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Published on Web 01/30/2003

Active-Site Structure and Electron-Transfer Reactivity of Plastocyanins

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Abstract: The active-site structures of Cu(II) plastocyanins (PCu's) from a higher plant (parsley), a seedless vascular plant (fern, *Dryopteris crassirhizoma*), a green alga (*Ulva pertusa*), and cyanobacteria (*Anabaena variabilis* and *Synechococcus*) have been investigated by paramagnetic ^{1}H NMR spectroscopy. In all cases the spectra are similar, indicating that the structures of the cupric sites, and the spin density distributions onto the ligands, do not differ greatly between the proteins. The active-site structure of PCu has remained unaltered during the evolutionary process. The electron transfer (et) reactivity of these PCu's is compared utilizing the electron self-exchange (ESE) reaction. At moderate ionic strength (0.10 M) the ESE rate constant is dictated by the distribution of charged amino acid residues on the surface of the PCu's. Most higher plant and the seedless vascular plant PCu's, which have a large number of acidic residues close to the hydrophobic patch surrounding the exposed His87 ligand (the proposed recognition patch for the self-exchange process), have ESE rate constants of $\sim 10^3$ M^{-1} s^{-1}. Removal of some of these acidic residues, as in the parsley and green algal PCu's, results in more favorable protein−protein association and an ESE rate constant of $\sim 10^4$ M^{-1} s^{-1}. Complete removal of the acidic patch, as in the cyanobacterial PCu's, leads to ESE rate constants of $\sim 10^5$−10^6 M^{-1} s^{-1}. The ESE rate constants of the PCu's with an acidic patch also tend toward $\sim 10^5$−10^6 M^{-1} s^{-1} at higher ionic strength, thus indicating that once the influence of charged residues has been minimized the et capabilities of the PCu's are comparable. The cytochromes and Fe−S proteins, two other classes of redox metalloproteins, also possess ESE rate constants of $\sim 10^5$−10^6 M^{-1} s^{-1} at high ionic strength. The effect of the protonation of the His87 ligand in PCu(I) on the ESE reactivity has been investigated. When the influence of the acidic patch is minimized, the ESE rate constant decreases at high [H^+].

Introduction

A detailed understanding of electron transfer (et) reactivity, in both biological and chemical systems, has been sought in recent years. Biological et catalysts are commonly metalloproteins in which the redox cofactor is protected by an elaborate protein envelope. This biological coat not only tunes the reactivity of the redox center but also imparts particular surface properties to the molecule, ensuring specificity in et reactions. The plastocyanins (PCu's) are the best studied member of the cupredoxin family of redox metalloproteins,[1,2] which possess a mononuclear type 1 copper ion as the redox center. The PCu's are involved in photosynthetic et between cytochrome *f* of the b_6f complex and $P700^+$ of photosystem I (PSI)[3−6] in plants, green algae, and cyanobacteria (blue green algae). The reactivity of PCu with its physiological partners has been investigated in detail.[7−20] In this study we have analyzed the active-site structure and et reactivity of PCu's from a variety of sources and assessed how these properties have been modified during evolution.

Plastocyanin was the first cupredoxin whose X-ray crystal structure was determined[21] and is one of the best structurally characterized et metalloproteins.[22−38] PCu consists of a β-barrel

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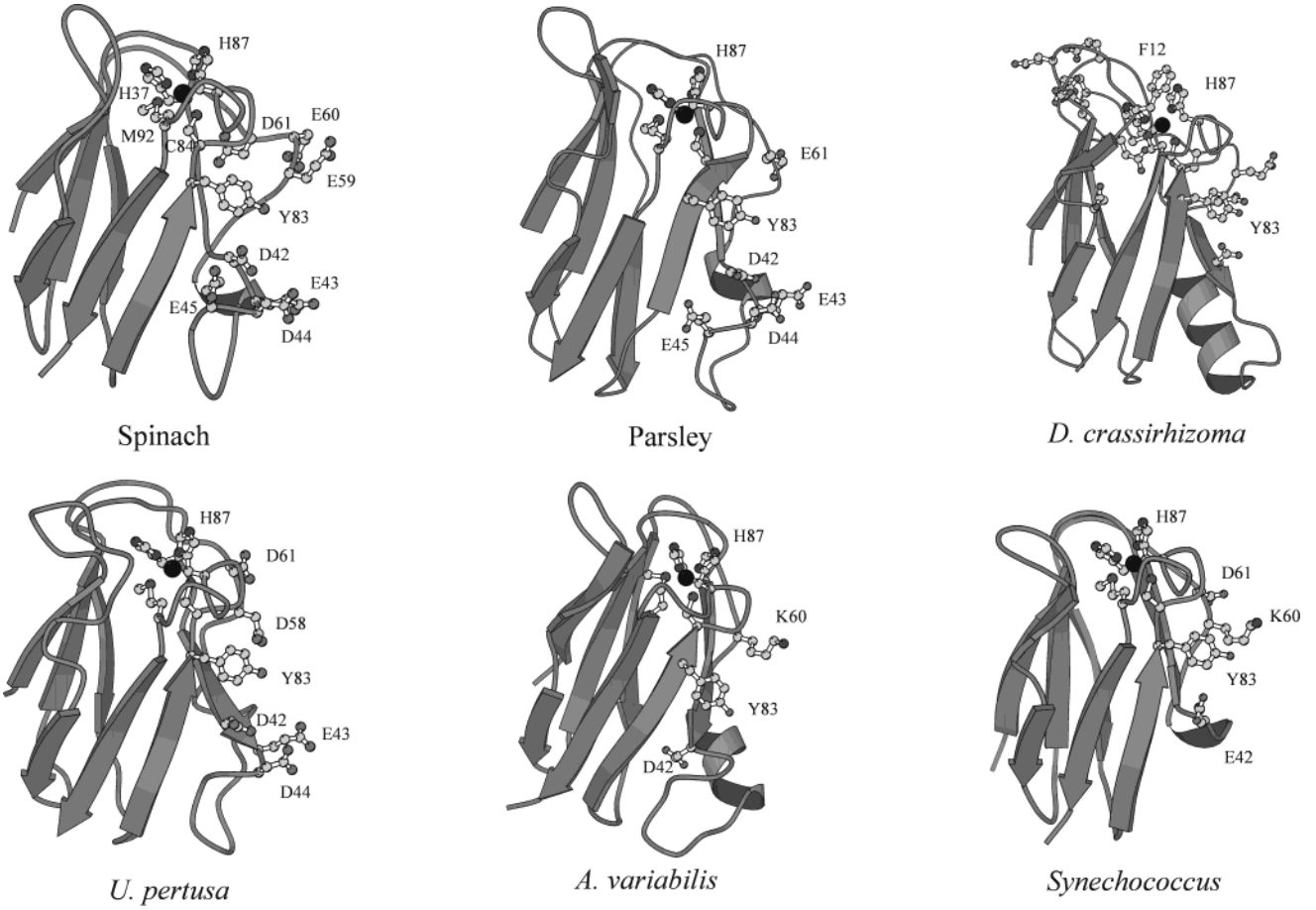

