Unusual Properties of Plastocyanin from the Fern *Dryopteris crassirhizoma*†

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ABSTRACT: The effect of pH on the ^{1}H NMR spectrum, reduction potential, and self-exchange rate constant of the novel plastocyanin (PCu) from the fern plant *Dryopteris crassirhizoma* has been studied. The results are compared with those for the higher-plant PCu from parsley. In the ^{1}H NMR spectrum of *D. crassirhizoma* PCu(I), there is no sign that either of the His ligands is protonated at pH* down to 5.4. The reduction potentials of *D. crassirhizoma* and parsley PCu are 382 and 379 mV, respectively, at pH 7.4. When the pH value is decreased, the reduction potential of parsley PCu is seen to increase quite dramatically, consistent with protonation at His87 in PCu(I). A pK_a of 5.8 is obtained from the electrochemistry data, consistent with a value of 5.6 determined by NMR. The reduction potential of *D. crassirhizoma* PCu exhibits a much less pronounced dependence on pH. The self-exchange rate constant of *D. crassirhizoma* PCu(I) is 3.4×10^3 M^{-1} s^{-1} at pH* 7.9. This is the smallest self-exchange rate constant reported to date for a PCu and can be rationalized by considering the altered distribution of charged residues on the surface of the *D. crassirhizoma* protein compared to the charge distributions of other higher-plant PCus. The self-exchange rate constant increases to 9×10^3 M^{-1} s^{-1} at pH* 5.4, consistent with enhanced protein−protein association at lower pH*, and the absence of His87 protonation in *D. crassirhizoma* PCu(I) in the accessible pH range.

Plastocyanin (PCu)[1] is a type 1 blue copper protein (cupredoxin) involved in photosynthetic electron transfer (et) between cytochrome *f* of the b_6f complex and P700$^+$ of PSI (*1*, *2*). Plastocyanin was the first cupredoxin to be structurally characterized (*3*) and consists of eight β-strands which constitute two β-sheets, giving the molecule an overall topology known as a β-barrel (see Figure 1) (*4*). The copper ion is buried approximately 6 Å from the protein surface and has a distorted tetrahedral geometry. Three ligands form strong bonds to the copper, namely, the thiolate sulfur of Cys84 and the N$^\delta$ atoms of His37 and His87 (*4*). The copper ion is slightly displaced from the plane of these three equatorial ligands toward the weakly coordinated thioether sulfur of Met92.

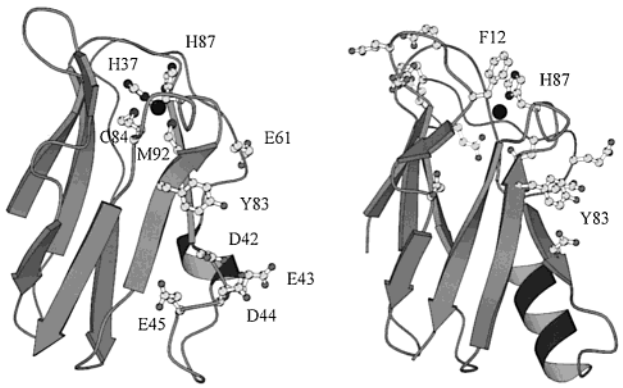

FIGURE 1: Representations of the structures of Cu(I) parsley (PDB entry 1PLB) and *D. crassirhizoma* (PDB entry 1KDI) PCus drawn with MOLSCRIPT (*78*). The copper ion is shown as a black sphere in both cases, and the side chains of the coordinating amino acids are included in the parsley structure. In the *D. crassirhizoma* structure, only the His87 ligand is shown as is the nearby Phe12 residue. Also shown are the acidic residues which surround Tyr83 in parsley PCu and which are concentrated around the hydrophobic patch in *D. crassirhizoma* PCu.

The structures of higher-plant and green algal PCus reveal two surface areas as potential binding sites for redox partners (*3*−*10*). The first is formed by a cluster of nonpolar side chains, which surround the solvent-exposed His87 ligand, and is known as the hydrophobic patch (see Figure 1). The second consists of a group of carboxylate side chains, which surround the exposed Tyr83, and has become known as the

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[1] Abbreviations: PCu, plastocyanin; LMCT, ligand to metal charge transfer; UV−vis, ultraviolet−visible; NMR, nuclear magnetic resonance; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 1D, one-dimensional; 2D, two-dimensional; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy; 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; *wt*, wild type; DEAE-SH, 2-(diethylamino)ethanethiol; PSI, photosystem I; et, electron transfer; $E^{\circ\prime}$, standard reduction potential.

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acidic patch (see Figure 1). The latter surface can be divided into the upper (E59, E60, and E61) and the lower (D42, E43, D44, and E45) acidic patches in higher-plant PCus. The size of the upper acidic patch is diminished in the higher-plant PCu from parsley (*10*) (see Figure 1) and in the green algal proteins (*7*−*9*). In the cyanobacterial PCus, the acidic patch is nonexistent (*11*−*15*). It has been found that the acidic and hydrophobic patches of higher-plant PCus are important for their interaction with both PSI and cytochrome *f* (*16*−*31*). In both cases, it appears that an electron-transfer pathway via the His87 ligand is used (*30*, *31*), and not one involving the Tyr83 residue which is adjacent to the Cys84 ligand.

