Kinetic Studies on 1:1 Electron-Transfer Reactions Involving Blue Copper Proteins. 3. Protonation Effects, Protein–Complex Association, and Binding Sites in Reactions of *Pseudomonas aeruginosa* Azurin with $Co(phen)_3^{3+}$, $Co(4,7\text{-DPSphen})_3^{3-}$, and $Fe(CN)_6^{3-}$ (Oxidants) and $Fe(CN)_6^{4-}$ (Reductant)†

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Abstract: Whereas the reaction of reduced *Pseudomonas aeruginosa* azurin, ACu^{I}, with $Co(4,7\text{-DPSphen})_3^{3-}$ is independent of pH in the range 6.3–9.0, the reactions with other oxidants $Co(phen)_3^{3+}$ and $Fe(CN)_6^{3-}$ are pH dependent, exhibiting behavior consistent with protonation at or near to the site on the protein at which the complex binds, i.e., associates. The oxidant $Co(phen)_3^{3+}$ binds at a site on ACu^{I} with a pK_a of 7.6, whereas $Fe(CN)_6^{3-}$ binds at a site with a pK_a of 7.1, which is shifted to $pK_a = 6.1$ for the reaction of ACu^{II} with $Fe(CN)_6^{4-}$. The sites are probably close to the two histidine groups of the azurin which are not bound to the copper. Protein–complex association (K) prior to electron transfer (k_{et}) has been observed in the reactions of $Co(phen)_3^{3+}$, $Co(4,7\text{-DPSphen})_3^{3-}$, and $Fe(CN)_6^{3-}$ with ACu^{I} and in the reaction of ACu^{II} with $Fe(CN)_6^{4-}$, the latter two reactions being less extensively studied here because of the existence of previous data. For $Co(phen)_3^{3+}$, pH 9.1, $I = 0.10$ M (NaCl), the association constant K (25 °C) = 457 M^{-1}, $\Delta H^\circ = -1.2$ kcal mol^{-1}, $\Delta S^\circ = 8.1$ cal K^{-1} mol^{-1}, and k_{et} (25 °C) = 21.3 s^{-1}, $\Delta H^{\ddagger} = 14.3$ kcal mol^{-1}, $\Delta S^{\ddagger} = -4.5$ cal K^{-1} mol^{-1}. For $Co(4,7\text{-DPSphen})_3^{3-}$, pH 7.0, $I = 0.10$ M (NaCl), K (25 °C) = 2750 M^{-1}, $\Delta H^\circ = -3.7$ kcal mol^{-1}, $\Delta S^\circ = 3.3$ cal K^{-1} mol^{-1}, and k_{et} (25 °C) = 0.21 s^{-1}, $\Delta H^{\ddagger} = 10.7$ kcal mol^{-1}, $\Delta S^{\ddagger} = -25.9$ cal K^{-1} mol^{-1}. The reaction of $Fe(CN)_6^{3-}$ with ACu^{I} is unaffected by the presence of $Co(4,7\text{-DPSphen})_3^{3-}$ bound to the protein, confirming that these two reactants use different binding sites. The various data obtained are compared with previous results for similar reactions of parsley plastocyanin.

Azurins are blue copper proteins obtained from certain bacteria, where they apparently function between cytochrome c_{551} and cytochrome oxidase in bacterial electron transport.[1] Information from various sources is consistent with a single Cu atom, utilizing oxidation states I and II, in a protein of molecular weight approximately 16 000 (ca. 130 amino acids) with very little if any associated carbohydrate.[2] A number of properties are similar to those of the plastocyanins, and the recent X-ray crystal structure of *Pseudomonas aeruginosa* azurin (3.0-Å resolution)[3a] has indicated that the Cu(II) coordination site is identical with that of popular plastocyanin, PCu^{II}.[3b] Structural homologues of the azurins and plastocyanins have been noted by Freeman et al.[3b]

Gray and colleagues have previously studied reactions of *Pseudomonas aeruginosa* azurin, ACu^{I}, with $Co(phen)_3^{3+}$, $Co(4,7\text{-DPSphen})_3^{3-}$,[4] and $Ru(NH_3)_5py^{3+}$ [5] and of ACu^{II} with Fe(edta)$^{2-}$.[6] Goldberg and Pecht[7] have studied the kinetics of the equilibration of ACu^{II}/ACu^{I} with $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. We report here studies on the reactions of ACu^{I} with $Co(phen)_3^{3+}$, $Co(4,7\text{-DPSphen})_3^{3-}$, and $Fe(CN)_6^{3-}$, and of ACu^{II} with $Fe(CN)_6^{4-}$. Our results are in agreement with those of the previous investigations, but we have been able to demonstrate that protein–complex association occurs prior to electron transfer in all these reactions, and that there are significant pH effects which give information about the nature and location of the binding sites.