Figure 1. The structures of Cu(II) spinach (PDB entry 1AG6),[31] Cu(I) parsley (PDB entry 1PLB),[28] Cu(II) *D. crassirhizoma* (PDB entry 1KDJ),[36,37] Cu(II) *U. pertusa* (PDB entry 1IUZ),[33] Cu(I) *A. variabilis* (PDB entry 1NIN)[29] and Cu(II) *Synechococcus* (PDB entry 1BXU)[34] PCu's drawn with MOLSCRIPT.[39] The copper ion is shown as a black sphere in all cases, and the side chains of the coordinating amino acids are included in all proteins and are labeled in the spinach structure. In the *D. crassirhizoma* structure the Phe12 residue, whose phenyl ring is involved in a $\pi-\pi$ interaction with the imidazole ring of the His87 ligand is also shown. Also included are the acidic residues which surround Tyr83 in spinach, parsley and *U. pertusa* PCu and which are concentrated around the hydrophobic patch (through which the His87 ligand protrudes in all of the PCu's) of the *D. crassirhizoma* protein. In the structures of the two cyanobacterial PCu's (*A. variabilis* and *Synechococcus*) the charged residues which are found close to Tyr83 are included.

with the copper ion buried approximately 6 Å from the protein surface in a distorted tetrahedral geometry (see Figure 1). Three ligands form strong bonds to the copper, namely the thiolate sulfur of Cys84 and the N^{δ} atoms of His37 and His87. The copper ion is slightly displaced from the plane of these three equatorial ligands toward the weakly coordinated thioether sulfur of Met92. The structure of higher plant PCu's reveal two surface areas as potential binding sites for redox partners.[21,22,26,28,31] These are the hydrophobic patch surrounding the exposed His87 ligand and the acidic patch which is more distant from the copper site (see Figure 1). In the fern (ferns form a division of the seedless vascular plants which are among the oldest terrestrial organisms known) *Dryopteris crassirhizoma* PCu the acidic region has relocated and surrounds the hydrophobic patch (see Figure 1).[36,37] In green algal PCu's the acidic patch is diminished,[24,25,27,33] while in the cyanobacterial PCu's it is nonexistent (see Figure 1).[29,30,32,34,38] It has been found that the

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acidic and hydrophobic patches of higher plant PCu's are important for the interaction with both physiological et partners.[7−20]

The electron self-exchange (ESE) reaction is an intrinsic property of all redox systems.[40] 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, and these values are of fundamental importance to Marcus theory. Furthermore, the reaction has no driving force and thus provides a relative measure of the et capabilities of the different members of a family of redox proteins. Therefore, we have used the self-exchange reaction to assess the relative et capabilities of PCu's from various (higher plant, seedless vascular plant, green algal, and cyanobacterial) sources.

The et reactivity of metalloproteins is carefully regulated by the environment provided by the protein for the redox center, and thus we have also assessed how the copper-site structure differs among the PCu's from various sources. The technique which we have chosen for this purpose is paramagnetic proton nuclear magnetic resonance (^{1}H NMR) spectroscopy as it has recently been shown to provide extremely detailed information about the active-site structure of Cu(II) cupredoxins, including the spin density distribution onto the ligands.[41−47]

Materials and Methods

Protein Isolation and Purification. *Dryopteris crassirhizoma*,[37] *Ulva pertusa*,[48] *Anabaena variabilis*,[49] and parsley[50] PCu's were isolated and purified as described previously. Purity ratios, which correspond to single bands on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, are; A_{278}/A_{590} of ≤1.5 for *D. crassirhizoma* PCu(II), A_{278}/A_{595} of ≤2.0 for *U. pertusa* PCu(II), A_{278}/A_{597} of ≤1.2 for *A. variabilis* PCu(II) and A_{278}/A_{597} of ≤1.7 for parsley PCu(II). Protein concentrations were determined from the following molar absorption coefficients at the wavelength given in parentheses: 4700 M^{-1} cm^{-1} (590 nm) for *D. crassirhizoma* PCu(II), 4900 M^{-1} cm^{-1} (595 nm) for *U. pertusa* PCu(II), and 4500 M^{-1} cm^{-1} (597 nm) for *A. variabilis* and parsley PCu(II).

For the production of *Synechococcus* sp. PCC 7942 PCu, *Escherichia coli* JM109 was transformed with a pKK223-3 derivative harboring the gene for this protein under the control of the *tac* promoter. A 10-mL culture of LB at pH 7.0 containing 100 μg/mL ampicillin was inoculated with a single colony and grown overnight at 37 °C. A 100-μL aliquot of the overnight culture was transferred into 500 mL of fresh LB containing 100 μM of $Cu(NO_3)_2$, 100 μg/mL of ampicillin, and 2 mL of glycerol (pH 7.0). Bacteria were grown aerobically for 6 h at 37 °C, and the harvested cells were frozen at −20 °C in 30 mM tris(hydroxymethyl)aminomethane (Tris) pH 8.0 containing 20% sucrose and 1 mM ethylenediaminetetraacetic acid (EDTA). The periplasmic proteins were removed by osmotic shock, and the resulting cell solution was centrifuged at 27000*g* for 20 min at 4 °C. The supernatant was incubated with (diethylamino)ethyl (DEAE) sepharose (Pharmacia), which had been equilibrated with 10 mM Tris pH 8.0, for 60 min at 4 °C with stirring. The bound proteins were eluted with 10 mM Tris pH 8.0 containing 400 mM NaCl. The protein solution was exchanged into 10 mM Tris pH 8.0 by ultrafiltration (Amicon stirred cell, 3 kDa MWCO membrane) and loaded onto a DEAE sepharose column equilibrated with the same buffer. The PCu was eluted with a 0−100 mM NaCl gradient in 10 mM Tris pH 8.0. The PCu-containing fractions were concentrated to less than 1 mL and loaded onto a G-50 sephadex gel filtration column (Sigma) equilibrated with 20 mM Tris pH 8.0 containing 150 mM NaCl. The PCu fractions from this column were combined and exchanged into 2 mM phosphate buffer pH 7.1 using ultrafiltration and loaded onto a DEAE sepharose column which had been equilibrated in the same buffer. Pure *Synechococcus* PCu(II) was eluted with a 2−25 mM phosphate (pH 7.1) gradient, had a A_{278}/A_{601} ratio of ≤2.4 and gave a single band on a 15% SDS-PAGE gel. The protein concentration was determined from the molar absorption coefficient of 4900 M^{-1} cm^{-1} at 601 nm.

