Kinetic Studies on 1:1 Electron-Transfer Reactions Involving Blue Copper Proteins. 2. Protonation Effects and Different Binding Sites in the Oxidation of Parsley Plastocyanin with $Co(4,7\text{-DPSphen})_3^{3-}$, $Fe(CN)_6^{3-}$, and $Co(phen)_3^{3+}$†

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Abstract: Strong protein-complex association (K) prior to electron transfer (k_{et}) is observed in the oxidation of parsley plastocyanin, PCu^{I}, with the 4,7-di(phenyl-4′-sulfonate)-1,10-phenanthroline complex, $Co(4,7\text{-DPSphen})_3^{3-}$. At 25 °C, I = 0.10 M (NaCl), K = 4600 M^{-1}, $\Delta H°$ = −4.2 kcal mol^{-1}, $\Delta S°$ = 2.7 cal K^{-1} mol^{-1}, k_{et} = 0.041 s^{-1}, $\Delta H^{\ddagger}_{et}$ = 13.2 kcal mol^{-1}, $\Delta S^{\ddagger}_{et}$ = −20.6 cal K^{-1} mol^{-1}. Rate constants are independent of pH in the range 5.2–7.5 investigated. This contrasts with the strong pH dependence observed with $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$ as oxidants ($pK_a \sim 6$). Addition of redox-inactive $Cr(phen)_3^{3+}$ (which forms a 1:1 adduct with the protein) blocks reaction with $Co(phen)_3^{3+}$, consistent with a single binding site being utilized by this oxidant. Rate constants for the $Fe(CN)_6^{3-}$ oxidation of PCu^{I} at pH 7.0 are unaffected by the presence of $Co(4,7\text{-DPSphen})_3^{3-}$ which is 1:1 associated (up to 70%) with the protein. It is concluded that $Co(4,7\text{-DPSphen})_3^{3-}$ and $Fe(CN)_6^{3-}$ use different binding sites on the protein. Moreover, for the reaction of $Co(phen)_3^{3+}$ with PCu^{I} it has been shown that on decreasing the pH from 7.5 to 5.5 K decreases significantly, whereas k_{et} increases slightly. For this reaction the decrease in overall rate constants to zero at pH ca. 5 is therefore due to $K \rightarrow 0$. Such an effect suggests that H^+ modifies the binding site on the PCu^{I}. A similar change is not observed with PCu^{II}. These results indicate that different binding sites have different reduction potentials.

The plastocyanins are copper proteins (mol wt ca. 10 500) containing type 1 copper,[1,2] which lie partially exposed on the surface of the thylakoid membrane,[3] where they are involved in electron transport from photosystem II to photosystem I.[4] They contain a single copper which utilizes oxidation states I and II. The molecular structure of poplar plastocyanin PCu^{II} has recently been determined,[5] and the Cu(II) shown to be bound by two histidines, one cysteine, and one methionine in a distorted tetrahedral arrangement. Similar structural features are expected to be retained in other PCu^{II} species, and also in the reduced protein PCu^{I}, the structure of which is at present being investigated.[5]

The work of Gray and colleagues[6,7] has helped establish the area of investigation of redox reactions of blue copper proteins using inorganic complexes as redox partners. Results with parsley and spinach plastocyanin previously reported from this laboratory[8] have provided evidence consistent with a mechanism involving association of the protein and metal complex prior to electron transfer.

$$PCu^{I} + \text{oxid} \overset{K}{\rightleftharpoons} PCu^{I}, \text{oxid} \quad (1)$$

$$PCu^{I}, \text{oxid} \xrightarrow{k_{et}} \text{products} \quad (2)$$

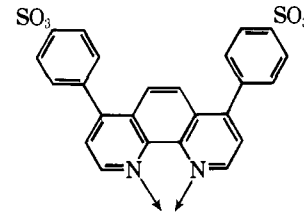
A similar mechanism is involved in the reduction of PCu^{II}. Factors influencing protein-complex association, protonation effects, and the nature of binding sites are further investigated in this paper.

Experimental Section

Protein. Plastocyanin was isolated from parsley leaves by the method of Plesničar and Bendall.[9] It was purified and stored (ca. 5 × 10^{-5} M) as previously described.[8] The oxidized form, PCu^{II}, gave an absorbance peak ratio A_{278}/A_{597} = 1.7 ± 0.1. To obtain the reduced protein, PCu^{I}, a few crystals of sodium dithionite (GPR grade, BDH), representing an excess of reductant, were added before dialysis. Protein solutions were dialyzed (21-mm diameter sacks, Sigma) against the appropriate buffer for 30 h at 0 °C.

