Redox Reactivity of the Type 1 (Blue) Copper Protein Amicyanin from *Thiobacillus versutus* with Inorganic Complexes†

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Electron-transfer reactions of the type 1 copper protein amicyanin from *Thiobacillus versutus* with $[Co(terpy)_2]^{2+}$ (terpy = 2,2':6',2''-terpyridine) as a reductant for $AmCu^{II}$, and $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) as oxidants for $AmCu^{I}$ have been explored by stopped-flow kinetic studies at 25 °C, I = 0.100 M (NaCl). The reaction with $[Co(terpy)_2]^{2+}$ is a straightforward single-stage process, whereas both the oxidations give biphasic kinetics. The first stages of the latter exhibit an active-site 'switch-off' mechanism previously assigned as a protonation (and dissociation) of the His-96 ligand of $AmCu^{I}$. From the studies with $[Co(phen)_3]^{3+}$ an acid dissociation constant pK_a of 6.6 and rate constant at the higher pH of 7.1 × 10^3 M^{-1} s^{-1} are obtained. The reaction with $[Fe(CN)_6]^{3-}$ gives similar behaviour with a pK_a of 6.6, and from the fitting procedure adopted, a rate constant at the higher pH of 8.3 × 10^6 M^{-1} s^{-1}, which is at the upper limit of the stopped-flow range. The second stage of the reaction is explained by the formation of a less-reactive form of $AmCu^{I}$ in amounts of between 5 and 40% depending on the pH. The rate law in the case of $[Co(phen)_3]^{3+}$ is independent of oxidant concentration, shows little dependence on pH and gives rate constants (a) in the range 0.07–0.12 s^{-1}. For the more reactive $[Fe(CN)_6]^{3-}$ the rate equation is $k_{2obs} = a + b[Fe(CN)_6^{3-}]$, with a varying between 0.13 and 0.15 s^{-1} and b = 680 M^{-1} s^{-1}. The similar values of a for the two reactions are consistent with a common rate-controlling intramolecular isomerisation step. The bimolecular rate constant b is ≈10^4 times smaller than the rate constant for the first stage of the $[Fe(CN)_6]^{3-}$ oxidation at pH ≈ 8. The nature of the structural differences between the two forms of $AmCu^{I}$ is considered.

Amicyanin is a type 1 (blue) copper protein which mediates electron transport between methylamine dehydrogenase and c-type cytochromes of certain methylotrophic bacteria.[1] The bacteria can utilise compounds that contain no carbon–carbon bond, *i.e.* C_1 compounds such as methylamine or methanol, as their sole source of carbon and energy. Methylamine is oxidised by the periplasmic methylamine dehydrogenase (MADH) as in equation (1),[2,3] and the electrons are transferred *via* amicyanin

$$CH_3NH_3^+ + H_2O \longrightarrow NH_4^+ + CH_2O + 2H^+ + 2e^- \quad (1)$$

and a c-type cytochrome to a membrane bound aa_3-type cytochrome oxidase. The terminal acceptor of the electrons is dioxygen.[4]

Amicyanin has been isolated from the methylotrophs *Methylobacterium extorques* AM1,[5] *Paracoccus denitrificans*,[2] *Thiobacillus versutus*[6] and an organism referred to as 4025.[7] Typical properties of amicyanin from *T. versutus* are M_r = 11 700 (106 amino acids), a copper(II) UV/VIS absorbance peak at 597 nm (ε = 3900 M^{-1} cm^{-1}), axial-type EPR parameters for the oxidised state and a reduction potential of 260 mV (*vs.* normal hydrogen electrode, NHE) at pH 7.0.[6]

The crystal structures of oxidised *P. denitrificans*[8] and *T. versutus*[9] amicyanin have been determined. The former has also been cocrystallised with its physiological partner MADH,[10] and with both MADH and cytochrome c_{551i}.[11] The polypeptide fold in amicyanin from *T. versutus* is based on the β-sandwich structure commonly found in the type 1 copper proteins. Amicyanin resembles plastocyanin in overall structure but differs from it with respect to an extra N-terminal strand of 22 residues. The copper active site is co-ordinated by four amino acids, His-54, Cys-93, His-96 and Met-99, arranged in a distorted-tetrahedral geometry. The His-96 residue is exposed at the surface and lies in the centre of a cluster of seven hydrophobic residues.[9] The crystal structure and the solution structure derived from NMR studies are in good agreement.[12,13] The His-96 residue of reduced amicyanin from *T. versutus* protonates,[14,15] and at lower pH becomes dissociated from the Cu^{I}. An acid dissociation pK_a of 6.9 has been determined by NMR spectroscopy (39 °C, I = 0.05 M). Similar effects have been observed in the case of plastocyanin[16] and pseudoazurin[17] among the type 1 copper proteins. Further support for the dissociation of the histidine ligand of reduced amicyanin at low pH values has been provided by extended X-ray absorption fine structure (EXAFS) data.[18]