Experimental Section

Azurin, extracted from a culture of *Pseudomonas aeruginosa*[8] and purified by standard chromatographic column techniques, was obtained (ca. 5×10^{-4} M) in ammonium acetate buffer from Microbiological Products. The absorbance peak ratio of A_{280}/A_{625} for the oxidized form was 1.67 (literature value 1.72[9]). The broad absorption band at 625 nm for the Cu(II) form, characteristic of the blue Cu center, has an absorption coefficient variously quoted as 5700[7] or 4800 M^{-1} cm^{-1}.[10] The reduced form of the protein, ACu^{I}, was generated by addition of a few crystals of sodium dithionite (G.P.R. grade BDH) representing an excess of reductant. Protein solutions were dialyzed (21-nm diameter sacks, Sigma) for at least 30 h at 0 °C.

The preparations of complexes tris(1,10-phenanthroline)cobalt(III) chloride, $[Co(phen)_3]Cl_3 \cdot 7H_2O$, sodium tris[4,7-di(phenyl-4′-sulfonate)-1,10-phenanthroline]cobalt(III), $Na_3[Co(4,7\text{-DPSphen})_3]$, and sources of $K_3[Fe(CN)_4]$ and $K_4[Fe(CN)_6] \cdot 3H_2O$ have been referred to previously.[11,12] Buffers used have also been described. In addition sodium borate (Analar, BDH) was used in this work for studies at pHs of around 9.

Buffer (1×10^{-2} M) was present in both reactant solutions, where the pH was adjusted to the required value ca. 30 min before mixing. For experiments in which the pH was varied, 2×10^{-2} M buffer at the required pH was present in the oxidant solution and the protein solution contained 1×10^{-3} M buffer at pH ca. 7. For runs at pH ca. 9 protein solutions in 1×10^{-3} M phosphate buffer, pH ca. 7, were mixed with a solution of the oxidant containing 2×10^{-2} M borate buffer at pH 9. The pH of all solutions was measured after mixing the reactants. Ionic strengths were adjusted to 0.10 M using NaCl. The procedure in kinetic experiments was as outlined previously,[11,12] where absorbance changes were monitored at the 625-nm peak for ACu^{II}. Concentrations of inorganic complex were in large (>tenfold) excess of the protein. Protein concentrations were in the range $(0.3\text{–}1.0) \times 10^{-5}$ M.

Treatment of Data. A nonlinear least-squares program[13] and subroutines were used throughout. Weighting factors were equivalent to $1/y$ for slower (and on stopped-flow time scales less accurate) reactions and to $1/y^2$ in all other cases.

Results

Oxidation of ACu^{I} with $Co(phen)_3^{3+}$. Second-order rate constants k, Table I,[14] determined with $[Co(phen)_3^{3+}]$ at a relatively low value in the range $(1\text{–}2) \times 10^{-4}$ M, gave a dependence on pH as illustrated in Figure 1. In order to cover the required pH range it was necessary to use three buffers, where results in different buffers are in general agreement with each

† No reprints available.

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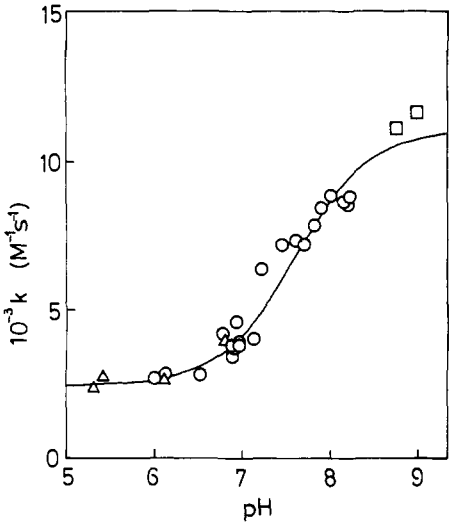

Figure 1. The variation of second-order rate constants, k, with pH for the oxidation of *Pseudomonas aeruginosa* azurin, ACu^{I} (ca. 10^{-5} M), with $Co(phen)_3^{3+}$ at 25 °C, I = 0.10 M (NaCl), 10^{-2} M phosphate (○), borate (□). and cacodylate (△) buffers.

other. The dependence was analyzed in terms of

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

where the constants are as defined in the equations

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

$$ACu^{I} + \text{oxidant} \xrightarrow{k_0} \text{products} \quad (3)$$

$$ACu^{I}H + \text{oxidant} \xrightarrow{k_H} \text{products} \quad (4)$$

A least-squares fit of data to (1) gives $k_0 = (11.1 \pm 0.6) \times 10^3$ M^{-1} s^{-1}, $k_H = (2.43 \pm 0.12) \times 10^3$ M^{-1} s^{-1}, and $K_H = (4.0 \pm 0.7) \times 10^7$ M^{-1} (corresponding to an acid dissociation pK_a of 7.6 ± 0.2) at 25 °C.