† No reprints available.

Complexes. The sodium salt of tris[4,7-di(phenyl-4′-sulfonate)-1,10-phenanthroline]cobalt(III), $Co(4,7\text{-DPSphen})_3^{3-}$, was prepared



4,7 DPSphen

as described elsewhere and purified by repeated precipitation from aqueous ethanol.[10,11] The dried, purified form had an absorbance maximum at 293 nm, ε 1.2 × 10^5 M^{-1} cm^{-1}, based on the formula $Na_3[Co(4,7\text{-DPSphen})_3]$. in agreement with the literature value.[11] Tris(1,10-phenanthroline)cobalt(III) chloride and perchlorate salts were prepared as previously described.[8] Tris(1,10-phenanthroline)-chromium(III) perchlorate, $[Cr(phen)_3](ClO_4)_3$, was prepared by a method based on that of Lee et al.[12] A solution of 1,10-phenanthroline monohydrate (0.60 g) in absolute ethanol (ca. 12 mL) was deoxygenated with N_2 using air-free techniques. Chromium(II) (ca. 0.8 M) in $HClO_4$ (ca. 0.8 M) was prepared by reduction of chromium(III) with amalgamated zinc shot under N_2, and 1.2 mL injected into the solution of ligand with stirring. A solution of I_2 (ca. 0.15 g) in ethanol (7 mL) was added, the mixture extracted with boiling water (ca. 120 mL), and $NaClO_4$ (2 g) added (in air). After the resulting solution was cooled the product was filtered off, washed with a little cold water and ethanol, and recrystallized from water. The pure product was washed with ethanol and dried in vacuo over P_2O_5. Analyses for C, H, and N were satisfactory. The UV-visible spectrum gave λ, nm (ε, M^{-1} cm^{-1}) at 430 sh (642), 320 sh (1.32 × 10^4), and 266 peak (6.37 × 10^4), in good agreement with the literature spectrum.[13] Potassium hexacyanoiron(III), $K_3Fe(CN)_6$ (BDH Analar). peaks λ, nm (ε, M^{-1} cm^{-1}) at 300 (1600) and 420 (1010), was used.

Buffers. Phosphate and cacodylate buffers were as described previously.[8] Acetate buffers were prepared from sodium acetate (Analar, BDH) and hydrochloric acid. Collidine (2,4,6-trimethylpyridine, Laboratory Reagent, BDH) was also used with hydrochloric acid. The buffer concentration after mixing was 1 × 10^{-2} M with phosphate, cacodylate, and acetate. With collidine, solutions were adjusted to keep

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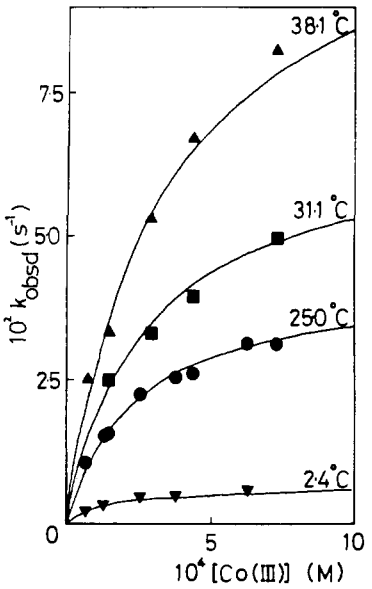

Figure 1. The variation of first-order rate constants, k_{obsd}, with Co(III) concentration for the $Co(4,7\text{-}DPSphen)_3^{3-}$ oxidation of reduced parsley plastocyanin, PCu^I, at pH 7.50 (10^{-2} M phosphate), I = 0.10 M (NaCl).

