# Reactivity of Pseudoazurin from *Achromobacter cycloclastes* with Inorganic Redox Partners and Related NMR and Electrochemical Studies

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The effect of pH on rate constants (25 °C) for the $[Co(dipic)_2]^-$ and $[Co(phen)_3]^{3+}$ oxidations of pseudoazurin pACu[1] have been studied in the pH range 3.5–8.7. From the trends observed (decrease in rate constants with decreasing pH) two $pK_a$'s are obtained, and from $^1H$ NMR these are confirmed as being associated with an active site His protonation/deprotonation, $pK_a = 4.84$ (average), and a protonation/deprotonation of the uncoordinated His6, $pK_a = 7.21$ (average). Alongside plastocyanin and amicyanin, pseudoazurin therefore provides a third example of a type 1 protein the Cu(I) state of which exhibits an active site protonation in the accessible pH range. The spacings of the active site His, Cys, and Met coordinated residues are noted and appear to relate to the magnitude of the $pK_a$ values observed. The rate constant for the oxidation with $[Fe(CN)_6]^{3-}$ is $>2.6 \times 10^6$ $M^{-1}$ $s^{-1}$ at pH 5.5, yielding an exceptionally high $k_{Fe}/k_{Co}$ ratio of $>7.8 \times 10^3$ at this pH, where $k_{Co}$ is for the $[Co(phen)_3]^{3+}$ oxidation. The effect of a number of conserved basic residues Lys38, Lys46, Lys57, and Lys77 (the last adjacent to the active site Cys78) on reactivity is discussed. The presence of these basic residues explains the relatively small value of $2.9 \times 10^3$ $M^{-1}$ $s^{-1}$ for the electron self-exchange rate constant at 25 °C, $I = 0.100$ M.

## Introduction

Pseudoazurin ($M_r \sim 14\,000$) is a single type 1 blue copper protein found in denitrifying bacteria and methylotrophs.[1-8] Pseudoazurins from *Alcaligenes faecalis* S-6,[6,9] *Achromobacter cycloclastes* IAM 1013,[4,5,10] and *Pseudomonas* AMI[7] have been characterized. From their amino acid sequences,[7,9,10] it is known that *A. cycloclastes* pseudoazurin has an extra C-terminal residue giving, 124 amino acids in total. The degree of homology is greatest between *A. cycloclastes* and *A. faecalis* S-6 pseudoazurins with 65% conservation of amino acid residues. If the *Pseudomonas* AMI sequence is included, this degree of homology decreases to 42%. The isoelectric point (pI) of *A. cycloclastes* pseudoazurin is 8.4, and the estimated charge (from the amino acid composition with Asp and Glu as −1, Lys and Arg as +1, and the uncoordinated His as zero charge) for the oxidized protein is +1 at pH ~7.0.

The three-dimensional structure of pseudoazurin from *A. faecalis* S-6 has been determined.[11-13] Like all other known type 1 blue copper protein structures, pseudoazurin has a β-barrel shape made up of two β-sheets. The folding pattern of pseudoazurin is similar to those of plastocyanin and azurin, but there seems to be a greater similarity to the former.[11] However pseudoazurin does not possess an acidic patch as in the case of the plastocyanins.

The copper at the active site of pseudoazurin is coordinated by His40, Cys78, His81, and Met86 in a distorted tetrahedral arrangement. The Cu–$S^\delta$ (Met86) bond is shorter (2.76 Å)[12] than the corresponding bonds in plastocyanin[14] and azurin.[15] As a result of this closer axial approach, the copper atom lies further out of the plane of the three equatorial ligands. This slightly less symmetrical copper environment is thought to be responsible for the X-band EPR spectra of oxidized pseudoazurin and some other type 1 blue copper proteins being rhombic.[16-20] The visible absorption spectrum shows strong peaks at *ca.* 450 and 600 nm,[4-6,8,16,21] which are assigned as Cys → Cu(II) charge-transfer transitions.[17] The increased intensity of the 450-nm peak in pseudoazurin as compared to plastocyanin and azurin is thought to be due to the less symmetrical active site.[17,20] These spectral properties are similar to those of cucumber basic protein,[22] which also has a short Cu–$S^\delta$ (Met86) bond (2.63 Å).[23]

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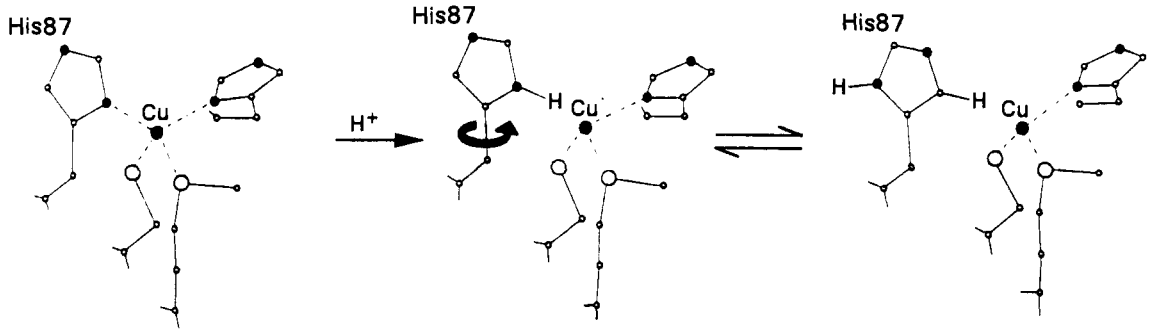


**Figure 1.** $H^+$-induced dissociation of the Cu(I)–N(His87) bond of plastocyanin and the existence of two conformers of the protonated form.

