.50	3.20	402
Tyr83Phe	0.71	2.64	26.9	1.80	2.86	374

* These values have been redetermined and are in agreement with those in ref. 13.

Table 2 The variation of second-order rate constants (25 °C) with pH for the oxidation of the Leu12Glu PCu(I) mutant (≈1 × 10^{-5} M) with $[Fe(CN)_6]^{3-}$ (≈5 × 10^{-4} M) or $[Co(phen)_3]^{3+}$ (≈5 × 10^{-4} M), I = 0.100 M (NaCl)

$[Fe(CN)_6]^{3-}$ as oxidant

pH	4.29	4.30	4.57	4.75	4.96			
$10^{-4}k_{Fe}$/M^{-1} s^{-1}	1.10	1.42	1.69	1.90	2.57			
pH	5.70	6.00	6.11	6.65	7.08	7.33		
$10^{-4}k_{Fe}$/M^{-1} s^{-1}	3.64	3.55	3.49	2.70	1.90	1.40		
pH	7.40	7.85	7.90	8.41	8.62			
$10^{-4}k_{Fe}$/M^{-1} s^{-1}	1.30	1.00	0.91	0.79	0.79			

$[Co(phen)_3]^{3+}$

pH	4.64	4.93	5.35	5.53	5.77	5.90	5.97	6.00
$10^{-3}k_{Co}$/M^{-1} s^{-1}	0.11	0.13	0.27	0.44	0.79	1.41	1.41	1.56
pH	6.13	6.25	6.35	6.48	6.63	6.65	7.15	
$10^{-3}k_{Co}$/M^{-1} s^{-1}	1.86	2.08	2.01	1.98	2.22	2.38	3.42	
pH	7.23	7.20	7.40	7.68	8.08	7.80	8.48	
$10^{-3}k_{Co}$/M^{-1} s^{-1}	3.7	3.7	4.4	4.8	5.0	4.8	5.0	

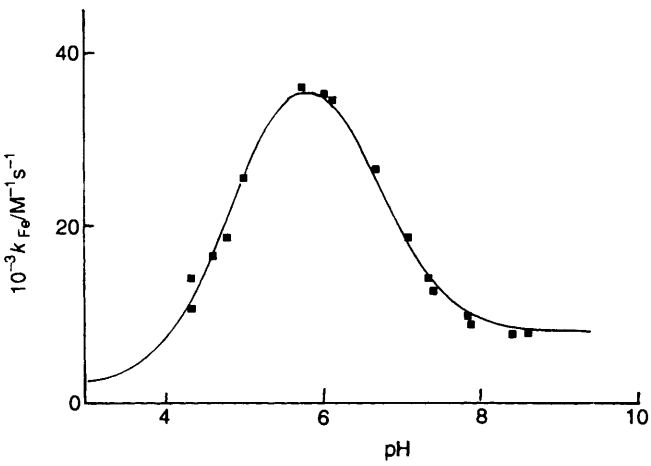

Fig. 2 Variation of second-order rate constants, k_{Fe} (25 °C), for the $[Fe(CN)_6]^{3-}$ oxidation of Leu12Glu PCu(I) with pH, I = 0.100 M (NaCl)

tion were obtained with (1–10) × 10^{-4} M $[Fe(CN)_6]^{3-}$, and (1–8) × 10^{-4} M $[Co(phen)_3]^{3+}$, consistent with the rate-law (6). Rate constants for the wild-type PCu(I) with $[Fe(CN)_6]^{3-}$

$$\text{Rate} = k[\text{PCu(I)}][\text{Oxidant}] \quad (6)$$

and $[Co(phen)_3]^{3+}$ are in satisfactory agreement with those for native protein.[13] These and other second-order rate constants k_{Fe} and k_{Co} at pH 7.5 for the PCu(I) mutants are summarised in Table 1.

Reduction of PCu(II) *Mutants at* pH 7.5.—Concentration-

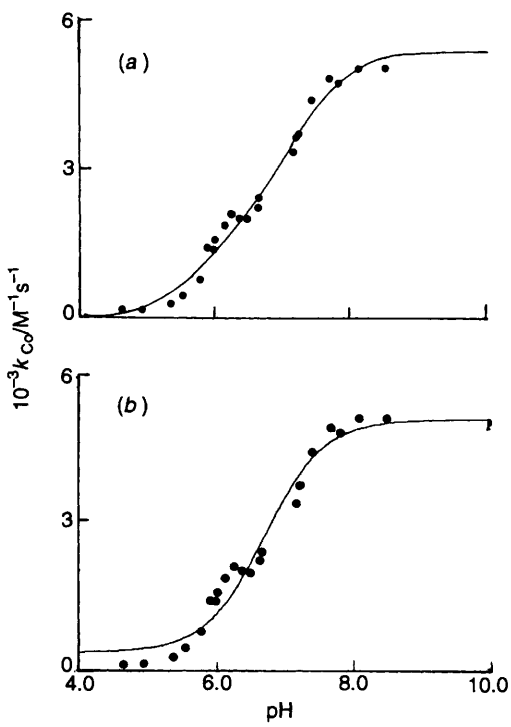

Fig. 3 Variation of second-order rate constants, k_{Co} (25 °C), for the $[Co(phen)_3]^{3+}$ oxidation of Leu12Glu PCu(I) with pH, I = 0.100 M (NaCl), (*a*) the best fit with two pK_a values, and (*b*) the less satisfactory fit with just one pK_a