The protonation and dissociation of His-96 results in a greater than two orders of magnitude decrease in the electron self-exchange rate constant.[15] It was therefore of interest whether this effect might be reflected in the reactivity of amicyanin, $AmCu^{I}$, with the redox partners $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline), equations (2)

$$AmCu^{I} + [Fe(CN)_6]^{3-} \longrightarrow AmCu^{II} + [Fe(CN)_6]^{4-} \quad (2)$$

and (3). Previously the use of inorganic redox partners has

$$AmCu^{I} + [Co(phen)_3]^{3+} \longrightarrow AmCu^{II} + [Co(phen)_3]^{2+} \quad (3)$$

resulted in the identification of important properties of

† *Non-SI unit employed:* M = mol dm^{-3}.

metalloproteins, at a time when no structural information was available.[16] In the present studies in aqueous solution two forms of $AmCu^{I}$ have been detected, whereas $AmCu^{II}$ exhibits uniphasic reactivity. Information regarding binding sites on metalloproteins used by redox partners can be explored by ^{1}H NMR line-broadening techniques, using redox-inactive paramagnetic chromium(III) complexes,[19-22] which is the subject of a further paper.[23]

Experimental

Isolation of Amicyanin.—The gene coding for amicyanin from *T. versutus* has previously been cloned behind the *lac* promoter in pUC18.[24] In this work *Escherichia coli* K12, strain JM101 was used as a host for the expression of amicyanin. Cells were grown in Luria broth (LB) medium supplemented with ampicillin (70 μg cm^{-3}) and $CuSO_4$ (100 μM) in a 40 l fermentor (NBS MPP40; New Brunswick Scientific, New Brunswick, USA). The fermentor was inoculated 1:100 with a starting culture grown on the same medium. After 3 h of growth, expression of amicyanin was induced by the addition of 100 μM isopropyl β-D-galactopyranoside. Growth was continued for another 4.5 h after which cells were harvested.

The bacterial paste was suspended in sucrose buffer [20% sucrose, 1 mM ethylenediaminetetraacetate (edta), 30 mM tris(hydroxymethyl)aminomethane (Tris), pH 8.0] and stirred for 15 min at room temperature. This resulted in amicyanin release from the periplasm. The cells were then removed by centrifugation and amicyanin was purified from the supernatant solution using the procedure described previously.[6] The final yield of amicyanin was approximately 30 mg per litre of bacterial culture. A feature of the UV/VIS spectrum of $AmCu^{II}$ is the intense peak at 597 nm with relatively little absorbance at ≈470 nm, Fig. 1, consistent with the axial EPR spectrum and with the Cu atom at the active site being in the plane of the three equatorial ligands.[25] Concentrations of oxidised protein $AmCu^{II}$ were determined from the absorbance at 597 nm (ε = 3900 M^{-1} cm^{-1}). The $AmCu^{I}$ form has no absorbance in the visible range. In order to make comparisons with wild-type protein (as above) a sample of native protein was obtained from *T. versutus* by the earlier procedure.[6]

Buffers.—Acetate–acetic acid buffer was used in the range pH 4.2–5.2, 2-morpholinoethanesulfonic acid (mes, Sigma), to which NaOH was added, for pH 5.2–6.9, Tris (Sigma), to which HCl was added, for pH 7.0–8.8, and 2-(cyclohexylamino)ethanesulfonic acid (ches, Sigma), to which NaOH was added, for pH 8.8–9.5. In all cases the ionic strength was adjusted to 0.100 M by the addition of appropriate amounts of NaCl (BDH, Analar).

Inorganic Complexes.—Preparations/sources were as described previously, and characterisation was by UV/VIS absorbance spectra, peak positions λ/nm (ε/M^{-1} cm^{-1}) being as follows: bis(2,2′:6′,2″-terpyridine)cobalt(II) perchlorate, $[Co(terpy)_2][ClO_4]_2$, 445 (1580), 505 (1400);[26] tris(1,10-phenanthroline)cobalt(III) chloride, $[Co(phen)_3]Cl_3 \cdot 7H_2O$, 330 (4460), 350 (3620), 450 (100);[27] potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, 300 (1600), 420 (1010) (BDH, Analar). Reduction potentials for the relevant redox couples are $[Co(terpy)]^{3+/2+}$ (270), $[Fe(CN)_6]^{3-/4-}$ (410) and $[Co(phen)_3]^{3+/2+}$ (370 mV).[28]

Purification of Protein prior to Kinetic Studies.—Oxidised amicyanin $AmCu^{II}$ was purified by fast protein liquid chromatography (FPLC) using a Mono-Q column at pH 7.5. The protein eluted as two bands and the major (first) fraction with absorbance (A) ratio A_{278}/A_{597} = 3.8:1 was used for kinetic studies. Pure amicyanin samples were dialysed against the appropriate buffer for 24 h before the stopped-flow experiments.

