Protein–Protein Cross-reactions involving Plastocyanin, Cytochrome f and Azurin: Self-exchange Rate Constants and Related Studies with Inorganic Complexes†

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Protein–protein cross-reactions involving acidic and basic plastocyanins, cytochrome f and azurin, and including reactions between two blue copper proteins, have been studied for the first time. The high reactivity of cytochrome f, and properties of the basic *Anabaena variabilis* plastocyanin, are noted. Reduction potentials for different plastocyanins have been obtained at pH 7–9 and are in the range 340–380 mV. The application of Marcus theory has enabled self-exchange rate constants (25 °C) for acidic parsley (3.3×10^3 M^{-1} s^{-1}) and basic *A. variabilis* (5.9×10^5 M^{-1} s^{-1}) plastocyanins to be calculated at pH 7.5, I = 0.10 M (NaCl). The influence of overall charge on the PCu(I)–PCu(II) self-exchange, estimated as −8, −7 in the case of parsley and +1, +2 for *A. variabilis*, is noted. A mechanism for electron transfer involving contact of adjacent hydrophobic regions on the two plastocyanin surfaces giving a Cu · · · Cu separation of 12–14 Å is proposed. Rate constants for the cytochrome f(II) reduction of four PCu(II) plastocyanins (acidic parsley, spinach, *Scenedesmus obliquus* and basic *A. variabilis*) are a factor of $\approx 10^2$ less in the case of the basic form. The rate constant for *Pseudomonas aeruginosa* azurin with cytochrome f has also been determined. The *A. variabilis* and azurin rate constants give a cytochrome f(II)–f(III) self-exchange of 5.0×10^5 M^{-1} s^{-1} at pH 7.5, whereas that obtained from the cross-reaction with the more highly charged parsley PCu(II) (2.3×10^8 M^{-1} s^{-1}) requires correction for work terms, and is predictably out of line. Only one out of four cross-reactions of *A. variabilis* plastocyanin and azurin with $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) and $[Fe(CN)_6]^{3-}$ gives a satisfactory fit to the Marcus theory, indicating an influence of more localised protein charge on these reactions. The absence of a fully developed acidic patch region on the surface of *A. variabilis* plastocyanin is discussed.

This paper is concerned with the electron-transfer reactivity of different plastocyanins and cytochrome f, which are physiological redox partners in photosynthetic electron transport between photosystems II and I in the chloroplast of higher plants and algae.[1,2] Plastocyanin (PCu) is a single (type 1) blue copper protein, characteristic features of which are its intense blue colour and narrow hyperfine coupling in the EPR spectrum of the copper(II) protein.[3–6] The reduction potential [E° *vs.* normal hydrogen electrode (NHE)] is generally assumed to be 375 mV, a value determined for the spinach PCu(II)–PCu(I) couple in the early work of Katoh *et al.*[7] and recently obtained from cyclic voltammetry at 25 °C, I = 0.10 M (NaCl), using neomycin as promoter.[8] The variability of E° for different plastocyanins is a subject addressed in this paper. Plastocyanin has 99 amino acids ($M_r \approx 10\,500$); in some cases *e.g.* parsley and green algal plastocyanins, there are deletions at 57 and 58,[3,4] and in one case, that of the blue-green algal plastocyanin from *Anabaena variabilis*, there are additional residues giving 104 in all.[9] There are now 25 full amino-acid sequences (see refs. 10–14 for recent reports), which have been summarised.[14] Of the 20 higher plant sequences (including spinach and parsley) 47 of the 99 residues are invariant. With the inclusion of four green algal sequences (including *Scenedesmus obliquus*) this number reduces to 28, and with the *A. variabilis* sequence it is 23.[14] The invariant residues include His37, Cys84, His87 and Met92, which co-ordinate the Cu at the active site.[15]

Cytochrome f is a monohaem protein ($M_r \approx 31\,000$, 285 amino acids),[16] with histidine and lysine as axial ligands,[17] and E° = 360 mV at pH 5–9.[18,19] Whereas plastocyanin is mobile in the inner thylakoid, cytochrome f is a component of the b_6f protein complex, and as such is membrane bound.[20] From the amino-acid sequence it has been deduced that a substantial part of cytochrome f exists as a highly charged globular section, which is outside the membrane.[16] A procedure has been described for isolating a non-aggregating water-soluble form of cytochrome f from cabbage leaves, *e.g. Brassica oleracea*, from which residues 250–285 have been cleaved.[18] The latter includes the hydrophobic section 250–271, which in the chloroplast is believed to be *trans*-membrane. The water-soluble protein is used in the present work.

In all but one case plastocyanin is acidic with the charge on PCu(I) estimated to be in the range (or close to) -9 ± 1 at pH $\approx$ 7. Reactions of plastocyanin have been assigned to remote (close to Tyr83) and adjacent (His87) sites.[21,22] Features of the former are the negatively charged 42–45 and 59–61 regions on two protruding sections of polypeptide either side of the solvent-exposed Tyr83.[15] Evidence has been obtained for cytochrome f reacting at this remote site.[19] Although cytochrome f(II) has an estimated overall charge of −1 at pH $\approx$ 7, the exposed haem edge is believed to have positive region(s) close by.[16,23] In the case of *A. variabilis* the negative charge at the remote site is not retained, and the overall charge on the PCu(I) form is +1 at pH $\approx$ 7.[9] Information regarding the reactivity of this plastocyanin is therefore of considerable interest.

