Kinetic Studies on 1:1 Electron-Transfer Reactions Involving Blue Copper Proteins. 1. Evidence for an Unreactive Form of the Reduced Protein (pH <5) and for Protein–Complex Association in Reactions of Parsley (and Spinach) Plastocyanin

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Abstract: Reactions involving the 1:1 oxidation of reduced parsley plastocyanin, PCu(I), with $Co(phen)_3^{3+}$, $Co(bpy)_3^{3+}$, and $Fe(CN)_6^{3-}$, and reduction of the blue PCu(II) with $Fe(CN)_6^{4-}$, $Ru(NH_3)_5Py^{2+}$, and $Ru(NH_3)_6^{2+}$, have been investigated, $I = 0.10 \pm 0.01$ M (NaCl), by the stopped-flow method. Whereas overall rate constants for reactions of PCu(II) with $Fe(CN)_6^{4-}$ show little variation (ca. 30% increase) on decreasing the pH from 7 to 5, there is a dramatic decrease for reactions of PCu(I) with all three oxidants corresponding to rapid (reversible) formation of a redox-inactive protonated form of the protein with acid dissociation constants, pK_a, in the range 5.7–6.1. Protonation at or near to the Cu(I) site seems likely. Overall activation parameters, pH >7, were determined for all but the $Ru(NH_3)_6^{2+}$ reaction, and in two cases it was possible to extend the range of complex concentrations sufficiently for (kinetic) detection of protein-complex association (K) prior to electron transfer (k_{et}). These were the reactions: PCu(I) + $Co(phen)_3^{3+}$, where at 25 °C, $K = 167$ M^{-1}, $\Delta H° = 10$ kcal mol^{-1}, $\Delta S° = 45$ cal K^{-1} mol^{-1}, $k_{et} = 17.9$ s^{-1}, $\Delta H_{et}^{\ddagger} = 4.3$ kcal mol^{-1}, $\Delta S_{et}^{\ddagger} = -39$ cal K^{-1} mol^{-1}; and $Fe(CN)_6^{4-}$ + PCu(II), $K = 110$ M^{-1}, $\Delta H° = -5.1$ kcal mol^{-1}, $\Delta S° = -7.8$ cal K^{-1} mol^{-1}, $k_{et} = 170$ s^{-1}, $\Delta H_{et}^{\ddagger} = 11.4$ kcal mol^{-1}, $\Delta S_{et}^{\ddagger} = -9.7$ cal K^{-1} mol^{-1}. Comparisons with previous data for the temperature-jump equilibration study of azurin ACu(I)/ACu(II) with $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ are made. Possible limitations imposed by consideration of overall kinetic parameters without reference to K and k_{et} are to be stressed. From ionic strength dependences and values of $\Delta H°$ and $\Delta S°$ as above the protein behaves as a negatively charged species, but it is not clear whether this corresponds to the charge at the binding site. A similar dependence on pH and evidence for association were obtained in the $Co(phen)_3^{3+}$ oxidation of spinach plastocyanin.

Plastocyanin is a blue copper (type 1) protein which is found in plant chloroplasts and other photosynthetic organisms.[1] It is an essential component in the photosynthetic electron-transport chain making use of the blue Cu(II) and colorless Cu(I) states.[2] While earlier papers have indicated that there are 2 Cu atoms per protein it has now been demonstrated[3,4] that there is only a single Cu atom per unit of molecular weight ca. 10 500. The Cu is bound in a distorted tetrahedral ligand environment,[5] which is not accessible to solvent H_2O.[6] Plastocyanins from different plant sources show only minor differences in amino acid composition and sequencing.[3,4] An isoelectric point is observed at pH ca. 4.2,[7] and at higher pHs the protein becomes appreciably negatively charged. Standard reduction potentials have been determined and are ca. 360 mV.[8,9]

As a means of understanding more fully features of the electron-transfer process involving such metalloproteins, reactions of parsley and (to a lesser extent) spinach plastocyanin have been investigated, and are reported herein. Comparisons with studies involving the blue copper protein azurin from bacterial sources are made. The abbreviations PCu(I)/PCu(II) and ACu(I)/ACu(II) are used to indicate different oxidation states of plastocyanin and azurin, respectively.

Experimental Section

Proteins. Plastocyanin was isolated from parsley leaves by the method of Plesničar and Bendall.[10] After preparation the parsley leaves were stored at ca. −15 °C for a minimum time (generally overnight) before use. The final stage in the procedure described, involving concentration of the protein, was omitted. The yield of PCu(II) was 20–40 mL of (4–6) × 10^{-5} M solution from 2.5 kg of cut leaves. This gave an absorbance (A) ratio at peak positions A_{278}/A_{597} of 1.7 ± 0.1, in good agreement with previous determinations.[8] The spectrum based on $\epsilon = 4.5 \times 10^3$ M^{-1} cm^{-1} at 597 nm[7,8] is as shown in Figure 1. Concentrations were determined from the absorbance at 597 nm. No significant (±5%) variation in kinetic parameters was observed using samples from different batches. Before use the protein solution was dialyzed (Sigma 21-mm dialysis sacks) against the required buffer for 24 h at ca. 0 °C. To obtain the reduced form, PCu(I), a few crystals of sodium dithionite (G.P.R. grade, B.D.H.), representing an excess of this reductant, were added before dialysis. Reduction is known to be rapid.[8] The reduced form has the same UV spectrum but no visible absorption.

Spinach plastocyanin was extracted from spinach leaves by the literature method.[11] After removal of ferredoxin as described, the 0.2 M Cl^- eluate of the first DEAE-cellulose (Whatman Ltd.) column was diluted 2×, loaded onto a column of DE23 cellulose (7 cm long, 5 cm diameter), and washed with 0.05 M sodium phosphate buffer (pH 6.9) and the plastocyanin PCu(I) was eluted with a 0.1 M solution of the same buffer. The protein was purified in the same way as for parsley on further columns of DEAE-cellulose. The spectrum is very similar to that of parsley, Figure 1, peak ratio $a_{278}/A_{597} = 1.69$. Spectra are compared with those of other blue Cu proteins in Table I.

Complexes. Tris(1,10-phenanthroline)cobalt(III) was prepared as the perchlorate salt, $[Co(phen)_3](ClO_4)_3 \cdot 2H_2O$,[12] which was percipitated out of solution by addition of saturated $NaClO_4$, and recrystallized from H_2O with the addition of a few drops of 5 M $HClO_4$. Peak positions [λ, nm (ε, M^{-1} cm^{-1})] were at 330 (4660), 350 (3620), and 450 (100) in good agreement with literature values. The chloride salt, $[Co(phen)_3]Cl_3 \cdot 7H_2O$,[13] was also used when the perchlorate salt was not sufficiently soluble. Replacement of Cl^- by ClO_4^- was shown to have no effect on rate constants (see e.g., Table III of the microfilm edition; see paragraph at end of paper). Tris(2,2′-bipyridine)cobalt(III), $[Co(bpy)_3](ClO_4)_3 \cdot 3H_2O$, was prepared,[14] and gave a spectrum with peak positions [λ, nm (ε, M^{-1} cm^{-1})] at 306 (34 100), 317 (30 700), and 450 (68.4) in agreement with previous values. Potassium hexacyanoiron(III), $K_3Fe(CN)_6$ (B.D.H, AnalaR), peak at 300 (1600) and 420 (1010), and potassium hexacyanoiron(II), $K_4[Fe(CN)_6] \cdot 3H_2O$ (Hopkin and Williams, AnalaR), peak 330 (330), were used as supplied. Solutions of $Fe(CN)_6^{4-}$ were used soon after preparation, i.e. within 30 min, or alternatively were stored under N_2. When this precaution was not observed significant air oxidation occurred, and there was an increase in experimentally determined rate constant due to formation of $Fe(CN)_6^{3-}$. Hexaammineruthenium(III) chloride, $[Ru(NH_3)_6]Cl_3$, was obtained from Johnson and Matthey and purified by the literature method.[15] Aqueous solutions (1–5) × 10^{-4} M were reduced (ca. 75% conversion at neutral pH) on an amalgamated zinc shot column (20