dependence studies for the reduction of PCu(II) by $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ confirmed the rate law (7). Second-order

$$\text{Rate} = k'[\text{Reductant}][\text{PCu(II)}] \quad (7)$$

rate constants k_{Fe}' and k_{Co}' at pH 7.5 are also listed in Table 1.

Effect of pH *on the Oxidation of* Leu12Glu PCu(I).—Assuming that the rate law (6) applies at all pH, first-order rate constants k_{obs} give second-order k_{Fe} and k_{Co} values, Table 2. In the case of the oxidation with $[Fe(CN)_6]^{3-}$ the pH dependence, Fig. 2, indicates two effects. That at lower pH corresponds to the active-site protonation/inactivation which has been the subject of numerous previous studies.[1] At higher pH the rate constants decrease with increasing pH indicating lower reactivity of the acid-dissociated glutamate form. Two acid dissociation processes are indicated in the best fit of data by equation (1),[14] when the following parameters are obtained: pK_{aA} (active site) = 4.8 ± 0.3, pK_{aH} (glutamate) = 6.7 ± 0.1, k_H = (1.9 ± 2.0) × 10^3 M^{-1} s^{-1} (*i.e.* zero), k_1 = (4.3 ± 1.0) × 10^4 M^{-1} s^{-1}, and k_0 = (8.2 ± 0.5) × 10^3 M^{-1} s^{-1}. In the case of the $[Co(phen)_3]^{3+}$ oxidant, second-order rate constants give a better fit with two pK_a values rather than one, Fig. 3.

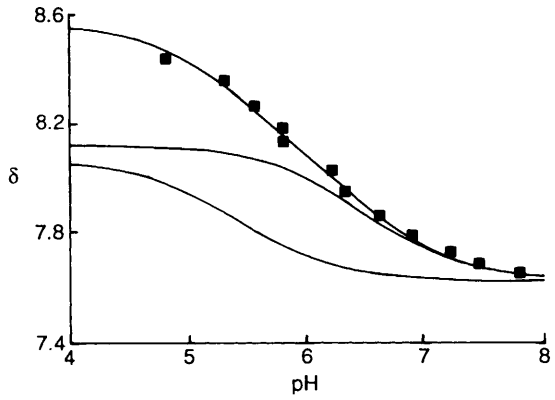

Fig. 4 Variation of the chemical shift δ of the $C^{\varepsilon}H$ His-87 proton with pH for the Leu12Glu mutant

Table 3 First-order rate constants (25 °C) for the oxidation of Leu12Glu PCu(I) ($\approx 1 \times 10^{-5}$ M) by $[Co(phen)_3]^{3+}$ (6×10^{-4} M) at pH 8.00 in the presence of $[Co_4(NH_3)_{12}(OH)_4(C_2O_4)]^{6+}$ as competitive inhibitor, $I = 0.100$ M (NaCl)

$10^4[Co^{III}_4]$/M	0	2.0	4.0	7.0	7.5
k_{obs}/s^{-1}	2.80	2.14	1.70	1.83	1.70

Contributions from the active-site and glutamic acid dissociations are therefore apparent to give the parameters: pK_{aA} (active site) = 5.8 ± 0.2 (an 'apparent' value including remote and active site effects), pK_{aH} (glutamate) = 7.2 ± 0.2, $k_H = (-0.08 \pm 0.01) \times 10^3$ M^{-1} s^{-1} (*i.e.* zero), $k_1 = (2.2 \pm 0.6) \times 10^3$ M^{-1} s^{-1} and $k_0 = (5.4 \pm 3.0) \times 10^3$ M^{-1} s^{-1}. The single pK_a fit gives a value of 6.68 which is not readily explained.