The effect of pH on the reactivity of type 1 blue copper proteins is well documented. In kinetic[24-27] and $^1H$ NMR studies,[28-30] reduced plastocyanin has been shown to participate in an active site protonation/deprotonation equilibrium, which for spinach plastocyanin gives an acid dissociation $pK_a$ of 4.9 from NMR studies[28] and a $pK_a$ of 4.78 from kinetic studies with $[Fe(CN)_6]^{3-}$ as oxidant.[27] The assignment of this active site protonation to His87 is supported by crystallographic data, which show that at low pH values the His87 of reduced plastocyanin protonates, resulting in a trigonally coordinated Cu(I) site (Figure 1).[31] The subsequent reorganization which has to occur for the Cu(I) form of the protein to be oxidized leads to an increase in the reduction potential of plastocyanin. In the case of amicyanin a similar Cu(I) active site protonation effect, involving His96,[32] has been demonstrated to occur. However in this case higher $pK_a$'s of 6.9 from NMR[33] and 6.59 from kinetic studies with $[Co(phen)_3]^{3+}$ [34] are obtained.

It has been noted in $^1H$ NMR studies on amicyanin and spinach plastocyanin that the protonated histidine ligand broadens under certain conditions. This is indicative of two forms of the protonated protein in an intermediate to slow exchange in amicyanin at 600 MHz and at temperatures below 27 °C,[33] whereas in spinach plastocyanin the protonated forms are exchanging at an intermediate rate at 470 MHz.[30]

No other type 1 blue copper protein so far studied has been shown to give a reversible active site protonation in the accessible range of pH. Kinetic studies to date have been carried out on azurin,[35] stellacyanin,[36] umecyanin,[37] and rusticyanin,[38] none of which have provided any evidence for such an effect. It was of interest therefore to consider the behavior of pseudoazurin in an attempt to identify features of type 1 blue copper proteins giving rise to the active site protonation effect. One particular aspect which we highlight is the number of amino acids separating the residues at the active site.

The effect of pH on the redox activity of metalloproteins is not solely confined to changes in geometry at the active site. Protonation/deprotonation can occur close to but not at the active site an can affect the reduction potential of the protein. The effect of pH on the reduction potential of azurins from five different sources has been reported.[39] It is concluded that the trends observed in this case are due to protonation/deprotonation of uncoordinated histidine residues. Other examples are the protonation of His59 in *S. obliquus* plastocyanin,[40] the protonation of His83 in the Tyr83His spinach plastocyanin mutant,[41] and effects seen also for umecyanin[37] and rusticyanin.[38]

The variation of the reduction potential ($E°'$) of pseudoazurin with pH has been demonstrated previously by electrochemical studies using a glassy-carbon electrode.[42a] In the present work we include a more thorough investigation of $E°'$ values of pseudoazurin using a bis(4-pyridyl) disulfide modified gold electrode (4-pyds/Au). We also report the effect of pH on the oxidation of pseudoazurin by inorganic complexes and on the $^1H$ NMR spectrum of pseudoazurin. A communication on this work has appeared.[42b]

## Experimental Section

**Isolation and Purification of *A. cycloclastes* IAM 1013.** The isolation and purification were carried out according to the method of Iwasaki and Matsubara,[4] with some modifications as described previously.[21] Pure pseudoazurin $pACu^{II}$ has a UV–vis absorbance ($A$) peak ratio $A_{277}/A_{593}$ of 1.4/1. Protein used for NMR and kinetic studies was repurified either on a CM52 column at pH 6.0 or on an FPLC Mono-S cation-exchange column under the same conditions. Both methods of purification resulted in pseudoazurin with the required purity ratio. The concentration of protein solutions was determined from the $pACu^{II}$ peak at 593 nm ($\epsilon$ = 3700 $M^{-1}$ $cm^{-1}$).[16]

**Buffers for Kinetics.** Acetate–acetic acid buffer was appropriate for the range pH 3.50–5.10. For the range pH 5.10–6.90 the buffer 2-morpholinoethanesulfonic acid (Mes; Sigma Chemicals), containing added NaOH (BDH, Convol), was used. For pH 7.00–8.70 tris(hydroxymethyl)aminomethane (Tris; Sigma), to which HCl (BDH, Convol) was added, was used. All the above buffers were adjusted to $I$ = 0.100 ± 0.001 M using NaCl.

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**Inorganic Complexes.** These were prepared according to literature methods and characterized from previously reported peak positions $\lambda$/nm ($\epsilon$/M$^{-1}$ cm$^{-1}$) as follows: tris(1,10-phenanthroline) cobalt(III) chloride, $[Co(phen)_3]Cl_3 \cdot 7H_2O$, 330 (4660), 350 (3620), 450 (100);[43] ammonium bis(pyridine-2,6-dicarboxylato)cobaltate(III), $NH_4[Co(dipic)_2] \cdot H_2O$, 510 (630).[44] Potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, 420 nm ($\epsilon$ 1010)[45] (BDH, AnalaR), was used without further purification. Reduction potentials of the relevant 1-equiv redox couples are as follows: $[Co(phen)_3]^{3+/2+}$, 370 mV;[46] $[Co(dipic)_2]^{-/2-}$, 747 mV;[47] $[Fe(CN)_6]^{3-/4-}$, 410 mV.[46]

**Kinetic Studies.** The oxidation of pACu$^{I}$ was monitored at 593 nm on a Dionex D-110 stopped-flow spectrophotometer at 25 °C, $I$ = 0.100 M (NaCl). The stopped-flow was interfaced to an IBM PC/AT-X computer for data acquisitions using software from On-Line Instruments Systems (Bogart, GA). All the rate constants are an average of at least five determinations using the same solutions. Investigation of the pH effects can use large quantities of protein, and so the "pH-jump" method was used as in previous studies.[27,41] In this procedure the protein is in low-concentration (2 mM) buffer, while the inorganic complex is in a controlling 40 mM buffer solution at the required pH. Using this method, one protein solution, e.g. at pH 7.0, can be used for studies in the range pH 6.0–8.0. Results obtained are in good agreement with those from experiments in which both the protein and oxidant were in 20 mM buffer at the same pH.

