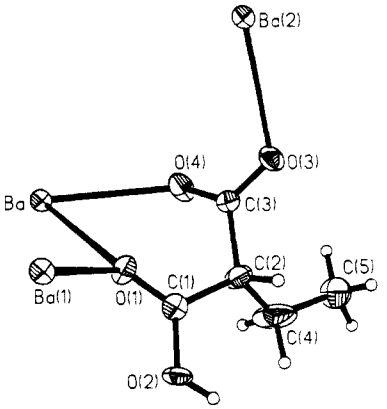

Figure 8. Binding of a single emalH$^-$ ion in $[Ba(emalH)_2]\cdot H_2O$. Atoms labeled Ba(1) and Ba(2) are related to the parent atom by the symmetry operations $x, y, z + 1$; and $x - {}^1/_2, {}^1/_2 - y, 1^1/_2 - z$, respectively.

The geometric parameters of the hydrogen ethylmalonate ion agree well with those in the related benzylmalonate ion (vide supra). One of the carboxyl groups [O(1)-C(1)-O(2)] is 7° out of the plane C(1)-C(2)-C(3) while the other forms an angle with the same plane of 57°, and the dihedral angle between carboxyl groups is 63°.

Discussion

The four structures presented here clearly demonstrate that the coordination of Ba^{2+} to Gla and Asa analogues is comparable to that of calcium but in contrast to that observed for the smaller alkaline-earth metals magnesium and beryllium. At present, the limited data available for Be^{2+} and Mg^{2+} suggest that the malonate mode may be the only mode that will be observed for these metals, since in $Be(mal)_2^{2-}$,[28] $Mg(malH)_2\cdot 2H_2O$,[25] and $Mg(Memal)(OH_2)_4$[15] no other mode is observed; there is no obvious a priori reason for this observation, however. For the larger alkaline-earth-metal ions, three coordination modes are possible, and in any given structure one, two, or all three of them might be observed. More significant, however, than the nature of the coordination around any given metal is the overall structure of the complex. For the smaller ions (Be^{2+}, Mg^{2+}), the overall structures are regular and *monomeric*, lacking any interactions between neighboring metal centers. The four barium structures presented here, along with the calcium complexes examined earlier,[15,17,18,23-25] are all *polymeric*, and it has been suggested that the ability of Gla and/or Asa to contribute additional carboxylate groups to these arrays is a major reason for their presence in calcium proteins.[15,17] The present result would suggest that, in principle, barium could bind in much the same way as calcium to such proteins, which would explain the observation that normal prothrombin adsorbs onto barium citrate but that abnormal prothrombin (which lacks the Gla residues) does not.[20-22]

Registry No. $[Ba(bzmalH)_2]\cdot H_2O$ (coordination compound entry), 114595-65-2; $[Ba(bzmal)]\cdot 3H_2O$ (coordination compound entry), 114584-18-8; $[Ba(dmal)]_2\cdot 5H_2O$ (coordination compound entry), 114584-19-9; $[Ba(emalH)_2]\cdot H_2O$ (coordination compound entry), 114584-20-2; $[Ba(bzmalH)_2]\cdot H_2O$ (salt entry), 114584-21-3; $[Ba(bzmal)]\cdot 3H_2O$ (salt entry), 114584-22-4; $[Ba(dmal)]_2\cdot 5H_2O$, 114584-23-5; $[Ba(emalH)_2]\cdot H_2O$, 114584-24-6; prothrombin, 9001-26-7.

Supplementary Material Available: Tables S1-S4, listing hydrogen atom positional parameters, and Tables S5-S8, listing anisotropic thermal parameters (8 pages); Tables S9-S12, listing observed and calculated structure amplitudes (38 pages). Ordering information is given on any current masthead page.

Contribution from the Department of Inorganic Chemistry, The University, Newcastle upon Tyne NE1 7RU, England, Department of Biochemistry, The University, Liverpool L69 3BX, England, and Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, California 92037

Kinetic Studies on 1:1 Electron-Transfer Reactions Involving Blue Copper Proteins. 16. Reactivity of Plastocyanin from the Green Alga *Scenedesmus obliquus* with Inorganic Redox Partners and Related NMR Studies

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The reactivity of plastocyanin from a green algal source, *Scenedesmus obliquus* (estimated PCu^I charge -9 at pH ~7), has been studied for the first time in order to further assess the effect of amino acid sequence variations in naturally occurring plastocyanins. At 25 °C, I = 0.10 M (NaCl), and pH <7.5, rate constants with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ as oxidants for PCu^I and with $[Co(phen)_3]^{2+}$ as reductant for PCu^{II} are similar in magnitude to those obtained previously for the higher plant plastocyanins and show similar dependences on pH. Attention is drawn to the possibility that in this and some other instances there may be a small residual reactivity of PCu^I with $[Fe(CN)_6]^{3-}$ at low pH. A novel feature at pH >7.5 is the increase in rate constants giving protein pK_a values of 7.6 ($[Fe(CN)_6]^{3-}$) and 8.2 ($[Co(phen)_3]^{3+}$), confirmed by NMR spectroscopy (pK_a = 7.8) as a deprotonation/protonation of His59, a residue not present in the higher plant plastocyanins. Also from NMR measurements a sensitivity to active-site protonation at His59 and to His59 protonation at the active site is indicated, although there is no direct link between the two and the imidazole ring of His59 is 10-12 Å from the Cu site. There is however no evidence for electron transfer from the 59-61 binding site. At pH 7.56 no saturation kinetic behavior is observed with $[Co(phen)_3]^{3+}$ ($K < 25$ M^{-1}), which is attributable to the lower (-3 instead of -4) charge at residues 42-45, but at pH 9.1, when His59 is acid dissociated, such behavior is observed (K = 50-100 M^{-1}). From ^1H NMR line-broadening experiments at pH 6.3 and 7.0 using Cr(III) analogue complexes, it has been demonstrated that $[Cr(CN)_6]^{3-}$ produces almost no specific broadening effects. With $[Cr(phen)_3]^{3+}$ binding to *S. obliquus* PCu^I is observed over a delocalized region of the protein surface including the negatively charged residues 42-44 and 60-61. Deletions at positions 57 and 58 in the aligned sequences bring these two regions sufficiently close together for reaction at the 42-44 site to be affected by the state of protonation of His59.

Introduction

Plastocyanin is a single blue (type 1) copper metalloprotein involved in electron transport ($E°$ = 370 mV at pH 7) between photosystems II and I of the chloroplasts in higher plants and algae.[1] Normally it consists of a single polypeptide chain of ca. 99 amino acids (mol wt 10 500). Plastocyanins from 15 higher plants, 3 green algae, and 1 blue-green alga have been fully sequenced. [The fully sequenced plastocyanins are as follows:

[†] The University, Newcastle upon Tyne.
[‡] The University, Liverpool.
[§] Research Institute of Scripps Clinic.

(1) Sykes, A. G. *Chem. Soc. Rev.* **1985**, 283.

