Alkaline transition of phytocyanins: a comparison of stellacyanin and umecyanin

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The effect of pH on Cu(I) and Cu(II) forms of the isolated soluble domain of the stellacyanin from *Rhus vernicifera* (SCu), the Japanese lacquer tree, has been studied by electronic and NMR spectroscopy and using direct electrochemical measurements. A pK_a value of 10.1–10.4 is observed for the alkaline transition in this oxidized phytocyanin and results in a slightly altered active-site structure, as indicated by changes in the visible and paramagnetic ¹H NMR spectra. Electrochemical studies show that the pK_a value for this transition in SCu(I) (reduced SCu) is 11.0. These results are compared with those

recently obtained for other phytocyanins, and in particular umecyanin. In all cases, the alkaline transition is caused by the deprotonation of the surface lysine residue adjacent to the axial ligand. This lysine residue is completely conserved in known phytocyanin sequences. Also highlighted in these studies are the remarkable active-site similarities between stellacyanin and umecyanin.

Key words: cupredoxin, electron transfer, paramagnetic NMR.

INTRODUCTION

Type 1 blue copper proteins (cupredoxins) function as electron transfer agents in both prokaryotes and eukaryotes. The phytocyanins form a subclass of the cupredoxins, which are found in plants [1–6] and have themselves been divided into three main groups [5]: the stellacyanins, the plantacyanins and the uclacyanins. Crystal structures are available for three phytocyanins, namely cucumber stellacyanin (CST) [3] and the plantacyanins from spinach [6] and cucumber (cucumber basic protein, CBP) [4]. These structural studies have demonstrated that the phytocyanins possess a number of unusual features compared with other cupredoxins (see Figure 1). The phytocyanins have a somewhat more open β -sandwich structure, which is stabilized by a disulphide bridge close to the active site [3,4,6]. The copper ion is strongly co-ordinated by the thiolate sulphur of a Cys residue and the imidazole nitrogens of two histidine residues, as in all other cupredoxins [3,4,6]. In CST, the ligand axial to the His₂Cys trigonal plane is a glutamine residue (see Figure 1), bonded via its side-chain amide oxygen, as compared with a methionine residue in most other cupredoxins [8], including the plantacyanins. Another unusual feature of the active site of the phytocyanins is that both of the His ligands are solvent-exposed [3,4,6], whereas in all other cupredoxins only the C-terminal His ligand protrudes through the surface of the protein [8]. The exact physiological function of the phytocyanins is unknown, although it is assumed that they act as redox catalysts.

The environment provided by the protein for the cupric ion at type 1 copper sites results in unique spectroscopic properties when compared with copper co-ordination compounds [9,10]. This includes an intense ligand to metal charge transfer (LMCT) absorption at approx. 600 nm (with a weaker LMCT band at approx. 450 nm) and an unusually small hyperfine coupling constant in the g_z region of their EPR spectra [9,10]. Both of these features have been attributed to the relatively short bond between the metal and the thiolate sulphur of the cysteine ligand [9,10]. A unique property of the phytocyanins, which was

established as early as 1967 [1] for stellacyanin from *Rhus vernicifera* (SCu), is that their visible spectra are dramatically altered on increasing the pH value [1,11–15]. This has been termed the ‘alkaline transition’ and results in a rearrangement of the copper site [12,14]. A recent study [14] on umecyanin (UCu), a phytocyanin isolated from horseradish roots, has indicated that protonation of a surface lysine residue, close to the copper site, is the cause of the alkaline transition.

Paramagnetic NMR is a technique which has been applied to Cu(II) cupredoxins [12,14,16–18] and their Co(II) and Ni(II) derivatives [12,19,20], providing detailed information about the active-site architecture. NMR investigations of the Co(II)-substituted SCu have shown that the alkaline transition results in a conformational change at the active site [12]. In the present study, we have reassessed the paramagnetic NMR spectrum of SCu(II) (oxidized SCu) and utilized electrochemical investigations to analyse further the alkaline transition of SCu. We compare the results with those recently obtained for UCu and other phytocyanins.

MATERIALS AND METHODS

Protein isolation and purification

The isolated soluble domain of the SCu from the acetone powder of the tree sap of the Japanese lacquer tree (*R. vernicifera*) was a gift from Professor A. G. Sykes (University of Newcastle upon Tyne, Newcastle upon Tyne, U.K.). The purified protein gives a single band on an SDS/PAGE gel.

Protein samples for UV–visible and paramagnetic ¹H NMR studies

For UV–visible and paramagnetic NMR experiments, the protein was exchanged into 10 mM potassium phosphate buffer. The NMR experiments were performed with the protein in either 90% H₂O/10% ²H₂O or 99.9% ²H₂O.

Abbreviations used: CBP, cucumber basic protein; CST, cucumber stellacyanin; LMCT, ligand to metal charge transfer; SCu, stellacyanin from *Rhus vernicifera*; UCu, umecyanin.

