# Kinetic, Spectroscopic, and Structural (Extended *X*-Ray Absorption Fine Structure) Studies on the Type 1 Blue Copper Protein Umecyanin†

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The effects of pH variations on the visible spectrum ($pK_a$ 9.46 ± 0.04), e.s.r. spectrum, extended *X*-ray absorption fine structure (EXAFS), and on the reactivity of the blue copper protein umecyanin have been investigated. The electron-transfer reactions of umecyanin have been studied with the complexes $[Co(dipic)_2]^-$ (dipic = dipicolinate) and $[Co(C_2O_4)_3]^{3-}$ as oxidants for UCu(I), and $[Ru(NH_3)_5(py)]^{2+}$ (py = pyridine) as a reductant for UCu(II), at 25 °C, $I$ = 0.10 M (NaCl). The oxidation of umecyanin with $[Fe(CN)_6]^{3-}$ is extremely rapid, consequently a limited study was carried out at a lower temperature. The oxidation of the protein by $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) was also studied under these conditions for comparison. Of interest are the $pK_a$ values observed for the oxidation of umecyanin by $[Co(C_2O_4)_3]^{3-}$ ($pK_a$ 9.68 ± 0.08) and the reduction of umecyanin by $[Ru(NH_3)_5(py)]^{2+}$ ($pK_a$ 9.50 ± 0.07). The low temperature (20 K) *X*-band e.s.r. spectrum of oxidised umecyanin ($g_{\|}$ = 2.32, $g_{\perp}$ = 2.06) shows little variation over the pH range 7.5—10.5. The Cu *K*-edge EXAFS of umecyanin in both oxidation states and at pH 7.5 and 10.5 were obtained. Analysis of data from the oxidised protein shows the Cu atom has two nitrogen ligands at 1.99 ± 0.03 Å and one sulphur at 2.13 ± 0.02 Å. In the reduced protein the bond lengths increase to 2.03 ± 0.03 Å and 2.21 ± 0.02 Å respectively. There was no detectable difference in co-ordination number or bond lengths at the different pH values, and no fourth ligand could be detected by the EXAFS technique. The results from these various studies are discussed in terms of changes at or near the active site of umecyanin.

Umecyanin from horseradish roots (function unknown) consists of a single polypeptide ($M$ 14 600)[1] of approximately 125 amino acids,[2] and a single Cu active site which exhibits the characteristic type 1 e.s.r. spectrum.[3] It has an isoelectric point of 5.85.[2] The sequence of the first 88 residues shows considerable homology with other blue copper proteins, 38% of the residues in umecyanin are identical to those found in stellacyanin.[4] Current spectroscopic and sequence data are consistent with two histidines and a cysteine co-ordinating to the copper.[3-5] The absence of a methionine after position 74 supports the idea that (like stellacyanin and unlike plastocyanin and azurin) umecyanin does not have methionine as a co-ordinated ligand.[5] The estimated charge for reduced umecyanin, UCu(I), from the amino-acid composition is 11− at pH 7. A redox potential of 283 mV (at pH 7.0) has been reported for the protein.[6]

All oxidised type 1 copper proteins have characteristic visible spectra with a prominent band at ~600 nm.[7,8] The visible spectrum of oxidised umecyanin, UCu(II), is dependent upon pH. This dependence was also observed in preliminary kinetic studies on the protein, using simple inorganic complexes as redox partners. With these results in mind it was decided to investigate further the reactivity of both UCu(I) and UCu(II), and to combine these studies with e.s.r. and extended *X*-ray absorption fine structure (EXAFS) investigations on the Cu site of the protein.

## Experimental

*Proteins.*—Umecyanin was isolated from horseradish roots (*Armoracia laphatifolia*), and purified to an absorbance peak ratio for UCu(II) of $A_{280}/A_{610}$ < 4.3 (usually 3.7) which has been shown to be characteristic of pure umecyanin.[9] The protein was reduced, when required, with ascorbate or dithionite followed by dialysis. Oxidation was performed similarly using $K_3[Fe(CN)_6]$. The protein was recovered from kinetic experiments by first dialysing out the inorganic complex, followed by concentrating the protein on a short DEAE52 column. After concentration the protein was repurified to the required absorbance ratio by passage down a G-75 gel filtration column.

