Kinetic Studies on 1:1 Electron-Transfer Reactions Involving Blue Copper Proteins. 15. The Reactivity of *Anabaena variabilis* Plastocyanin with Inorganic Complexes and Related NMR Studies

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Abstract: To test further the effect of variations in plastocyanin composition on reactivity we here compare the reactions of plastocyanin PCu^{I} from the prokaryotic blue-green alga *A. variabilis*, pI 7.8 (charge +2 at pH ~7), with those of higher plant plastocyanins, pI 4.2 (charge -9 ± 1 at pH ~7). As in the latter studies, active site protonation of PCu^{I} gives a redox inactive form, acid dissociation $pK_a = 5.0$ (25 °C) from studies with $[Fe(CN)_6]^{3-}$ as oxidant, in accordance with an NMR value of 5.1. Similarly $[Co(phen)_3]^{3+}$ gives a higher *apparent* pK_a of 5.4, which suggests contributions from a second process $pK'_a = 5.65$ arising from the protonation of acidic residues. Two such residues at 42 and 85 are close to each other and to Tyr83, which for the higher plant plastocyanins is near to a binding site for $[Co(phen)_3]^{3+}$. The reduction potential for *A. variabilis* PCu^{II}/PCu^{I} (343 mV) is similar to that for higher plant plastocyanins (370 mV). Rate constants (M^{-1} s^{-1}; 25 °C) at pH 7.5, $I = 0.10$ M (NaCl), for *A. variabilis* PCu^{I} are greater for $[Fe(CN)_6]^{3-}$ (6.5×10^5) and less for $[Co(phen)_3]^{3+}$ (630), ratio 1060, compared with ratios for parsley (36), spinach (32), and French bean (12) plastocyanins, in accordance with the different overall charge on *A. variabilis* PCu^{I}. Other oxidants $[Co(dipic)_2]^-$ and $[Co(C_2O_4)_3]^{3-}$ and reductants $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ for PCu^{II} have also been studied. None of the *A. variabilis* investigations give saturation kinetic behavior, and there is no competitive inhibition by redox inactive $[Pt(NH_3)_6]^{4+}$ on the $[Co(phen)_3]^{3+}$ oxidation of PCu^{I}. Proton NMR studies with the redox inactive analogue complex $[Cr(phen)_3]^{3+}$ do not give any broadening of protein resonances. With $[Cr(CN)_6]^{3-}$ NMR shows association at sites in the vicinity of His59/Lys60 and Lys9/Lys33, in the "northern" part of the molecule, with only a slight shift in His87 resonances. With negatively charged oxidants and reductants no effect of pH in the range 6.0–8.0 consistent with His59/Lys60 participation in electron transfer was detected ($pK_a = 7.3$ for His59). Participation of the Lys9/Lys33 site is not ruled out; however, competitive inhibition from $[Zr(C_2O_4)_4]^{4-}$ and $[Mo(CN)_8]^{4-}$ is not observed.

The prokaryotic blue-green algae were among the first oxygen-evolving photosynthetic organisms.[1-3] Their photosynthetic apparatus resembles more closely that of the chloroplast than photosynthetic bacteria. As in eukaryotic green algae and higher plants the low molecular weight (~10 500) metalloprotein plastocyanin, with a single copper atom in a type 1 ligand environment, participates in photosynthetic electron transport.

Plastocyanins from higher plant sources have been the subject of much recent research.[4,5] Full amino acid sequences have been determined for plastocyanins from 15 higher plant (99 residues), 3 green, and 1 blue-green algal sources.[5-11] The latter from *A. variabilis* (105 residues) is the subject of this study.[10] Although *A. variabilis* plastocyanin has many similarities to other plastocyanins including the Cu-coordinating residues at His37, Cys84, His87, and Met92, there are some differences, most notably those centering around the overall charge and distribution of charge on the molecule. In the absence of other information the structure for poplar plastocyanin (Figure 1) is assumed to be relevant. The six additional amino acids of *A. variabilis* plastocyanin are accommodated at the beginning and end of the sequence, with two extra residues inserted at position 75.[10]

Of the 15 higher plant sequences, 50 residues are invariant. Sequence homologies are not as strong if the three green algae are included (33), and with the inclusion of plastocyanin from *A. variabilis* only 26 residues remain invariant. Estimates of the overall charge for PCu^{I} at pH ~7 from amino acid composition indicate conservation at −9 (±1) for the higher plant and green algal plastocyanins (pI ~4.2),[5] whereas for *A. variabilis* plastocyanin it is +2. The latter positive value is supported in this work by pI determinations by isoelectric focussing. Interestingly the actual number of charged residues on *A. variabilis* (21) is similar to those for many higher plant plastocyanins, but the distribution between + and − charge is quite different. In particular, only one of the highly conserved acidic residues at 42–45 and 59–61, both regions close to Tyr83, is retained, and there are no two consecutive acidic residues in the sequence for *A. variabilis* plastocyanin. Previous work on higher plant plastocyanins has suggested that acidic residues (those at 42–45 in particular)[13,14] on the right-hand side of the structure in Figure 1 (sometimes referred to as the "east" face)[15] are important as binding sites in the reactions with positively charged redox reagents including the

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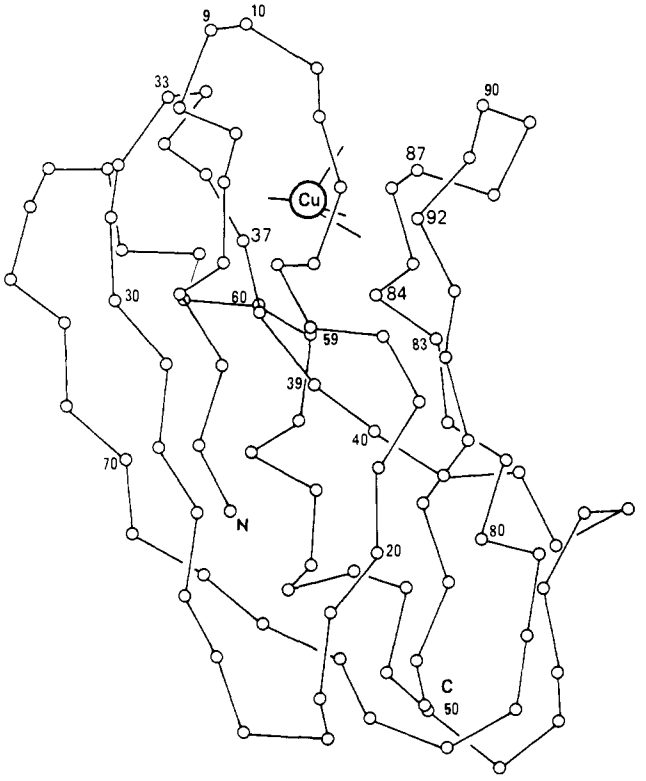

Figure 1. The structure of poplar plastocyanin, PCu^{II}, as determined by Guss and Freeman.[15] The α-carbon framework and the location of the Cu, which is coordinated to residues 37 and 87 (histidines), 84 (cysteine), and 92 (methionine), is shown.

natural partner cytochrome *f*.[16] The quite different charge distribution on *A. variabilis* plastocyanin (e.g., His59 and Lys60 are basic residues giving rise to a 2+ charge at pH <ca. 7) makes it important to investigate reactions of this protein in order to further assess the role of charge in determining binding sites. In addition the "northern" surface of the molecule around His87 (Figure 1), which represents the closest approach to the Cu, and in the case of the higher plant plastocyanins is the binding site for negatively charged oxidants such as $[Fe(CN)_6]^{3-}$, remains essentially nonpolar and hydrophobic for *A. variabilis*, but with some positively charged residues not too distant and in positions of possible influence.