0020-1669/88/1327-2306$01.50/0

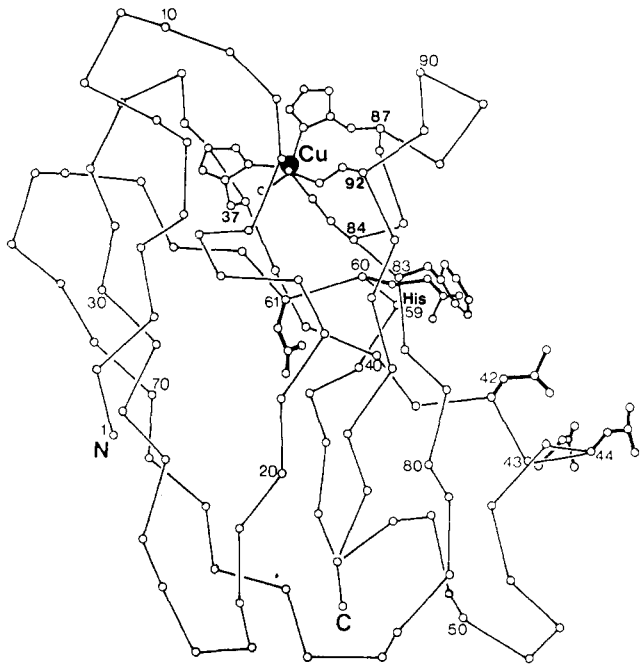

Figure 1. Structure of poplar plastocyanin, PCu^{II}, as reported by Guss and Freeman,[9] showing the α-carbon framework and side chains at the Cu site. The acidic residues indicated at positions 42–44 and 60–61 are observed in *S. obliquus*; deletions at positions 57 and 58 for *S. obliquus* have the effect of placing the α-carbon of His59 in approximately the position indicated here for residue 57 and of decreasing the size of the 57–61 loop.

spinach, French bean, potato, elder, marrow, broad bean, lettuce, dog's mercury, shepherd's purse, solanum, dock, poplar, cucumber, parsley, and campion (the 15 higher plant sources); *Scenedesmus obliquus*, *Chlorella fusca*, and *Enteromorpha prolifera* (green algae); and *Anabaena variabilis* (blue-green alga). Details of the sequences are to be found in ref 1–7.] Of the 15 higher plant sequences, 50 residues are invariant. Sequence homologies are not as strong if the three green algae are included (33 invariant residues), and with the inclusion of plastocyanin from the blue-green alga *A. variabilis* only 26 residues remain invariant. The latter include His37, Cys84, His87, and Met92, which coordinate the Cu and constitute the active site. For higher plant and green algal plastocyanins, charge is conserved at -9 ± 1 for PCu^{I} at pH ca. 7, which compares with a charge of +1 for PCu^{I} from *A. variabilis*.[8]

It is of interest to consider the effect of these various changes in order to understand more fully the reactivity of plastocyanin. Structural information available from X-ray diffraction studies on poplar PCu^{II}[9] and PCu^{II}[10] makes plastocyanin an appropriate choice for such investigations. Figure 1 shows the α-carbon skeleton of plastocyanin based on the poplar PCu^{II} structure. Two-dimensional NMR studies of *S. obliquus* PCu^{I} show that the overall tertiary structure is very similar to that of the poplar protein.[11]

	42	43	44	45------57	58	59	60	61------82	83
S. obliquus	Asp	Glu	Asp	Ala------*	*	His	Asp	Asp------Tyr	Phe
C. fusca	Asp	Glu	Asp	Glu------*	*	His	Glu	Asp------Tyr	Phe
E. prolifera	Asp	Glu	Asp	Ala------*	*	Ala	Glu	Asp------Val	Tyr
Parsley	Asp	Glu	Asp	Glu------*	*	Gln	Pro	Glu------Phe	Tyr
Poplar	Asp	Glu	Asp	Ser------Met	Ser	Glu	Glu	Asp------Phe	Tyr
Spinach	Asp	Glu	Asp	Glu------Met	Ser	Glu	Glu	Asp------Phe	Tyr
Fr. bean	Asp	Glu	Asp	Glu------Met	Pro	Glu	Glu	Glu------Phe	Tyr
A. variabilis	Asp	Ala	Ala	Leu------Leu	Ser	His	Lys	Gln------Phe	Tyr

Figure 2. Portions of the amino acid sequence of various plastocyanins[1,4] (* indicates deletion).

Previous work has implicated two sites on the plastocyanin molecule where electron transfer can take place.[1,9,12,13] The first of these is close to the exposed edge of the Cu ligand His87, which is located in a hydrophobic pocket at the top of the molecule viewed as in Figure 1. This site (the north site) represents the closest approach (ca. 6 Å) to the copper atom. The second (east) site is located on the right-hand side of Figure 1 and includes the conserved negative patch of acidic residues 42–45 close to the aromatic residue Tyr83. Residues 59–61 are acidic in most higher plant PCu's and are also close to Tyr83. To date, the evidence for the involvement of the 59–61 locality either as part of the east site or as a separate negatively charged binding site has remained uncertain.[1] Negatively charged electron-transfer agents such as $[Fe(CN)_6]^{3-}$ use the His87 site, while positively charged reagents such as $[Co(phen)_3]^{3+}$ and the natural partner cytochrome *f* use the east site.[14] In the case of small positive inorganic redox partners, reaction has been demonstrated to be not exclusively at the negative patch, some 15–45% occurring at some other site or sites on the protein.[15,16] The active site of reduced plastocyanin is known to be susceptible to protonation, and as the pH is lowered, the His87 ligand becomes protonated and dissociated from the copper, leaving the Cu(I) planar and 3-coordinate.[10b] This effect, which is not observed for the Cu(II) protein, is manifest as a dramatic "switch off" in reactivity of the protein as the pH decreases.[1,17] Protonation of His87 is also observed in the ^1H NMR spectrum of the reduced protein, from which the pK_a can be determined.[18]

In order to study the reactivity of a green algal plastocyanin, *S. obliquus* was selected since the full sequence is known.[3] The overall charge on *S. obliquus* PCu^{I} is estimated to be −9 at pH ~7 excluding His59. A comparison of sequence information with that of other plastocyanins is made in Figure 2. Noteworthy features of the *S. obliquus* sequence include first the deletions at positions 57 and 58, in common with the other green algae *C.*