Recently, the structure of a PCu from the fern (vascular higher plant) *Dryopteris crassirhizoma* has been determined (see Figure 1) (*32*, *33*). The acidic patch around Tyr83 (note that the residues in *D. crassirhizoma* PCu are numbered here according to the accepted convention, and thus, ignore the three inserted residues found in its sequence compared to typical higher-plant PCus, vide infra) in this PCu is restricted to two carboxylate side chains (Asp42 and Glu59). This region of the amino acid sequence possesses additional amino acids, compared to other higher-plant PCus, which form an α-helical section (see Figure 1). The *D. crassirhizoma* PCu still possesses a large number of acidic residues on its surface, but the majority of these form an arc around the edge of the hydrophobic patch of the protein (see Figure 1). In fact, 11 of the 15 acidic residues which *D. crassirhizoma* PCu possesses are found in this area of the protein. This results in a molecule having electrostatic surface properties drastically different from those of all other higher-plant PCus.

Certain cupredoxins, including PCu (*34*−*38*), pseudoazurin (*39*−*41*), and amicyanin (*42*−*44*), exhibit fluxionality of their active sites at low pH in their reduced forms. This is a consequence of the protonation of the exposed C-terminal His ligand (in all structurally characterized cupredoxins, the His ligand corresponding to His87 of PCu is solvent-exposed and is surrounded by a hydrophobic surface) and results in the reduced copper adopting an almost trigonal planar geometry in which the Cu−S(Met) bond is shortened (except in the case of amicyanin) (*38*, *41*, *44*). The pK_a value of the histidine ligand is in the range of 5−6 for PCus (*45*), ∼5 for pseudoazurins (*40*), and ∼7 for the amicyanins (*42*, *43*). The *D. crassirhizoma* protein is unique among the PCus in that crystallographic studies of the oxidized and reduced proteins at pH 4.5 have indicated very similar active site structures and thus the absence of protonation of the His87 ligand in PCu(I) (*32*).

The electron self-exchange reaction is an intrinsic property of all redox systems (*46*). In the case of redox metalloproteins, it is an extremely useful reaction to study because the structure of only one protein needs to be considered when interpreting the rate constants. Furthermore, the reaction has no driving force and thus provides a measure of the electron-transfer capabilities of the different members of a family of redox proteins. Another reason for determining self-exchange rate constants is their fundamental importance to Marcus theory. NMR spectroscopy provides the only routine method available for the study of this process and has been used to measure the self-exchange rate constants of a number of cupredoxins, with values ranging from 10^3 to 10^6 M^{-1} s^{-1} (*47*). In the case of the PCus, self-exchange rate constants of 5.0×10^4 and 3.2×10^5 M^{-1} s^{-1} have been determined for the proteins from parsley (*45*) and *Anabaene variabilis* (cyanobacterium) (*48*), respectively. The smaller rate constant in the case of parsley PCu has been attributed (*45*) to the presence of the acidic patch in this protein, which hinders protein−protein association. The absence of an acidic patch in the cyanobacterial PCu facilitates protein−protein association, resulting in a larger second-order self-exchange rate constant. The effect of pH on the self-exchange rate constant of parsley PCu has also been investigated (*45*). Interestingly, at pH* 5.6, a value at which half of the PCu(I) molecules are protonated at His87 (pK_a* = 5.6), the self-exchange rate constant is almost identical to that determined at pH* 7.5. This has been rationalized by the fact that although at pH* 5.6 protonation of His87 in PCu(I) would hinder et, this effect is counterbalanced by the enhanced association of two parsley PCus at lower pH* due to the partial protonation of the protein's acidic patch.

In this study, we investigate the effect of lowering the pH on the ^{1}H NMR spectrum of *D. crassirhizoma* PCu(I), the reduction potential of the protein, and the self-exchange rate constant. The behavior is compared directly with that of parsley PCu. Conclusions from all of these experiments are consistent with the crystallographic data on *D. crassirhizoma* PCu which indicate that His87 does not protonate in this protein in the accessible pH range. The reasons for the absence of this behavior in this one member of the PCus are discussed. A number of interesting conclusions are also drawn concerning the effect of the distribution of surface charges on the et reactivity of *D. crassirhizoma* PCu as compared to other higher-plant PCus.

EXPERIMENTAL PROCEDURES

Protein Isolation and Purification. PCus from *D. crassirhizoma* (*32*) and parsley (*45*) were isolated and purified using methods described previously. Pure *D. crassirhizoma* PCu possesses a peak A_{278}/A_{590} ratio of ≤1.5, whereas the pure protein from parsley has an A_{278}/A_{597} ratio of ≤1.7. Both pure PCus gave a single band on an SDS−PAGE gel.

NMR Sample Preparation for pH Titrations. PCu was fully reduced by the addition of 1 equiv of sodium ascorbate, and the protein was exchanged using ultrafiltration (Amicon, 5000 MWCO membrane) into potassium phosphate buffer (99.9% D_2O). The sample was transferred to an NMR tube and the tube flushed with nitrogen. A small amount of sodium ascorbate was added to the sample to maintain the protein in the reduced form.

NMR Sample Preparation for Self-Exchange Rate Constant Measurements. For self-exchange rate constant measurements, the sample was exchanged into either 36 mM phosphate at pH* 7.9, 57 mM phosphate at pH* 6.8, 86 mM phosphate at pH* 5.8, or 94 mM phosphate at pH* 5.4 (all with $I = 0.10$ M). PCu(I) was produced as described above, with the excess reductant exchanged out by ultrafiltration. The reduced sample was placed in an NMR tube and the tube flushed with nitrogen and sealed. Fully oxidized protein was obtained by the addition of a sufficient volume of 20 mM $[Fe(CN)_6]^{3-}$, and the excess oxidant was removed by ultrafiltration. Small amounts of the oxidized protein were added to the reduced sample. The concentration of the oxidized protein in the sample was determined by transferring the mixed sample to a 2 mm UV−vis cuvette and measuring

the absorbance at 590 nm (ϵ = 4700 M^{-1} cm^{-1}) (*32*). Readings were taken before and after the acquisition of NMR spectra, with an average of the two values used for all subsequent calculations.

