Electron-Transfer Properties and Active-Site Structure of the Type 1 (Blue) Copper Protein Umecyanin

Christopher Dennison, Gonzalez Van Driessche, Jos Van Beeumen, William McFarlane and A. Geoffrey Sykes*

Abstract: The electron self-exchange rate constant for the Type 1 blue copper protein umecyanin from horseradish roots has been determined as $6.1 \times 10^3\ M^{-1}s^{-1}$ at pH 7.5, $I = 0.100$ M, 25 °C by an NMR line-broadening method. The value obtained is one of the lower self-exchange rate constants determined for this class of protein; this is attributed to the presence of positively charged residues near to the electron-transfer site. The self-exchange rate constants calculated by means of a Marcus analysis of data for the cross-reactions (25 °C) of umecyanin with azurin and cytochrome c_{551} (both from *Pseudomonas aeruginosa*) are substantially less at $8.0\ M^{-1}s^{-1}$ and $13.9\ M^{-1}s^{-1}$, respectively, and are independent of pH in the range 7.0–8.0, $I = 0.100$ M. The discrepancy between the self-exchange rate constants obtained by these two different methods can be rationalised if it is assumed that umecyanin reacts with the two proteins employed in the cross-reaction studies through the same site, but that this site is different from that used for the self-exchange process. A comparison of the primary structure of umecyanin with those of other Type 1 copper proteins has revealed that a glutamine rather than a methionine is likely as the fourth ligand of Cu at the active site. Other comparisons are made with stellacyanin, and the electron-transfer reactivity of the two proteins is discussed.

Keywords
copper proteins · cross-reactions · electron-transfer reactions · kinetics · metalloproteins

Introduction

The structures of five different Type 1 copper proteins, namely, plastocyanin, azurin, cucumber basic protein (CBP), amicyanin and pseudoazurin, have been determined by X-ray crystallography.[1–6] The Cu at the active site is coordinated by two histidines (N donors) and a cysteine (S donor). In all these cases the thioether of a methionine provides a further weak axial interaction with the copper, and in the case of azurin there is a second axial interaction with the carbonyl oxygen of a glycine. The blue copper protein stellacyanin does not have a methionine in its primary structure,[7, 8] and different active site coordination is therefore implied.

The crystal structure of CBP is noteworthy because the sequence is 46% homologous to that of stellacyanin,[9] and has been used as a starting point to create a model of stellacyanin.[10] Such studies indicate that stellacyanin may have a glutamine as its fourth ligand. The coordination of the copper by the oxygen of a glutamine is also proposed[10] as the reason for stellacyanin having the lowest known reduction potential of any blue copper protein (184 mV).[11] Site-directed mutagenesis studies of azurin have demonstrated that the axial methionine is not essential for the creation of a Type 1 blue copper site.[12, 13] Similar studies of plastocyanin[13] and the Type 1 copper site of nitrite reductase[14] have so far failed to produce a Cu^{II} binding active site. The fact that the Met121Gln azurin mutant appears to possess many of the properties of oxidised stellacyanin[15] also indicates the presence of an axial glutamine ligand in stellacyanin.

The copper protein umecyanin from horseradish roots (*Armoracia laphatifolia*), first isolated by Paul and Stigbrand,[16] has not been studied in such detail. The Cu^{II} state has characteristic properties of a Type 1 protein, namely a strong absorption in the visible spectrum, in this case at 610 nm, and an X-band EPR spectrum with narrow hyperfine coupling.[17] The reduction potential for the Cu^{II}/Cu^{I} couple is 283 mV at pH 7.0.[18]

The electron-transfer reactivities of umecyanin[19] and stellacyanin[20–22] with small inorganic complexes have been investigated. It is interesting to note that stellacyanin generally displays a high reactivity, with no apparent discrimination between cationic and anionic complexes. This suggests that both types of reactant use the same binding site on the protein and that this is uncharged. The self-exchange rate constant of stellacyanin at pH 7.0 has been determined as $1.2 \times 10^5\ M^{-1}s^{-1}$ at 20 °C.[23] Umecyanin, on the other hand, exhibits a distinct preference for anionic reactants.[19] In order to investigate the electron-transfer properties of umecyanin further, we have determined the self-exchange rate constant directly by an NMR method and indirectly by studying cross-reactions.

An earlier report found umecyanin to consist of 125 residues.[24] The sequence of the first 88 residues indicated some homologies with stellacyanin and the possibility that methion-

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0947-6539/96/0201-0104 $ 10.00+.25/0

ine was not involved in coordination of the copper. The complete sequence has now been determined, and His44, Cys85, His90 and Gln95 have been proposed as the amino acids coordinating the Cu.[25] We discuss here some of the wider implications of this proposal and compare the sequence with those of other Type 1 copper proteins.

Results

Assignment of proton resonances: Umecyanin has three histidine residues; that at position 65 is uncoordinated. The two singlets in the aromatic region of the ^{1}H NMR spectrum of reduced umecyanin whose chemical shifts are dependent upon pH can be assigned to this residue. The singlets were selected in the way described previously for pseudoazurin.[26] The C^ε proton is assigned on the basis of the greater chemical shift difference between its resonance positions in the protonated and deprotonated forms of the residue.