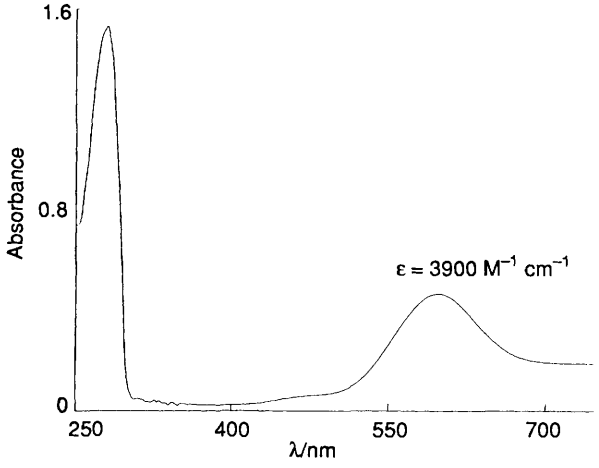

Fig. 1 The UV/VIS spectrum of the oxidised form of amicyanin, $AmCu^{II}$, from *T. versutus* (0.1 mM protein in 50 mM phosphate buffer, pH 7.5, at 25 °C, 1.0 cm cell)

The dialysis buffer was changed three times over this period. In order to reduce or oxidise amicyanin, small amounts of sodium ascorbate (BDH, Analar) or $[Fe(CN)_6]^{3-}$ were added to the protein solution and any excess removed subsequently by dialysis. Solutions which in kinetic studies display biphasic behaviour give only a single band.

Kinetic Studies.—All kinetic runs were monitored at 597 nm on a Dionex D-110 stopped-flow spectrophotometer at 25.0 ± 0.1 °C, I = 0.100 ± 0.001 M (NaCl). The spectrophotometer was interfaced to an IBM PC/AT-X computer for data acquisition using software from On-Line Instruments Systems (Bogart, GA, USA). All quoted rate constants are an average of at least five determinations using the same solutions. In the case of the $[Fe(CN)_6]^{3-}$ oxidation of $AmCu^{I}$, which is at the limit of the stopped-flow range, check runs were carried out on an Applied Photophysics stopped-flow instrument in Dr. S. K. Chapman's laboratory at the University of Edinburgh (for which we are most grateful).

Treatment of Data.—The standard treatment for a biphasic reaction involves first the evaluation of k_{2obs} from the linear section of plots of absorbance changes $\ln(A_\infty - A_t)$ *vs.* time (t). The intercept on the $\ln(A_\infty - A_t)$ axis (y) enables $\ln[A_\infty - A_t - \exp(y - k_{2obs}t)]$ against t to be plotted for the first part of the reaction, the slope of which gives k_{1obs}.

For studies on the oxidation of $AmCu^{I}$ by $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$, variations of the second-order rate constants k for the first stage with pH in the range 4.3–9.3 were fitted by use of equation (4), which is derived from the kinetic scheme (5)–(7)

$$k = (k_o K_a + k_H[H^+])/(K_a + [H^+]) \quad (4)$$

$$AmCu^{I}H^+ \overset{K_a}{\rightleftharpoons} AmCu^{I} + H^+ \quad (5)$$

$$AmCu^{I} + oxid \overset{k_o}{\rightleftharpoons} AmCu^{II} + red \quad (6)$$

$$AmCu^{I}H^+ + oxid \overset{k_H}{\rightleftharpoons} AmCu^{II}H^+ + red \quad (7)$$

where oxid and red are the oxidant and reduced species respectively. Rate constants and their standard deviations were obtained from an unweighted least-squares treatment of the data.

Results

Reduction of $AmCu^{II}$ *by* $[Co(terpy)_2]^{2+}$.—The reaction (8)

$$[Co(terpy)_2]^{2+} + AmCu^{II} \longrightarrow [Co(terpy)_2]^{3+} + AmCu^{I} \quad (8)$$

between $AmCu^{II}$ and $[Co(terpy)_2]^{2+}$ was investigated in order to check whether reduction takes place in two stages as is the case for the oxidation of $AmCu^{I}$ (see below). Uniphasic traces were obtained for the reduction of $AmCu^{II}$ at four different pH values, Table 1. No pH dependence was observed and, at pH between 6.6 and 8.3, $k = (1.8 \pm 0.1) \times 10^3$ M^{-1} s^{-1}.

Oxidation of $AmCu^{I}$ *by* $[Fe(CN)_6]^{3-}$.—The reaction occurs in two stages. First-order rate constants k_{1obs} and k_{2obs} were obtained from the standard biphasic treatment already indicated. A protonation switch-off mechanism is observed for the first stage. At pH > 7.0 rate constants k_{1obs} are the limit of the stopped-flow range and the high-pH plateau region is therefore ill defined. At pH 5.2 first-order rate constants k_{1obs}, Table 2, suggest an approach to saturation kinetic behaviour. Owing to the rapidity of the reactions (for the two faster runs only the latter half of the reaction was monitored), no quantification is here attempted. However from NMR and kinetic studies carried out in the presence of redox-inactive $[Cr(CN)_6]^{3-}$ this effect will be further developed in a subsequent paper. Second-order rate constants k_{Fe} at different pH values, obtained from runs with a sufficiently small (> 10-fold) excess of $[Fe(CN)_6]^{3-}$, are listed in Table 3, and the pH dependence is illustrated in Fig. 2. Some care is required in fitting the data of Fig. 2 by equation (4). If all points are included the pK_a defined by equation (5) is ≈ 6 which is substantially less than the value of 6.6 determined for the $[Co(phen)_3]^{3+}$ reaction. However we note that the rate constants for the three highest pH values 7.09, 7.64 and 7.84 are obtained from absorbance changes recorded over the last 13, 7 and 7% of reaction respectively. It is therefore unlikely that these points are as reliable as the others for which much more of the reaction was monitored. From a fit of the data excluding these three points the parameters $pK_a = 6.6 \pm 0.05$, $k_o = (8.3 \pm 0.2) \times 10^6$ M^{-1} s^{-1} and $k_H = (1.4 \pm 1.6) \times 10^5$ M^{-1} s^{-1} (assumed to be zero), as defined in equations (5)–(7) are obtained.