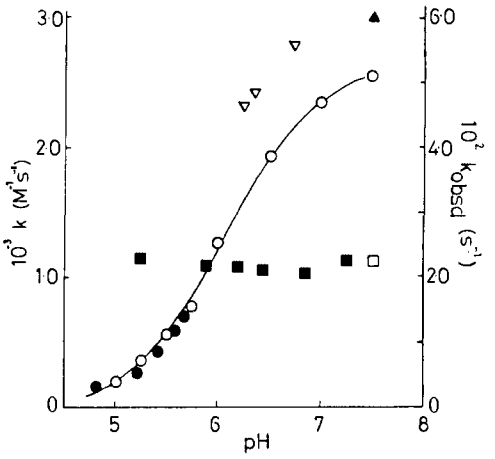

Figure 2. The dependence of rate constants on pH for the oxidation of reduced parsley plastocyanin, PCu^I, with cobalt(III) complexes at 25 °C. Left-hand ordinate: second-order rate constants for the reaction with $Co(phen)_3^{3+}$ in cacodylate (○), acetate (●), collidine (△), and phosphate (▼). Right-hand ordinate: first-order rate constants for the reaction with $Co(4,7\text{-}DPSphen)_3^{3-}$ (2.5×10^{-4} M) in phosphate (□) and cacodylate (■) buffers.

the concentration of the protonated form of the buffer constant at 1×10^{-2} M. Normally 1×10^{-2} M buffer was present in both reactant solutions and the pH was adjusted to the required value at least 30 min before mixing. For a series of experiments in which the pH was varied, 2×10^{-2} M buffer at the desired pH was present in the oxidant solution, and the protein solution contained 1×10^{-3} M buffer at pH ca. 7. The pH of solutions after mixing was measured using a Radiometer (PHM 4d) instrument with a combined electrode type GW 2322C.

Kinetic Studies. Ionic strengths (I) were adjusted to 0.10 M using NaCl. Reactions were monitored at the visible absorption maximum for PCu^{II} at 597 nm, ϵ 4.5×10^3 M^{-1} cm^{-1},[8] using a Durrum-Gibson stopped-flow spectrophotometer. For the slower reactions the added precaution of closing all taps to the absorption cell after mixing substantially prevented any diffusion. Traces were photographed from a Tektronix (RM564) storage oscilloscope and at least three traces were analyzed for each run. A large excess (>tenfold) of oxidant was used in all runs. Plots of log ($A_\infty - A_t$) against time were generally linear for at least 3 half-lives and first-order rate constants, k_{obsd}, were obtained from the slopes (×2.303). For some $Co(4,7\text{-}DPSphen)_3^{3-}$ runs the Guggenheim method was used.[14]

Treatment of Data. A nonlinear least-squares program[15] and subroutines were used. Weighting factors were $1/y$ for the slower (less accurate on stopped-flow time scale) reactions and $1/y^2$ in all other cases.

Results

Oxidation of PCu(I) with $Co(4,7\text{-}DPSphen)_3^{3-}$. First-order rate constants k_{obsd} (pH 7.50), Table I,[16] are dependent on $[Co(4,7\text{-}DPSphen)_3^{3-}]$ as shown in Figure 1. Plots of $(k_{obsd})^{-1}$ against $[Co(4,7\text{-}DPSphen)_3^{3-}]^{-1}$ are linear with positive intercepts, consistent with a mechanism as in (1) and (2). A nonlinear least-squares fit to the derived rate law

$$k_{obsd} = \frac{Kk_{et}[Co(III)]}{1 + K[Co(III)]} \quad (3)$$

gave K(25 °C) = 4600 M^{-1}, $\Delta H^\circ = -4.2 \pm 1.8$ kcal mol^{-1}, $\Delta S^\circ = 2.7 \pm 5.9$ cal K^{-1} mol^{-1}, and k_{et} (25 °C) = 0.041 s^{-1}, $\Delta H^\ddagger_{et} = 13.2 \pm 0.8$ kcal mol^{-1}, $\Delta S^\ddagger_{et} = -20.6 \pm 2.5$ cal K^{-1} mol^{-1}. Rate constants, k_{obsd}, show no dependence on $[H^+]$ over the pH range 5.2-7.5, Figure 2, and no effect on changing the buffer from cacodylate to phosphate.

Oxidation of PCu(I) with $Co(phen)_3^{3+}$. Previous studies have indicated that in cacodylate buffer there is a strong pH dependence of the reaction between PCu^I and $Co(phen)_3^{3+}$, Figure 2. The absence of a comparable dependence with $Co(4,7\text{-}DSPphen)_3^{3-}$ has prompted further experiments, particularly as the acid dissociation pK_a of 6.1 indicated by the previous data is not far removed from the pK_a for cacodylic acid. The behavior observed in acetate buffer (pH 4.8-5.7) is in agreement with the cacodylate data (Figure 2 and Table II[16]). As a further check the pH of a number of protein solutions was adjusted to the final pH value (rather than being kept at pH 7.5 prior to mixing) without any observable change in rate constants. With collidine (Table II[16]) and phosphate (pH 7.5),[8] rate constants are some 30 and 10%, respectively, higher than in cacodylate.