The aromatic regions of the ^{1}H NMR spectra of UCu^I, UCu^{II} and of a 1:1 mixture of the two proteins are shown in Figure 1.

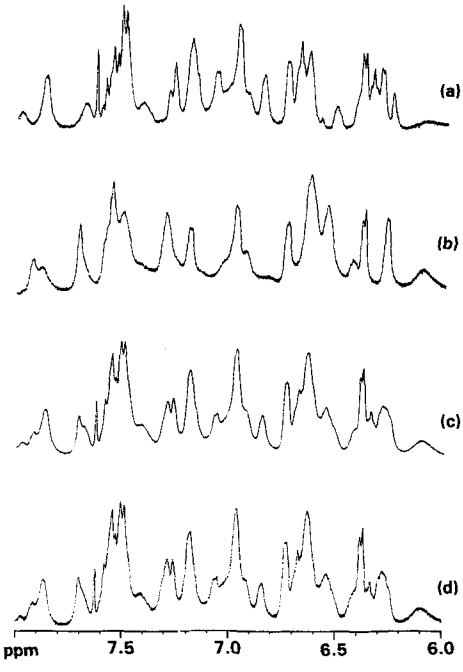

Fig. 1. Part of the aromatic region of the ^{1}H NMR spectrum (25 °C) of umecyanin at pH 7.50, $I = 0.100$ M: a) reduced protein, b) oxidised protein, c) 1:1 mixture of oxidised and reduced protein, d) sum of spectra a and b.

Along with the titrating His65 resonance at $\delta = 7.62$ (at pH 7.50) there are many other singlets in the aromatic region of the reduced form of umecyanin, including resonances at $\delta = 7.59$, 7.51 and 7.26. Upon oxidation of the protein many of these are broadened and only three readily observable singlets remain. Two of these are from His65 and the third must be from a C^δ proton of one of the Trp residues. This is most probably Trp23 as the homologous residues to Trp11 have been shown to be close to the active site in both CBP[3] and stellacyanin.[10]

In the reduced protein it is possible to identify four singlets at $\delta = 2.10$, 2.05, 2.03 and 2.01 that are all $C^\varepsilon H_3$ resonances of Met residues (Fig. 2). In the oxidised protein three singlet resonances are seen at $\delta = 2.03$, 2.02 and 2.00. The resonance at $\delta = 2.10$ has no counterpart in the oxidised protein and therefore must arise from protons that are within 10 Å of the Cu^{II} at the active site. It follows that this resonance arises from the $C^\varepsilon H_3$ of Met43, which is adjacent to His44, one of the proposed copper ligands. There is also a signal at $\delta = 1.96$ in the reduced protein that appears to be two singlets very close to each other. In the oxidised protein these two resonances have shifted to $\delta = 1.91$.

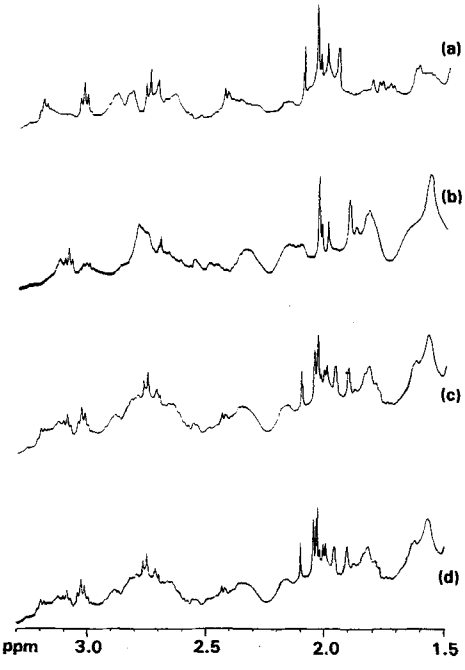

Fig. 2. Part of the aliphatic region of the ^{1}H NMR spectrum (25 °C) of umecyanin at pH 7.50, $I = 0.100$ M: a) reduced protein, b) oxidised protein, c) 1:1 mixture of oxidised and reduced protein, d) sum of spectra a and b.

Differences are also observable between the ^{1}H NMR spectra of reduced and oxidised umecyanin in the region around $\delta = 3$ where the $C^\varepsilon H_2$ and $C^\delta H_2$ residues of lysine and arginine characteristically appear. In the reduced protein there are obvious triplets at $\delta = 3.20$ and 3.03. In the oxidised protein the resonance at $\delta = 3.03$ is broadened whilst that at $\delta = 3.20$ is shifted to $\delta = 3.09$. Either of these resonances could be from Lys96, which is conserved in the amino-acid sequences of umecyanin, stellacyanin and CBP (Fig. 3) and is adjacent to the proposed Gln95 ligand (in umecyanin), or from Lys12, which is also thought to be close to the active site.[25]