**NMR Studies.** For the acquisition of proton NMR spectra in the range pH 5.8-9.5, the protein was exchanged into 99.9% deuterated 45 mM phosphate buffer at pH 7.00 ($I$ = 0.100 M). For studies at the lower pH values (pH ≤5.7), the protein was exchanged into 99.9% deuterated 88.5 mM phosphate buffer at pH 5.70 ($I$ = 0.100 M). In both cases, this was achieved by ultrafiltration (Amicon, YM3 membrane). For the preparation of reduced pseudoazurin, protein solutions, typically 1 mM, were transferred to NMR tubes and flushed with argon. The samples were reduced by the addition of cooled aliquots of 0.10 M sodium dithionite ($Na_2S_2O_4$; Fluka) in 99.9% $D_2O$ (0.10 M NaOD). The samples were then flushed again with argon, and the tubes were sealed. Fully oxidized pseudoazurin NMR samples were prepared using a 0.10 M solution of $K_3[Fe(CN)_6]$ in 99.9% $D_2O$. The excess oxidant was exchanged out by ultrafiltration, and protein at the required concentration was transferred to an NMR tube. The pH's of the NMR samples were measured using a narrow CMAWL/3.7/180 pH probe (Russell) in combination with a Radiometer PHM62 pH meter which was calibrated using aqueous buffers. The pH of a protein solution was adjusted immediately prior to the acquisition of an NMR spectrum using NaOD or DCl (0.10 M), and no correction was made for the deuterium isotope effect. The pH of the solution was also measured after acquisition of the NMR data, and the values used are an average of these two readings.

Most proton NMR spectra were acquired at 500.14 MHz on a Bruker AMX500 spectrometer at 25 °C using samples in 5 mm o.d. borosilicate glass tubes. Some spectra were also acquired at 300.13 MHz on a Bruker WM300WB spectrometer. Typically, 512 free induction decays were accumulated into 16K data points and transformed into 32K data points after zero-filling. The residual HDO resonance was suppressed by presaturation at its resonant frequency. All chemical shifts are cited in parts per million (ppm) relative to internal dioxane at $\delta$ 3.74 ppm.

**Electrochemistry.** Cyclic voltammetry (CV) was carried out using a BAS Model CV 50-W voltammetric analyzer (Bioanalytical Systems Inc.) with a Faraday cage. Prior to each experiment, the gold working electrode was polished with an alumina-coated film (3-$\mu$m particle size, Sumitomo 3M) and then sonicated for 1 min and rinsed thoroughly with distilled water. Promoter-modified gold electrodes were obtained by dipping the freshly polished gold electrode into a saturated solution of the bis(4-pyridyl) disulfide promoter. After 15–20 min, the gold electrode was removed and rinsed thoroughly with distilled water.

The working compartment was made air-free by passing argon through the electrochemical cell for 15 min. A single-compartment electrochemical cell was used with an Ag/AgCl reference electrode (Bioanalytical Systems) and a platinum wire counter electrode which was separated from the working solute using a Vycor glass tip. All potentials were corrected to give values vs NHE using the value for the Ag/AgCl reference electrode (+209mV vs NHE at 25 °C).

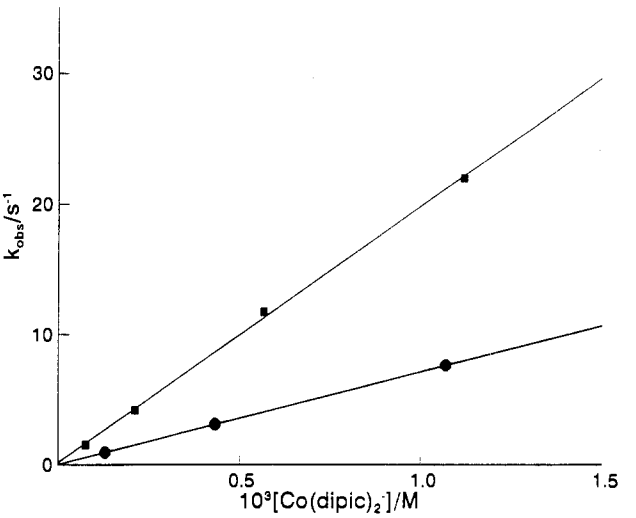


**Figure 2.** Dependence of first-order rate constants (25 °C) on concentration for the oxidation of pACu$^{I}$ by $[Co(dipic)_2]^-$ at pH 7.5 (■) and 5.5 (●), $I$ = 0.100 M (NaCl).

## Results

**Oxidation of pACu$^{I}$.** Linear plots of first-order rate constants, $k_{obs}$ ($s^{-1}$), against oxidant concentrations (1–10) × $10^{-4}$ M $[Co(dipic)_2]^-$ (Figure 2) and (1–20) × $10^{-4}$ M $[Co(phen)_3]^{3+}$ (Figure 3) are consistent with the rate law (1). The dependence

$$\text{rate} = k[\text{pACu}^{I}][\text{oxidant}] \quad (1)$$

of second-order rate constants $k$ on pH showed two protonation effects, and the data were analyzed using eq 2 derived from the reaction scheme in (3). In this scheme P represents reduced

$$k = \frac{k_H + (k_0 - k_H)K_{aH} + (k_1 - k_H)K_{aA}[H^+] - k_H K_{aH}[H^+]}{k_{aA}K_{aH} + K_{aH}[H^+] + K_{aA}[H^+] + [H^+]^2} \quad (2)$$

$$\begin{array}{ccc} H^+ + HP_A & \xrightleftharpoons{K_{aA}} & 2H^+ + P \\ \Big\updownarrow K_{aH} & & K_{aH} \Big\updownarrow \\ H_2P_{AH}^{2+} & \xrightleftharpoons{K_{aA}} & H^+ + HP_H^+ \end{array} \quad (3)$$

pseudoazurin which can protonate at two different sites $HP_A^+$ (active site) and $HP_H^+$ (His6), where $K_{aA}$ and $K_{aH}$ are the acid dissociation constants for these two sites, respectively. It is assumed that protonation at one of these sites does not affect protonation at the other. This assumption and the absence of any specific effects from the oxidant are supported by the agreement of p$K_a$ values obtained from the kinetic data and $^1$H NMR studies. The relevant oxidation steps are as defined in eqs 4–6. Assuming that the rate law (1) applies at all pH values, the