Experimental Section

Growth of Alga. A slope of *Anabaena variabilis* (strain 27892), provided by Dr. Noel Carr at the University of Liverpool, was grown in a salt medium (initially 200 mL in a 500 mL vessel) according to Kratz and Meyers[17] with minor modifications. These included aerating (by bubbling) with 3–5% CO_2 in air, and using more $NaHCO_3$ (×1.3) to maintain the pH in the range 7.3–7.6. Glass aspirators (20-L volume) and growth media were sterilized by standard bulk autoclaving. During growth, illumination of up to four aspirators was provided by six 2 m long 65/80W Grolux discharge lamps (Thorn, U.K.) fitted to a 2 × 0.5 × 0.5 m frame. The vessels were heated externally by four 150 W spotlight lamps, and solutions were maintained at a temperature of 30 ± 1 °C. Cells were harvested by allowing the solutions to settle, decanting off the supernatant solution, and centrifuging for 10 min at 5000 r.p.m. to obtain a paste which was stored at −17 °C. Fresh slopes were used after every fourth harvest. *A. variabilis* cells were also obtained from SP Inc., Salem, MA.

Isolation of Plastocyanin. Extraction and purification of plastocyanin was by the method of Ellefson et al.[18] with some modifications. Instead of breaking the cells with a homogeniser, a sonicator (Rapidis Soniprobe) was used. A suspension of cells in phosphate buffer (pH 7, 50 mM) was sonicated in 100-mL batches for 2 × 10 min with cooling in an ice/water/acetone bath to keep the temperature <10 °C. The penultimate column in the procedure[18] was not used. After dialysis against 1 mM phosphate a trace of $[Fe(CN)_6]^{3-}$ was added to the PCu^{II} (to keep the protein in the oxidized form), which was then loaded onto a C50-120 Sephadex column (Sigma) (25 cm × 1 cm diameter) and eluted in 1–50 mM phosphate buffer gradient, total volume 1000 mL (also containing 0.1 mM $[Fe(CN)_6]^{3-}$). Dialysis against 1 mM phosphate at pH 6.5, followed by concentration on a CM52 Whatman cellulose column (3 × 1 cm), removed the $[Fe(CN)_6]^{3-}$ and consistently yielded a peak ratio A_{278}/A_{597} of <1.5 often 1.15 ± 0.1. The plastocyanin obtained was homogeneous by isoelectric focussing and cation-exchange chromatography. Rate constants from different isolations were in satisfactory agreement. The visible spectrum is similar to that of plastocyanin from higher plants with a prominent peak at 597 nm. An ε of 4500 M^{-1} cm^{-1} is normally used for higher plant plastocyanin,[10] and for *A. variabilis* it was here confirmed as 4650 M^{-1} cm^{-1} by ICP atomic emission.

Complexes. These were prepared as previously, and characterized by absorbance peak positions λ/nm (ε/M^{-1} cm^{-1}): tris(1,10-phenanthroline)cobalt(III) chloride, $[Co(phen)_3]Cl_3 \cdot 7H_2O$, 330 (4660), 350 (3620) 450 (100);[19] ammonium bis(pyridine-2,6-dicarboxylato)cobaltate(III), $NH_4[Co(dipic)_2]$, 510 (630);[20] potassium tris(oxalato)cobaltate(III), $K_3[Co(C_2O_4)_3] \cdot 3.5H_2O$, 245 (2.22 × 10^4), 420 (221), 596 (167);[21] hexaammineplatinum(IV) chloride, $[Pt(NH_3)_6]Cl_4$, 260 (129);[22] potassium tetrakis(oxalato)zirconate(IV), $K_4[Zr(C_2O_4)_4] \cdot 5H_2O$, by titration oxalate 51.5 (calcd 51.1%);[23] potassium octacyanomolybdate(IV), $K_4[Mo(CN)_8] \cdot 2H_2O$, 367 (170);[24] potassium hexacyanochromate(III), $K_3[Cr(CN)_6]$, 308 (58), 378 (83.5);[25] and tris(1,10-phenanthroline)-chromium(III) perchlorate, $[Cr(phen)_3](ClO_4)_3$, 320 sh (1.32 × 10^4), 430 sh (64.2).[26] A solution of $[Co(phen)_3]^{2+}$ was obtained as previously described by addition of phenanthroline (in 4-fold excess over Co) to a solution of $CoCl_2 \cdot 6H_2O$ (BDH, Analar).[27] 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)} (both BDH, Analar) were used as supplied. Solutions of $[Fe(CN)_6]^{4-}$ were used within 30 min of preparation.

Buffers. Acetate/acetic acid buffer (0.040 M total concentration) was used for pHs in the range 4.4 to 5.4. The pH of run solutions was adjusted in many cases by the pH-jump method, thus avoiding dialysis of the protein at low pH. For pH 5.1 to 6.9 a 0.020 M solution of 2-morpholinoethanesulfonic acid, Mes (Sigma), to which NaOH was added, and for pH >7.0 a 0.020 M solution of tris(hydroxymethyl)-aminomethane, Tris (Sigma), to which HCl was added, were used. No dependence on buffer identity was observed in adjacent regions of pH.

NMR Studies. Protein with an A_{278}/A_{597} ratio of 1.36 was used for proton NMR studies. Solutions (~0.4 mM) of the desired pH were prepared in deuteriated phosphate buffer (10 mM) in ^2H_2O and reduced with dithionite. Samples were equilibrated under argon for 10 min. To ensure that there was no oxidation the samples were placed in NMR tubes sealed with rubber septums. Solutions of Cr(III) complexes in ^2H_2O for NMR titration experiments were freshly prepared. The pH was adjusted to the appropriate value with NaO^2H and ^2HCl, and the solution was flushed with argon. For NMR titrations aliquots of the Cr(III) complexes were injected directly through the septum cap into the NMR tube.[28] The pH was measured before and after titration experiments. Spectra were recorded for solutions at 25 °C with a Bruker AM500 spectrometer equipped with an Aspect 3000 computer. Chemical shifts were referenced to internal dioxan at 3.75 ppm. The resolution was enhanced by Lortentz–Gaussian transformation. Gated water suppression was used to reduce the intensity of the residual $^1HO^2H$ peak.