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Figure 1 Representation of the structure of CST (PDB entry 1JER) [3] drawn with MOLSCRIPT [7]

The active site is shown in detail with the copper ion as a magenta sphere, and the corresponding ligating residues in SCu are His⁴⁶, Cys⁸⁷, His⁹² and Gln⁹⁷. Also indicated are the disulphide bridge close to the active site and Lys¹⁰⁰, which is adjacent to the axial Gln ligand and corresponds to Lys⁹⁸ in SCu.

Adjustment of the pH of protein samples

The pH values of protein solutions were measured and corrected as described previously [14]. The pH values quoted in deuterated solutions were not corrected for the deuterium isotope effect and are indicated by pH*.

UV–visible spectrophotometry

UV–visible spectra were recorded at 25 and 30 °C on a Shimadzu UV-2101PC spectrophotometer.

NMR spectroscopy

The ¹H NMR spectra were recorded on a Jeol Lambda 500 spectrometer as described previously [14,18]. The pH* titrations were all performed at 30 °C, and paramagnetic spectra of SCu(II) were also measured at 25 °C. All chemical shifts are quoted in p.p.m. relative to the residual HDO signal using the relationship $\delta_{\text{HDO}} = -0.012t + 5.11$, where t is the temperature in °C [16].

Electrochemistry of SCu

Direct measurement of the reduction potential of SCu was performed using an electrochemical set-up described previously [14]. The gold working electrode was modified as described

previously [14], as were the buffers used (usually at $I = 0.10$ M, NaCl) over a particular pH range [14].

RESULTS

Effect of pH on the UV–visible spectrum of SCu(II)

We have confirmed previous observations [1,12,22] that the visible spectrum of SCu(II) is pH-dependent (see Figure 2). The effect of pH over the range approx. 7–11 on the positions of the two LMCTs at 605 and 452 nm (at pH 7) can be fitted to the equation

$$\lambda = (K_a \lambda_H + [H^+] \lambda_L) / (K_a + [H^+]) \quad (1)$$

yielding pK_a values of 10.1 and 10.2 respectively. The intensity of the 605 nm band increases on increasing the pH (the intensity of the other LMCT band is almost unaffected), resulting in the A_{452}/A_{605} ratio of 0.22 at pH 7.0, decreasing to an A_{438}/A_{588} ratio of 0.19 at pH 11.2. Almost identical results are obtained at both 25 and 30 °C.

The ¹H NMR spectrum of SCu(II)

The paramagnetic ¹H NMR spectrum of SCu(II) at pH* 7 (and at 25 °C) shown in Figure 3 is remarkably similar to that of oxidized CST, for which all of the isotropically shifted resonances have been assigned [17]. The spectra of both proteins possess the same number of directly observed isotropically shifted resonances with very similar relaxation properties, and from a comparison we can assign the resonances in the spectrum of SCu(II). The broad signals in the 20–70 p.p.m. region in SCu(II) arise from the imidazole ring protons of the two co-ordinated His ligands. The peaks at 57 and 49 p.p.m. are assigned to the C^βH protons of the two His ligands (His⁴⁶ and His⁹²), whereas the broad resonance at approx. 30 p.p.m. is made up of the two overlapping C^αH peaks. It has been reported [12] that the peak at 49 p.p.m. in SCu(II) is an exchangeable resonance (this led to its assignment as the N^εH proton of the Gln⁹⁷ ligand). However, we have observed this peak in ²H₂O (as well as in 90% ²H₂O/10% ¹H₂O), demonstrating that it is not exchangeable and supporting its assignment as a His C^βH resonance. Furthermore, no axial Gln ligand resonances experience sizeable Fermi-contact shifts in

Figure 2 Part of the UV–visible spectra (25 °C) of SCu(II) at pH 7.0 and 11.2 in 10 mM phosphate buffer

Figure 3 ¹H NMR spectra

Spectra (30 °C) of SCu(II) (500 MHz) in 10 mM phosphate buffer: (A) in 90% H₂O/10% ²H₂O at pH 4.5; (B) in ²H₂O at pH* 7.0. Also shown are spectra of UCu(II) in 10 mM phosphate buffer: (C) in 90% H₂O/10% ²H₂O at pH 4.6; (D) in ²H₂O at pH* 7.8.

the spectrum of CST [17] and, thus, we do not expect them to do so in SCu(II).