Adjustment of the pH of Protein Samples for NMR Spectroscopy. 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. The quoted pH values are uncorrected for the deuterium isotope effect and thus are denoted by pH*.

NMR Spectroscopy. All proton NMR spectra were acquired at 500.16 MHz on a JEOL Lambda 500 spectrometer at 25 °C. Standard 1D spectra were acquired with a spectral width of 8 kHz and employing presaturation of the HDO resonance during the relaxation delay. Free induction decays were accumulated into 16K data points and zero filled to give 32K points for transformation. All chemical shifts (δ) are quoted in parts per million (ppm) relative to water at 4.76 ppm. 1D spectra for the assignment of singlet resonances were acquired using the Hahn spin-echo (90°$-\tau-$180°$_y-\tau-$) (τ = 60 ms) and Carr−Purcell−Meiboom−Gill [90°$-\tau-$(180°$_y-2\tau)_n-$180°$_y-\tau$] (n = 59, τ = 1 ms) pulse sequences. 2D TOCSY and NOESY spectra of PCu(I) were acquired using a spectral width of ca. 8 kHz with 2048 points for t_2 and 256−512 t_1 increments. Mixing times of 70 and 200 ms were used for the TOCSY and NOESY spectra, respectively. Spin−lattice (T_1) relaxation times were determined using a standard inversion recovery sequence ($d-$180°$-\tau_D-$90°−acq). The values of τ_D ranged from 10 ms to 10 s, with the total relaxation delay (d + acq) always being greater than 5 times the T_1 of the resonances being analyzed. The solvent peak was irradiated during d and τ_D. An exponential fit of a plot of the peak intensity against τ_D, for a particular proton, yielded its T_1 value. Spin−spin (T_2) relaxation times were derived from peak widths at half-height using the relation $\nu_{1/2} = (\pi T_2)^{-1}$.

UV−Vis Spectrophotometry. UV−vis spectra were acquired at 25 °C on either a Shimadzu UV-2101PC or a Philips PU8740 spectrophotometer.

Electrochemistry of D. crassirhizoma and Parsley PCu. The direct measurement of the reduction potential of PCu 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) (*49*).

Preparation of the Gold Working Electrode. Prior to 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 a 0.03 μm film. The electrode was further polished on a slurry of Al_2O_3 (0.015 μm particle size) on fresh Buehler cloth. After being polished, the electrode was sonicated for at least 1 min in deionized water. The electrode was rinsed thoroughly with deionized water and then modified by immersion in a 1 M solution of 2-(diethylamino)ethanethiol (DEAE-SH) for <5 min. The electrode was thoroughly rinsed after modification.

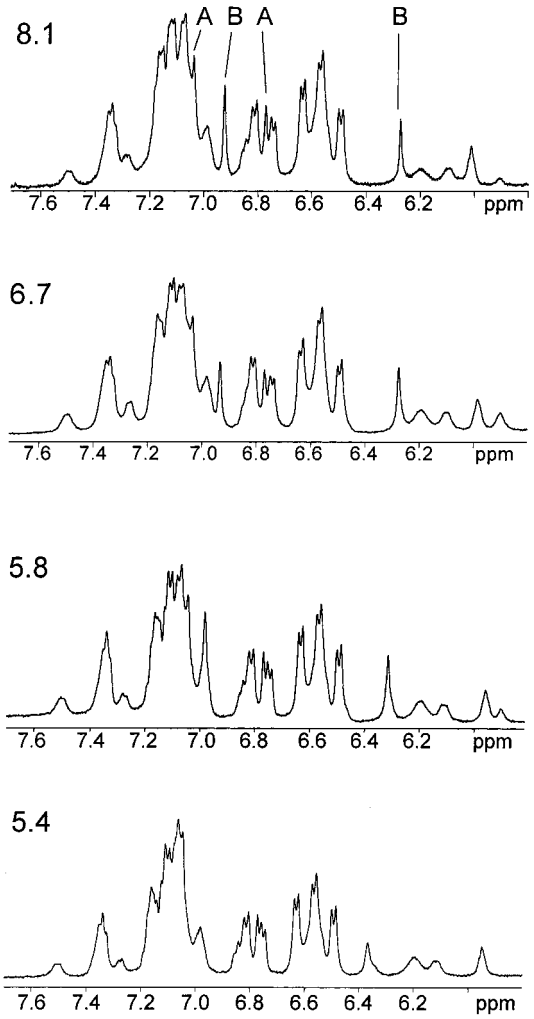

FIGURE 2: Part of the aromatic region of the ^{1}H NMR spectrum of *D. crassirhizoma* PCu(I) at 25 °C. The numbers beside the spectra are the pH* values at which the spectra were obtained. The spectra at pH* 8.1 and 6.7 are of samples in 20 mM phosphate, while those at pH* 5.8 and 5.4 are of the protein in 100 mM phosphate buffer. The resonances assigned to the two histidine ligands are indicated in the pH* 8.1 spectrum.

pH and Buffers for Electrochemical Studies. A pH-jump method was used 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 buffer [I = 0.1 M (NaCl)]. For the studies in the pH range of 4.4−5.0, sodium acetate buffer was utilized; for the pH range of 5.1−6.9, MES buffer was used, and TRIS was used in the pH range of 7.0−7.6. All buffers were at an ionic strength (I) of 0.10 M (NaCl). When checked, the final pH value after mixing was within 0.02 pH unit of that of the diluting buffer.