Proton NMR Titration of His-87 *of* Leu12Glu PCu(I).—The experiments carried out repeat earlier titrations of the active-site His-87 of native protein,[15] when protonation and dissociation from the Cu^I are observed ($pK_a = 4.9$). Thus the chemical shift of the $C^{\varepsilon}H$ of His-87 is monitored as a function of pH.[16] The variation of δ with pH, Fig. 4, gives a curve which can be reasonably fitted to pK_a values of 5.4 and 6.5. A single pK_a fit gives a value of 6.1 which is regarded as less satisfactory in chemical terms. The smaller pK_a value can be assigned to the active-site protonation/deprotonation, and the higher value to the titration of Glu-12. The former (5.4) is greater than the independently determined value for native protein of 4.9, while the latter (6.5) is less than the kinetic values which average at 6.9. The quality of the two pK_a fit may be a contributing factor. We note however that the mutation Leu12Glu does affect the chemical shift of residues as far away as Tyr-83. A pK_a of ≈5 has been determined for the His-87 of the Tyr83His mutant,[17] and the shifts observed for the Leu12Glu mutant (if real) may be specific to this form. The pK_a of the other histidine ligand (His-37) has also been determined and is 4.3 ± 0.1. This is in good agreement with the value <4.5 obtained for the native protein,[15] and provides further confirmation that the structural changes in the case of the Leu12Glu mutant are local.

Competitive Inhibition Studies on the $[Co(phen)_3]^{3+}$ *Oxidation of* Leu12Glu PCu(I).—The oxidation of Leu12Glu PCu(I) (1×10^{-5} M) by $[Co(phen)_3]^{3+}$ (6×10^{-4} M) at pH 8.00 was studied in the presence of varying amounts of $[Co_4(NH_3)_{12}(\mu\text{-}OH)_4(\mu_4\text{-}C_2O_4)]^{6+}$. Competitive inhibition by the tetranuclear cobalt(III) complex is observed. First-order rate constants at different concentrations of the 6+ complex are given in Table 3. The dependence of k_{obs} on concentration $[Co^{III}_4]$ of the tetranuclear cobalt(III) complex is shown in Fig. 5.

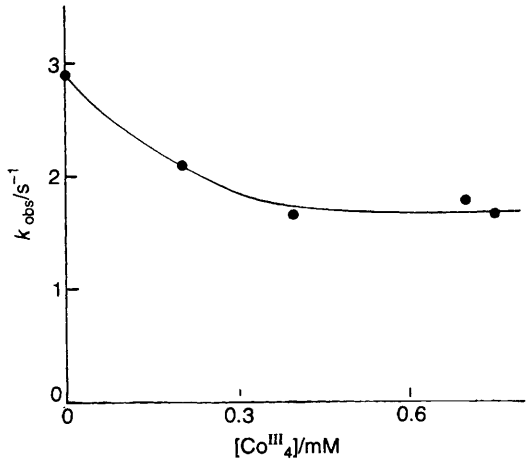

Fig. 5 The effect of $[Co_4(NH_3)_{12}(OH)_4(C_2O_4)]^{6+}$ on first-order rate constants, k_{obs} (25 °C), for the oxidation of Leu12Glu PCu(I) ($\approx 1.0 \times 10^{-5}$ M) with $[Co(phen)_3]^{3+}$ (6.0×10^{-4} M)

5. From this plot it can be seen that at the higher concentrations when the remote, acidic patch of PCu(I) is fully blocked there remains significant (63%) reaction. This is presumably occurring at the adjacent site which now incorporates the negatively charged Glu-12 residue. In this treatment we assume that there is no inhibition of the Glu-12 site by the cobalt complex. From simple electrostatic calculations based on a remote site 7− with 6+ interaction as compared to an adjacent site 1− with 6+ interaction, and taking note of association constants obtained using the Fuoss–Eigen treatment,[18] it can be concluded that association at the remote site is dominant, and at least an order of magnitude more favourable. It is possible that the Co^{III}_4 complex associated at the remote site has some long-range inhibitory effect on reactions at the adjacent site.[19] However we have no means of taking this into account, and assume that the effect is low key.

Biphasic Kinetics for the $[Fe(CN)_6]^{3-}$ *and* $[Co(phen)_3]^{3+}$ *Oxidation of* Leu12Glu PCu(I).—A second kinetic phase is observed for the oxidation of Leu12Glu PCu(I) by $[Fe(CN)_6]^{3-}$ (pH 4.30–6.00) and $[Co(phen)_3]^{3+}$ (pH 5.60–7.80). The first-order rate constants k_{2obs} are ≈30 and ≈20 times smaller than for the first phase (k_{obs}). The variation of k_{2obs} with the concentration of oxidant was studied by non-'pH-jump' kinetic studies (*i.e.* with the pH of both reactants at the same value) at pH 7.20 for $[Co(phen)_3]^{3+}$ and pH 5.08 for $[Fe(CN)_6]^{3-}$. In both cases an oxidant-independent reaction is observed, and the full dependence is as in equation (8). Thus