Spinach PCu was expressed in *E. coli* TG1 using a pTrc99A derivative harboring the gene for this protein behind the *Pseudomonas aeruginosa* azurin transit peptide (this construct was provided by Professor P. Schürmann, Université de Neuchâtel, Switzerland) under the control of the *lac* promoter. The growth of the bacteria was carried out as for the *Synechococcus* protein (vide supra) except that 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the 50-mL culture after it had reached an OD_{600} of ∼0.6−0.8 (ca. 2 h after inoculation) and was then grown for a further 5 h. The isolation and purification of spinach PCu was identical to that used for the *Synechococcus* protein. PCu(II) with an A_{278}/A_{597} ratio of ≤1.1 gave a single band on an SDS-PAGE gel, and the protein concentration was determined from the molar absorption coefficient of 4500 M^{-1} cm^{-1} at 597 nm.

PCu(I) Samples for pH Titration Studies by ^{1}H NMR Spectroscopy. PCu was fully reduced by the addition of sodium ascorbate. The excess reductant was removed, and the protein was exchanged into 10 mM phosphate buffer (99.9% D_2O), using ultrafiltration. The sample (usually 1−2 mM) was transferred to an NMR tube and flushed with nitrogen. A small amount of sodium ascorbate (100−200 μM) 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 KCMAW11) with an Orion 420A pH meter. The pH of the samples was adjusted using 1 M NaOD or DCl in deuterated solutions and 1 M NaOH and HCl in H_2O solutions. The pH values quoted for deuterated solutions are uncorrected for the deuterium isotope effect and thus are indicated by pH*.

Sample Preparation for ESE Rate Constant Measurements. For ESE rate constant measurements the PCu's were exchanged into 99.9% deuterated phosphate buffer (usually at $I = 0.10$ M). To maintain the ionic strength, as the pH* values were altered, the phosphate concentration was modified in the following manner. For *A. variabilis* PCu, experiments were carried out in 73 mM phosphate at pH* 6.2, 100 mM phosphate at pH* 5.1 and 4.8. For *U. pertusa* PCu, measurements were made in 35 mM phosphate at pH* 8.0, 80 mM phosphate at pH* 6.0, and 100 mM phosphate at pH* 5.0. For the ESE rate constant determinations of spinach and *Synechococcus* PCu the protein was in 35 mM phosphate at pH* 8.0. PCu(I) was produced as above, with the excess reductant exchanged out by ultrafiltration. The reduced sample (usually 1−2 mM) was placed in an NMR 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

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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 ultraviolet/visible (UV/vis) cuvette and measuring the absorbance at the appropriate wavelength (vide supra) using a Perkin-Elmer λ35 spectrophotometer. UV/vis readings were taken before and after the acquisition of NMR spectra, with an average of the two values used for all subsequent calculations. The ESE rate constant of *U. pertusa* PCu was also measured in 10 mM phosphate buffer (in 99.9% D_2O) plus 500 mM NaCl at pH* 8.0 and 5.3 (the pH* values were adjusted prior to the addition of NaCl). The ESE rate constant of spinach PCu was also measured in 10 mM phosphate plus 2.0 M NaCl at pH* 8.0.

Samples for Paramagnetic ^{1}H NMR Studies. Paramagnetic ^{1}H NMR spectra of fully oxidized PCu's were obtained with the proteins in phosphate buffer at pH* 8.0 in 99.9% D_2O. The *A. variabilis* and parsley PCu's were exchanged into 10 and 20 mM phosphate buffer respectively, while *D. crassirhizoma*, *Synechococcus* and *U. pertusa* PCu's were all exchanged into 35 mM phosphate. Spectra were also obtained of the PCu's in 90% H_2O/10% D_2O in 10 mM phosphate (20 mM phosphate in the case of parsley PCu) at pH 8.0 and 5.0 (pH 5.7 for *Synechococcus* PCu).

To assign the paramagnetic ^{1}H NMR spectra of the PCu's saturation transfer difference spectra were obtained of a 1:1 mixture of PCu(I) and PCu(II) in 35 mM phosphate buffer at pH* 8.0 in 99.9% D_2O. These spectra were obtained for *D. crassirhizoma* and *U. pertusa* PCu's, and the PCu(I) and PCu(II) concentrations were typically 4−5 mM.

NMR Spectroscopy. All proton NMR spectra were acquired on a JEOL Lambda 500 spectrometer. Standard diamagnetic one-dimensional (1D) spectra were obtained by employing a spectral width of 8 kHz and 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 are quoted in parts per million (ppm) relative to water using the relationship $\delta_{HDO} = -0.012t + 5.11$ ppm, where t is the temperature in °C.[42] 1D and 2D spectra used for the assignment of diamagnetic spectra were carried out as described previously.[47] Spin−lattice (T_1) relaxation times were determined using a standard inversion recovery sequence (d − 180° − τ_D − 90° − acq, where d is the relaxation delay and acq the acquisition time). The values of τ_D ranged from 0.5 ms to 13 s, with the total relaxation delay (d + acq) always 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 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 $\nu_{1/2} = (\pi T_2)^{-1}$. All of the NMR measurements made for ESE rate constant determinations were at 25 °C.

Paramagnetic ^{1}H NMR spectra of PCu(II)s were acquired with the super-WEFT[51] pulse sequence (d − 180° − τ_D − 90° − acq) which selects fast relaxing resonances. Spectra were acquired with a τ_D of ca. 40 ms, a total relaxation delay (d + acq) of 45−55 ms, spectral widths of ∼100 kHz, and were processed with 40−50 Hz exponential line broadening as apodization. Saturation-transfer experiments on 1:1 mixtures of PCu(I) and PCu(II) were acquired in difference mode using a standard one-pulse experiment with irradiation of the paramagnetic signals during the relaxation delay. Typically, a relaxation delay of 50 ms was used with an acquisition time of approximately 50−100 ms.

When measuring ESE rate constants, spectra of *A. variabilis*, *Synechococcus*, and *U. pertusa* PCu were also obtained using the super-WEFT sequence. This approach can be used to observe resonances of PCu(I) in the presence of small amounts of PCu(II) (2−20% used typically herein) which experience paramagnetic relaxation enhancement as a consequence of the ESE reaction (and due to their proximity to the copper site).[52,53] Typically the interpulse delay was 40 ms, and the total relaxation delay ca. 50 ms. The number of data points was 2048, corresponding to a spectral width of 20 kHz. Free induction decays were zero-filled to give 8−16K data points for transformation. Spin−spin (T_2) relaxation times were derived from peak widths at half-height using the relation $\nu_{1/2} = (\pi T_2)^{-1}$.