The peak at 14.8 p.p.m. has a T_1 value (the longitudinal relaxation time) of approx. 5 ms and an analogous signal has been observed in a similar position in all studies to date on Cu(II) cupredoxins. This peak has been assigned in plastocyanin [16], azurin [17], CST [17] and pseudoazurin [18] as the C²H resonance of the Asn residue whose backbone amide hydrogen bonds to the thiolate sulphur of the co-ordinated Cys residue. A similar hydrogen bond is observed in the crystal structures of all cupredoxins [8], including CST [3], and can thus be anticipated at the active site of SCu. We therefore assign the peak at 14.8 p.p.m. in the spectrum of SCu(II) to the C²H of Asn⁴⁷. This peak was previously judged by Fernández et al. [12] to be a composite signal arising from the two C²H protons of the co-ordinated Gln⁹⁷ (but no resonances belonging to this residue are expected to experience a significant Fermi-contact shift, see above). In the up-field region of the spectrum of SCu(II), there are two shifted peaks and the broader resonance at -7.6 p.p.m. is assigned to the C²H of Cys⁸⁷. This assignment is based on previous studies [16–18] and, in particular, on the observation that the C²H of the co-ordinated Cys⁸⁹ has been detected at -7.5 p.p.m. in the spectrum of Cu(II) CST [17]. The signal at approx. -2 p.p.m. probably arises from the C²H resonance of one of the His ligands.

Figure 4 The pH* dependence (25 °C) of the chemical shift of the C²H resonance of Asn⁴⁷ in the ¹H NMR spectrum of SCu(II) in 10 mM phosphate buffer

The solid line shows a fit of the data to eqn. (2).

Two exchangeable signals are identified in the paramagnetic ¹H NMR spectrum of SCu(II). One of these, at 12.2 p.p.m. in a sample in 90% H₂O/10% ²H₂O at pH 7.0 (and 25 °C), is sharp (see Figure 3A), has a relatively long T_1 value (approx. 35 ms), and has previously been attributed to the C²H proton of the co-ordinated Cys⁸⁷ [12]. However, this cannot be the case and this peak probably belongs to a backbone amide group in the vicinity of the copper site (with the amide of Cys⁸⁷ being a probable candidate). The second exchangeable resonance (at 23.7 p.p.m.) is only observed in H₂O at more acidic pH values (pH 4.5). This signal has a shorter T_1 value (approx. 4 ms) and is assigned to the N²H of the more buried His⁴⁶ ligand. No additional exchangeable resonances were observed on cooling the sample at pH 4.5 (to 5 °C), and thus the N²H of the His⁹² ligand is in fast exchange with the bulk solvent under these conditions and is broadened beyond detection.

Effect of pH on the ¹H NMR spectrum of SCu(II)

As described previously [12], the paramagnetic ¹H NMR spectrum of SCu(II) is influenced by the alkaline transition, and in the present study we make a more detailed investigation of this effect. The C²H resonance of Asn⁴⁷ in SCu(II) shifts in an up-field direction from 14.6 p.p.m. at approx. pH* 8 (and 30 °C) to 13.4 p.p.m. at pH* 11.1. This indicates a pH-dependent equilibrium in which exchange between the two forms is fast on the NMR time scale. The chemical shift of this resonance as a function of pH* is shown in Figure 4 and can be fitted to the equation

$$\delta = (K_a \delta_H + [H^+] \delta_L) / (K_a + [H^+]) \quad (2)$$

where δ_H and δ_L are the chemical shifts at high and low pH respectively, yielding a pK_a^* value of 10.4. The C²H resonance of Asn⁴⁷ is unaffected on taking SCu(II) to acidic pH* values (see Figures 3 and 4). The positions of the other isotropically shifted signals are almost unchanged by altering the pH* value.

Figure 5 The dependence of the reduction potential (E_m) of SCu at $I = 0.10$ M (NaCl) on pH

Table 1 Properties of the phytocyanins SCu, UCu, mavicyanin and CBP, including the effect of the alkaline transition

The λ values are given in nm, E_m in mV and δ in p.p.m.; n.d., not determined.

Parameter	Phytocyanin			
	SCu*	UCu†	Mavicyanin‡	CBP§
Axial ligand	Gln	Gln	Gln	Met
$\lambda_{\max,1}$ (pH ~ 7)	605	606	599	595
$\lambda_{\max,2}$ (pH ~ 7)	452	455	448	441
$A_{\sim 460}/A_{\sim 600}$ (pH ~ 7)	0.22	0.12	0.20	0.53
$\lambda_{\max,1}$ (pH ~ 11)	588	584	577	580
$\lambda_{\max,2}$ (pH ~ 11)	438	439	431	422
$A_{\sim 460}/A_{\sim 600}$ (pH ~ 11)	0.19	0.12	0.19	0.43
EPR	Rhombic	Axial	Rhombic	Rhombic
E_m (pH ~ 8)	175	287	221	302
E_m (pH ~ 11)	136	271	~ 130	n.d.
δ Asn C ² H (pH ~ 8)	14.6	14.7	n.d.	n.d.
δ Asn C ² H (pH ~ 11)	13.4	13.3	n.d.	n.d.
$pK_a^{\text{ox}} \P$	10.4	9.5	9.0	n.d.
$pK_a^{\text{red}} \P$	11.0	9.9	10.6	n.d.

* Present study.

† See [14].

‡ See [15,23].

§ See [11,24].

|| See Figure 6.

\P Determined from electrochemistry experiments.

