Investigations of the Alkaline and Acid Transitions of Umecyanin, a Stellacyanin from Horseradish Roots†

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ABSTRACT: The effect of pH on Cu(I) and Cu(II) umecyanin (UCu), a phytocyanin obtained from horseradish roots, has been studied by electronic and NMR spectroscopy and using direct electrochemical measurements. A pK_a value of ∼9.5−9.8 is observed for the alkaline transition in UCu(II), and this leads to a slightly altered active site structure, as indicated by the changes in the paramagnetic ^{1}H NMR spectrum. Electrochemical studies show that the pK_a value for this transition in UCu(I) is 9.9. The alkaline transition is caused by the deprotonation of a surface lysine residue, with Lys96 being the most likely candidate. The isotropically shifted resonances in the ^{1}H NMR spectrum of UCu(II) also shift upon lowering the pH (pK_a 5.8), and this can be assigned to the protonation of the surface (noncoordinating) His65 residue. This histidine titrates in UCu(I) with a pK_a of 6.3. The reduction potential of the protein in this range is also dependent on pH, and pK_a values matching those from NMR, for the two oxidation states of the protein, are obtained. There is no evidence for either of the active site histidines (His44 and His90) titrating in UCu(I) in the pH range studied (down to pH 3.7). Also highlighted in these studies are the remarkable active site similarities between umecyanin and the other phytocyanins which possess an axial Gln ligand.

Type 1 blue copper proteins (cupredoxins) function as electron-transfer agents in both prokaryotes and eukaryotes. They possess a mononuclear copper site which always involves coordination of the metal ion by two histidines, via their N$^\delta$ atoms, and the thiolate group of a cysteine (*1*−*3*). The copper ion is usually displaced from the plane of these three equatorial ligands in the direction of the thioether moiety of a weak axial methionine ligand. In certain cupredoxins the axial methionine ligand is replaced by a glutamine (*4*−*11*). Type 1 copper centers have unique spectroscopic features in the cupric state as a consequence of their active site coordination geometry (*1*, *12*). This includes an intense S(Cys) → Cu(II) ligand to metal charge transfer (LMCT)[1] transition band at approximately 600 nm in their visible spectra, with a second LMCT band at around 450 nm. The EPR spectra of cupredoxins are characterized by having unusually small hyperfine coupling constants in the g_z region, due to the highly covalent nature of the Cu−S(Cys) bond (*12*).

The phytocyanins are a subclass of the cupredoxins which all originate from plants. Recently, the phytocyanins have been grouped into three subfamilies: the stellacyanins (the stellacyanin from *Rhus vernicifera* is one of the best studied phytocyanins and from now on will be denoted by the abbreviation RST), the plantacyanins (including cucumber basic protein, CBP), and the uclacyanins (*6*). This subdividing of the phytocyanins is based on the domain organization of the proteins, the glycosylation state, the nature of the coordination site, and spectroscopic properties. Stellacyanins are identified by the presence of an axial Gln ligand, by having associated carbohydrate, and by them being chimeric proteins consisting of a copper-binding (cupredoxin) domain and a cell-wall anchoring domain. The crystal structure of the cupredoxin domain of cucumber stellacyanin (CST) has been solved and demonstrates that the axial ligand to the copper is the side chain amide oxygen of a glutamine (Figure 1) (*5*). Umecyanin (UCu) is a little studied phytocyanin which is isolated from horseradish roots (*13*−*18*). The amino acid sequence of UCu is known (*7*), and from alignments to those of other phytocyanins the presence of an axial Gln ligand is indicated (see Figure 2). According to the classification of Nersissian et al. (*6*), UCu belongs in the stellacyanin subclass of the phytocyanins. As already mentioned, the spectroscopic features of the phytocyanins have been used in the subclassification of the proteins, which can lead to confusion. The presence of an axial Gln ligand does indeed result in certain spectroscopic features unique to the stellacyanins. However, the visible and EPR spectra of UCu exhibit subtle differences to those of CST and RST. In the case of CST and RST the EPR spectra are rhombic, and their visible spectra have a relatively intense absorption band at ∼450 nm (*4*, *19*). Umecyanin has an axial EPR spectrum

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[1] Abbreviations: UCu, umecyanin; LMCT, ligand to metal charge transfer; RST, stellacyanin from *Rhus vernicifera*; CBP, cucumber basic protein; CST, cucumber stellacyanin; UV/vis, ultraviolet/visible; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 1D, one dimensional; TOCSY, total correlation spectroscopy; WEFT, water-suppressed equilibrium Fourier transform; NHE, normal hydrogen electrode; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MES, 2-morpholinoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; pH*, pH meter reading uncorrected for the deuterium isotope effect; HSE, Hahn spin−echo; CPMG, Carr−Purcell−Meiboom−Gill; wt, wild type.

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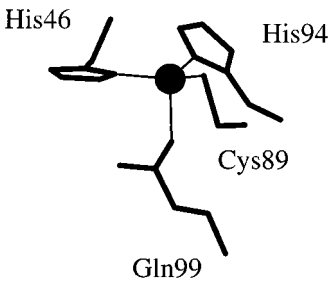

FIGURE 1: Representation of the active site of cucumber stellacyanin (CST) (*2*). The copper ion is shown as a black sphere. The corresponding ligating residues in UCu are His44, Cys85, His90, and Gln95.