There are at present no measured or reliably calculated self-exchange rate constants for plastocyanin or cytochrome f. In this work it has been possible to obtain values for an acidic plastocyanin (from parsley), and for the basic plastocyanin from *A. variabilis*, by studying cross-reactions and applying Marcus theory.[24] It is not easy to allow for work terms in the case of protein molecules having a substantial and non-uniform

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

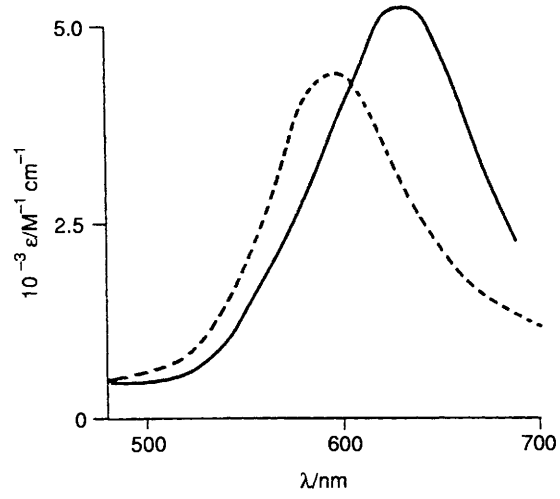

Fig. 1 A comparison of visible-range absorption spectra for the copper(II) forms of *P. aeruginosa* azurin (——) and spinach (– – – –) plastocyanin

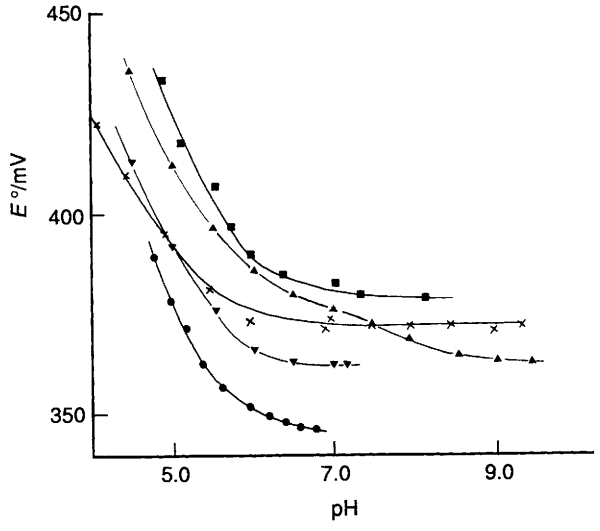

Fig. 2 The variability of reduction potentials ($E°$) with pH for different plastocyanins at 25 °C, I = 0.10 M (NaCl). Values were obtained from rate constants k_f and k_b, equation (2), with $[Co(phen)_3]^{3+/2+}$ [*S. obliquus* (▲), spinach (×), poplar (▼), *A. variabilis* (●)] and $[Fe(CN)_6]^{3-/4-}$ [parsley (■) and spinach (×)] as redox partners

distribution of charge on the surface. The approach here has been to use the blue copper protein azurin (M_r 14 000, 128 amino acids) as redox partner. The net charge on *Pseudomonas aeruginosa* ACu(I) at pH ≈ 7 is −1. From the structure it is known that there is extensive pairing of charged residues on the surface.[25,26] Work terms are minimised therefore. We have also determined rate constants for the reactions of the four plastocyanins and *P. aeruginosa* azurin with cytochrome f. Using the same approach, rate constants for the cytochrome f self-exchange reaction are calculated. Finally we consider rate constants for the oxidation of both *A. variabilis* PCu(I) and *P. aeruginosa* ACu(I) with $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) and $[Fe(CN)_6]^{3-}$ as oxidants, and test the application of Marcus theory to these reactions.

Experimental

Isolation of Plastocyanins.—Samples of parsley,[21,27] spinach,[28] *Scenedesmus obliquus*[29] and *Anabaena variabilis*[30,31] plastocyanins were obtained as previously described. Final plastocyanin purifications were to UV/VIS absorbance (A) peak ratios A_{278}/A_{597} for parsley (1.68), spinach (<1.14), *S. obliquus* (3.0) and *A. variabilis* (1.15:1). The final purification was done by Pharmacia fast protein liquid chromatography (FPLC) using a Mono-Q anion-exchange column (parsley, spinach and *S. obliquus*), and a Mono-S cation-exchange column for *A. variabilus* plastocyanin. Concentrations were determined from the PCu(II) peak at 597 nm (ε = 4500 M^{-1} cm^{-1}).[3]

Isolation of Cytochrome f.—Water-soluble cytochrome f (residues 1–250) was obtained from cabbage (*Brassica*) leaves by the procedure of Gray.[18] As previously the product was purified to A_{554}/A_{280} = 0.85 ± 0.10:1 for the reduced form.[32] The absorbance maximum for cytochrome f(II) at 554 nm (ε = 32 000 M^{-1} cm^{-1}) was used to determine concentrations.[33] The UV/VIS spectra of cytochrome f(II) (pink) and cytochrome f(III) (orange-pink) forms were as illustrated in a previous paper.[32]

Isolation of Azurin.—Azurin was obtained from *Pseudomonas aeruginosa* as previously described.[34] Samples of ACu(II) were purified in the final stages to A_{280}/A_{625} ratio of 1.67–1.72:1 by Pharmacia FPLC using a Mono-S cation-exchange column. Concentrations of ACu(II) were determined from the absorbance at the 625 nm peak (ε = 5200 M^{-1} cm^{-1}).*

Absorption Spectra of PCu(II) *and* ACu(II).—Although both active sites have HisCysHisMet co-ordination, azurin has the peptide carbonyl O atom of Gly45 3.13 Å from the Cu^{II} and is therefore five-co-ordinate.[26] The Cu^{II} in plastocyanin is 0.34 Å from the plane defined by the N, S and N atoms of the co-ordinating HisCysHis residues,[15] whereas in azurin the corresponding distance is 0.1 Å.[26] Differences in the relative positions of the S(Cys)→Cu^{II} charge-transfer bands of plastocyanin and azurin are noted in Fig. 1.

Reduction Potentials of the Plastocyanins.—Accurate values are required in applying the Marcus theory. In previous work the closeness of the reduction potentials of $[Co(phen)_3]^{3+}$–$[Co(phen)_3]^{2+}$ (370 mV) and $[Fe(CN)_6]^{3-}$–$[Fe(CN)_6]^{4-}$ (410 mV)[3] to those of the plastocyanin PCu(I)–PCu(II) couple has enabled the separate determination of forward and back rate constants [equation (1)]. We can now draw on these extensive

$$PCu(I) + ox \rightleftharpoons PCu(II) + red \quad (1)$$

data at 25 °C, I = 0.10 M (NaCl), to calculate reduction potentials ($E°$) for five different plastocyanins, four of which are relevant to this work, equation (2).[21,28,29b,31,37–39]

$$\log_{10}(k_f/k_b) = \Delta E°/0.059 \quad (2)$$

In the case of spinach plastocyanin information is available for both inorganic redox couples and the $E°$ values are in good agreement (±3%).[28] Results obtained are indicated in Fig. 2. We note that a spread in $E°$ values of ≈40 mV is observed for the different plastocyanins at pH ≈ 7.5. Interesting in the case of *S. obliquus* is the inflexion at pH ≈ 7.5 due to the influence of protonation/deprotonation of His59,[29b] which we conclude is transmitted to the active site (and $E°$). An $E°$ has been determined for *A. variabilis* by titration with $[Fe(CN)_6]^{3-/4-}$,[31] and at pH 7.5 is 340 mV. A value has also been determined for *S. obliquus* plastocyanin at pH 7.0 (376 mV) by direct cyclic voltammetry, which is again in good agreement with that indicated in Fig. 2. The accuracy of $E°$ determinations is estimated to be ±5 mV.

