Type 1 Blue Copper Protein Amicyanin from *Thiobacillus versutus*: Line-broadening Effects of Chromium(III) Complexes and Related Studies†

Christopher Dennison,[a] Panayotis Kyritsis,[a] Arnout P. Kalverda,[b] Gerard W. Canters,[b] William McFarlane*,[a] and A. Geoffrey Sykes*,[a]

[a] *Department of Chemistry, The University of Newcastle, Newcastle upon Tyne NE1 7RU, UK*

[b] *The Department of Chemistry, Gorlaeus Laboratories, Leiden University, Leiden 2300-RA, The Netherlands*

The effect of redox-inactive cationic and anionic paramagnetic chromium(III) complexes on the ^1H NMR spectrum of the reduced type 1 blue copper protein amicyanin, $AmCu^I$, from *Thiobacillus versutus* has been studied as a means of defining sites for association at the protein surface. With $[Cr(CN)_6]^{3-}$ two sites are detected, one at the adjacent hydrophobic patch close to the exposed imidazole of the co-ordinated His-96, and the other at Phe-92 which has Lys-59, Lys-60, Arg-69 and Arg-100 in close proximity and is adjacent to the active site-co-ordinated Cys-93. In contrast, the cationic complexes $[Cr(NH_3)_6]^{3+}$ and $[Cr(en)_3]^{3+}$ (en = ethane-1,2-diamine) cause no significant line broadening and no preferred sites for association are detected. Kinetic stopped-flow studies on the competitive inhibition by $[Cr(CN)_6]^{3-}$ of the $[Fe(CN)_6]^{3-}$ oxidation of $AmCu^I$ indicate that $[Fe(CN)_6]^{3-}$ reacts at two sites, one of which is inhibited by $[Cr(CN)_6]^{3-}$ and the other is unaffected by $[Cr(CN)_6]^{3-}$. It is suggested that the first of these corresponds to reaction at the Phe-92 site, contributing 25% to the reaction, and the second to reaction at His-96. Therefore, in its reaction with $[Fe(CN)_6]^{3-}$ amicyanin has adjacent and remote binding sites.

Amicyanin, $M_r \approx 11\,000$ (106 amino acids),[1] a component of the methylotroph *Thiobacillus versutus*, mediates electron transfer from the periplasmic protein methylamine dehydrogenase (MADH) to cytochrome c_{550}.[2] Methylamine reacts directly with the tryptophan tryptophylquinone (TTQ) co-factor of MADH with the release of two electrons to two molecules of amicyanin [equation (1)].[3,4] Details of the transfer of the

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

electrons through to cytochrome oxidase, where the reduction of molecular oxygen occurs, and the properties of individual components in the electron-transport chain are of continuing interest.[5]

Oxidised amicyanin from *Paracoccus denitrificans*[6] and *T. versutus*[7] has structural similarities to plastocyanin[8] and pseudoazurin,[9] although sequence homologies of these proteins are not extensive. The active site is very similar to that of plastocyanin with the Cu co-ordinated to His-54, Cys-93, His-96 and Met-99 in *T. versutus* amicyanin. As with plastocyanin[8] and azurin,[10,11] the C-terminal histidine ligand is solvent-exposed in a hydrophobic area of the protein.

The ^1H NMR spectrum of reduced *T. versutus* amicyanin has been assigned,[12] and a three-dimensional solution structure has been obtained[13] which is very similar to the crystal structure of oxidised *T. versutus* amicyanin.[7] The hydrophobic patch around the exposed His-96 includes residues Met-29, Met-52, Pro-53, Met-72, Pro-95, Pro-97 and Phe-98.[7] All of these except Met-52 are conserved in the three known amicyanin amino-acid sequences.[6] In the solution structure from NMR studies the distance of the copper to the N^{τ} of His-96 is ≈4.2 Å. From both kinetic[14] and NMR[12,15,16] investigations it has been demonstrated that the reduced form of amicyanin $AmCu^I$ from *T. versutus* undergoes reversible active-site protonation at His-96 with a pK_a of 6.7. This is greater than the corresponding values for plastocyanin (4.7–5.1)[17] and pseudoazurin (4.7).[18,19] The larger pK_a for amicyanin is relevant to its function[14] since the physiological pH is in this case close to 7.0.