(2) Boulter, D.; Haslett, B. F.; Peacock, D.; Ramshaw, J. A. M.; Scawen, M. D. In *Plant Biochemistry II*; Northcote, D. H., Ed.; University Park Press: Baltimore, MD, 1977; Vol. 13, p 1040.
(3) Unpublished work of Dr. R. P. Ambler, Department of Molecular Biology, University of Edinburgh, quoted in ref 1, p 288. It has now been established from 2D NMR spectra that sequence 94–99 in *S. obliquus* should read Gly Lys Val Ile Val Gln. All other residues in the sequence previously reported are confirmed.[24]
(4) Ramshaw, J. A. M. In *Encyclopaedia of Plant Physiology*; Boulter, D., Parther, B., Eds.; Springer Verlag: Berlin, 1982; Vol. 14A, p 229.
(5) Ramshaw, J. A. M.; Felton, A. A. *Biochemistry* **1982**, *21*, 1317.
(6) Smeekins, S.; de Groot, M.; van Binsbergen, J.; Weisbeek, P. *Nature (London)* **1985**, *231*, 456.
(7) Simpson, R. J.; Moritz, R. L.; Nice, E. C.; Grego, B.; Yoshizaki, F.; Sugimura, Y.; Freeman, H. C.; Murata, M. *Eur. J. Biochem.* **1986**, *157*, 497.
(8) Aitken, A. *Biochem. J.* **1975**, *149*, 675. An estimate of charge was previously given as +2.
(9) Guss, J. M.; Freeman, H. C. *J. Mol. Biol.* **1983**, *169*, 521.
(10) (a) Freeman, H. C. In *Coordination Chemistry-21*; Laurent, J. L., Ed.; Pergamon: Oxford, England, 1981; p 29. (b) Guss, J. M.; Harrowell, P. R.; Murata, M.; Norris, V. A.; Freeman, H. C. *J. Mol. Biol.* **1986**, *192*, 361.
(11) Moore, J. M.; Case, D. A.; Chazin, W. J.; Gippert, G.; Havel, T. F.; Powls, R., Wright, P. E., unpublished observations.
(12) Cookson, D. J.; Hayes, M. T.; Wright, P. E. *Nature (London)* **1980**, *283*, 682.
(13) Handford, P. M.; Hill, H. A. O. Lee, R. W.-K.; Henderson, R. A.; Sykes, A. G. *J. Inorg. Biochem.* **1980**, *13*, 83.
(14) Beoku-Betts, D.; Chapman, S. K.; Knox, C. V.; Sykes, A. G. *Inorg. Chem.* **1985**, *24*, 1677.
(15) King, G. C.; Wright, P. E., unpublished work.
(16) McGinnis, J.; Sinclair-Day, J. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1986**, 2007.
(17) Segal, M. J.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 4584.
(18) Markley, J. L.; Ulrich, E. L.; Berg, S. P.; Krogmann, D. W. *Biochemistry* **1975**, *14*, 4428.

fusca and *E. prolifera* and also (surprisingly) with parsley plastocyanin. Second, the presence of an uncoordinated histidine (His59) is noted. Such a histidine is also present in the algal plastocyanins from *C. fusca* and *A. variabilis*. Third, the highly conserved −4 negative patch of residues 42–45 for higher plant plastocyanins is −3 only for *S. obliquus*, with residue 45 uncharged (Ala45). This is a feature shared with poplar plastocyanin, where residue 45 is serine. Finally, residues 82 and 83, Phe and Tyr in higher plants, are interchanged in *S. obliquus*.

Kinetic and NMR studies on *S. obliquus* plastocyanin are reported in this paper. Results are compared with those obtained for other plastocyanins and where possible attributed to sequence differences such as those referred to above.

Experimental Section

S. obliquus was grown heterotrophically in the dark at 28 °C on nitrate medium supplemented with 0.5% glucose and 0.25% yeast extract.[19] Extraction and preliminary purification of plastocyanin were as described,[20] followed by gel filtration on Ultrogel AcA54. This yielded protein with an absorbance (A) peak ratio A_{278}/A_{597} of ca. 3.7, which was further purified as follows. The protein was oxidized with a slight excess of $[Fe(CN)_6]^{3-}$, dialyzed against 50 mM phosphate buffer at pH 7, and loaded onto a DEAE-Sephadex A-50 column (20–25 × 1.6 cm, suitable for 50–100 mg of protein) previously equilibrated with 50 mM buffer at pH 7. After being washed with buffer, the column was eluted with a linear ionic strength gradient from 50 to 200 mM phosphate at pH 7 (total volume 400 mL) at a flow rate of <18 mL/h. Protein fractions having an absorbance ratio $A_{278}/A_{597} = 3.0 \pm 0.1$ were pooled for use in kinetic experiments. Protein was recovered after use by dialyzing against 1 mM phosphate at pH 7 and then concentrating on a Whatman DE52 column. The protein was eluted with 20 mM phosphate at pH 7 containing 0.2 M NaCl and stored in this form prior to a final (repeat) purification on Sephadex A-50 as above.

The kinetic approach, linearity of plots, and treatment of data are similar to those described previously.[1,21,22] Solutions of $[Co(phen)_3]^{2+}$ were obtained by addition of 1,10-phenanthroline (in 4- to 6-fold excess over Co) to a solution of $CoCl_2 \cdot 6H_2O$ (BDH, AnalaR).

Protein with $A_{278}/A_{597} = 3.5$ was used for ^1H NMR experiments. No peaks from denatured material were obtained in the NMR spectrum.

Solutions of plastocyanin (0.5 mM) for pH titration were prepared in deuteriated phosphate buffer (0.1 M) in D_2O containing 0.1 mM EDTA. Samples were reduced with dithionite and flushed with argon to avoid oxidation. The pH was adjusted by addition of small aliquots of DCl or NaOD. Solutions were flushed again with argon and the tubes sealed with a rubber septum. The pH was measured both before and after NMR measurements. Values of the pH are quoted as direct meter readings with no correction for deuterium isotope effects. To obtain pK_a values, a nonlinear least-squares fitting procedure assuming a one-proton dissociation was used.

Solutions of plastocyanin for titration with Cr(III) complexes were prepared as described above except that the buffer was omitted. Solutions of Cr(III) complexes were freshly prepared in D_2O, and the pH was adjusted with DCl or NaOD. The solutions were then flushed with argon and the tubes sealed with a rubber septum. Aliquots of Cr(III) complexes were withdrawn with a Hamilton syringe and injected directly into the NMR tube containing plastocyanin at the same pH. Titrations with $[Cr(CN)_6]^{3-}$ and $[Cr(phen)_3]^{3+}$ (20 μM) were carried out at pH 6.3 and 7.0, respectively.

NMR spectra were recorded at 25 °C with Bruker AM-300 and AM-500 spectrometers. Gated proton decoupling was used to reduce spectral contributions from residual HOD. Chemical shift values are referenced to internal dioxane at 3.75 ppm.

Results

Kinetics with $[Fe(CN)_6]^{3-}$ as Oxidant. At a fixed pH, pseudo-first-order rate constants k_{obsd} (Table I)[23] give a linear dependence on $[Fe(CN)_6^{3-}]$ from which second-order rate constants k can be evaluated. The variation of k with pH is illustrated in Figure 3, from which it is apparent that *two* protonation equilibria

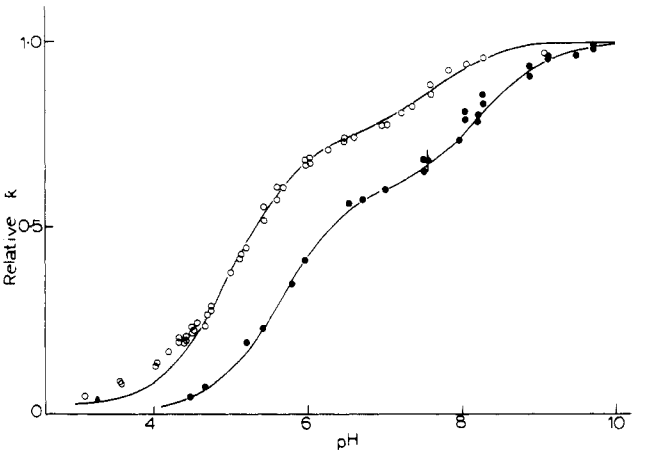

Figure 3. Effect of pH (3.1–9.7) on second-order rate constants (relative scale) for the oxidation of *S. obliquus* PCu^I with $[Fe(CN)_6]^{3-}$ (○) and $[Co(phen)_3]^{3+}$ (●).

are effective in this reaction, in contrast to the case for all other plastocyanins so far studied. The data can be fitted to

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

which can be derived from

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

involving active site (subscript A) and one other (subscript H) acid dissociation pK_a. Relevant kinetic steps are as defined in (3)–(5). This scheme is similar to the double-protonation scheme

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

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

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

proposed previously[24] but has been modified to allow for a possible nonzero value of k_H. Kinetic contributions from HP_A^+ have been neglected as K_{aH} is at least 2 orders of magnitude less than K_{aA}.