In all cases at the lower pH values, $E°$ values are seen to increase due to protonation and dissociation of His87 from the Cu^{I}, giving trigonal-planar Cu^{I} with substantially less redox reactivity.[21,40] Acid dissociation constants (K_a) are as defined

* This value from ref. 35. A value of 5800 M^{-1} cm^{-1} is given in ref. 36.

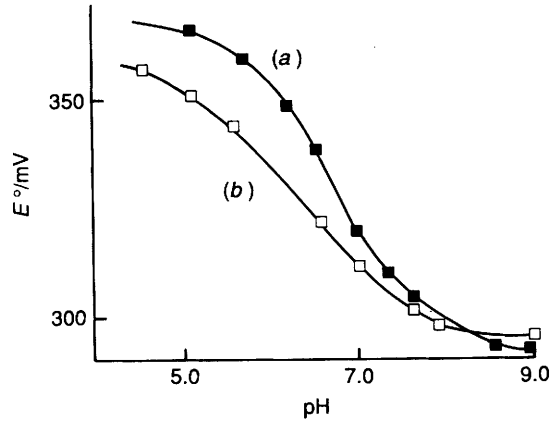

Fig. 3 Variation of reduction potential ($E°$) with pH for the *P. aeruginosa* azurin ACu(II)–ACu(I) couple from (*a*) titration with $[Fe(CN)_6]^{3-/4-}$ (ref. 44) and (*b*) rate constants for the ACu(I) + $[Fe(CN)_6]^{3-}$ and $[Fe(CN)_6]^{4-}$ + ACu(II) redox reactions [ref. 34(*b*)], average value taken

Table 1 Rate constants (25 °C), I = 0.10 M (NaCl), for the azurin ACu(I) (reactant in >10-fold excess) reduction of parsley and *A. variabilis* PCu(II) ($\approx 1 \times 10^{-5}$ M)

(*a*) Parsley PCu(II)

pH	6.40, 6.81, 6.96, 7.03, 7.45, 7.55, 7.93, 8.18
$10^{-5}k$/M^{-1} s^{-1}	2.30, 1.98, 2.10, 2.00, 2.25, 2.06, 2.44, 2.10

(*b*) *A. variabilis* PCu(II)

pH	6.84, 7.22, 7.40, 7.82
$10^{-6}k$/M^{-1} s^{-1}	1.44, 1.47, 1.44, 1.28

Table 2 Rate constants k (10 °C) for the cytochrome f(II), (0.3–1.0) × 10^{-6} M, reduction of four different plastocyanins, PCu(II), present in >10-fold excess, (1.0–2.0) × 10^{-5} M, I = 0.20 M (NaCl)

	$10^{-5}k$/M^{-1} s^{-1}	
Source	pH 5.0	pH 7.5
Spinach	115	163
Parsley	110	210[a]
S. obliquus	78	—[b]
A. variabilis	2.8	4.0[c]

[a] The effect of pH here is more extensive than in the case of *A. variabilis* PCu(II) (footnote *c*), and is assigned to the protonation of the remote site on PCu(II) (ref. 19). The rate constant for the corresponding reaction with parsley cytochrome f has been reported to be 360 × 10^5 M^{-1} s^{-1} at 25 °C, I = 0.100 M; see, for example, ref. 19. [b] Significantly faster than at pH 5.0. [c] The smaller effect of pH (and slightly higher pK_a) here is assigned to contributions from the cytochrome f and is in the direction observed for the $[Co(phen)_3]^{3+}$ oxidation of cytochrome f(II).[46]

in equation (3), where H^+PCu(I) is the trigonal form. Values

$$H^+PCu(I) \rightleftharpoons H^+ + PCu(I) \quad (3)$$

of pK_a determined by NMR and kinetic methods (in close agreement) are respectively for parsley (5.7, 5.5),[21,41] *S. obliquus* (5.4, 5.0),[29b] spinach (4.9, 4.8)[28,38,42] and *A. variabilis* (5.1, 5.0).[31,43] It is noted that parsley and *S. obliquus* with deletions at positions 57 and 58 have higher pK_a values. In turn this is seen to have an influence on the incidence of higher $E°$ values as the pH is decreased, Fig. 2.

Reduction Potential of Azurin.—Variations of $E°$ with pH for *P. aeruginosa* azurin are indicated in Fig. 3.[34b,44] At the lower pH values, where the two sets of data diverge most, average values have been taken.

Kinetic Studies.—All the reactions were monitored on a Dionex D-110 stopped-flow spectrophotometer. The ACu(I) reductions of PCu(II) and cytochrome f(III) were at 25 °C, I = 0.10 M (NaCl). To slow down rates and make stopped-flow studies possible the reaction of cytochrome f(II) with PCu(II) was carried out at 10 °C, I = 0.20 M (NaCl). The stopped-flow spectrophotometer was interfaced with an IBM computer PC/AT-X for data acquisition and analysis using software from On Line Instruments Systems (Jefferson, GA, USA). All rate constants were an average of four or five determinations using the same solutions. Concentrations of protein monitored were varied by factors of at least two in any one particular study.

Self-exchange rate constants. The Marcus equations,[24] (4) and (5), were used, where k_{11} and k_{22} are self-exchange

$$k_{12}^2 = k_{11}k_{22}K_{12}f \quad (4)$$

$$\log f = (\log K_{12})^2/4 \log (k_{11}k_{22}/Z^2) \quad (5)$$

rate constants (for azurin and plastocyanin respectively), k_{12} is the rate constant for the cross-reaction with equilibrium constant K_{12}, and Z is the collision frequency $\approx 10^{11}$ M^{-1} s^{-1}. As $\Delta E° \rightarrow 0$ so the value of $f \rightarrow 1$. In the present studies f was included although it has values >0.90 which have little effect.