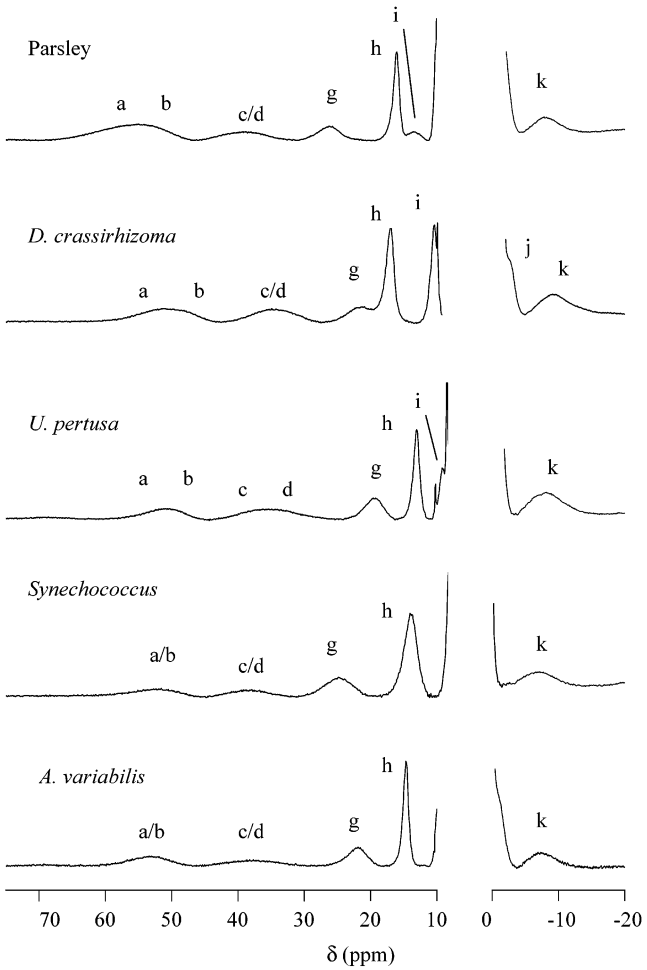

Figure 2. ^{1}H NMR spectra of PCu(II)s (500 MHz) in 99.9% deuterated phosphate buffer at pH* 8.0. All of the spectra were acquired at 25 °C except for that of *D. crassirhizoma* PCu(II) which was obtained at 30 °C. The parsley PCu(II) sample was in 20 mM phosphate buffer while *D. crassirhizoma*, *U. pertusa* and *Synechococcus* PCu(II)s were in 35 mM phosphate buffer and *A. variabilis* PCu(II) was in 10 mM phosphate buffer.

Electrochemistry of PCu's. The direct measurement of the reduction potential was carried out at ambient temperature (21 ± 1 °C) using an electrochemical setup described previously.[54] Measurements were carried out at scan rates of typically 20 mV/s. All reduction potentials were referenced to the normal hydrogen electrode (NHE) and voltammograms were calibrated using the $[Co(phen)_3]^{3+/2+}$ couple (370 mV vs NHE). The gold working electrode was modified with 2-diethylaminoethanethiol (DEAE-SH) as described previously.[54] A pH jump method was used for the electrochemical studies as described previously.[54]

Results

Paramagnetic ^{1}H NMR Spectra of PCu(II)s and their Assignment. The paramagnetic ^{1}H NMR spectra of the five PCu's studied herein in 99.9% deuterated buffer are shown in Figure 2. In all cases an additional exchangeable resonance is

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observed in 90% H_2O/10% D_2O at pH 8.0, with a second exchangeable resonance present at acidic pH (data not shown). Therefore, the PCu(II)s have eight or nine directly observed downfield-shifted resonances and one or two upfield-shifted signals. The temperature dependence of the positions of the hyperfine shifted signals (data not shown) indicates that all of these resonances exhibit Curie-type behavior (increasing shift with decreasing temperature). The spectrum of the higher plant PCu from parsley is very similar to that which has been assigned for spinach PCu.[42] The spectra of both of these proteins possess the same number of directly observed isotropically shifted resonances with very similar relaxation properties, and thus the signals in parsley PCu(II) can be readily assigned. Furthermore, the spectra for the cyanobacterial PCu's from *Synechococcus* and *A. variabilis* studied herein are comparable to that of *Synechocystis* PCC6803 (also a cyanobacterium), which has been assigned previously,[46] and the assignment of the isotropically shifted resonances in the former two proteins is therefore straightforward. A PCu(II) from a fern plant has not been investigated using paramagnetic ^{1}H NMR previously; likewise, neither has that from a green algal (for example *U. pertusa*). As a result, we have assigned these spectra using saturation transfer difference experiments on 1:1 mixtures of PCu(II) and PCu(I). The broad hyperfine shifted resonances of PCu(II) are irradiated, and saturation transfer is observed to their diamagnetic counterparts in PCu(I) as a consequence of the ESE reaction. We have assigned certain resonances in the PCu(I)s using conventional approaches (vide infra). In other cases, where diamagnetic assignments are not available, we have used the chemical shifts observed in the reduced proteins, as compared to typical values for PCu(I)s, for assignment of the PCu(II) signals (this approach is strongly supported by the previously paramagnetic NMR studies on PCu[42,46]). The saturation transfer difference spectra obtained when irradiating peaks a, b, g, h, and i of *D. crassirhizoma* PCu(II) are shown in Figure 3. Listed in Tables S1 and S2 (see Supporting Information) are results of all of the saturation transfer experiments and the assignments that have been made for *D. crassirhizoma* and *U. pertusa* PCu's, respectively. The data for all of the PCu(II)s, including those for the spinach[42] and *Synechocystis*[46] proteins are summarized in Table 1.

Active-Site Protonation in Reduced Plastocyanins. The C-terminal His87 ligand in PCu(I),[23,55−58] and in certain other reduced cupredoxins,[59−62] is known to protonate resulting in a three-coordinate cuprous site.[23,61,62] In these studies we have investigated, in detail, the effect of this pH-induced alteration at the active site on the et reactivity of PCu's. Consequently, it has been necessary to determine the pK_a* (pK_a* indicates a pK_a determined in D_2O solutions for which pH readings were not corrected for the deuterium isotope effect) of this His ligand,