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Table I. A Comparison of Visible Spectra [λ (nm), ε (M^{-1} cm^{-1})] of Blue Copper Proteins, Oxidized From Cu(II), in Aqueous Solution, pH ~7

Protein	Source	λ (ε)	λ (ε)	λ (ε)
Plastocyanin[a]	Parsley	460 (600)	597 (4500)	770 (1500)
Plastocyanin[b]	Spinach	460 (590)	597 (4900)	770 (1650)
Azurin[b]	Pseudomonas A	459 (464)	625 (5700)	781 (521)
Stellacyanin[b]	*Rhus vernicifera*	448 (554)	604 (3820)	845 (700)

[a] This work; see also ref 7 and 8. [b] R. Malkin and B. G. Malmström, *Adv. Enzymol.*, **33**, 177 (1970). The protein unit is assumed to contain 1 Cu (mol wt 10 500). Azurin ε values as in ref 32.

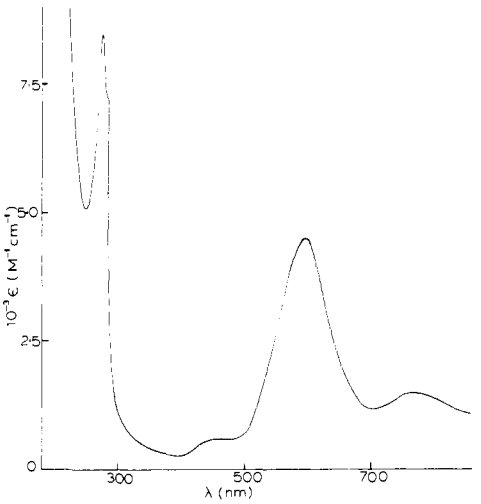

Figure 1. UV-visible spectrum of parsley plastocyanin PCu(II) in aqueous solution, pH 7. The reduced form PCu(I) has the same spectrum, <300 nm.

cm long, 2 cm diameter) under argon gas, immediately prior to use. Concentrations were determined by reacting with μ-superoxo-bis[pentaamminecobalt(III)], when there is a rapid 1:1 reaction, and measuring the absorbance change at 670 nm (ε 890 M^{-1} cm^{-1}). Crystalline pyridinopentaammineruthenium(II) perchlorate, $[Ru(NH_3)_5py](ClO_4)_2$, was prepared by the literature method,[16] starting from $[Ru(NH_3)_6]Cl_3$. Peak positions λ, nm (ε, M^{-1} cm^{-1})] were at 244 (4.8×10^3) and 407 (7.24×10^3).

Buffers. Solutions were prepared from mono- and disodium hydrogen phosphates, hydrochloric acid, sodium acetate, and acetic acid (all AnalaR, B.D.H.), sodium cacodylate, $Na[(CH_3)_2AsO_2]$ (Laboratory Reagent, B.D.H.), and tris(hydroxymethyl)aminomethane (Trizma) here referred to as Tris (Sigma Chemicals). For experiments in which the pH variation was investigated, 10^{-2} M sodium cacodylate, pH adjusted to 5.0-7.5 by addition of HCl, was used. Tris was added to 2×10^{-3} M HCl to give solutions pH 7.0-9.0, and 5×10^{-3} M sodium acetate with HCl was used to obtain a pH of 4.3. Rate constants in Tris were found to be 20% higher than those in cacodylate for the region where the pH ranges overlapped (see, e.g., Table IX[23]). To investigate complex concentration dependencies, and for the determination of activation parameters, 10^{-2} M sodium phosphate ($Na_2HPO_4 + NaH_2PO_4$) pH 7.0 or 7.5 was used. Cacodylate was also used to check concentration dependence at pH 5.0. Rate constants in phosphate were also 10-20% higher than those in cacodylate. For ionic strength variations 10^{-3} M phosphate buffer was used.

Redox Potentials. The standard reduction potential for the PCu(II)/PCu(I) couple is 0.36 V at pH 7.5.[6] The high reactivity of the protein permitted only a narrow range of redox reagents to be investigated. Reduction potentials for complexes used are: $Co(phen)_3^{3+}/Co(phen)_3^{2+}$ (0.37 V),[12] $Co(bpy)_3^{3+}/Co(bpy)_3^{2+}$ (0.37 V),[18] $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (0.41 V),[19] $Ru(NH_3)_5py^{3+}/Ru(NH_3)_5py^{2+}$ (0.253 and 0.273 in ref 17, but 0.310 in ref 20), $Ru(NH_3)_6^{3+}/Ru(NH_3)_6^{2+}$ (0.051 V).[20]

Stoichiometry. Potential measurements of Katoh et al.[9] have indicated a one-electron transfer in the redox equilibration of spinach plastocyanin with $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$. An equilibrium constant of 5.0 determined from rate constants for PCu(I) + $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ + PCu(II) (present study) is in good agreement with a value of ca. 7 obtained from redox potentials, thus supporting a one-electron reaction. McArdle et al.[21] have previously studied the oxidation of PCu(I) with $Co(phen)_3^{3+}$ and analyzed their data in terms of a 1:1 reaction. Spectrophotometric titration of PCu(I) with $Co(phen)_3^{3+}$ indicated a 1:1 reaction with an equilibrium constant for PCu(I) + $Co(phen)_3^{3+} \rightleftharpoons$ PCu(II) + $Co(phen)_3^{2+}$ of 1.6 in close agreement with the electrode potentials quoted above, pH 7.0 (phosphate), I = 0.10 M (NaCl).