RESULTS

Dependence on pH of the ^{1}H NMR Spectrum of D. crassirhizoma PCu.* Part of the aromatic region of the ^{1}H NMR spectrum of *D. crassirhizoma* PCu(I) is shown in Figure 2. Singlets, which can only arise in this region of the spectrum from the imidazole ring protons of the two histidine ligands (the only His residues found in *D. crassirhizoma*

PCu), are identified at 6.28, 6.77, 6.93, and 7.04 ppm at pH* 8.1 (it should be noted that these imidazole protons are actually unresolved doublets). In a TOCSY spectrum, the peaks at 6.28 and 6.93 ppm show a cross-peak as do those at 6.77 and 7.04 ppm. Therefore, these two sets of signals can be assigned to ligands HisB and HisA, respectively (sequence specific assignments have not been carried out as they are not required for this investigation). In a NOESY spectrum obtained at pH* 6.7 (each of the imidazole peaks has the same chemical shift at this pH* value as at pH* 8.1), the singlets at 7.04 and 6.93 ppm exhibit a cross-peak. Such a dipolar connectivity is usually observed in cupredoxins between the $C^{\epsilon 1}H$ protons of the two His ligands. Also, a strong dipolar connectivity is observed between the HisA resonance at 7.04 ppm and an aromatic proton belonging to a Phe spin system, and thus, the latter is assigned to Phe12 which is situated close to the active site (see Figure 1).

As the pH* value is reduced to 5.4, there is almost no change in the aromatic region of the ^1H NMR spectrum of *D. crassirhizoma* PCu(I) (see Figure 2). The only significant chemical shift change is that the $C^{\delta 2}H$ and $C^{\epsilon 1}H$ resonances of HisB move slightly downfield to 6.37 and 7.05 ppm, respectively, at pH* 5.4. Additionally, these two singlets are broader at pH* 5.4 than at pH* 8.1. The amount of broadening of these two peaks was found to depend on the concentration of phosphate, with the effect diminished at higher concentrations of the buffer. At the lower pH* values, the signals from HisB continue to experience small changes in their chemical shift and broaden quite considerably (even at high phosphate concentrations). By pH* 5.0, the imidazole ring protons associated with this histidine have moved into crowded regions of the spectrum. At pH* 5.0, the $C^{\delta 2}H$ and $C^{\epsilon 1}H$ resonances of HisA are found at 6.78 and 7.05 ppm and thus have hardly been affected by the decrease in pH*. The same is also true for most other peaks in the aromatic region of the spectrum, including those of Phe12. At pH* values of <5.3, *D. crassirhizoma* PCu(I) becomes quite unstable at the concentrations required for the NMR experiments (1−2 mM) and starts to precipitate. At pH* values of <5.0, it is not possible to obtain NMR spectra of *D. crassirhizoma* PCu(I).

Dependence on pH of the Reduction Potential of D. crassirhizoma and Parsley PCu. Both *D. crassirhizoma* and parsley PCu yield good, quasi-reversible, responses on a DEAE-SH-modified gold electrode in the pH range of 4.3−7.7. In all cases, the anodic and cathodic peaks are of equal intensity, and peak separations of 60−80 mV were obtained for parsley PCu, while values of ∼60 mV were found for *D. crassirhizoma* PCu, at all pH values studied and at scans rates of typically 20 mV/s. The peak currents are proportional to the square roots of the scan rates in the range of 2−120 mV/s. Given the electrochemical behavior of the PCus studied herein, the average of the anodic and cathodic peak potentials can be reasonably assumed to be the $E^{\circ\prime}$ value. At pH values of <4.3−4.4, the electrochemical response of both PCus deteriorated considerably regardless of the conditions that were used. The variations with pH of the reduction potentials of *D. crassirhizoma* and parsley PCu are shown in Figure 3. In the case of the parsley protein, a reduction potential of 378 mV is found at pH 7.7. As the pH value is lowered, the reduction potential increases, and a value of 471 mV is obtained at pH 4.3. The data at low pH (<5.0) have a slope of approximately −60 mV/pH unit. This indicates that the reduction of the protein is accompanied by the uptake of a proton at the active site. This pH dependence can be fit (two parameters, nonlinear least squares) to eq 1:

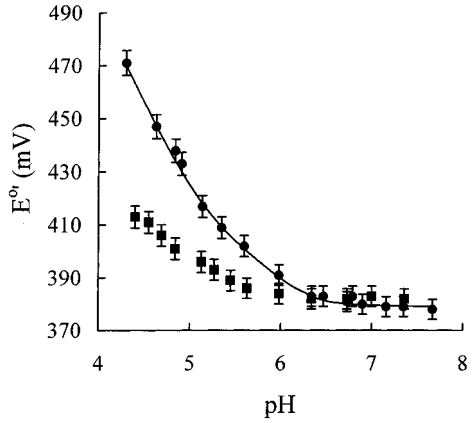

FIGURE 3: Dependence on pH of the reduction potential ($E^{\circ\prime}$) of parsley (●) and *D. crassirhizoma* (■) PCu at an ionic strength of 0.10 M (NaCl). The line shown is obtained from a fit of the data for parsley PCu to eq 1.

$$E^{\circ\prime}(\text{pH}) = E^{\circ\prime}(\text{high pH}) + \frac{RT}{nF}\ln\left(1 + \frac{[\text{H}^+]}{K_a^{\text{red}}}\right) \qquad (1)$$

where $E^{\circ\prime}$(pH) is the measured reduction potential, $E^{\circ\prime}$(high pH) is the reduction potential at high pH, K_a^{red} is the protein dissociation constant for the residue in the reduced protein which affects $E^{\circ\prime}$(pH) as the pH value is lowered, and the other symbols have their usual meanings (*43*). The fit of the data to this equation yields a pK_a^{red} value of 5.8.