CBP	Tyr	Phe	Ile	Cys	Asn	Phe	Pro	Gly	His	Cys	Gln	Ser	Gly	Met	Lys	90
SCu	Tyr	Tyr	Ile	Cys	Gly	Val	Pro	Lys	His	Cys	Asp	Leu	Gly	Gln	Lys	98
CPC	Tyr	Phe	Val	Cys	Thr	Val	Gly	Thr	His	Cys	Ser	Asn	Gly	Gln	Lys	100
UCu	Tyr	Tyr	Ile	Cys	Thr	Val	Gly	Asp	His	Cys	Arg	Val	Gly	Gln	Lys	96

Fig. 3. Part of the C-terminal sequence of umecyanin (UCu) aligned with those of cucumber basic protein (CBP), stellacyanin (SCu) and cucumber peeling cupredoxin (CPC). With the exception of Met89 of CBP, the residues shown inside a border are conserved in all four sequences whilst those inside a double border are proposed ligands.

  0947-6539/96/0201-0105 $ 10.00+.25/0

Determination of the electron self-exchange rate constant of umecyanin by NMR: At pH 7.50 the His65 $C^{\varepsilon}H$ resonance is a sharp signal at $\delta = 7.62$ in the reduced protein (Fig. 1a). In the oxidised protein this resonance appears at a different chemical shift position as one of the three singlets in the aromatic region of the spectrum, and in a 1:1 mixture of the two forms at a concentration of 0.66 mM all four resonances are still present (Fig. 1). It therefore follows that the rate of self-exchange at this concentration is relatively low on an NMR timescale. An indication of the self-exchange rate constant can be gained from the fact that the $C^{\varepsilon}H_3$ resonances at $\delta = 2.01$ and 2.00 of one of the Met residues in the reduced and oxidised protein, respectively, have not coalesced in the 1:1 mixture (Fig. 2). At the coalescence point in a 1:1 mixture of the two forms of the protein the self-exchange rate constant k_{ese} can be obtained from Equation (1).[27] With

$$k_{ese} = \frac{\pi \nu_o |\delta^{ox} - \delta^{red}|}{\sqrt{2[UCu^{II}]}} \quad (1)$$

$\nu_o|\delta^{ox}-\delta^{red}| = 5.0$ Hz an upper limit of $2 \times 10^4\,M^{-1}s^{-1}$ is obtained for the self-exchange rate constant of umecyanin. The spectrum of the 1:1 mixture of the two forms of the protein and the sum of the spectra of the oxidised and reduced forms, Figures 1 and 2, show a close similarity to each other, but a small amount of broadening of several resonances in the former. This broadening is due to the self-exchange process $UCu^{I} + UCu^{II} \rightleftharpoons UCu^{II} + UCu^{I}$, and from the amount of broadening the self-exchange rate constant k_{ese} can in principle be determined from Equation (2), where k_{obs} can be determined from the

$$k_{ese} = \frac{k_{obs}}{[UCu^{II}]} \quad (2)$$

broadening of certain resonances $(\pi\Delta\nu_{1/2})$.[26, 28–31] The results obtained by measuring peak widths are given in Table 1.

Because the rate of self-exchange in umecyanin was so low, it seemed appropriate to determine it more accurately by measuring T_2 values of suitable resonances by means of spin-echo experiments on fully reduced protein and comparing them with the T_2 values of the corresponding peaks in a 1:1 mixture of the two forms of the protein. In this case,[26, 28–31] $k_{obs} = |T_{2red}^{-1} - T_{2ox}^{-1}|$. A plot of $\ln(I_t)$ against $2t$ for the Met43 $C^{\varepsilon}H_3$ resonance in the fully reduced protein and in a 1:1 mixture with the concentration of UCu^{II} at 0.66 mM is shown in Figure 4.

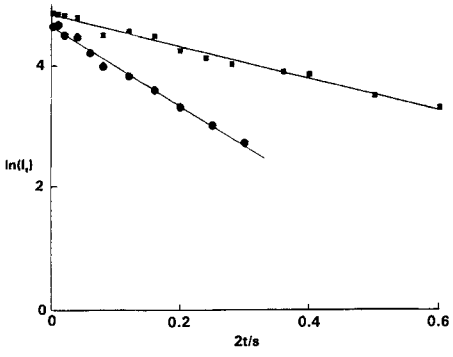

Fig. 4. Plot of $\ln(I_t)$ against $2t$ in the spin-echo experiment (25 °C) for the Met43 $C^{\varepsilon}H_3$ resonance ($\delta = 2.10$) in UCu^{I} (■) and in a 1:1 mixture with $[UCu^{II}]$ equal to 0.66 mM (●) at pH 7.50, $I = 0.100$ M.

Table 2. Self-exchange rate constants k_{ese} (25 °C) for umecyanin from T_2 determinations with a spin-echo pulse sequence at pH 7.50, $I = 0.100$ M.