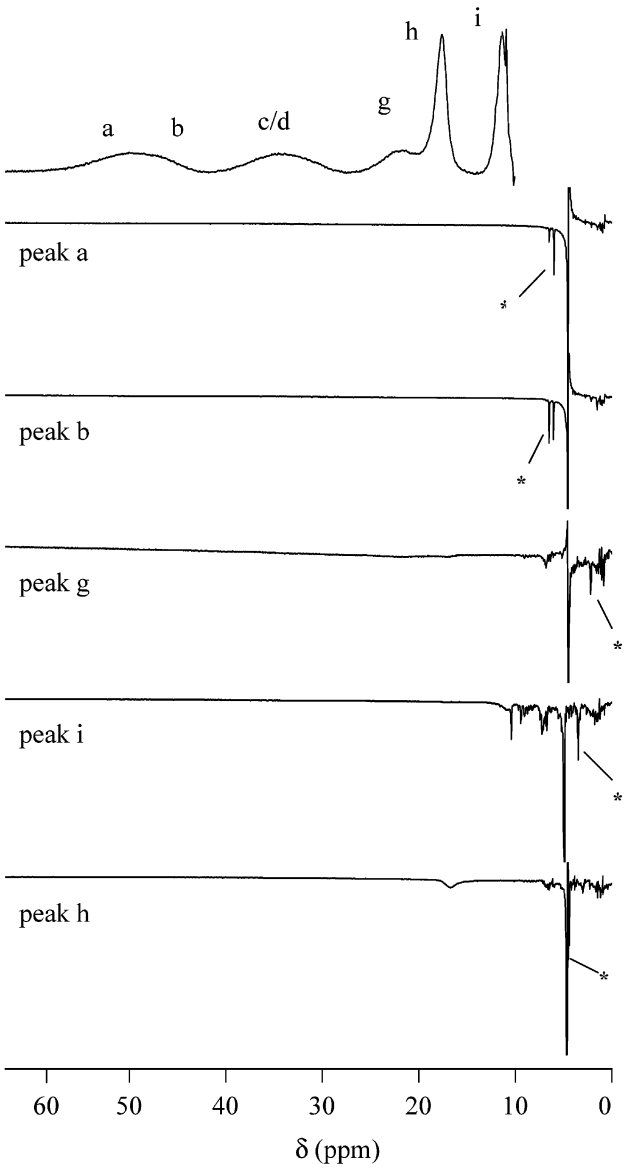

Figure 3. ^{1}H NMR saturation transfer difference spectra (30 °C) of a 1:1 mixture of *D. crassirhizoma* PCu(I) and PCu(II) in 35 mM phosphate buffer (99.9% D_2O) at pH* 8. The top spectrum is that of PCu(II) and those below are the saturation transfer difference spectra in which the peaks indicated were irradiated. The observed saturation transfer peaks in PCu(I) are shown by an asterisk.

using ^{1}H NMR spectroscopy, in the various PCu's studied. The His ligand resonances in *A. variabilis* PCu(I) have been assigned previously,[29] and a pK_a* of 5.1 for His87 has been determined.[63] From the pH* dependence of the chemical shift (δ) of the His87 $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances (exchange between the protonated and deprotonated forms of His87 is fast on the NMR time scale) in this PCu(I) we obtain a pK_a* of 5.0 for His87 (see ref 50 for the equation used in the pK_a determination).

The *U. pertusa* and *Synechococcus* PCu's have not been studied by NMR spectroscopy previously. We have assigned the imidazole ring resonances of both His ligands in these proteins and have studied their pH* dependence. In the case of *U. pertusa* PCu(I) pK_a* values of 6.0 are obtained from the dependence on pH* of the chemical shift of the $C^{\epsilon 1}H$ and $C^{\delta 2}H$ resonances of both His ligands (His37 and His87). The His87

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Table 1. Assigned Hyperfine Shifted Resonances in the ^{1}H NMR Spectra of the Various PCu(II)s

		δ_{obs} (ppm) in PCu(II)[a]						
peak	assignment[b]	spinach[c]	parsley	*D. crassirhizoma*	*U. pertusa*	*Synechocystis*[d]	*Synechococcus*	*A. variabilis*
a	His87 $C^{\delta 2}H$	51.6	57.1	51.7	52.1	52.6	52.8	52.8
b	His37 $C^{\delta 2}H$	47.1	53.1	48.2	50.2	51.1	52.8	52.8
c	His37/87 $C^{\epsilon 1}H$	35.6	39.1	34.9	38.0	38.5	39.3	37.8
d	His37/87 $C^{\epsilon 1}H$	35.6	39.1	34.9	34.3	35.7	39.3	37.8
e	His87 $N^{\epsilon 2}H$		42.2	44.3	40.6	42.9	43.8	41.9
f	His37 $N^{\epsilon 2}H$	31.4	32.2	31.2	33.7	31.1	30.6	34.0
g	Met92 $C^{\gamma 2}H$	23.5	26.1	22.3	20.7	24.0	25.9	21.7
h	Asn38 $C^{\alpha}H$	17.0	15.7	17.6	14.4	14.7	15.1	14.5
i	Met92 $C^{\gamma 1}H$	13.0	13.1	11.0	10.6			
j	His $C^{\beta}H$	−1.5		−1.6		−2.7		−2.2
k	Cys84 $C^{\alpha}H$	−8.0	−7.6	-8.0	−7.5	-7.8	−7.6	−7.8

[a] In all cases the hyperfine shifts quoted are those at 25 °C, except for *D. crassirhizoma* PCu(II) for which data was recorded at 30 °C and the *Synechocystis* protein which was measured at 22 °C. Peak positions are quoted at pH 8.0 except for peak e which was observed at pH 5.7 in *Synechococcus* PCu(II) and at pH 5.0 in all of the other oxidized proteins, and all of the *Synechocystis* data which was recorded at pH 5.2. [b] Small sequence differences exist between the PCu's which alters residue numbering, but herein we have numbered the proteins in all cases as in spinach PCu. [c] Taken from ref 42. [d] Taken from ref 46. The peaks at 38.5 and 35.7 ppm have been assigned to the His87 $C^{\epsilon 1}H$ and His37 $C^{\epsilon 1}H$ protons respectively.

resonances experience much larger chemical shift differences (with the $C^{\epsilon 1}H$ proton being much more affected than the $C^{\delta 2}H$ resonance) between low and high pH*. This is consistent with His87 being the protonating residue and the effect at His37 being a consequence of the resulting change in active-site geometry. In the *Synechococcus* PCu the pH* dependence of the chemical shift of the $C^{\epsilon 1}H$ resonance of His87 yields a pK_a* of ∼5.3 (the studies on this protein are hampered by its instability at acidic pH). It has been demonstrated previously that in *D. crassirhizoma* PCu(I) His87 does not become protonated in the accessible pH range,[54] while in parsley PCu(I) His87 has a pK_a* of 5.6.[50]

The pK_a* determined above for His87 in the *U. pertusa* protein is high for a PCu(I). To confirm this value we studied the effect of pH on the reduction potential using protein electrochemistry. *U. pertusa* PCu yields a good, quasi-reversible response on a DEAE-SH-modified gold electrode in the pH range 4.1−7.7 (the variation with pH of the reduction potential of *U. pertusa* PCu is shown in Figure S1 in the Supporting Information). A reduction potential of 355 mV is found at pH 7.7 and increases to a value of 470 mV at pH 4.2. The data at low pH (<5.3) has a slope of approximately −60 mV/pH, and thus the reduction of the protein is accompanied by the uptake of a proton at the active site (protonation of His87). The pH dependence of the reduction potential gives a pK_a^{red} value of 6.1 (see ref 54 for the equation used) in very good agreement with that determined by NMR spectroscopy (vide supra). Attempts were also made to study the effect of pH on the reduction potential of *Synechococcus* PCu. However, for this protein we could not obtain a good response at a gold electrode customized with a variety of electrode-surface modifiers with different properties (anionic, cationic, and nonpolar).

It is interesting to note that in the paramagnetic ^{1}H NMR studies of PCu(II) (vide supra) there is no evidence of His87 protonating in experiments carried out at low pH. Due to protein instability it was not possible to obtain spectra at pH values <3.5, but even in the case of *U. pertusa* PCu(II) (which has the highest pK_a* for His87 in the Cu(I) protein) there was no sign of any modification of the active-site structure under these conditions.