Recent work[14] has focused on the oxidation of amicyanin $AmCu^I$ by the one-electron oxidants $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) (370 mV) and $[Fe(CN)_6]^{3-}$ (410 mV). We now consider selective broadening of certain ^1H NMR resonances by redox-inactive paramagnetic chromium(III) complexes arising from their association at specific regions on the protein.[20–29] In the case of the plastocyanins,[20,21,25–27] studies with $[Cr(CN)_6]^{3-}$ and $[Cr(phen)_3]^{3+}$ have helped to demonstrate dual-site reactivity.[17,30] We now report similar investigations on amicyanin from *T. versutus* using the complexes $[Cr(CN)_6]^{3-}$, $[Cr(NH_3)_6]^{3+}$ and $[Cr(en)_3]^{3+}$ (en = ethane-1,2-diamine). This work was extended to include kinetic studies on the ability of $[Cr(CN)_6]^{3-}$ to inhibit oxidation of $AmCu^I$ by $[Fe(CN)_6]^{3-}$.

Experimental

Isolation and Purification of Amicyanin.—Protein samples were obtained by the procedures previously described.[1,14] Purification of oxidised protein $AmCu^{II}$ was by Pharmacia fast protein liquid chromatography (FPLC) using a Mono-Q column at pH 7.5 to give protein with an absorbance ratio A_{278}/A_{596} = 3.8:1. Concentrations of $AmCu^{II}$ protein were determined at 596 nm (ε = 3900 M^{-1} cm^{-1}).

Kinetic Studies.—The oxidation of $AmCu^I$ by $[Fe(CN)_6]^{3-}$ was monitored at 596 nm on a Dionex D-110 stopped-flow spectrophotometer at 25.0 ± 0.1 °C as was the inhibition of this reaction by the redox-inactive complex $[Cr(CN)_6]^{3-}$. All of the reactions were carried out at pH 4.70 in acetate–acetic acid buffer. The ionic strength of the buffer was adjusted to I = 0.100 ± 0.001 M using NaCl. The stopped-flow apparatus was interfaced to an IBM PC/AT-X computer for data acquisition

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

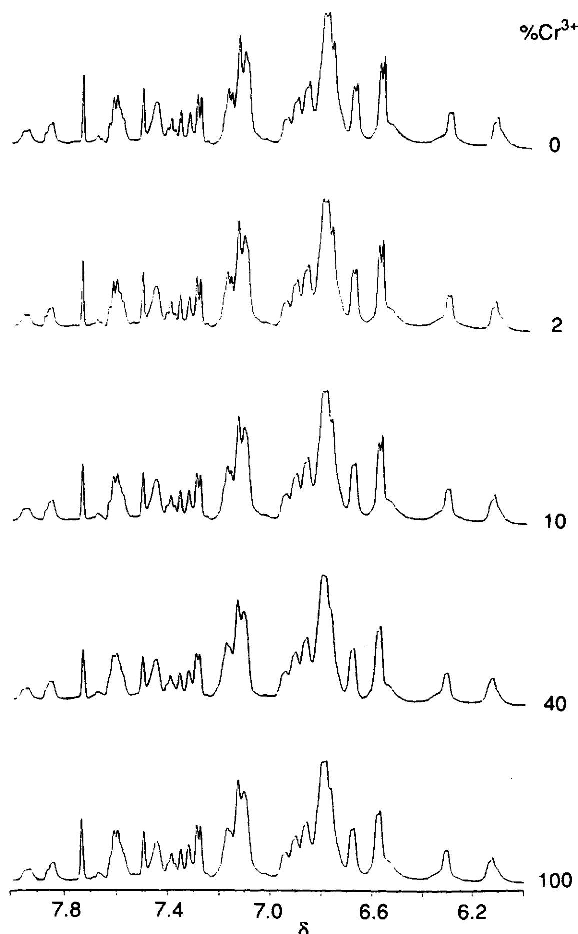

Fig. 1 Part of the aromatic region of the ^1H NMR spectrum (25 °C) of *T. versutus* $AmCu^I$ showing the effect of increasing concentrations of $[Cr(NH_3)_6]^{3+}$ at pH 7.4 (I = 0.10 M)