At pH 6.9, the dependence of k_{obsd}, Table II,[14] on $[Co(phen)_3^{3+}]$ is linear even at relatively high concentrations (ca. 4×10^{-3} M), Figure 2. However at pH 9.1 (Table III[14]) curvature is observed, Figure 2, consistent with protein-complex adduct formation (5) prior to electron transfer (6).

$$ACu^{I} + \text{oxidant} \overset{K}{\rightleftharpoons} ACu^{I}, \text{oxidant} \quad (5)$$

$$ACu^{I}, \text{oxidant} \xrightarrow{k_{et}} \text{products} \quad (6)$$

For this reaction sequence the equation

$$k_{obsd} = \frac{Kk_{et}[\text{oxidant}]}{1 + K[\text{oxidant}]} \quad (7)$$

can be derived, and plots of $(k_{obsd})^{-1}$ against $[Co(phen)_3^{3+}]^-$ for data, Table III, at pH 9.1 are linear, Figure 3. These give K (25 °C) = 457 M^{-1}, $\Delta H°$ = −1.2 ± 3.9 kcal mol^{-1}, $\Delta S°$ = 8.1 ± 13.7 cal K^{-1}, k_{et} (25 °C) = 21.3 s^{-1}, $\Delta H^{\ddagger}_{et}$ = 14.3 ± 2.5 kcal mol^{-1}, $\Delta S^{\ddagger}_{et}$ = −4.5 ± 8.8 cal K^{-1} mol^{-1}. From the data at pH 6.9 (absence of curvature in Figure 2) it is concluded that $K < 40$ M^{-1}.

Oxidation of ACu^{I} with $Co(4,7\text{-DPSphen})_3^{3-}$. First-order rate constants k_{obsd} for the oxidation of ACu^{I} (ca. 10^{-5} M) with $Co(4,7\text{-DPSphen})_3^{3-}$ (2.4×10^{-4} M) at 25 °C, I = 0.10 M (NaCl), exhibit little or no dependence (±8%) on pH over the range studied, pH 6.3-9.0, using phosphate, cacodylate, and borate buffers (10^{-2} M), Table IV.[14] Because it is difficult

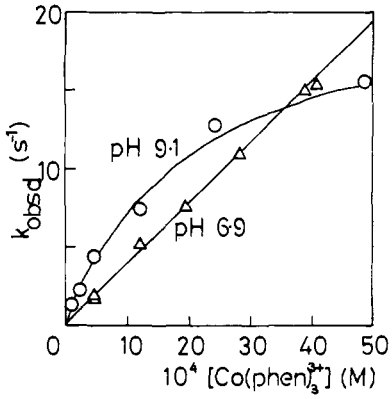

Figure 2. The variation of first-order rate constants, k_{obsd}, for the oxidation of *Pseudomonas aeruginosa*, ACu^{I} (ca. 10^{-5} M), with $Co(phen)_3^{3+}$ on concentration of $Co(phen)_3^{3+}$ at 25 °C, pH 6.9 ± 0.2 (10^{-2} M phosphate buffer) and 9.1 ± 0.1 (10^{-2} M borate buffer), I = 0.10 M.

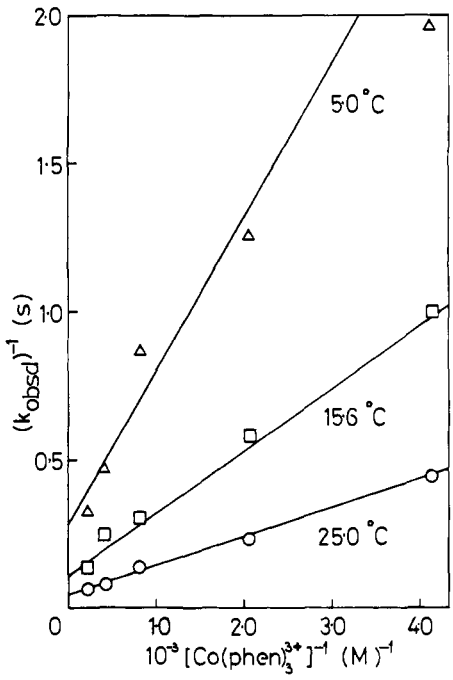

Figure 3. The linear dependence of reciprocal first-order rate constants, k_{obsd}, on $[Co(phen)_3^{3+}]^-$ for the oxidation of *Pseudomonas aeruginosa* azurin, ACu^{I} (ca. 10^{-5} M), with $Co(phen)_3^{3+}$, pH 9.1 ± 0.1 with 10^{-2} M borate buffer, I = 0.10 M (NaCl).