Azurin from *Pseudomonas aeruginosa* (used as a model protein in the EXAFS study) was supplied by Microbiological Products, Porton, U.K. and purified by standard chromatographic techniques to an absorbance peak ratio for the oxidised protein of $A_{280}/A_{625}$ = 1.70.[10]

*Complexes.*—These were obtained and purified to known spectra [λ/nm (ε/$M^{-1}$ $cm^{-1}$)] by procedures already described: penta-ammine(pyridine)ruthenium(II) perchlorate, $[Ru(NH_3)_5(py)][ClO_4]_2$, 407 (7 800);[11] potassium tris(oxalato)cobaltate(III), $K_3[Co(C_2O_4)_3]\cdot 3.5H_2O$, 596 (167), 420 (221), 245 ($2.2 \times 10^4$);[12] ammonium bis(dipicolinato)cobaltate(III), $NH_4[Co(dipic)_2]\cdot H_2O$, 510 (630);[13] potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (B. D. H. AnalaR), 300 (1 600), 420 (1 010); tris(1,10-phenanthroline)cobalt(III) chloride, $[Co(phen)_3]Cl_3\cdot 7H_2O$, 330 (4 660), 350 (3 620), and 450 (100). Redox potentials for these complexes are listed in Table 1.

*Buffers and* pH.—Tris(hydroxymethyl)aminomethane (Sigma Chemicals) ('Tris') (20 mM), borate (12.5 mM), carbonate (25 mM), and glycine (10 mM) buffers were prepared by published procedures.[14] The ionic strength of these buffer

† *Non-S.I. units employed:* M = mol $dm^{-3}$, eV ≈ $1.60 \times 10^{-19}$ J.

**Table 1.** Reduction potentials relevant to the present studies

| Complex | $E^{\circ}$/mV | Ref. |
|---|---|---|
| $[Ru(NH_3)_5(py)]^{2+}$ | 273 | *a* |
| $[Co(C_2O_4)_3]^{3-}$ | 570 | *b* |
| $[Co(dipic)_2]^{-}$ | 747 | *c* |
| | 400 | 13 |
| $[Fe(CN)_6]^{3-}$ | 410 | *d* |
| $[Co(phen)_3]^{3+}$ | 370 | *d* |

[a] D. Cummins and H. B. Gray, *J. Am. Chem. Soc.*, 1977, **99**, 5158. [b] H. F. Lee and W. C. E. Higginson, *J. Chem. Soc. A*, 1967, 298. [c] N. H. Williams and J. K. Yandell, *Aust. J. Chem.*, 1983, **36**, 2377. [d] S. K. Chapman, C. V. Knox, P. Kathirgamanathan, and A. G. Sykes, *J. Chem. Soc., Dalton Trans.*, 1984, 2769.

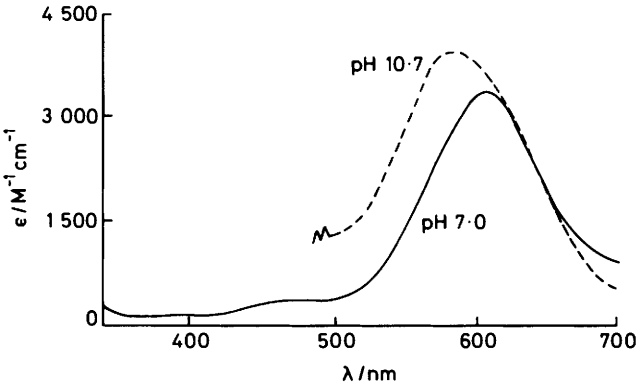


**Figure 1.** The effect of pH on the visible spectrum of oxidised umecyanin, UCu(II)

solutions was adjusted to a final value of 0.10 M with NaCl. All pH values were checked using a Radiometer (PHM 62) pH-meter fitted with a Russell (CWR/322) glass electrode. The pH of frozen solutions (used in e.s.r. and EXAFS experiments) was determined by the indicator dye method of Orii and Morita.[15]

*Kinetics.*—All solutions were at an ionic strength of 0.10 M (NaCl). The inorganic complex was in at least 10-fold excess over the protein. Reactions were monitored at the umecyanin absorbance peak (~600 nm depending on pH) using a Dionex D-110 stopped-flow spectrophotometer. The absorbance changes were stored digitally using a Datalab DL901 transient recorder interfaced to a Commodore PET 2001-16K desk-top computer. A simple program displayed first-order rate constants, $k_{obs.}$, and plots of $\ln|A_t - A_\infty|$ against time. All absorbance changes were consistent with 1:1 stoicheiometries as in equations (1) and (2). First-order plots were linear to at

$$UCu(I) + Co^{III} \longrightarrow UCu(II) + Co^{II} \quad (1)$$

$$Ru^{II} + UCu(II) \longrightarrow Ru^{III} + UCu(I) \quad (2)$$

least four half-lives. The computation of $pK_a$ values was carried out using an unweighted non-linear least-squares program.[16]

*E.S.R.*—E.s.r. spectra were collected at around 9.1 GHz on a Varian E-9 spectrometer fitted for low temperature (20 K in this case) operation. Samples were frozen in liquid $N_2$ before data collection.