Kinetic Studies. Ionic strengths (I) were adjusted to 0.10 M using NaCl. For reactions in 10^{-2} M sodium phosphate at pH 7.0 we have followed Wood[8] in using 0.09 M NaCl. With the phosphate contribution this yields a total ionic strength of ca. 0.108 M. Reactions were monitored using a Durrum stopped-flow spectrophotometer. Traces were photographed from a Tektronix storage oscilloscope, where at least two traces were analyzed for each run (agreement ±5%). We attempted to study the equilibration of $Fe(CN)_6^{4-}$ with PCu(II) by temperature-jump techniques, but encountered stability problems with the protein, which was decolorized after a series of jumps. Instead it was found that the reactions of PCu(I) with $Fe(CN)_6^{3-}$, and $Fe(CN)_6^{4-}$ with PCu(II), could be studied independently by the stopped-flow method by having reactants $Fe(CN)_6^{3-}$ (>tenfold) and $Fe(CN)_6^{4-}$ (>30-fold), respectively, in excess. In all experiments the formation or decay of the blue Cu protein absorbance at the 597-nm was monitored with time. Plots of log ΔA against time were linear for at least 3 half-lives. First-order rate constants were obtained from slopes (×2.303). Air-free conditions (Ar gas) were necessary for the $Ru(NH_3)_5py^{2+}$ and $Ru(NH_3)_6^{2+}$ studies. The latter reduction is very rapid and had to be studied under second-order conditions with $[Ru(NH_3)_6^{2+}] = (1\text{-}6) \times 10^{-6}$ M and $[PCu(II)] = (7\text{-}10) \times 10^{-6}$ M to obtain meaningful traces. Under these conditions rate constants obtained were lacking in precision and only an approximate rate constant is indicated.

Treatment of Data. A nonlinear least-squares program[22] and subroutines based on this were used. Weighting factors were in all cases equivalent to $1/y^2$. For computation of data in connection with eq 1, k_0, k_H, and K_H were independent variables; with eq 7, $\Delta H°$, $\Delta S°$ (for K), $\Delta H_{el}^{\ddagger}$, and $\Delta S_{el}^{\ddagger}$ (for k_{et}) were independent variables.

Results

Parsley plastocyanin was used throughout except for the oxidation of PCu(I) with $Co(phen)_3^{3+}$ when the reaction (25 °C) with spinach plastocyanin was also studied.

Oxidation of PCu(I) with $Co(phen)_3^{3+}$. Second-order rate constants, k, for the reactions of both parsley and spinach plastocyanin with $Co(phen)_3^{3+}$ ($<5 \times 10^{-4}$ M) are dependent on $[H^+]$ for the pH range 5.0-7.5, Table II[23]. The dependence, Figure 2, is of the form:

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

where the constants are as defined in eq 2-4:

$$PCu(I) + H^+ \overset{K_H}{\rightleftharpoons} HPCu(I) \quad (2)$$

$$PCu(I) + Co(phen)_3^{3+} \xrightarrow{k_0} \text{products} \quad (3)$$

$$HPCu(I) + Co(phen)_3^{3+} \xrightarrow{k_H} \text{products} \quad (4)$$

A least-squares best fit to eq 1 gives a small negative value to

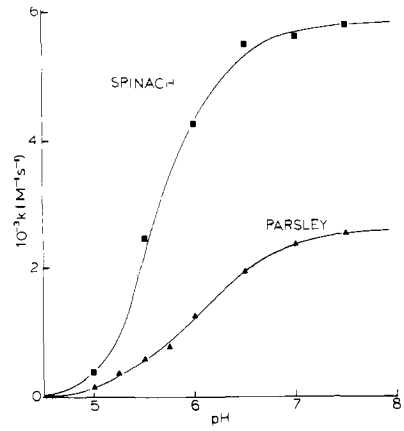

Figure 2. The variation of rate constants (25 °C) for the $Co(phen)_3^{3+}$ oxidation of parsley and spinach PCu(I) with pH, I = 0.10 M (NaCl).

Table IV. Dependence of First-Order Rate Constants (25 °C) for the Oxidation of PCu(I) Spinach Plastocyanin (10^{-5} M) with $Co(phen)_3^{3+}$ on Concentration of Oxidant (pH 7.5 Sodium Cacodylate/HCl Buffer, I = 0.10 M (NaCl))

$10^3[Co(phen)_3^{3+}]$, M	k_{obsd}, s^{-1}	$10^3[Co(phen)_3^{3+}]$, M	k_{obsd}, s^{-1}
0.20	1.16	1.00	4.78
0.30	1.77	2.00	6.95
0.40	2.31	3.00	9.40
0.60	3.14	4.00	10.0

k_H. We have therefore fixed this parameter at zero which then yields $K_H = (1.27 \pm 0.11) \times 10^6$ M^{-1} and $k_0 = (2.69 \pm 0.15) \times 10^3$ M^{-1} s^{-1} for parsley PCu(I). For spinach plastocyanin, $K_H = \sim 5 \times 10^5$ M^{-1}[= K_1] $= \sim 5.9 \times 10^3$ M^{-1} s^{-1}. Attempts to monitor the reactions at pH 4.3 (acetate buffer) by the same stopped-flow method failed due to the slowness of the redox process. The parsley protein was held at pH 5 for several hours and the reactivity to oxidation fully restored on raising the pH. Both the protonation and deprotonation processes are rapid relative to the redox step, and cannot be observed on the stopped-flow time scale.

By extending the range of $[Co(phen)_3^{3+}]$ to $(0.1\text{-}4.8) \times 10^{-3}$ M at pH 7.5 under which condition PCu(I) is deprotonated and $k = k_0$, a nonlinear dependence of first-order rate constant k_{obsd} on oxidant is observed. Plots of k_{obsd}^{-1} against $[Co(phen)_3^{3+}]^{-1}$ are linear, as illustrated in Figure 3, which is consistent with a reaction sequence (eq 5 and 6):

$$PCu(I) + Co(phen)_3^{3+} \overset{K}{\rightleftharpoons} PCu(I), (Co\ phen)_3^{3+} \quad (5)$$

$$PCu(I), Co(phen)_3^{3+} \xrightarrow{k_{et}} PCu(II) + Co(phen)_3^{2+} \quad (6)$$

and a dependence as in eq 7:

$$k_{obsd} = \frac{k_{et}K[Co(phen)_3^{3+}]}{1 + K[Co(phen)_3^{3+}]} \quad (7)$$

The temperature was varied 10.8-31.1 °C for parsley and a least-squares fit of data in Table III gave K(25 °C) = 167 ± 20 M^{-1}, ΔH° = 10 ± 3.5 kcal mol^{-1}, ΔS° = 45 ± 11 cal K^{-1} mol^{-1}, k_{et}(25 °C) = 17.9 ± 1.7 s^{-1}, $\Delta H_{et}^{\ddagger}$ = 4.3 ± 2.9 kcal mol^{-1}, $\Delta S_{et}^{\ddagger} = -39 \pm 10$ cal K^{-1} mol^{-1}. For spinach, Table IV, data at 25 °C gave K = 389 ± 25 M^{-1} and k_{et} = 16.7 ± 0.8 s^{-1}. The dependence of k for parsley on ionic strength was investigated (see Table V).[23]

Oxidation of PCu(I) with $Co(bpy)_3^{3+}$. The dependence of second-order rate constants on pH (Table VI) exhibited more

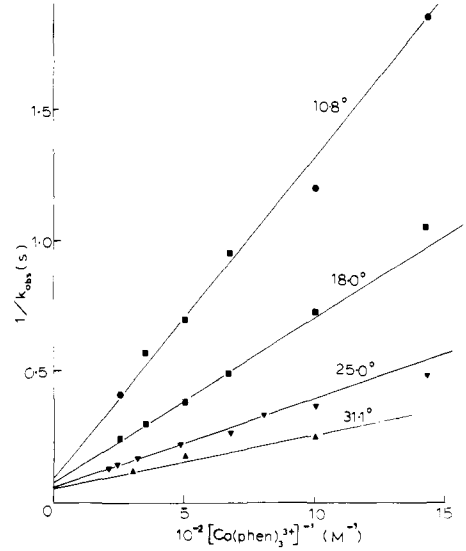

Figure 3. The linear dependence of reciprocal first-order rate constants for the $Co(phen)_3^{3+}$ oxidation of parsley PCu(I) on $[Co(phen)_3^{3+}]^{-1}$ at pH 7.5, I = 0.10 M (NaCl).