The reduction potential of *D. crassirhizoma* PCu is 382 mV at pH 7.4. As the pH value is lowered to ∼6, the reduction potential remains almost constant (see Figure 3). As the pH is decreased further, the reduction potential increases slightly and a value of 413 mV is found at pH 4.4 (see Figure 3). The data for *D. crassirhizoma* PCu have not been fitted to eq 1, and the reason for this will be discussed below.

Determination of the Electron Self-Exchange Rate Constant of D. crassirhizoma PCu by ^1H NMR Spectroscopy. In a mixture of PCu(I) and PCu(II), the slow-exchange condition (*45*, *48*, *50*−*52*) applies to protons which obey the following relationship:

$$k[\text{PCu}]_T \ll 1/T_{i,\text{ox}} - 1/T_{i,\text{red}} \qquad (2)$$

where k is the second-order self-exchange rate constant, $[PCu]_T$ is the total concentration of protein, and $T_{i,ox}$ and $T_{i,red}$ ($i = 1$ or 2) are the relaxation times for the oxidized and reduced forms, respectively. Under these circumstances, for dilute solutions containing only a small ($<10\%$) proportion of the oxidized form of the protein, it can be shown that eq 3 applies (*45*, *48*, *50*−*52*)

$$1/T_i = (1/T_{i,\text{red}}) + k[\text{PCu(II)}] \qquad (3)$$

where T_i is the observed relaxation time of the resonance in the reduced protein and [PCu(II)] is the concentration of PCu(II). Thus, a plot of T_i^{-1} against [PCu(II)] will give a

Table 1: Summary of the Self-Exchange Rate Constants (25 °C) Derived from T_1 (k_1) and T_2 (k_2) Data[a] in Phosphate Buffer (I = 0.10 M)

	HisB (6.97 ppm)			HisB (6.32 ppm)			HisA (6.77 ppm)	Phe12 (6.75 ppm)	
pH*	k_1 (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})	k_2/k_1	k_1 (M^{-1} s^{-1})	k_2 (M^{-1} s^{-1})	k_2/k_1	k_1 (M^{-1} s^{-1})	k_1 (M^{-1} s^{-1})	average k_1 (M^{-1} s^{-1})
7.9	3.3×10^3	2.3×10^3	0.7	3.1×10^3	3.0×10^3	1.0	3.4×10^3	3.8×10^3	3.4×10^3
6.8	4.1×10^3	4.3×10^3	1.0	4.1×10^3	3.0×10^3	0.7	5.0×10^3	5.1×10^3	4.6×10^3
5.8	nd[b]	nd[b]	–	6.8×10^3	6.8×10^3	1.0	6.8×10^3	7.3×10^3	7.0×10^3
5.4	nd[b]	nd[b]	–	8.3×10^3	8.1×10^3	1.0	9.0×10^3	9.8×10^3	9.0×10^3

[a] The estimated error in the k_1 values is ±10%, while that for the k_2 values is ±20%. [b] Not determined due to overlap with other peaks at this pH* value.

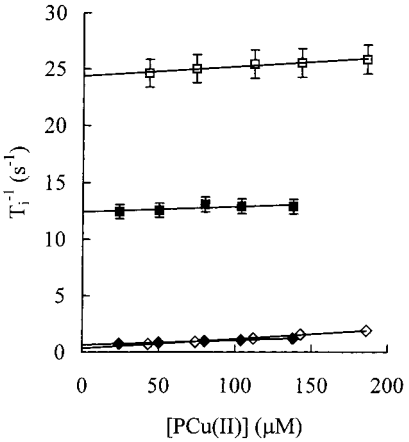

FIGURE 4: Plots of T_1^{-1} (■) and T_2^{-1} (◆) vs [PCu(II)] for the $C^{\epsilon 1}H$ signal of HisB (6.97 ppm) at pH* 6.8. Also shown are plots of T_1^{-1} (□) and T_2^{-1} (◇) vs [PCu(II)] for the $C^{\delta 2}H$ resonance of HisB (6.32 ppm) at pH* 5.4. The error bars in the case of the T_1^{-1} data are smaller than the symbols.

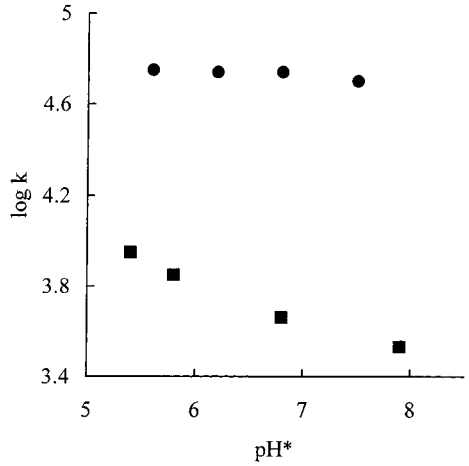

FIGURE 5: Dependence (25 °C) on pH* of log k (k is the self-exchange rate constant) in phosphate buffer [I = 0.10 M (NaCl)] of *D. crassirhizoma* PCu (■) and parsley PCu (●).

straight line with a slope of k. It should be noted that protons which are situated close to the paramagnetic center are more likely to satisfy the slow-exchange condition (*48*, *52*).