*EXAFS.*—*X*-Ray absorption measurements were carried out at the Cornell High Energy Synchrotron Source (CHESS) which runs in parasitic mode from the 8 GeV Cornell Electron Storage Ring (CESR) at the Wilson laboratory of Cornell University.[17] All experiments were performed on the C-1 beam line. The separated Si(III) crystal monochromator was controlled by a CAMAC system yielding a useful energy range of 3.2—21.0 keV, scannable in 0.2 eV steps. A series of monochromator cave and hutch slits were adjusted to achieve 2 eV energy resolution. The intensity of the incident radiation ($I_0$) was measured with a $N_2$ flow ionisation chamber. Samples were loaded into a cryostat unit (from Johnson Research Foundation, University of Pennsylvania), and kept at *ca.* 150 K under a He atmosphere with a liquid $N_2$ cooled heat-exchange apparatus. Fluorescence photon intensities were counted by an Ar flow detector (EXAFS Co., Seattle).[18] A nickel foil (12.7-μm thick, McKay Metals) was placed directly before the soler slits. Control of individual experimental parameters was achieved with an LSI-11/23 computer. EXAFS spectra were collected, typically, over a period of 5—10 min, with no significant sample deterioration. The *X*-ray absorption data were analysed by the method of Teo and co-workers.[19-22] Summed raw absorption data were normalised to the edge jump and fitted with a three-sectioned third-order polynomial spline function to remove low frequency contributions. The theoretical amplitude and phase functions of Teo and Lee[21] were used in conjunction with model compounds as outlined in the BFBT and FABM methods.[22,23] Azurin was chosen as a model blue copper protein because of the already available crystallographic[24,25] and EXAFS[26] information. As well as the protein, a copper(II)–α-aminoisobutyric acid tripeptide complex,[27] supplied by Professor D. W. Margerum (Purdue University, Indiana), and copper(II) sulphide (used as a precipitate suspended in a glycerol–water mixture) were used as models.

## Results

UCu(II) *Visible Spectrum.*—The visible spectrum of oxidised umecyanin, UCu(II), is dependent upon pH. The peak position shifts from 610 nm at pH 7.0 (ε 3 300 $M^{-1}$ $cm^{-1}$) to 580 nm at pH 10.7 (ε 4 000 $M^{-1}$ $cm^{-1}$), see Figure 1, consistent with earlier observations.[28] Changes are reversible and give rise to an observed $pK_a$ of 9.46 ± 0.04, provided that an excess of $[Fe(CN)_6]^{3-}$ (~$5 \times 10^{-4}$ M) is present to keep the protein fully oxidised. Interconversions were found to be complete within the mixing time (~5 ms) of the stopped-flow. At pH values > 11.2 absorbance changes are no longer reversible.

*Kinetics.*—Observed rate constants, $k_{obs.}$, gave linear first-order dependences on the concentration of inorganic complex in all cases (over the concentration ranges studied). The simple rate law (3) applies. The variation of rate constants, $k$, with pH

$$\text{Rate} = k[UCu][Complex] \quad (3)$$

are interpreted in terms of the sequence (4)—(6). Equation (7) may be derived from this sequence.

$$H^+UCu \overset{K_a}{\rightleftharpoons} UCu + H^+ \quad (4)$$

$$UCu + Complex \xrightarrow{k_o} Products \quad (5)$$

$$H^+UCu + Complex \xrightarrow{k_H} Products \quad (6)$$

$$k = \frac{k_o K_a + k_H[H^+]}{K_a + [H^+]} \quad (7)$$

Rate constants (25 °C) for the reduction of UCu(II) by $[Ru(NH_3)_5(py)]^{2+}$ are dependent on pH (Table 2) and this dependence can be seen in Figure 2. A good fit to equation (7)

**Table 2.** Rate constants for the $[Ru(NH_3)_5(py)]^{2+}$ reduction of umecyanin UCu(II) ($\sim 1.0 \times 10^{-5}$ M) at 25 °C, $I = 0.10$ M (NaCl). Kinetic runs at pH > 8.5 were by the pH-jump method

| pH | Buffer | $10^4[Ru^{II}]$/M | $k_{obs.}$/s$^{-1}$ | $10^{-3}k$/M$^{-1}$ s$^{-1}$ |
|---|---|---|---|---|
| 7.39 | Tris | 2.20 | 1.40 | 6.4 |
| | | 4.82 | 2.93 | 6.1 |
| 8.12 | Tris | 2.56 | 1.59 | 6.2 |
| | | 5.32 | 3.29 | 6.2 |
| 8.55 | Tris | 2.60 | 1.59 | 6.1 |
| | | 5.74 | 3.41 | 5.9 |
| 9.09 | Tris | 4.50 | 2.42 | 5.4 |
| 9.50 | Borate | 1.04 | 0.51 | 4.9 |
| 9.95 | Borate | 0.70 | 0.27 | 3.9 |
| 10.50 | Borate | 0.41 | 0.15 | 3.7 |
| | | 0.77 | 0.27 | 3.5 |
| 10.65 | Borate | 0.80 | 0.28 | 3.5 |