Dependence of the reduction potential of SCu on pH

SCu yields good, quasi-reversible, responses on a 4,4-dithiopyridine-modified gold electrode over the pH range 4.4–11.2. In most cases, the cathodic and anodic peaks are of equal intensity with a separation of 60–80 mV at scan rates of typically 20–30 mV/s. The peak currents are proportional to the (scan rate)^{1/2} at pH 7.4 ($I = 0.10$ M, NaCl) over the range 3–120 mV/s. The influence of pH on the reduction potential (E_m) of SCu is shown in Figure 5. The reduction potential at pH 7.0 (189 mV) is in

good agreement with the literature value for SCu [21,22]. As the pH is lowered from approx. 7, the reduction potential gradually increases, and a value of 211 mV is observed at pH 4.4. When the pH value is increased above 7, the reduction potential gradually starts decreasing and reaches a value of 136 mV at pH 11.2. The pH dependence of the reduction potential above pH 9 can be fitted to the equation

$$E_m(\text{pH}) = E_m(\text{low pH}) + (RT/nF) \ln[(K_a^{\text{red}} + [\text{H}^+])/(K_a^{\text{ox}} + [\text{H}^+])] \quad (3)$$

where $E_m(\text{pH})$ is the measured reduction potential, $E_m(\text{low pH})$ the reduction potential at low pH, R the gas constant, T the absolute temperature, n the number of electrons, F the Faraday constant and K_a^{red} and K_a^{ox} are the proton dissociation constants for the residue which influences the reduction potential over this pH range in the reduced and oxidized proteins respectively. A fit to the data yields a pK_a^{ox} value of 10.4 and a pK_a^{red} value of 11.0.

The results obtained for the effect of the alkaline transition in SCu are summarized in Table 1. Also included in this Table are the corresponding results for UCu [14], mavicyanin [15,23] and CBP [11,24] (all phytocyanins).

DISCUSSION

Paramagnetic ¹H NMR spectrum of SCu(II)

The paramagnetic ¹H NMR spectrum of SCu(II) reported in the present study is remarkably similar to the one published for oxidized CST [17] and oxidized UCu [UCu(II)] [14]. No proton resonances from the axial Gln ligand experience sizeable Fermi-contact shifts in the spectra of oxidized phytocyanins. This has been attributed [17] to the fact that this ligand does not contribute to the highest occupied molecular orbital in which the unpaired electron is located in these proteins. The spectrum of SCu(II) is discussed below in more detail in comparison with that of UCu(II).

Effect of pH on the UV-visible and ¹H NMR spectra of SCu

The shift in the positions of the two LMCT bands in the visible spectrum of SCu(II), together with the changes in intensity, indicates that the geometry of the active site is different at alkaline pH as compared with pH 7.0. The smaller $A_{\sim 450}/A_{\sim 600}$ ratio at higher pH suggests a slightly weaker axial interaction as a consequence of the alkaline transition [14,18].

In the paramagnetic ¹H NMR spectrum of SCu(II), the isotropic shift of the Asn⁴⁷ C²H resonance is dependent on pH*. This resonance shifts up-field on increasing the pH* from 7.8 (14.6 p.p.m.) to 11.1 (13.4 p.p.m.), with a pK_a^* of 10.4. Thus the alkaline transition also results in a decreased Cu–S(Cys) interaction at the active site (the isotropic shift of the Asn⁴⁷ C²H proton depends on the amount of spin density on the thiolate sulphur of the Cys ligand). A similar conclusion has also been reached from paramagnetic ¹H NMR studies of Co(II)-substituted SCu [12]. No other paramagnetically shifted peaks are significantly affected by increasing the pH* value and none are influenced by lowering the pH* of the sample. This is slightly different from what has been found in UCu(II), where the protonation of the non-co-ordinated His⁶⁵ [approx. 14 Å (1 Å = 10^{−10} m) from the copper ion] influences the paramagnetic NMR spectrum and thus the active-site structure [14]. SCu has two surface His residues at positions 32 and 100 with pK_a values of approx. 6.7 and 7.3 respectively in both oxidation states of the protein (results not shown), but protonation of these does not influence the active-site structure.

Effect of pH on the reduction potential of SCu

The low-reduction potential (for a cupredoxin) exhibited by SCu throughout the pH range studied is a consequence of its solvent-exposed active site, and the presence of an axial oxygen ligand [which has a preference for Cu(II)]. Between approx. pH 8 and 4.4, there is a general increase in the reduction potential of SCu due to stabilization of the Cu(I) form of the protein as the pH is lowered. The protonation of the non-co-ordinated His³² and His¹⁰⁰ residues has no distinct influence on the reduction potential of the protein. This is consistent with the observed similarity between the pK_a^{red} and pK_a^{ox} values of these two residues (results not shown).