It is therefore imperative, when determining the self-exchange rate constant of a cupredoxin by ^1H NMR spectroscopy, that the protons used be in the slow-exchange regime. In these studies, we have used the resolved imidazole ring protons of HisB, one of the HisA imidazole protons (both HisA and HisB are copper ligands), and a Phe12 resonance (this residue is situated very close to the copper center; see Figure 1). Experiments were carried out at $[PCu]_T$ values of ~1−2 mM, and the k values are approximately $3-9 \times 10^3$ M^{-1} s^{-1} (vide infra). The $T_{1,red}^{-1}$ values for the resonances used are in the range of 0.3−0.7 s^{-1}, while the $T_{1,ox}^{-1}$ values of the $C^{\delta 2}H$ and $C^{\epsilon 1}H$ resonances of the His ligands in spinach PCu range from 370 to >1000 s^{-1} (*53*). The $T_{2,red}^{-1}$ values of the *D. crassirhizoma* PCu peaks used in this study range from 12 to 24 s^{-1}, while the $T_{2,ox}^{-1}$ values are $>7 \times 10^3$ s^{-1} (*53*). Therefore, inequality 2 applies to both the T_1 and T_2 data for the peaks used in this study in all of the experiments that are described. Verification that the protons used belong to the slow-exchange regime is provided by the observation that very similar values of k are found for both the T_1 and T_2 data in the cases where they were both measured (vide infra) (*45*, *48*, *52*). Furthermore, the observed T_i values are independent of $[PCu]_T$ as would be expected to be the case in the slow-exchange regime. In this study, as in a previous investigation (*45*), we have found that eq 3 remains valid when a much higher proportion (>10%) of PCu(II) is present.

Plots of T_i^{-1} against [PCu(II)] for the $C^{\epsilon 1}H$ signal of HisB at pH* 6.8 (6.97 ppm) and for the $C^{\delta 2}H$ peak of HisB at pH* 5.4 (6.32 ppm) are shown in Figure 4. The slopes of these plots (k values) are listed in Table 1 along with the results from all of the self-exchange experiments that were carried out. From the values shown, it is clear that the available k_2/k_1 ratios {where k_1 and k_2 are the slopes of the plots of T_1^{-1} and T_2^{-1}, respectively, against [PCu(II)]} are all very close to 1, highlighting the fact that the resonances used in this study are indeed in the slow-exchange regime [k_2/k_1 ratios in excess of 5−10 are expected for protons in the fast-exchange regime (*48*, *52*)]. Due to the small self-exchange rate constant of *D. crassirhizoma* PCu (in particular, at neutral pH*), the effect of increasing [PCu(II)] on T_2^{-1} is extremely difficult to measure. However, precise measurements of the self-exchange rate constant are readily achieved by measuring the effect of [PCu(II)] on the T_1^{-1} values, and thus, the k_1 values provide the more reliable data. The dependence of the self-exchange rate constant of *D. crassirhizoma* PCu on pH* is shown in Figure 5. Also included in Figure 5 are the corresponding data for the PCu from parsley.

DISCUSSION

Effect of pH on the ^1H NMR Spectrum of D. crassirhizoma PCu(I).* Lowering the pH* has very little effect on the aromatic region of the ^1H NMR spectrum of *D. crassirhizoma* PCu(I). This is in stark contrast to what is typically found for other reduced PCus (*35*, *36*, *45*). For example, in the case of parsley PCu(I), we have recently (*45*) observed an ~1 ppm downfield movement in the chemical shift of the $C^{\epsilon 1}H$ proton of His87 when the pH* value is lowered from 7.6 to 5.0. The $C^{\delta 2}H$ signal shifts also but by a much smaller amount. This behavior indicates that the His87 ligand

becomes protonated at the N^{δ} atom and is no longer coordinated to Cu(I), and that exchange between the deprotonated and protonated forms of the histidine is fast on the NMR time scale. A pK_a* value of 5.6 is obtained (25 °C) for His87 in parsley PCu(I). The chemical shift of a number of other resonances in the ^{1}H NMR spectrum of parsley PCu(I) are also affected by the protonation of His87, most notably, the imidazole ring protons of the other His ligand (His37) and a number of signals arising from residues in the hydrophobic patch of the protein. These quite widespread effects are a consequence of the structural rearrangement at the Cu(I) site upon protonation of His87. The only real effect which is observed in the ^{1}H NMR spectrum of *D. crassirhizoma* PCu(I) when the pH* is lowered is a very small shift in the resonances of HisB (<0.1 ppm in the pH* range of 8.2−5.4). The magnitudes of these shifts and the absence of an effect on any other resonances in the spectrum, in particular, those of the ligand HisA and Phe12, the latter of which is situated very close to the active site (see Figure 1 and vide infra), indicate that His87 does not become protonated under these conditions. This conclusion is supported by the fact that both of the HisB imidazole resonances shift by exactly the same amounts. The broadening of the imidazole ring protons of HisB at even lower pH* values, and the dependence of the broadening on phosphate concentration, is not completely understood at the moment and is the subject of further studies. The instability of the reduced protein at these lower pH* values makes more detailed investigations under these conditions difficult. The observed small shifts indicate that there is a pH-induced conformation change (affecting only one His ligand) and that exchange is fast on the NMR time scale. One possibility is that the protonation of one or more of the acidic residues situated close to the active site are responsible for this (vide infra).