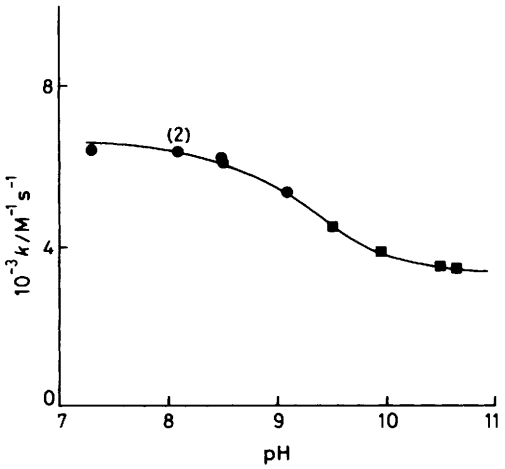


**Figure 2.** The pH dependence of rate constants, $k$, for the reduction of umecyanin, UCu(II), by $[Ru(NH_3)_5(py)]^{2+}$ at 25 °C, $I = 0.10$ M (NaCl). Buffers: Tris (●) or borate (■). The line corresponds to the non-linear least-squares fit of points. Number of runs shown in parentheses

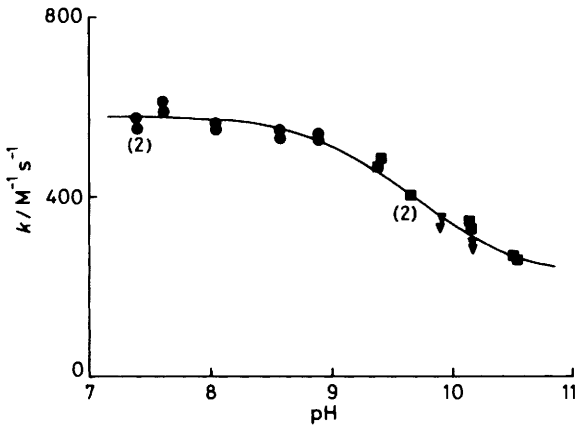


**Figure 3.** The pH dependence of rate constants, $k$, for the oxidation of umecyanin, UCu(I), by $[Co(C_2O_4)_3]^{3-}$ at 25 °C, $I = 0.10$ M (NaCl). Buffers: Tris (●), borate (■), or carbonate (▼). The line corresponds to the non-linear least-squares fit of points. Number of runs shown in parentheses

is obtained with $k_H = (6.3 \pm 0.1) \times 10^3$ M$^{-1}$ s$^{-1}$, $k_o = (3.2 \pm 0.1) \times 10^3$ M$^{-1}$ s$^{-1}$, and a p$K_a$ of 9.50 ± 0.07. Rate constants (25 °C) for the oxidation of UCu(I) by $[Co(C_2O_4)_3]^{3-}$ are also dependent on pH (see Table 3, Figure 3). Values from the fit to equation (7) are $k_H = 580 \pm 10$ M$^{-1}$ s$^{-1}$, $k_o =$

**Table 3.** Rate constants for the $[Co(C_2O_4)_3]^{3-}$ oxidation of umecyanin UCu(I) ($\sim 1 \times 10^{-5}$ M) at 25 °C, $I = 0.10$ M (NaCl). Kinetic runs at pH > 9.0 were by the pH-jump method

| pH | Buffer | $10^4[Co^{III}]$/M | $k_{obs.}$/s$^{-1}$ | $k$/M$^{-1}$ s$^{-1}$ |
|---|---|---|---|---|
| 7.40 | Tris | 7.8 | 0.44 | 570 |
| | | 14.3 | 0.79 | 550 |
| | | 19.2 | 1.06 | 550 |
| 7.62 | Tris | 2.8 | 0.17 | 610 |
| | | 5.7 | 0.33 | 590 |
| 8.05 | Tris | 5.2 | 0.29 | 570 |
| | | 8.9 | 0.49 | 550 |
| 8.57 | Tris | 3.2 | 0.18 | 540 |
| | | 6.3 | 0.34 | 530 |
| 8.90 | Tris | 3.8 | 0.20 | 540 |
| | | 8.0 | 0.42 | 530 |
| 9.38 | Borate | 7.3 | 0.34 | 460 |
| 9.41 | Borate | 5.0 | 0.24 | 480 |
| 9.65 | Borate | 4.5 | 0.18 | 400 |
| | | 8.4 | 0.33 | 400 |
| 9.87 | Carbonate | 3.6 | 0.12 | 330 |
| 9.89 | Carbonate | 5.2 | 0.18 | 350 |
| 10.15 | Borate | 2.7 | 0.09 | 330 |
| | | 4.9 | 0.17 | 340 |
| 10.15 | Carbonate | 4.0 | 0.12 | 300 |
| | | 8.0 | 0.23 | 290 |
| 10.52 | Borate | 5.0 | 0.13 | 269 |
| 10.57 | Borate | 6.9 | 0.19 | 267 |