$[UCu^{I}] = [UCu^{II}] = 3.7 \times 10^{-4}$ M		$[UCu^{I}] = [UCu^{II}] = 6.6 \times 10^{-4}$ M	
Resonance (δ)	$10^{-3} k_{ese}/M^{-1}s^{-1}$	Resonance (δ)	$10^{-3} k_{ese}/M^{-1}s^{-1}$
His65 $C^{\varepsilon}H$ (7.62)	6.7	His65 $C^{\varepsilon}H$ (7.62)	6.4
Met43 $C^{\varepsilon}H_3$ (2.10)	6.8	Met43 $C^{\varepsilon}H_3$ (2.10)	6.0
Met $C^{\varepsilon}H_3$ (2.05)	6.0	Met $C^{\varepsilon}H_3$ (2.05)	5.0

All the self-exchange rate constants obtained by this method are shown in Table 2.

Oxidation of UCu^{I} with ACu^{II}: Relevant UV-Vis spectra are shown in Figure 5. First-order rate constants k_{obs} (25 °C) obtained for the oxidation of UCu^{I}, concentration $(5-10) \times 10^{-5}$ M, with ACu^{II} ($\approx 5 \times 10^{-6}$ M), are consistent with the rate law, Equation (3). Second-order rate constants k_{12} were

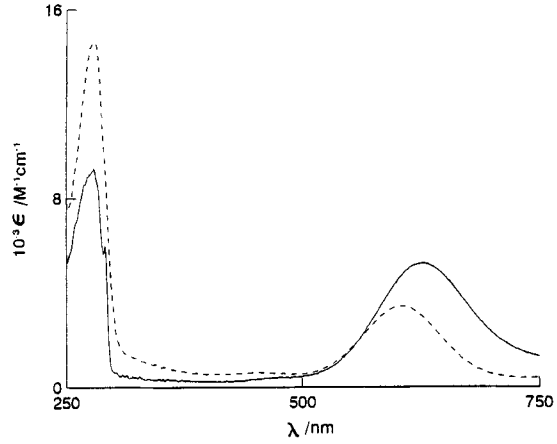

Fig. 5. UV/Vis absorption spectra of ACu^{II} (—) and UCu^{II} (- - -).

$$\text{rate} = k_{obs}[UCu^{I}] = k_{12}[UCu^{I}][ACu^{II}] \quad (3)$$

found to be independent of pH in the range 7.0–8.1, $I = 0.100$ M (NaCl) (Table 3).

Table 3. Second-order rate constants k_{12} (25 °C) for the oxidation of UCu^{I}, $(5-10) \times 10^{-5}$ M, by ACu^{II} ($\approx 5 \times 10^{-6}$ M) in the pH range 7.0–8.1, $I = 0.100$ M (NaCl).

pH	7.02	7.07	7.45	7.55	7.57	8.06
$10^{-3} k_{12}/M^{-1}s^{-1}$	3.9	4.2	3.9	4.3	4.8	4.2

Reduction of UCu^{II} with ferrocytochrome c_{551}: A similar dependence of first-order rate constants k_{obs} on $[UCu^{II}]$ for the reduction of UCu^{II} (concentration $6-13 \times 10^{-6}$ M) with ferrocytochrome c_{551} ($\approx 5 \times 10^{-7}$ M) was observed. Second-order rate constants k_{12} (25 °C) showed no dependence on pH in the range 7.0–8.4 (Table 4).

Table 1. Self-exchange rate constants k_{ese} (25 °C) for umecyanin obtained from NMR peak-width measurements at pH 7.50, $I = 0.100$ M.

$[UCu^{I}] = [UCu^{II}] = 3.3 \times 10^{-4}$ M		$[UCu^{I}] = [UCu^{II}] = 3.7 \times 10^{-4}$ M		$[UCu^{I}] = [UCu^{II}] = 4.0 \times 10^{-4}$ M		$[UCu^{I}] = [UCu^{II}] = 6.6 \times 10^{-4}$ M	
Resonance (δ)	$10^{-3} k_{ese}/M^{-1}s^{-1}$	Resonance (δ)	$10^{-3} k_{ese}/M^{-1}s^{-1}$	Resonance (δ)	$10^{-3} k_{ese}/M^{-1}s^{-1}$	Resonance (δ)	$10^{-3} k_{ese}/M^{-1}s^{-1}$
His65 $C^{\varepsilon}H$ (7.61)	4.9	His65 $C^{\varepsilon}H$ (7.62)	7.1	His65 $C^{\varepsilon}H$ (7.62)	7.5	His65 $C^{\varepsilon}H$ (7.61)	5.0
Met43 $C^{\varepsilon}H_3$ (2.10)	5.3	Met43 $C^{\varepsilon}H_3$ (2.10)	6.5	Met43 $C^{\varepsilon}H_3$ (2.10)	6.7	Met43 $C^{\varepsilon}H_3$ (2.10)	5.9

0947-6539/96/0201-0106 $ 10.00+.25/0 Chem. Eur. J. 1996, 2, No. 1

Table 4. Second-order rate constant k_{12} (25 °C) for the reduction of UCu^{II} ($\approx 7 \times 10^{-6}$ M) by ferrocytochrome c_{551} ($\approx 5 \times 10^{-7}$ M) in the pH range 7.0–8.4, $I = 0.100$ M (NaCl).

pH	7.02	7.19	7.55	7.60	7.65	8.03	8.38
$10^{-4} k_{12}/M^{-1}s^{-1}$	1.25	1.25	1.32(4) [a]	1.29	1.41	1.47	1.26

[a] First-order rate constants give a linear dependence on 10^5[UCu^{II}] with the latter in the range $(0.6–1.3) \times 10^{-5}$ M.