Protein	Amino acid sequence										
UCu	Cys^{85}	Thr	Val	Gly	Asp	His^{90}	Cys	Arg	Val	Gly	Gln^{95} Lys
CST	Cys^{89}	Thr	Val	Gly	Thr	His^{94}	Cys	Ser	Asn	Gly	Gln^{99} Lys
RST	Cys^{87}	Gly	Val	Pro	Lys	His^{92}	Cys	Asp	Leu	Gly	Gln^{97} Lys
CBP	Cys^{79}	Asn	Phe	Pro	Gly	His^{84}	Cys	Gln	Ser	Gly	Met^{89} Lys

FIGURE 2: Alignment of the amino acid sequences of the ligand-containing loop of umecyanin (UCu), cucumber stellacyanin (CST), stellacyanin from *R. vernicifera* (RST), and cucumber basic protein (CBP) (*5*, *7*, *66*). In all cases the copper ligands are numbered.

and very little absorption at 450 nm in its visible spectrum (*14*). In other subfamilies of the cupredoxins similar spectroscopic differences also exist which can be attributed to small alterations in ligand−Cu bond lengths and also to relatively minor angular changes in the positions of the ligating residues (*20*−*25*).

An interesting feature of all phytocyanins studied to date is the transition that occurs at high pH values in the oxidized protein and which results in the protein going from a blue to a violet color. Extensive studies of this alkaline transition have been carried out on RST (*19*, *26*, *27*) and CST (*4*). The main visible absorption band for RST occurs at 604 nm at neutral pH and shifts to ∼584 nm at pH 11. The weaker electronic absorption band at lower wavelength also shifts from 452 to 440 nm upon increasing pH. For both bands a pK_a of 10.2 is obtained for the transition. Alkaline transitions have previously been observed in UCu (*13*, *16*, *28*), CST (*4*) and its Gln99Met (axial ligand) variant (*4*), and also in the plantacyanins (*29*). Although numerous studies have been carried out on the alkaline transition of the phytocyanins, the exact cause of this effect has not been found. Initial suggestions (*8*, *30*) that there exists a pH-induced change of the coordination mode of the glutamine ligand appear to be incorrect. The fact that the alkaline transition is observed in the plantacyanins, which have an axial Met ligand (*29*), and also in the Gln99Met variant of CST (*4*) suggests that the glutamine ligand is not responsible for this effect. Furthermore, the Met121Gln azurin (*31*) and Met99Gln amicyanin (*32*) variants do not display this feature. More recent suggestions (*27*, *31*, *33*, *34*) have pointed toward a surface lysine residue, close to the copper site, as being responsible for the alkaline transition. The effect of the alkaline transition on the reduction potential of a phytocyanin has not previously been investigated.

Paramagnetic NMR is a technique which has been applied to Cu(II) cupredoxins and also to their Co(II) and Ni(II) derivatives (*11*, *27*, *35*−*45*). NMR investigations of Cu(II) and Co(II) RST have been used to study the alkaline transition (*27*). The conclusions of these studies are that a conformational change does occur at the active site at high pH but that this is not due to a change in the coordination mode of the axial glutamine ligand. The transition affects most of the ligating residues and highlights the flexibility of the active site in RST. Interestingly, ^{1}H NMR studies of Co(II) and Ni(II) azurin at lower pH values have shown that the protonation of His35, a noncoordinating residue (pK_a ∼5.7−5.9), also results in subtle changes at the active site (*35*, *37*).

Certain cupredoxins have been shown to undergo an active site protonation in the reduced protein, which involves the C-terminal histidine ligand. This occurs in reduced amicyanin (pK_a 6.6) (*46*−*49*), in pseudoazurin (pK_a 4.8) (*50*−*52*), and in most plastocyanins (pK_a 4.9−5.7) (*53*−*57*). Protonation of the same ligand in the oxidized protein, which presumably occurs at much more acidic pH values, has not been observed. The protonation of the His ligand in the reduced protein results in a dramatic increase in the reduction potential due to the preference of Cu(I) for the three-coordinate site formed when one of the His ligands dissociates (*48*, *51*, *55*, *58*−*61*). This protonation also results in a large increase in the reorganization energy of the site (*46*, *57*, *62*). A number of papers have addressed the question of whether one of the His ligands in the reduced phytocyanins becomes protonated and dissociates from the copper **(***26*, *63*−*65***)**. This has been attributed, in part, to the exposed nature of the copper site in phytocyanins [both of the His ligands are solvent exposed in the phytocyanins (*5*, *66*, *67*) whereas in other cupredoxins only the imidazole ring of the C-terminal His protrudes]. The only phytocyanins for which there is any convincing experimental evidence for an active site protonation are the plantacyanins from cucumber (CBP) and spinach. Electrochemical studies on CBP have shown that at pH values below 4.0 the reduction potential of the protein increases quite dramatically (*64*), as in the other cupredoxins which exhibit an active site protonation. In the case of spinach plantacyanin a similar increase in the reduction potential is observed at higher pH values, resulting in a pK_a of 5.7 for the reduced protein. Due to the exposed nature of the copper site (*66*, *67*) either of the coordinated His residues could become protonated in the reduced plantacyanins at low pH. In a recent phytocyanin paper (*6*) a reference was made to the structure of reduced CST, which is said to have an altered copper coordination geometry compared to the oxidized protein; however, no details of the differences were given.

EXPERIMENTAL PROCEDURES

Protein Isolation and Purification. UCu from horseradish roots (*Armoracia laphatifolia*) was purchased from Sigma and used without further purification. This protein gives two bands on an SDS−PAGE gel. Sequencing and mass spectrometry studies (*7*) have shown that the two bands are both UCus of slightly different length (106 and 115 amino acids).

Protein Samples for UV/Vis and Paramagnetic ^{1}H NMR Studies. For UV/vis and paramagnetic NMR experiments the protein was fully oxidized using a solution of $K_3[Fe(CN)_6]$. The excess oxidant was removed using an ultrafiltration cell (Amicon, 5 kDa cutoff membrane). The protein was usually exchanged into 10 mM potassium phosphate buffer. For the

paramagnetic NMR experiments UCu(II) samples were prepared in both 90% H_2O/10% D_2O and 99.9% D_2O and typically contained 2 mM protein.