The data give a good fit to (1) with $pK_{aA} = 5.0 \pm 0.1$, $pK_{aH} = 7.6 \pm 0.1$, $k_H = (0.2 \pm 0.3) \times 10^4$ M^{-1} s^{-1}, $k_I = (9.0 \pm 0.1) \times 10^4$ M^{-1} s^{-1}, and $k_0 = (12.1 \pm 0.2) \times 10^4$ M^{-1} s^{-1}.

The protonation K_{aH} is assigned to His59, for which a pK_a of 7.83 has been determined by NMR spectroscopy; see below.

Kinetics with $[Co(phen)_3]^{3+}$ as Oxidant. At a fixed pH pseudo-first-order rate constants k_{obsd} (Table II)[23] show a linear dependence on $[Co(phen)_3^{3+}]$ up to 1×10^{-3} M, from which second-order rate constants k can be obtained. The variation of k with pH is illustrated in Figure 3, from which it can be seen that the protonation of His59 has an effect on this reaction also.

The curve drawn is derived from an equation of the same form as (1) with $pK_a = 5.65$, $pK_{aH} = 8.25$, $k_H = 0$, $k_I = 1850$ M^{-1} s^{-1}, and $k_0 = 3050$ M^{-1} s^{-1}. Estimated errors from manual curve fitting procedures are ±0.1 for pK_a's and ±100 M^{-1} s^{-1} for rate constants. As in earlier studies, the apparent pK_a of 5.65 is believed to be a composite value made up of contributions from the active-site protonation (pK_{aA}) and protonation of the negative-patch binding site (pK_{aB}). A value of pK_{aB} (which has been denoted as pK'_a in previous work) was obtained by refitting the data at pH <7 to a curve of the same form as (1). With $pK_{aA} = 5.0$ (from the $[Fe(CN)_6]^{3-}$ reaction), $k_H = 0$, and $k_0 = 1850$ M^{-1} s^{-1} (i.e. k_I obtained above from the data over the full pH range), a reasonable

(19) Kessler, A.; Arthur, W.; Brugger, J. B. *Arch. Biochem. Biophys.* **1957**, *71*, 326.
(20) Rowell, P.; Powls, R. *Biochim. Biophys. Acta* **1976**, *423*, 65.
(21) E.g.: Chapman, S. K.; Sanemasa, I.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1983**, 2549.
(22) Sinclair-Day, J. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1986**, 2069.
(23) See paragraph at end of paper regarding supplementary information.
(24) Sinclair-Day, J. D.; Sisley, M. J.; Sykes, A. G.; King, G. C.; Wright, P. E. *J. Chem. Soc., Chem. Commun.* **1985**, 505.

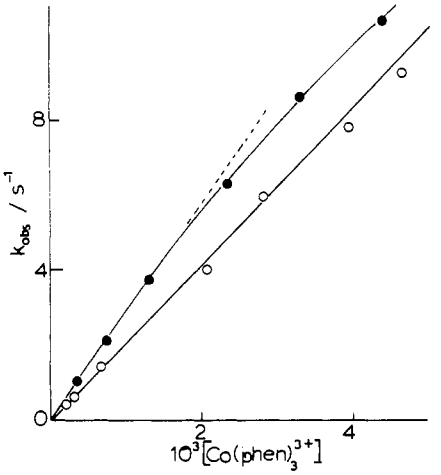

Figure 4. Variation of pseudo-first-order rate constants k_{obsd} for the $[Co(phen)_3]^{3+}$ oxidation of *S. obliquus* PCu^I with oxidant concentration at pH 7.56 (○) and 9.12 (●).

fit is obtained with $pK_{aB} = 5.8$ and rate constant k_I (for the reaction of PCu^I protonated at the negative patch) of 750 M^{-1} s^{-1}. However, the data can also be fitted with $pK_{aA} = 5.45$ (the NMR value) to give $k_0 = 1850$ M^{-1} s^{-1}, $pK_{aB} = 5.5$, and $k_I = 990$ M^{-1} s^{-1}. Of the two interpretations, we favor the latter, to which we will refer henceforth. This gives k_I equal to the rate constant at maximum inhibition by $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ (see below). It has been found in previous studies that the effect of protonation at the negative patch is approximately the same as that of inhibitor complexes, suggesting that protonation totally inhibits reaction at this site.

At pH 7.56 a plot of k_{obsd} vs $[Co(phen)_3^{3+}]$ remains linear up to $[Co(phen)_3^{3+}] = 4.6 \times 10^{-3}$ M (Figure 4). This is in contrast to the behavior of other plastocyanins, where such plots generally begin to show curvature at $[Co(phen)_3^{3+}] > 5 \times 10^{-4}$ M.[1,15,17] From individual values in Table II we note that there may be some slight decrease in apparent second-order rate constants at the highest oxidant concentrations, but this is virtually within experimental error and is too small to permit a meaningful analysis (K for association <25 M^{-1}). This behavior can be explained in terms of the reduced negative charge in the 42–45 region. In view of the effect of His59 protonation, it was of interest to explore the possibility of limiting kinetics at pH >9 also. At pH 9.1 a plot of k_{obsd} vs $[Co(phen)_3^{3+}]$ is curved (Figure 4), indicating increased binding of the positive reagent following deprotonation of His59. As previously,[16] for reaction assigned to the east face (63%), it is possible to interpret in terms of association of $[Co(phen)_3]^{3+}$ ($K = 76 \pm 20$ M^{-1}) prior to electron transfer ($k_{et} = 26 \pm 8$ s^{-1}).

The data were fitted by an unweighted nonlinear least-squares procedure to (6), where k_2 represents the contribution to the

$$k_{obsd} - k_2[Co(phen)_3^{3+}] = \frac{Kk_{et}[Co(phen)_3^{3+}]}{1 + k[Co(phen)_3^{3+}]} \quad (6)$$

reaction arising from electron-transfer site(s) other than the east face. This value was estimated from a limited competitive inhibition study at pH 9.1 (data not included in this report).