to study the reaction under second-order conditions (see evidence below for strong protein-complex association), first-order rate constants (k_{obsd}) are listed, where all runs were at an initial $Co(4,7\text{-DPSphen})_3^{3-}$ concentration of 2.4×10^{-4} M. The nonlinear dependence of k_{obsd} (Table V)[14] on $[Co(4,7\text{-DPSphen})_3^{3-}]$ at pH 7.1 is illustrated in Figure 4. Plots of $(k_{obsd})^{-1}$ against $[Co(4,7\text{-DPSphen})_3^{3-}]^{-1}$ are linear, consistent with the reaction sequence (5)-(6) involving association. From the temperature dependence K (25 °C) = 2750 M^{-1}, $\Delta H°$ = −3.7 ± 2.2 kcal mol^{-1}, $\Delta S°$ = 3.3 ± 7.6 cal K^{-1} mol^{-1}, k_{et} (25 °C) = 0.21 s^{-1}, $\Delta H^{\ddagger}_{et}$ = 10.7 ± 1.6 kcal mol^{-1}, and $\Delta S^{\ddagger}_{et}$ = −25.9 ± 5.6 cal K^{-1} mol^{-1}.

Oxidation of ACu^{I} with $Fe(CN)_6^{3-}$. The dependence of second-order rate constants k, Table VI,[14] on pH is shown in Figure 5. At I = 0.10 M (NaCl) a fit of data to (1) gives at 25 °C k_0 = (0.49 ± 0.016) × 10^4 M^{-1} s^{-1}, k_H = (1.82 ± 0.07) × 10^4 M^{-1} s^{-1}, K_H = (1.15 ± 0.18) × 10^7 M^{-1} (corresponding to a pK_a of 7.06 ± 0.16). The reaction is unaffected by the presence of $Co(4,7\text{-DPSphen})_3^{3-}$, which binds strongly to the protein but reacts much more slowly than $Fe(CN)_6^{3-}$. For runs

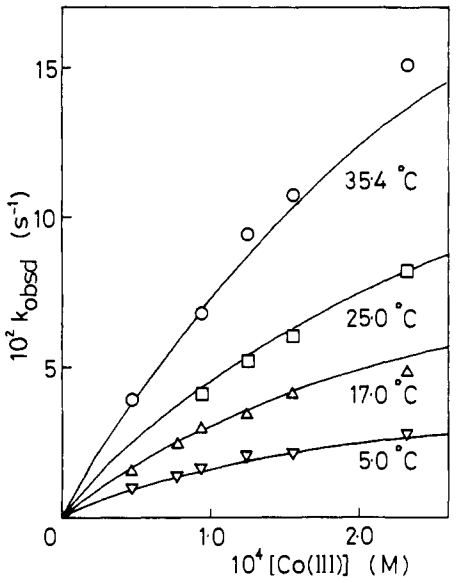

Figure 4. The variation of first-order rate constants, k_{obsd}, for the oxidation of *Pseudomonas aeruginosa*, ACu^{I} (ca. 10^{-5} M), with $Co(4,7\text{-}DPSphen)_3^{3-}$ on concentration of oxidant, pH 7.1 ± 0.1, I = 0.10 M (NaCl), 10^{-2} M phosphate buffer.

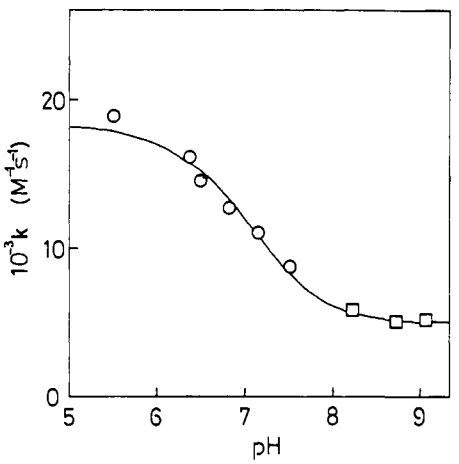

Figure 5. The variation of second-order rate constants, k, with pH for the oxidation of *Pseudomonas aeruginosa* azurin, ACu^{I} (ca. 10^{-5} M), with $Fe(CN)_6^{3-}$ (1.03×10^{-4} M), I = 0.10 M (NaCl), 10^{-2} M phosphate (○) and borate (□) buffers.

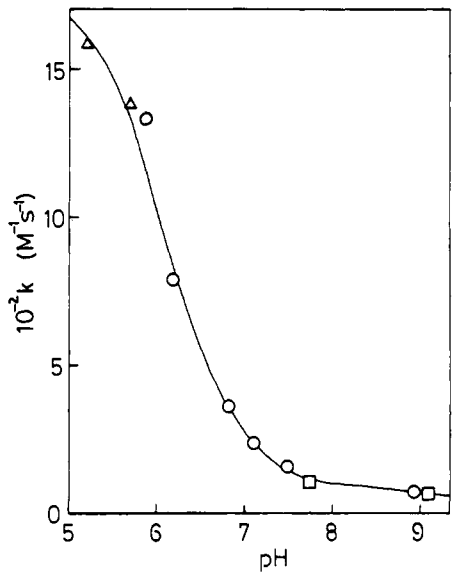

Figure 6. The variation of second-order rate constants, k, with pH for the reduction of *Pseudomonas aeruginosa* azurin, ACu^{II} (ca. 10^{-5} M), with $Fe(CN)_6^{4-}$ (1.0×10^{-3} M) at 25 °C, I = 0.10 M (NaCl), 10^{-2} M phosphate (○), borate (□), and cacodylate (△) buffers.