Association of $Co(phen)_3^{3+}$ with PCu^I has been observed previously in kinetic studies at pH 7.5, I = 0.10 M, and from a treatment as in (3) values $K = 167 \pm 20$ M^{-1} and $k_{et} = 17.9 \pm 1.7$ s^{-1} were reported.[8] Information regarding the pH dependences of K and k_{et} has now been obtained. At pH 5.5, rate constants k_{obsd} (Table III)[16] give a linear plot with $[Co(phen)_3^{3+}]$, Figure 3, from which K is estimated to be <50 M^{-1}. At pH 6.5, Table III and Figure 3, curvature is observed. The reciprocal plot is linear and from the computer fit $K = 92 \pm 20$ M^{-1} and $k_{et} = 25 \pm 5$ s^{-1}.

A series of experiments was carried out with the addition of the redox-inactive complex $Cr(phen)_3^{3+}$, which was found to inhibit the reaction of PCu^I with $Co(phen)_3^{3+}$. These experiments were at 25 °C, pH 7.5 (10^{-2} M phosphate), with constant concentrations of $Co(phen)_3^{3+}$ (2.0×10^{-4} M) and PCu^I (1×10^{-5} M). Rate constants k_{obsd}, Table IV, gave a linear plot of $(k_{obsd})^{-1}$ against $[Cr(phen)_3^{3+}]$, Figure 4. Because the observed trend was small, and the low solubility of the perchlorate salt of $Cr(phen)_3^{3+}$ ruled out higher concentrations, a further series of runs was carried out at I = 0.06 M, when association of both complexes with the protein is expected to be enhanced. The data was analyzed according to the scheme

$$PCu^I + Co(III) \overset{K}{\rightleftharpoons} PCu^I, Co(III) \quad (4)$$

$$PCu^I + Cr(III) \overset{K_{Cr}}{\rightleftharpoons} PCu^I, Cr(III) \quad (5)$$

$$PCu^I, Co(III) \overset{k_{et}}{\rightleftharpoons} PCu^{II} + Co(II) \quad (6)$$

The observed rate constants were fitted to the expression

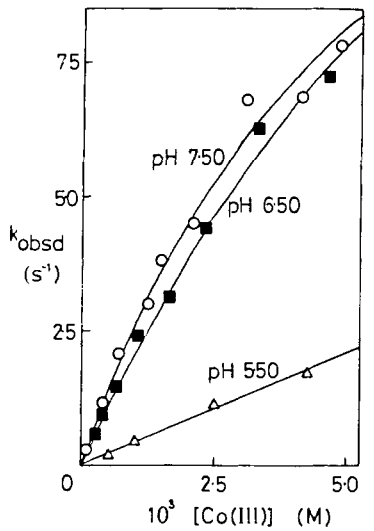

Figure 3. The dependence (25 °C) of first-order rate constants, k_{obsd}, on Co(III) concentration for the $Co(phen)_3^{3+}$ oxidation of reduced parsley plastocyanin, PCu^I, at different pHs. I = 0.10 M.

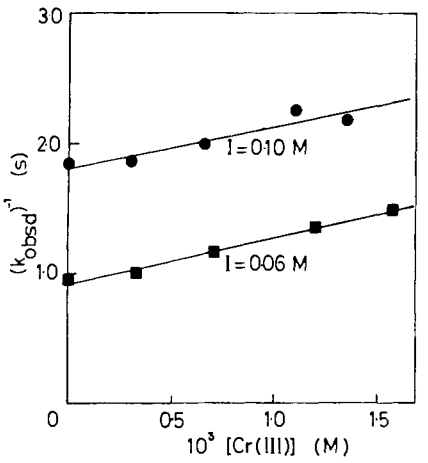

Figure 4. Reciprocal first-order rate constants, k_{obsd}^{-1}, at 25 °C for the $Co(phen)_3^{3+}$ oxidation of reduced parsley plastocyanin, PCu^I, pH 7.5 (10^{-2} M phosphate). I = 0.10 and 0.06 M (NaCl), and the inhibition produced by addition of redox-inactive $Cr(phen)_3^{3+}$.

$$k_{obsd} = \frac{k_0}{1 + K_{Cr}[Cr(III)]} \quad (7)$$

where k_0 is the rate constant observed in the absence of $Cr(phen)_3^{3+}$. At I = 0.10 M (NaCl) a value of K_{Cr} = 176 ± 38 M^{-1} was obtained, while at I = 0.06 M (NaCl) K_{Cr} = 367 ± 33 M^{-1}.