Inhibition of the $[Co(phen)_3]^{3+}$ Oxidation by $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$. Data for the dependence of second-order rate constants on inhibitor concentration at pH 7.56 are shown in Table III.[23] The dependence is of the type described by (7), which can be derived from (8)–(10), where k_C is the rate

$$k = \frac{k_C + k_B K_B[B]}{1 + K_B[B]} \quad (7)$$

$$P + C \xrightarrow{k_C} \text{products} \quad (8)$$

$$P + B \rightleftharpoons P,B \quad (9)$$

$$P,B + C \xrightarrow{k_B} \text{products} \quad (10)$$

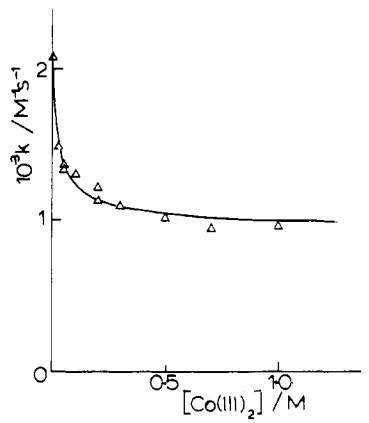

Figure 5. Effect of $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ concentration on rate constants for the $[Co(phen)_3]^{3+}$ oxidation of *S. obliquus* PCu^I at pH 7.56.

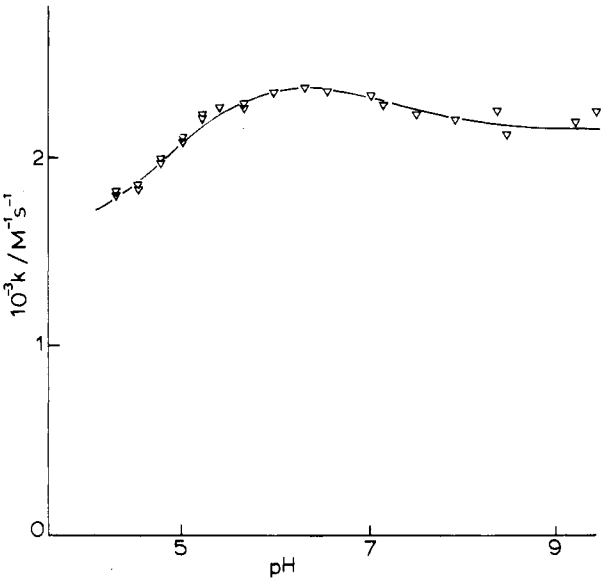

Figure 6. Effect of pH (4.3–9.2) on second-order rate constants for the reduction of *S. obliquus* PCu^{II} with $[Co(phen)_3]^{2+}$.

constant at pH 7.56 in the absence of inhibitor and B is the inhibitor complex. A fit of the data to (7) is shown in Figure 5. This gives $K_B = (3.5 \pm 0.6) \times 10^4$ M^{-1} and $k_B = 990 \pm 40$ M^{-1} s^{-1}. Association of inhibitor complex therefore results in a 52% reduction in reactivity at pH 7.56. There is a small discrepancy here due to the increase in reactivity relative to pH 7 as a consequence of His59 deprotonation.

Kinetics with $[Co(phen)_3]^{2+}$ as Reductant. Rate constants for the reduction of PCu^{II} by $[Co(phen)_3]^{2+}$ are shown in Table IV[23] and Figure 6. The data at pH < 6.5 give a good fit to (11), which

$$k = \frac{k'_0 K'_{aB} + k'_H[H^+]}{K'_{aB} + [H^+]} \quad (11)$$

is derived from the single-protonation scheme used previously.[1,17] Parameters for the oxidized protein carry a superscript dash notation. Equation 11 gives $pK'_{aB} = 4.8 \pm 0.1$, $k'_H = (1.58 \pm 0.05) \times 10^3$ M^{-1} s^{-1}, and $k'_0 = (2.39 \pm 0.02) \times 10^3$ M^{-1} s^{-1}. The ratio k'_H/k'_0 indicates some 35% reactivity at the east face binding site.

Upon extension of the pH range out to pH >9, a small ~10% decrease in rate is observed consequent to His59 deprotonation. There are insufficient data to give a good fit to (11), but inspection of Figure 6 suggests a pK'_{aH} of ~7.

From the ratio of rate constants for the $[Co(phen)_3]^{3+/2+}$ reactions in Tables II and IV, assuming a value 370 mV for the $[Co(phen)_3]^{3+/2+}$ couple, the reduction potential for PCu^{II}/PCu^I decreases: pH 7.5 (373 mV), pH 8.5 (368 mV), pH 9.5 (363 mV).

Active-Site and His59 PCu^I pK_a's from NMR Data. The pH dependences of the chemical shifts of the resolved resonances of

Table V. Summary of Acid Dissociation (pK_a) and Rate Constant Parameters for Plastocyanin from Different Sources

	pK_{aA} from NMR data	pK_{aA} from kinetics with $[Fe(CN)_6]^{3-}$	pK_a from kinetics with $[Co(phen)_3]^{3+}$ [a]	pK_{aB} for PCu^{I} [b]	pK'_{aB} for PCu^{II}	k_{Fe}, M^{-1} s^{-1} [c]	k_{Co}, M^{-1} s^{-1} [c]	k_{Fe}/k_{Co}	10^{-3} K_B, M^{-1}	% blocking
parsley	5.7	5.5	6.1	5.8	5.0	94 000	3000	31	16.0	61
spinach	4.9	4.8	5.6	5.6	5.3	85 000	2500	34	6.8	75
french bean	4.7	4.6	5.4	5.5		58 000	4700	12		
poplar		4.7	5.2	5.3	5.1	69 000	2900	25	5.7	70
S. obliquus	5.4	5.0	5.6	5.5	4.8	90 000 [d]	1850 [d]	49	35.0	52

[a] Apparent value. [b] Assuming that two processes pK_a (determined by NMR measurements or with $[Fe(CN)_6]^{3-}$ as oxidant) and pK'_a contribute with $[Co(phen)_3]^{3+}$ as oxidant. [c] pH 7.5. [d] Value obtained from curve fitting excluding contributions from the deprotonation of His59 (pK_{aH} = 8.2).

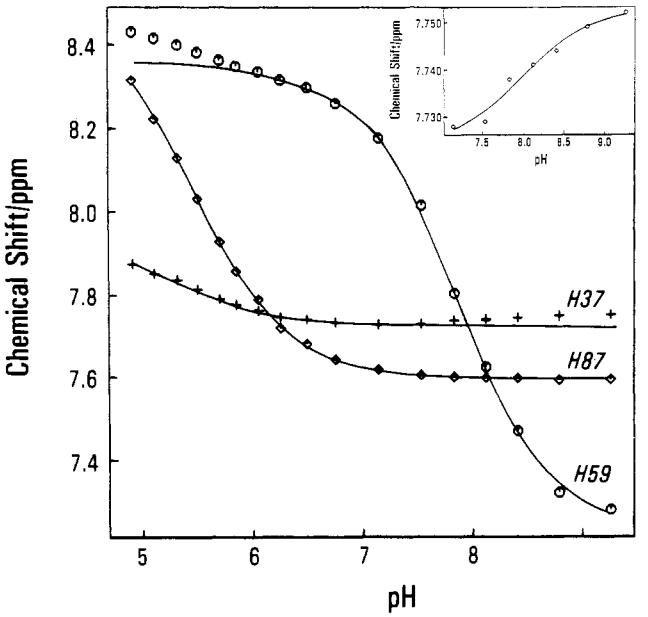

Figure 7. NMR–pH titration curves for *S. obliquus* plastocyanin. Spectra were recorded at 25 °C for solutions of PCu^{I} in 0.1 M potassium phosphate buffer. Inset shows titration data for His37 above 7.0.