The pH dependence of the reduction potential of SCu over the pH range 9–11.2 allows us to determine the pK_a values for the alkaline transition in both the reduced and oxidized forms of the protein. The pK_a^{ox} value of 10.4 from the electrochemical studies is in good agreement with the values determined from UV–visible (10.1–10.2) and ¹H NMR experiments (10.4). Electrochemistry has proved to be the sole method of determining the pK_a^{red} value for the alkaline transition in phytocyanins, and the value obtained for reduced SCu [SCu(I)] is 11.0. The pH value at which the alkaline transition occurs indicates that the residue responsible is a lysine. The difference in the pK_a values for SCu(I) and SCu(II) (0.6 pH unit) is similar to that observed in UCu [14] (see Table 1), and suggests that the Lys residue responsible for the alkaline transition must be close to the active site. In zucchini mavycyanin, the difference in the pK_a for the alkaline transition between the two oxidation states of the protein is 1.6 pH units [15] and the ionizable group responsible for the effect in this phytocyanin must either be closer to the active site than that in SCu and UCu or is less solvent-exposed.

A comparison of SCu, UCu and other phytocyanins

The amino acid sequences of 24 phytocyanins [2,5,25–31] are compared in Figure 6 (some of these have only been identified by interrogation of DNA databases using tblastn). These proteins have been split into the three main groups as described by Nersissian et al. [5], based on the domain organization of the proteins, the glycosylation state, the nature of the co-ordination site and spectroscopic properties. The first group comprising the stellacyanins (sequences 1–9 in Figure 6), is identified by the presence of an axial Gln ligand (see above), by having associated carbohydrate and by the presence of a copper-binding (cupredoxin) domain, followed by a cell-wall-anchoring domain and finally a C-terminal hydrophobic extension (all of the phytocyanins possess an N-terminal endoplasmic reticulum targeting signal peptide). The phytocyanins from capsicum and morning glory, sequences 10 and 11 respectively (see Figure 6), possess a glutamine which aligns with the axial ligand in CST. However, the Cys ligand is missing in these two proteins and are thus not included in this group (it should be noted that the morning glory phytocyanin [26] also has the N-terminal His ligand replaced by an Asp). The second division of the phytocyanins are the uclacyanins (sequences 12–18 in Figure 6), which are very similar to the stellacyanins, except that the axial ligand is a Met rather than a Gln residue. The bread wheat phytocyanin (TAP, sequence 18) is unusual in having a slightly extended loop between the His and Met ligands. The final subgroup of the phytocyanins comprises the plantacyanins (sequences 19–23 in Figure 6), which lack the two C-terminal domains, are not glycosylated and have an axial Met ligand. The tomato phytocyanin (sequence 24) is plantacyanin-like, but has a Val residue in place of the Met/Gln. This is possibly the first example of a cupredoxin, which

has a non-co-ordinating residue at the axial position, although the tomato phytocyanin protein has not been isolated. It should be remembered that certain fungal laccases and ceruloplasmin do possess cupredoxin subdomains in which the axial ligand is either a Leu or a Phe residue [32]. A number of amino acids, excluding the ligating residues and the cysteines of the disulphide bridge, are conserved in the 24 sequences included in Figure 6. In the context of the present investigation, the absolute conservation of the lysine residue at position 98 (in SCu) is of utmost interest. It should be noted that in TAP, the corresponding lysine residue is still found adjacent to the axial ligand (Met) but is slightly offset in the sequence alignment (see Figure 6).

The structural, spectroscopic and functional properties of four phytocyanins are listed in Table 1. All of these proteins undergo the alkaline transition, which has a similar effect on their spectroscopic properties. The observation of an alkaline transition in the plantacyanins indicates that the axial glutamine is not responsible for this effect. In light of the present study, and that published previously [14] for UCu, the most probable cause of the alkaline transition in phytocyanins is the conserved lysine residue close to the copper site (Lys⁹⁸ in SCu; see Figures 1 and 6). This conclusion is supported by theoretical studies, which have shown that the visible spectral shifts observed for the phytocyanins can be caused by a change in the electrostatic field near the copper ion [13]. Furthermore, attributing the alkaline transition in phytocyanins to the lysine residue is corroborated by studies on the Met⁴⁴ → Lys azurin variant in which the introduced basic residue is close to the copper site in this cupredoxin [33]. The pH dependence (> pH 9) of the spectral properties and the reduction potential of this mutant are very similar to those observed for the phytocyanins. Of the four phytocyanins included in Table 1, SCu and UCu have been studied in great detail, and it is on these two that we now focus.