Oxidation of PCu^I with $Fe(CN)_6^{3-}$. Previous studies of this reaction indicated that at 25 °C and pH 7.0 the association constant K is <360 M^{-1}. At pH 5.2 (10^{-2} M cacodylate) the reaction remains first order in each reagent, five runs with concentrations of $Fe(CN)_6^{3-}$ in the range (2-10) × 10^{-4} M, and K < 200 M^{-1}

Experiments reported above have indicated that the complex $Co(4,7\text{-}DPSphen)_3^{3-}$ (K = 4600 M^{-1}) binds to PCu^I much more strongly than does $Fe(CN)_6^{3-}$ (K < 360 M^{-1}), but that the overall rate of oxidation of the protein by $Co(4,7\text{-}DPSphen)_3^{3-}$ is some 500 times slower. Since these two oxidants do not interact with each other it is possible to test whether $Co(4,7\text{-}DPSphen)_3^{3-}$ affects the $Fe(CN)_6^{3-}$ oxidation of PCu^I. Experiments were at 25 °C, I = 0.10 M (NaCl), pH 7.0 (phosphate), with [PCu^I] = 1.0 × 10^{-5} M, and [$Fe(CN)_6^{3-}$] = 2.0 × 10^{-4} M. Seven runs with [$Co(4,7\text{-}DPSphen)_3^{3-}$] in the range (0-6) × 10^{-4} M gave rate constants k = (9.9 ± 0.3) × 10^4 M^{-1} s^{-1} with no detectable trend as the Co(III) concentration is varied. The presence of the complex $Co(4,7\text{-}DPSphen)_3^{3-}$ does not affect the reaction of $Fe(CN)_6^{3-}$ with PCu^I.

Discussion

The oxidation of PCu^I with inorganic complexes is consistent with a reaction sequence (1) and (2), which involves protein-complex association (K) prior to electron transfer (k_{et}). Oxidation with $Co(4,7\text{-}DPSphen)_3^{3-}$ is also consistent with this reaction sequence. The association constant K = 4600 M^{-1} is the largest so far observed for reactions of inorganic complexes with blue copper proteins. The reaction shows no pH dependence (pH range 5.2-7.5) and there is little effect on changing the buffer from cacodylate to phosphate. In a less extensive study of the oxidation of bean PCu^I with $Co(4,7\text{-}DPSphen)_3^{3-}$, McArdle et al.[7] did not detect the strong association and report a much smaller value of k ($= Kk_{et}$) of 25.9 M^{-1} s^{-1} at pH 7.0, I = 0.1 M $(NH_4)_2SO_4$, compared with a value 189 M^{-1} s^{-1} obtained in the present work. It is possible that the differences for bean and parsley plastocyanin may be explained by differences in the reaction medium. We have found that amine buffers (Tris and collidine) do to some extent affect rates of reaction of PCu(I); see, e.g., Figure 2.

The absence of a pH dependence in the reaction of $Co(4,7\text{-}DPSphen)_3^{3-}$ with PCu^I contrasts sharply with the marked influence of pH on the reactions of $Co(phen)_3^{3+}$, $Fe(CN)_6^{3-}$, and (less extensively studied) $Co(bpy)_3^{3+}$ with PCu^I. With these latter oxidants there is a dramatic switch-off in reactivity between pH 7 and 5 with kinetic acid dissociation pK_a values of 6.1 for $Co(phen)_3^{3+}$ and 5.7 for $Fe(CN)_6^{3-}$. This type of switch-off behavior is also observed in the pH range 4.5-6.0 for the oxidation of parsley PCu^I with its physiological redox partner, the protein P700.[17] It has been demonstrated that such pK_a values are not due to the cacodylate buffer used in earlier studies, identical results now being obtained in acetate buffer.