Isoelectric Focussing. Thin-layer electrofocussing was performed on LKB equipment over the pH range 3.5–9.5 with a 2117 Multiphor, 2103 Power supply and 1818 Ampholine solution as described in the LKB application notes.[29] The pH was measured at 10-mm intervals across

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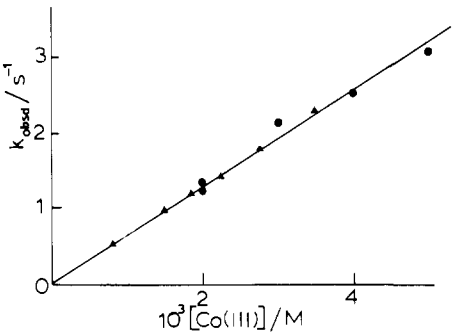

Figure 2. The linear dependence of first-order rate constants, k_{obsd} (25 °C), on oxidant concentration, $[Co(phen)_3^{3+}]$, for the reaction with *A. variabilis* PCu^I at pH 7.5 (Tris/HCl), I = 0.10 M (NaCl), (●), and 0.050 M (NaCl) (▲).

the gel with use of a narrow diameter (6 mm) Russell pH electrode (CWR/322). Protein applied as either PCu^I or PCu^{II} tended to equilibrate as 95% PCu^I during focussing. The pI values obtained were 7.8 ± 0.2 for PCu^I and 8.5 ± 0.2 for PCu^{II}.

Reduction Potential. This was determined for *A. variabilis* plastocyanin at 25 °C by spectrophotometric titration at 597 nm with $[Fe(CN)_6]^{4-}$ (pH 7.5 Tris/HCl), I = 0.10 M (NaCl). After dialysis and immediately prior to titration, the protein was fully oxidized with $[Fe(CN)_6]^{3-}$, which was then removed on a DE52 column (0.5 × 1.0 cm) equilibrated in the same buffer solution. The eluted protein (9.3×10^{-5} M) gave A_{278}/A_{597} = 1.44. A fresh 2.0 mM solution of $[Fe(CN)_6]^{4-}$ in the same buffer was added in small aliquots with use of a Hamilton gas-tight microsyringe to the PCu^{II} (initially 1.0 mL) in a small volume 1 cm path length quartz cell. After mixing, the absorbance at 597 nm was determined on a Perkin-Elmer Lambda 5 spectrophotometer. The final volume was 1.6 mL and the titration was completed within 34 min. Plastocyanin PCu^{II} was measured at 597 nm, which enabled other relevant concentrations in (1) to be calculated. These gave for (1), K = (7.1

$$[Fe(CN)_6]^{4-} + PCu^{II} \rightleftharpoons [Fe(CN)_6]^{3-} + PCu^{I} \quad (1)$$

± 0.4) × 10^{-2}. With use of a reduction potential of 410 mV for $[Fe(CN)_6]^{3-,4-}$ that for the PCu^{II}/PCu^I couple is 339 ± 5 mV (vs. nhe). If instead of an absorption coefficient of 4650 M^{-1} cm^{-1} for PCu^{II} at 597 nm a value of 4500 M^{-1} cm^{-1} is used then the reduction potential is 342 ± 5 mV. Therefore the reduction potential of *A. variabilis* plastocyanin is taken as 340 mV, which compares with a value of 373 mV determined by the same method for spinach plastocyanin.[30] Kinetic studies[31] give a value of 370 mV for parsley plastocyanin.

Kinetic Studies. The protein in 1 mM phosphate at pH 6 was reduced to PCu^I by addition of minimal amounts of sodium ascorbate. The protein was loaded onto a short (0.5 × 1 cm) CM52 column previously equilibrated in the same buffer and washed with buffer until all ascorbate (UV absorbtion at 265 nm) had been removed. The protein (absorbance at ~ 280 nm) was then eluted with 1 mM phosphate at pH ~7, I = 0.10 M (NaCl). Protein solutions of the required pH were prepared by dialysis for 24 h with changes as appropriate for the volumes of solution employed. Absorbance changes were consistent with 1:1 oxidation of PCu^I to PCu^{II}. All kinetic runs were monitored on a Dionex D-110 stopped-flow spectrophotometer at the 597-nm peak for PCu^{II}. The molarity of buffers was in the range (10–20) × 10^{-2} M, with ionic strength adjusted to 0.100 ± 0.003 M with NaCl. Inorganic complexes were in large >10-fold excess of the protein, which was in the range (0.5–2.0) × 10^{-5} M. First-order rate constants, k_{obsd}, were obtained from the slope of ln $(A_\infty - A_t)$ against time. Variations in second-order rate constants, k, with $[H^+]$ were fitted to expression 2,[30] where the protein

$$k = \frac{k_0 + k_H K_a [H^+]}{1 + K_a [H^+]} \quad (2)$$

acid dissociation constant K_a and rate constants are as defined in eq 3–5.

$$HP^+ \overset{K_a}{\rightleftharpoons} H^+ + P \quad (3)$$

$$P + C \xrightarrow{k_0} \text{products} \quad (4)$$

$$HP^+ + C \xrightarrow{k_H} \text{products} \quad (5)$$

In this sequence P designates the protein and C the complex.

Treatment of Data. Unweighted nonlinear least-squares and standard least-squares fitting procedures were used as appropriate.

Table I. The Dependence of Rate Constants (25.0 °C) for Oxidation of *A. variabilis* Plastocyanin, PCu^I (~5 × 10^{-6} M), by $[Fe(CN)_6]^{3-}$ on pH and Oxidant Concentration (I = 0.10 M (NaCl))

pH	$10^5[Fe(CN)_6]^{3-}$ (M)	k_{obsd} (s^{-1})	pH	$10^5[Fe(CN)_6]^{3-}$ (M)	k_{obsd} (s^{-1})
4.50	19.5	25.9	5.25	11.3	50
4.55	15.8	28.6		7.2	33.5
	9.9	15.8	5.65	7.0	40
4.65	5.4	8.9		5.0	28.9
4.82	17.2	40	6.21	10.9	71
	10.3	22.4		7.7	52
5.10	15.7	55	6.85	11.5	80
	10.8	40		8.4	58

Table II. First-Order Rate Constants, k_{obsd} (25 °C), for the $[Co(phen)_3]^{3+}$ Oxidation of *A. variabilis* Plastocyanin, PCu^I (5 × 10^{-6} M), at pH 7.5 (Tris/HCl)