Results

ACu(I) *Reductions of* PCu(II).—Rate constants k_{12} for the ACu(I) reduction of parsley and *A. variabilis* PCu(II), equation (6), were determined from absorbance changes at 660 nm with

$$ACu(I) + PCu(II) \longrightarrow ACu(II) + PCu(I) \quad (6)$$

ACu(I) present in large excess, Table 1. Variations in pH 6.4–8.2 had little or no effect on k_{12}. At pH 7.5 k_{12} = (2.2 ± 0.2) × 10^5 M^{-1} s^{-1} (parsley) and (1.4 ± 0.1) × 10^6 M^{-1} s^{-1} (*A. variabilis*). With $E°$ for azurin at 305 mV $\Delta E°$ is 75 mV for the parsley and 35 mV for the *A. variabilis* plastocyanin reactions. *P. aeruginosa* azurin has a self-exchange ACu(I) + ACu(II) rate constant (25 °C) of 7.5 × 10^5 M^{-1} s^{-1} at pH 7.5 from values determined at pH 4.5 and 9.0 by the NMR line-broadening method.[45] Application of the Marcus equations gives k_{22} of (3.3 ± 0.6) × 10^3 M^{-1} s^{-1} for parsley and (5.9 ± 0.5) × 10^5 M^{-1} s^{-1} for *A. variabilis* plastocyanin.

Cytochrome f(II) *Reductions of* PCu(II).—Reactions were monitored at 422 nm, Δε 166 mM^{-1} cm^{-1}, for the cytochrome f(II)–f(III) interconversion. Conditions were 10 °C and I = 0.20 M (NaCl) to slow down the reaction so that rate constants could be accurately determined. Rate constants with four different plastocyanins (reactant in >10-fold excess) are listed in Table 2. The entries with parsley PCu(II) as oxidant are as previously reported.[19] For the other plastocyanins the rate constant trends at pH 7.5 and 5.0 are in agreement with the full pH profile reported for parsley PCu(II). The $E°$ for the cytochrome f(III)–f(II) couple is 360 mV over this pH range.[19,32,33] Rate constants for *A. variabilis* PCu(II) are significantly smaller than for the three other plastocyanins. For the conditions adopted the *A. variabilis* plastocyanin reaction is 84–93% complete, which in the context of the present studies does not have a significant effect.[47] Using data at different temperatures an approximate cytochrome f(II)–f(III) self-exchange rate constant from equations (4) and (5) is (5.8 ± 0.8) × 10^5 M^{-1} s^{-1} at pH 7.5.

ACu(I) *Reduction of Cytochrome* f(III).—Rate constants (25 °C) determined at I = 0.10 M (NaCl) with ACu(I) ($\approx 1.0 \times 10^{-5}$ M) and cytochrome f(III) (10^{-7}–10^{-6} M) give a

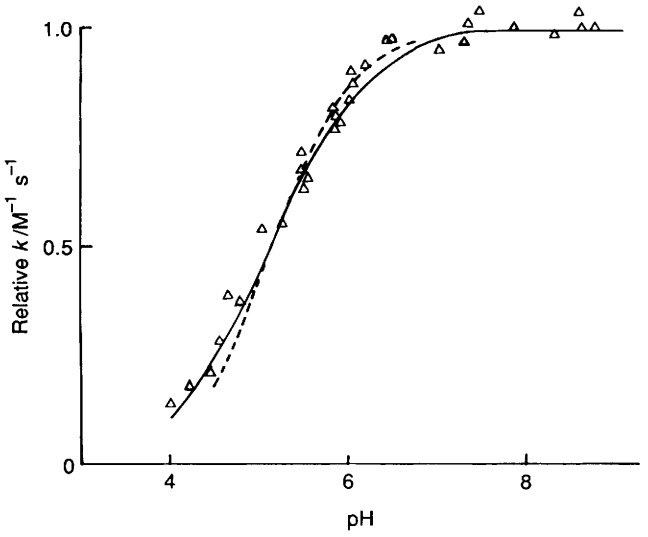

Fig. 4 A comparison of the dependence of second-order rate constants (25 °C) (relative scale) on pH for the $[Co(phen)_3]^{3+}$ oxidation of *A. variabilis* PCu(I). The broken line is the corresponding curve generated for $[Fe(CN)_6]^{3-}$

Table 3 Rate constants for electron transfer between azurin (reactant in excess) and cytochrome f, concentration in the range 10^{-7}–10^{-6} M, monitored at 422 nm

(*a*) ACu(I) + cyctochrome f(III): 25 °C, pH 7.5, I = 0.10 M (NaCl)

10^6[ACu(I)]/M	2.5, 4.3, 4.6, 5.5, 5.6, 8.6
k_{obs}/s^{-1}	4.3, 7.0, 7.7, 8.7, 9.3, 14.1

(*b*) Cytochrome f(II) + ACu(II): 10 °C, pH 5.0, I = 0.20 M (NaCl)

10^5[ACu(II)]/M	1.85, 3.25, 6.70
k_{obs}/s^{-1}	6.8, 12.6, 26.0

'bell shaped' dependence on pH.[48] The maximum rate constant observed is $(1.6 \pm 0.05) \times 10^6$ M^{-1} s^{-1} at pH 7.5, Table 3. From equations (4) and (5) a cytochrome f(II)–f(III) self-exchange rate constant of $(4.1 \pm 0.2) \times 10^5$ M^{-1} s^{-1} is obtained. As a further check the rate constant (10 °C) for the reaction of cytochrome f(II) with ACu(II) was determined at pH 5.0, I = 0.20 M (NaCl), when a value $(3.75 \pm 0.36) \times 10^5$ M^{-1} s^{-1} was obtained indicating little effect of temperature. At pH 5.0 azurin has an $E°$ of ≈360 mV. This gives a self-exchange rate constant of $(1.5 \pm 0.3) \times 10^5$ M^{-1} s^{-1}.