$$k_{2obs} = a + b[\text{oxidant}] \quad (8)$$

with $[Co(phen)_3]^{3+}$ and $[Fe(CN)_6]^{3-}$ respectively values of a (0.05 and 0.28 s^{-1}) and b (97.5 and 1608 M^{-1} s^{-1}) are obtained. The oxidant-independent pathway accounts for ≈15% of the total absorption change in the $[Co(phen)_3]^{3+}$ oxidation and ≈25% in the case of $[Fe(CN)_6]^{3-}$. It is clear that the changes observed for the second phase are less precise, and with only limited amounts of the mutant it is difficult to improve on the accuracy of data. The behaviour observed suggests that in the reaction with $[Fe(CN)_6]^{3-}$ a less reactive unprotonated form of Glu-12, and in the reaction with $[Co(phen)_3]^{3+}$ a less reactive protonated form are held back. In other words reformation of the more reactive form of the protein is in part rate determining.

Effect of pH *on the Oxidation of* Tyr83His PCu(I).—The trends in second-order rate constants k_{Fe} and k_{Co}, Table 4, are shown in Figs. 6 and 7 respectively. In order to explain the effect of pH on the $[Fe(CN)_6]^{3-}$ oxidation (in particular

Table 4 The variation of second-order rate constants (25 °C) with pH for the oxidation of the Tyr83His PCu(I) mutant ($\approx 1 \times 10^{-5}$ M) with $[Fe(CN)_6]^{3-}$ ($\approx 5 \times 10^{-4}$) or $[Co(phen)_3]^{3+}$ ($\approx 5 \times 10^{-4}$ M), I = 0.100 M (NaCl)

$[Fe(CN)_6]^{3-}$ as oxidant

pH	4.31	4.36	4.75	4.77	4.97		
$10^{-4}k_{Fe}/M^{-1}\ s^{-1}$	1.42	1.23	1.81	1.86	2.12		
pH	5.16	5.60	6.10	6.56	6.88		
$10^{-4}k_{Fe}/M^{-1}\ s^{-1}$	2.04	2.59	2.77	3.1	3.4		
pH	7.32	7.48	8.13	8.51	8.70	9.16	9.42
$10^{-4}k_{Fe}/M^{-1}\ s^{-1}$	3.6	3.7	4.5	5.0	5.0	5.1	5.2

$[Co(phen)_3]^{3+}$ as oxidant

pH	4.39	4.61	5.04	5.66	6.10	6.54	6.88
$10^{-3}k_{Co}/M^{-1}\ s^{-1}$	0.06	0.12	0.24	0.37	0.46	0.54	0.66
pH	7.00	7.15	7.33	7.59	8.08	8.47	8.65
$10^{-3}k_{Co}/M^{-1}\ s^{-1}$	0.71	0.64	0.70	0.89	1.06	1.25	1.47
pH	9.07	9.37	9.47				
$10^{-3}k_{Co}/M^{-1}\ s^{-1}$	1.66	1.80	1.85				

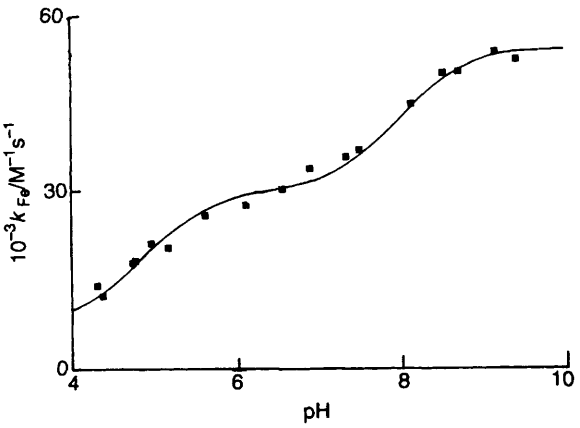

Fig. 6 Variation of second-order rate constants k_{Fe} (25 °C) with pH for the $[Fe(CN)_6]^{3-}$ oxidation of Tyr83His PCu(I), I = 0.100 M (NaCl)

the further increase in rate constants as the pH increases in the range 7.0–9.0) we suggest that the effect of protonation/deprotonation at His-83 is transmitted to the active copper site. Thus, at high pH, deprotonation of His-83 results in a lower Cu^{I}–Cu^{II} potential at this site and, therefore, the driving force for the oxidation by $[Fe(CN)_6]^{3-}$ is larger. This would then explain the larger rate constants at high pH values. If the effect was electrostatic only in origin then one would have expected that at high pH the reactivity with $[Fe(CN)_6]^{3-}$ should have been little affected. From similar fits involving two acid dissociation constants as in reaction scheme (2) we obtained the following parameters for the $[Fe(CN)_6]^{3-}$ oxidation: pK_{aA} (active site) 4.9 ± 0.1, pK_{aH} (His-83) = 7.9 ± 0.1, k_H = (7.3 ± 1.5) × 10^3 M^{-1} s^{-1}, k_1 = (3.1 ± 0.01) × 10^4 M^{-1} s^{-1} and k_0 = (5.4 ± 0.8) × 10^4 M^{-1} s^{-1}. In the case of $[Co(phen)_3]^{3+}$: pK_1 (active site) = 5.6 ± 0.1, pK_2 (His-83) = 8.4 ± 0.1, k_H = (0.03 ± 0.01) × 10^3 M^{-1} s^{-1} (*i.e.* close to zero), k_1 = (0.60 ± 0.03) × 10^3 M^{-1} s^{-1} and k_0 = (1.94 ± 0.05) × 10^3 M^{-1} s^{-1}. A summary of the data is given in Table 5.