I = 0.100 M (NaCl)	
$10^3[Co(phen)_3^{3+}]/M$	1.0, 2.0, 2.0, 3.0, 4.0, 5.0
k_{obsd}/s^{-1}	0.72, 1.23, 1.36, 2.13, 2.51, 3.07
I = 0.050 M (NaCl)	
$10^3[Co(phen)_3^{3+}]/M$	0.79, 1.48, 1.85, 2.25, 2.76, 3.48
k_{obsd}/s^{-1}	0.52, 0.99, 1.19, 1.41, 1.79, 2.30

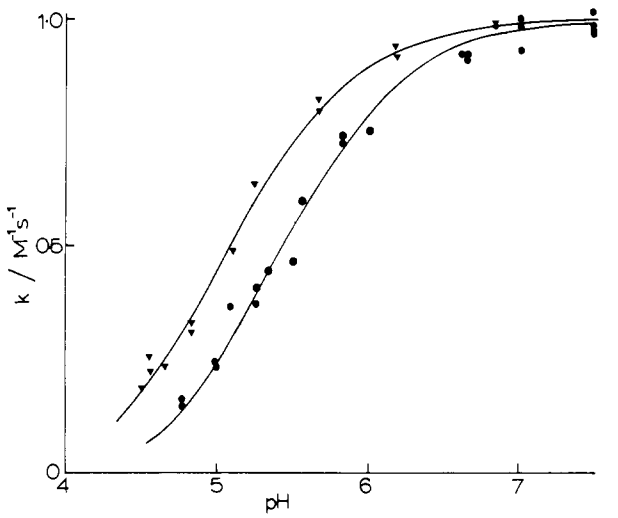

Figure 3. A comparison of the dependences of second-order rate constants (25 °C) (relative scale) on pH for the $[Fe(CN)_6^{3-}]$ (▼) and $[Co(phen)_3]^{3+}$ (●) oxidations of *A. variabilis* PCu^I, I = 0.10 M (NaCl).

Results

Oxidation of PCu^I with $[Fe(CN)_6]^{3-}$. The reaction was too fast to monitor with $[Fe(CN)_6^{3-}] > 5 \times 10^{-4}$ M. Second-order rate constants (k) obtained from k_{obsd} are listed in Table I. The variation of k with pH is illustrated in Figure 3. From a fit to (2), pK_a = 5.04 ± 0.05, k_0 = (7.1 ± 0.1) × 10^5 M^{-1} s^{-1}, and k_H = (−0.3 ± 0.3) × 10^5 M^{-1} s^{-1} (indicating little or no contribution from the protonated form) at 25 °C.

Oxidation of PCu^I with $[Co(phen)_3]^{3+}$. Rate constants k_{obsd} at pH 7.5 (Table II) give a linear dependence on $[Co(phen)_3^{3+}]$ in the range (0.8–5.0) × 10^{-3} M. Second-order rate constants, k, obtained from the slopes exhibit little dependence on ionic strength, and are 627 ± 18 M^{-1} s^{-1} at I = 0.10 M and 651 ± 15 M^{-1} s^{-1} at I = 0.050 M (Figure 2). The dependence of second-order rate constants k on pH (Table III) is illustrated in Figure 3. From a fit of data (25 °C) to (2), the apparent pK_a = 5.41 ± 0.05, k_0 = 630 ± 7 M^{-1} s^{-1}, and k_H = −9 ± 25 M^{-1} s^{-1}. Again, the latter indicates a value at or close to zero. From NMR studies Kojiro and Markley[32] have assigned a pK_a of 5.1 to protonation/de-

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Table III. The Dependence of First-Order Rate Constants, k_{obsd} (25.0 °C), for the $[Co(phen)_3]^{3+}$ Oxidation of *A. variabilis* Plastocyanin (~1 × 10^{-5} M) on pH and Oxidant Concentration (I = 0.10 M (NaCl) Except As Stated)[a]

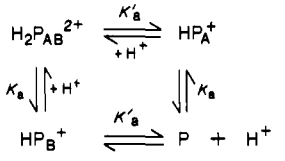
pH	$10^3[Co(phen)_3^{3+}]$ (M)	k_{obsd} (s^{-1})	pH	$10^3[Co(phen)_3^{3+}]$ (M)	k_{obsd} (s^{-1})
4.77	1.00	0.103	5.83	1.95	0.92
	2.05	0.202		3.8	1.74
4.97	1.34	0.207	6.02	1.60	0.76
	2.61	0.41	6.40	1.60	0.93
5.08	5.1	1.20	6.65	1.25	0.73
5.25	1.58	0.41	6.67	1.68	0.98
	2.50	0.59		3.1	1.77
5.31	1.29	0.36	7.02	1.06	0.62
5.50	3.9	1.15		2.04	1.27
5.55	1.40	0.53		4.0	2.57

[a] Additional data are in Table II.

Table IV. The Dependence of First-Order Rate Constants, k_{obsd} (9.6 °C), for the $[Fe(CN)_6]^{4-}$ Reduction of *A. variabilis* PCu^{II} (~5 × 10^{-6} M) on pH and Oxidant Concentration (I = 0.10 M (NaCl))

pH	$10^3[Fe(CN)_6^{4-}]$ (M)	k_{obsd} (s^{-1})	pH	$10^3[Fe(CN)_6^{4-}]$ (M)	k_{obsd} (s^{-1})
6.13	0.75	23.5	8.10	0.75	20.5
	1.50	45.3		1.50	41.7
6.61	0.75	24.1	8.21	0.75	21.3
	1.50	46.1		1.50	39.2
7.50	0.75	23.3	8.64	0.75	21.8
	1.00	31.5		1.50	44.5
	1.50	48.1	8.84	0.75	22.0
				1.50	41.9

protonation of the N^{δ} of His87 of *A. variabilis* PCu^{I}, which at the lower pHs dissociates from the Cu^{I}. As previously indicated[33] the higher pK_a value observed with $[Co(phen)_3]^{3+}$ can be accounted for by active site (A) protonation of His87 to give a redox inactive form at low pH (pK_a), and in addition a binding site (B) protonation (pK_a'). Assuming the two protonations to be independent the modified scheme is



(6)

which gives

$$k = \frac{k_0K_aK'_a + k'_HK'_a[H^+]}{K_aK'_a + K'_a[H^+] + K_a[H^+] + [H^+]^2} \quad (7)$$

where k'_H is the second-order rate constant for protein protonated at the second site (B). When pK_a 5.1 as determined by NMR is used,[32] pK_a' = 5.65 ± 0.15, k_0 = 640 ± 15 M^{-1} s^{-1}, decreasing to k'_H = 350 ± 30 M^{-1} s^{-1}, which is 45% of k_0. Rate constants (M^{-1} s^{-1}) determined at pH 5.8 (for maximum effectiveness)[13] in the presence of redox inactive $[Pt(NH_3)_6]^{4+}$ were with $10^3[Pt^{IV}]$ = 0 (506), 0.93 (488), and 1.55 (490). There is no meaningful trend and it is concluded that there is no measurable inhibition.