**Table 4.** Rate constants for the $[Co(dipic)_2]^-$ oxidation of umecyanin UCu(I) ($\sim 1.0 \times 10^{-5}$ M) at 25 °C, $I = 0.10$ M (NaCl). Kinetic runs at pH > 9.0 were by the pH-jump method

| pH | Buffer | $10^4[Co^{III}]$/M | $k_{obs.}$/s$^{-1}$ | $10^{-4}k$/M$^{-1}$ s$^{-1}$ |
|---|---|---|---|---|
| 7.20 | Tris | 4.9 | 39.0 | 8.0 |
| 8.06 | Tris | 2.0 | 16.5 | 8.3 |
| | | 7.3 | 58.3 | 8.0 |
| 8.90 | Tris | 1.4 | 11.1 | 8.1 |
| | | 2.6 | 20.8 | 8.1 |
| | | 7.7 | 60.2 | 7.9 |
| 9.63 | Borate | 1.3 | 9.7 | 7.4 |
| 9.65 | Borate | 3.2 | 24.5 | 7.7 |
| 9.87 | Carbonate | 2.1 | 17.0 | 8.0 |
| | | 3.0 | 22.7 | 7.7 |
| 10.12 | Borate | 1.5 | 11.0 | 7.3 |
| 10.17 | Borate | 3.7 | 29.5 | 8.0 |
| 10.47 | Borate | 1.8 | 13.5 | 7.7 |
| | | 6.1 | 48.1 | 7.9 |

223 ± 19 M$^{-1}$ s$^{-1}$, with a p$K_a$ of 9.68 ± 0.08. The variation of rate constants (25 °C) for the oxidation of UCu(I) by $[Co(dipic)_2]^-$ (dipic = pyridine-2,6-dicarboxylate) appears to be only very slightly dependent on pH (Table 4). The dependence is so mild that an accurate determination of the p$K_a$ was not possible, however it would appear to be in the region of 9.5. The oxidation of UCu(I) by $[Fe(CN)_6]^{3-}$ is so rapid that only a limited stopped-flow study was possible at a lower temperature, 5.8 °C. At this temperature the second-order rate constant (pH 7.55) was approximately $2.8 \times 10^6$ M$^{-1}$ s$^{-1}$. Under the same conditions the second-order rate constant for the oxidation of UCu(I) by $[Co(phen)_3]^{3+}$ (phen = 1,10-phenanthroline) was 301 ± 25 M$^{-1}$ s$^{-1}$ (Table 5).

UCu(II) *E.S.R. Spectrum.*—The e.s.r. spectrum (9.1 GHz, 20 K, 0.5 mW) of oxidised umecyanin at pH 7.5 is shown in Figure 4 ($g_{\|} = 2.32$ and $g_{\perp} = 2.06$) and is very similar to that obtained by Stigbrand *et al.*[3] at pH 5.7 ($g_{\|} = 2.317$ and $g_{\perp} = 2.05$). At pH 7.5—10.5 the e.s.r. spectrum shows little change, and over this pH range the spectrum remains completely axial.

**Table 5.** Rate constants for the $[Co(phen)_3]^{3+}$ oxidation of umecyanin UCu(I) ($\sim 1.0 \times 10^{-5}$ M) at 5.8 °C, pH 7.55, $I = 0.10$ M (NaCl); $k = 301 \pm 25$ $M^{-1}$ $s^{-1}$.

| $10^3[Co^{III}]/M$ | $k_{obs.}/s^{-1}$ |
|---|---|
| 0.23 | 0.101 |
| 0.76 | 0.197 |
| 1.33 | 0.36 |
| 2.54 | 0.73 |
| 3.25 | 0.91 |
| 3.7 | 1.23 |
| 4.5 | 1.36 |