Determination of the self-exchange rate constant from cross-reaction studies: In Marcus calculations with Equation (4),[32–33] a value of 311 mV was used as the reduction potential of azurin,[34] and a value of $7.5 \times 10^5 M^{-1}s^{-1}$ was used for the azurin self-exchange rate constant (k_{11}),[35] both at pH 7.0. For the

$$k_{12}^2 = k_{11} k_{22} K_{12} f \quad (4)$$

reaction involving cytochrome c_{551} a reduction potential for this protein of 260 mV (pH 7.0)[36] and a self-exchange rate constant (k_{11}) of $4.6 \times 10^6 M^{-1}s^{-1}$ at 25 °C and pH 7.0 were used.[37] From these values and a reduction potential of 283 mV for umecyanin (pH 7.0) the calculated self-exchange rate constants for umecyanin (k_{22} in this instance) are $8.0 M^{-1}s^{-1}$ and $13.9 M^{-1}s^{-1}$, from the studies with azurin and cytochrome c_{551}, respectively. The driving force of the reactions is small and consequently f in Equation (4) is close to 1. Since the rate constants in Tables 3 and 4 do not vary over the pH range 7.0–8.0, the self-exchange rate constants obtained are assumed to apply over at least this range of pH.

Discussion

The self-exchange rate constant for umecyanin determined by NMR methods is $6.1 \times 10^3 M^{-1}s^{-1}$. This value, obtained from an average of data obtained with UCu^{II} at 0.66 mM (Table 1), is relatively small compared with values for other Type 1 blue copper proteins. The lack of structural information for umecyanin hinders interpretation, but some valid points can be made. From the amino-acid sequence, the charge on UCu^{II} at neutral pH is -4, and it therefore seems unlikely that the overall charge on umecyanin is responsible for the small self-exchange rate constant. The rate constant for the oxidation of UCu^{I} by $[Co(phen)_3]^{3+}$ is small, $300 M^{-1}s^{-1}$ at pH 7.55 and 5.8 °C,[19] for a driving force ($\Delta E^{\circ\prime}$) of 87 mV. The rate constant (25 °C) for the same oxidant with stellacyanin is very large ($1.8 \times 10^5 M^{-1}s^{-1}$, driving force of 186 mV); it has been suggested that this is due to the presence of a more accessible active site in the case of stellacyanin.[10, 20, 21] However, for stellacyanin the oxidation of SCu^{I} by $[Fe(CN)_6]^{3-}$ is too fast to monitor by stopped-flow spectrophotometry.[21b, 22] In the case of umecyanin the reaction with $[Fe(CN)_6]^{3-}$ is also fast and could only be monitored at 5.8 °C ($2.8 \times 10^6 M^{-1}s^{-1}$).[19] The ratio of rate constants (k_{Fe}/k_{Co}) for the oxidation of UCu^{I} by $[Fe(CN)_6]^{3-}$ and $[Co(phen)_3]^{3+}$ is of interest alongside the slow self-exchange rate constant. At 5.8 °C this ratio is 9.3×10^3 for umecyanin,[19] which compares with that for the Type 1 copper protein pseudoazurin,[38] $k_{Fe}/k_{Co} > 7.8 \times 10^3$. The small self-exchange rate constant for pseudoazurin ($2.9 \times 10^3 M^{-1}s^{-1}$)[26] has been attributed to the presence of lysine residues at the surface hydrophobic patch adjacent to the Cu. It is interesting to note that in azurin the Met44Lys[27, 39] and Met64Glu[40] mutants, which incorporate single positive and negative charges in the adjacent hydrophobic region, respectively, have drastic effects on the rate of self-exchange. It is therefore not unreasonable to assume that in umecyanin the basic residues Lys12 and Lys96 are close to the site used for self-exchange. In stellacyanin the lack of a preference for cationic or anionic reactants is consistent with the relatively large self-exchange rate constant.[23]