Oxidation of PCu^{I} with $[Co(dipic)_2]^-$ and $[Co(C_2O_4)_3]^{3-}$. The reactions were of interest to further explore the possibility of effects of pH over the range 6–8 attributable to protonation/deprotonation of His59. Rate constants ($10^{-3}k/M^{-1}$ s^{-1}) were independent of $[Co(dipic)_2]^-$ concentrations (0.85–2.5) × 10^{-4} M and were as follows (pH in brackets): 1.50 (5.90), 1.85 (6.68), 1.97 (6.93), 2.05 (7.43), and 2.20 (8.45). From a less extensive conventional spectrophotometric (initial slopes) method with (1.2–2.3) × 10^{-4} M $[Co(C_2O_4)_3]^{3-}$, rate constants ($10^5k/M^{-1}$ s^{-1}) were 1.72 (6.81) and 1.88 (8.08). The trend observed is in each case as expected from the active site pK_a, with no contribution from a His59 pK_a.

Reduction of PCu^{II} with $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$. Both reactions are thermodynamically uphill. Rate constants, k_{obsd}, for the $[Fe(CN)_6]^{4-}$ reaction were determined at 9.6 °C (Table IV). The reaction proceeds to the required >90% completion with $[Fe(CN)_6^{4-}]$ in excess of 0.75 × 10^{-3} M. The reverse reaction contributes significantly to equilibration kinetics at lower concentrations. No evidence is obtained for saturation kinetics (Figure 5). Second-order rate constants over the pH range 6.13–8.84 are within ±5% of each other. At 25 °C and pH 7.50 the second-order rate constant is (6.3 ± 0.3) × 10^4 M^{-1} s^{-1} from three runs with 10^3 $[Fe(CN)_6^{4-}]$ = 0.8–1.2 M. On addition of redox inactive complexes $[Zr(C_2O_4)_4]^{4-}$ (3.6 × 10^{-3} M) and [Mo-

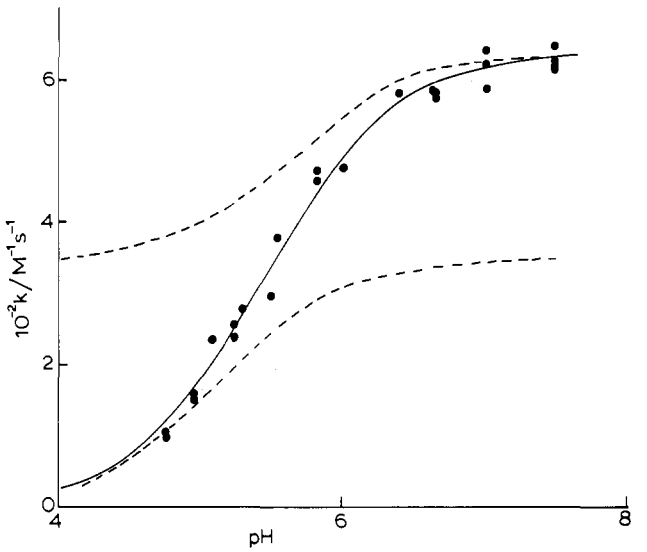


Figure 4. The dependence of second-order rate constants, k (25 °C), on pH for the $[Co(phen)_3]^{3+}$ oxidation of *A. variabilis* PCu^{I} at I = 0.10 M (NaCl). The solid line represents the best fit of experimental data to eq 7 and the broken lines the respective contributions of binding site (upper) and active site (lower) protonations.

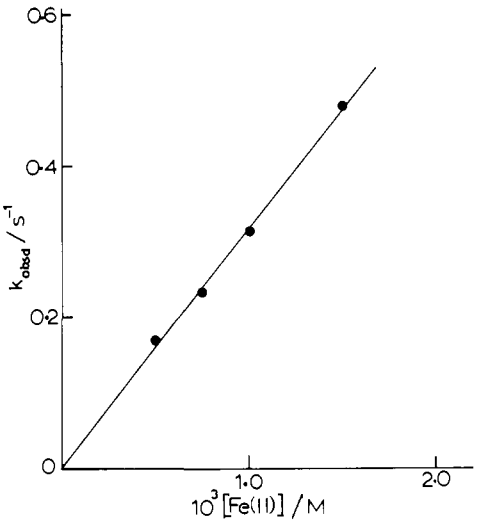


Figure 5. The linear dependence of first-order rate constants k_{obsd} (9.6 °C) on $[Fe(CN)_6]^{4-}$ for the reduction of *A. variabilis* PCu^{II} at pH 7.5 (Tris/HCl), I = 0.10 M (NaCl).

Table V. The Dependence of First-Order Rate Constants, k_{obsd} (25 °C), for the $[Co(phen)_3]^{2+}$ Reduction of *A. variabilis* Plastocyanin, PCu^{II} (~1 × 10^{-5} M), on pH and Reductant Concentration (I = 0.10 M (NaCl))

pH	$10^3[Co(phen)_3^{2+}]$ (M)	k_{obsd} (s^{-1})	pH	$10^3[Co(phen)_3^{2+}]$ (M)	k_{obsd} (s^{-1})
4.62	0.25	0.054	6.20	0.25	0.057
	0.50	0.104		0.33	0.072
5.34	0.25	0.056		0.50	0.105
	0.50	0.106	7.50	0.25	0.059
5.72	0.25	0.059		0.33	0.069
	0.33	0.073		0.50	0.104
	0.50	0.105			

(33) Sinclair-Day, J. D.; Sisley, M. J.; Sykes, A. G.; King, G. C.; Wright, P. E. *J. Chem. Soc., Chem. Commun.* **1985**, 505.