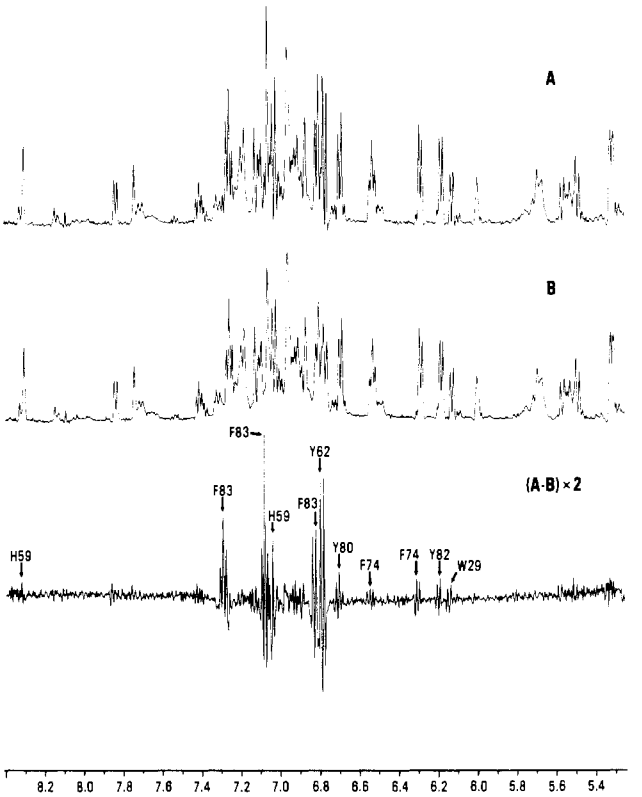

Figure 8. NMR spectra of the aromatic region of *S. obliquus* PCu^{I} (0.5 mM) at pH 7.0: (A) no $[Cr(phen)_3]^{3+}$ present; (B) $[[Cr(phen)_3]^{3+}]$ = 20 μM. The difference spectrum (A–B) is with the vertical scale expanded 2-fold. Broadened resonances are labeled.

the three histidine residues of *S. obliquus* PCu^{I} are shown in Figure 7. Fitting of the data for His87, His37, and His59 yields pK_a values of 5.45 (pK_{aA}), 5.42 (pK_{aA}), and 7.83 (pK_{aH}), respectively. For His59 there is an additional inflection below pH 6, which presumably arises from protonation of the active site His87. For His37 there is an additional inflection with $pK_a \sim 8.0$, which we believe reflects deprotonation at His59 (inset, Figure 7). The His87 pK_{aA} value of 5.45 is higher than that reported for spinach, French bean, and *A. variabilis* plastocyanins but lower than the value of 5.7 reported for parsley plastocyanin.

Effect of Cr(III) Analogues on the ^1H NMR Spectra. Further information concerning binding sites of the electron-transfer reagents $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ was obtained by measuring the broadening of ^1H resonances of *S. obliquus* PCu^{I} by the redox-inactive Cr(III) complexes $[Cr(CN)_6]^{3-}$ and $[Cr(phen)_3]^{3+}$. Complete sequential NMR assignments for *S. obliquus* PCu^{I} will be reported elsewhere.[25]

The aromatic region of the NMR spectrum of *S. obliquus* PCu^{I} is shown in Figure 8A. Titration with $[Cr(phen)_3]^{3+}$ results in selective broadening of ^1H resonances (Figure 8B), which is most readily seen in the difference spectrum. The most affected resonances are those of the C4H and C3,5H protons of Phe83 and the C3,5H protons of Tyr62, with somewhat lesser broadening of the resonances of the His59 C4H and the Phe83 C2,6H protons. Slight but significant broadening of the C2,6H and C3,5H resonances of Phe74, the C2,6H resonance of Tyr82, the C3,5H resonance of Tyr80, and the His59 C2H and Trp29 C7H proton resonances is also evident. Resonances of several methyl groups are also broadened, but since they occur in a highly crowded region of the spectrum, these cannot be unambiguously identified from the present one-dimensional paramagnetic difference spectra.

The binding affinity of *S. obliquus* PCu^{I} for the negatively charged electron-transfer reagent $[Fe(CN)_6]^{3-}$ was examined by titration with the redox-inactive analogue $[Cr(CN)_6]^{3-}$. In contrast to the results observed with *A. variabilis* PCu^{I},[26] no specific broadening of aromatic resonances was observed, even at high concentrations of $[Cr(CN)_6]^{3-}$ (with a $[Cr(CN)_6]^{3-}$:Pcu(I) ratio of 2). At this $[Cr(CN)_6]^{3-}$ concentration a number of aliphatic resonances were slightly broadened, but none of these effects can be unambiguously identified from one-dimensional spectra.

Discussion

A significant finding in the case of *S. obliquus* plastocyanin is that at alkaline pH deprotonation of His59, a residue not present in higher plant plastocyanins, has an influence on the kinetics. Also, from the comparison of parameters at pH <7 with those for higher plant plastocyanins (Table V), some similarities with parsley plastocyanin are noted. In the latter case the active-site pK_a from both NMR and kinetic studies is out of line with that for other higher plant plastocyanins.[22,27] One possible explanation

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could be the amino acid sequence deletions at positions 57 and 58, which are unique to parsley among higher plants but occur also in *S. obliquus*.

From NMR measurements on *S. obliquus* PCu^{I} in 0.1 M phosphate buffer, an acid dissociation pK_a of 5.45 is obtained for the Cu ligand His87. Protonation of His87 results in its dissociation from the copper,[10b] and the resultant alteration of the active site has a significant effect on the reactivity of plastocyanin.[1,17] The same pK_a should be observed in kinetic studies with $[Fe(CN)_6]^{3-}$. However, the kinetic data obtained with $[Fe(CN)_6]^{3-}$ give a pK_a of 5.0, substantially lower than the NMR value. It can be seen from Table V that pK_a's obtained with $[Fe(CN)_6]^{3-}$ are generally up to 0.2 unit lower than those found by NMR. Possible explanations for this include the effects of (i) different buffers (phosphate in NMR work), (ii) ionic strength (not as precisely controlled in NMR titrations), and (iii) the use of D_2O (instead of H_2O) in the NMR studies. No correction for junction or isotope effects has been made to pH values determined in D_2O. It has also been shown previously[28] that ionic strength variations (0.01–0.10 M) have little effect on the pK_a of parsley plastocyanin, as determined from $[Fe(CN)_6]^{3-}$ kinetics. From the kinetic viewpoint, as carboxylates protonate and the overall (negative) charge on the protein is reduced, rate constants with $[Fe(CN)_6]^{3-}$ could give a lower (apparent) pK_a for His87. Such factors might account for the generally small discrepancy between $[Fe(CN)_6]^{3-}$ and NMR pK_a's. We have no explanation as to why the discrepancy should be greater in the case of *S. obliquus*. As in previous studies, we have used the His87 NMR values as a more specific measure of the pK_a in question.