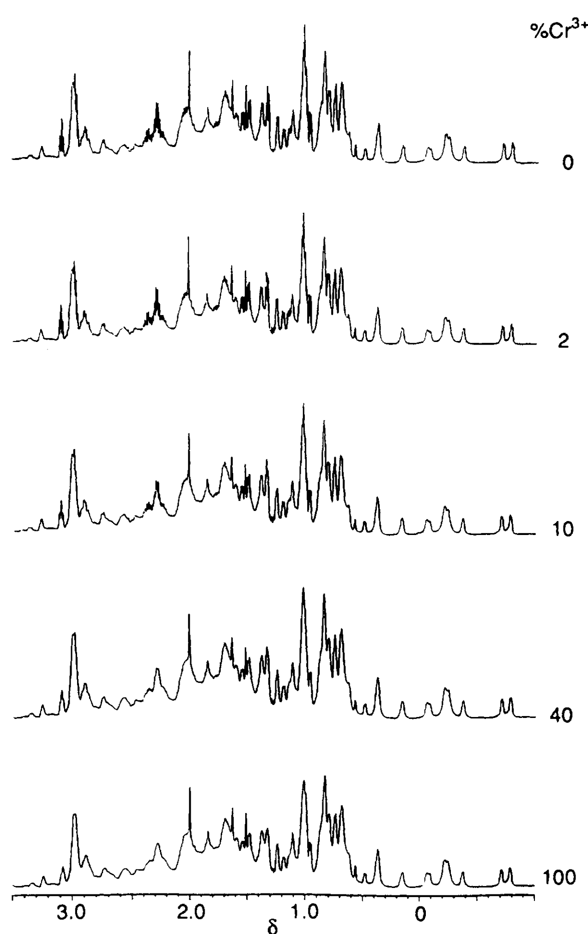

Fig. 2 Part of the aliphatic region of the ^1H NMR spectrum (25 °C) of *T. versutus* $AmCu^I$ showing the effect of increasing concentrations of $[Cr(NH_3)_6]^{3+}$ at pH 7.4 (I = 0.10 M)

using software from On-Line Instrument Systems (Bogart, GA, USA). All rate constants quoted are an average of at least five determinations using the same solutions.

Inorganic Complexes.—Preparations/sources and characterisations by UV/VIS absorbance spectra, peak position λ/nm (ε/M^{-1} cm^{-1}) were as follows: potassium hexacyanochromate(III), $K_3[Cr(CN)_6]$, 351 (63), 458 (73);[31] hexaamminechromium(III) chloride, $[Cr(NH_3)_6]Cl_3{\cdot}H_2O$, 462 (40), 350 (33);[32] tris(ethane-1,2-diamine)chromium(III) chloride, $[Cr(en)_3]Cl_3{\cdot}H_2O$, 457 (73), 351 (63);[33] potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$, 300 (1600), 420 (1010) (BDH, Analar).[34]

NMR Studies.—The protein was exchanged by ultrafiltration (Amicon filter with YM5 membrane) into 99.9% deuteriated 38.2 mM phosphate buffer at pH 7.4 (I = 0.10 M). Some studies were also carried out with $AmCu^I$ in 50 mM phosphate buffer at pH 6.8. Protein solutions, typically 2 mM, were transferred to an NMR tube and flushed with argon. Reduction was by the addition of cooled aliquots of 0.1 M sodium dithionite ($Na_2S_2O_4$, Fluka) in 99.9% D_2O (0.10 M NaOD). The pH of the sample was measured using a narrow CMAWL/3.7/180 pH probe in combination with a Radiometer PHM62 pH probe and was adjusted using NaOD or DCl (0.10 M) as necessary. No corrections were made to the pH for the deuterium isotope effect.

Solutions of the chromium(III) complexes used in the NMR studies (see below) were made up in the deuteriated buffer. Typically, solutions which were 1, 5 and 20 mM were prepared. The stability of chromium(III) solutions in the presence of $Na_2S_2O_4$ was investigated prior to the NMR experiments, and all complexes used were found to be stable for at least 12 h. The chromium(III) solutions were deaerated prior to the NMR experiments and were stored away from light. Appropriate volumes of these solutions were added to the reduced protein, under air-free conditions, to produce the required chromium(III) concentration in the NMR tube (typically in the range 10–2000 μM).

All proton NMR spectra were acquired at 500.14 MHz on a Bruker AMX500 spectrometer at 25 °C described previously.[19] Chemical shifts are in ppm relative to internal dioxane at δ 3.74.

Results

Titration of $AmCu^I$ *with* $[Cr(NH_3)_6]^{3+}$ *and* $[Cr(en)_3]^{3+}$.—Figs. 1 and 2 show the aromatic and δ 3.5 to −1.0 regions of the ^1H NMR spectrum of $AmCu^I$ at increasing concentrations of $[Cr(NH_3)_6]^{3+}$. It is apparent that any broadening effects are very small, even at high concentrations of the chromium complex. Experiments using $[Cr(en)_3]^{3+}$ as well as $[Cr(NH_3)_6]^{3+}$ at pH 6.8 gave similar results.