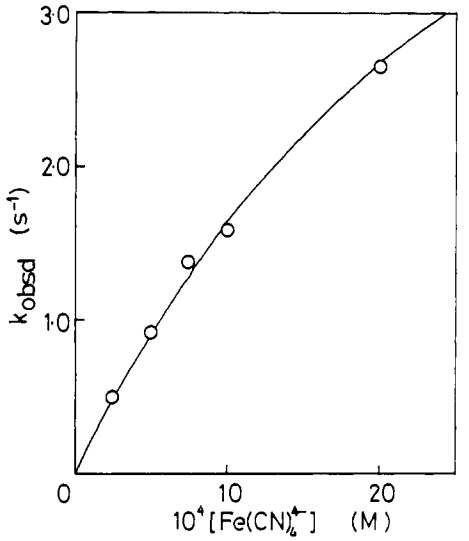

Figure 7. The variation of first-order rate constants, k_{obsd}, with concentration of $Fe(CN)_6^{4-}$ for the reduction of *Pseudomonas aeruginosa* azurin, $ACu(II)$ (ca. 10^{-5} M), with $Fe(CN)_6^{4-}$ at pH 5.2 ± 0.1, I = 0.10 M (NaCl), 10^{-2} M cacodylate buffer.

at 25 °C, pH 7.15 (10^{-2} M phosphate), with $[Fe(CN)_6^{3-}] = 1.0 \times 10^{-4}$ M, $10^4[Co(4,7\text{-}DPSphen)_3^{3-}] = 0, 0.78, 1.56, 3.11, 3.67$, and 5.42 rate constants $10^{-4}k$ were 0.97, 0.97, 1.00, 1.02, 1.01, and 1.08 M^{-1} s^{-1}, respectively.

Reduction of ACu^{II} with $Fe(CN)_6^{4-}$. Although slightly unfavorable thermodynamically, this reaction could be studied by having the reductant in sufficient excess for reaction to proceed essentially to completion (always >90%). Second-order rate constants k (Table VII)[14] exhibit a dependence on pH, Figure 6. It was demonstrated that contributions from the term $K[Fe(CN)_6^{4-}]$ corresponding to protein-complex association, eq 5, of up to 30% at pH 5.2 (see below) have little effect on the pK_a determination. A fit to (1) using the data in Table VII gives $k_0 = 65.7 \pm 4.1$ M^{-1} s^{-1}, $k_H = (1.80 \pm 0.14) \times 10^3$ M^{-1} s^{-1}, $pK_a = 6.12 \pm 0.14$. If the data are corrected for protein-complex association making the assumption that the change in k_{obsd} is mainly determined by the change in K and not k_{et}, which is essentially invariant (an assumption suggested by the similarity of k_{et} reported in this paragraph with the value of Goldberg and Pecht[7]), then $k_H = 2.38 \times 10^3$ M^{-1} s^{-1} and $pK_a = 6.01$. First-order rate constants k_{obsd} (Table VIII) at pH 5.2 give a nonlinear dependence on $[Fe(CN)_6^{4-}]$, Figure 7, where from a plot of $(k_{obsd})^{-1}$ against $[Fe(CN)_6^{4-}]^{-1}$ values (25 °C) $K = 290 \pm 20$ M^{-1} and $k_{et} = 7.3 \pm 0.4$ s^{-1} are obtained.

Discussion

Rate constants at pH 7 are in good agreement with those obtained previously by McArdle et al.[4] for the oxidation of ACu^{I} with $Co(phen)_3^{3+}$ and $Co(4,7\text{-}DPSphen)_3^{3-}$, and by Goldberg and Pecht[7] for the reactions of ACu^{I} and ACu^{II} with $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$, respectively, bearing in mind that different ionic strengths were used. No pH variations were reported in these earlier investigations, however, and it is this aspect of our studies, and the implications which such variations carry with regard to the nature of binding sites, which we wish to emphasize. In addition, protein-complex association has been detected for the ACu^{I} reaction with $Co(phen)_3^{3+}$ at pH values above the acid dissociation, $pK_a = 7.6$. This asso-