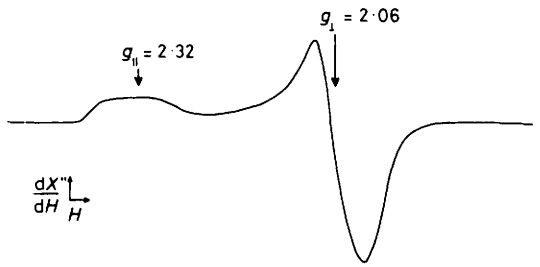


**Figure 4.** The second derivative e.s.r. spectrum of umecyanin, UCu(II), at pH 7.5. Conditions: temperature 20 K, microwave power 0.5 mW, frequency 9.1 GHz

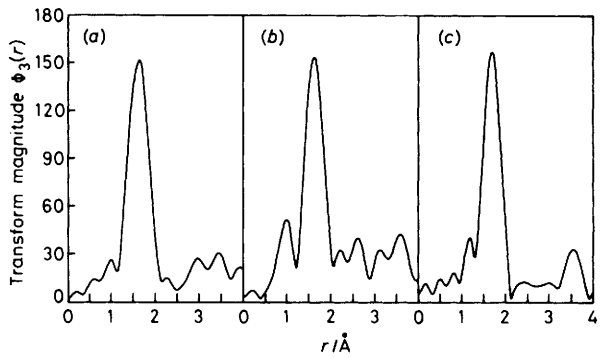


**Figure 5.** Fourier transforms of EXAFS data for: (*a*) umecyanin, UCu(II), at pH 7.5 (Tris); (*b*) umecyanin, UCu(II), at pH 10.5 (glycine); (*c*) azurin, ACu(II), at pH 7.5 (Tris). Notice the strong similarity of the transform magnitude and position of the major peak in each case. [$\varphi_3(r)$ is the relative transform magnitude]

*EXAFS*.—The Fourier transforms of the EXAFS data for oxidised umecyanin at pH 7.5 and 10.5 can be seen in Figure 5 (*a*) and (*b*). Figure 5(*c*) shows the Fourier transform of oxidised azurin (pH 7.5). There are very strong similarities in terms of magnitudes and positions of the peaks in these Fourier transforms. The Fourier filtered data from umecyanin in oxidised and reduced states, and at both pH values, could only be fitted well to a combination of both nitrogen and sulphur terms. An example of these fits may be seen in Figure 6. The Cu–N and Cu–S distances and co-ordination numbers are listed in Table 6. Both the Cu–N and Cu–S bond lengths increase upon reduction of UCu(II) to UCu(I), 1.99 to 2.03 for Cu–N and 2.13 to 2.21 Å for Cu–S (see Table 6). This results in a change in the phase of the data which can be seen clearly in a comparison of the filtered data for UCu(I) and UCu(II) shown in Figure 7.

The potential model compounds examined by EXAFS gave fits in good agreement with previous structural characterisations [azurin,[24-26] copper(II) tripeptide,[27] copper(II) sulphide[29]] and provided the characteristic values used in the FABM method.[22] The striking similarity between the Cu sites in azurin and umecyanin is evident from Table 6, the largest difference in bond lengths being only 0.02 Å.

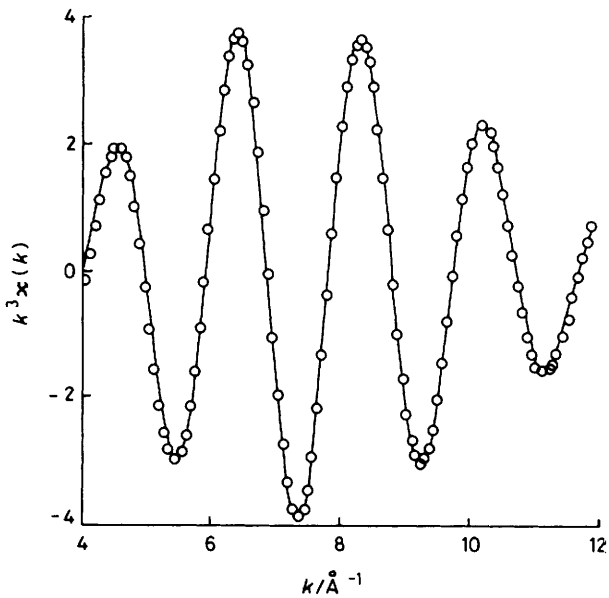


**Figure 6.** Typical Fourier filtered data(——) for umecyanin UCu(II), showing the excellent fit (○) obtained using a combination of both nitrogen and sulphur terms. The numerical results of such fits may be seen in Table 6 (*k* represents the wave factor)

No significant differences were seen in distance or co-ordination number around the Cu in umecyanin at pH 10.5 and 7.5. Also no fourth ligand to the Cu was detected by the EXAFS technique. This was also the case for azurin where the long Cu–S(methionine) bond ($\sim 2.9$ Å) was not detected.