Table VI. Dependence of Rate Constant k(25 °C) on pH for the Oxidation of PCu(I) Parsley Plastocyanin (10^{-5} M) with $Co(bpy)_3^{3+}$ (6.0×10^{-4} M) (10^{-2} M Sodium Cacodylate/HCl Buffer, I = 0.10 M (NaCl))[a]

pH	k, M^{-1} s^{-1}	pH	k, M^{-1} s^{-1}
5.0	33	6.5	283
5.5	96	7.0	383
6.0	200	7.0[c]	367
6.0[b]	260	7.5	575

[a] The plastocyanin was kept at pH 7.5 prior to mixing. [b] Protein was stored at pH 5.0 prior to mixing. The small difference in k is possibly due to different degrees of association of protein with buffer. [c] 2×10^{-3} M HCl + Tris.

complicated behavior than $Co(phen)_3^{3+}$. The sigmoidal shape, pH <6.5, originates from reaction steps as in eq 2 and 3, and gives $K_H = \sim 9.7 \times 10^5$ M^{-1} and $k_0 = \sim 390$ M^{-1} s^{-1}. The further increase in rate constants at higher pH values is believed to stem from side reactions of the oxidant.[24] Aquation of the complex contributes at pH >7.5. Rate constants, k, at pH 7 (Table VII) give an estimate for k_0. A least-squares fit of data at pH 7, 10.0-29.6 °C, gives k(25 °C) = 322 M^{-1} s^{-1}, $\Delta H^{\ddagger}$ = 9.7 ± 4 kcal mol^{-1}, $\Delta S^{\ddagger} = -14.6 \pm 1.5$ cal K^{-1} mol^{-1}. On increasing the range of $[Co(bpy)_3^{3+}]$ to 4.0×10^{-3} M no evidence for association as in eq 5-6 was obtained. Table VIII gives the variation of k with ionic strength.[23]

Oxidation of PCu(I) with $Fe(CN)_6^{3-}$. Second-order rate constants, k (Table IX[23]) give a dependence on pH, Figure 4, similar to that for $Co(phen)_3^{3+}$. A fit of data to the same type of reaction sequence yields $K_H = (5.2 \pm 0.2) \times 10^5$ M^{-1}, $k_0 = (7.8 \pm 0.2) \times 10^4$ M^{-1} s^{-1}. The concentration of $Fe(CN)_6^{3-}$ was increased to the limit possible for stopped-flow studies without being able to detect a less than first-order dependence on oxidant $[Fe(CN)_6^{3-}]$. It was not possible therefore to evaluate K and k_{et}. From the temperature dependence 10.5-33.4 °C, Table X, k(25 °C) = (9.4 ± 0.4) × 10^4 M^{-1} s^{-1}, $\Delta H^{\ddagger} = -3.4 \pm 0.1$ kcal mol^{-1}, and $\Delta S^{\ddagger} = -47 \pm 0.3$ cal K^{-1} mol^{-1}. The variation of k with I was investigated (Table XI[23]).

Reduction of PCu(II) with $Fe(CN)_6^{4-}$. Only a small variation in rate constant k with pH was observed, Figure 4 (Table

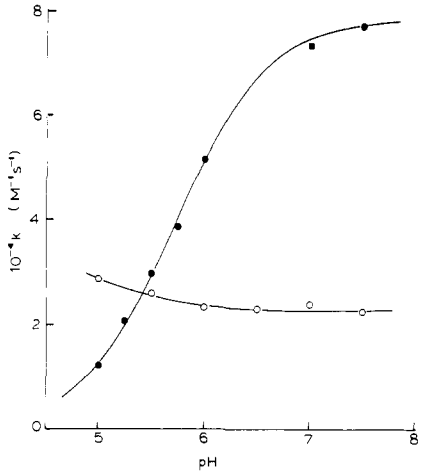

Figure 4. The variation of rate constants (25 °C) for the reactions of parsley plastocyanin PCu(I) + $Fe(CN)_6^{3-}$ (●) and $Fe(CN)_6^{4-}$ + PCu(II) (○) with pH, I = 0.10 M (NaCl).

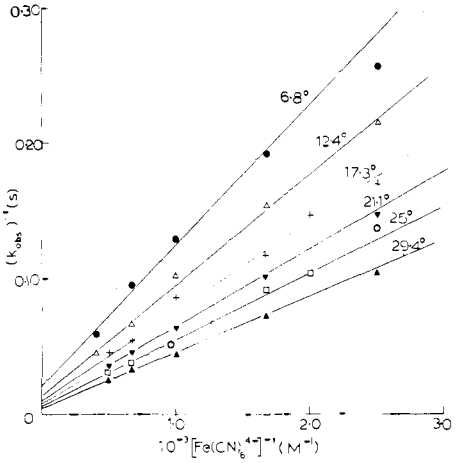

Figure 5. The linear dependence of reciprocal first-order rate constants for the $Fe(CN)_6^{4-}$ reduction of parsley PCu(II) on $[Fe(CN)_6^{4-}]$ at pH 7.0, I = 0.108 M (NaCl).

Table VII. Second-Order Rate Constants for the Oxidation of PCu(I) Parsley Plastocyanin (10^{-5} M) with $Co(bpy)_3^{3+}$ at pH 7.0[a] and pH 5.0[b]

Temp, °C	pH	$10^3[Co(bpy)_3^{3+}]$, M	k, M^{-1} s^{-1}
29.6	7.0	1.50	412
25.0		0.20	313
		0.60	297
		1.00	310 (2)
		1.50	326
		2.00	317 (2)
		3.00	342
		4.00	354
	5.0	0.60	45.4
		1.00	52.0
		1.50	52.3
		2.00	52.0
20.4	7.0	1.50	261
15.2	7.0	1.50	173
10.0	7.0	1.50	129

[a] 10^{-2} M sodium phosphate, I = 0.108 M (NaCl). [b] 10^{-2} M sodium cacodylate + HCl, I = 0.10 M (NaCl).

Table X. Second-Order Rate Constants for the Oxidation of PCu(I) Parsley Plastocyanin (10^{-5} M) with $Fe(CN)_6^{3-}$ at pH 7.0[a] and pH 5.0[b]

Temp, °C	pH	$10^4[Fe(CN)_6^{3-}]$, M	$10^{-4}k$, M^{-1} s^{-1}
33.4	7.0	1.28	7.58
29.5	7.0	1.28	8.20
25.0	7.0	1.28	8.75
	7.0	2.54	9.24
	7.0	4.35	9.85
	7.0	5.55	9.63
18.6	7.0	1.28	9.61
10.5	7.0	1.28	11.1
25.0	5.0	1.00	2.29[c]
	5.0	2.00	2.19
	5.0	4.00	2.09
	5.0	7.00	2.10

[a] Buffer 10^{-2} M sodium phosphate; I = 0.108 M (NaCl). [b] Buffer 10^{-2} M sodium cacodylate + HCl; I = 0.10 M (NaCl); PCu(I) stored at pH 5.0. [c] [PCu(I)] = 5×10^{-6} M.