Titration of $AmCu^I$ *with* $[Cr(CN)_6]^{3-}$.—The complex $[Cr(CN)_6]^{3-}$ has a much more pronounced effect on the ^1H NMR spectrum of $AmCu^I$ than do the cationic chromium(III) complexes. In the aromatic region, Fig. 3 resonances from His-54 (C^2H at δ 7.50 and C^5H at δ 7.35), His-96 (C^2H at δ 7.72 and C^5H at δ 7.32) and Phe-98 (C^3H at δ 7.61, C^4H at δ 7.39 and C^2H at δ 7.28) are all broadened by the addition of appreciable amounts of $[Cr(CN)_6]^{3-}$. The resonances of His-54 broaden substantially less than those of the other two residues. A

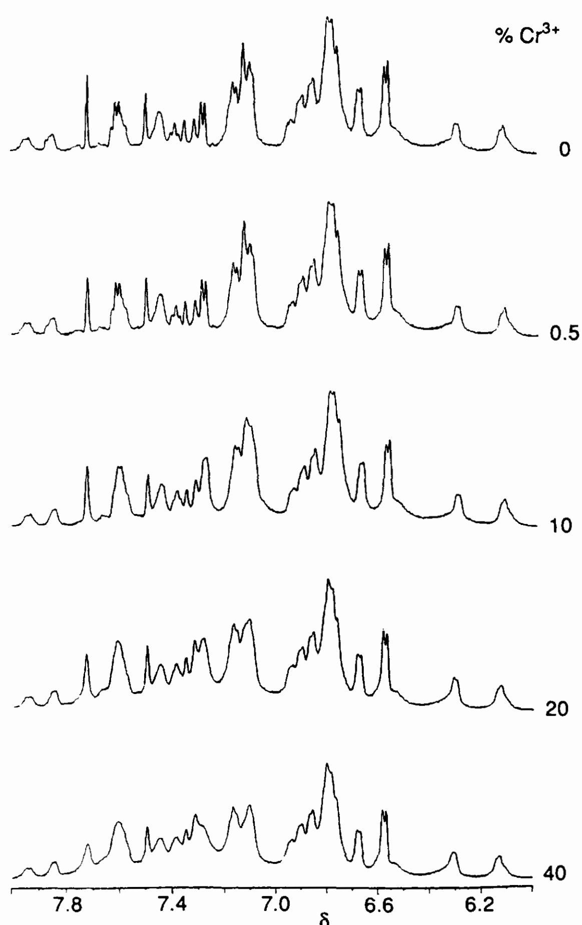

Fig. 3 Part of the aromatic region of the ^{1}H NMR spectrum (25 °C) of *T. versutus* AmCu^I showing the effect of increasing concentrations of $[Cr(CN)_6]^{3-}$ at pH 7.4 (I = 0.10 M)

resonance from Phe-92 (δ ≈7.2) is less well resolved but is probably also broadened. It is important to realise that the effects on the resonances of His-54, His-96 and Phe-98 are specific as shown by the absence of significant effects on aromatic resonances from δ 6 to 7, even at the higher concentrations of $[Cr(CN)_6]^{3-}$.

The broadening effect of $[Cr(CN)_6]^{3-}$ is also greater on the resonances near δ 3 (Fig. 4) which arise from either the $C^{\epsilon}H_2$ or $C^{\delta}H_2$ groups of lysine or arginine residues respectively. A particularly striking effect is seen on the resonances of Lys-59 and Lys-60 (δ 2.74 and 3.08 respectively) when $[Cr(CN)_6]^{3-}$ is present at a level of ≈0.5% of the protein concentration. At 10% $[Cr(CN)_6]^{3-}$ these two resonances are no longer observable. At the higher $[Cr(CN)_6]^{3-}$ concentrations there are noticeable effects on the $C^{\epsilon}H_3$ resonance of Met-29 (δ 2.01) and the resonances at δ 1.63, 1.51, 0.96 and 0.95. However it is evident that the $C^{\epsilon}H_3$ resonance of Met-92 (δ 0.57) is not broadened by $[Cr(CN)_6]^{3-}$. In this region of the ^{1}H NMR spectrum of AmCu^I higher $[Cr(CN)_6]^{3-}$ concentrations also cause broadening of the $C^{\gamma}H_2$ resonance at δ 0.49 which could belong to a C^{γ} signal of Ile-38 or the $C^{\beta}H_3$ of Ala-57. It should be noted that no other upfield-shifted methyl resonances are broadened, even at the higher concentrations of $[Cr(CN)_6]^{3-}$.