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

$$HP_H^+ + \text{oxidant} \xrightarrow{k_1} \text{products} \quad (5)$$

$$H_2P_{AH}^{2+} + \text{oxidant} \xrightarrow{k_H} \text{products} \quad (6)$$

second-order rate constants of Tables 1 and 2 are obtained for

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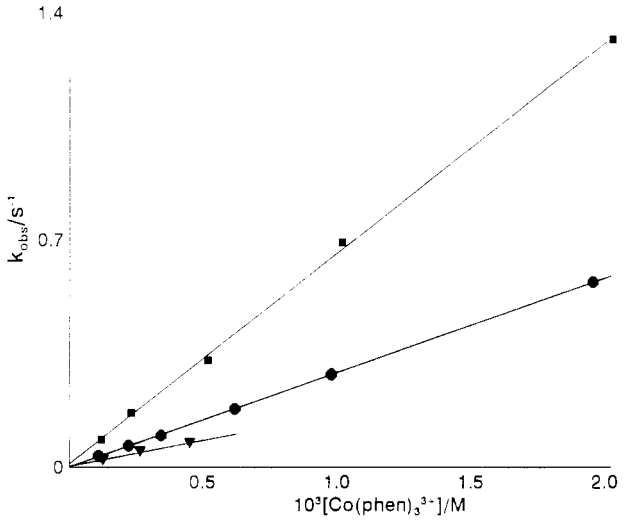


**Figure 3.** Dependence of first-order rate constants (25 °C) on concentration for the oxidation of pACu$^I$ by $[Co(phen)_3]^{3+}$ at pH 7.5 (■), 5.5 (●), and 4.5 (▼), $I$ = 0.100 M (NaCl).

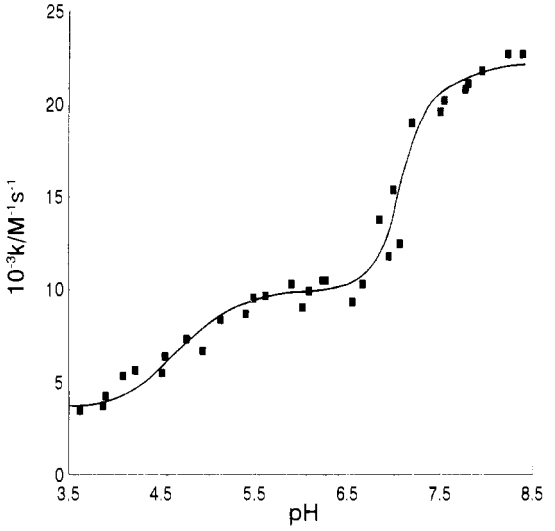


**Figure 4.** Variation of second-order rate constants (25 °C) with pH for the $[Co(dipic)_2]^-$ oxidation of pACu$^I$, $I$ = 0.100 M (NaCl).

**Table 1.** Variation of Second-Order Rate Constants (25 °C) with pH for the Oxidation of pACu$^I$ (~1 × $10^{-5}$ M) by $[Co(dipic)_2]^-$ at (1–10) × $10^{-4}$ M, $I$ = 0.100 M (NaCl)

| pH | $10^{-4}k/M^{-1}s^{-1}$ | pH | $10^{-4}k/M^{-1}s^{-1}$ |
|---|---|---|---|
| 3.62 | 0.34 | 6.23 | 1.05 |
| 3.87 | 0.37 | 6.26 | 1.04 |
| 3.90 | 0.42 | 6.55 | 0.94 |
| 4.09 | 0.53 | 6.66 | 1.03 |
| 4.22 | 0.56 | 6.84 | 1.38 |
| 4.51 | 0.55 | 6.94 | 1.18 |
| 4.54 | 0.64 | 6.99 | 1.54 |
| 4.77 | 0.73 | 7.06 | 1.25 |
| 4.94 | 0.67 | 7.19 | 1.90 |
| 5.13 | 0.84 | 7.50 | 1.96 |
| 5.40 | 0.87 | 7.54 | 2.02 |
| 5.48 | 0.95 | 7.77 | 2.08 |
| 5.61 | 0.97 | 7.80 | 2.11 |
| 5.89 | 1.03 | 7.95 | 2.18 |
| 6.01 | 0.90 | 8.23 | 2.27 |
| 6.08 | 0.99 | 8.39 | 2.27 |

the $[Co(dipic)_2]^-$ and $[Co(phen)_3]^{3+}$ oxidation of pseudoazurin, respectively. The pH dependencies (Figures 4 and 5) indicate two effects, where as mentioned previously that at the lower pH's is due to protonation at or near the active site and that at a higher pH is due to the protonation of His6. For the $[Co(dipic)_2]^-$ oxidation of pACu$^I$, $pK_{aA}$(active site) = 4.70 ± 0.11, $pK_{AH}$(His6) = 7.25 ± 0.11, $k_H$ = (3.3 ± 0.4) × $10^3$ $M^{-1}$ $s^{-1}$, $k_1$ = (9.1 ± 1.1) × $10^3$ $M^{-1}$ $s^{-1}$, and $k_0$ = (2.4 ± 0.1) × $10^4$ $M^{-1}$ $s^{-1}$. In the case of the $[Co(phen)_3]^{3+}$ oxidation of pACu$^I$, the following parameters are obtained: $pK_{aA}$ = 4.65 ± 0.10, $pK_{AH}$ = 7.27 ± 0.07, $k_H$ = 74 ± 18 $M^{-1}$ $s^{-1}$, $k_1$ = 320 ± 30 $M^{-1}$ $s^{-1}$, and $k_0$ = 825 ± 19 $M^{-1}$ $s^{-1}$. These five-parameter fits were carried out using an iterative program with no weighting factor. Separate three-parameter fits for the high and low $pK_a$'s were also determined using a least squares procedure and gave almost identical values.