XII[23]). On extending the range of $[Fe(CN)_6^{4-}]$ to (0.3-2.0) $\times 10^{-3}$ M, first-order rate constants, k_{obsd} (Table XIII[23]), gave a nonlinear dependence on $[Fe(CN)_6^{4-}]$. Plots of k_{obsd}^{-1} against $[Fe(CN)_6^{4-}]^{-1}$, Figure 5, are linear, consistent with the reaction sequence of eq 8 and 9, which is similar to eq 5 and 6:

$$Fe(CN)_6^{4-} + PCu(II) \overset{K}{\rightleftharpoons} Fe(CN)_6^{4-}, PCu(II) \quad (8)$$

$$Fe(CN)_6^{4-}, PCu(II) \xrightarrow{k_{et}} Fe(CN)_6^{3-} + PCu(I) \quad (9)$$

From a least-squares fit of data, 6.8-29.4 °C, K(25 °C) = 110 ± 30 M^{-1}, $\Delta H°$ = −5.1 ± 2.6 kcal mol^{-1}, $\Delta S°$ = −7.8 ± 9.0 cal K^{-1} mol^{-1}, k_{et}(25 °C) = 170 ± 40 s^{-1}, $\Delta H_{et}^{\ddagger}$ = 11.4 ± 2.3 kcal mol^{-1}, $\Delta S_{et}^{\ddagger}$ = −9.7 ± 8.0 cal K^{-1} mol^{-1}. The ionic strength dependence was also investigated (Table XIV[23]).

Reduction of PCu(II) with $Ru(NH_3)_5py^{2+}$ and $Ru(NH_3)_6^{2+}$. Of prime interest was whether a nonlinear dependence on reductant could be detected. Unfortunately both reactions were too rapid for a sufficiently extensive range of reductant concentrations to be investigated. Overall activation parameters were obtained from studies on the reaction of $Ru(NH_3)_5py^{2+}$ (7.1-25 °C), Table XV. These are k(25 °C) = (1.1 ± 0.1) × 10^6 M^{-1} s^{-1}, $\Delta H^{\ddagger}$ = 9.7 ± 0.8 kcal mol^{-1}, $\Delta S^{\ddagger}$ = 1.6 ± 2.9 cal K^{-1} mol^{-1}. Rate constants for the $Ru(NH_3)_6^{2+}$ reduction of PCu(II) at 5 °C were ca. 2.4 × 10^6 M^{-1} s^{-1} in Tris buffer (pH 7.0).

Discussion

Gray and coworkers[25] have previously investigated the effect of pH on the $Fe(edta)^{2-}$ reduction of bean PCu(II) and found there to be a (relatively) small effect in the range pH 5-8. For the oxidation of PCu(I) no similar investigation of pH has been reported. A similar dependence to that for PCu(II) would be consistent with the report by Katoh et al.[9] that the reduction potential for spinach PCu(II)/PCu(I) is invariant until pH <5.4. In accordance with Gray[25] we find that for the reduction of PCu(II) with $Fe(CN)_6^{4-}$ there is only a mild (30%) increase in rate constant with a decrease in the pH from 7 to 5. Our data do not give a sigmoidal shape, Figure 4, and we have not attempted, therefore, to calculate a protonation constant K_H. It is not clear to us that the case for a single protonation step has been clearly established.

The effect of pH on the oxidation of PCu(I) by $Fe(CN)_6^{3-}$, $Co(phen)_3^{3+}$, and $Co(bpy)_3^{3+}$ has now been investigated, and a quite dramatic variation in rate constants is observed. Comparisons of parsley and spinach plastocyanin with Co-

Table XV. Rate Constants for the Reduction of PCu(II) Parsley Plastocyanin (10^{-5} M) with $Ru(NH_3)_5py^{2+}$ at pH 7.0 in 10^{-2} M Sodium Phosphate Buffer, I = 0.108 M (NaCl)

Temp, °C	$10^4[Ru(NH_3)_5py^{2+}]$, M	k_{obsd}, s^{-1}
25.0	1.80	179
16.2	0.31	18.6
	1.80	131
	3.05	210
7.1	0.45	14.0
	1.80	63
	3.15	117
	4.50	161

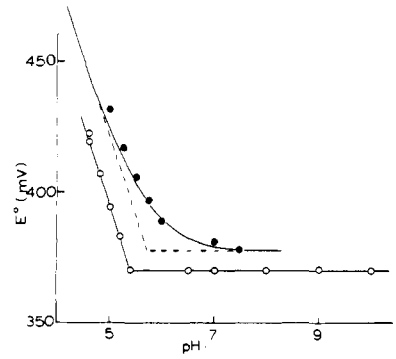

Figure 6. The variation of reduction potential for the PCu(II)/PCu(I) couple with pH. Points (●) are as derived from kinetic data for the respective reactions with $Fe(CN)_6^{4-}$ and $Fe(CN)_6^{3-}$. The line drawn deviates from these points at low pH assuming that the PCu(II) + $Fe(CN)_6^{3-}$ reaction is independent of pH and that the rate constant pH 7 applies. The points (○) are taken from Katoh et al. (ref 9).

$(phen)_3^{3+}$ as oxidant indicate analogous pH behavior, and in view of the similar physical properties of plastocyanins from a wide range of sources,[1] we have no reason to suspect that the effects observed for parsley plastocyanin are nontypical. The pH dependence was investigated with oxidant concentrations at the lower end of the range employed, under which condition reactions conform to rate laws first order in oxidant. Although a complication is observed with $Co(bpy)_3^{3+}$ at pH >7, the behavior can otherwise be satisfactorily accounted for in terms of a simple protonation as in eq 2 with the protonated form redox inactive. Rate constants for the $Fe(CN)_6^{3-}$ oxidation of PCu(I) decrease from 7.9×10^4 M^{-1} s^{-1} at pH >7 to zero at pH <5. The effects do not correspond to a denaturing of the protein. Thus, after storing at pH 5.0 for several hours the reactivity to oxidation is fully restored on raising the pH. Both the protonation and deprotonation are rapid relative to the redox steps and could not be observed on the stopped-flow time scale. When different buffers are used only small differences are observed. Thus, rate constants obtained in 10^{-2} M phosphate and Tris buffers are in very good agreement, but rate constants determined in cacodylate buffer give rate constants ca. 20% less. Differences in k_0 as determined in cacodylate buffer and k determined in phosphate at pH ≥7 are apparent therefore. The pH profiles, Figures 2 and 4, give pK_a values ($K_a = 1/K_H$) of 6.1 and 5.7 with $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$, respectively, as oxidants. The difference in these values is sufficiently large as to prompt the question whether binding of the oxidant to the protein prior to electron transfer may influence the protonation which then becomes dependent on the identity of the oxidant. Further studies are required for confirmation of this possibility. The quite different pH profiles observed for PCu(I) and PCu(II), Figure 4, clearly require the reduction potential to vary with pH. Values of the electrode potential, $E°$, at different pH values have been calculated from the kinetically determined equilibrium constant obtained from rate constants k for the reactions PCu(I) + $Fe(CN)_6^{3-}$ and PCu(II) + $Fe(CN)_6^{4-}$. These are shown in Figure 6. The results obtained by Katoh et al.[9] for spinach plastocyanin by static equilibrium measurements are also shown for comparison. Their observations indicate a pK_a value of ca. 5.4 in the presence of a mixture of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$.