Related Kinetic Studies.—Increasing amounts of redox-inactive $[Cr(CN)_6]^{3-}$ were found to decrease the second-order rate constant k_{Fe} (25 °C) for the first phase of the oxidation of AmCu^I (≈1 × 10^{-5} M) by $[Fe(CN)_6]^{3-}$ (1.4 × 10^{-4} M) at pH 4.7, I = 0.100 M as shown in Table 1. The inhibiting effect of $[Cr(CN)_6]^{3-}$ is illustrated in Fig. 5.

A mechanism for the inhibition by $[Cr(CN)_6]^{3-}$ of the first stage of the $[Fe(CN)_6]^{3-}$ oxidation of AmCu^I in terms of prior association of $[Fe(CN)_6]^{3-}$ at a single reaction site on the surface of the protein and blocking of this site by $[Cr(CN)_6]^{3-}$ was tested. However, a plot of $(k_{Fe})^{-1}$ against $[Cr(CN)_6{}^{3-}]$ is non-linear and hence this single-site mechanism can be excluded.

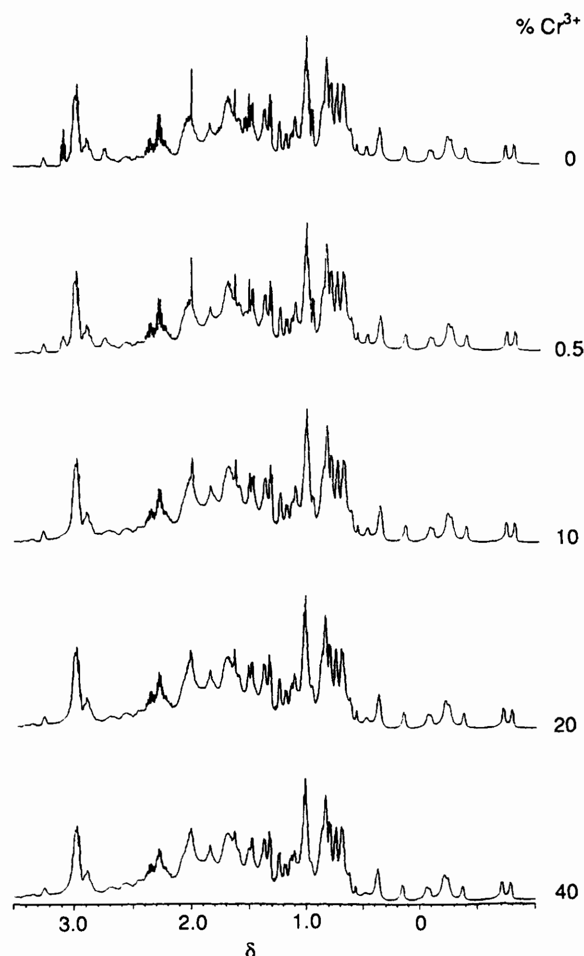

Fig. 4 Part of the aliphatic region of the ^{1}H NMR spectrum (25 °C) of *T. versutus* AmCu^I showing the effect of increasing concentrations of $[Cr(CN)_6]^{3-}$ at pH 7.4 (I = 0.10 M)

Table 1 Effect of increasing amounts of redox-inactive $[Cr(CN)_6]^{3-}$ on the second-order rate constants (25 °C) for the first stage of the oxidation of AmCu^I by $[Fe(CN)_6]^{3-}$ at pH 4.7, I = 0.100 M (NaCl)

$10^3[Cr(CN)_6{}^{3-}]$/M	$10^{-5}k_{Fe}$/M^{-1} s^{-1}
0.00	1.84
0.25	1.72
0.60	1.60
0.85	1.62
1.00	1.55
1.30	1.52
1.40	1.53
1.60	1.50
1.80	1.53
1.90	1.40
2.20	1.43

An alternative two-site mechanism involves initial association of the chromium(III) complex at one site on the amicyanin (site 1) with an equilibrium constant K_{Cr} [equation (2)]. Reaction

$$\mathrm{AmCu^I + Cr^{III} \underset{}{\overset{K_{Cr}}{\rightleftharpoons}} AmCu^I, Cr^{III}} \qquad (2)$$