Discussion

From a comparison of the data in Table 1, it is apparent that major differences in reactivity are observed in the case of the Leu12Glu, Leu12Asn and Tyr83His mutants. The other two mutants (Tyr83Phe and Asp42Asn) and the wild-type protein show similar reactivity to that of the native protein. Rate constants determined for wild-type plastocyanin, are within 9% {for $[Fe(CN)_6]^{3-}$} and 6% {for $[Co(phen)_3]^{3+}$} of values obtained for native protein, Table 1.

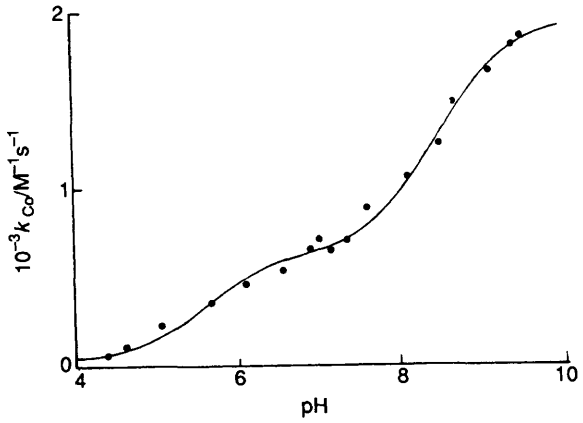

Fig. 7 Variation of second-order rate constants k_{Co} (25 °C) with pH for the $[Co(phen)_3]^{3+}$ oxidation of Tyr83His PCu(I), I = 0.100 M (NaCl)

Effects of Mutations on Structures.—Before analysing in detail the results obtained, it is appropriate to comment on the effects of the mutations on the tertiary structure of the protein. The ideal situation would of course be that a single mutation did not affect the overall structure. The changes in reactivity observed in the case of the mutants, as compared to the native protein, can then be attributed solely to structural effects close to the mutation. Evidence for retention of the overall structure and only minor effects of the mutants arises from spectroscopic studies. All the mutant forms show indistinguishable UV/VIS and EPR spectra, which suggests that the copper site remains intact.[6c]

Furthermore the NMR spectrum of the Leu12Glu PCu(I) mutant at pH 7.4, Fig. 1, shows very little difference to those of native (spinach)[16] and the wild-type protein.[6c] The resonance of the C^{δ}-methyl protons of Leu-12 is missing as expected. Modi *et al.*[6c] have found that all mutations at position 12 affect the chemical shift of the $N^{\varepsilon}H$ of His-37 and the Asn-38, Ser-85 and Tyr-83 backbone amides. However these changes and the changes observed in other mutants are minor and the overall similarity of the mutant spectra to that of the wild-type protein indicates that no major conformational changes result from the mutations.

The mutants of other blue copper proteins have been studied. In the case of *Pseudomonas aeruginosa* azurin X-ray crystallographic studies have been carried out on the His35Gln and His35Leu mutants[20] and in the case of pseudoazurin (from *Alcaligenes faecalis* S-6) on the Pro80Ala mutant.[21] These studies show that the mutations have mainly local effects and that the overall structure of the protein is retained. In the case of plastocyanin more detailed X-ray crystal structure studies on the mutants are required to identify smaller perturbations on the overall tertiary structure.