In previous studies the use of azurin as the partner in Marcus cross-reaction studies has proved extremely successful, and for pseudoazurin, amicyanin, a higher plant plastocyanin and a blue-green algal plastocyanin has yielded self-exchange rate constants which are within a factor of two of those determined by NMR.[26, 28, 29, 31, 41, 42] The success of the method relates to the uniformity of the charge distribution on azurin. The self-exchange rate constants calculated for umecyanin by a Marcus analysis of the cross-reactions with azurin and cytochrome c_{551} are substantially ($\approx 10^3$) smaller than that obtained by NMR. The only reasonable explanation is that azurin and cytochrome c_{551} react with umecyanin at an alternative site that differs from that used by umecyanin for the self-exchange process. To date, azurin appears to have only one electron-transfer site, its hydrophobic patch,[27, 39, 40] which is presumably the site it utilises in the reaction with umecyanin. Cytochrome c_{551}, which is less highly charged than cytochrome c, has a large self-exchange rate constant that is independent of ionic strength,[37] indicating the involvement of a hydrophobic surface in this process. In view of the similar self-exchange rate constants obtained (8.0 and $13.9 M^{-1}s^{-1}$) it is not unrealistic to assume that cytochrome c_{551} may react with umecyanin at the same site as azurin. An unusual feature observed in the crystal structure of CBP is that both of the active-site histidines are exposed to the solvent.[3] This observation may account for the existence of two electron-transfer sites on umecyanin, with electron transfer in the one case (with azurin and cytochrome c_{551}) through one of the histidines, and electron self-exchange at the other.

The question of inhomogeneity of protein samples has been raised in a previous paper.[25] As far as the above kinetic studies are concerned we have not experienced any inconsistencies from one sample to another which might be attributed to such effects, which would seem to imply that the C-terminus peptide chain has no major influence on reactivity.

The similarity of the amino-acid sequence of umecyanin to those of stellacyanin, cucumber basic protein (CBP) and cucumber peeling cupredoxin (CPC) is of interest with regard to the identity of the fourth copper ligand in these proteins. The amino-acid sequence of CPC, which has recently been published,[43] suggests properties in common with stellacyanin.[44] This protein may also possess a glutamine as its fourth ligand. Like stellacyanin and umecyanin, which possess 40% and 4% carbohydrate, respectively,[7] CPC is a glycoprotein with approximately 10% associated carbohydrate. It is interesting to note that all the multi-copper oxidases which have been characterised are also associated with carbohydrate.[45] From the sequence alignments (Fig. 3), it appears that umecyanin, stellacyanin and CPC possess a glutamine as their fourth active site ligand. This has also been shown in a model of stellacyanin from the crystal structure of CBP,[10] which successfully explains a number of experimental observations made previously for stellacyanin.

The visible spectrum of umecyanin shows a reversible change with pH in the range 7 to 11; the 610 nm peak shifts to lower wavelengths upon increasing the pH.[19, 46] A pK_a of 9.5 has been determined for this transition. The visible spectrum of stellacyanin is similarly affected by pH, $pK_a \approx 10.5$,[47] as is that of the Type 1 copper site of laccase (which may also be lacking a methionine[45]), $pK_a \approx 10.2$.[47b, c] In the case of stellacyanin it has been proposed that this change is due to the deprotonation of the side-chain amide of the coordinated glutamine, with a concurrent switch from oxygen to nitrogen coordination as in Figure 6.[10] However, the EPR spectra of stellacyanin[47] and

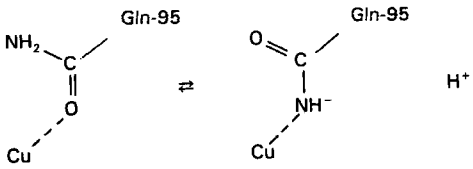

Fig. 6. The different coordination modes at low and high pH of the glutamine (Gln95) at the Cu active site of umecyanin and stellacyanin.

umecyanin[19] are almost unchanged in the pH range 7–11. If such a drastic change in coordination was occurring, it would be expected to affect the EPR spectrum, which is a highly sensitive indicator of Cu^{II} geometry. At pH values above 11 the EPR spectrum of stellacyanin shows superhyperfine splitting, and this change is irreversible.[47] Studies on the Cu^{II} complexes of 3,8-dimethyl-4,7-diazadeca-3,7-dieneamine in solution[48] demonstrate that a pH-induced change from oxygen to nitrogen coordination of an amide group is accompanied by an increase in the number of superhyperfine lines in the EPR spectrum. Irreversible effects are also observed in the visible spectrum of umecyanin at higher pH values.[46] However, studies of stellacyanin with ENDOR[49] and the EPR results mentioned above indicate that the change in coordination at the active site occurs at higher pH values ($\approx$11). The effects seen at lower pH values (9–11) on the visible spectra of umecyanin and stellacyanin are therefore attributed to the protonation/deprotonation of another residue, one possibility being an adjacent lysine. This argument is supported by studies on the effect of pH on visible spectra of rusticyanin[50] and CBP[51] in the pH range 9–11, which are attributed to the deprotonation of a basic amino-acid residue (or a tyrosine). Moreover a similar effect of pH on the visible spectrum of the Met44Lys mutant of azurin is seen that is not present in the wild-type protein.[27] Since all of these proteins have a Met as their fourth ligand, the suggestion of Freeman et al[10] may need modifying. From Figure 3, a potential candidate as the protonating/deprotonating species in the case of umecyanin is Lys96, which is adjacent to the proposed Gln95 ligand. We note that a lysine is conserved in all of the sequences in Figure 3. Alternatively Lys12 of umecyanin, which is conserved in the sequences of rusticyanin and stellacyanin and is known to be close to the active site from the sequence alignments with CBP,[25] might also be considered.