Table X. Comparison of Association Constants (K) and Thermodynamic Parameters $\Delta H°$ and $\Delta S°$ for the Reactions of *Pseudomonas aeruginosa* Azurin and Parsley Plastocyanin with Inorganic Complexes, I = 0.10 M (NaCl), Except as Stated

reaction	pH	K (25 °C), M^{-1}	$\Delta H°$, kcal mol^{-1}	$\Delta S°$, cal K^{-1} mol^{-1}	conditions/ ref
ACu^{I} + $Co(phen)_3^{3+}$	9.1	457	−1.2	8.1	this work
	7.0	<40			this work
PCu^{I} + $Co(phen)_3^{3+}$	7.5	167	10	45	11
	6.5	92			12
	5.5	<50			12
ACu^{I} + $Co(4,7\text{-DPSphen})_3^{3-}$	6.3–9.0	2750	−3.7	3.3	this work
PCu^{I} + $Co(4,7\text{-DPSphen})_3^{3-}$	5.2–7.5	4600	−4.2	2.7	12
ACu^{I} + $Fe(CN)_6^{3-}$	9.1	<120			this work
	7.0	610	−7.7	−13.1	I = 0.22 M, ref 7
	5.0	large (?)			this work
PCu^{I} + $Fe(CN)_6^{3-}$	7.0	≤360			11
	5.2	≤200			12
$Fe(CN)_6^{4-}$ + ACu^{II}	9.1	small (?)			this work
	7.0	54	−5.5	−10.5	I = 0.22 M, ref 7
	5.2	290			this work
$Fe(CN)_6^{4-}$ + PCu^{II}	7.0	110	−5.1	−7.8	11
	5.0	large (?)			11

ciation could not be detected at pH 6.9. The results in Figure 2 demonstrate that, as K decreases on going from pH 9.1 to 6.9, k_{et} (the limiting rate constant at high oxidant concentrations) increases. In contrast, available data (this work and ref 7) suggest that in the reactions of azurin with $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ the trend in K values with pH is in the opposite direction.

The isoelectric point for *Pseudomonas aeruginosa* azurin is at pH 5.4,[15] indicating a negative overall charge on the protein at higher pHs. Estimates of the charge at pH 7 from the amino acid composition give an approximate value of −1.[16] The effects of $[H^+]$ observed in the present studies indicate changes in this overall charge, which, however, can be assumed to remain small. Interestingly, the trends observed in all three pH profiles, Figures 1, 5, and 6 (where variations in K are a major contributing factor), are consistent with the influence which the 1+ charge of an H^+ will have on the electrostatics. The apparent charge on the protein must be small for a single H^+ to have so much influence.

The pK_a values obtained from the pH dependences are now considered, and their implications with regard to defining different binding sites on the protein. With $Co(phen)_3^{3+}$ as oxidant the kinetics (Figure 1) give a pK_a of 7.6, whereas with $Fe(CN)_6^{3-}$ (Figure 5) the pK_a is 7.1. *Pseudomonas aeruginosa* azurin is known to contain four histidines.[16] From NMR studies on ACu^{I} one of the histidines titrates with a pK_a of 7.57 and a second with a pK_a of ca. 7.[18,19] The two other histidines protonate at much lower pH values because they are coordinated to the Cu.[20] A small shift in pK_a of the first histidine from 7.57 to 7.35 has been observed on oxidation to ACu^{II}, and Ugurbil et al.[18] have suggested that this histidine is far removed from the Cu site. The second histidine, although not coordinated to the Cu, is near enough for it to be affected by the change in oxidation state of the copper, although no pK_a value for ACu^{II} was reported from the NMR studies.

The pK_a values for ACu^{I} determined by NMR are sufficiently close to those detected in the kinetics as to suggest involvement of the same histidines (for the free amino acids only histidine titrates at a pH of around 7). Thus $Co(phen)_3^{3+}$ binds at a site on the protein close to the histidine with a pK_a = 7.6; the reaction is sensitive as to whether this group is protonated or not, and the $Fe(CN)_6^{3-}$ utilizes a site influenced by the histidine with a pK_a = 7.1. The reactants $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ must use the same site on the protein (microscopic reversibility). The kinetics show that the pK_a of 7.1 for ACu^{I} shifts to 6.1 for ACu^{II}, consistent with a histidine close to the Cu site. The X-ray crystal structure[3a] has indicated the positions of Hist-35 and Hist-83, both of which appear close to but not actually on the surface of the protein. Present information is that the former is significantly nearer to the Cu, which suggests that $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ use a binding site influenced by Hist-35, whereas $Co(phen)_3^{3+}$ uses another site influenced by Hist-83.[21] Assuming that the protein retains the same shape in solution, and that the complexes do not penetrate the peptide sheath, then electron transfer could well be occurring over quite large distances. Some caution must be exercised, however, since further resolution of the crystal structure is desirable, and there is a danger of overinterpreting existing data.