Determination of the ESE Rate Constants of the PCu's by ^{1}H NMR Spectroscopy. Herein we determine the ESE rate constants of the PCu's from *U. pertusa* and *Synechococcus* and have reinvestigated this feature of the spinach and *A. variabilis* proteins. We have assessed the dependence of the ESE rate constant on ionic strength for spinach and *U. pertusa* PCu's. Furthermore, the influence of pH* on the ESE reactivity of the *U. pertusa* and *A. variabilis* proteins has been studied, to determine the effect of the protonation of the His87 ligand on the et reactivity of PCu's.

The measurement of the ESE rate constant of a cupredoxin by ^{1}H NMR relies on the analysis of resonances which satisfy the slow-exchange condition,[64,65] in a mixture of the oxidized and reduced proteins. It has been shown[49,50,54,59,66] that this condition is usually satisfied by the imidazole ring protons of the two His ligands. For these resonances, and for dilute solutions containing only a small (<10%) proportion of the oxidized form of the protein, it can be shown that expression 1 applies;[49,66]

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

where T_i (i = 1 or 2) is the observed relaxation time of the resonance in the reduced protein in the presence of PCu(II), $T_{i,\mathrm{red}}$ is the relaxation time of the resonance in PCu(I) and [PCu(II)] is the concentration of PCu(II). Thus, a plot of T_i^{-1} against [PCu(II)] will give a straight line of slope k (k is the ESE rate constant and we use k_1 and k_2 to define the slopes of the plots of T_1^{-1} and T_2^{-1} respectively, against [PCu(II)]). This approach (the "standard method") has been used herein and in all cases both T_1 and T_2 data for His ligand resonances analyzed. In situations where the ESE rate constant is small the effect of increasing [PCu(II)] on peak widths can be difficult to determine precisely[50] and in such situations the k_1 values provide more reliable results (it should be noted that T_1 measurements are also more precise when self-exchange is faster and thus all quoted ESE rate constants obtained with this method herein are an average of the available k_1 values).

It has been reported that, due to spectral overlap in the diamagnetic region of a reduced cupredoxin, a better approach for determining the ESE rate constant is to utilize the super-WEFT sequence to select fast relaxing signals in the Cu(I) protein in the presence of small concentrations of the Cu(II)

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Table 2. ESE Rate Constants (25 °C) of *A. variabilis* PCu at Various pH* Values (all at $I = 0.10$ M), Derived from T_1 (k_1) and T_2 (k_2) Values[a]

pH*	resonances	k_1/M^{-1} s^{-1}	k_2/M^{-1} s^{-1}	k_2/k_1	$k_{2,WEFT}$/M^{-1} s^{-1}
6.2	Leu12 C$^{\delta 2}$H	nd[b]	nd		1.7×10^5
	His37 C$^{\alpha}$H	nd	nd		1.7×10^5
	His37 C$^{\delta 2}$H	2.3×10^5	2.1×10^5	0.9	2.0×10^5
	Cys84 C$^{\alpha}$H	nd	nd		2.3×10^5
	His87 C$^{\delta 2}$H	2.5×10^5	2.2×10^5	0.9	1.6×10^5
	Average	2.4×10^5	2.2×10^5		1.9×10^5
5.1	His37 C$^{\alpha}$H	nd	nd		1.2×10^5
	His37 C$^{\delta 2}$H	1.4×10^5	1.2×10^5	0.9	1.1×10^5
	Cys84 C$^{\alpha}$H	nd	nd		1.3×10^5
	His87 C$^{\epsilon 1}$H	nd	nd		1.2×10^5
	Average	1.4×10^5	1.2×10^5		1.2×10^5
4.8	His37 C$^{\delta 2}$H	8.6×10^4	7.8×10^4	0.9	nd

[a] Also included are values derived from T_2 data in super-WEFT spectra ($k_{2,WEFT}$). [b] Not determined.

form.[52,53] This approach (the "super-WEFT" method) has been implemented by Ma et al. using *A. variabilis* PCu[53] and also by Salgado et al. with amicyanin.[52] The resonances which are selected are close to the copper ion and are more likely to belong to the slow-exchange regime. Due to the smaller number of resonances observed with this approach (compared to the case in a conventional 1D spectrum) the chances of spectral overlap are greatly diminished. However, this approach is only of any use if the ESE rate constant is approximately 10^5 M^{-1} s^{-1} or larger, as we demonstrate in this study.

Another approach for determining the ESE rate constants of cupredoxins has also been reported very recently, and again *A. variabilis* PCu has been used to test it.[67] This method does not require assignment of peaks to the slow-exchange regime and has been implemented using exchangeable amide protons close to the copper site. As we can unambiguously assign all of the resonances that we have used herein to the slow-exchange limit, we have not had a need to use this approach. It should be noted that another method for measuring the ESE rate constants by NMR spectroscopy exists which involves the analysis of saturation transfer experiments. However, this approach is only appropriate for lifetimes in the range of the T_1 values, and thus the ESE reactions of the cupredoxins are generally too fast for this method to give reliable results.

For *A. variabilis* PCu we have determined the ESE rate constant at pH* 6.2, 5.1, and 4.8 ($I = 0.10$ M) using the standard method and monitoring the effect of increasing [PCu(II)] on the T_1 and T_2 values of the C$^{\delta 2}$H proton of His37, and His87 at pH* 6.2, which are well resolved. The available k_2/k_1 ratios are all very close to 1 (see Table 2), highlighting the fact that the resonances used belong to the slow-exchange regime [k_2/k_1 ratios in excess of 5−10 are expected for protons in the fast-exchange regime[49,66]]. At pH* 6.2 and 5.1 we have also measured the ESE rate constant using the super-WEFT approach, and the results obtained are also listed in Table 2. For the cyanobacterial PCu from *Synechococcus* we have determined the ESE rate constant at pH* 8.0 ($I = 0.10$ M) using only the super-WEFT method (see Figure 4), due to the absence of well-resolved His ligand imidazole proton resonances in the 1D spectrum of the PCu(I). Values of $k_{2,WEFT}$ (the slopes of plots of T_2^{-1} obtained from WEFT spectra against [PCu(II)]) ranging from 3.3×10^5 to 3.9×10^5 M^{-1} s^{-1} were obtained for the ligand proton resonances labeled in Figure 4, giving an average ESE rate constant of 3.5×10^5 M^{-1} s^{-1}. The ESE rate constant of this PCu could not be studied at acidic pH* values due to the instability of the protein (both oxidation states of the protein have a tendency to precipitate at pH < 6.0).