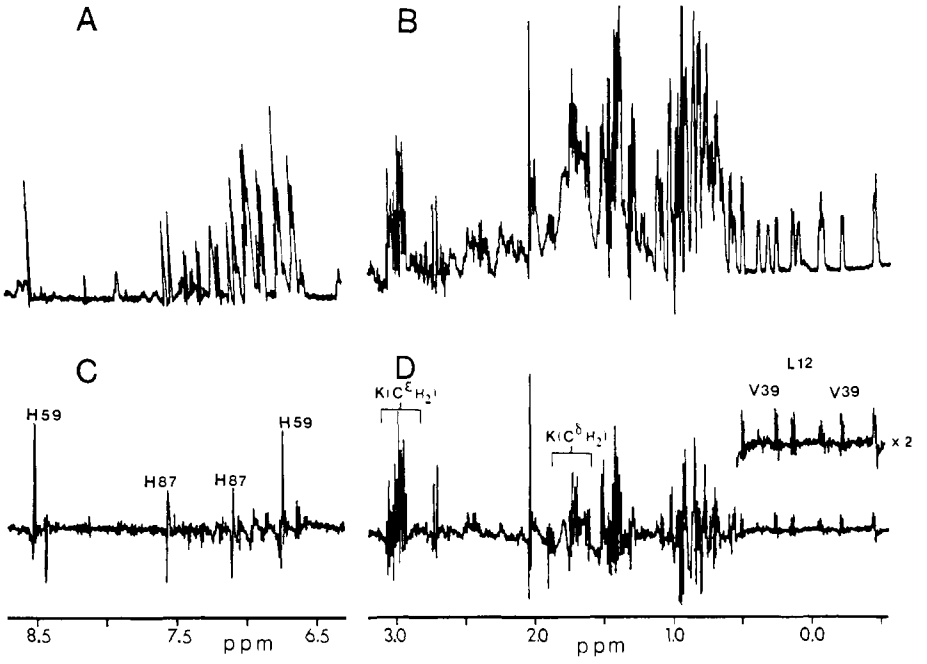


Figure 6. Effects of $[Cr(CN)_6]^{3-}$ on ^1H NMR spectrum of 0.4 mM *A. variabilis* PCu^I at pH 6.3, 25 °C: (A) aromatic region, (B) aliphatic region (vertical scale × $^1/_2$), (C) difference spectrum obtained by subtraction of spectrum of *A. variabilis* PCu^I + 0.6 mM $[Cr(CN)_6]^{3-}$ from spectrum in (A), (D) difference spectrum obtained by subtraction of spectrum of *A. variabilis* PCu^I + 0.6 mM $[Cr(CN)_6]^{3-}$ from spectrum in (B). Assignments of some of the resonances are indicated for histidine (H), lysine (K), valine (V), and leucine (L) residues.

$(CN)_8]^{4-}$ (3.6×10^{-3} M) no evidence was obtained for inhibition.

Rate constants k_{obsd} with $[Co(phen)_3]^{2+}$ are listed in Table V. No effect of $[H^+]$ is apparent over the pH range 4.62–7.50 studied. The second-order rate constant at 25 °C is 192 ± 8 M^{-1} s^{-1}.

Protein Reduction Potential. From rate constants at 25 °C for the $[Co(phen)_3]^{3+}$ (630 M^{-1} s^{-1}) and $[Co(phen)_3]^{2+}$ (192 M^{-1} s^{-1}) reactions (pH 7.5), we calculate a reduction potential of 340 mV for *A. variabilis* PCu^{II}/PCu^I assuming a value of 370 mV for the $[Co(phen)_3]^{3+/2+}$ couple.[5] Similarly from rate constants (25 °C) for $[Fe(CN)_6]^{3-}$ (6.7×10^5 M^{-1} s^{-1}) and $[Fe(CN)_6]^{4-}$ (6.3×10^4 M^{-1} s^{-1}) reactions (pH 7.5) a reduction potential of 348 mV is obtained assuming a value of 410 mV for the $[Fe(CN)_6]^{3-,4-}$ couple.[5] These values compare with 340 mV from the spectrophotometric titration of PCu^{II} with $[Fe(CN)_6]^{4-}$ as described in the Experimental Section.

NMR Studies with $[Cr(CN)_6]^{3-}$. Figure 6 (A and B) shows the spectrum of *A. variabilis* PCu^I at pH 6.3. The spectrum has many features in common with those of French bean[34] and poplar[35] plastocyanins, particularly for residues close to the active site,[36] indicating similar structures. Detailed assignments of the proton resonances will be published elsewhere.[36] The resonances of the two histidine ligands have been identified from earlier ^{13}C and ^1H NMR studies.[32]

A. variabilis PCu^I was titrated with $[Cr(CN)_6]^{3-}$, a redox inactive analogue of $[Fe(CN)_6]^{3-}$. Specific broadening of several resonances is observed, as shown in the difference spectra of Figure 6. In the aromatic region, the C-2H and C-4H resonances of His59 at 8.54 and 6.76 ppm, respectively, begin to broaden upon addition of relatively small concentrations of $[Cr(CN)_6]^{3-}$. The resonances are essentially broadened beyond detection once a 1:1 $[Cr(CN)_6]^{3-}$:plastocyanin ratio is reached. At pHs higher than the His59 pK_a of 7.3,[32] higher concentrations of $[Cr(CN)_6]^{3-}$ are required to produce comparable broadening. The C-2H and C-4H resonances of His87 at 7.59 and 7.12 ppm, respectively, shift slightly downfield upon addition of $[Cr(CN)_6]^{3-}$. At $[Cr(CN)_6]^{3-}$:plastocyanin ratios greater than 2.5:1 some broadening of the Phe82 2,6H resonance is observed. Slight broadening of some other aromatic proton resonances also occurs. However, these resonances are in highly overlapped regions of the spectrum and a clear identification of the residues involved is not possible at present.

Quite dramatic line broadening effects are observed in the aliphatic region. At low concentrations of $[Cr(CN)_6]^{3-}$ the resonances most affected are those of the lysine $C^{\epsilon}H_2$ and $C^{\delta}H_2$ protons near 3.0 and 1.7 ppm, respectively. At higher concentrations of the chromium complex the $C^{\gamma}H_3$ resonances of Val39 at 0.26 and −0.21 ppm and one of the $C^{\delta}H_3$ resonances of Leu12 at 0.15 ppm begin to broaden. Resonances of other aliphatic protons are also affected but cannot yet be assigned reliably to specific amino acid residues.

NMR Studies with $[Cr(phen)_3]^{3+}$. Previously the redox inactive $[Cr(phen)_3]^{3+}$ analogue of the electron-transfer reagent $[Co(phen)_3]^{3+}$ has been found to bind to the negative patch of the higher plant plastocyanins close to Tyr83.[28,37] This was apparent from the broadening of protein resonances, particularly those of Tyr83, due to efficient dipolar coupling with the relatively slowly relaxing spin of the Cr(III) ion.

These experiments have been repeated with PCu^I from *A. variabilis* with entirely different results. No specific broadening of protein resonances occurred at pH 6.3 up to ratios of $[Cr(phen)_3]^{3+}$:PCu^I as high as 2.5:1. In contrast, detectable broadening of the Tyr83 resonances of French bean PCu^I occurred at $[Cr(phen)_3]^{3+}$:PCu^I as small as 1:20.[28] Hence specific binding of $[Cr(phen)_3]^{3+}$ to *A. variabilis* PCu^I must be at best very weak. It would appear that the negative patch close to Tyr83 has been changed to such an extent that strong binding of positively charged complexes is no longer favorable.