A. variabilis PCu(I) *with* $[Co(phen)_3]^{3+}$ *and* $[Fe(CN)_6]^{3-}$.—Previous work[31] was carried out at pH <7.5. Here we were particularly anxious to check rate constants for the $[Co(phen)_3]^{3+}$ oxidation of *A. variabilis* PCu(I) in the pH range 7–9. Of interest is the possible influence of acid dissociation of protonated His59 on rate constants. In similar kinetic studies on *S. obliquus* PCu(I) a well defined pK_a of 7.6 (7.83 by NMR spectroscopy) was observed.[29b] With *A. variabilis* at pH 7.0–8.8 erratic behaviour was at first observed, with a random scatter of rate constants, up to 66% greater than those indicated at pH ≈ 7.0. With freshly isolated protein, and with Dowex 50W-X2 column-purified $[Co(phen)_3]^{3+}$, self-consistent data were obtained, Fig. 4, with no apparent effect stemming from the protonation of His59 (pK_a previously determined as 7.3 by NMR spectroscopy).[31] What is important is the value of the plateau rate constant at pH > 7.0. A smaller value of 568 ± 7 (k_0) instead of 630 M^{-1} s^{-1} is now indicated. We have carried out more runs at pH 4.06–5.10, and fitted the data by use of equation (7).[31] At the lower pH values there is a

$$k = \frac{k_0 + k_H K_a [H^+]}{1 + K_a [H^+]} \quad (7)$$

tendency for plastocyanin to denature. By including six entries k = 0 at pH 2.0 as weighting factor, the rate constant for the protonated form H^+PCu(I), $k_H = 17 \pm 9$ M^{-1} s^{-1} (close to zero), and active site pK_a = 5.15 ± 0.04, give a satisfactory fit. The pK_a compares with the value 5.1 reported from NMR studies.[31] Moreover (relative) rate constant values pH 4.0–7.0 now superimpose with those for the $[Fe(CN)_6]^{3-}$ oxidation of *A. variabilis* PCu(I), Fig. 4. For the latter, with additional rate constants included, $k_0 = (7.2 \pm 0.1) \times 10^5$ M^{-1} s^{-1}, $k_H = (-0.01 \pm 0.3) \times 10^5$ M^{-1} s^{-1} (close to zero) and pK_a = 5.13 ± 0.48. A complete listing of rate constants is given in Table 4. It is concluded that the pK_a determined from the $[Co(phen)_3]^{3+}$ kinetic studies corresponds to the active-site protonation and there is no contribution to be associated with the acidic residues, in this case Asp42 and Glu85, at or near the remote site. Indeed there is no remaining evidence for reaction of *A. variabilis* PCu(I) with $[Co(phen)_3]^{3+}$ at the remote site.

Self-exchange rate constants have been reported for the $[Fe(CN)_6]^{4-/3-}$ (1.9×10^4 M^{-1} s^{-1})[49] and $[Co(phen)_3]^{2+/3+}$ (12 M^{-1} s^{-1})[50] couples. From studies at pH 7.5 with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ as oxidants for *A. variabilis* PCu(I), self-exchange rate constants for the PCu(I)–PCu(II) couple were calculated and are 2.0×10^6 and 9.3×10^3 M^{-1} s^{-1} respectively. Only the first of these is in reasonable agreement with the self-exchange rate constants indicated above. From the rate constants (25 °C) at pH 7.5 for ACu(I) with $[Fe(CN)_6]^{3-}$ (8.5×10^3 M^{-1} s^{-1}) and $[Co(phen)_3]^{3+}$ (6.1×10^3 M^{-1} s^{-1}), values of the ACu(I)–ACu(II) self-exchange rate constant have been calculated and are 630 and 7.1×10^4 M^{-1} s^{-1} respectively. The agreement here is less satisfactory (NMR measured value 7.5×10^5 M^{-1} s^{-1}).[45]

Discussion

Self-exchange rate constants for parsley PCu(I) + PCu(II) (charges −8 and −7) and *A. variabilis* PCu(I) + PCu(II) (+1 and +2)* have been calculated at 25 °C, I = 0.100 M (NaCl) from cross-reaction studies with azurin, and are 3.3×10^3 and 5.9×10^5 M^{-1} s^{-1} respectively. These are listed, Table 5, alongside values for other blue copper proteins determined by rapid-freeze EPR and line-broadening NMR methods.[45,51–53] The smaller parsley self-exchange rate constant is consistent with the value $<3.0 \times 10^3$ M^{-1} s^{-1} reported from NMR studies on spinach plastocyanin.[54] Work terms are expected to contribute in the case of experimentally determined self-exchange rate constants for the more highly charged plastocyanins. The calculated rate constant 3.3×10^3 M^{-1} s^{-1} will not include such contributions. From NMR studies the *A. variabilis* plastocyanin self-exchange rate constant is $(1–4) \times 10^5$ M^{-1} s^{-1} at 25 °C, I = 0.100 M (NaCl),[35] in satisfactory agreement with the calculated value $(5.9 \times 10^5$ M^{-1} $s^{-1})$. In other words the selective approach of using azurin as redox partner to minimise work terms gives good agreement and provides a satisfactory approach. Self-exchange rate constants for cytochromes have been reported in an earlier study using three different azurins, but no rationale of the approach was indicated.[55]

The difference in self-exchange values for parsley and *A. variabilis* plastocyanins can be understood in terms of charge. Thus the self-exchange rate constants for *A. variabilis* plastocyanin and azurin, both of which have small overall charge and therefore small work terms, are of similar magnitude, Table 5. Stellacyanin, estimated charge +7 for the copper(I) state, has a self-exchange rate constant of 1.2×10^5 M^{-1} s^{-1}, which might at first seem quite large. However one contributing factor is the

* Here and elsewhere overall charges have been estimated from amino-acid sequences assuming Asp and Glu contribute −1, while Lys, Arg and unco-ordinated His contribute +1. At the active site Cys present as a thiolate is −1, but the His residues have zero charge.