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Table 3. Summary of the ESE Rate Constants (25 °C) of *U. pertusa* PCu Derived from T_1 (k_1) and T_2 (k_2) Data

	His37 C$^{\delta 2}$H			His87 C$^{\epsilon 1}$H		
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
8.0[a]	3.2×10^4	3.1×10^4	1.0	3.8×10^4	3.1×10^4	0.8
6.0[a]	3.5×10^4	3.5×10^4	1.0	4.6×10^4	3.7×10^4	0.7
5.0[a]	2.3×10^4	2.8×10^4	1.2	2.5×10^4	2.6×10^4	1.0
8.0[b]	1.2×10^5	1.2×10^5	1.0	1.7×10^5	1.4×10^5	0.8
5.3[b]	3.4×10^4	4.0×10^4	1.2	nd[c]	nd	

[a] Determined at $I = 0.10$ M. [b] Measured in 10 mM phosphate buffer plus 0.50 M NaCl. [c] Not determined due to this resonance not being resolved under these conditions.

The ESE rate constant of the green algal PCu from *U. pertusa* has been determined at pH* 8.0, 6.0 and 5.0 ($I = 0.10$ M) using the standard approach and utilizing the His37 C$^{\delta 2}$H and the His87 C$^{\epsilon 1}$H resonances. The ESE rate constants of *U. pertusa* PCu were also determined at pH* 8.0 and 5.3 in 10 mM phosphate buffer plus 0.50 M NaCl and plots of T_i^{-1} against [PCu(II)] for the His37 C$^{\delta 2}$H signal under these conditions are shown in Figure 5. The slopes of these plots (k values) are listed in Table 3 along with the results from all of the other ESE experiments carried out on this PCu. Again the available k_2/k_1 ratios are all very close to 1, which emphasizes that the resonances used are in the slow-exchange regime. We have measured the ESE rate constant of *U. pertusa* PCu at pH* 6.0 ($I = 0.10$ M) at two different total protein concentrations (data not shown). The results are almost identical, further confirming that the protons studied belong to the slow-exchange regime (only resonances in the fast-exchange limit will exhibit a dependence on total protein concentration[49,66]). Attempts to study the ESE reactivity of *U. pertusa* PCu (at $I = 0.10$ M) using the super-WEFT method proved unsuccessful as no PCu(I) signals were selected in partially oxidized samples due to the relatively small ESE rate constant for this PCu (see Figure 4).

The ESE rate constant of spinach PCu at pH* 8.0 was measured at both $I = 0.10$ M (35 mM phosphate) and $I = 2.03$ M (10 mM phosphate plus 2.0 M NaCl) using the standard approach and monitoring the His37 C$^{\delta 2}$H and His87 C$^{\epsilon 1}$H resonances. The k_2/k_1 ratios are all ∼1, and the k_1 values for the two resonances studied are within 10% of each other. The ESE rate constants obtained (the average of the k_1 values) are 2.5×10^3 M^{-1} s^{-1} at moderate ionic strength and 9.0×10^4 M^{-1} s^{-1} at $I = 2.03$ M.

All of the ESE results are summarized in Figure 6. Also included in this Figure are data for parsley PCu[50] and for the fern protein from *D. crassirhizoma*.[54]

Discussion

Surface Properties of PCu's from Various Sources. The key question which this study addresses is the influence of the structure of the PCu's from various sources on their et reactivity. The PCu's from the four different types of photosynthesizing

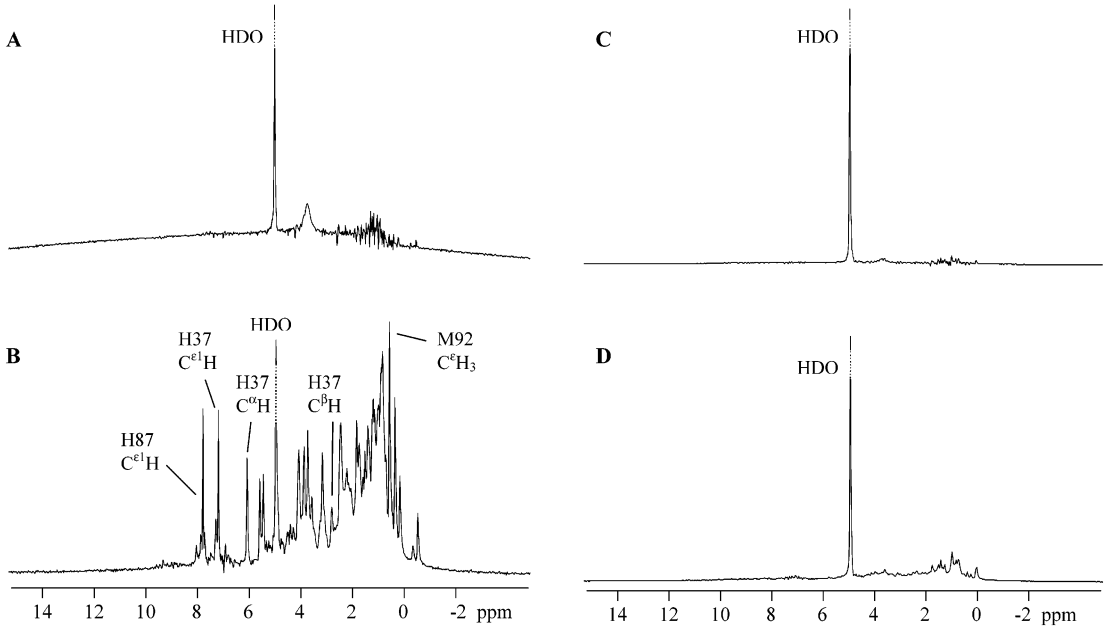

Figure 4. ^{1}H NMR spectra (25 °C) of *Synechococcus* PCu in 35 mM phosphate buffer (99.9% D_2O) at pH* 8.0 and *U. pertusa* PCu in 80 mM phosphate buffer (99.9% D_2O) at pH* 6.0 obtained using the super-WEFT pulse sequence (at 500 MHz). A is a spectrum of *Synechococcus* PCu(I) (2.0 mM), while in B 1.4% PCu(II) is present. The spectrum of *U. pertusa* PCu(I) (1−4 mM) is shown in C and in D is a spectrum of the sample containing 4.3% PCu(II). In all cases the interpulse delay was 40 ms and the total relaxation delay ca. 50 ms.

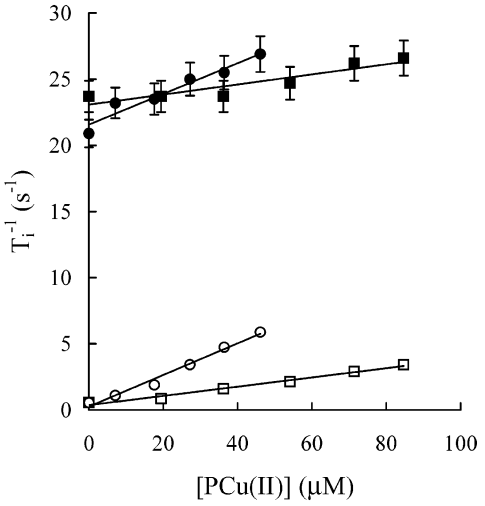

Figure 5. Plots (25 °C) of T_1^{-1} (○) and T_2^{-1} (●) against [PCu(II)] for the His37 $C^{\delta 2}H$ resonance of the *U. pertusa* protein in 10 mM phosphate buffer (99.9% D_2O) plus 0.50 M NaCl at pH* 8.0. Also shown are plots (25 °C) of T_1^{-1} (□) and T_2^{-1} (■) against [PCu(II)] for the same resonance measured in 10 mM phosphate buffer (99.9% D_2O) plus 0.50 M NaCl at pH* 5.3. The error bars in the case of the T_1^{-1} data are smaller than the symbols.