UCu(I) Samples for ^{1}H NMR Investigations. UCu was fully reduced by the addition of 1 equiv of sodium ascorbate, and the protein was exchanged into 10 mM potassium phosphate buffer (99.9% D_2O). The sample was transferred to an NMR tube and flushed with nitrogen. A small amount of sodium ascorbate was added to the sample to maintain the protein in the reduced form.

Adjustment of the pH of Protein Samples. The pH values of protein solutions were measured using a narrow pH probe (Russell CMAWL/3.7/180) with an Orion 420A pH meter. The pH of the sample was adjusted using NaOD or DCl in deuterated solutions and NaOH and HCl in H_2O solutions. The pH values quoted in deuterated solutions are uncorrected for the deuterium isotope effect and are indicated by pH*.

UV/Vis Spectrophotometry. UV/vis spectra were acquired at 25 °C on either a Perkin-Elmer λ 6 or a Philips PU8740 spectrophotometer.

NMR Spectroscopy. The ^{1}H NMR spectra were acquired on either a Varian Unity 500 or a JEOL Lambda 500 spectrometer usually at 25 °C, using either a standard one-pulse sequence employing presaturation of the H_2O or HDO resonance during the relaxation delay or using the super-WEFT pulse sequence (*68*). Diamagnetic 1D spectra were acquired with a spectral width of ca. 8 kHz. One-dimensional spectra for the assignment of singlet resonances were acquired using the Hahn spin−echo (HSE) [90°$-\tau-$180°$_y-\tau-$] (τ = 60 ms) and Carr−Purcell−Meiboom−Gill (CPMG) [90°$-\tau-$(180°$_y-2\tau)_n-$180°$_y-\tau$] (n = 59, τ = 1 ms) pulse sequences. Two-dimensional TOCSY spectra of UCu(I) were acquired with mixing times of 40 and 60 ms, using a spectral width of ca. 8 kHz with 2048 points for t_2 and 256−512 t_1 increments. Paramagnetic ^{1}H NMR spectra were acquired with spectral widths ranging from 20 to 100 kHz and were processed with 10−50 Hz exponential line broadening as apodization. Spin−lattice (T_1) relaxation times of hyperfine-shifted resonances were determined using the super-WEFT sequence at pH* 6.5 and 32 °C and at pH 4.6 and 7 °C as described previously (*69*).

Electrochemistry of UCu. The direct measurement of the reduction potential of UCu was carried out using a Princeton Applied Research Model 173 potentiostat operated using software from EG&G. The electrochemical cell consisted of a three-electrode system: a gold working electrode (which was in direct contact with 100 μL of a protein containing solution), a platinum auxiliary electrode, and a Ag/AgCl reference electrode. Measurements were carried out at ambient temperature (21 ± 1 °C) at scan rates of typically 20 mV/s. All reduction potentials were referenced to the NHE, and voltammograms were calibrated using the $[Co(phen)_3]^{3+/2+}$ couple (370 mV vs NHE) (*70*).

Preparation of the Gold Working Electrode. Previous direct electrochemical investigations of UCu have utilized a modified gold working electrode (*71*, *72*). Studies have shown that an electrode modified with either 4,4-dithiodipyridine or 2-(diethylamino)ethanethiol results in a good voltammetric response (*71*). In these studies we have used both of these modifiers and in some cases have found that a combination of the two gives an improved response. Before each measurement the gold electrode underwent a series of polishing steps crucial to the voltammetric response and was then chemically modified. The electrode was polished using Al_2O_3-coated films, starting with a particle size of 0.3 μm and followed by 0.03 μm film. The electrode was further polished on a slurry of Al_2O_3 (particle size = 0.015 μm) on fresh Buehler cloth. After polishing, the electrode was sonicated for at least 1 min. The electrode was rinsed thoroughly with deionized water and then, for protein samples in the range of pH 3.7−9.0, modified by immersion in a saturated solution of 4,4-dithiodipyridine for 2 min (*73*). For the more alkaline studies a combination of two promoters was used. First, the gold electrode was immersed in a 2.5 M solution of 2-(diethylamino)ethanethiol for l min, washed thoroughly with deionized water, and soaked in a saturated solution of 4,4-dithiodipyridine for 2 min. The electrode was thoroughly rinsed after modification.

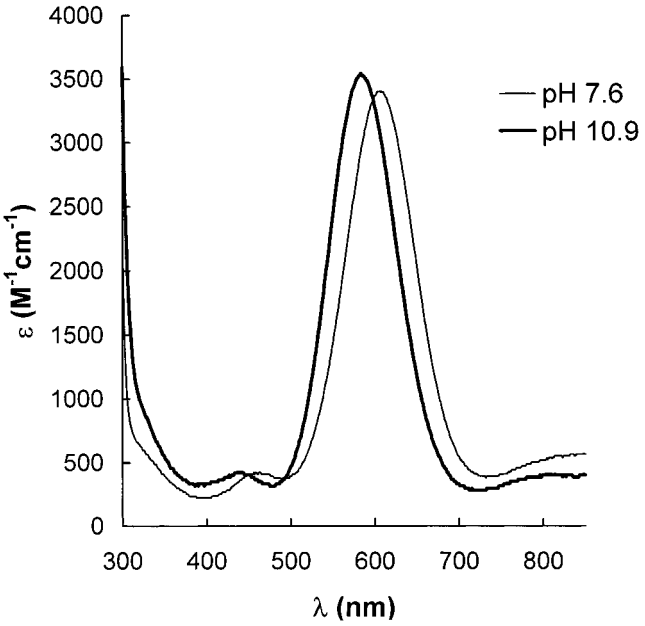

FIGURE 3: Part of the UV/vis spectra (25 °C) of UCu(II) at pH 7.6 and 10.9 in 10 mM phosphate.