There are three ways whereby the protonation of the protein may render it inactive: (i) by preventing the binding of oxidant to the protein surface; (ii) by preventing electron transfer between the bound oxidant and the protein redox site; (iii) by making the copper ion redox inactive. The absence of a major pH effect on the reactivity of PCu(II) indicates that protonation occurs at a site greatly affected by the copper oxidation state, and so at or near the Cu coordination sphere. Further studies are in progress to determine the effects of pH on K, (i) above, and k_{et}, (ii) and (iii) above.

It is possible that protonation of an amino acid close to the Cu could induce a change in coordination sufficient to account for the above effect. However, an alternative explanation which we find attractive is that addition of a proton may bring about dissociation of a ligand from the metal. Crystallographic evidence for coordination of the Cu to one cysteinyl sulfur, two histidine nitrogens, and one methionine at a distorted tetrahedral site 6 Å from the surface of the protein has been obtained.[5] In the absence of a crystallographic study the same structure is assumed to apply for PCu(I), which is in keeping with the extremely efficient interconversion of PCu(I) and PCu(II) at pH ≥7 involving minimal reorganizational requirements. A distorted tetrahedral geometry is believed to be acceptable to both oxidation states.[27] From its coordination chemistry Cu(I) exhibits 2, 3, and 4 (tetrahedral) coordination. Coordination numbers of 3[28] and 4[29] are only generally acceptable, however, in the presence of Π-acceptor ligands in view of the $3d^{10}$ configuration. Dissociation of the methionine ligand would not involve a proton since the sulfur atom remains unprotonated. Retention of the cysteine ligand as a stabilizing influence seems likely. From NMR studies protonation of the two histidines of PCu(I) has been reported with pK_a values of 4.9 and <4.5.[30] If ligand dissociation occurs we feel that this is most probably a histidine where it is of course possible that the pK_a has been shifted to a higher value by the presence of oxidant. We have been unable to test the possibility of a change in coordination number in terms of variations in spectra because PCu(I) has no visible absorbance, and the spectrum <300 nm, which is the same as for PCu(II) (Figure 1), is probably not helpful in diagnosing a change in geometry.

If a PCu(I) species of decreased coordination number is formed then it is possible that other species in solution might under favorable circumstances coordinate to the metal, thus hindering rapid re-formation of reactive PCu(I) on increasing the pH. This possibility was tested for at 25 °C in 10^{-2} M cacodylate buffer, I = 0.10 M (NaCl), by addition of NaSCN (10^{-2} M) which was found to have negligible (<10%) effect on the $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$ oxidation of PCu(I) at pH 5. The thiocyanate was added to the protein solution 1–2 h before mixing on the stopped flow. These experiments are consistent with the Cu site being inaccessible to the aqueous environment even when the protein is protonated.

It should be emphasized in the context of pH variation that for $[H^+]$ dependencies of the type observed, eq 1, ambiguities do exist in the interpretation. According to the treatment of Newton and Baker,[31] alternative mechanisms besides those indicated in eq 2–4 are kinetically feasible. While we favor the mechanism shown, which is by far the simplest, the evidence at this stage is not unequivocal.

Any detailed analysis of electron-transfer processes must

Table XVI. A Comparison of Overall Activation Parameters ($k = Kk_{et}$)

	k,[a] M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal K^{-1} mol^{-1}	Ref
PCu(I) (parsley) + $Co(phen)_3^{3+}$	3.0×10^3	14.3	6.0	This work[b]
PCu(I) (bean) + $Co(phen)_3^{3+}$	4.9×10^3	14.0	5.0	21[c]
ACu(I)(*Ps. aer.*) + $Co(phen)_3^{3+}$	3.2×10^3	14.3	5.0	21[d]
PCu(I) (parsley) + $Fe(CN)_6^{3-}$	9.4×10^4	−3.3	−47	This work[e]
ACu(I)(*Ps. aer.*) + $Fe(CN)_6^{3-}$	2.7×10^4	−4.1	−52	32[f]
$Ru(NH_3)_5py^{2+}$ + PCu(II)(parsley)	1.1×10^6	9.7	1.6	This work[e]
$Fe(CN)_6^{4-}$ + PCu(II)(parsley)	1.9×10^4	6.3	−17.5	This work[e]
$Fe(CN)_6^{4-}$ + PCu(II) spinach)	2.0×10^4 (20 °C)	8.8	−9.1	*g*
$Fe(CN)_6^{4-}$ + PCu(II) (bean)	1.9×10^4	8.4	−10.6	*g*
$Fe(CN)_6^{4-}$ + ACu(II) (*Ps. aer.*)	345	5.9	−27.1	32[f]

[a] $k = Kk_{et}$ as in eq 5 and 6, 25 °C. [b] $I = 0.10$ M (NaCl), pH 7.5. [c] $I = 0.10$ M (NH_4^+ sulfate), pH 7.0. [d] $I = 0.20$ M (NaCl), pH 7.0. [e] $I = 0.108$ M (NaCl), pH 7.0. [f] $I = 0.22$ M (K^+ phosphate), pH 7.0. [g] D. Fenson and H. B. Gray, unpublished work, cited in ref 2; $I = 0.2$ M (acetate), pH 6.0.

Table XVII. Summary of Protein-Complex and Complex-Complex Association Constants (K), Rate Constants for the Electron Transfer (k_{et}), and Corresponding Enthalpic and Entropic Terms at 25 °C

Reactants	K, M^{-1}	$\Delta H°$, kcal mol^{-1}	$\Delta S°$, cal K^{-1} mol^{-1}	k_{et}, s^{-1}	$\Delta H_{et}^{\ddagger}$, kcal mol^{-1}	$\Delta S_{et}^{\ddagger}$, cal K^{-1} mol^{-1}	Ref
PCu(I)[a] + $Co(phen)_3^{3+}$	167	10	45	17.9	4.3	−39	This work[e]
PCu(I)[b] + $Co(phen)_3^{3+}$	389			16.7			This work[e]
ACu(I)[c] + $Co[4,7\text{-}(PhSO_3)_2phen]_3^{3-}$ [d]	700			0.37			Ref 21[f]
ACu(I)[c] + $Fe(CN)_6^{3-}$	610	−7.7	−13.1	45	3.6	−38.9	Ref 32[g]
$Fe(CN)_6^{4-}$ + PCu(II)[a]	110	−5.1	−7.8	170	11.4	−9.7	This work[h]
$Fe(CN)_6^{4-}$ + ACu(II)[c]	54	−5.5	−10.5	6.4	11.4	−6.6	Ref 32[g]
$Fe(CN)_6^{4-}$ + $Co(NH_3)_5py^{3+}$	2400	0	15	0.015	21.7	5.8	Ref 34[i]