For the second stage, first-order rate constants k_{2obs}, Table 4, conform to equation (9). There is no detectable dependence on

$$k_{2obs} = a + b[Fe(CN)_6^{3-}] \quad (9)$$

pH in the range 6.00–8.10, Fig. 3. The intercept $a = 0.14 \pm 0.01$ s^{-1} and $b = 680 \pm 130$ M^{-1} s^{-1}.

The second fraction of protein obtained on FPLC treatment of $AmCu^{II}$ exhibited identical behaviour to that described above. Native amicyanin also gave biphasic kinetics with rate constants as listed in Tables 3 and 4 in good agreement (± 5%) with those of the wild-type protein.

Oxidation of $AmCu^{I}$ *by* $[Co(phen)_3]^{3+}$.—At pH 6.92 a linear dependence of the first-order rate constant k_{1obs}/s^{-1} *vs.* oxidant concentration $\{10^4[Co(phen)_3^{3+}]\}$ is observed [0.7 (1.67), 1.8 (3.65), 2.2 (4.15), 3.1 s^{-1} (6.50 M)], consistent with the rate law (10). The dependence of the second-order rate

$$\text{Rate} = k_{Co}[AmCu^{I}][Co(phen)_3^{3+}] \quad (10)$$

constant k_{Co} on pH, Table 5, is illustrated in Fig. 4. A satisfactory fit by equation (4) is obtained and gives $pK_a = 6.59 \pm 0.05$, $k_o = (7.1 \pm 0.1) \times 10^3$ M^{-1} s^{-1} and $k_H = -120 \pm 206$ M^{-1} s^{-1} which is assumed to be zero. Rate constants k_{2obs} for the second phase, Table 6, are independent of $[Co(phen)_3^{3+}]$ in the range $(4.2–13.3) \times 10^{-4}$ M, with first-

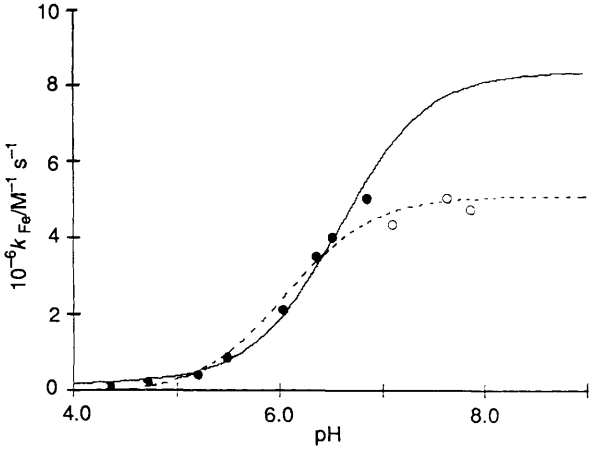

Fig. 2 Variation of second-order rate constants k_{Fe} (25 °C) with pH for the first stage of the $[Fe(CN)_6]^{3-}$ oxidation of $AmCu^{I}$, $I = 0.100$ M (NaCl). The solid line fit (shaded circles) gives a pK_a of 6.6, whereas the broken line (all circles) gives a $pK_a \approx 6.0$

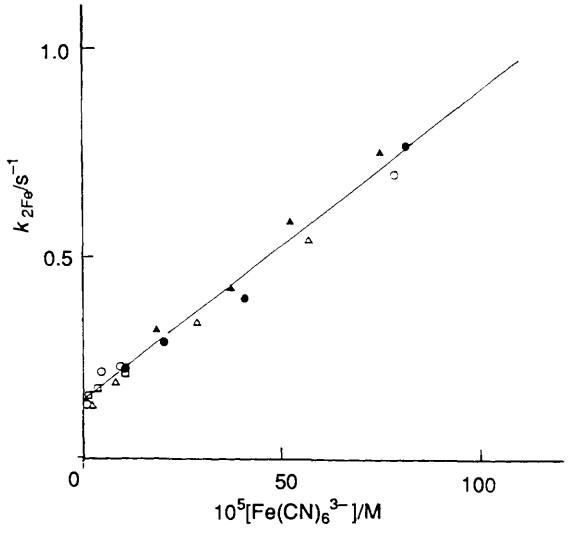

Fig. 3 Oxidant concentration dependence of the first-order rate constants k_{2Fe} for the second stage of the $[Fe(CN)_6]^{3-}$ oxidation of $AmCu^{I}$, at pH 6.00 (○), 6.03 (□), 7.12 (△), 7.48 (●) and 8.10 (▲), $I = 0.100$ M (NaCl)

