Reversible Active Site Protonation and Electron-transfer Properties of *Achromobacter cycloclastes* Pseudoazurin: Comparisons with other Type 1 Copper Proteins

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Kinetic and NMR studies on the copper(I) form of *A. cycloclastes* pseudoazurin provide evidence for an active site protonation (and dissociation) equilibrium process involving His81, pK_a 4.9 (average); the three type 1 copper proteins now known to exhibit this behaviour have just two amino acids separating the ligating Cys and His residues in the C-terminal region.

From the crystal structures of type 1 copper proteins,[1–5] it has been established that the active site geometry is that of a distorted tetrahedron, with closer approaches by two histidines and a cysteine in an equatorial plane, and a longer axial bond to a methionine. In the case of azurin a second axial ligand (the carbonyl O-atom of Gly45) is also implicated, resulting in a trigonal bipyramidal geometry. Of the four most common ligands one His is always towards the *N*-terminal region of the primary sequence, and the other three are within 11 amino acids of each other in the C-terminal region.

Reversible protonation and dissociation of the active site His87 in reduced plastocyanin is now well established.[6] However, no similar process is observed in the cases of azurin,[7] stellacyanin,[8] umecyanin,[9] and rusticyanin.[10] A second example of reversible active site protonation has been reported for *Thiobacillus versutus* amicyanin, in which the solvent-exposed His96 ligand protonates with an unusually high pK_a of 6.9.[11]

We now report studies on *Achromobacter cycloclastes* IAM 1013 pseudoazurin which provide a third example of reversible active site protonation of the Cu^I but not the Cu^{II} state. Pseudoazurin from this source (M_r *ca.* 14000) has 124 amino acids in its primary structure. This includes three histidines, two of which are ligands to the copper at the active site, while the third at position 6 (pK_a = 7.11) is uncoordinated.[12] The protein was isolated and purified as described previously.[13] The final stage of purification was on an FPLC Mono S column giving protein with an absorbance (A) ratio A_{277}/A_{593} of 1.4:1.

The oxidation of pACu^I (*ca.* 10 μM†) by $[Co(phen)_3]^{3+}$ in large > tenfold excess was studied by stopped-flow spectrophotometry in the pH range 3.5 to 6.5 at 25 °C, I = 0.100 M (NaCl). The buffers used were acetate, pH range 3.5–5.5, and 2-[morpholino]ethane sulfonic acid (Mes), pH 5.5–6.5. The ^{1}H NMR spectrum of pACu^I was studied at 500.14 MHz, with the protein initially exchanged into 88.5 mM phosphate buffer at pH 5.7. Adjustment of pH to values in the same range as for the kinetics, using DCl or NaOD (0.10 M), was carried out immediately prior to measurements. All pH values were determined using an electrode calibrated with aqueous buffers. No corrections were made for the deuterium isotope effect.

The variation with pH of the second-order rate constants (k) for the oxidation of pACu^I by $[Co(phen)_3]^{3+}$ is illustrated in Fig. 1. The dependence on $[H^+]$ was analysed in terms of eqn. 1,

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

where the various constants are as defined in eqns. (2)–(4).

$$pACu^I H^+ \overset{K_a}{\rightleftarrows} pACu^I + H^+ \quad (2)$$

$$pACu^I + Co^{III} \overset{k_o}{\rightarrow} pACu^{II} + Co^{II} \quad (3)$$

$$pACu^I H^+ + Co^{III} \overset{k_H}{\rightarrow} pACu^{II} + Co^{II} + H^+ \quad (4)$$

A least-squares fit of the data to (1) gives k_o = 353 ± 11 $M^{-1}s^{-1}$, k_H = 74 ± 19 $M^{-1}s^{-1}$, and a pK_a of 4.63 ± 0.11. The reaction with $[Co(dipic)_2]^-$ (dipic = 2,6 dicarboxylatopyridine) as oxidant gave similar behaviour with pK_a = 4.77 ± 0.11. A feature in both cases is that k_H, although very much smaller than k_o, is not zero.