Effect of pH on the Reduction Potential of D. crassirhizoma and Parsley PCu. The influence of pH on the reduction potential of parsley PCu is consistent with the protonation of the His87 ligand in the reduced protein. This results in a three-coordinate Cu(I) site which is a preferred coordination number for this oxidation state of copper (*54*), and thus, an increase in the reduction potential is observed (*34*, *37*, *55*, *56*). The pK_a value of 5.8 obtained from a fit of the data to eq 1 is in good agreement with the pK_a* value of 5.6 for His87 obtained from NMR studies (*45*). The reduction potential of *D. crassirhizoma* PCu at neutral pH is very similar to that of parsley PCu regardless of the drastically different distribution of charged amino acid residues close to the copper center. When the pH value is lowered, there is a very limited effect on the reduction potential. The data cannot be fit in a satisfactory manner to eq 1, indicating that the observed effect is not due to protonation at the active site. The slope of the data in the low pH range (−22 mV/pH unit) is also consistent with the lack of uptake of a proton at the active site upon reduction. The observed increase in the reduction potential at lower pH values is most likely due to the protonation of one or more of the acidic residues which are found close to the active site of *D. crassirhizoma* PCu. An alternative explanation is that the small effect of pH* on the reduction potential and the ^{1}H NMR spectrum could be due to the onset of protonation of His87. This would mean that the pK_a* value of this His in fern PCu(I) is ≪5, a pH* value at which this oxidation state of the protein is not stable.

*Self-Exchange Rate Constant of D. crassirhizoma PCu and the Influence of pH**. The self-exchange rate constant of 3.4×10^3 M^{-1} s^{-1} for *D. crassirhizoma* PCu at pH* 7.9 (I = 0.10 M) is the smallest value determined for a member of this subclass of the cupredoxins. In the case of all cupredoxins, it is thought that the hydrophobic area surrounding the exposed histidine ligand is the surface patch through which two molecules associate to form the encounter complex required for the self-exchange process (*47*, *57*−*61*). In the case of the PCus, the absence of any charged residues in the vicinity of the hydrophobic patch, as in the case of the cyanobacterial proteins, results in a self-exchange rate constant of $\sim 3 \times 10^5$ M^{-1} s^{-1} (*48*, *62*). The introduction of an acidic patch which is relatively remote from the exposed His87 ligand (the supposed conduit for et), as in the higher-plant PCu from parsley, results in a 1 order of magnitude decrease in self-exchange reactivity (5×10^4 M^{-1} s^{-1} at pH* 7.5) (*45*). This can be attributed to a decrease in the association constant for two PCu molecules as a consequence of enhanced electrostatic repulsion. The self-exchange rate constant of *D. crassirhizoma* PCu is an additional 1 order of magnitude smaller than that of parsley PCu. The acidic residues in the *D. crassirhizoma* PCu are not found concentrated in the region surrounding Tyr83, but are located in an arc on the edge of the hydrophobic patch of the protein. This results in an increase in the amount of negative charge (at neutral pH) close to the exposed His87 ligand. This leads to a decrease in the association constant for two PCu molecules and thus a smaller self-exchange rate constant (it cannot be totally discounted that the observed effects are, in part, due to alterations in the distance over which et occurs in the different PCus due to variations in the arrangement of the proteins in the encounter complex, or that the proteins exhibit different reorganization energies). When the pH* value is lowered, the acidic residues on the surface of *D. crassirhizoma* PCu start to protonate and thus the negative charge is partially neutralized. The protonation of these acidic residues has an effect on the protein's reduction potential (vide supra) and also the reactivity with small inorganic complexes (*32*) (and also may be the reason for the minor effects seen in the NMR spectra). The observed increase in the self-exchange rate constant, upon reduction of the pH* value, is consistent with the hydrophobic patch being used for self-exchange and also with the absence of protonation of the His87 ligand in *D. crassirhizoma* PCu(I) in the accessible pH range (the protonation of His87 would be expected to cause a decrease in the self-exchange rate constant, due mainly to an increased reorganization energy).

Active Site (His Ligand) Protonation in Cupredoxins. The active site of all structurally characterized cupredoxins contains a C-terminal His ligand whose imidazole ring protrudes through a conserved hydrophobic patch on the surface of the protein (*63*). In reduced plastocyanin (*34*−*38*), amicyanin (*42*−*44*), and pseudoazurin (*39*−*41*), it has been demonstrated that this His ligand can dissociate from the cuprous ion and become protonated. In all of the proteins, this results in a three-coordinate, almost planar, Cu(I) site (*38*, *41*, *44*). The pK_a value for the His ligand differs in the three classes of cupredoxins. The reason only certain cupredoxins exhibit this behavior and the factors controlling the pK_a value of the His ligand are not well understood. The PCu from the fern plant studied herein provides an intriguing

new dimension to this analysis in that it is the first PCu which does not exhibit protonation of His87 in the accessible pH range.

A theory that was put forward some years ago (*64*) implicated the length and structure of the C-terminal ligand-containing loop, which runs from the Cys to the Met ligand and contains the exposed His ligand, as a major factor in controlling active site protonation in cupredoxins. It was noticed (*64*) that in all of the proteins which exhibit an active site protonation there are only two intervening residues between the Cys and His ligands. The number of residues between the His and Met ligands appeared to control the pK_a value. Loop-directed mutagenesis experiments on amicyanin, the cupredoxin with the highest active site pK_a value, and the shortest C-terminal ligand-containing loop, have been carried out to investigate this proposal further (*65*). The introduction of extended sequences between the His and Met ligands results in a decrease in the pK_a value of the histidine as predicted. However, increasing the number of residues between the Cys and His ligands only reduced the pK_a value of the His and did not abolish active site protonation. Very recently (*66*), the introduction of the C-terminal ligand-containing loop from the acidophilic cupredoxin rusticyanin into amicyanin results in a variant in which the His ligand does not protonate. Clearly, the length of the C-terminal loop does influence the ability of the His ligand to become protonated.