Table 1 Second-order rate constants k (25 °C) for the $[Co(terpy)_2]^{2+}$ ($\approx 6.5 \times 10^{-5}$ M) reduction of *T. versutus* $AmCu^{II}$ (6.5×10^{-6} M), $I = 0.100$ M (NaCl)

pH	$10^{-3}k/M^{-1}$ s^{-1}
6.63	1.7
7.08	1.8
7.52	1.9
8.30	1.7

Table 2 First-order rate constants k_{1obs} (25 °C) for the first stage of the oxidation of *T. versutus* $AmCu^{I}$ ($\approx 1.0 \times 10^{-5}$ M) with $[Fe(CN)_6]^{3-}$ at pH 5.20, illustrating saturation kinetic behaviour *

$10^3[Fe(CN)_6^{3-}]/M$	k_{1obs}/s^{-1}
0.27	109
0.55	227
1.15	452
2.44	659
3.04	671

* Ionic strengths are 0.100, 0.102, 0.103, 0.107 and 0.109 M respectively for the above runs. These variations are not expected to have a significant effect.

order rate constants varying from $\approx 0.07\ s^{-1}$ at low pH and increasing to a value of $0.12\ s^{-1}$ at high pH, Fig. 5. It seems likely therefore that the intercept a in the $[Fe(CN)_6]^{3-}$ study, equation (9), and the value observed here are for the same intramolecular process.

The native protein also gives biphasic behaviour and rate constants, Tables 5 and 6, were again in good (± 3%) agreement with those for wild-type protein.

Relative contributions of the first and second stages in terms of percentage contributions to the total absorbance change are indicated in Fig. 6. No effect on the relative percentage contributions of the two phases was observed for amicyanin stored frozen for different lengths of time, or for solutions used over a 24 h period.

Table 3 Variation of second-order rate constants $k_{Fe}/M^{-1}\ s^{-1}$ (25 °C) with pH for the first stage of the oxidation of *T. versutus* $AmCu^{I}$ ($\approx 2.0 \times 10^{-5}$ M) with $[Fe(CN)_6]^{3-}$ ($\approx 2 \times 10^{-4}$ M), $I = 0.100$ M (NaCl)

pH	$10^{-6}k_{Fe}$	pH	$10^{-6}k_{Fe}$
4.35	0.08	6.35	3.50
4.72	0.70	6.50	4.00
5.12	0.30*	6.84	5.00
5.20	0.40	7.09	4.40
5.48	0.85	7.64	5.00
6.03	2.10	7.84	4.70
6.24	2.80*		

* Native $AmCu^{I}$. From three or four runs at different oxidant concentrations. Not included in fitting procedure.

Table 4 Variation of first-order rate constants k_{2obs} (25 °C) with pH and oxidant concentration for the second stage of the $[Fe(CN)_6]^{3-}$ oxidation of *T. versutus* $AmCu^{I}$, $I = 0.100$ M (NaCl)

pH	$10^4[Fe(CN)_6^{3-}]/M$	k_{2obs}/s^{-1}
6.00	0.16	0.14
	0.49	0.21
	0.98	0.23
	7.80	0.69
6.03	0.15	0.16
	0.34	0.18
	1.03	0.21
6.24*	1.07	0.25
	2.13	0.35
	4.27	0.42
	8.53	0.69
7.12	0.36	0.14
	0.81	0.20
	2.80	0.34
	5.66	0.55
7.30*	1.84	0.30
	3.68	0.42
	7.35	0.63
7.48	1.01	0.22
	2.01	0.29
	4.03	0.39
	8.06	0.75
8.10	1.85	0.32
	3.70	0.42
	5.18	0.58
	7.40	0.75

* Native $AmCu^{I}$. Not included in fitting procedure.

Discussion

Amicyanins from methylotrophic bacteria show many similarities to higher-plant plastocyanins, despite the eukaryotic origin of the latter. The most interesting is the occurrence in both proteins of an active-site protonation, His-87 in plastocyanin[16] and His-96 in *T. versutus* amicyanin.[14,15] A third example of a type 1 copper protein showing the same effect in the accessible range of pH is pseudoazurin from *Achromobacter cycloclastes*.[17]

The present kinetic studies were undertaken in order to check whether pH effects are observed in the reaction of amicyanin with different redox partners. This is the first study concerning the electron-transfer reactivity of amicyanin from *T. versutus* with non-physiological redox partners. The complexes $[Fe(CN)_6]^{3-}$ ($E^{\circ\prime}$ 410 mV) and $[Co(phen)_3]^{3+}$ ($E^{\circ\prime}$ 370 mV) were chosen because they have suitable redox properties as compared to amicyanin ($E^{\circ\prime} = 260$ mV), and kinetic data have been reported for their reactions with other type 1 Cu proteins.[16]

It is found that the $[Co(phen)_3]^{3+}$ and $[Fe(CN)_6]^{3-}$ oxidations of $AmCu^{I}$ take place in two stages, whereas the $[Co(terpy)_2]^{2+}$ (270 mV) reduction of $AmCu^{II}$ occurs in one stage. It is therefore concluded that the two-stage reactivity pattern is due to structural effects occurring at the copper site of reduced amicyanin. The kinetic data for the first stage of the $[Co(phen)_3]^{3+}$ and $[Fe(CN)_6]^{3-}$ oxidation of $AmCu^{I}$ illustrate two points: first a remarkable loss in electron-transfer reactivity at low pH values and secondly a much higher reactivity with these inorganic redox partners as compared with other type 1 Cu proteins.