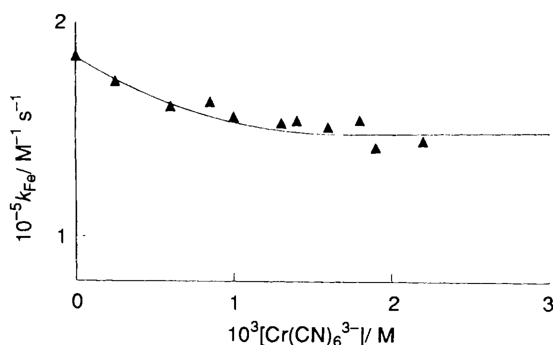

Fig. 5 Dependence of the second-order rate constant (25 °C) for the first stage of the oxidation of *T. versutus* $AmCu^I$ by $[Fe(CN)_6]^{3-}$ on the concentration of $[Cr(CN)_6]^{3-}$ at pH 4.7, I = 0.10 M (NaCl)

with the oxidant at this site can occur only with unbound amicyanin [equation (3)]. Unblocked site 2 can then react

$$AmCu^I + Fe^{III} \xrightarrow{k_1} AmCu^{II} + Fe^{II} \qquad (3)$$

irrespective of whether amicyanin has $[Cr(CN)_6]^{3+}$ bound at site 1, which can be expressed as in equation (4) where $AmCu^I{}_T$

$$AmCu^I{}_T + Fe^{III} \xrightarrow{k_2} AmCu^{II}{}_T + Fe^{II} \qquad (4)$$

refers to the sum of $AmCu^I$ and $AmCu^I, Cr^{III}$. These equations lead to (5).[17] With $k_2 = (1.38 \pm 0.2) \times 10^5$ M^{-1} s^{-1} (from Fig. 5) a plot of $(k_{Fe} - k_2)^{-1}$ against $[Cr(CN)_6{}^{3-}]$ is linear, thus

$$k_{Fe} = \frac{k_1}{1 + K_{Cr}[Cr^{III}]} + k_2 \qquad (5)$$

supporting this mechanism. The plot yielded $(4.8 \pm 0.3) \times 10^4$ M^{-1} s^{-1} and 1780 ± 200 M^{-1} for k_1 and K_{Cr} respectively. The observation that at high concentrations of $[Cr(CN)_6]^{3-}$ the rate constants remain at 75% of the level in the absence of $[Cr(CN)_6]^{3-}$ indicates that 25% of the first phase of the reaction of $AmCu^I$ with $[Fe(CN)_6]^{3-}$ occurs at the site which is blocked by the redox-inactive complex.

In contrast, $[Cr(CN)_6]^{3-}$ increases the first-order rate constant (defined as k_{2Fe} in earlier work [14]) for a less-important second phase of reaction, Table 2 and Fig. 6. It has been demonstrated previously[14] that the second phase of the oxidation of $AmCu^I$ by $[Fe(CN)_6]^{3-}$ has $[Fe(CN)_6{}^{3-}]$-dependent and $[Fe(CN)_6{}^{3-}]$-independent terms as in equation (6). These two terms are attributed to reaction of a less-reactive

$$k_{2obs} = a + b[Fe(CN)_6{}^{3-}] \qquad (6)$$

form of $AmCu^I$ present in amounts between 5 and 40% so that in the presence of $[Cr(CN)_6]^{3-}$ equations (7) and (8) can be

$$Am'Cu^I + Cr^{III} \overset{K'_{Cr}}{\rightleftharpoons} Am'Cu^I, Cr^{III} \qquad (7)$$

$$Am'Cu^I, Cr^{III} \xrightarrow{k} Am''Cu^I, Cr^{III} \qquad (8)$$

invoked where $Am''Cu^I$ may be a product in (8). On the assumption that the acceleration of the second phase by $[Cr(CN)_6]^{3-}$ (Fig. 6) is due to an increase in a then equation (9)

$$k_{eff} = \frac{kK_{Cr}[Cr^{III}]}{1 + K_{Cr}[Cr^{III}]} \qquad (9)$$

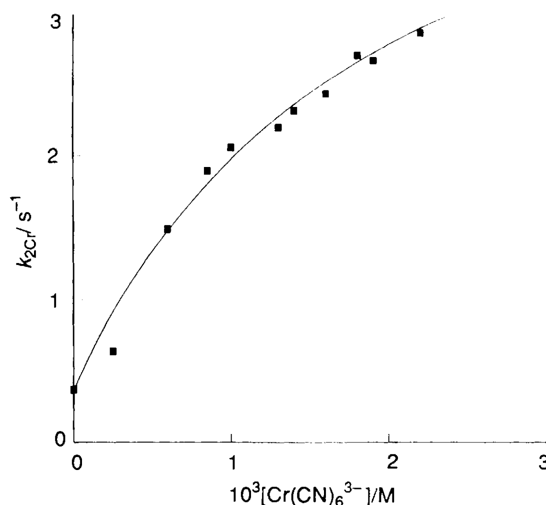

Fig. 6 Dependence of the first-order rate constant (25 °C) for the second stage of the oxidation of *T. versutus* $AmCu^I$ by $[Fe(CN)_6]^{3-}$ on the concentration of $[Cr(CN)_6]^{3-}$ at pH 4.7, I = 0.10 M (NaCl)