Reduction Potentials.—Values of $E_f°$ have been obtained for the different mutants from rate constants at pH 7.5 for the forward and reverse reactions as defined in equations (9) and (10). Values calculated from equations (9) and (10) are in good

$$PCu(I) + [Fe(CN)_6]^{3-} \rightleftharpoons PCu(II) + [Fe(CN)_6]^{4-}\ (k_{Fe}, k_{Fe}') \quad (9)$$

$$PCu(I) + [Co(phen)_3]^{3+} \rightleftharpoons PCu(II) + [Co(phen)_3]^{2+}\ (k_{Co}, k_{Co}') \quad (10)$$

agreement (± 3 mV), and average values are indicated in Table 1. There is also good agreement with values obtained from

Table 5 Summary of data (25 °C) for the oxidation of native protein and Leu12Glu and Tyr83His mutants of spinach plastocyanin, PCu(I), with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$, I = 0.100 M (NaCl). The pK_{aA} values from the studies with $[Co(phen)_3]^{3+}$ are 'apparent' values resulting from both active and remote site effects

	Native PCu(I)		Leu12Glu PCu(I)		Tyr83His PCu(1)	
	$[Fe(CN)_6]^{3-}$	$[Co(phen)_3]^{3+}$	$[Fe(CN)_6]^{3-}$	$[Co(phen)_3]^{3+}$	$[Fe(CN)_6]^{3-}$	$[Co(phen)_3]^{3+}$
pK_{aA}	4.9	5.7	4.8	5.8	4.9	5.6
pK_{aH}			6.7	7.2	7.9	8.4
$10^{-3}k_H/M^{-1}\ s^{-1}$			0	0	7.3	(0.03)*
$10^{-3}k_1/M^{-1}\ s^{-1}$			43	2.2	31	0.6
$10^{-3}k_0/M^{-1}\ s^{-1}$	85	2.5	8.2	5.4	54	1.94
pK_a (NMR)	4.9 (His-87)			5.4, 6.5 (His-87)	< 5.2 (His-87), 8.4 (His-83)	

* Close to zero.

potentiometric titration of Leu12Glu (358 mV) and Tyr83His (419 mV) at pH 7.5.[5] Overall there is a spread in values either side of the $E_f°$ for native protein (375 mV). Reduction potentials for the Leu12Glu and Tyr83His are at either extreme, which may relate at least in part to the negative charge on Glu-12 and positive charge on His-83 at pH 7.5. As the pH is varied $E_f°$ is expected to vary, as is the case for the His-59 protonation/deprotonation on *Scenedesmus obliquus* PCu(I).[14]

Reactions of the Leu12Asn *and* Asp42Asn *Mutants*.—The five-fold enhancement in rate constants for the $[Fe(CN)_6]^{3-}$ oxidation of Leu12Asn PCu(I) as compared to native protein suggests that the more polar $-CONH_2$ group in the hydrophobic region favours this reaction. On the other hand there is no effect on the oxidation by $[Co(phen)_3]^{3+}$. This is in line with the observation that native spinach PCu(I) reacts with $[Co(phen)_3]^{3+}$ predominantly at the remote site.[1] Studies on Leu12Glu PCu(I) demonstrate conclusively the effects that charge can have on electron transfer at the adjacent site. The small effect of replacement of Asp-42 by Asn on rate constants for the reaction with $[Co(phen)_3]^{3+}$ is unexpected since the Asp-42 residue is invariant in thirty known plastocyanin sequences.[22] Interestingly Modi *et al.*[6c] also report no effect on rate constants for the Asp42Asn PCu(II) oxidation of cytochrome f(II). A possible explanation relates to the less extensive protrusion of Asp-42 from the plastocyanin surface as compared to Glu-43 and Asp-44. This observation suggests that studies on other mutants are desirable, in order to designate the involvement of these residues and specificities at the remote site. Also of interest is the reactivity of *Anabaena variabilis* plastocyanin, which retains only Asp-42 and has little or no reactivity with $[Co(phen)_3]^{3+}$ at the remote site.[4b] The presence of basic residues His-59 (pK_a 7.3), Lys-60 and Arg-88 (the side chain of which appears to bridge Asp-42 and Glu-85) further minimises negative charge at this site. It appears, therefore, that the identity of residue 42 and whether it is acidic or not has little or no influence on the reactivity with $[Co(phen)_3]^{3+}$ and (presumably) other cationic redox partners.

Reactions of the Tyr83Phe *Mutant*.—Significantly in the case of Tyr83Phe there is again no effect on the reactivity with $[Co(phen)_3]^{3+}$. However He *et al.*[6a] have reported an effect on the cytochrome f(II) reduction of this PCu(II) mutant. More specifically the association step (K) which precedes electron transfer (k) is eight times less favourable than for the native protein (with k unchanged). In the corresponding studies with cytochrome c(II) as reductant no significant differences are observed.[6b] Therefore, the phenolic group of Tyr-83 appears to be of direct benefit in the reaction with cytochrome f, most probably by forming a hydrogen bond. It is surprising that the reaction of cytochrome f(II) with *S. obliquus* plastocyanin PCu(II), which has a sequence Tyr82–Phe83 as compared to Phe82–Tyr83 in other plastocyanins, shows no apparent diminution in the overall observed rate constant (which incorporates K).[4b] This effect is particularly interesting since *S. obliquus* plastocyanin has deletions at (or in the region of) positions 57 and 58, with introduction of a surface-exposed tyrosine at position 62. The latter may have a compensating effect and be able to enhance association with cytochrome f instead of Tyr-83. Of the thirty known plastocyanin sequences,[22] nine have these deletions and in each case a tyrosine is incorporated at 62. However only *S. obliquus* and *Chlorella fusca* have the Tyr82–Phe83 sequence change. It is not surprising that an inorganic complex such as $[Co(phen)_3]^{3+}$ with no physiological role to play, and incapable of forming hydrogen bonds, should not be sensitive to the identity of residue 83, and whether this is Tyr or Phe.