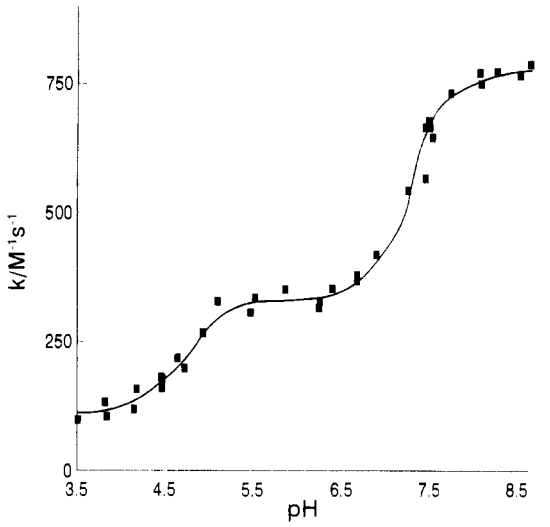


**Figure 5.** Variation of second-order rate constants (25 °C) with pH for the $[Co(phen)_3]^{3+}$ oxidation of pACu$^I$, $I$ = 0.100 M (NaCl).

**Table 2.** Variation of Second-Order Rate Constants (25 °C) with pH for the Oxidation of pACu$^I$ (~1 × $10^{-5}$ M) by $[Co(phen)_3]^{3+}$ at (1–20) × $10^4$ M, $I$ = 0.100 M (NaCl)

| pH | $k/M^{-1}s^{-1}$ | pH | $k/M^{-1}s^{-1}$ |
|---|---|---|---|
| 3.51 | 98 | 6.39 | 353 |
| 3.82 | 133 | 6.67 | 368 |
| 3.84 | 105 | 6.67 | 379 |
| 4.15 | 119 | 6.89 | 419 |
| 4.18 | 158 | 7.25 | 544 |
| 4.46 | 181 | 7.45 | 567 |
| 4.47 | 178 | 7.45 | 666 |
| 4.47 | 160 | 7.49 | 678 |
| 4.64 | 218 | 7.50 | 665 |
| 4.72 | 198 | 7.53 | 646 |
| 4.93 | 267 | 7.74 | 732 |
| 5.09 | 328 | 8.07 | 772 |
| 5.47 | 326 | 8.09 | 750 |
| 5.52 | 335 | 8.27 | 774 |
| 5.86 | 351 | 8.53 | 766 |
| 6.24 | 316 | 8.65 | 790 |
| 6.25 | 329 | | |

The oxidation of pACu$^I$ by $[Fe(CN)_6]^{3-}$ was found to be too fast to monitor at two pH's (7.50 and 5.60) by the stopped-flow method under the same conditions. This indicates a first-order rate constant in excess of 200 $s^{-1}$, and at the oxidant concentration used (7.8 × $10^{-5}$ M) a second-order rate constant of >2.6 × $10^6$ $M^{-1}$ $s^{-1}$ is obtained at pH 5.6.

**NMR Titration of His6.** The resonances belonging to the $C^\delta$ and $C^\epsilon$ protons of His6 have been assigned[21] in both the reduced ($C^\delta H$ at δ 7.04 ppm and $C^\epsilon H$ at δ 7.92 ppm) and oxidized ($C^\delta H$ at 7.01 ppm and $C^\epsilon H$ at δ 7.70 ppm) forms of pseudoazurin at pH 7.50. The difference in the chemical shifts of these resonances in the different oxidation states of the protein at the same pH indicates that the $pK_a$ of His6 is different in the two oxidation states. The change in chemical shift of these two resonances in both oxidation states of the protein as a function of pH has been studied. Figure 6 shows the dependence of the chemical shift of the $C^\delta H$ and $C^\epsilon H$ resonances of His6 in reduced pseudoazurin on pH. From a least squares fit of the data shown $pK_a$'s of 7.08 ± 0.02 and 7.11 ± 0.01 are obtained for the $C^\delta H$ and $C^\epsilon H$

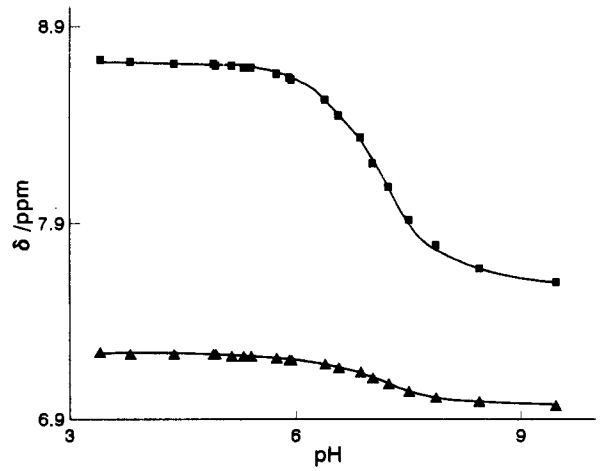


**Figure 6.** Variation with pH of δ (ppm) of the C$^{\delta}$H (▲) and C$^{\epsilon}$H (■) resonances of His6 in pACu$^{I}$.

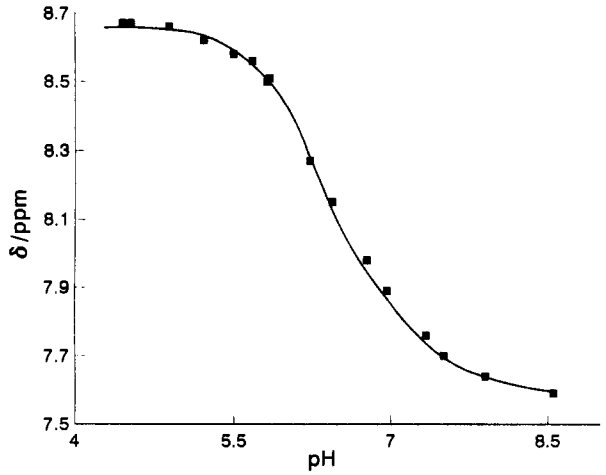


**Figure 7.** Variation with pH of δ (ppm) of the C$^{\epsilon}$H resonance of His6 in pACu$^{II}$.

resonances, respectively. In Figure 7 the variation of the chemical shift of the C$^{\epsilon}$H resonance of His6 in oxidized pseudoazurin is shown and gives a p$K_a$ of 6.50 ± 0.02. The C$^{\delta}$H resonance shifts so little with pH for the oxidized protein (<0.1 ppm) that an accurate p$K_a$ fit is difficult.