[a] Parsley. [b] Spinach. [c] *Pseudomonas aeruginosa*. [d] 4,7-$(PhSO_3)_2$phen is 4,7-diphenylsulfonate 1,10-phenanthroline. [e] $I = 0.10$ M (NaCl), pH 7.5. [f] $I = 0.20$ M (NaCl), pH 7.0. [g] $I = 0.22$ M (K^+ phosphate), pH 7.0 [h] $I = 0.108$ M (NaCl). [i] $I = 0.10$ M ($NaClO_4$).

take into account the nature of intermediates in the reaction. Since the Cu is known to be in a hydrophobic environment,[5,6] an outer sphere model is assumed for the PCu(II)/PCu(I) interchange. Three steps may be considered (eq 10):

$$\text{PCu(I)} + \text{M(III)} \overset{K_1}{\rightleftharpoons} \text{PCu(I),M(III)}$$

$$\overset{k_{ox}}{\underset{k_{red}}{\rightleftharpoons}} \text{PCu(II),M(II)} \overset{1/K_2}{\rightleftharpoons} \text{PCu(II)} + \text{M(II)} \quad (10)$$

Goldberg and Pecht[32] have obtained data consistent with such a mechanism in a temperature-jump study on the reaction of azurin ACu(I)/ACu(II) with $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$. In the present work we have used a sufficiently large excess of inorganic complexes, so that each reaction is driven essentially to completion. The constant K is used in this paper to refer to association steps K_1 and K_2, and k_{et} for k_{ox} and k_{red}.

Two other mechanisms could account for the dependence as in eq 7. These are PCu(I) + $Co(phen)_3^{3+}$ ⇌ PCu(I), $Co(phen)_3^{3+}$ (nonreactive), followed by PCu(I) + $Co(phen)_3^{3+}$ → products, and, secondly, PCu(I) ⇌ PCu(I)* to give a reactive form, followed by PCu(I)* + $Co(phen)_3^{3+}$ → products. We cannot rule out the first of these possibilities, although it clearly represents a more complicated situation since two binding sites are required, one of which leads to a "switching off" of redox activity. We note, however, that this is precisely what is required of H^+ in eq 2. The second of the above mechanisms can be excluded since it would give identical rate constants for the PCu(I) → PCu(I)* step independent of the identity of the oxidant. This is clearly not the case since the measured quantity k_{et} varies with oxidant.

A summary of overall rate constants for the oxidation of PCu(I) and ACu(I), and reductions of PCu(II) and ACu(II), is given in Table XVI. For the $Fe(CN)_6^{4-}$ reduction of Cu(II) close agreement of data not only with parsley, spinach, and bean plastocyanin, but with *Pseudomonas aeruginosa* azurin (mol wt 13 900 as compared to ca. 10 500 for plastocyanin), is particularly interesting. With other redox reagents there is a wide range of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values which is at first difficult to explain. The parameters fall into readily defined groups depending on whether the oxidant is positive, $Co(phen)_3^{3+}$, or negative, $Fe(CN)_6^{3-}$, and likewise also for the reductants. It has been possible in two cases, the reactions with $Co(phen)_3^{3+}$ and $Fe(CN)_6^{4-}$, to split k into K and k_{et} terms, Table XVII.[33] Other relevant information is included in this table. With redox partners $Co(bpy)_3^{3+}$, $Fe(CN)_6^{3-}$, and $Ru(NH_3)_5py^{2+}$, we were unable to extend the range of complex concentrations sufficiently for diagnostic kinetic behavior consistent with association (less than first-order dependence on complex) to be observed. Upper limits for K of <50 M^{-1} (25 °C) for $Co(bpy)_3^{3+}$, <360 M^{-1} (25 °C) for $Fe(CN)_6^{3-}$, and <450 M^{-1} (7.1 °C) for $Ru(NH_3)_5py^{2+}$ have been deduced from data obtained.

The results for plastocyanin and azurin, Table XVII, complement each other in a highly satisfactory manner, and certainly substantiate the complicated temperature-jump interpretation necessary in the azurin study. It is reasonable, therefore, to discuss features of the enthalpic and entropic terms corresponding to K and k_{et}. The corresponding parameters are also available for the reaction between the two inorganic complexes, $Fe(CN)_6^{4-}$ and $Co(NH_3)_5py^{3+}$,[34] and these are compared in Table XVII. We suggest that a major factor contributing to $\Delta S°$ values is the charge.[35] For reactants of opposite charge, charge neutralization occurs on going to the transition state, water of solvation is released, and $\Delta S°$ is positive, whereas for reactions of like charge an increase in solvation occurs and $\Delta S°$ is negative. On this basis the effective charge on both proteins is negative, which agrees with the results obtained from the variation of ionic strength (see below). This conclusion is opposite to that of Goldberg and Pecht[32] whose reasoning is not clear to us. The numerically large values of $\Delta H°$ obtained for the protein reactions suggest specific

complex-protein interactions, e.g., possibly H-bonding in the case of cyanide ligands, or interactions of the phenanthroline ligand with organic groups on the protein.[36] An x-ray crystal structure of (poplar) PCu(II) has indicated that plastocyanin is barrel shaped with the Cu atom situated near to one end and ca. 6 Å from the surface.[5] A histidine group is interposed between the Cu at its closest approach to the surface, and a "stacking" type interaction between the imidazole and phenanthroline (oxidant) rings is a possibility. The buffers used might, in addition, associate with the protein. Evidence for binding of phosphate (and sulfate) to cytochrome *c* has, for example, been reported.[37] Until a wider range of ligands have been tested, a more clear-cut and satisfactory rationalization of $\Delta H°$ values is difficult. In the case of the $Fe(CN)_6^{4-}$ reactions, the favorable $\Delta H°$ outweighs the unfavorable $\Delta S°$ due to the interaction of like charges, and results in a significant K value comparable to that observed for the reactions of $Co(phen)_3^{3+}$. Interestingly, the reaction of PCu(I) with cytochrome *f*, its natural redox partner, occurs with an overall positive entropy of activation,[8] presumably due to the interaction of oppositely charged groups on the reactants. Also, for a number of antibody-antigen interactions it has been observed that $\Delta H°$ and $\Delta S°$ fall within the ranges −14 to −17 kcal mol^{-1} and −18 to −25 cal K^{-1} mol^{-1}, respectively.[38] The large $\Delta H°$ values have again to be satisfactorily accounted for.