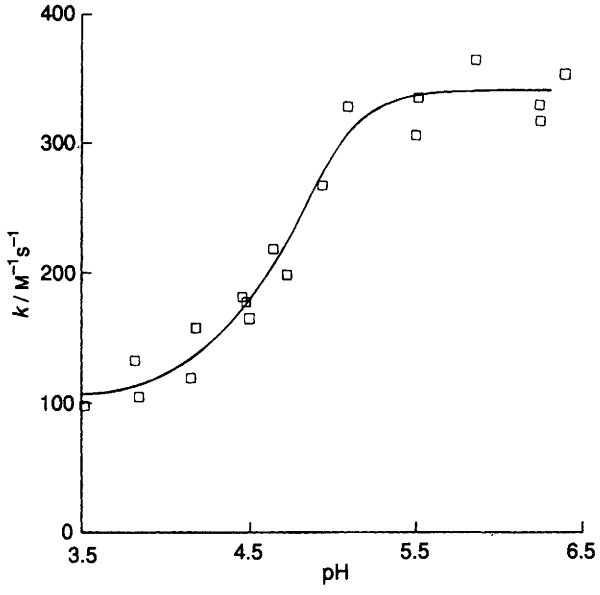

Fig. 1 Variation of second-order rate constants k (25 °C) for the $[Co(phen)_3]^{3+}$ oxidation of *A. cycloclastes* pseudoazurin pACu^I with pH, I = 0.100 M (NaCl)

Table 1 Spacing of the ligating amino acids in the sequences of different type 1 blue copper proteins.

Protein	Number of intervening amino acids
Plastocyanin	His (46) Cys (2) His (4) Met
Azurin	His (65) Cys (4) His (3) Met
Pseudoazurin	His (37) Cys (2) His (4) Met
CBP[a]	His (39) Cys (4) His (4) Met
Amicyanin[b]	His (38) Cys (2) His (2) Met
Rusticyanin[c]	His (52) Cys (4) His (4) Met
Stellacyanin	His (40) Cys (4) His (4) Gln[d]

[a] Cucumber basic protein. [b] *T. versutus*. [c] Sequence information from R. P. Ambler and J. W. Ingledew (unpublished results); M. Ronk, J. E. Shively, E. A. Shute and R. C. Blake II, *Biochemistry*, 1991, **30**, 9435; T. Yano, Y. Fukumori and T. Yamanaka, *FEBS Lett.*, 1991, **288**, 159. [d] As proposed in ref. 3 and B. A. Fields, J. M. Guss and H. C. Freeman, *J. Mol. Biol.*, 1991, **222**, 1053.

In the NMR spectra of pACu^{I} at different pH values the resonances of the C^{δ} and C^{ε} protons of the two histidine ligands shift slightly and then disappear as the pH is lowered. Moreover, the resonances re-appear when the pH is raised indicating a reversible equilibrium at or near the active site, which is dependent on pH. This is consistant with slow exchange between the unprotonated and more than one protonated form, with exchange involving the latter occurring at an intermediate rate, so that their resonances are not observed as previously found for plastocyanin,[14] and amicyanin.[11] The $C^{\varepsilon}H$ ligated histidine resonance at δ 7.54 is well resolved from other peaks and its behaviour is therefore easiest to follow. Integration relative to the $C^{\varepsilon}H$ resonance of His6 at different pH values gives a pK_a of 5.18 ± 0.06.

From the comparisons in Table 1 it appears that for the active site of a type 1 protein in the Cu^{I} state to show reversible protonation two intervening amino acids are required between the cysteine and the second coordinating histidine. The similarity between the active site pK_as of plastocyanin and pseudoazurin could also be due to their having the same number of amino acids between the C-terminal histidine and methionine. The fact that amicyanin has a higher active site pK_a may be related to the observation that there are only two amino acids between the latter two coordinating residues. The tightness of the loop in the case of amicyanin may confer greater strain on the active site making the histidine easier to protonate.

We thank the Leverhulme Trust (W. McF.) and the SERC (C. D.) for financial support.

Received, 8th November 1993; Com. 3/06704A

Footnote

† M is a non-IUPAC symbol for mol dm^{-3}.

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