From the difference between the p$K_a$'s of His6 in the two oxidation states of pseudoazurin it is possible to determine, using eq 7,[39,48] the effect the protonation of this residue has on the

$$E^{\circ\prime}(\text{pH}) = E^{\circ\prime}(\text{low pH}) + (RT/nF)\ln(K_a^{\text{red}}/K_a^{\text{ox}}) \quad (7)$$

reduction potential of pseudoazurin. From (7), using a value of 249 mV (vs NHE) as the reduction potential of pseudoazurin at pH 8.0 (as determined electrochemically), a value of 285 mV is obtained as the reduction potential of pseudoazurin at the lower pH values, when His6 will be protonated and the active site unprotonated.

**Active Site Protonation Studies by NMR.** The resonances in reduced pseudoazurin of the C$^{\delta}$ and C$^{\epsilon}$ protons of the two histidine ligands (40 and 81) have been assigned previously and are at δ 7.54, 7.20, 7.01, and 6.84 ppm (pH 7.5).[21] On lowering of the pH of reduced pseudoazurin below 6.0, it is apparent that the resonances at δ 7.20 and 7.01 ppm (at pH 7.50), which belong to the same histidine ligand, shift slightly downfield. As the pH is lowered further (<5.5), the histidine ligand resonances disappear (Figure 8). When the pH of a reduced pseudoazurin NMR sample is raised from 4.2 to 6.5, the histidine ligand resonances are seen to reappear (Figure 9), indicating a pH-dependent equilibrium at the active site of pseudoazurin. The C$^{\delta}$H ligand resonance at δ 7.54 ppm is the most resolved of these resonances, and so its behavior is easiest to follow. Integration of this peak relative to the His6 C$^{\epsilon}$H resonance (assumed to be of one proton intensity

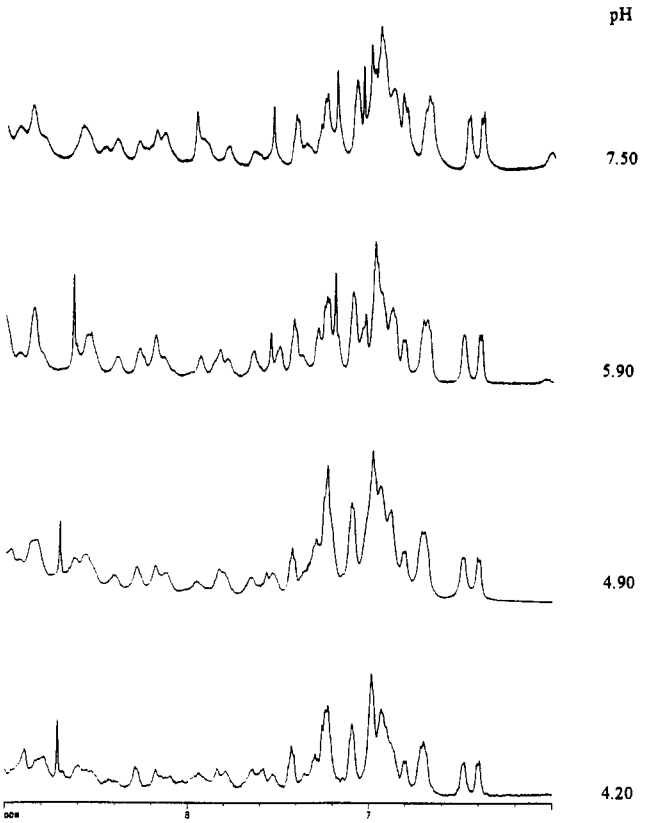


**Figure 8.** Effect of decreasing the pH (7.5 to 4.2) on the aromatic region of the $^{1}$H NMR spectrum of pACu$^{I}$.

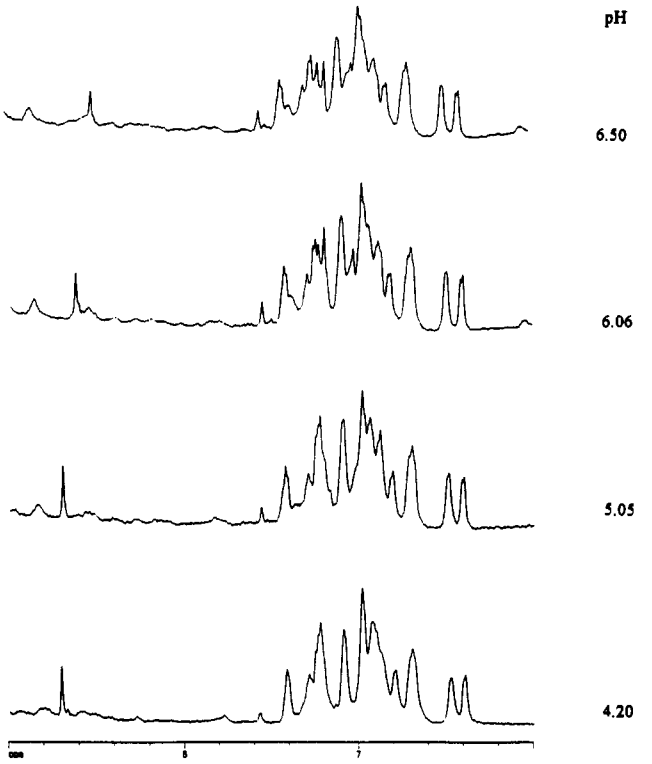


**Figure 9.** Effect of increasing the pH (4.2 to 6.5) on the aromatic region of the $^{1}$H NMR spectrum of pACu$^{I}$.

(48) (a) Pettigrew, G. W.; Leitch, F. A.; Moore, G. R. *Biochim. Biophys. Acta* **1983**, *725*, 409. (b) Bashford, D.; Karplus, M.; Canters, G. W. *J. Mol. Biol.* **1988**, *203*, 507.