The introduction of a non-aromatic residue (Tyr83Leu) in pea plastocyanin[6a] gives a more dramatic 40-fold decrease in the overall rate constant for reaction with cytochrome f, and a lower intrinsic rate constant and weaker binding constant (K) are to be noted. It seems therefore that the existence of an aromatic residue connected to the Cu-bound ligand Cys-84 is crucial for efficient electron transfer from the remote site. It is interesting that in the multicopper enzyme ascorbate oxidase a similar electron-transfer pathway Cys–His is thought to be the route of intramolecular electron transfer between the type 1 and the trinuclear type 2/type 3 copper sites.[23] Resonance-Raman[24] and theoretical studies[25,26] also support Cys84–Tyr83 as a likely route for electron transfer between the Cu and the remote site of plastocyanin.

Oxidation of Tyr83His- *and* Leu12Glu-PCu(I) *in the High* pH *Range*.—The responses of rate constants k_{Fe} and k_{Co} for the oxidation of Tyr83His PCu(I) to pH is similar to that observed for *S. obliquus* PCu(I) which has a histidine residue at position 59.[14] The behaviour observed with $[Fe(CN)_6]^{3-}$ as oxidant is unusual in so far as a reactant at the adjacent site would not be expected to respond to protonation of a histidine at position 83. The protonation/deprotonation is believed to be transmitted to the active site and effects $E_f°$, with the result that reactions at both the adjacent and remote sites are influenced to about the same extent. In fact, the relative change in rate constants over the range pH 7.0–9.5 is greater for $[Co(phen)_3]^{3+}$ than for $[Fe(CN)_6]^{3-}$ in keeping with an additional influence of the electrostatics at the remote site. A similar interpretation applies in the case of His-59 of *S. obliquus* PCu(I).[14] What is remarkable in the latter is that protonation at His-59 can be transmitted to the active site when there is no direct covalent link, while in the case of the ruthenium-modified His-59 derivative no favourable electron-transfer pathway exists from Ru^{II} to Cu^{II}.[27] The His-83 influence in spinach plastocyanin is more understandable since the imidazole of the latter is bonded directly to the cysteinyl sulfur atom of Cys-84. The kinetic pK_a values of 7.9 and 8.4 are in satisfactory agreement with the NMR titration value of 8.4.[17] The high pK_a value assigned to

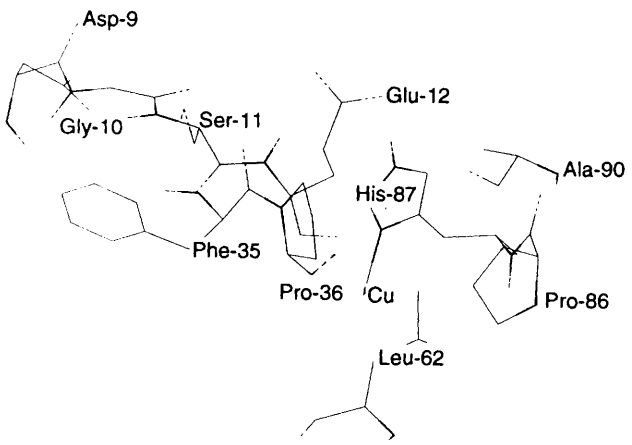

Fig. 8 The adjacent site of plastocyanin showing the proximity of Glu-12 to Ser-11 and His-87 using information provided by the poplar plastocyanin structure (ref. 2)

His-83 (a histidine residue might normally be expected to have $pK_a \approx 6.0$) suggests proton sharing with a carboxylate at positions 42–45 or 59–61.