The paramagnetic ¹H NMR spectrum of SCu(II) exhibits remarkable similarity to that of UCu(II) (see Figure 3). Many isotropically shifted resonances in SCu(II) have counterparts in the spectrum of UCu(II), at very similar positions, and thus the active sites, and in particular the spin density distribution on the ligands, must be analogous in both proteins. Furthermore, the alkaline transition results in the Asn⁴⁷ [SCu(II)]/Asp⁴⁵ [UCu(II)] C²H resonance at approx. 14.6–14.7 p.p.m. at neutral pH* shifting to approx. 13.3–13.4 p.p.m. at pH* approx. 11 in both proteins. In SCu(II), the pK_a for this transition is slightly higher when compared with that in UCu(II). The high degree of similarity between the paramagnetic NMR spectra of SCu(II) and UCu(II) is slightly surprising, as the EPR spectrum of SCu(II) is rhombic and that of UCu(II) is axial. This suggests that the dissimilar EPR spectra arise as a consequence of different interactions of the cupric ion with the axial Gln ligand in the two proteins, for which we have no direct information from the paramagnetic NMR spectra of the Cu(II) proteins. We are currently studying Co(II)-substituted UCu to obtain additional information about the active-site structure, and in particular the nature of the axial interaction at the active site of this phytocyanin.

Another similarity between the paramagnetic NMR spectra shown in Figure 3 is that both possess an exchangeable N²H proton belonging to the more buried His ligand, which is found at 23.7 and 28.3 p.p.m. in SCu(II) and UCu(II) respectively. The signal has similar T_1 values in the two proteins and is in fast exchange with the bulk solvent at neutral pH (and thus is broadened beyond detection under these conditions), but is observed at pH 4.5. It would therefore appear that the degree of exposure to the solvent of this His ligand is similar in these two proteins. The similarity of the chemical shift values of the N²H