Further evidence for oxygen being the normal donor atom at the fourth coordination site in umecyanin and stellacyanin (at low pH) comes from a plot of g_{11} vs A_{11} for the X-band EPR spectra of Type 1 copper proteins.[52] The spectral parameters for these two proteins are similar and differ from those for Type 1 proteins with a methionine coordinated to the copper. Active-site mutants of azurin[15, 53] and amicyanin[54] in which an oxygen ligand has been introduced result in an oxidised protein whose EPR parameters are similar to those of stellacyanin and umecyanin.

Although stellacyanin and umecyanin appear to have the same residues coordinating the copper there are some differences at the active site. The visible spectrum of umecyanin is more like those for plastocyanin and azurin (Fig. 5), with the band at $\approx$450 nm relatively weak compared with that for stellacyanin[47] and CBP.[9] There are also differences between the X-band EPR spectrum of umecyanin, which is axial,[17, 19] and spectra of stellacyanin[47] and CBP,[9] which are rhombic. These two features, axial EPR and a relatively weak absorption at $\approx$450 nm in Type 1 copper proteins, are thought to be due to a longer axial ligand–copper bond.[12, 15, 55, 56] Hence the copper-to-oxygen (Gln95) bond in umecyanin must be longer than the corresponding Gln97 bond in stellacyanin (2.54 Å from the model).[10] This assumption finds some support from EXAFS studies, which have identified a fourth ligand in stellacyanin,[57, 58] whereas in umecyanin no such ligand was identified.[19] It is also in agreement with reduction potentials of 184 and 283 mV for stellacyanin and umecyanin, respectively. The stabilising effect of the proximal oxygen atom on the Cu^{II} form is accordingly diminished in umecyanin.

Another feature of umecyanin compared with other Type 1 copper proteins is that the Asn residue, which is usually present immediately after the first His ligand,[6] is replaced by an Asp. The side chain of the Asn makes several hydrogen-bonding interactions that are thought to stabilise the copper active site. The role of this residue has been demonstrated in the Asn 47 Leu mutant of *Alcaligenes denitrificans* azurin, which has decreased reduction potential and stability as compared with the wild-type protein.[59] Rusticyanin, on the other hand, which is stable at pH < 2 and has the highest reduction potential among this class of proteins (680 mV), has a Ser residue at the corresponding position.[60]

The question as to whether stellacyanin and CBP form a sub-class among the Type 1 blue copper proteins has been raised previously.[3] It must now be concluded that umecyanin and cucumber peeling cupredoxin (CPC) also belong to this class (Fig. 3). Stellacyanin, CBP, umecyanin and CPC are all found in plants, with functions as yet unknown. In a recently published phylogenetic tree for small blue proteins[61] the Type 1 copper proteins from bacteria occur alongside plastocyanin. The phytocyanins, including stellacyanin and CBP, are at some distance from the bacterial proteins. Umecyanin and CPC, though not included in this analysis, would presumably be located along with the phytocyanins. However the fact that stellacyanin, umecyanin and CPC all appear to possess a Gln as the fourth ligand suggests that a sub-class of Type 1 copper proteins exists with a different active site structure.

Experimental

Isolation and purification of umecyanin from horseradish roots: Umecyanin (UCu) was isolated from horseradish roots (*Armoracia laphatifolia*) by a modified version of the method used by Paul and Stigbrand [16]. Approximately 20 kg of horseradish roots were washed and chopped into approximately 2 cm^3 portions and blended (Waring Blender, 1 gallon capacity) with cooled distilled water (4 °C) for 2 × 2 min. In the published method it is reported that only the peelings of the horseradish roots were used, and this approach was attempted initially but resulted in a low yield of umecyanin. The homogenate from the blender was strained through coarse cheesecloth and then through two layers of muslin cloth. The final stage of purification of UCu^{II} was carried out on an FPLC system (Pharmacia, Mono-Q column) at pH 7.5 and gave protein with a UV/Vis absorption (A) peak ratio A_{280}/A_{610} of 5:1. Umecyanin concentrations were determined from the UCu^{II} peak at 610 nm in the visible spectrum (ε = 3400 $M^{-1} cm^{-1}$) [16]. Umecyanin from Sigma was also used and gave a purity ratio of <4.0:1. Under identical conditions the UCu^{II} from Sigma eluted on the FPLC system with the same retention time as the UCu^{II} isolated in Newcastle. No difference in kinetic behaviour was observed.