A further question with the $[Fe(CN)_6]^{3-}$ kinetic data centers around the apparent retention of as much as 12% reactivity at the low-pH limit. Similar discrepancies have been noted previously,[21,22] but satisfactory fits have generally been obtained with rate constants constrained to zero at low pH, consistent with the view that the protonated active site containing 3-coordinate Cu(I) is redox inactive.[1,10b] However it has been noted that electron transfer from PCu^{I} to its natural partner P700 is optimal at pH 4,[29] and rapid electrochemical oxidation at low pH has also been observed.[30] In the present case, because of the large magnitude of the apparent low-pH rate constant ($\sim 10^4$ M^{-1} s^{-1}), studies were extended for the first time into the range pH 3–4 by using the pH-jump method. Rate constants measured even at pH 3.1 were still around 5×10^3 M^{-1} s^{-1}, about 6% of the pH 7 value.

The effect of pH on the reduction potential of the PCu^{I}/PCu^{II} couple should be considered in this context. The $E°$ of spinach and parsley plastocyanins is known to increase from 370 mV at pH 7.5 to a value of >420 mV (still increasing) as the pH decreases below 5.[1,17,31] As the $E°$ of the $[Fe(CN)_6]^{3-/4-}$ couple is 410 mV, it is possible that at lower pH's the reaction is significantly "uphill". If this is the case equilibration kinetics may be observed, which if not allowed for could lead to erroneously high values of rate constants.[32] The $[Fe(CN)_6]^{4-}$ product can also exhibit protonation, reported pK_a values ranging from 2.33 to 4.28,[33] resulting in some change in the $E°$ value.[34] Runs were carried out at pH 4.4 and 4.7 with $[Fe(CN)_6]^{3-}$ in large (100-fold) excess of the protein, sufficient to ensure that an equilibration reaction proceeds to >90% completion even if the $E°$ of the protein is as high as 480 mV. The same rate constants were obtained as when a 10- to 20-fold excess was used, and there was no significant decrease in optical change for any runs at pH >4. For the runs at pH 3.1 and 3.6, however, optical changes were substantially lower than expected, consistent with incomplete reaction; the rate constants at these pH's should therefore be taken as an upper limit, and the best fit line in Figure 3 has been determined accordingly. There are no similar difficulties in interpreting the $[Co(phen)_3]^{3+}$ studies, though the corresponding $E°$ value for the complex is less and is equal to that of the protein at pH 7. In this case the lability of the product $[Co(phen)_3]^{2+}$ (in the absence of excess phen ligand) may prevent the true equilibrium from being attained.

The active-site (His87) pK_a of *S. obliquus* PCu^{I} is higher than the typical higher plant values (though not by very much when values derived from $[Fe(CN)_6]^{3-}$ kinetics are compared) but not as high as the anomalous parsley value. Thus, it seems likely that the local structural changes resulting from the deletions at positions 57 and 58 are having the effect of increasing the active-site pK_a but that other sequence differences are responsible for the further increase on going from *S. obliquus* to parsley. We note for example that parsley has a proline residue at position 60, which may have some influence.

The behavior of *S. obliquus* plastocyanin with $[Co(phen)_3]^{3+}$ at pH <7 is in accord with that of other plastocyanins. An apparent pK_a of 5.65 is observed, which is higher than that found with $[Fe(CN)_6]^{3-}$ as oxidant. This is assigned to a combination of the effects of active-site protonation (pK_{aA}) and protonation at the 42–44 negative patch (pK_{aB}). The latter protonation prevents reaction from occurring at the acidic patch, and the remaining reactivity is believed to be at some other site or sites on the protein.

If a pK_{aA} of 5.0 is taken for the active site, pK_{aB} can be assigned a value of 5.8, or if the active site pK_{aA} is taken as the NMR value of 5.43, pK_{aB} can be assigned a value of 5.5. We have expressed a preference for the latter interpretation above. In terms of sequence variation, we note that poplar plastocyanin with a −3 negative patch has the lowest pK_{aB},[27] while parsley with deletions at positions 57 and 58 has the highest. *S. obliquus*, with both these features, has a pK_{aB} in line with typical higher plant values observed for spinach and French bean.

No active-site pK_a is observed for PCu^{II}, as shown by the invariance in the PCu^{II} crystal structure down to pH 4.2[9] and the small effect of pH on the rate of reduction of parsley PCu^{II} with $[Fe(CN)_6]^{4-}$.[17] Therefore, pH effects on the reduction of PCu^{II} with positively charged reductants can be assigned to protonation at the acidic patch binding site, pK'_{aB}. The pH profile for reduction with $[Co(phen)_3]^{2+}$ (Figure 6) gives a value of 4.8 for pK'_{aB} in the oxidized protein, compared with 5.5 (or higher) in the reduced protein. The large shift of 0.7 unit is to be compared with that of 0.8 unit found with parsley plastocyanin, while the typical higher plant plastocyanin from spinach gives a shift of only 0.3 unit. We have previously suggested[1,35] that the protein oxidation-state dependence of pK_{aB} may provide a mechanism to enhance binding of PCu^{II} to the natural reductant cytochrome *f* prior to electron transfer and ensure that there is less effective binding of PCu^{I} to cytochrome *f* after electron transfer.

The effect of redox-inactive $[(NH_3)_5CoNH_2Co(NH_3)_5]^{5+}$ on the reaction with $[Co(phen)_3]^{3+}$ at pH 7.56 is in line with observations on higher plant plastocyanins. The maximum effect of blocking is 52%, consistent with the view that $[Co(phen)_3]^{3+}$ reacts at one (or more) other site on the protein, which does not bind the (+5) complex. With *S. obliquus* the extent of blocking is the lowest so far observed, while the association constant K_B is the highest. A high K_B is also found for parsley plastocyanin, and the sequence deletions may therefore be implicated.

The ^1H NMR titrations with $[Cr(phen)_3]^{3+}$ indicate at least three regions of interaction on *S. obliquus* PCu^{I}. The two most populated sites clearly involve the negatively charged residues 42–44 (causing broadening of resonances of Phe83 and Tyr82)

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and Asp60 and Asp61 (broadening of resonances of Tyr62). Weaker binding at an additional site, probably involving acidic residues 75 and/or 79 and the C-terminus, causes a smaller degree of broadening of resonances of Phe74 and Tyr80.