## Discussion

The shift in peak position in the visible spectrum of UCu(II) with change in pH (Figure 1) must correspond to a change at or near the copper site sufficient to affect the chromophore. One possibility is that a residue close to the Cu site (an amine?) may deprotonate and move closer to the copper. It is important to point out however that the change in the visible spectrum of UCu(II) with pH (7.5—10.5) is no greater than the difference between the visible spectra of plastocyanin and azurin. It is known from crystallographic data that these two proteins have very similar copper active sites.[24,30] In a crystallographic study of azurin from *Alcaligenes denitrificans* it has been noted that the O atom of a peptide carbonyl group approaches to within 3.2 Å of the Cu (there is a large error associated with this value).[25,*] A similar type of effect may occur in the case of umecyanin. The fact that changes in the visible spectrum of UCu(II) are irreversible above pH 11.2 is most probably due to the formation of a denatured biuret-type of $Cu^{2+}$ complex, as noticed in the case of stellacyanin by Malmstrom *et al.*[31]

The pH dependences noted for the oxidation and reduction of umecyanin with inorganic complexes (Figures 2 and 3) are mild when compared to those observed for the oxidation of plastocyanin and similar to those observed for other blue copper proteins.[32,33] In addition, the oxidation state of the Cu in umecyanin has surprisingly little influence on the observed

* *Note added in proof*: This structural feature has been confirmed and better defined in a recent paper: G. E. Norris, B. F. Anderson, and E. N. Baker, *J. Am. Chem. Soc.*, 1986, **108**, 2784.

**Table 6.** EXAFS structural parameters, according to FABM [22] (fit adjustment based on models), obtained from Cu–N and Cu–S fits to filtered data from umecyanin (and azurin for comparison) in oxidised and reduced states. Data obtained for umecyanin at pH 7.5 (Tris) and 10.5 (glycine) gave identical results to within error limits. Data for azurin were collected at pH 7.5 (Tris) only

| | Cu–N | | Cu–S | |
|---|---|---|---|---|
| | Co-ordination number | Bond length $r$/Å | Co-ordination number | Bond length $r$/Å |
| Oxidised umecyanin UCu(II) | 2.2 ± 0.5 | 1.99 ± 0.03 | 1.2 ± 0.5 | 2.13 ± 0.02 |
| Reduced umecyanin UCu(I) | 2.1 ± 0.6 | 2.03 ± 0.03 | 1.1 ± 0.3 | 2.21 ± 0.02 |
| Oxidised azurin ACu(II) | 2.0[a] | 1.97 ± 0.02[b] | 1.0[a] | 2.12 ± 0.02[b] |
| Reduced azurin ACu(I) | 2.3 ± 0.6 | 2.00 ± 0.03 | 1.2 ± 0.4 | 2.22 ± 0.02 |

[a] Known from crystallographic studies (the methionine known to be at 2.9 Å was not detected by EXAFS) (refs. 24, 25). [b] This value for oxidised azurin is the same (within error limits) as that previously reported in ref. 26.

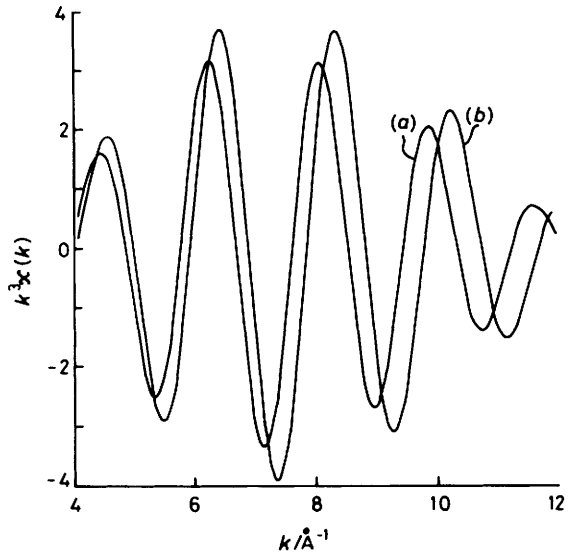


**Figure 7.** Comparison of Fourier filtered data for (*a*) reduced umecyanin UCu(I), and (*b*) oxidised umecyanin, UCu(II). The difference in phase between the two waves can be clearly seen, and arises because of the increase in Cu–N and Cu–S bond lengths upon reduction of the Cu in umecyanin

p$K_a$ values (9.5 for $Cu^{II}$ and 9.68 for $Cu^{I}$). These effects of pH on reactivity are consistent with the idea that the deprotonation in umecyanin is occurring without any residues adjacent to the Cu becoming fully co-ordinated, which would be expected to produce a much larger effect. The small pH effect seen with umecyanin may be compared to the large effect noted with reduced plastocyanin (in the pH range 4.0—7.0),[32] which is known to correspond to a change in co-ordination from four to three.[34] We found no evidence for a corresponding switch off in the reactivity of UCu(I), in fact, with $[Co(C_2O_4)_3]^{3-}$ as an oxidant, rate constants increase (rather than decrease) upon lowering the pH to 4.3.