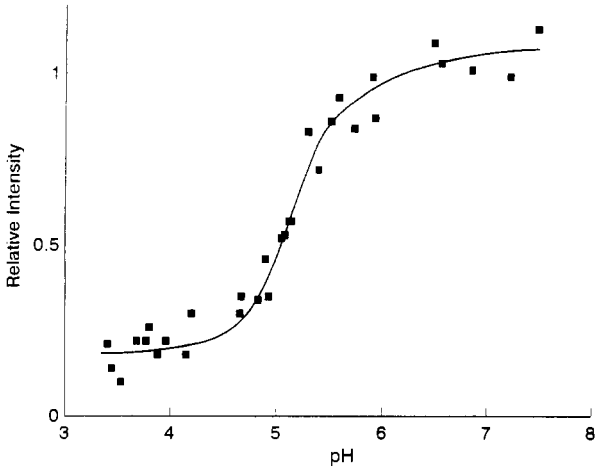


**Figure 10.** Variation with pH of the intensity of the $C^{\epsilon}H$ ligand resonance at $\delta$ 7.54 ppm relative to the $C^{\epsilon}H$ resonance of His6.

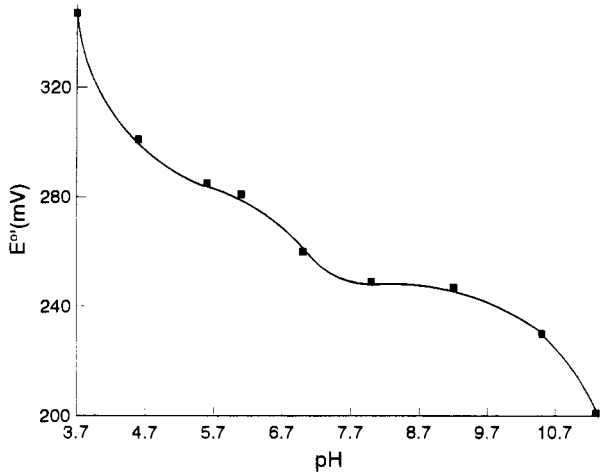


**Figure 11.** Variation of the reduction potential (25 °C) of pseudoazurin vs NHE with pH in 0.10 M phosphate.

**Table 3.** Variation of $E°'$ (25 °C) vs NHE with pH for the Pseudoazurin Cu(II)/Cu(I) Couple in 0.10 M Phosphate

| pH | $E°'$/mV | pH | $E°'$/mV |
|---|---|---|---|
| 3.7 | 347 | 8.0 | 249 |
| 4.6 | 301 | 9.2 | 247 |
| 5.6 | 285 | 10.5 | 230 |
| 6.1 | 281 | 11.3 | 201 |
| 7.0 | 260 | | |

at all pH values) as a function of pH gives the profile shown in Figure 10 and a $pK_a$ of 5.18 ± 0.06.

**Electrochemical Studies on Pseudoazurin.** The determination of the reduction potential of pseudoazurin by electrochemical techniques has been carried out at various pH values and gives the data shown in Table 3. If the steep increase in the $E°'$ of pseudoazurin at very low pH values is ignored, since it is attributable to protonation of the bis(4-pyridyl) disulfide modified gold electrode, then the electrochemical data in the range pH 4.6–9.2 can be fitted to two $pK_a$'s of 5.01 ± 0.20 and 6.68 ± 0.05 (Figure 11). It should be noted that when the electrochemical measurements were carried out using square-wave voltammetry, the effect of pH on the reduction potential in the range 6–8 was not as clear-cut.

## Discussion

Active site protonations have been observed previously for the reduced forms of plastocyanin and amicyanin among the type 1 blue copper proteins. It is interesting to note that in the case of spinach plastocyanin the second histidine ligand (His37) has been shown to have a $pK_a$ of <4.5 compared to a value of 4.9 for His87,[28] whereas in plastocyanin from *S. obliquus* His37 has a $pK_a$ of 5.42 compared to a value of 5.45 for His87.[40] The structural influence of the deletions at positions 57 and 58 in the sequence of *S. obliquus* plastocyanin is thought to result in the higher $pK_a$ of His87 and may also be responsible for both active site histidines having similar $pK_a$ values.

**Table 4.** Spacing of the Ligating Amino Acids in the Sequences of Different Type 1 Blue Copper Proteins[a]

| protein | coordinating amino acids | | | | intervening amino acids |
|---|---|---|---|---|---|
| plastocyanin | His37 | Cys84 | His87 | Met92 | 46, 2, 4 |
| azurin | His46 | Cys112 | His117 | Met121 | 65, 4, 3 |
| pseudoazurin | His40 | Cys78 | His81 | Met86 | 37, 2, 4 |
| CBP[b] | His39 | Cys79 | His84 | Met89 | 39, 4, 4 |
| amicyanin[c] | His54 | Cys93 | His96 | Met99 | 38, 2, 2 |
| rusticyanin[d] | His85 | Cys138 | His143 | Met148 | 52, 4, 4 |
| stellacyanin | His46 | Cys87 | His92 | Gln97[e] | 40, 4, 4 |

[a] Active site $pK_a$'s corresponding to His deprotonation/protonation have now been determined for all plastocyanins (at least six) so far studied, pseudoazurin, and amicyanin (see text) but are not observed in the other cases. [b] Cucumber basic protein. [c] *T. versutus*. [d] Alternatives for His85 are His39 and His57. Sequence information from: Ambler, R. P.; Ingledew, J. W. Unpublished work. Ronk, M.; Shively, J. E.; Shute, E. A.; Blake, R. C., II. *Biochemistry* **1991**, *30*, 9435. Yano, T.; Fukumori, Y.; Yamanaka, T. *FEBS Lett.* **1991**, *288*, 159. [e] As proposed in ref 23 and: Guss, J. M.; Merritt, E. A.; Phizackerley, R. P.; Hedman, B.; Murata, M.; Hodgson, K. V.; Freeman, H. C. *Science* **1988**, *241*, 806.