	1	10	20	30	40	50	60	70	80	90	100																						
1. SCU	--TVYTVGDS	-AGWKVPFFG	VDYDQW	KWASNKTF	HIGDVLV	FKYDRR	FHNVDKVT	-QKNYQSC	NDTTPIAS	YNTG-NNR	INLKT	VGQK-YYIC	GVPKH	CDLQ	GQKH	INVT	VRS-----																
2. CST	QSTVHIVGDN	-TGWSPSS	-PNFYS-QWA	AGKTFRV	GDLSLQ	FNFPANA	HNVHEMET	KQSFDA	CNFVNS	DNDVERTS	SPVIER	LDELGMH	-YFVCT	VGTHCS	NGQK	LSIN	VVAANA-----																
3. UCU	--EDYDVGGD	-MEWKRP	-DPKFIYI	-TWTGKT	FRVGD	ELFDF	AGMDVAVVT	-KDAFNC	CKKENP	ISHMTTP	-PVKIM	LNTTGPQ	-YYICT	VGDC	HRVQ	GKLS	INNVGAGGAG--																
4. BCB	-AEDYDVGGD	-TEWTRPM	-DPEFYT	-SWATGKT	FRVGD	ELFDF	AGMDVAVVS	-EAFENCE	KEKPI	SHMTVP	-PVKIM	LNTTGPQ	-YFICT	VGDC	HRVQ	GKLS	ITVVAAGATGGAT																
5. MAV	-ATVHKVGD	-TGWTTLP	VPDYA---	KWASNK	FHVGDSL	FNFPANA	HNVHVLQVD	-QQFCS	NSSP	ASYS	TSG-ADSI	PLKRP	GTGF-YFIC	GP	GH	CQK	QV	VEIKVPD---G-----															
6. TDC	AQQTHVVGDT	-LGWTV	PNGGAASYS	-TWAAGK	SFVVD	ILVFN	FRSGSHVAEVS	-KGAFD	SCNTSSPIS	-ISTNG	PTNITL	SSAGSH	-YLC	TPF	SHCT	LQK	LAINVSGSASPAPQ-																
7. NTP	AQTAHVVGDN	-EGWTIP	SSGASAYT	-NWAAGK	TFMVGD	TLVFN	MTNTHDVLQVP	-KASFD	GCSSQNA	IGSAIV	SGPANVT	LDSAGER	-YYICT	FGRH	CQNG	QKLA	ITVSSSTG----																
8. NAP	NAAIYVVG	GDG-N	GWTFGVS	N-----	WPNGK	NFKAGD	VLVFKY	PKGVH	NVIVN	-KANYG	TCNASG	-RTLSS	G-NDR	VTLGK	-GT	-YFIC	GP	PGH	C	SG	QKLS	VTAKLTNK-----											
9. PTS	AATTYAVG	GN-TG	WTIPASNA	KLYT-DW	VKARTF	KLGDIL	VLFKATN	VHNVYRVS	-KADYD	CVTTSP	LEKYETG	-PASIT	LNTTGH	-YYIC	AVSGH	CAAG	QKLS	IKVSTAAAFGS--															
10. PST	AQTVHVG	VDN-TG	WVPSNGAA	AYT-NW	ADRKTF	RVGDTL	VFNFTN	QHDLVQVQ	-KSSFD	GCNSQNA	VSGPIL	GRTSKY	NTQLH	LEITTI	ISTFGR	HCLNG	QKLA	IRVSSSTS-----															
11. INP	EARDHLV	GKPD	AWKIPSS	PSDSL	N-NWAQ	KTRFLP	GDLSLV	WKYDGK	ADAVLEVS	-KRDYV	TCNISL	PIGAMVDG	-TRSIV	LE	RS	GPY	-YFIS	GAEGH	CQKQ	KVIVVVLSEKH	TRKFL												
12. PEA	LATVYTVGDT	-SGW--	-VIGGDS--	--TWASD	KTF	AVGDSL	VFN	YAGAG	HTVDEVK	-ESDYK	SC	TSGNS	SISTD	STG-ATTI	PLK	KAGKH	-YFIC	GV	PGH	CTG	GMKLS	IKV	KASSG-----										
13. UCCI	VATDHTIGGP	-SGWTV	GASLR-----	--TWAAG	QTF	AVGDN	LVFSY	PAAFH	DVVEVT	-KPEF	DS	QAVK	PLITF	FANG-NSL	VPLT	TPGKR	-YFIC	GM	PGH	CSQ	GMK	LEV	NVVP	TATVAPTA									
14. UCCII	VAVTYTI	-----	EWTTG	VDYS--	--GWAT	GKT	FRVGD	ILE	FKYG	-SSHT	VDVVD	-KAGYD	GC	DASS	TENHSDG	-DTKID	LKT	VGIN	-YFIC	STP	PGH	CSG	GMK	LAV	NV	VAGSADLRP-							
15. UCCIII	FAATFKV	GDI-SG	WTSNL	DIY-----	VWLT	GKT	FRVGD	TL	EFYV	G-LSH	SVSVVD	-KAGYD	NC	SSGAT	QNFADG	-DTKID	LT	VTGT	M-HFL	CP	TG	HCK	NG	GMK	LAV	PVLAAPSPSTP							
16. PINE	-----	-----	-----	-----	-----	ELV	FKY	TPG	AHNTLV	VN-KA	AYD	ACTLT	NAL	ATY	TSG-NDT	ISL	NS	TGAK	-YYIC	GP	PGH	CSG	GMK	L	TV	VAAKSN	NGTA-						
17. OSUIII	FAVDYTV	GDT-SG	WSSG	VDYV-----	TWAKS	KTF	SVGDSL	VP	QYS-M	MTVAEVS	-SADY	SACS	ASNSI	QSYSDQ	-NTK	IAL	TKP	GTR	-YFIC	CT	SGH	CSG	GMK	LAV	MV	AAAA	TPTP						
18. TAP	SAVTVN	VEQ-G	WWT	LNTDYS	-----	SWVSG	KFN	VGD	ELV	FKY	SAH	DVVEVS	-KAGYD	SC	ISD	GAINT	FKSG	-NDVI	PL	SAT	GTR	-YFIC	GT	PGH	CSG	GMK	LAV	MV	AAAA	TPTP			
19. CBP	--AVYVVG	S-GW	TFTES	-----	WPKGR	F	FRAGD	ILL	FN	YNP	XMH	NVNVVN	-QGGF	ST	CTN	PAG	KVY	TSG-RD	QIK	L	PK	-G	QS-YFIC	NP	FGH	CSG	GMK	LAV	MV	AAAA	TPTP		
20. PNC	LAAYVNI	G-----	WSFNV	N-----	GARGK	S	FRAGD	VLV	FKY	IKG	QHN	VAVN	-GRGY	AS	CAP	RGARTY	SSG-QD	RIL	KTR	-G	QN-YFIC	S	FP	GH	CSG	GMK	LAV	MV	AAAA	TPTP			
21. ARPN	QAATYTV	GDS-GI	WTF	NAV-----	WPKG	H	FRAGD	VLV	FN	YNP	XMH	NVNVVN	-SGS	YNN	CKT	PTG	AKPY	TSG-KD	RIL	SK	-G	QN-YFIC	NP	FGH	CSG	GMK	LAV	MV	AAAA	TPTP			
22. CAP	QAATYTV	GGA-GW	TFTNTIA	-----	WPNGK	N	FKAGD	TLV	FN	YS	P	GAH	NVAVS	-KAGYD	SC	CTP	PRG	KVY	RS	-KD	QIR	LAR	-G	QN-YFIC	NP	FGH	CSG	GMK	LAV	MV	AAAA	TPTP	
23. MSP	NAETVTV	GGP-KG	WTFGIK	-----	WPNGK	S	FAVGD	VLV	DFG	YNP	XMH	NVNVVN	-QTGYD	K	CT	PEG	SKV	RTG	-SD	QIL	VK	-G	DN-YFIC	NP	FGH	CSG	GMK	LAV	MV	AAAA	TPTP		
24. LEP	NAATYPA	GDG-KG	WGFNMNG	-----	WPNGK	T	FA	GDVIE	FKY	KVDE	HNVVKS	-QEEYD	SC	TS	-GGQ	V	FNSG	-DDQ	I	P	L	E	K	-G	TS-YFIC	T	FGH	CSG	GMK	LAV	MV	AAAA	TPTP