Table 4 The variation of rate constants (25 °C) with pH for the oxidation of *A. variabilis* plastocyanin ($\approx 5 \times 10^{-6}$ M) with (*a*) $[Co(phen)_3]^{3+}$ and (*b*) $[Fe(CN)_6]^{3-}$, the oxidant in large excess, $I = 0.10$ M (NaCl)

(*a*) *A. variabilis* PCu(I) + $[Co(phen)_3]^{3+}$

pH	$10^{-2}k/M^{-1}\ s^{-1}$	pH	$10^{-2}k/M^{-1}\ s^{-1}$
4.06	0.80	5.87	4.5
4.24	1.05	6.02	4.8
4.47	1.22	6.04	5.2
4.57	1.58	6.07	5.0
4.67	2.23	6.21	5.3
4.80	2.11	6.39	5.6
5.10	3.1	6.45	5.7
5.28	3.2	7.04	5.4
5.44	3.7	7.33	5.6
5.50	3.6	7.36	5.8
5.51	4.1	7.48	6.0
5.51	3.8	7.85	5.8
5.55	3.8	8.32	5.7
5.83	4.7	8.60	6.0
5.83	4.6	8.62	5.8
5.84	4.4	8.75	5.8

(*b*) *A. variabilis* PCu(I) + $[Fe(CN)_6]^{3-}$

pH	$10^{-5}k/M^{-1}\ s^{-1}$	pH	$10^{-5}k/M^{-1}\ s^{-1}$
4.50	1.3	6.21	6.5
4.55	1.8	6.34	6.8
4.55	1.6	6.85	7.0
4.65	1.6	6.85	6.9
4.82	2.1	7.00	7.2
4.82	2.2	7.00	7.1
5.10	3.5	7.07	7.1
5.10	3.7	7.25	7.0
5.21	3.5	7.36	7.4
5.25	4.4	7.41	7.1
5.25	4.6	7.51	7.2
5.65	5.7	7.56	7.6
5.65	5.8	7.70	7.1
5.77	5.4	7.79	7.5
5.77	6.1	8.02	7.5
6.06	6.1	8.05	7.1
6.21	6.7	8.09	7.6

Table 5 Self-exchange rate constants (25 °C, except as indicated) for the reactions between the copper-(I) and -(II) forms of different blue copper proteins

Source	$k/M^{-1}\ s^{-1}$ (pH)	Technique	Ref.
Azurin (*P. aeruginosa*)	9.6×10^5 (4.5)[a]	NMR	45
	7.0×10^5 (9.0)		
	2.4×10^6 (5.0)[b]	EPR	51
Azurin (*A. denitrificans*)	4.0×10^5 (6.7)	NMR	52
Plastocyanin (*A. variabilis*)	5.9×10^5 (7.5)	Calc.	This work
Amicyanin (*T. versutus*)	1.3×10^5 (8.6)[c]	NMR	52
Stellacyanin (*R. vernicifera*)	1.2×10^5 (7.0)[d]	EPR	53
Plastocyanin (parsley)	3.3×10^3 (7.5)	Calc.	This work

[a] I not indicated. [b] ±40%, 22 °C. [c] $I = 0.05$ M. [d] $I = 0.22$ M, 20 °C.

charge density which is less ($M_r \approx 20\,000$, 107 amino acids, 40% carbohydrate).[56] It has been suggested that the azurin self-exchange occurs by contact of two 'hydrophobic' adjacent surfaces giving a Cu···Cu separation of ≈14 Å.[45] This is supported by site-directed mutagenesis experiments in which the single mutation Met44→Lys,[35] with introduction of a 1+ charge, results in a decrease in self-exchange rate constant. Results now presented are consistent with *A. variabilis* plastocyanin self-exchange occurring by a similar mechanism, and it is possible that this applies also in the case of the parsley plastocyanin self-exchange. The alternative is for reaction to occur *via* the two remote, highly charged, acidic patch regions one on each protein. From simple electrostatic considerations this would be expected to be much less favourable. Instead exchange *via* the two adjacent hydrophobic patches, with a smaller rate constant due to the overall charges, seems more likely.

We have re-examined the kinetic data for the oxidation of *A. variabilis* PCu(I) with inorganic complexes. Rate constants, Fig. 4, with $[Co(phen)_3]^{3+}$ as oxidant give no variations at pH > 7, and on this evidence we conclude that there is no influence of protonation/deprotonation of His59, pK_a 7.3,[31] on reactivity. At first rate constants were spuriously high (up to 66%) with $[Co(phen)_3]^{3+}$ at pH > 7. We have no satisfactory explanation for this behaviour. Protein isolated within the 2 weeks before kinetic runs, and stored frozen at −20 °C for a minimum time, and ion-exchange-column purified $[Co(phen)_3]^{3+}$ gave the behaviour illustrated in Fig. 4. Also unexplained is the different behaviour of *A. variabilis* and *S. obliquus* PCu(I) both of which have a histidine at position 59. In previous work on *A. variabilis* PCu(I) it was reported that neither of the oxidants $[Co(dipic)_2]^-$ (dipic = pyridine-2,6-dicarboxylate) or $[Co(C_2O_4)_3]^{3-}$ gave a pH dependence in the range 6–8.[31]

Additional rate constants have been obtained also with $[Fe(CN)_6]^{3-}$ as oxidant. The observation, Fig. 4, that the pH profiles for these two reactions now overlay is in line with previous experiments in which it was noted that *A. variabilis* PCu(I) does not give saturation kinetics with $[Co(phen)_3]^{3+}$ or competitive inhibition in the presence of redox-inactive $[Pt(NH_3)_6]^{4+}$.[31] All these findings support the view that there is less (possibly zero) participation of the remote region in the electron-transfer reactivity of *A. variabilis* plastocyanin. From molecular graphics using the poplar plastocyanin crystal structure coordinates,[15] making amino-acid substitutions appropriate to *A. variabilis* and applying energy-minimisation programs to the resulting structure, the basic residue Arg88 is seen to be located close to and about midway between the remote site Asp42 and Glu85 residues. The possibility that Asp42 and Glu85 together constitute a remote site pK_a of 5.65 as suggested previously[31] is unlikely therefore, and this pK_a is no longer included in the treatment.

The argument that there is little or no involvement of the remote site in *A. variabilis* PCu(I) electron transfer is reinforced by rate constants obtained for the cytochrome f(II) reduction of PCu(II), Table 2. Rate constants with parsley, spinach and *S. obliquus* plastocyanin are of similar magnitude (and large), whereas that for *A. variabilis* PCu(II) is two orders of magnitude smaller, in spite of the $\approx 10^3$ times more favourable *A. variabilis* self-exchange rate constant. From the PCu(II)–PCu(I) $E°$ values at pH 7.5, parsley (380 mV), spinach (375 mV), *S. obliquus* (375 mV), with cytochrome f(II)–f(III) at 360 mV, equilibrium constants K_{12} are ≈2.0. For *A. variabilis* PCu(II)–PCu(I) (343 mV) the equilibrium constant (≈0.5) and therefore driving force is less favourable, but not sufficiently different to introduce other than a marginal effect. The three plastocyanins with remote acidic patches react more favourably with cytochrome f than does *A. variabilis* plastocyanin. We are hesitant to say that there is no reaction at the remote site of *A. variabilis* plastocyanin because the conserved Tyr83 and Asp42 residues remain. Certainly contributions are very much decreased.