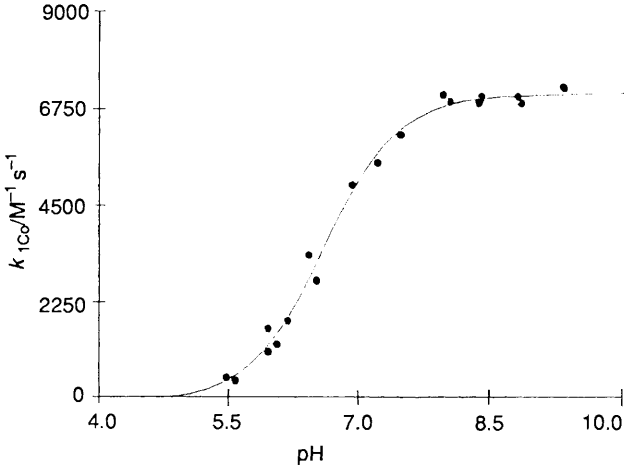

Fig. 4 Variation of second-order rate constants k_{1Co} (25 °C) with pH for the first stage of the $[Co(phen)_3]^{3+}$ oxidation of $AmCu^{I}$, $I = 0.100$ M (NaCl)

Table 5 Variation of second-order rate constants $k_{Co}/M^{-1}\ s^{-1}$ (25 °C) with pH for the first stage of the oxidation of $AmCu^{I}$ ($\approx 1.0 \times 10^{-5}$ M) with $[Co(phen)_3]^{3+}$ ($\approx 5.0 \times 10^{-4}$ M), $I = 0.100$ M (NaCl)

pH	$10^{-3}k_{Co}$	pH	$10^{-3}k_{Co}$	pH	$10^{-3}k_{Co}$	pH	$10^{-3}k_{Co}$
5.47	0.46	6.17	1.8	7.47	6.1	8.81	7.0
5.57	0.40	6.42	3.3	7.95	7.1	8.85	6.9
5.95	1.60	6.51	2.7	8.03	6.9	9.33	7.2
5.95	1.1	6.92	5.0	8.37	6.9		
6.05	1.2	7.20	5.6*	8.39	7.0		
		7.21	5.6				

* Native $AmCu^{I}$. From three runs at different oxidant concentrations (2.7–10.7) $\times 10^{-4}$ M. Not included in fitting procedure.

From the fitting procedures described the $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ oxidants both give an $AmCu^I$ pK_a of 6.6. In the case of the plastocyanin with the same two oxidants different pK_a values are observed, *e.g.* 4.8 and 5.5 respectively for spinach plastocyanin;[16] NMR studies give a pK_a of 4.9,[29] in agreement with the first of these values. The larger value from the kinetic studies (5.5) is attributed to the involvement of carboxylate side chains at the remote acidic patch of plastocyanin, which is used in the case of the $[Co(phen)_3]^{3+}$ oxidant, resulting in a combined (apparent) pK_a value. With amicyanin no similar shift is observed or likely since the nearby charged residues are basic (see below), and these remain protonated over the pH range investigated.

Making allowances for the different conditions employed the acid dissociation pK_a value of 6.6 from kinetic studies is in satisfactory agreement with the value of 6.9 from NMR studies in D_2O at 39 °C, I = 0.05 M and at 20 °C in 100 mM phosphate buffer.[15] The much higher pK_a value as compared to plastocyanin (4.9) reflects differences in the two copper sites. It is interesting that the average pK_a value for amicyanin (6.7) is in the same range as that for a free histidine (5.0–8.0). Comparisons between the amino-acid sequences of different type 1 copper proteins have revealed differences in the spacing between the three ligands (Cys, His and Met) which are located in the C-terminal region of the protein, Table 7.[17] In particular it is noted that the three proteins plastocyanin, pseudoazurin and amicyanin which exhibit active-site protonation/deprotonation in the accessible range of pH have just two amino acids separating the His and Cys residues at the C-terminal end. In addition it is clear that amicyanin is characterised by the smallest spacing (also two) between the histidine and methionine residues. Thus a tighter structure in the active-site region might also be the reason for a higher pK_a value of His-96 (6.7) compared to the corresponding pK_a values for plastocyanin His-87 (4.9) and pseudoazurin His-81 (4.8). The newly identified type 1 protein halocyanin with two residues between the His and Cys residues, Table 7, is a further example which might be used to further test these observations. With plastocyanin a component of the inner thylakoid of the chloroplast at pH < 5,[34,35] active-site protonation and 'switch-off' in reactivity occur after receiving an electron from cytochrome f. This provides protection against electron loss during transport to photosystem I, and is clearly of considerable physiological significance. No similar explanation of the dependence on pH has been forthcoming in the case of pseudoazurin. In the case of amicyanin the oxidation of methylamine to formaldehyde releases two protons as well as two electrons, equation (1). Since the periplasm of methylotrophic bacteria has a pH of ≈7.0, the transfer of electrons from MADH to amicyanin could be accompanied by a proton transfer from the quinol group of the MADH tryptophan tryptophylquinone (TTQ) cofactor. In other words the amicyanin pK_a of 6.7 may have been tuned to enable such a physiological role.