pH and Buffers for Electrochemical Studies. A pH-jump method was used in the range 3.7−9.0 by diluting the protein (10-fold) with 20 mM buffer, I = 0.1 M (NaCl). Stock protein solutions (~1 mM) were stored in 1 mM HEPES at pH = 7.1 [I = 0.1 M (NaCl)]. For the studies in the pH range 3.7−5.0, sodium acetate buffer was utilized, while for pH 5.1−6.7, MES buffer was used, and Tris was used for the pH range 7.0−10.6. For measurements at the more alkaline pH values (9.0−10.6), the protein was first diluted into 20 mM Tris, pH 9.0 (I = 0.1 M), and the pH of the protein sample was adjusted by direct addition of 1 M NaOH or 1 M HCl. Aliquots were removed, and the reduction potential was measured. As this approach results in small changes in the ionic strength, the dependence of the reduction potential of UCu as a function of this parameter was measured at pH 10.2 and 10.5. We found that the reduction potentials at these two pH values were almost independent of ionic strength between 0.1 and 0.3 M.

RESULTS

Effect of pH on the UV/Vis Spectrum of UCu(II). In Figure 3 the UV/vis spectra of UCu(II) at pH 7.6 and 10.9 are

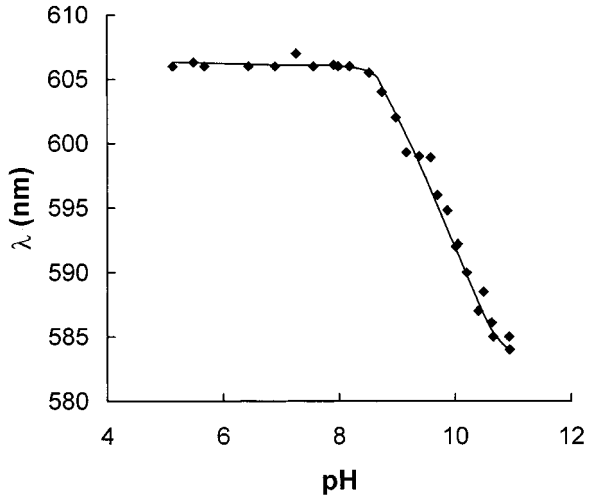

FIGURE 4: Dependence on pH (25 °C) of the wavelength of the main visible absorption band of UCu(II) in 10 mM phosphate.

shown. The main visible absorption band is at 606 nm at neutral pH. When the pH is increased to 10.9, this band shifts to 584 nm. This process is completely reversible, as upon lowering the pH of the sample to 7.6, the band returns to 606 nm. Figure 4 shows the dependence of the position of this visible absorption band upon pH. The data can be fit (three parameters, nonlinear least squares) to eq 1 corresponding to a two-state pH-dependent equilibrium:

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

where λ_H and λ_L are the wavelengths of the visible absorption band at high and low pH, respectively, yielding a pK_a value of 9.8. The position of the second LMCT band is also pH dependent and shifts from 455 nm at pH 7.6 to 439 nm at pH 10.9, giving a pK_a of 9.7. The intensities of these two bands are not greatly affected by the transition, and thus the A_{455}/A_{606} ratio at pH 7.6 is almost identical to the A_{439}/A_{584} ratio at pH 10.9 (0.12). The very broad peak at ~800 nm, which is due to ligand field transitions (*20*, *34*), shifts to lower wavelength, and its intensity diminishes when the pH is increased. However, the broadness of this peak precludes an accurate pK_a determination. The visible spectrum of UCu(II) is unaffected by lowering the pH below 7.6 (see Figure 4).

^{1}H NMR Spectrum of UCu(II). The ^{1}H NMR spectrum of UCu(II) at pH* 7.8 is shown in Figure 5B. From a comparison to published spectra for other cupredoxins (*38*−*40*, *43*), the very broad resonances in the 20−60 ppm region can be assigned to imidazole ring protons from the two coordinated histidine ligands. In the upfield-shifted region there are two partially overlapping peaks. The sharper peak probably originates from a C$^\beta$H signal of a His ligand while the broader resonance is most likely due to the C$^\alpha$H of the Cys85 ligand (*40*). The relatively sharp peak at 14.7 ppm has a T_1 relaxation time of 5.5 ms. A similar sharp resonance, which is the least paramagnetic in the spectrum, has been observed in a very similar region in all ^{1}H NMR studies on oxidized cupredoxins (*38*−*40*, *43*). This peak has been assigned in plastocyanin, azurin, and CST as the C$^\alpha$H resonance of the Asn whose backbone amide group makes a hydrogen bond to the thiolate group of the coordinated Cys (*39*, *40*). A similar hydrogen bond is observed in all of the crystal structures of cupredoxins (*2*, *3*) and can thus be anticipated to be present at the active site of UCu. In the case of UCu the corresponding residue is an aspartic acid (Asp45). We therefore assign this resonance to the C$^\alpha$H of Asp45 in UCu(II). This assignment is confirmed by studies on the Met99Gln amicyanin and Met121Gln azurin variants (*74*). In ^{1}H NMR studies on the Cu(II) forms of these variants a single resonance is found in the 10−20 ppm region of the spectrum which has a relatively long T_1 value, as is also the case in the wt proteins. In all cases this single peak must belong to the same resonance, the C$^\alpha$H resonance of the corresponding Asn residue.