A quite remarkable feature of the cytochrome f(II) reductions of parsley, spinach and *S. obliquus* PCu(II) is the order of magnitude of rate constant ($10^7\ M^{-1}\ s^{-1}$ at pH 7.5) for reactions with little or no thermodynamic driving force. This suggests

little reorganisation energy, and an extraordinary specificity of the two reactants for electron transfer. Experimental rate constants can be considered as composite and equal to $k_{et}K$. In a recent study K for association of plastocyanin with cytochrome f prior to electron transfer (k_{et}) has been reported to be $\approx 9890\ M^{-1}$.[57]

Self-exchange rate constants for cytochrome f(II)–f(III) calculated from the azurin (25 °C, $I = 0.10$ M) and *A. variabilis* plastocyanin (10 °C, $I = 0.20$ M) cross-reactions are in satisfactory agreement (average $5.0 \times 10^5\ M^{-1}\ s^{-1}$), but understandably out of line with the value obtained from the negatively charged parsley plastocyanin cross-reaction ($2.3 \times 10^8\ M^{-1}\ s^{-1}$). The rate constant determined for the *A. variabilis* plastocyanin cross-reaction at 10 °C is not expected to be more than three times less than the value at 25 °C, $I = 0.10$ M (NaCl) (see for example, Table V in ref. 19). This is a small effect in the application of the Marcus equations. The value of $5.0 \times 10^5\ M^{-1}\ s^{-1}$ for the cytochrome f self-exchange falls between those for cytochrome c with a high (7+, 8+) overall positive charge (10^2–$10^4\ M^{-1}\ s^{-1}$) and for cytochrome c_{551} with small overall charge ($\approx 10^7\ M^{-1}\ s^{-1}$).[58] Electrostatic repulsion between positively charged residues may make some contribution in the case of the cytochrome f self-exchange.

Our attempts further to extend the application of Marcus equations (4) and (5) to the reactions of *A. variabilis* PCu(I) and azurin ACu(I) with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ were less successful. Thus for these four cross-reactions only the $[Fe(CN)_6]^{3-}$ ($2.0 \times 10^6\ M^{-1}\ s^{-1}$) oxidation of *A. variabilis* PCu(I) gave a self-exchange rate constant in satisfactory agreement (factor of 3) with that obtained from the ACu(I) reduction of PCu(II). Localised electrostatic interactions of the small inorganic complexes with charged amino-acid residues most likely account for this behaviour.

The similar behaviour of rate constants for parsley, spinach and *S. obliquus* plastocyanins with cytochrome f gives no reason for supposing that there is any special effect resulting from the use of *Brassica* instead of the corresponding physiological cytochrome f partner for each reaction. Present information suggests that important features at the remote site of plastocyanin are its overall negative charge, together with the aromatic residue at 83, which is bonded directly to the active-site ligated Cys84. The existence of a similar HisCys combination connecting the type 1 and type 3 coppers in ascorbate oxidase is noted.[59] The presence of an aromatic residue in close proximity to the copper-thiolate may be important in both cases.