With regard to the identification of possible electron-transfer sites on amicyanin we note that His-96 is exposed to the solvent in a hydrophobic area on the protein.[8–10,13] The corresponding C-terminal histidine ligand is exposed in all type 1 copper

Table 6 Variation of first-order rate constants k_{2obs} (25 °C) with pH and oxidant concentration for the second stage of the $[Co(phen)_3]^{3+}$ oxidation of *T. versutus* $AmCu^I$, I = 0.100 M (NaCl)

pH	$10^4[Co(phen)_3^{3+}]$/M	k_{2obs}/s^{-1}
5.47	3.30	0.072
	6.60	0.072
5.57	3.10	0.075
	6.20	0.076
6.05	5.10	0.071
	10.2	0.090
6.50	3.80	0.071
	8.30	0.071
7.10	4.20	0.073
	8.40	0.068
	9.10	0.069
	13.3	0.072
7.20*	2.67	0.068
	5.35	0.071
	10.7	0.075
7.48	2.60	0.080
	5.10	0.080
	9.10	0.078
8.10	4.80	0.095
	7.69	0.102
	9.75	0.096
8.60	3.20	0.114
	6.60	0.108
8.80	6.60	0.106

* Native $AmCu^I$.

Table 7 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
Cucumber basic protein	His(39)Cys(4)His(4)Met
Amicyanin	His(38)Cys(2)His(2)Met
Rusticyanin[a]	His(52)Cys(4)His(4)Met
Stellacyanin	His(40)Cys(4)His(4)Gln[b]
Halocyanin	His(39)Cys(2)His(4)Met[c]

[a] Sequence information from ref. 30. [b] As proposed in refs. 31 and 32. [c] From ref. 33.

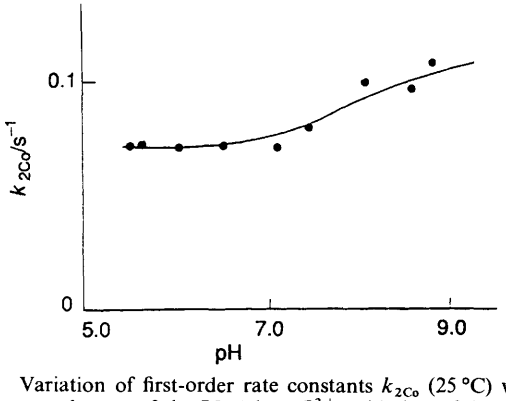

Fig. 5 Variation of first-order rate constants k_{2Co} (25 °C) with pH for the second stage of the $[Co(phen)_3]^{3+}$ oxidation of $AmCu^I$, I = 0.100 M (NaCl)

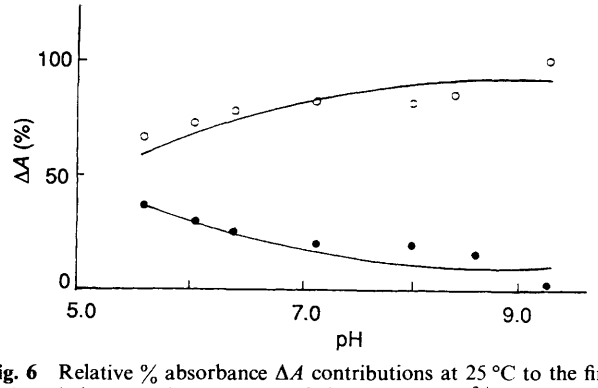

Fig. 6 Relative % absorbance ΔA contributions at 25 °C to the first (○) and the second stages (●) of the $[Co(phen)_3]^{3+}$ oxidation of $AmCu^I$ at different pH values, I = 0.100 M (NaCl)

Table 8 Reduction potentials $E^{o\prime}$/mV and second-order rate constants k_{Fe} and k_{Co}/M^{-1} s^{-1} (25 °C unless otherwise stated) for the oxidation by $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ respectively of copper(I) proteins: plastocyanin from spinach and *Anabaena variabilis*, pseudoazurin from *Achromobacter cycloclastes* (pACu), azurin from *Pseudomonas aeruginosa* (ACu), umecyanin from horseradish (UCu), stellacyanin from *Rhus vernicifera* (SCu), and the first stage for amicyanin from *T. versutus* (AmCu) at pH 7.5, *I* 0.100 M (NaCl)

Protein	$E^{o\prime}$	Charge[a]	$10^{-5}k_{Fe}$	$10^{-3}k_{Co}$	k_{Fe}/k_{Co}	Ref.
PCu (spinach)	375	−9	0.85	2.50	34	16
PCu (*A. variabilis*)	340	+1	7.2	0.57	1260	39
pACu	250[b]	+1	> 26[b]	0.34[b]	> 7600	40
ACu	305	−1	0.08	6.10	1.3	41
UCu	280	−4	28[b,c]	0.30[c]	9300	42
SCu	184	+7[d]	*e*	180[f]	—	43
AmCu	260[f]	−4	83	6.4	1300	This work