The question as to whether K and/or k_{et} are affected by changes in pH has been investigated. Results obtained for the reaction of PCu^I with $Co(phen)_3^{3+}$ clearly show that as the pH is decreased from 7.5 to 6.5 the value of K decreases from 167 to 92 M^{-1}, and at pH 5.5, when association between protein and complex could not be detected, K is <50 M^{-1}. The value of k_{et} is 17.9 ± 1.7 s^{-1} at pH 7.5 and 25 ± 5 s^{-1} at pH 6.5. It is concluded that for $Co(phen)_3^{3+}$ the decrease in the overall rate constant k to a value of, or close to, zero at pH <5 is due to the decrease in association constant K. Below pH 5 it is known from NMR studies that the histidines bound to the copper in PCu^I become protonated,[18] so we did not extend our studies below this pH.

The switch-off in reactivity has been discussed previously[8] in terms of protonation at or near to the copper coordination site, leading to dissociation of one of the copper ligands. Clearly this explanation cannot now hold as the $Co(4,7\text{-}DPSphen)_3^{3-}$ reaction is unaffected by protonation. The simplest explanation is that $Co(4,7\text{-}DPSphen)_3^{3-}$ binds to the protein at a different site from the oxidants $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$. Experiments to test this hypothesis were performed under conditions where as much as 70% of the protein PCu^I was present, associated with $Co(4,7\text{-}DPSphen)_3^{3-}$. Under these conditions the rate of oxidation with $Fe(CN)_6^{3-}$ is unaffected. This is consistent with the belief that these two complexes are not using the same binding site.

A second series of experiments was carried out to test whether the redox-inactive complex $Cr(phen)_3^{3+}$ affects the oxidation of PCu^I with $Co(phen)_3^{3+}$. Inhibition was observed and the association constant for the binding of $Cr(phen)_3^{3+}$ to the protein (K = 176 M^{-1}) was very similar to that observed

Table V. Summary of the Overall Kinetic Parameters ($k = k_{et}K$) for the Oxidation of Reduced Parsley Plastocyanin, PCu^{I}, at 25 °C, pH ~7.5 (Where Relevant), I = 0.10 M (NaCl)

oxidant	k, M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal K^{-1} mol^{-1}	ref
$Co(4,7\text{-DPSphen})_3^{3-}$	1.9×10^2	9.0	−18	*a*
$Co(phen)_3^{3+}$	3.0×10^3	14.3	6	8
$Fe(CN)_6^{3-}$	9.4×10^4	−3.3	−47	8

[a] This work.

Table VI. Summary of Association Constants K (25 °C), Equation 1, and Related Thermodynamic Parameters for Reactions of Parsley Plastocyanin, I = 0.10 M (NaCl), pH ca. 7

reaction	K, M^{-1}	$\Delta H°$, kcal mol^{-1}	$\Delta S°$, cal K^{-1} mol^{-1}
$PCu^{I} + Co(4,7\text{-DPSphen})_3^{3-}$ [a]	4600	−4.2	2.7
$PCu^{I} + Co(phen)_3^{3+}$ [b]	167	10	45
$PCu^{I} + Co(bpy)_3^{3+}$ [b]	<50		
$PCu^{I} + Fe(CN)_6^{3-}$ [b]	<360		
$Fe(CN)_6^{4-} + PCu^{II}$ [b]	110	−5.1	−7.8

[a] This work. [b] Reference 8.

for $Co(phen)_3^{3+}$ (K = 167 M^{-1}). It is concluded that $Cr(phen)_3^{3+}$ and $Co(phen)_3^{3+}$ are competing for a single binding site on the protein.

The pH effects with $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$ are attributed to inhibition of complex binding ($K \rightarrow 0$) by protonation at or near the oxidant binding site. To produce such a dramatic switch-off in K, some modification in structure at the binding site seems likely, and NMR studies to check whether there is any evidence for a conformational change in PCu^{I} in this region of pH would be of interest. The pK_a values obtained for $Co(phen)_3^{3+}$ (6.1) and $Fe(CN)_6^{3-}$ (5.7) raise the question as to whether the same protonation site is involved in each case.[19] From the effects reported here it is possible that the pK_a for $Co(phen)_3^{3+}$ oxidation is dependent on the nature of the buffer and varies slightly with the identity of buffer used. If some association of buffer with the protein occurs it is not clear whether differently charged oxidants will respond in the same way. At this stage, therefore, we feel that the observed pK_a values could well correspond to the same protonation, suggesting that binding sites for $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$ are in the same locality of the protein. This does not imply that the two binding sites are identical; some differences are to be expected since we believe that electrostatic binding is important.