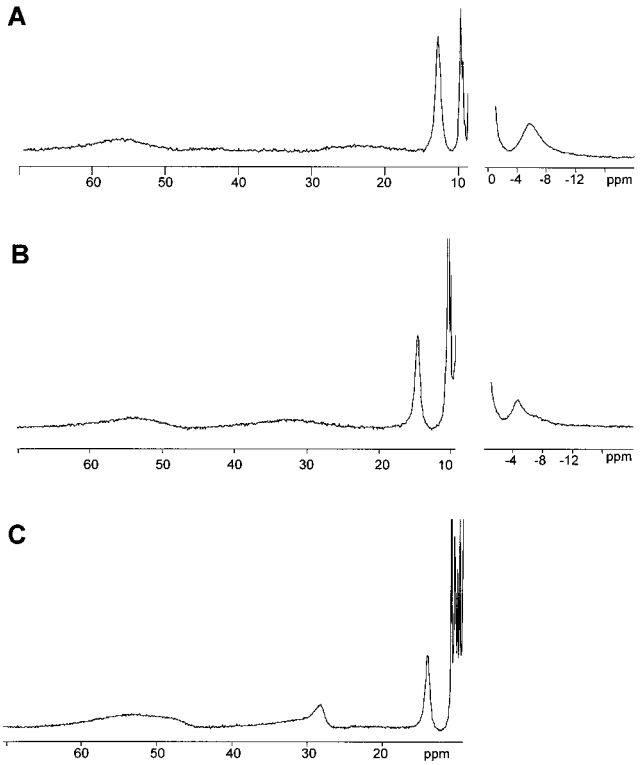

FIGURE 5: ^{1}H NMR spectra (25 °C) of UCu(II) (500 MHz) in 10 mM phosphate: (A) in D_2O at pH* 10.5; (B) in D_2O at pH* 7.8; (C) in 90% H_2O/10% D_2O at pH 4.6.

Effect of Alkaline pH on the ^{1}H NMR Spectrum of UCu(II). When the pH* is increased, the C$^\alpha$H resonance of Asp45 shifts in an upfield direction from 14.7 ppm at pH* 7.8 to 13.3 ppm at pH* 10.9 (Figure 5A). 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 6. The data above pH* 7.8 can be fit 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 9.9. The pH titration of UCu(II) was repeated in 90% H_2O/10% D_2O in the same pH range, and a pK_a value of 9.8 was obtained.

The relatively sharp upfield-shifted peak at −4.5 ppm at pH* 7.8 shifts to −5.8 ppm when the pH* is increased to 10.9 (see Figure 5A,B). From the shift of this resonance a pK_a* of 9.7 is obtained (a similar shift and pK_a are observed in 90% H_2O/10% D_2O). The very broad peaks at ~32 and ~53 ppm (pH* 7.8) are also affected when the pH* is

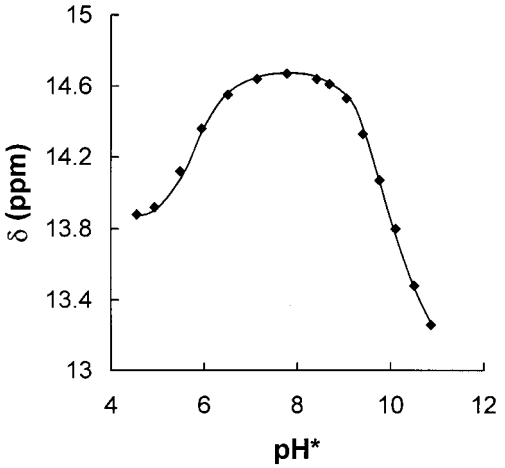

FIGURE 6: Dependence on pH* (25 °C) of the chemical shift of the $C^{\alpha}H$ resonance of Asp45 in the ^1H NMR spectrum of UCu(II) in 10 mM phosphate. The solid line shows a fit of the data to eq 2.

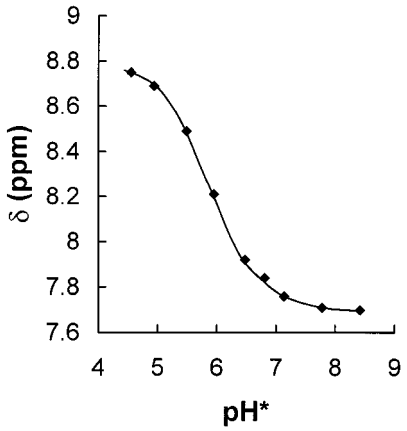

FIGURE 7: Dependence on pH* of the chemical shift of the His65 $C^{\epsilon 1}H$ resonance in the ^1H NMR spectrum of UCu(II) in 10 mM phosphate (25 °C). The solid line shows a fit of the data to eq 2.

increased (see Figure 5A,B) and shift to ~25 and ~58 ppm at pH* 10.9, giving pK_a* values of 9.9 and 9.8, respectively.

Effect of Acidic pH on the ^1H NMR Spectrum of UCu(II). When the pH* is lowered, the Asp45 $C^{\alpha}H$ peak shifts to 13.9 ppm at pH* 4.6 (see Figure 5B,C). The dependence on pH* in this range is also shown in Figure 6, and a fit of the data below pH* 7.8 to eq 2 yields a pK_a* value of 5.7 (a pK_a value of 5.8 is obtained from the titration carried out in 90% H_2O/10% D_2O). The positions of certain other paramagnetic resonances are also dependent on pH in this range, but in all cases the shifts are too small to allow accurate pK_a determinations. In the diamagnetic region of the ^1H NMR spectrum of UCu(II) the $C^{\epsilon 1}H$ resonance of a histidine can be identified (at 7.71 ppm at pH* 7.8; data not shown). This can only belong to His65, as the other two histidines in the UCu sequence are copper ligands and their imidazole ring resonances are very broad and shifted (vide supra). This resonance shifts by ca. 1 ppm in the pH* range 8−4.5, giving a pK_a* value of 5.9 (see Figure 7). The $C^{\delta 2}H$ proton of His65 is found at 6.64 ppm at pH* 7.8 and shifts very little with pH.