With $[Co(phen)_3]^{3+}$ it is not always clear whether the negative residues 42–44 and 60–61 form two distinct binding sites or whether they give rise to a diffuse region of negative charge on the protein surface to which cationic reagents can bind nonspecifically. Similar findings have been reported recently from NMR studies on spinach plastocyanin, where again it was impossible to distinguish between $[Cr(phen)_3]^{3+}$ binding at distinct sites or "rolling around" on a broad area of negatively charged protein surface.[36] One way in which the sequence deletions at positions 57 and 58 might affect the reactivity of *S. obliquus* (and parsley) plastocyanin is by bringing the residues 59–61 into close proximity with the electron-transfer site at residues 42–44. From 2D NMR this is further established as a strong possibility.[25]

The kinetic studies with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ both show an additional pH effect at pH >7, pK_a = 7.6 and 8.2, respectively. This is attributed to deprotonation of His59, for which the pK_a determined by NMR measurements is 7.8. In the case of $[Co(phen)_3]^{3+}$ this effect can in part be attributed to increased binding at the east face electron-transfer site consequent to His59 deprotonation, consistent with the idea that, as a result of the deletions at residues 57 and 58, residues 59–61 are close enough to residues 42–44 to give a significant combined effect. Further evidence for increased binding of $[Co(phen)_3]^{3+}$ comes from saturation kinetic studies at different pH's. At pH 7.56 no curvature of the plot of k_{obsd} against $[Co(phen)_3^{3+}]$ is detectable up to 4.6 mM ($K < 25$ M^{-1}), in contrast to the behavior of higher plant plastocyanins, where such plots show distinct curvature above 0.5 mM. Considering only the 42–45 locality this is understandable as there is one less negative charge in the *S. obliquus* protein. Poplar plastocyanin which is also −3 in this region does not give limiting kinetics either for the range of $[Co(phen)_3^{3+}]$ normally employed.[27] For *S. obliquus* at pH 9.1, however, mild but definite limiting kinetic behavior is observed (Figure 4) and K is in the range 50–100 M^{-1}. The state of protonation of His59 therefore appears to influence binding over a broad region of negatively charged surface involving residues 42–44, 59–61, and possibly Glu85, an additional acidic residue present in *S. obliquus* and parsley plastocyanins, which it has been suggested is relevant in the studies on *A. variabilis* plastocyanin.[26]

An increase in binding affinity for positively charged complexes cannot be the only effect of His59 deprotonation; otherwise, the $[Fe(CN)_6]^{3-}$ reaction would not be affected. In fact, the oxidation rate with $[Fe(CN)_6]^{3-}$ increases by approximately 33% between pH 7 and 9.5 (Figure 3). NMR experiments detected no specific binding of the analogue complex $[Cr(CN)_6]^{3-}$ anywhere on the protein surface, not even at the exposed His87 ligand, where $[Fe(CN)_6]^{3-}$ is believed to react. The negative charge around His59 makes it a most unfavorable locality for interaction with $[Fe(CN)_6]^{3-}$. This is in marked contrast to *A. variabilis* PCu^I,[26] where most of the acidic residues in the negative patch have been replaced; NMR data show preferred binding of $[Cr(CN)_6]^{3-}$ in the vicinity of His59 for the *A. variabilis* protein. Thus, it seems that the only explanation for the effect of the His59 pK_a on the $[Fe(CN)_6]^{3-}$ reaction is that the state of protonation of His59 is transmitted to the active site and affects it, resulting in a decrease in the $E°$ of the protein. Significantly, the NMR titration experiments have demonstrated a sensitivity of the His59 to active-site protonation and of the active site to His59 protonation. It is not clear how these effects are transmitted since there is no direct polypeptide link between the two, the nearest ligand His37 being 20 residues away from His59 after allowing for the deletions. Direct electrostatic and/or H-bonding would seem the most likely explanation(s). The imidazole ring of His59 is some 10–12 Å from the Cu site.

The more favorable binding (K) of $[Co(phen)_3]^{3+}$ to the acidic east site at pH 9.1 when His59 is deprotonated is expected to contribute to the increase in reactivity between pH 7 and 9.5. It is able to explain the greater increase in reactivity for $[Co(phen)_3]^{3+}$ (65%) compared with that for $[Fe(CN)_6]^{3-}$ (33%) over this range. Since $[Fe(CN)_3]^{3-}$ cannot associate at positions 59–61, it seems likely that this locality does not contribute directly to electron transfer. Electron transfer does not have to be invoked for $[Co(phen)_3]^{3+}$, even though $[Cr(phen)_3]^{3+}$ has been shown to associate close to residues 60–61. An interpretation in which there is no electron transfer from the 59–61 site is able to explain all the present observations.

We consider now the His59 pK_{aH} values from the kinetic studies with $[Fe(CN)_6]^{3-}$ (7.6) and $[Co(phen)_3]^{3+}$ (8.2) and from NMR measurements (7.8). While there is reasonable agreement between the $[Fe(CN)_6]^{3-}$ kinetic and NMR values, the $[Co(phen)_3]^{3+}$ value is significantly higher. It is difficult to conceive of any other acid dissociation process that could be effective in this pH range, and it may be that binding of the positive reagent at the east face has the effect of increasing the pK_a of His59. We note also that the His59 pK_a appears to be substantially affected by the oxidation state of the copper, being as far as we can tell 1 pH unit lower for the $[Co(phen)_3]^{2+}$ reduction of PCu^{II} than for the corresponding oxidation of PCu^I. It has to be emphasized that the effect observed with $[Co(phen)_3]^{2+}$ is small however and the pK_a less precise.

There seems to be little or no effect on the reactivity of *S. obliquus* resulting from the interchanging of Phe and Tyr at positions 82 and 83, respectively, as compared to the case of the higher plant plastocyanins.[1] Conservation of these two residues is extensive throughout the plastocyanin series (Figure 2).

Conclusion

It has been demonstrated from NMR measurements that $[Cr(phen)_3]^{3+}$ and therefore (it can be presumed) $[Co(phen)_3]^{3+}$ associate close to residues 60 and 61 on the east face of *S. obliquus* plastocyanin and close to residues 42–44. Also, deprotonation of His59, by providing a greater concentration of negative charge, enhances association of $[Co(phen)_3]^{3+}$ at the east face. There is however no firm evidence for contributions to electron transfer from the 59–61 site with either $[Co(phen)_3]^{3+}$ or $[Fe(CN)_6]^{3-}$. This conclusion is supported by studies on *S. obliquus* plastocyanin modified at His59 by attachment of $Ru(NH_3)_5$ when at the most only a small Ru(II) to Cu(II) intramolecular electron transfer rate constant is observed.[38,39] On deprotonation of His59 at pH >7, the greater increase in reaction rate for $[Co(phen)_3]^{3+}$ (~63%) as compared to that for $[Fe(CN)_6]^{3-}$ (~33%) is satisfactorily accounted for by the more favorable electrostatics and more favorable association in the region of residues 42–44 (K increases from <25 to 50–100 M^{-1}). From ^1H NMR data for PCu^I the active site His37 is sensitive to His59 deprotonation (pK_a = 7.8), and likewise His59 is sensitive to protonation of the active site His87. It appears that deprotonation of His59 decreases the reduction potential of the Cu active site by some 7–15 mV.

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Registry No. $[Co(phen)_3]^{3+}$, 18581-79-8; $[Co(phen)_3]^{2+}$, 16788-34-4; $[Fe(CN)_6]^{3-}$, 13408-62-3; His, 71-00-1.

Supplementary Material Available: Listings of rate constants (Tables I–IV) (8 pages). Ordering information is given on any current masthead page.

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