The ratio between the rate constants for the $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ oxidation of umecyanin ($k_{Fe}/k_{Co}$) is $10^4$. This is the highest value yet seen with blue copper proteins (for parsley plastocyanin it is 30). The ratio is important in the case of plastocyanin where it is believed that two sites for electron transfer exist on the protein, one used by negatively charged complexes *e.g.* $[Fe(CN)_6]^{3-}$, the other by positively charged complexes *e.g.* $[Co(phen)_3]^{3+}$.[33] A comparison of this ratio for a whole series of metalloproteins will be the topic of a future report.

The e.s.r. spectrum of umecyanin (Figure 4) is clearly typical of a type 1 blue copper protein. The fact that the spectrum changes little over the pH range 7.5—10.5 is consistent with the idea, already mentioned, of only a small change occurring at the Cu site. Also the e.s.r. spectrum of umecyanin is completely axial over this pH range which would suggest a Cu site more similar to azurin than stellacyanin.[3]

Results from the EXAFS of umecyanin, UCu(II), at pH 7.5 and 10.5 are essentially identical (within error analysis). This supports the idea that the three closest ligands, two histidines and one cysteine, are relatively unaffected by the deprotonation as the pH increases. Unfortunately it was not possible to detect a fourth ligand in umecyanin using the EXAFS technique, which is not surprising when we consider that the long Cu–S(methionine) bonds present in azurin and plastocyanin are not detected by EXAFS.[26,35] Similarly the possibility of a group moving closer to the copper on deprotonation would not be detected by EXAFS unless the movement brought the group into direct co-ordination with the copper at a distance of < 2.9 Å. Thus the EXAFS results are consistent with the spectroscopic and reactivity studies already mentioned in supporting the idea of only a slight change at the Cu site upon increasing the pH.

A dramatic change in the phase of the EXAFS spectrum is seen upon reduction of umecyanin (Figure 7). Analysis of the data has shown that this arises from increased Cu–N and Cu–S bond lengths (Table 6). This type of change has also been seen (with EXAFS) in the Cu sites of plastocyanin and stellacyanin.[36] These observations may help us to explain some of the unusual properties of the blue copper proteins such as their high redox potentials.

The similarity of the Cu site in umecyanin to that in azurin can be seen by comparing the EXAFS results listed in Table 6. These two proteins have almost identical circular dichroism spectra over the whole wavelength region (at neutral pH).[9] The redox potential of umecyanin has been reported to be 283 mV at pH 7.0,[6] which lies within the values reported for a range of

azurins at this pH (*P.aeruginosa* azurin 300 mV,[37] *Pseudomonas denitrificans* 230 mV[38]). Umecyanin also has marked similarities to stellacyanin including a high degree of amino acid sequence homology (38% of residues identical between the two proteins).[4] Both umecyanin and stellacyanin contain some carbohydrate, however the amounts are quite different (40% carbohydrate for stellacyanin,[39] but only 3.7% for umecyanin[2]). It is possible that umecyanin may come from the same phylogenetic branch as stellacyanin in the family tree of type 1 blue copper proteins.

The nature of a possible fourth ligand to the copper of umecyanin has not yet been resolved (as is also the case with stellacyanin) and as we have already seen EXAFS is unable to provide us with the answer. In the case of stellacyanin it has been suggested that the fourth ligand may be a disulphide group and this could not be ruled out by resonance Raman studies.[40] However if, as has been suggested, this disulphide bond is formed during preparation of samples for sequence determination, then two free cysteine residues may be available for co-ordination to the copper.[40,41] If a cysteinyl sulphur is indeed a ligand it is presumably at such a distance from the copper that it does not contribute significantly to the 600 nm band in the visible spectrum.[40] In the case of umecyanin, carboxymethylation experiments are consistent with there being a disulphide bridge between residues 87 and 93.[4] Consequently we are left in a similar situation to that of stellacyanin, and a disulphide or a cysteinyl sulphur remain as possible fourth ligands to the copper.

## Acknowledgements

We should like to thank Dr. B. K. Teo and Dr. E. C. Niederhoffer for help with the EXAFS analysis. A portion of this work was supported by USPH (U. S. Public Health). We are grateful to the S.E.R.C. for postdoctoral (to S. K. C. and J. McG.) and postgraduate (to J. D. S-D.) support.

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*Received 2nd October* 1985; *Paper* 5/1708