An interesting feature of the ^1H NMR spectrum of UCu(II) in H_2O at low pH is the presence of two additional resonances at 48.3 (T_1 1.3 ms) and 28.3 ppm (T_1 1.7 ms) (see Figure 5C). Spectra were also obtained at pH 4.6 and 7 °C (data not shown), when these two peaks increased in intensity. No additional signals were observed at the lower temperature. The two exchangeable protons can arise from the $N^{\epsilon 2}H$ signals of the two His ligands or from the side chain amide group of the axial Gln ligand. From the crystal structure of CST (*4*) one of the Gln $N^{\epsilon 2}$ protons should be very close to the copper ion (3.13 Å in CST) and will be very broad. In previous NMR studies of Cu(II) cupredoxins the $N^{\epsilon 2}H$ signal of the more buried His ligand is usually observed in the 20−30 ppm region of the spectrum (*38*−*40*, *43*). The proton belonging to the more exposed histidine ligand is in fast exchange with bulk water and is broadened beyond detection. In studies on CST the $N^{\epsilon 2}H$ signal of His46 is observed at 26 ppm at pH 6.0 (*40*). No resonance from the axial Gln ligand of CST experiences a sizable Fermi contact shift. A signal from an exchangeable proton is observed at ~48 ppm in RST and has been tentatively assigned to an axial Gln $N^{\epsilon 2}$ proton (*27*). It is therefore most likely that the signals due to exchangeable protons in the spectrum of UCu(II) belong to the $N^{\epsilon 2}H$ signals of His44 and His90. An alternative assignment is that the peak at 28.3 ppm is due to His44 while that at 48.3 ppm belongs to the $N^{\epsilon 2}H$ of the axial Gln95 ligand.

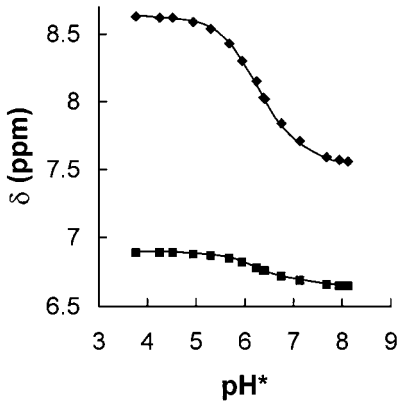

FIGURE 8: Dependence on pH* of the chemical shifts of the His65 $C^{\epsilon 1}H$ (◆) and $C^{\delta 2}H$ (■) resonances in the ^1H NMR spectrum of UCu(I) in 10 mM phosphate (25 °C). The solid lines show the fits of the data to eq 2.

Effect of pH on the ^1H NMR Spectrum of UCu(I). Singlets belonging to His resonances have been assigned in the ^1H NMR spectrum of UCu(I) using the HSE and CPMG pulse sequences. The two singlets at 7.59 and 6.66 ppm at pH* 7.7 show a cross-peak in a TOCSY spectrum and thus can be assigned to the same His residue. These peaks are still present in the spectrum of UCu(II) and must arise from His65. The chemical shifts of these two peaks are dependent on pH* in the range 8.0−4.5. The resonance at 7.59 ppm experiences a much larger overall shift with pH and is thus assigned as the $C^{\epsilon 1}H$ peak, while that at 6.66 ppm is the $C^{\delta 2}H$ signal. The dependence on pH* of the chemical shift of these two peaks is shown in Figure 8. The data can be fit to eq 2, which yields pK_a values of 6.3 for both peaks. The singlets at 7.51 and 7.28 ppm and at 7.50 and 7.26 ppm exhibit cross-peaks in a TOCSY spectrum. These singlets are absent in the diamagnetic region of the spectrum of UCu(II) and are thus assigned to the imidazole ring protons of the two histidine ligands. These ligand histidine signals are unaffected by changes in pH*, even at pH* values down to 3.7.

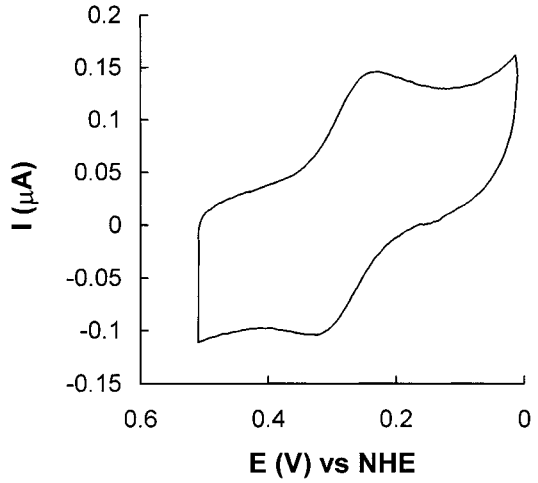

FIGURE 9: Cyclic voltammogram of UCu in 20 mM Tris, pH 7.2 [I = 0.10 M (NaCl)], using a scan rate of 50 mV/s.

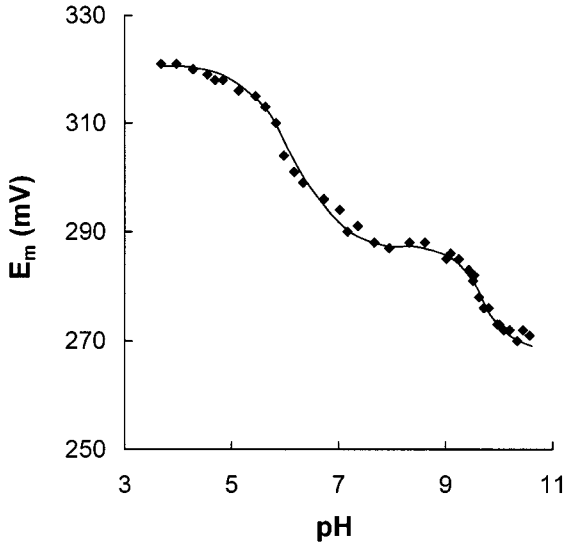

FIGURE 10: Dependence on pH of the reduction potential (E_m) of UCu at I = 0.10 M (NaCl). The line shown is obtained from a fit of the data to eq 3.