Turning now to the electron-transfer step, the close agreements of $\Delta H_{et}^{\ddagger}$ and $\Delta S_{et}^{\ddagger}$ values firstly for reactions of Cu(I) and secondly for the Cu(II) proteins are to be noted. These values are seen to be independent of charge on the complex, which in the case of the Cu(I) reactions is 3+ in one case and 3− in the other. Values of $\Delta S_{et}^{\ddagger}$ of around zero or, for two oppositely charged reactants, small and positive (consistent with charge neutralization) might have been expected. Taube and colleagues[39] have studied intramolecular electron-transfer reaction within binuclear complexes of the type $[(NH_3)_5Co(III)L\cdots LRu(II)(NH_3)_4H_2O]^{5+}$, where the L···L bridging ligand is a bipyridine or bispyridyl derivative, and found $\Delta S_{et}^{\ddagger}$ values close to zero, consistent with such a view. Values of $\Delta S_{et}^{\ddagger}$ for both $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$ oxidation of Cu(I) protein are large and negative and one possible explanation is a conformational (or coordination number) change involving the Cu(I) protein. Stellwagen and Shulman[40] have previously suggested that $\Delta S_{et}^{\ddagger} = -31.4$ cal K^{-1} mol^{-1} for the oxidation of cytochrome *c*(II) with $Fe(CN)_6^{3-}$ is consistent with a conformational change. Furthermore, values of $\Delta H_{et}^{\ddagger}$ as high as 11.4 kcal mol^{-1} might be considered exceptional if the geometries of the Cu(I) and Cu(II) states are closely matching unless protein rearrangement is also relevant. However, the unexpectedly high barriers to electron transfer may result from the distance between the redox sites, and the absence of an efficient conducting pathway between them.

The trend of rate constants with ionic strength for the reactions of PCu(I) and PCu(II) is illustrated in Figure 7. From the slopes observed with positively and negatively charged complexes it is again concluded that the effective charge on the protein is negative. Isoelectric points for plastocyanin (pH 4.2)[7] and azurin (pH 4.9)[41] indicate an overall negative charge on the proteins for the conditions investigated. It is not immediately clear, however, whether it is this total charge or the charge of the localized binding site which is relevant. Further investigations with stellacyanin, which has an isoelectronic point at 9.86,[42] will, it is hoped, yield further information on this question. Estimates of total protein charge from known charges on individual amino acids at pH ca. 7 give widely differing values for the plastocyanins and azurin. Since similar $\Delta H°$ and $\Delta S°$ values are observed for reactions of PCu(II) and ACu(II), Table XVII, it is possible that local rather than overall charge is influential in these reactions.

Overall activation parameters for the $Ru(NH_3)_5py^{2+}$ +

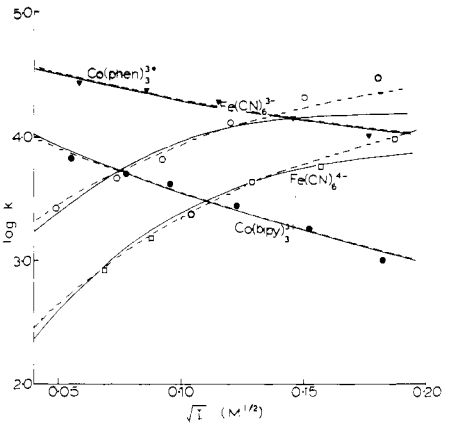

Figure 7. The dependence of rate constants k on ionic strength (I). Experimental points are indicated. The solid line has been obtained from eq 11 using a value $R = 15$ Å for the total protein radius, and the broken line using a value $R_1 = 5$ Å, corresponding to the radius of a localized site.

PCu(II) reaction, Table XVI, are consistent with trends identified in other reactions, and, although K was not determined, positive $\Delta H°$ and $\Delta S°$ contributions in keeping with a positive-negative charge interaction seem perfectly reasonable. The kinetics for the $Co(bpy)_3^{3+}$ oxidation of PCu(I) gave no evidence for a less than first-order dependence on oxidant although a range of concentrations similar to that used for $Co(phen)_3^{3+}$ was employed. Here, overall activation parameters ($\Delta H^{\ddagger} = 9.7$ kcal mol^{-1}; $\Delta S^{\ddagger} = -14.6$ cal K^{-1} mol^{-1}) suggest smaller (positive) $\Delta H°$ and $\Delta S°$ contributions. However, the possibility of small (ca. 15%) contributions due to the incidence of the second deprotonation pH ≥7 (which we do not fully understand) limits further discussion of these parameters.

Following Gray and coworkers,[43] we have briefly tried to fit experimental data as in Figure 7 to equations of the Debye-Hückel type[44] in order to try and ascertain the numerical value of the effective protein charge. The theory of the effect of ionic strength on activity coefficients derived by Brønsted and Bjerrum leads to the following equation (11) at low ionic strengths:

$$\log k = \log k' - \frac{Z_1^2 a\sqrt{I}}{1 + bR_1\sqrt{I}} - \frac{Z_2^2 a\sqrt{I}}{1 + bR_2\sqrt{I}} + \frac{(Z_1 + Z_2)^2 a\sqrt{I}}{1 + bR_{12}\sqrt{I}} \quad (11)$$

where k is the rate constant at ionic strength I, k' is the rate constant with $I \rightarrow 0$, a (0.509) and b (0.329) are constants (25 °C), Z_1 and Z_2 are the charges on the reactants, R_1 and R_2 are their radii, and R_{12} is the radius of the activated complex (angstroms). This equation is based on the assumption that each reagent behaves as a sphere with symmetrical distribution of charge. For the complexes used this is a reasonable assumption and the radii can be determined or estimated with reasonable accuracy. Also, R_{12} can be taken as $R_1 + R_2$. We have tried to use this equation to fit our data in a number of ways, (a) allowing k', Z_1, and R_1 to float, and fixing Z_2 and R_2 for the complex, (b) assuming R_1 for the protein is fixed at 15 Å, and (c) assuming the local protein charge applies over a nominal sphere of arbitrary radius 5 Å, and that this represents the protein reactant. No self-consistent interpretation emerged from any one of these approaches and the values of Z_2 deduced varied considerably. We doubt very much whether equations such as 11, which have been shown to apply for inorganic complexes under the stipulated ionic strength conditions, apply to the present type of problem. The application of eq 11 is critically dependent on the range of I (<0.01 M).[44] We

have restricted ourselves to a lower range of I than that used by Gray.[43]

There is a further very good reason as to why it is difficult to fit the data with $Co(phen)_3^{3+}$ and $Co(bpy)_3^{3+}$ as oxidants. Close inspection of the experimental points in Figure 7 reveals that there is a slight trend showing a downward concavity in both cases. Assuming k_{et} is not very much affected by I it is concluded that K increases appreciably with decreasing I to exceed $K = 2 \times 10^3\ M^{-1}$ for $Co(phen)_3^{3+}$ at the lowest experimental I. Such a value would implicate considerable protein-complex association even at the low reactant concentration used, so that a rate law first order in oxidant no longer applies. Values of k are, therefore, suppressed at low I. Since K will decrease with decreasing I for the negatively charged reactants, no similar argument applies in the case of the $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ reactions.

Finally, with regard to the application of the Marcus theory,[45] we feel some caution is required in view of the magnitude of K values clearly applying in at least some protein-complex reactions. Three overall rate constants, k_{12}, k_{11}, and k_{22} (where k_{12} is for the cross-reaction and k_{11} and k_{22} are for related self-exchange processes), are generally considered in relation to this theory. Each of these values is the product of K for association and k_{et} for electron transfer. The association parameters tend to balance out in many cases when it is legitimate to use overall rate constants. However, it only requires one of the rate constants to be influenced by a specific, i.e., nonelectrostatic interaction of magnitude now detected for the correlation to no longer apply.

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Supplementary Material Available: A listing of rate constants, Tables II, III, V, VIII, IX, XI, XII, XIII, and XIV (8 pages). Ordering information is given on any current masthead page.

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