Discussion

The difference in composition between *A. variabilis* plastocyanin and the higher plant plastocyanins is the greatest observed in nature, and it is reflected in the overall charges on PCu^I of +2 and −9 ± 1, respectively. The same active site is retained, and there remains appreciable amino acid conservation (26 residues) in the series as a whole as already indicated. Also at pH 7.5 the reduction potential for *A. variabilis* plastocyanin (343 mV; average of different determinations) is very similar to that of higher plant

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(35) King, G. C.; Wright, P. E. *Biochemistry* **1986**, *25*, 2364.
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Table VI. Acid Dissociation pK_a and pK_a' Values for Plastocyanin PCu^I from Different Sources As Determined by NMR and Kinetic Studies with $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ as Oxidants (25.0 °C)[c]

PCu^I source	pK_a NMR	pK_a $[Fe(CN)_6]^{3-}$	pK_a $[Co(phen)_3]^{3+}$[a]	pK_a'[b]
parsley	5.7	5.5	6.1	5.8
spinach	4.9	4.9	5.5	5.6
French bean	4.8	4.6	5.4	5.5
A. variabilis	5.1	5.0	5.4	5.7

[a] Apparent pK_a only. [b] From more detailed fit of $[Co(phen)_3]^{3+}$ data to eq 7, assuming pK_a is as obtained in the NMR studies. [c] Data as in ref 31 with further refined values for *A. variabilis* from this study.

plastocyanins (370 mV), indicating that surrounding charge has little effect. The properties displayed by *A. variabilis* plastocyanin, in particular the solution NMR (see also ref 32), suggests that Figure 1 can for the present be used as a guide to the structure.

In the reactions with inorganic redox partners, close similarities are observed in the behavior of the *A. variabilis* and higher plant active sites. At low pH (<4.5) *A. variabilis* PCu^I becomes redox inactive with the different oxidants. From the effect of pH on rate constants with $[Fe(CN)_6]^{3-}$ the pK_a obtained (5.0) is in good agreement with the value from NMR studies (5.1), which has been assigned to the process involving protonation and dissociation of His87 from the Cu^I.[32] This property is exhibited by all higher plant plastocyanins so far studied (parsley,[5,33] spinach,[14] French bean[38]), and it has been confirmed as His87 dissociation in crystallographic studies on poplar PCu^I.[39] The behavior is in contrast to that of other single (type 1) blue Cu proteins (azurin,[40] stellacyanin,[41] and rusticyanin[43]), and it is believed to be related to the role of plastocyanin in photosynthesis.[39,44] The *apparent* pK_a of 5.4 with $[Co(phen)_3]^{3+}$ as oxidant for *A. variabilis* PCu^I is sufficiently different to require interpretation in terms of a second protonation alongside the active site protonation.[33] Equation 7 derived from the reaction scheme in (6) can be used to fit pK_a and pK_a' values to the data for $[Co(phen)_3]^{3+}$. An active site pK_a of 5.1 has been used in this treatment, which gives a value for pK_a' of 5.65. A comparison of pK_a and pK_a' values in Table VI suggests that the behavior of *A. variabilis* is very similar to that of the higher plant plastocyanins, and in fact it resembles spinach and French bean PCu^I more closely than does parsley PCu^I.

In the case of the higher plant plastocyanins protonation of carboxylate(s) at the acidic patch 42–45 (and/or 59–61) has provided the most acceptable explanation for pK_a'. This explanation carries less weight in the case of *A. variabilis* PCu^I (overall charge 2+) because there are fewer carboxylates, and the extensive negative charge at residues 42–45 and 59–61 is no longer conserved. Residue Asp42 is in fact the only one of these acidic residues retained in *A. variabilis*, and residues His59 and Lys60 both carry positive charge at pH >ca. 7. An additional carboxylate Glu85 not present in the higher plant sequences is close by, and with Asp42 it could provide a significant level of negative charge. However, in the NMR experiments no $[Cr(phen)_3]^{3+}$ line broadening of Tyr83 or any other protein resonances was observed. This indicates that any binding and association of $[Cr(phen)_3]^{3+}$ to the protein is very weak.

Table VII. A Comparison of Rate Constants (M^{-1} s^{-1}, 25.0 °C) for the $[Fe(CN)_6]^{3-}$ (k_{Fe}) and $[Co(phen)_3]^{3+}$ (k_{Co}) Oxidation of Plastocyanin PCu^I from Different Sources at pH 7.5 (I = 0.10 M (NaCl))

source	k_{Fe}	k_{Co}	k_{Fe}/k_{Co}
parsley	81000	2600	36
spinach	81000	2500	32
French bean	58000	4700	12
A. variabilis	670000	630	1060

It is clear that contributions from the Asp42/Glu85 site (if any) are not as pronounced as for the higher plant 42–45 site. First, there is no saturation kinetic behavior for *A. variabilis* PCu^I with $[Co(phen)_3]^{3+}$ (ionic strengths 0.10 and 0.050 M were tested), and no competitive inhibition is observed on addition of redox inactive $[Pt(NH_3)_6]^{4+}$. Association constants K for 3+ and 4+ complexes are therefore smaller than observed for the higher plant plastocyanins. Second, from the treatment given, it appears that no more than ~45% of the reaction at pH 7.5 is at the "east" face, which compares with values of 72% for spinach and 63% for parsley PCu^I.[45] As suggested previously,[45] it is possible that the remaining reaction of $[Co(phen)_3]^{3+}$ is at or near the "north" site, incorporating His87 which has the advantage of being closer to the Cu.

Consistent with the influence of overall charge on the reactivity of *A. variabilis* compared with higher plant PCu^I, rate constants are larger with $[Fe(CN)_6]^{3-}$ and smaller with $[Co(phen)_3]^{3+}$ (Table VII). The order of magnitude faster rates with $[Fe(CN)_6]^{3-}$ suggests some possible benefits from the positively charged lysines at positions 9 and 33 (see below). Because rate constants with $[Fe(CN)_6]^{3-}$ are close to or at the limit of the stopped-flow range, it was not possible to test for saturation kinetics with concentrations of $[Fe(CN)_6]^{3-}$ in excess of 10^{-4} M. However with $[Fe(CN)_6]^{4-}$ (up to 1.5×10^{-3} M) as a reductant for PCu^{II}, no saturation kinetic behavior was detected (at 9.6 °C). Also the 4– redox inactive complexes $[Zr(C_2O_4)_4]^{4-}$ and $[Mo(CN)_8]^{4-}$ (both at 3.6×10^{-3} M) do not inhibit the $[Fe(CN)_6]^{4-}$ reaction, and they do not therefore associate significantly with PCu^{II}.