pH Dependence of the Reduction Potential of UCu. UCu yielded good, quasi-reversible, responses on a 4,4-dithiodipyridine-modified gold electrode in the pH range 4.6−10.6. The response at pH values above 9.0 was improved by using a working electrode which had been modified with both 4,4-dithiodipyridine and 2-(diethylamino)ethanethiol. In all cases the anodic and cathodic peaks were of equal intensity, and their separation was approximately 60−80 mV at a scan rate of typically 20 mV/s. The peak currents are proportional to the (scan rate)$^{1/2}$ in the range 3−140 mV/s at pH 7.4 [I = 0.10 M (NaCl)], and a typical cyclic voltammogram is shown in Figure 9. At pH values above 10.6 the electrochemical response of UCu at the gold electrode deteriorated regardless of which modifiers are used. At pH values below 4.6 the electrochemical response is not ideal, but relatively accurate reduction potentials can be measured down to pH 3.7. The variation of the reduction potential of UCu with pH is shown in Figure 10. The reduction potential at pH 7.0 [I = 0.10 M (NaCl)] is 294 mV, consistent with a previously measured value (*72*). As the pH value is increased, the reduction potential gradually decreases and a value of 271 mV is found at pH 10.6. As the pH is lowered from neutral, the reduction potential increases and a value of 321 mV is obtained at pH 3.7. The pH dependence of the reduction potential of UCu can be fit to the following equation:

$$E_m(\text{pH}) = E_m(\text{low pH}) + \frac{RT}{nF}\ln\left(\frac{[H^+]^2 + [H^+]K_a^{red,1} + K_a^{red,1}K_a^{red,2}}{[H^+]^2 + [H^+]K_a^{ox,1} + K_a^{ox,1}K_a^{ox,2}}\right) \quad (3)$$

where E_m(pH) is the measured reduction potential, E_m(low pH) is the reduction potential at low pH, $K_a^{red,1}$ and $K_a^{ox,1}$ are the proton dissociation constants for the residue in the reduced and oxidized proteins, respectively, which affect E_m(pH) at lower pH, $K_a^{red,2}$ and $K_a^{ox,2}$ are the proton dissociation constants for the residue in the reduced and oxidized proteins, respectively, which affect E_m(pH) at higher pH, and the other symbols have their usual meaning (*75*). The fit of the data yields a p$K_a^{red,1}$ of 6.3, a p$K_a^{ox,1}$ of 5.8, a p$K_a^{red,2}$ of 9.9 and a p$K_a^{ox,2}$ of 9.5

DISCUSSION

Effect of pH on the UV/Vis Spectrum of UCu(II). The UV/vis spectrum of UCu(II) at pH 7.6 reported herein is slightly different from that usually quoted in the literature (*76*). The main visible absorption band is at 606 nm compared to the value of 610 nm usually used. It should be noted that in the original studies on UCu (*13*) this band was also reported to be at 606 nm. A second LMCT transition is found at 455 nm, and the ratio of the absorptions at these wavelengths (A_{455}/A_{606}) is 0.12. The corresponding bands are found at 604 and 450 nm in oxidized RST at neutral pH, and the A_{450}/A_{604} ratio is 0.24 (*27*). In CST they are at 600 and 450 nm with an A_{450}/A_{600} ratio of 0.17 (*6*). The blue shifts observed for the two LMCT bands in UCu(II) when the pH is increased give a pK_a of ∼9.8. This is very similar to that observed for the same transition in RST (10.2) (*27*). To further investigate the alkaline transition of UCu, we have carried out ^{1}H NMR and electrochemical studies on the protein at various pH values.

^{1}H NMR Spectrum of Cu(II) Umecyanin and Effect of pH. The ^{1}H NMR spectrum of UCu(II) exhibits a number of hyperfine-shifted resonances for which assignments can be made. When the pH is increased from neutral values, all of these resonances shift and yield pK_a values of 9.7−9.9. This indicates that the alkaline transition alters the active site geometry, affecting the ligating residues.

When the pH is lowered from 7, the Asp45 C$^\alpha$H signal also moves in an upfield direction, though the total shift is approximately half that observed in the pH range 7−11. Additionally, most of the other isotropically shifted resonances are affected in this pH range. The effect observed on lowering the pH* can be assigned to the protonation of the noncoordinated His65 residue. Paramagnetic ^{1}H NMR studies on the Co(II)- and Ni(II)-substituted forms of *Pseudomonas aeruginosa* azurin (*35*, *37*) have shown that protonation of the noncoordinated His35 residue, which is situated >8 Å from the copper ion (*77*), has an effect on the structure of the active site. Again, in this case most of the isotropically shifted resonances are affected by protonation of His35, indicating a slightly altered active site geometry at low pH caused by the protonation of the surface His

residue. In crystallographic studies on Cu(II) azurin at low and high pH no discernible difference was found at the active site (*77*). Therefore, the magnitude of the active site changes at acidic and alkaline pH values in UCu, which could be due to a combination of changes in both bond lengths and the orientation of coordinating residues, is probably quite small (about a few tenths of an angstrom or a few degrees).

Influence of pH on the ^{1}H NMR Spectrum of UCu(I). The ^{1}H NMR spectrum of UCu(I) exhibits the singlet resonances from the three His residues present in the protein. Only two of these resonances are affected by pH, and both give the same pK_a value of 6.3. These two resonances are also present in the ^{1}H NMR spectrum of UCu(II), indicating that they belong to the noncoordinated His65 residue. The imidazole ring resonances of His44 and His90 are not affected by pH, even at a pH* value of 3.7. This indicates that neither of the His ligands is protonated in the reduced protein at lower pH values, a feature which has been found to occur in a number of the cupredoxins (*46*−*55*). This conclusion is further supported by electrochemical studies on UCu at low pH values (vide infra). Various studies have been interpreted to imply that active site protonation is a feature common to the phytocyanins (*26*, *63*−*65*). Furthermore, investigations of the Met121Gln azurin variant have shown that this mutation leads to a weakening of one of the Cu−N(His) bonds (*31*). However, we show that in UCu(I), and possibly in all of the other phytocyanins with an axial Gln ligand, neither of the ligand His resonances becomes protonated at low pH.