A different binding site is indicated for the oxidation of ACu^{I} with $Co(4,7\text{-DPSphen})_3^{3-}$ since little or no dependence on pH is observed. This is supported by the observation that under conditions when as much as 60% of ACu^{I} is bound to $Co(4,7\text{-DPSphen})_3^{3-}$ (which oxidizes the protein only slowly) the reaction with $Fe(CN)_6^{3-}$ continues unimpeded. The special nature of the 4,7-DPSphen ligand (most probably its hydrophobic nature) requires this complex to use a different site on the protein. A similar conclusion was reached for the corresponding oxidations of parsley PCu^{I} [12]

The rate constants for the reactions of ACu^{I} with $Co(phen)_3^{3+}$ do not appear to respond in any way to the pK_a of 7.1 observed for the $Fe(CN)_6^{3-}$ reaction, and vice versa for the pK_a of 7.6. Since the overall charge on the azurin is small and removal of a proton creates an appreciable change in this parameter, clearly reactions are little influenced by overall charge but are susceptible to changes in local binding site charge. An alternative interpretation is that protonation induces some local structural change which affects only those reactants using that particular binding site.

Kinetic parameters, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, corresponding to overall rate constants k, Table IX, are seen to be very similar for like reactions of *Pseudomonas aeruginosa* azurin and parsley plastocyanin. With one very notable exception this similarity holds when parameters corresponding to K for associations (Table X) and k_{et} for electron transfer (Table XI) are compared. The exception is with $Co(phen)_3^{3+}$ as oxidant. The association step for the $Co(phen)_3^{3+}$ oxidation of ACu^{I} at pH 9.1 gives a value for $\Delta S°$ of 8 cal K^{-1} mol^{-1}, which indicates that electrostatics play a minor role compared to the corresponding reaction of parsley plastocyanin, which has a much larger $\Delta S°$ (45 cal K^{-1} mol^{-1}).[11] Parsley plastocyanin has a higher overall negative charge (estimated to be −7)[12] than azurin, and, since local binding site charges are now believed to be more relevant, the local charge on plastocyanin must also

Table XI. Comparison of Rate Constants (k_{et}) and Activation Parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ for the Reaction of *Pseudomonas Aeruginosa* Azurin and Parsley Plastocyanin with Inorganic Complexes, I = 0.10 M (NaCl), Except as Stated

reaction	pH	k_{et}, s^{-1}	$\Delta H^{\ddagger}_{et}$, kcal mol^{-1}	$\Delta S^{\ddagger}_{et}$, cal K^{-1} mol^{-1}	conditions/ ref
ACu^{I} + $Co(phen)_3^{3+}$	9.1	21.3	14.3	−4.5	this work
PCu^{I} + $Co(phen)_3^{3+}$	7.5	17.9	4.3	−39.0	11
ACu^{I} + $Co(4,7\text{-DPSphen})_3^{3-}$	6.3–9.0	0.21	10.7	−25.9	this work
PCu^{I} + $Co(4,7\text{-DPSphen})_3^{3-}$	5.2–7.5	0.041	13.2	−20.6	12
ACu^{I} + $Fe(CN)_6^{3-}$	7.0	45	3.6	−38.9	I = 0.22 M, ref 7
PCu^{I} + $Fe(CN)_6^{3-}$	7.0	270			11
$Fe(CN)_6^{4-}$ + ACu^{II}	7.0	6.4	11.4	−16.6	I = 0.22 M, ref 7
$Fe(CN)_6^{4-}$ + PCu^{II}	7.0	170	11.4	−9.7	11

be more negative for this reaction. Activation parameters corresponding to k_{et} for the $Co(phen)_3^{3+}$ oxidation of ACu^{I} at pH 9.1, Table XI, suggest that a different pathway is operating compared to that proposed for the plastocyanin reaction. The more positive $\Delta S^{\ddagger}_{et}$ value is indicative of electron transfer through a more compact assembly. What is remarkable (and presumably a coincidence) is that the different K and k_{et} values for plastocyanin and azurin should mutually compensate to give such similar overall parameters.

The possibility has been considered that, as the pH varies and protonation occurs at one site on azurin, completely inhibiting the reaction, the oxidant $Co(phen)_3^{3+}$ reverts to an alternative site which is not similarly affected by pH. It would appear that no such duality of binding sites occurs in this case since the magnitude of rate constants at the different pH values inverts with changing oxidant concentration, Figure 2. Unless the single proton simultaneously activates one site while deactivating the other, reaction at a single site is implied. Therefore one thinks in terms of removal of a proton at pH 7.6 which makes the $Co(phen)_3^{3+}$ bind more strongly at the same site, but makes the electron-transfer process (k_{et}) slower. Whether the latter effect is due to a decrease in conjugation or π overlap or some other reason remains to be seen.

Similar parameters have been obtained, Tables IX–XI, for the reactions of $Co(4,7\text{-DPSphen})_3^{3-}$ and $Fe(CN)_6^{3-,4-}$ with azurin and plastocyanin. Specific interactions have been suggested previously for the plastocyanin reactions,[11,12] and we see no reason to modify this view in the light of the azurin data. One factor which must be emphasized, however, is the fundamentally different response of the ACu^{I} and PCu^{I} oxidations with $Fe(CN)_6^{3-}$ to pH. In the case of PCu^{I} protonation induces a dramatic switch-off in reactivity ($K \rightarrow 0$) suggesting a possible conformation change, whereas with ACu^{I} there is an increase in rate consistent with the influence of the 1+ charge of H^+ on the electrostatics.