Table 2 Effect of increasing amounts of redox-inactive $[Cr(CN)_6]^{3-}$ on the first-order rate constant (25 °C) for the second stage of the oxidation of $AmCu^I$ by $[Fe(CN)_6]^{3-}$ at pH 4.7, I = 0.100 M (NaCl)

$10^3[Cr(CN)_6{}^{3-}]$/M	k_{2Cr}/s^{-1}
0.00	0.37
0.25	0.64
0.69	1.50
0.85	1.90
1.00	2.06
1.30	2.20
1.40	2.32
1.60	2.44
1.80	2.72
1.90	2.68
2.20	2.88

can be derived. Here $k_{eff} = k_{2Cr} - k_{2obs}$, where k_{2Cr} and k_{2obs} are the first-order rate constants in the presence and absence respectively of $[Cr(CN)_6]^{3-}$, and corresponds to the acceleration observed. A plot of $k_{eff}{}^{-1}$ against $[Cr(CN)_6{}^{3-}]^{-1}$ gives a straight line from which a value of K'_{Cr} = 620 M^{-1} is obtained. Although we have quantified this effect it is not considered further because the true origin of the second phase remains uncertain.

Discussion

The NMR and kinetic experiments were carried out at different pH values since at the protein concentrations needed for the NMR studies $AmCu^I$ is unstable below pH 6.5, while at pH > 4.7 the rate of oxidation of $AmCu^I$ by $[Fe(CN)_6]^{3-}$ becomes too fast for the stopped-flow range. Furthermore, saturation kinetic behaviour[14] demonstrates the association of $AmCu^I$ at pH 5.2, and at pH 4.7 the rate constants for the oxidation of amicyanin by $[Fe(CN)_6]^{3-}$ are no longer critically dependent upon pH.[14]

It appears that selective association of cationic complexes with $AmCu^I$ is not significant, even though there was some broadening at high chromium(III) concentrations. Notwithstanding a charge balance of −4 at pH 7.4 there appears to be a much greater affinity of $AmCu^I$ for the anionic complex $[Cr(CN)_6]^{3-}$ suggesting a region of localised positive charge on the protein. Rate constants for the oxidation of $AmCu^I$ (E° 260 mV) by $[Fe(CN)_6]^{3-}$ (410 mV) indicate a fast reaction which might be partially due to a favourable association

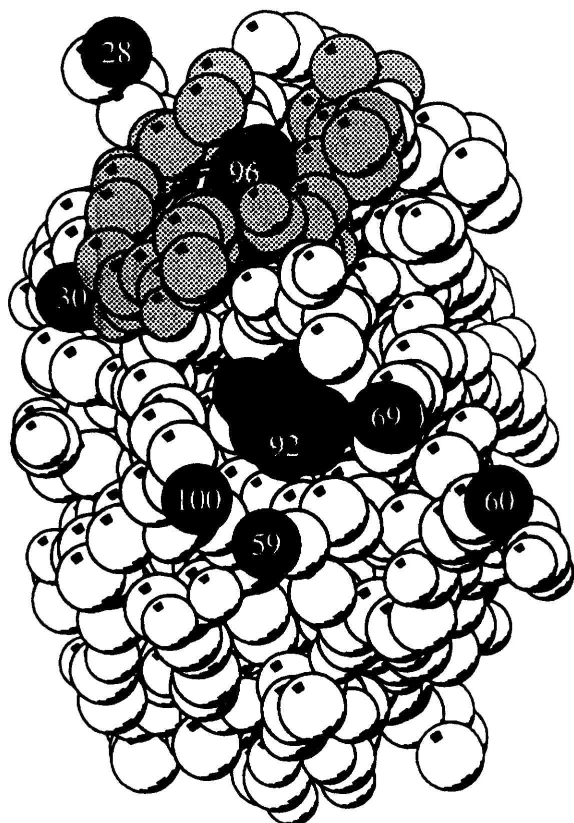

Fig. 7 Space-filling representation of a solution structure of *T. versutus* AmCu^I.[16] Lysine residues have their N$^\zeta$ atoms in black as do the N$^{\eta 1}$ atoms of arginine residues; Phe-92 and His-96 are dark grey, and the hydrophobic patch around His-96 is light grey

constant. Amicyanin has a number of basic residues on the surface surrounding His-96 at the adjacent site, Fig. 7, but these are not close (> 10 Å) to the exposed imidazole ring. There are several positively charged residues close to Phe-92, the residue which aligns with the exposed Tyr-83 at the acidic patch on plastocyanin. The role of the negative charge in this area in the reactivity of plastocyanin has been discussed.[17,30,35,36] The positive charges in the case of amicyanin make this area electrostatically favourable for association with $[Fe(CN)_6]^{3-}$ and $[Cr(CN)_6]^{3-}$.