Effect of pH on the Reduction Potential of UCu. UCu exhibits a distinct dependence of its reduction potential on pH. This can be assigned to the ionization of two groups reasonably close to the active site. The effect at low pH is due to the protonation/deprotonation of His65. The pK_a values obtained from NMR and electrochemical studies for this residue in UCu(I) and UCu(II) are in very good agreement. From the structure of CST (*5*) and sequence alignments (*7*), the residue which corresponds to His65 of UCu (Val69) is 13.5 Å from the active site (distance quoted is that from Cu to the C$^\alpha$ atom).

The observation that the protonation of a surface histidine residue probably >10 Å from the copper center has a significant effect on the active site structure of UCu(II) indicates that the alkaline transition can also be assigned to the deprotonation of a surface residue. The effect of increasing the pH above 8.0 on the reduction potential of UCu not only gives rise to the pK_a for the alkaline transition in UCu(II) but also provides, for the first time, the pK_a value for this effect in a reduced phytocyanin. Given the pH range for this transition, it would appear that a lysine has to be the most likely candidate (*78*). Umecyanin possesses seven Lys residues whereas RST has ten. Cucumber stellacyanin, for which an X-ray structure exists (*5*), has only three Lys residues, two of which are conserved among the different phytocyanin primary structures. These are Lys29 and Lys100 (CST numbering), which are 22.5 and 8.9 Å, respectively, from the copper ion (distances are to the C$^\alpha$ atoms) (*5*). It would therefore appear that the most likely candidate as the cause of the alkaline transition in UCu is Lys96 (Lys100 in CST) and that the corresponding residue (i.e., the Lys residue which is adjacent to the axial ligand) is responsible for the effect in all other phytocyanins (see Figure 2). An alkaline transition has also been observed in the blue copper protein pseudoazurin, which is not a phytocyanin (*43*, *79*). In this case the cause of this effect has also been attributed to a Lys residue close to the active site. The Met44Lys azurin variant (*80*), in which a basic residue is introduced into the center of the protein's hydrophobic patch, exhibits very similar effects at high pH values as compared to the phytocyanins.

At low pH values there is no indication of the reduction potential of UCu steadily increasing (by 60 mV/pH unit), a feature which has been found in all cupredoxins which exhibit an active site protonation at their C-terminal His ligand (*48*, *50*, *58*−*61*). This confirms the conclusion of the ^{1}H NMR studies at low pH values in which none of the His ligand resonances were seen to titrate in UCu(I).

Comparison of Umecyanin, R. vernicifera Stellacyanin, and Cucumber Stellacyanin. The ^{1}H NMR spectrum of UCu(II) exhibits remarkable similarity to that of RST (*27*) and CST (*40*). The spectra of UCu(II) and oxidized RST both possess an exchangeable resonance at ∼48 ppm, which has been tentatively assigned to the N$^{\epsilon 2}$H of the axial glutamine ligand in RST (*27*). An alternative assignment suggested here is that this resonance belongs to the N$^{\epsilon 2}$H of the more exposed His90 ligand in UCu(II). This conclusion is consistent with the fact that in the spectrum of CST no resonances from the axial Gln ligand experience sizable Fermi contact shifts. This has been attributed (*40*) to the fact that the axial Gln ligand does not contribute to the HOMO in which the unpaired electron is located (*20*). A number of the other isotropically shifted resonances in UCu(II) have counterparts in the spectra of RST and CST, at very similar positions, highlighting the remarkable similarity between the active site architectures in the proteins. Another intriguing similarity between the ^{1}H NMR spectra of the oxidized forms of UCu and RST is the effect of the alkaline transition. In both proteins a peak at ∼14.6−14.7 ppm at neutral pH is shifted to ∼13.2−13.3 ppm at pH ∼11.

A number of differences do exist between the ^{1}H NMR spectra of UCu(II), RST, and CST. The most interesting is the presence of two signals attributed to solvent-exchangeable protons in UCu(II), while in the other two proteins only one such signal is observed (*27*, *40*). This points toward a more buried active site in UCu. The reduction potential of UCu is 294 mV at pH 7.5 and is higher than those of RST (187 mV) (*72*) and CST (260 mV) (*4*), possibly as a consequence of a more buried active site in UCu (*81*). Confirmation of this conclusion must await further detailed structural information on these proteins.

CONCLUSIONS

These investigations demonstrate that the active site of UCu is very similar to those of RST and CST which further justifies the classification of this protein as a stellacyanin. This indicates that the name umecyanin is now obsolete and this protein should be referred to as the stellacyanin from horseradish roots. Spectroscopic differences do exist between the stellacyanins. These variations could be due to small changes in the bond lengths and angular arrangements at the copper site (*20*−*25*, *82*), and therefore spectroscopy alone should not be used as a criterion when subclassifying the phytocyanins (or any subfamily of the cupredoxins). UCu

exhibits both an acid and an alkaline transition. The former only has an effect on the paramagnetic NMR spectrum while the alkaline transition also alters the visible spectrum. The acid transition is due to the protonation of the surface His65 residue while the alkaline transition can be attributed to Lys96. Both effects alter the reduction potential of the protein by 20−30 mV. This may be of importance to the physiological function of the protein which is presumed to be in electron transfer. We demonstrate by both electrochemical and NMR studies that UCu does not exhibit an active site (ligand His) protonation in the pH range investigated. This is probably also true for all of the other stellacyanins.

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