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References

1 W. Haehnel, *Annu. Rev. Plant. Physiol.*, 1984, **35**, 659.
2 J. Barber, *Plant Cell Environment*, 1983, **6**, 311.
3 A. G. Sykes, *Chem. Soc. Rev.*, 1985, **14**, 283.
4 A. G. Sykes, *Struct. Bonding (Berlin)*, 1991, **75**, 175.
5 See, for example, *Copper Proteins and Copper Enzymes*, ed. R. Lontie, CRC Press, Boca Raton, FL, 1984, vol. 1.
6 L. Ryden, *Coord. Chem. Rev.*, 1989, **94**, 157.
7 S. Katoh, I. Shiratori and A. Takamiya, *J. Biochem. (Tokyo)*, 1962, **51**, 32.
8 F. A. Armstrong, J. N. Butt, K. Govindaraju, J. McGinnis, R. Powls and A. G. Sykes, *Inorg. Chem.*, 1990, **29**, 4858.
9 A. Aitken, *Biochem. J.*, 1975, **149**, 675.
10 H. Yanu, M. Kano, A. Tsugita, K. Aso and Y. Nozu, *Protein Sequences Data Anal.*, 1989, **2**, 385.
11 D. I. Last and J. C. Gray, *Plant Mol. Biol.*, 1989, **12**, 655.
12 F. Yoshizaki, T. S. Fukazawa, Y. Mishina and Y. Sugimura, *J. Biochem.*, 1989, **106**, 282.
13 R. J. Simpson, R. L. Moritz, E. C. Nice, B. Grego, F. Yoshizaki, Y. Sugimura, H. C. Freeman and M. Murata, *Eur. J. Biochem.*, 1986, **157**, 497.
14 A. G. Sykes, *Adv. Inorg. Chem.*, 1991, **36**, 377.
15 J. M. Guss and H. C. Freeman, *J. Mol. Biol.*, 1983, **169**, 521.
16 D. L. Willey, A. D. Auffret and J. C. Gray, *Cell*, 1984, **36**, 555.
17 D. J. Davis and M. K. Frame, *Biochim. Biophys. Acta*, 1988, **936**, 61.
18 J. C. Gray, *Eur. J. Biochem.*, 1978, **82**, 133.
19 D. Beoku-Betts, S. K. Chapman, C. V. Knox and A. G. Sykes, *Inorg. Chem.*, 1985, **24**, 1677.
20 G. Hauska, *Encycl. Plant Physiol. New Series*, 1986, **19**, 496; G. Hauska, W. Nitschke and R. G. Hermann, *J. Bioenerg. Biomemb.*, 1988, **20**, 211.
21 M. G. Segal and A. G. Sykes, *J. Am. Chem. Soc.*, 1978, **100**, 4585.
22 D. G. Cookson, M. T. Hayes and P. E. Wright, *Biochim. Biophys. Acta*, 1980, **591**, 162; P. M. Handford, H. A. O. Hill, R. W.-K. Lee, R. A. Henderson and A. G. Sykes, *J. Inorg. Biochem.*, 1980, **13**, 83.
23 Z. Adam and R. Malkin, *Biochim. Biophys. Acta*, 1989, **975**, 158.
24 R. A. Marcus, *J. Phys. Chem.*, 1963, **67**, 853; *Annu. Rev. Phys. Chem.*, 1964, **15**, 155; R. A. Marcus and N. Sutin, *Biochim. Biophys. Acta*, 1985, **811**, 265.
25 E. T. Adman, in *Metalloproteins*, ed. P. M. Harrison, McMillan, London, 1985, Part 1, ch. 1.
26 (*a*) G. E. Norris, B. F. Anderson and E. N. Baker, *J. Mol. Biol.*, 1983, **165**, 504; (*b*) E. N. Baker, *J. Mol. Biol.*, 1988, **203**, 1071; (*c*) W. E. B. Sheppard, B. F. Anderson, D. A. Lewandoski, G. E. Norris and E. N. Baker, *J. Am. Chem. Soc.*, 1990, **112**, 7817; (*d*) H. Nar, A. Messerschmidt, R. Huber, M. van de Kamp and G. W. Canters, *J. Mol. Biol.*, 1991, **218**, 427.
27 M. Plesničar and D. S. Bendall, *Biochim. Biophys. Acta*, 1970, **216**, 192.
28 H. E. M. Christensen, J. Ulstrup and A. G. Sykes, *Biochim. Biophys. Acta*, 1990, **1039**, 94.
29 (*a*) A. Kessler, W. Arthur and J. B. Brugger, *Arch. Biochem. Biophys.*, 1957, **71**, 326; (*b*) J. McGinnis, J. D. Sinclair-Day, A. G. Sykes, R. Powls, J. Moore and P. E. Wright, *Inorg. Chem.*, 1988, **27**, 2306.
30 W. L. Ellefson, E. A. Ulrich and D. W. Krogmann, *Methods Enzymol.*, 1980, **69**, 223.
31 M. P. Jackman, J. D. Sinclair-Day, M. J. Sisley, A. G. Sykes, L. A. Denys and P. E. Wright, *J. Am. Chem. Soc.*, 1987, **109**, 6443.
32 D. Beoku-Betts and A. G. Sykes, *Inorg. Chem.*, 1985, **24**, 1142.
33 T. Takabe, S. Niwa, H. Ishikawa and K. Takenaka, *J. Biochem.*, 1980, **88**, 1167.
34 (*a*) R. P. Ambler and M. Wynn, *Biochemistry*, 1978, **17**, 485; (*b*) A. G. Lappin, M. G. Segal, D. C. Weatherburn, R. A. Henderson and A. G. Sykes, *J. Am. Chem. Soc.*, 1979, **101**, 2302.
35 M. Van de Kamp, R. Floris, F. C. Hali and G. W. Canters, *J. Am. Chem. Soc.*, 1990, **112**, 907.
36 B. G. Karlson, R. J. Aasa, B. G. Malmstrom and L. G. Lundberg, *FEBS Lett.*, 1989, **253**, 99.
37 J. McGinnis, J. D. Sinclair-Day and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1986, 2011.
38 J. D. Sinclair-Day and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1986, 2069.
39 M. P. Jackman, J. McGinnis, A. G. Sykes, C. A. Collyer, M. Murata and H. C. Freeman, *J. Chem. Soc., Dalton Trans.*, 1987, 2573.
40 J. M. Guss, P. R. Harrowell, M. Murata, V. A. Norris and H. C. Freeman, *J. Mol. Biol.*, 1986, **192**, 361.
41 J. D. Sinclair-Day, M. J. Sisley, A. G. Sykes, G. C. King and P. E. Wright, *J. Chem. Soc., Chem. Commun.*, 1985, 505.
42 J. L. Markley, E. L. Ulrich, S. P. Berg and D. W. Krogmann, *Biochemistry*, 1975, **14**, 4428.
43 C. L. Kojiro and J. L. Markley, *FEBS Lett.*, 1983, **162**, 54.
44 G. W. Pettigrew, F. A. Leitch and G. R. Moore, *Biochim. Biophys. Acta*, 1983, **725**, 409.
45 C. M. Groeneveld and G. W. Canters, *J. Biol. Chem.*, 1988, **263**, 167; C. M. Groeneveld, M. C. Ouwerling, C. Erkelens and G. W. Canters, *J. Mol. Biol.*, 1988, **200**, 189.
46 D. G. A. H. de Silva, Ph.D. Thesis, University of Newcastle upon Tyne, 1987.
47 J. Butler, D. M. Davies and A. G. Sykes, *J. Inorg. Biochem.*, 1981, **15**, 41.
48 D. Beoku-Betts, Ph.D. Thesis, University of Newcastle upon Tyne, 1985, ch. 2.
49 A. Haim and N. Sutin, *Inorg. Chem.*, 1976, **15**, 476.

50 R. L. Warren, A. G. Lappin, B. V. Mehta and H. M. Neumann, *Inorg. Chem.*, 1990, **29**, 4185.
51 C. M. Groeneveld, S. Dahlin, B. Reinhammer and G. W. Canters, *J. Am. Chem. Soc.*, 1987, **109**, 3247.
52 A. Lommen and G. W. Canters, *J. Biol. Chem.*, 1970, **265**, 2768.
53 S. Dahlin, B. Reinhammar and M. T. Wilson, *Biochem. J.*, 1984, **218**, 609.
54 F. A. Armstrong, P. C. Driscoll and H. A. O. Hill, *FEBS Lett.*, 1985, **190**, 242.
55 S. Wherland and I. Pecht, *Biochemistry*, 1978, **17**, 2585.
56 C. Bergman, E.-K. Gandvik, P. O. Nyman and L. Strid, *Biochem. Biophys. Res. Commun.*, 1977, **77**, 1052.
57 S. He, S. Modi, D. S. Bendall and J. C. Gray, *Embo J.*, 1991, **10**, 4011.
58 G. R. Moore, C. G. S. Eley and G. Williams, *Adv. Inorg. Biol. Mech.*, 1984, **3**, 11.
59 A. Messerschmidt, A. Rossi, R. Ladenstein, R. Huber, M. Bolognesi, G. Gatti, A. Marchesini, T. Petruzzelli and A. Finazzi-Agro, *J. Mol. Biol.*, 1989, **206**, 513.

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