Figure 6 Multiple sequence alignment of the cupredoxin domains from 24 members of the phytoecyanin family (see [2,5] for the original sources of data which are not cited here)

The abbreviations refer to the following proteins: BCB, *A. thaliana* blue copper-binding protein [25]; MAV, green zucchini mavicyanin; TDC, tomato dicyanin (EMBL accession no. AF243180); NTP, tobacco phytoecyanin, which is called 'Nt-SA1-1' on the EMBL database entry (EMBL accession no. AB041517); NAP, Persian tobacco phytoecyanin, which is called 'putative S-RNase-binding protein p11 precursor' on the EMBL database entry (EMBL accession no. AF172853); PTS, *Pinus taeda* stellacyanin, which is called 'PtaPHY2' on the EMBL database entry (EMBL accession no. AF101788); PST, stellacyanin from *Capsicum annuum*, which is called 'CASL1' on the EMBL database entry (EMBL accession no. AF291179); INP, morning glory blue copper protein (EMBL accession no. AB035146) [26]; PEA, pea pods blue copper protein; UCCI, UCCII, UCCIII, *A. thaliana* uclacyanin I, II and III [5]; PINE, loblolly pine phytoecyanin [27,28]; OSUIII, rice uclacyanin III-like protein (EMBL accession no. CAC39044, [29]); TAP, bread wheat phytoecyanin, which is called 'S85' on the EMBL database entry (EMBL accession no. AF031195); PNC, spinach plantacyanin; ARPN, *A. thaliana* plantacyanin [5]; CAP, chickpea phytoecyanin [30]; MSP, alfalfa phytoecyanin [31]; LEP, tomato phytoecyanin (EMBL accession no. AF243181). Where appropriate, we have used the abbreviations of Nersissian et al. [5]. The amino acid sequences are grouped based on the criteria described in Nersissian et al. [5]. Residue numbering is indicated for SCU and the invariant residues are indicated by asterisks. The residues known, or postulated, to be involved in metal-ion co-ordination are indicated in boldface, and the cysteine residues known, or thought, to be involved in disulphide-bond formation are underlined. The alignment was produced using ClustalW (<http://www2.ebi.ac.uk/clustalw/>) and dashes indicate gaps introduced for optimal alignment.

resonances of this His highlights the comparable spin density distribution on to this ligand in the two proteins.

A number of differences do exist between the ^1H NMR spectra of SCU(II) and UCU(II). The most interesting one is the presence of an additional exchangeable resonance in UCU(II) at approx. 48 p.p.m., which is not observed in the spectrum of SCU(II), and is assigned to the N^{H} of the more exposed His⁹⁰ ligand. The observation of the N^{H} signals of both His ligands in UCU(II) indicates that its active site is more protected from the solvent when compared with that of SCU(II), which is responsible for the higher reduction potential observed in UCU (294 mV) compared with that of SCU (189 mV).

It is clear that SCU and UCU are remarkably similar proteins (see Table 1 and Figures 3 and 6). On the basis of the results described in the present study, we reinforce the previous suggestion [14] that UCU should actually be referred to as the stellacyanin from horseradish roots. It would follow that phytoecyanins 1–9 included in Figure 6 should also be referred to in this way, and thus mavicyanin (MAV, sequence 5) should be called the stellacyanin from zucchini.

The exact function of the phytoecyanins is an issue which is as yet unresolved. Although experimental evidence for the function of any phytoecyanin is lacking, we, and others [5], have noted that the stellacyanin, uclacyanin and plantacyanin families of phytoecyanins share significant sequence similarity (see Figure 6). Functional information for any one of the phytoecyanins is

probably informative for some, if not all, members of this protein family. Of particular note is the relatively high sequence identity (approx. 80%) between UCU and the product of the *Arabidopsis thaliana* bcb gene (Figure 6, sequences 3 and 4 respectively). It would therefore seem probable that UCU and BCB perform a similar function in their respective organisms. Given that (i) the *A. thaliana* genome has been sequenced and (ii) BCB transcript abundance/localization has been studied in some detail [25,34–36], the study of BCB function in *A. thaliana* seems particularly attractive.

Conclusions

The alkaline transition in SCU can be assigned to the solvent-exposed Lys⁹⁸ residue, which is situated adjacent to the Gln⁹⁷ ligand. This Lys residue is completely conserved in all available phytoecyanin sequences and will presumably result in similar effects on the spectral properties of these proteins at pH > 8. Confirmation of the assignment of the alkaline transition to this Lys residue is currently being sought by investigating site-directed mutants of UCU and CBP. The alkaline transition in SCU(II) results in a slightly less rhombic active site than at neutral pH, as indicated by the changes in the UV-visible spectrum, and a decrease in the Cu–S(Cys) interaction, as demonstrated by the paramagnetic NMR studies. Lowering the pH does not influence the active-site structure of SCU(II). The active sites of SCU and

UCu are very similar, with that of the former being slightly more solvent-exposed, resulting in it having a lower reduction potential.

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