The NMR experiments with $[Cr(CN)_6]^{3-}$ at $I \sim 0.025$ M suggest at least two possible binding sites. One is clearly in the immediate vicinity of His59. When protonated this residue together with the adjacent Lys60 (residue 61 is Gln) appears to form a positive binding site for $[Cr(CN)_6]^{3-}$; the Val39 resonances are most likely broadened by binding at this site. However, as far as we can tell, it is not one which contributes to electron transfer at I = 0.100 M (there is no pH effect attributed to His59), and there is no evidence for saturation kinetic behavior indicating extensive association with redox partners $[Fe(CN)_6]^{3-,4-}$. Also the effects of pH on rate constants for the $[Co(dipic)_2]^-$ and $[Co(C_2O_4)_3]^{3-}$ oxidations of PCu^I and the $[Fe(CN)_6]^{4-}$ and $[Co(phen)_3]^{2+}$ reductions of PCu^{II} are small (<6%) and do not indicate any influence from protonation/deprotonation of His59, pK_a 7.3.[32] From the NMR studies, binding of $[Cr(CN)_6]^{3-}$ at the His59 site is pH dependent between 7 and 8, and at pH 7.2 much higher concentrations of $[Cr(CN)_6]^{3-}$ are required to produce appreciable broadening of the C-2H and C-4H resonances. This apparent contradiction suggests that $[Fe(CN)_6]^{4-}$ (and $[Fe(CN)_6]^{3-}$) associates at His59 but does not contribute appreciably to electron transfer, i.e., k_{et} is small. The distance of the imidazole N^δ of His59 from the Cu is ~14 Å.[46] On the basis of the popular crystal structure the Lys60 is further from the active site than His59.

The dramatic broadening of lysine resonances by $[Cr(CN)_6]^{3-}$ indicates additional interactions. Resonance overlaps prevent the direct assignment of lysine residues involved, but Lys9 and Lys33 (α-carbon atoms ~14 and ~11 Å, respectively, from the Cu) are possible candidates. Thus the broadening of the $C^\delta H_3$ reso-

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(46) We thank Professor H. C. Freeman and colleagues for this information.

nance of Leu12 could be due to binding of $[Cr(CN)_6]^{3-}$ at this site. No inhibition with redox inactive $[Mo(CN)_8]^{4-}$ is observed, however, and there is no extensive association and/or no electron transfer from this site.

The slight broadening of the 2,6H resonance of Phe82 is possibly due to binding at the His59/Lys60 site or a site involving Lys22 and Lys95. Unlike higher plant PCu^1 there is no observable specific broadening of His87 resonances with up to a 6-fold excess of $[Cr(CN)_6]^{3-}$, although small downfield shifts are observed. The higher rate constants for *A. variabilis* PCu^{11} with $[Fe(CN)_6]^{3-}$ are consistent with a positively charged locality having an influence on the reaction.

To summarize, the reactivity of *A. variabilis* PCu^1 appears to be similar to that of higher plant PCu^1 with regard to active site effects, but with a less clearcut picture with regard to the identity of binding sites. This and other studies on the reactions of plastocyanin with inorganic complexes emphasize the following: (i) charge and its distribution on the protein are important; (ii) for any one complex association can occur at more than one site; (iii) not all of these sites need contribute to electron transfer, since there may be preferred highly directional routes for electron transfer; and (iv) association (K) and electron transfer (k_{et}) can both have a controlling influence on a particular reaction. Physiologically relevant protein redox partners are expected to have greater specificity in their binding than that exhibited by inorganic complexes.

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Registry No. $[Co(phen)_3]^{3+}$, 18581-79-8; $[Co(dipic)_2]^-$, 71605-21-5; $[Co(C_2O_4)_3]^{3-}$, 15053-34-6; $[Fe(CN)_6]^{3-}$, 13408-62-3; $[Cr(CN)_6]^{3-}$, 14875-14-0; $[Co(Phen)_3]^{2+}$, 16788-34-4; $[Fe(CN)_6]^{4-}$, 13408-63-4.

Sulfur Does Not Form Double Bonds in Phosphorothioate Anions

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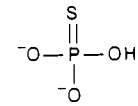
Abstract: Crystal structure, NMR, IR and pK_a measurements have recently been used (P. A. Frey and R. D. Sammons, *Science (Washington, D.C.)* **1985**, *228*, 541) to challenge the longstanding assumptions concerning the distribution of charge and bond orders in phosphorothioate anions. Results from ab initio electronic structure calculations are employed to critically evaluate their hypothesis. The claim of appreciable phosphorus–oxygen double bond character along with a correpsonding absence of phosphorus–sulfur double bonds is confirmed, but a systematic variation in the ratio of sulfur–phosphorus to oxygen–phosphorus multiple bonding with anionic charge is also obtained. Thus, singly charged anions are mesomeric and progress to a triply charged anion that possesses a sulfur–phosphorus single bond and a sulfur with unit negative charge.

I. Introduction

Sulfur substitution for oxygen in nucleotides makes possible the generation of substrate analogues with a chiral phosphate, and for several reasons it has become a favored approach in the stereochemical study of nucleotide–enzyme interactions. Thus, (1) these substrate analogues are generally easy to synthesize, and their configurations are easy to determine;[1] (2) since Mg^{2+} coordinates predominantly to the phosphate oxygen while Cd^{2+} to sulfur, a single isomer of a chiral nucleotide phosphorothioate can produce both of the screw sense isomers by choosing the proper cations,[2,3] (3) individual steps in a reaction sequence can frequently be identified because sulfur analogues have relatively slow rates in enzymatic reactions. The recent article by Frey and Sammons,[3] which challenges the conventional structural representation of phosphorothioate anions, is therefore of widespread and fundamental importance to the use and interpretation of its results. They question the localization of charge on oxygen and the assignment of a phosphorus–sulfur double bond in the conventional structural formula



suggesting instead a single-bonded sulfur bearing a negative charge and a PO Lewis bond order greater than one. Their conclusions for this example and related anions are based on a review of bond length data from X-ray crystallography and electron diffraction, ^{18}O and ^{31}P NMR chemical shifts, the pH dependence of ^{17}O NMR chemical shifts, the vibrational spectra of thiophosphate di- and trianions, and pK_a values of phosphoric and thiophosphoric acids.

The Frey and Sammons work calls attention to two longstanding basic questions concerning the differences between first and second row elements: (1) Which of the congeners, sulfur or oxygen, has the greater ability to stabilize negative charge in an anion? (2) Does a second row atom, like phosphorus, prefer to form a double bond with a first row atom, like oxygen, or rather with a second row atom like sulfur? In this article we have studied the compounds $(OH)_3PS$, H_3PO_4, $H_2PO_3S^-$, $H_2PO_4^-$, HPO_3S^{2-}, HPO_4^{2-}, PO_3S^{3-}, and PO_4^{3-} to address these two questions and to test the conclusions of Frey and Sammons. Ab initio electronic structure calculations are employed as data for our investigation (results with use of an experimentally calibrated semiempirical scheme are also included). Measured bond lengths are critically examined,

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