The results obtained for Leu12Glu are particularly interesting in that the reaction of $[Fe(CN)_6]^{3-}$ should be so sensitive to the presence of the 1 – charged glutamate. As the glutamic acid residue dissociates, rate constants decrease from 4.3×10^4 M^{-1} s^{-1} at pH 5.4 to 0.82×10^4 M^{-1} s^{-1} at pH > 8.0. The extent of the effect suggests that His-87 is a fairly specific contact region for electron transfer, so much so that the existence of a 1 – charge at the adjacent Glu-12 residue markedly inhibits reaction with $[Fe(CN)_6]^{3-}$. Conversely, $[Co(phen)_3]^{3+}$ switches its allegiances and instead of reacting 75:25 at the remote site (native protein),[13] a 37:63 reactivity pattern favouring reactivity at the adjacent site in the presence of the 1 – glutamate residue is observed. The ratio of rate constants $10^{-4}k_o/M^{-1}$ s^{-1} (25 °C)[1,13] at high pH for native:Leu12Glu:Tyr83His with $[Fe(CN)_6]^{3-}$ (8.5:0.86:5.0) and $[Co(phen)_3]^{3+}$ (0.25:0.55:0.20) is of interest. These values illustrate a particularly striking influence of the Leu12Glu mutation which is unfavourable to $[Fe(CN)_6]^{3-}$ (order of magnitude) and favourable to $[Co(phen)_3]^{3+}$ (factor of 2.2).

Shifts in the ^1H NMR peaks for the copper-ligated His-37 $N^{\varepsilon}H$ and residues as far as Tyr-83 plus slight changes in the EPR parameters on the same mutant[6c] are also noted. Fig. 8 shows a section of the hydrophobic surface from molecular graphics, with Glu-12 inserted into the poplar plastocyanin structure and orientated to minimise interactions with other groups. Fig. 8 suggests that the Ser-11 and/or His-87 are sufficiently close for one or other to interact with Glu-12. This also has the effect of retaining the glutamic acid proton to higher pH. The agreement of the pK_a values obtained for the glutamic acid from the two pK_a fits at 6.7 and 7.2 is considered satisfactory. These give an average value of 6.9 which is however high for a carboxylic acid group. Another factor that might stabilize the protonated form is the hydrophobicity of the site. In chicken lysozyme, the hydrophobicity of Trp-108 has been proposed as a contributing factor to the Glu-35 pK_a of 6.1.[28] Large shifts in the pK_a of glutamic acid residues (values of 4.3, 4.7, 6.3, 7.7 and 8.1) have been observed recently for five different polypeptides.[29] These remarkable increases (3.8 pH units overall) have been attributed to the hydrophobicity of adjacent phenylalanine residues.

Reactions of Tyr83His *and* Leu12Glu *in the Low*-pH *Range*.—The active-site pK_a values of 4.7 and 4.9 obtained for the $[Fe(CN)_6]^{3-}$ oxidations of the Leu12Glu and Tyr83His mutants respectively are in good agreement with values of 4.9 (NMR) and 4.8 (kinetics) obtained for native protein.[13,15] With $[Co(phen)_3]^{3+}$ as oxidant higher 'apparent' pK_a values of 5.8 and 5.6 are obtained as compared with 5.7 for native protein.[1,13] These are a result of reaction at the remote site, and combined effects of active- and remote-site pK_a values. Attempted fits with the Leu12Glu NMR values of 6.1 for the active-site pK_a are not acceptable for the $[Fe(CN)_6]^{3-}$ reaction. The NMR pK_a of 6.1 is believed to be a combination of protonation at the His-87 and Glu-12 residues, and a fit to two pK_a values gives an estimate of these two effects.

Finally as in other studies on plastocyanin PCu(I) with $[Fe(CN)_6]^{3-}$,[14] we note particularly in the case of the Tyr83His mutant a significant retention of reactivity at low pH, whereas in the case of $[Co(phen)_3]^{3+}$ a more complete 'switch-off' in reactivity is apparent. At lower pH, due to protonation of His-87, the electrostatics become more favourable with $[Fe(CN)_6]^{3-}$.

Conclusion

The studies described help further to establish reactivity patterns at the two sites of plastocyanin. The most important points to make are as follows.

The sensitivity of the reactions of the Tyr83His mutant to protonation is of interest in view of the evidence for the Tyr83–Cys84 pathway as a biologically relevant through-bond electron-transfer route.[6,23-26,30]

The responses of the Leu12Glu mutant, and the effect of the 1 – glutamate on reactivity are of interest. Translated into an effect on self-exchange *via* the adjacent hydrophobic surfaces of PCu(I) and PCu(II),[4b,31] it is likely that this will give a 2–3 orders of magnitude effect similar to that observed for the azurin Met44Lys[7] which introduces a 1+ charge at the corresponding surface.

The importance of negatively charged residues at the remote site, and the absence of any effect of the Asp42Asn mutation on reactivity, requires further study with mutant forms of other nearby acidic residues *e.g.* 43 and 44. The beneficial effects of the conserved Tyr-83 as opposed to mutant Phe-83 observed in the reaction with cytochrome f are not seen in the studies with small inorganic redox partners. Attention is drawn to the availability of an alternative surface Tyr-62 residue in green algal plastocyanins, in particular *S. obliquus* plastocyanin, which in the native form has Phe-83 in place of the Tyr-83 residue.

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