A further intriguing aspect of this and the plastocyanin studies is that experimentally determined reduction potentials for a protein, which are generally determined against an inorganic redox couple, include protein-complex association constants. They will therefore vary with pH and identity of the redox couple used. Assuming a reduction potential of 410 mV for the $Fe(CN)_6^{3-,4-}$ couple,[22] overall rate constants for the $Fe(CN)_6^{3-,4-}$ reactions give reduction potentials which vary from 360 (pH 5.0) to 320 (pH 7.0) and 300 mV at pH 9.1. If, on the other hand, it were possible to determine the reduction potential using the $Co(4,7\text{-DPSphen})_3^{3-,4-}$ couple (340 mV),[4] the potential of the azurin would probably be invariant with pH. With the $Co(phen)_3^{3+,2+}$ couple it is conceivable that either an increase or decrease in potential with pH could result depending on the concentration of cobalt used (Figure 2). In this context we note that the potential of laccase measured in the presence of $Fe(CN)_6^{3-,4-}$ was found to be dependent on the concentration of the complexes used.[23] It is hardly surprising that the Marcus theory applied to protein-complex reactions (and making use of such reduction potentials) gives varying protein self-exchange rate constants.[5]

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

References and Notes

(1) J. E. Coleman, *Prog. Bioorg. Chem.*, **1**, 159 (1971).
(2) J. A. Fee, *Struct. Bonding (Berlin)*, **23**, 1 (1975).
(3) (a) E. T. Adman, R. E. Stenkamp, L. C. Sieker, and L. H. Jensen, *J. Mol. Biol.*, **123**, 35 1978; (b) P. M. Colman, H. C. Freeman, J. M. Guss, M. Murata, V. A. Norris, J. A. M. Ramshaw, and M. P. Venkatappa, *Nature (London)*, **272**, 319 (1978).
(4) J. V. McArdle, C. L. Coyle, H. B. Gray, G. S. Yoneda, and R. A. Holwerda, *J. Am. Chem. Soc.*, **99**, 2483 (1977).
(5) D. Cummins and H. B. Gray, *J. Am. Chem. Soc.*, **99**, 5158 (1977).
(6) R. C. Rosenberg, S. Wherland, R. A. Holwerda, and H. B. Gray, *J. Am. Chem. Soc.*, **98**, 6364 (1976).
(7) M. Goldberg and I. Pecht, *Biochemistry*, **15**, 4197 (1976).
(8) R. P. Ambler and M. Wynn, *Biochemistry*, **17**, 485 (1978).
(9) K. Ugurbil, R. S. Norton, A. Allerhand, and R. Bersohn, *Biochemistry*, **16**, 886 (1977).
(10) D. L. Tennent and D. R. McMillin, personal communication.
(11) M. G. Segal and A. G. Sykes, *J. Am. Chem. Soc.*, **100**, 4585 (1978).
(12) A. G. Lappin, M. G. Segal, D. C. Weatherburn, and A. G. Sykes, *J. Am. Chem. Soc.*, submitted.
(13) R. H. Moore and R. K. Zeigler, Los Alamos Report LA 2367, 1959, and addenda.
(14) See paragraph at end of paper regarding supplementary material.
(15) T. Horio, I. Sekuzu, T. Higashi, and K. Okunuki, "Haematin Enzymes", IUB Symposium 19, 1959, p 302, quoted in ref 2.
(16) Approximate protein charges can be calculated for a pH of ca. 7 assuming that amino acid residues aspartate and glutamate contribute 1− and that lysine arglnine and uncoordinated histidine are 1+. Such a summation takes no account of hydrogen bonding within the protein and the possibility that protons are shared.
(17) R. P. Ambler, "Recent Developments in the Chemical Study of Protein Structure", A.J.-F. Previers and M.-A. Colette-Previers, Eds., Paris, 1971, p 289. See also L. Ryden and J. Lundgren, *Nature (London)*, **261**, 344 (1976).
(18) K. Ugurbil and R. Bersohn, *Biochemistry*, **16**, 3016 (1977).
(19) H. A. O. Hill, J. C. Leer, B. E. Smith, C. B. Storm, and R. P. Ambler, *Biochem. Biophys. Res. Commun.*, **70**, 331 (1976).
(20) E. L. Ulrich and J. L. Markley, *Coord. Chem. Rev.*, in press.
(21) A. G. Lappin, M. G. Segal, D. C. Weatherburn, and A. G. Sykes, *J. Chem. Soc., Chem. Commun.*, 38 (1979).
(22) I. M. Kolthoff and W. J. Tomsicek, *J. Phys. Chem.*, **39**, 945 (1935).
(23) B. Reinhammer, *Biochim. Biophys. Acta*, **275**, 245 (1972).