The $Fe(CN)_6^{4-}$ reduction of PCu^{II} does not respond to pH in the same way as the $Fe(CN)_6^{3-}$ reaction with PCu^{I}. We have repeated our previous experiments and have duplicated the results, which confirm that for the pH range 7.5 down to 5.0 overall rate constants for the $Fe(CN)_6^{4-}$ reduction of PCu^{II} are invariant until pH ca. 6.0. On further decrease of pH rate constants increase by ca. 30% and the pK_a is probably considerably less than 5.5. From microscopic reversibility $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ must be utilizing the same site on the protein, so that protonation must be influenced by the oxidation states of the Cu.

Rosenberg et al.[6] have obtained a fairly well-defined pK_a of 6.1 (ca. 50% trend in k's at pH 7.87-5.73, I = 0.2 M, phosphate buffer) for the reaction of bean PCu^{II} with $Fe(edta)^{2-}$. Assuming that bean and parsley PCu^{II} have similar structures, this implies that $Fe(CN)_6^{4-}$ and $Fe(edta)^{2-}$ must be using different binding sites. It could furthermore be argued that the pK_a of 6.1 for the $Fe(edta)^{2-}$ reduction of PCu^{II} is of the same origin as that observed in the reaction of

Table VII. Summary of Rate Constants k_{et} (25 °C), Equation 2, and Related Activation Parameters for Reactions of Parsley Plastocyanin, I = 0.10 M (NaCl), pH ≥ 7

reaction	k_{et}	$\Delta H^{\ddagger}_{et}$	$\Delta S^{\ddagger}_{et}$
$PCu^{I} + Co(4,7\text{-DPSphen})_3^{3-}$	0.041	13.2	−20.6
$PCu^{I} + Co(phen)_3^{3+}$ [b]	17.9	4.3	−39
$Fe(CN)_6^{4-} + PCu^{II}$ [b]	170	11.4	−9.7

[a] This work. [b] Reference 8.

PCu^{I} with $Co(phen)_3^{3+}$, and that the pK_a of 6.1 is unaffected by the oxidation state of the copper. For the present, however, we are reluctant to pursue this possibility both because the observed effect with $Fe(edta)^{2-}$ and PCu^{II} is so much smaller and because the change in oxidation state of the copper would be expected to have some effect on the pK_a.

An assignment of a pK_a ca. 6.0 to a particular group on the protein is not straightforward. Histidine is the only amino acid which has a pK_a in this region when free in solution, and the basic nitrogen atoms of both histidines in this protein are coordinated to the copper. Evidence from NMR studies gives pK_a values of 4.9 and <4.5 for these histidines.[18] However, lysozyme is known to give a pK_a of about 6, which has been assigned to a glutamic acid residue no. 35.[20] Hydrogen bonding to a negatively charged group can account for abnormally high pK_a values of carboxylic acids in protein environments. On present evidence it can be concluded that the plastocyanins from parsley,[8] spinach,[8,21] and marrow[22] have pK_as of around 6.0.

Our results allow us to comment on the alternative mechanism

$$PCu^{I} + \text{oxid} \rightleftharpoons PCu^{I}, \text{oxid} \quad (8)$$

$$PCu^{I} + \text{oxid} \rightarrow \text{products} \quad (9)$$

which gives a dependence of the same empirical form as (3). The basis of this mechanism is that the adduct formed in (8) is redox inactive. Since extensive association of $Co(4,7\text{-DPSphen})_3^{3-}$ does not affect the reaction with $Fe(CN)_6^{3-}$, for this mechanism to apply, the adduct PCu^{I}, $Co(4,7\text{-DPSphen})_3^{3-}$ must be unreactive with a second $Co(4,7\text{-DPSphen})_3^{3-}$, but react with $Fe(CN)_6^{3-}$ at exactly the same rate as unassociated PCu^{I}. Much seems to be asked of the protein for it to behave in this extremely versatile fashion. General applicability of the mechanisms (8) and (9) seems extremely unlikely, although isolated examples cannot of course be ruled out.