[a] Estimated for copper(I) protein at pH ≈ 7.5 (Asp/Glu as 1−, Arg/Lys as 1+, unco-ordinated His as zero). [b] pH 5.6. [c] 5.8 °C. [d] Carbohydrate (40% of total mass) attached to protein. [e] Too fast to monitor by stopped-flow spectrophotometry. [f] pH 7.0.

proteins the crystal structures of which have been determined (plastocyanin,[36] azurin,[37] pseudoazurin,[38] cucumber basic protein[32]). From the crystal structure,[9] and the solution structure derived from NMR data,[12,13] the adjacent His-96 ligand protrudes from the protein surface and is surrounded by hydrophobic residues (three Met, three Pro and one Phe). This feature is also observed in the crystal structure of the MADH–amicyanin complex of *P. denitrificans*.[10] The importance of the adjacent site in the latter case has been substantiated by the observation that His-95 (corresponding to His-96 in *T. versutus*) is located between the MADH redox factor TTQ and the copper centre of amicyanin providing a possible route for electron transfer.[10] It must be pointed out that the same orientation between TTQ and Cu is also maintained in the crystal structure of the (ternary) complex between MADH, amicyanin and cytochrome c_{551i} of *P. denitrificans*.[11]

Second-order rate constants k_{Fe} and k_{Co} at pH ≈ 7.5, I = 0.100 M (NaCl), are compared in Table 8 for the oxidation of different type 1 copper proteins including two plastocyanins, pseudoazurin, azurin, umecyanin, stellacyanin and amicyanin. Reduction potentials and the net charge balance of negative/positive amino acids at pH ≈ 7.5 for the reduced forms of the proteins are also listed. It is clear that the reactivity of amicyanin with both $[Co(phen)_3]^{3+}$ and $[Fe(CN)_6]^{3-}$ is among the highest of the type 1 proteins listed. Similar high reactivity of $[Fe(CN)_6]^{3-}$ is observed also for umecyanin, stellacyanin and pseudoazurin. One contributing factor is the larger driving force for the oxidations of these proteins, Table 8. On the other hand reorganisation-energy requirements at the copper site are expected to be very similar for the different proteins. We are not able to detect any correlation of rate constants (k_{Fe} and k_{Co}) with the overall charge balance on the proteins. This can be explained in terms of different distributions of charge on each protein as well as structural effects such as hydrogen bonding which will affect the estimated charge. It is the local charge (or lack of it) at the reaction site that is more important in the interaction of the protein with small inorganic redox partners.

It has been noted that the nearest charged residues to the adjacent hydrophobic patch on amicyanin are a number of basic residues.[12,13] The distances of the charge on these residues from the N^{ε} (N^{τ}) of His-96 are Arg-69 (≈ 15), Arg-100 (≈ 13), Lys-28 (≈ 12) and Lys-30 (≈ 12 Å). Although somewhat distant the charge distribution favours the reaction with $[Fe(CN)_6]^{3-}$. A second possible reaction site is at or close to Phe-92 with electron transfer occurring *via* Cys-93.[23] The Phe-92 is exposed at the surface of amicyanin and has a more immediate surrounding of basic residues Lys-59, Lys-60, Lys-69 and Arg-100. Because of the close proximity of basic residues this site may better account for the saturation kinetic behaviour here interpreted as association prior to electron transfer. The Phe-92 residue is however further from the copper active site, with a direct Cu to C^{δ} (C^{2}) (Phe-92) distance of 9.1 Å based on the NMR solution structure. A dual-site reactivity has been established previously for plastocyanin,[16] but in this case it is an acidic patch to which the positively charged oxidant $[Co(phen)_3]^{3+}$ associates.[16] Further evidence for $[Fe(CN)_6]^{3-}$ association with $AmCu^{I}$ has been obtained by investigating the effect of redox-inactive paramagnetic $[Cr(CN)_6]^{3-}$ on the ^1H NMR spectrum and the kinetics of the $[Fe(CN)_6]^{3-}$ oxidation.[23] Thus amicyanin is unusual in that it has an overall 4-charge balance, but basic residues positioned near the reaction sites are more influential on reactivity.

The existence of a second stage in the oxidation of $AmCu^{I}$ by both $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ is more difficult to explain. Amicyanin is the only type 1 copper protein exhibiting this sort of biphasic kinetic behaviour, although a similar effect has been noted for the Leu12Glu mutant of spinach plastocyanin.[44] It has to be stressed that in the case of $[Co(phen)_3]^{3+}$ the second stage is independent of oxidant and with $[Fe(CN)_6]^{3-}$ there is also an oxidant-independent term (*a*) which together suggest an intramolecular process. This may relate to the active-site protonation and the smaller number of amino acids between the ligated His, Cys and Met residues at the active site, Table 7. Thus one possibility which needs to be examined is that the deprotonation and reco-ordination of His-96 results in a different conformation of the amino acid linking His-96 to Cys-93 and/or Met-99 respectively. The linear component (*b*) for the $[Fe(CN)_6]^{3-}$ arises from the bimolecular oxidation of the second-less-reactive form of the protein. This step is 7.4×10^3 times slower than the bimolecular first stage (k_{Fe}), indicating a severe impairment of redox capability.

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