Even at low concentrations of $[Cr(CN)_6]^{3-}$ the resonances from Lys-59 and Lys-60 are broadened. In the three-dimensional structure of the protein[13] these two residues are adjacent to Phe-92, Fig. 7. In addition, at the higher concentrations of $[Cr(CN)_6]^{3-}$ a resonance from Phe-92 may be broadened, supporting this region of amicyanin as a site for association of $[Cr(CN)_6]^{3-}$. The Arg-100 residue is also close to Phe-92, but its C^2H_2 resonance is unaffected by high $[Cr(CN)_6]^{3-}$ concentrations. The residue Phe-92 is not conserved in the three known amicyanin sequences which suggests that *in vivo* this region has at most variable contributions to make in the context of electron transfer.

It is clear that $[Cr(CN)_6]^{3-}$ has a much greater affinity than $[Cr(NH_3)_6]^{3+}$ for the adjacent site of AmCu^I. However, it should be noted that the association is not as favourable as that involving $[Cr(CN)_6]^{3-}$ with Phe-92. The broadening of the resonance at δ 0.49 in the presence of $[Cr(CN)_6]^{3-}$ may indicate a third binding site involving Ile-38 despite an ambiguity of assignment, but this is too far removed from the active site to be involved in electron-transfer processes of low driving force. If the resonance arises from Ala-57, which is adjacent to Phe-92, the complete broadening by $[Cr(CN)_6]^{3-}$ provides further evidence for a highly favoured interaction of the complex at this site on the protein.

Kinetic experiments on the blocking by $[Cr(CN)_6]^{3-}$ of the $[Fe(CN)_6]^{3-}$ oxidation of AmCu^I at pH 4.7 also indicate the presence of two sites on the surface of the protein able to interact with anionic complexes. {An alternative mechanism in which $[Fe(CN)_6]^{3-}$ reacts at a single site on the protein, which is blocked by $[Cr(CN)_6]^{3-}$, was tested and found not to be applicable.} It was demonstrated that one of the sites contributes 25 and the second 75% to the reaction observed. From the NMR studies these are the adjacent site (near to His-96) and the region close to Phe-92.

The NMR studies indicate that the association of $[Cr(CN)_6]^{3-}$ with AmCu^I is more favourable at the Phe-92 site. We therefore believe that this is the site blocked in the kinetic experiments and we assign approximately 25% of the first stage of the reaction of AmCu^I with $[Fe(CN)_6]^{3-}$ to reaction at the remote Phe-92. This remote site implies an electron-transfer pathway from Phe-92 *via* Cys-93 to copper. A similar pathway has been identified involving the remote acidic site in plastocyanin (generally Cu–Cys-Tyr but in at least one case Cu–Cys-Phe[17]). Analogous intramolecular electron-transfer pathways are also found in the multicopper enzymes ascorbate oxidase (Cu-Cys-His–Cu_3)[37] and nitrite reductase (Cu–Cys-His–Cu).[38] Theoretical studies further support dual-site reactivity in blue copper proteins[39,40] as do resonance-Raman measurements which indicate a highly conserved Cu–Cys moiety in this class of proteins.[41] Crystallography shows a coplanar arrangement of the five atoms (Cu–S^γ–C^β–C^α–N) involved, indicating that this orientation is optimised for electron transfer.

The second site on amicyanin at which electron transfer occurs with $[Fe(CN)_6]^{3-}$ is the adjacent one involving the exposed His-96. All structurally characterised blue copper proteins possess, to some degree, a hydrophobic patch close to the active site, through which a histidine ligand protrudes.[6–11] The site has been implicated in electron-transfer processes,[36,42–50] and in the case of amicyanin X-ray crystallographic evidence has been obtained for interactions with MADH at this site.[46–48] A kinetic study of electron transfer between amicyanin and MADH from *P. denitrificans* has recently been reported,[49] and indicates an electron-transfer pathway involving Trp-108 of MADH. In this approach it is assumed that the electrons are delocalised over both ring systems of TTQ and there is a 3.6 Å through-space jump involving Pro-94 and His-95 of amicyanin.

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