We have identified an active site protonation in pseudoazurin which has a $pK_a$ value (4.84) similar to that for plastocyanin. From the number of intervening residues between the C-terminal ligands (Table 4), it is apparent that, in order for a type 1 blue copper protein to exhibit an active site protonation in the accessible pH range (i.e. pH 4–10), it needs to have two residues between the Cys and His ligands. The higher $pK_a$ observed in the case of amicyanin (6.8 average from NMR and kinetic studies) may be a consequence of there being only two intervening amino acids between the Cys and His and between the His and Met ligands. The tightness of the loop containing the C-terminal copper ligands may impose some sort of effect on the active site of amicyanin making the histidine easier to protonate. The newly identified type 1 copper protein halocyanin has two amino acids between the Cys and His ligands,[49] and could provide a further example of this phenomenon.

Although only empirical, a recent publication has highlighted the influence of this aspect of protein structure on the properties of proteins containing Fe–S clusters.[50] It has been noted that the DNA repair enzyme endonuclease III as well as the ferredoxin and high-potential [4Fe–4S] electron-transfer proteins have similar [4Fe–4S] clusters but very different redox properties. The spacings between the coordinating Cys residues are quite different in the three cases. In the case of endonuclease III the [4Fe–4S] cluster is attached to the protein by cysteines which have six, two, and five intervening amino acids. Three of the coordinating cysteines of the ferredoxins are close together (typically two intervening residues between each), with the fourth cysteine far removed. This contrasts with the high-potential iron–sulfur proteins in which the coordinating cysteines have spacings of two, sixteen, and thirteen amino acids.

The NMR behavior of the histidine ligand resonance upon lowering the pH indicates that there is a slow exchange between the protonated and deprotonated forms of the protein at 500 MHz. The pH titration of reduced pseudoazurin at pH values <6.0 was also carried out at 300 MHz, and again the exchange process was slow on the 300-MHz time scale. However, in circumstances of slow exchange it should also be possible to observe

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(50) Prince, R. C.; Grossman, M. J. *Trends Biochem. Sci.* **1993**, *18*, 153.

resonances of the protonated histidine ligand. The absence of such resonances indicates that the protonated protein must exist in two forms which are exchanging at an intermediate rate. This is analogous to the situations in plastocyanin[30] and amicyanin,[33] except that in these cases the exchange between the protonated and deprotonated forms is fast.

The histidine ligand which protonates must be the more solvent-exposed His81. However the resonance at $\delta$ 7.54 ppm belongs to His40 since it gives a correlation to a peak at $\delta$ 10.49 ppm, as does the $C^{\delta}$ proton of this histidine residue, in a COSY spectrum of pACu$^{I}$ in 90% $H_2O$ (10% $D_2O$). The fact that the resonances belonging to both the histidine ligands show similar behaviors indicates that protonation affects the overall structure of the active site and thus changes the environment of His40 also.

As in previous studies of this kind,[26,27,34,40,51] the active site p$K_a$ from the kinetic studies is lower than that from the NMR determination. It should be noted that the NMR p$K_a$ in this case is not as accurate as in previous studies due to the difficulties and errors involved in the integration of protein $^1$H NMR spectra. The discrepancy between the NMR and kinetic active site p$K_a$ values could be due to the fact that the two studies were carried out in different buffers but this seems unlikely. The ionic strengths in the NMR experiments at pH $\leq$5.7 were always 0.10 M, so this cannot be a source of the discrepancy. The most likely explanation is that the NMR work was carried out in $D_2O$ with no correction for the deuterium isotope effect. It should also be noted that the active site p$K_a$ determined electrochemically is between the values from the NMR and kinetic studies. For the p$K_a$ of His6 there is also a difference between the NMR and kinetic values (in this case with the kinetic value higher), but this is not as significant.

The effects seen in the kinetic, NMR, and electrochemical studies at higher pH values are indicative that the protonation of His6 has a direct effect on the reduction potential of the protein. This is supported by the similarity of the kinetic and NMR p$K_a$'s. Also the value of the reduction potential of pseudoazurin at low pH (when His6 is protonated) determined from the difference between the p$K_a$ values of His6 in the oxidized and reduced proteins is in excellent agreement with the electrochemically determined reduction potential at the appropriate pH value.

Pseudoazurin has a large number of basic residues around the area where the histidine ligands are exposed. These include Lys38, Lys46, Lys57, and Lys77 (adjacent to Cys78) which are conserved in the three known pseudoazurin sequences. This feature of pseudoazurin has been postulated as being responsible for the slow rate of self-exchange in this metalloprotein.[21] The presence of the basic residues presumably makes the association constant for the interaction of pACu$^{I}$ with $[Fe(CN)_6]^{3-}$ large, and this fact along with the relatively large driving force explains why the reaction is too fast to monitor by stopped-flow spectrophotometry. The effect of the basic residues is also evident in the $k_{Fe}/k_{Co}$ ratio ($k_{Co}$ is the rate constant for the oxidation by $[Co(phen)_3]^{3+}$) which at pH $\sim$5.5 is $>7.8 \times 10^3$ and is most probably the highest seen in studies on a type 1 blue copper protein. The positive charges on pseudoazurin may be important for its interaction with the physiological redox partner nitrite reductase, which is known to be an acidic protein.[52]

Interesting in these studies is the fact that pseudoazurin appears to retain electron-transfer capabilities even at low pH. This has been observed previously in the case of certain studies on plastocyanin.[41,53] Therefore it is possibly inappropriate to refer to the low-pH forms of these proteins as redox inactive. Certainly the redox potential of the protein increases at lower pH's and is less compatible with efficient electron transfer.

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