NMR sample preparation: For the acquisition of proton NMR spectra umecyanin was exchanged into 99.9% deuterated 37.3 mM phosphate buffer at pH 7.50 (I = 0.100 M) by ultrafiltration through a YM3 membrane. Protein samples were reduced by the addition of cooled aliquots of 0.10 M $Na_2S_2O_4$ in 99.9% D_2O (0.1 M NaOD), and any excess reductant was exchanged out of samples used for rate-constant determinations. Oxidised samples of umecyanin were obtained with a 0.10 M solution of $K_3[Fe(CN)_6]$ in 99.9% D_2O, and likewise any excess was exchanged out of the solution with an appropriate buffer. Protein solutions of known concentration were transferred to an NMR tube which was flushed with argon and sealed. Half-oxidised mixtures of umecyanin were obtained by adding oxidised protein to reduced protein (both at approximately the same concentration) under air-free conditions. The concentration of oxidised protein present was determined by transferring the protein solution to a 2 mm UV/Vis spectrophotometer cell under air-free conditions and monitoring the absorbance at 610 nm. The pH of the NMR samples was measured with a narrow CMAWL/3.7/180 pH probe in combination with a Radiometer pH meter calibrated by means of aqueous buffers. The pH of the samples was adjusted with NaOD or DCl (0.10 M) as necessary, and no correction was made for the deuterium isotope effect.

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NMR spectra: All ^{1}H NMR spectra were acquired at 500.14 MHz on a Bruker AMX 500 spectrometer at 25 °C with samples in 5 mm o.d. borosilicate glass tubes. Free induction decays were accumulated into 16 K data points and transformed into 32 K data points after zero-filling. The residual HDO resonance was suppressed by presaturation at its resonant frequency. All chemical shifts are cited in parts per million (ppm) relative to internal dioxane at $\delta = 3.74$ ppm. Combinations of spectra obtained by Hahn Spin-Echo (HSE) [$90^\circ_x - t - 180^\circ_y - t$] ($t = 60$ ms) [62], and Carr–Purcell–Meiboom–Gill (CPMG) [$90^\circ_x - t - (180^\circ_y - 2t)_n - 180^\circ_y - t$] ($n = 59$, $t = 1$ ms) pulse sequences [63] were used to identify singlets in the ^{1}H NMR spectra of umecyanin. The HSE pulse sequence was also used to determine T_2 (spin–spin) relaxation times of certain resonances from plots of $\ln(I_t)$, where I_t is the intensity of a particular resonance when a delay time t is used in the HSE pulse sequence against $2t$, which give a straight line of slope $-T_2^{-1}$.

Isolation and purification of azurin and cytochrome c_{551}: Azurin (ACu) and cytochrome c_{551} were obtained from *Pseudomonas aeruginosa* by a method previously described [64–66]. Samples of ACu^{II} were purified to give a A_{280}/A_{625} ratio of 1.67–1.72:1 by FPLC on a Mono-S cation-exchange column. Azurin concentrations were determined from the ACu^{II} visible peak at 625 nm ($\varepsilon = 520\,M^{-1}\,cm^{-1}$). Ferrocytochrome c_{551} was purified by FPLC in a Mono-Q anion-exchange column to give protein with a A_{551}/A_{280} ratio of (1.10 ± 0.03):1. Ferrocytochrome c_{551} concentrations were determined from the α band at 551 nm ($\varepsilon = 2.86 \times 10^4\,M^{-1}\,cm^{-1}$).

Visible absorption spectra of ACu^{II} and UCu^{II}: Differences in the absorption spectra of ACu^{II} and UCu^{II} are indicated in Figure 5. These are probably the consequence of differing coordination at the active sites as already indicated. The differences make it possible to monitor the kinetics of the reaction of UCu^{I} with ACu^{II}.

Kinetic studies: All the reactions were monitored on a Dionex D-110 stopped-flow spectrophotometer at 25.0 ± 0.1 °C and $I = 0.100 \pm 0.001$ M (NaCl). The oxidation of UCu^{I} (reactant in >10-fold excess) with ACu^{II} was studied in tris[hydroxymethyl]aminomethane (tris)/HCl buffer (pH 7.0–8.1), and absorbance changes monitored at 650 nm. The reduction of UCu^{II} (reductant in >10-fold excess) with ferrocytochrome c_{551} was carried out in tris/HCl buffer (pH 7.0–8.4) and monitored at 416 nm where the $\Delta\varepsilon$ for the transition from ferro- to ferricytochrome c_{551} is $6.25 \times 10^4\,M^{-1}\,cm^{-1}$. The stopped-flow was interfaced to an IBM PC/At-X computer for data acquisitions with software from On Line Instruments Systems (Bogart, GA., USA). First-order rate constants k_{obs} were obtained by averaging at least five different determinations with the same solutions. The pH-jump method was used, in which solutions at low and high buffer concentrations were mixed, with the result that the pH of the latter prevailed. The proteins were either fully reduced or oxidised (as required) prior to kinetic studies and dialysed against the appropriate buffer. In the case of UCu^{I} and ferrocytochrome c_{551} dialyses were carried out under a nitrogen atmosphere with degassed buffer. The stopped-flow runs were under air-free conditions.

Acknowledgements: We thank the Leverhulme Trust (W. McF.), the UK Engineering and Physical Sciences Research Council (C. D.) and Flemish Concerted Research Action (J. V. B.) for financial support, and are most grateful to Colman's of Norwich for their generous gift of horseradish roots.

Received: February 4, 1995 [F83]

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