The C-terminal ligand-containing loop of *D. crassirhizoma* PCu is identical in length to those of other members of this class of cupredoxins. The sequence of the loop is slightly different from that normally observed in PCus, but only five of the nine residues in this loop are conserved among known PCu sequences. It therefore seems unlikely that the loop structure plays a significant role in preventing protonation of His87 in *D. crassirhizoma* PCu(I). Furthermore, residue 86 is a proline in the *D. crassirhizoma* protein as in all PCus. The presence of a Pro in this position, which cannot form the NH···S(Cys) hydrogen bond as seen in azurin (*67*), for example (a cupredoxin in which the exposed His ligand does not protonate), has been proposed as a possible destabilizing factor which could result in dissociation of the C-terminal His ligand in the reduced protein at low pH (*68*). Clearly, this argument also breaks down in the case of *D. crassirhizoma* PCu. The inability of His87 to protonate in *D. crassirhizoma* PCu(I) has been attributed (*32*) to the $\pi-\pi$ stacking interaction between the imidazole ring of the histidine and the phenyl ring of Phe12 (see Figure 1). This could stabilize the bound form of the histidine in *D. crassirhizoma* PCu(I). The corresponding position in all other sequenced PCus is a leucine [except in the PCu from *Prochlorothrix hollandica* where it is a Pro (*14*)], the side chain of which is not capable of such an interaction with His87. However, the situation is more complex than the presence of a Phe at position 12 being the sole requirement for preventing protonation, as in the Leu12Phe spinach PCu variant His87 has a higher pK_a value than in the *wt* protein (*69*). The packing of the side chain of the His117 ligand between the bulky side chains of Met13 and Phe114 has been suggested (*70*) as the reason for the absence of an active site protonation in azurin. It should be noted in these discussions that the histidine ligand will probably protonate in all reduced cupredoxins, but in many cases, this will occur at a pH value at which the protein is no longer stable. Recently, it has been calculated that in *wt* azurin the pK_a of His117 in the reduced protein is ~2 (*71*). Interestingly, the Phe114Ala mutation in azurin which removes the phenyl ring results in a 50 mV increase in the reduction potential (at neutral pH) and an ~0.4 Å increase in the Cu(II)−N(His117) bond length (*72*).

Another feature of *D. crassirhizoma* PCu which is unusual with respect to all other PCus, and which could contribute to the absence of an active site protonation, is the presence of a glycine at position 36 in the amino acid sequence. This position, which is adjacent to the N-terminal His37 ligand, is occupied by a proline in all other sequenced PCus. Crystallographic studies on poplar PCu(I) at various pH values (*38*) have shown that Pro36 flips from a C^{γ}-exo conformation at pH 7.8 to a C^{γ}-endo conformation in the low-pH structure. The high degree of flexibility at Pro36 is thought to be important in allowing the protonated imidazole of His87 to rotate away from the copper. A Pro residue adjacent to the N-terminal His ligand is a feature that is present in all sequenced amicyanins (*73*) which also exhibit protonation of their C-terminal His ligand. However, pseudoazurin, another cupredoxin in which the C-terminal ligand protonates, has a glycine in this position [except in the protein from *Thiosphaera pantotropha* where it is a Ser (*74*)]. Additionally, studies (*69*) of the Pro36Gly spinach PCu variant show that the nature of the residue at this position has little effect on the pK_a of His87.

Another factor which has been suggested to possibly influence the ability of the C-terminal His ligand to protonate in reduced cupredoxins is the solvent accessibility of the active site (*75*). The proximity of His87 in *D. crassirhizoma* PCu to Phe12 could help to protect the histidine from the solvent. This conclusion is not supported by studies on stellacyanin and umecyanin, which have very exposed active sites (*76*) and which do not exhibit protonation of their C-terminal His ligands in the reduced proteins (*77*).

From the above discussion, it would appear that the most likely reason for the absence of His87 protonation in the accessible pH range in *D. crassirhizoma* PCu(I) is the presence of a $\pi-\pi$ stacking interaction between the imidazole moiety and the phenyl ring of Phe12. This interaction must stabilize the bound form of the His in the Cu(I) protein [it has been shown in the case of the His117Gly azurin variant that the dissociation constant for imidazole is approximately 5 orders of magnitude larger in the reduced protein than in the cupric form (*71*)] and thus lower the pK_a to below a pH value at which the Cu(I) protein is stable. It appears that different factors are important in the various cupredoxins for controlling the ability of the His ligand to protonate. It has been suggested that the protonation of the His ligand may have some physiological relevance (*44*). However, the absence of active site protonation in *D. crassirhizoma* PCu and the apparent lack of a clear strategy for controlling this effect in the different cupredoxins argues against this. It may be that the lability of the His ligand in certain reduced cupredoxins is just an artifact of the particular protein's structure.

CONCLUSIONS

In this study, we have shown that the His87 ligand in *D. crassirhizoma* PCu(I) does not become protonated in the

accessible pH range in solution. This is completely consistent with the data from crystallographic experiments on the oxidized and reduced proteins at pH 4.5. Furthermore, we have investigated the et capabilities of this novel PCu by studying the self-exchange reaction. The self-exchange rate constant of *D. crassirhizoma* PCu determined herein at neutral pH is the smallest reported to date for a PCu. This is in agreement with the accepted idea that the hydrophobic patch surrounding His87 is the surface area that two PCu molecules utilize for the self-exchange reaction. The presence of an arc of acidic residues close to the hydrophobic patch of *D. crassirhizoma* PCu hinders protein−protein association and thus results in the small self-exchange rate constant.

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