The overall kinetic parameters summarized in Table V are widely divergent and difficult to assess. Thermodynamic parameters corresponding to K, and activation parameters for k_{et}, for reactions of parsley PCu^{I} and PCu^{II}, where these are known, Tables VI and VII, are probably more meaningful. As pointed out previously[8] it is possible to rationalize the entropy for association ($\Delta S°$) using a simple electrostatic approach. Thus the entropy change for the reaction of $Co(phen)_3^{3+}$ with PCu^{I} (45 cal K^{-1} mol^{-1}) indicates that solvent molecules are released in an association step involving oppositely charged species. We estimate that the overall charge on PCu^{I} may be as high as −7 at pH 7.[23] The association of PCu^{II} with $Fe(CN)_6^{4-}$, on the other hand, gives an unfavorable $\Delta S°$ (−7.8) consistent with an interaction of like-charged species. For the reaction of $Co(4,7\text{-DPSphen})_3^{3-}$ with PCu^{I} the value of $\Delta S°$ is positive (2.7), possibly reflecting the greater charge delocalization of the complex. Since this complex shows no variation in rate over a pH range where the charge on the protein is changing by at least one unit, it would seem that the local effective charge at the binding site rather than the overall charge on the protein is relevant. This belief is reinforced by the observation that the $Co(4,7\text{-DPSphen})_3^{3-}$,$PCu^{I}$ adduct

reacts at the same rate with $Fe(CN)_6^{3-}$ as does PCu^I.

There is a trend in $\Delta H°$ values which reflects the charge on the oxidant, but values of $\Delta H°$ are numerically bigger than might be expected on the basis of simple electrostatics. Specific protein-complex interactions, such as hydrogen bonding with $Fe(CN)_6^{3-}$ and hydrophobic-hydrophobic interactions with $Co(phen)_3^{3+}$ and $Co(4,7\text{-DPSphen})_3^{3-}$, are likely to be important, and may well be different at different sites. Desolvation effects, over and above those stemming from the electrostatic nature of interactions, are a further possibility for such protein reactions. Thus regions of high solvation, possibly solvent pockets, close to a binding site on the protein may desolvate on association of the complex.

The complexes $Co(4,7\text{-DPSphen})_3^{3-}$ and $Co(phen)_3^{3+}$ have very similar reduction potentials (340 and 370 mV, respectively),[7] but undergo electron transfer with PCu^I at substantially different rates, Table VII. The very large negative $\Delta S^{\ddagger}_{et}$ values for $Co(4,7\text{-DPSphen})_3^{3-}$ (−21 cal K^{-1} mol^{-1}) and $Co(phen)_3^{3+}$ (−39 cal K^{-1} mol^{-1}) are not consistent with intramolecular electron transfer within a compact assembly requiring minimal reorganization. Since the oxidants bind at various sites at the surface of the protein, the distances between the redox centers is fairly large, and different for the different oxidants. Even if the electron transfer were fully adiabatic, this may account for the magnitude and variation of activation parameters. The partial molar entropies of the reactants should also be considered. These have been calculated for the $Co(phen)_3^{3+}/Co(phen)_3^{2+}$ (25 cal K^{-1} mol^{-1})[24] and $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (48 cal K^{-1} mol^{-1})[25] couples, but the calculations relate to complexes in solution, whereas the species involved here are associated with the protein. An alternative explanation in which k_{et} values are identified with specific protein conformational changes[26] to allow closer approach of the inorganic complex and the blue copper center is also possible. Such changes would differ for different binding sites and might explain the large negative $\Delta S^{\ddagger}$ values.

Reduction potentials for PCu^{II}/PCu^I, and other blue copper protein couples, have been estimated by measuring the position of equilibrium in the presence of another couple, typically $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$.[22] For this couple it is now known that as the pH is varied the plastocyanin potential varies.[8] This pH dependence can be ascribed[8] to a change in the rate of the reaction of reduced plastocyanin with $Fe(CN)_6^{3-}$. Since no comparable change is noted in the reaction with $Co(4,7\text{-DPSphen})_3^{3-}$, it is reasonable to suggest that the different binding sites exhibit different potentials.

It is interesting to speculate on the possible location of the oxidant binding sites. In addition to the two sites in the vicinity of histidine-87 and tyrosine-83 considered by Freeman et al.,[5] there are at least two other regions of the poplar PCu^{II} structure both at some distance from the copper, around glutamate-43 and glutamate-59, worthy of consideration. The presence of several carboxylate residues in each region could account for the observed pK_a values. It is noteworthy that these localities of negative charge are generally conserved in the plant plastocyanins.[27]

The existence of different binding sites and of different reduction potentials has to be taken into account before considering the application of the Marcus theory to these reactions.

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Supplementary Material Available: A listing of rate constants, Tables I-IV (4 pages). Ordering information is given on any current masthead page.

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