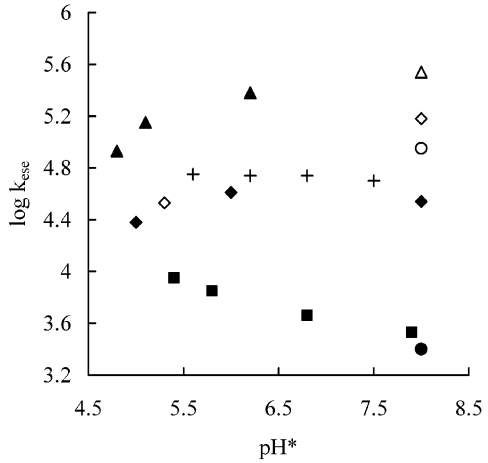

Figure 6. Dependence (25 °C) on pH* of log k_{ese} (k_{ese} is the ESE rate constant) of *U. pertusa* PCu in phosphate buffer at $I = 0.10$ M (◆), *U. pertusa* PCu in 10 mM phosphate buffer plus 0.50 M NaCl (◇), *A. variabilis* PCu in phosphate buffer at $I = 0.10$ M (▲), parsley PCu in phosphate buffer at $I = 0.10$ M (+) and *D. crassirhizoma* PCu in phosphate buffer at $I = 0.10$ M (■). Also included are data at pH* 8.0 for spinach PCu in phosphate buffer at $I = 0.10$ M (●), spinach PCu in 10 mM phosphate buffer plus 2.0 M NaCl (○) and *Synechococcus* PCu in phosphate buffer at $I = 0.10$ M (Δ).

organisms studied herein have distinct surface properties (see Figure 1). All of the PCu's have a hydrophobic patch surrounding the exposed His87 ligand, which is a conserved feature of all structurally characterized cupredoxins,[1,2] and is the preferred route for et reactions including self-exchange.[8,13,16,17,68−71] In higher plant PCu's there is a second binding site made up of Asp and Glu residues, which surround the solvent-exposed Tyr83, and is known as the acidic patch. This surface can be divided into upper (E59, E60, and E61) and lower (D42, E43, D44, and E45) acidic patches in higher plant PCu's (see Figure 1). In parsley PCu, the upper acidic patch consists of a single glutamate and is thus almost nonexistent (see Figure 1) as a consequence of deletions at positions 59 and 60 in the amino acid sequence. A diminished upper acidic patch is also a feature of green algal PCu's again due to deletions in this region (see Figure 1). In the cyanobacterial proteins, both acidic patches are absent (see Figure 1). The *D. crassirhizoma* PCu has some

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negative charge around Tyr83, but a large number of Asp and Glu residues are found in an arc around the edge of the hydrophobic patch (see Figure 1).

Paramagnetic ^{1}H NMR of the PCu(II)s and their Active-Site Structures. The 500-MHz paramagnetic ^{1}H NMR spectra of the five PCu(II)s studied in this work are very similar to each other (see Figure 2) and also to those published previously for the spinach[42] and *Synechocystis*[46] proteins (see Table 1). In particular the spectrum of parsley PCu(II) is almost identical to that of the protein from spinach, and those of *A. variabilis* and *Synechococcus* PCu(II) are comparable to the assigned *Synechocystis* spectrum. We report here for the first time the assignment of the directly observed resonances in the paramagnetic ^{1}H NMR spectrum of the PCu(II) from a green alga and for the novel protein from a fern plant. The observed shifts (δ_{obs}) for the signals of the PCu(II)s listed in Table 1 arise from three contributing factors as shown in the equation:

$$\delta_{obs} = \delta_{dia} + \delta_{pc} + \delta_{Fc} \quad (2)$$

where δ_{dia} is the shift in an analogous diamagnetic system (PCu(I)), δ_{pc} is the pseudocontact (through space) contribution, and δ_{Fc} is the Fermi-contact (through-bond) contribution. The pseudocontact shifts are small for protons at a cupric type 1 site,[41−47] due to the small anisotropy of the **g** tensor, and range from ∼2 to −3 ppm for the resonances listed in Table 1.[42,46] Therefore, the δ_{obs} value minus the δ_{dia} value (see Tables S1 and S2 in the Supporting Information for typical δ_{dia} values) for a particular proton listed in Table 1 provide a good estimate of δ_{Fc}, which is a measure of the spin density present at that particular proton.

In all of the proteins listed in Table 1 the ligand His signals exhibit similar δ_{obs} (and therefore δ_{Fc}) values. This is also true if data for other type 1 centers are included in this comparison,[41,43−45,47] demonstrating that the spin density distribution onto the two His ligands is remarkably similar in all PCu's and also other cupredoxins. The backbone amide of Asn38, a residue which is completely conserved in PCu's, is hydrogen-bonded to the thiolate sulfur of the Cys84 ligand, explaining the relatively large Fermi-contact shift of this resonance. The δ_{obs} of this resonance and the Cys84 $C^{\alpha}H$ signal, which possesses negative spin density, provide information about the strength of the interaction of the metal with the Cys ligand in the different PCu(II)s, and the similarity of their positions (see Table 1) indicates that the Cu−S(Cys) bond is comparable in all of the proteins. Further information about this interaction can be obtained from the hyperfine shifts of the Cys $C^{\beta}H$ protons, which are too broad to be observed directly. Bertini et al. have located these signals at 650 and 489 ppm in spinach PCu[42] and at 614 and 517 ppm in the *Synechocystis* protein,[46] thus confirming the similarity of the Cu−S(Cys) interaction in the various members of this class of metalloproteins.

The largest differences between the spectra of the PCu(II)s arises from protons associated with the axial Met92 ligand. In all cases the Met92 $C^{\gamma 2}H$ proton is observed at ∼26−20 ppm. The Met92 $C^{\gamma 1}H$ proton is only shifted outside of the diamagnetic envelope in the PCu(II)s from parsley, *D. crassirhizoma* and *U. pertusa* and also spinach[42] and is not observed in the cyanobacterial PCu(II)s. The Fermi-contact shifts exhibited by the Met92 $C^{\gamma}H$ signals depends on the amount of spin density on these protons, which is influenced by the strength of the Cu−S(Met) bond and also the Cu−S^{δ}−C^{γ}−H^{γ} dihedral angles. Analysis of the available structures for the PCu's studied[28,29,31,33,34,36,37] shows that the bond lengths between the copper and the thioether sulfur of the axial Met ligands range from ∼2.6 to 3.0 Å. The Cu−S^{δ}−C^{γ}−$H^{\gamma 1}$ dihedral angle ranges from ∼−170° to −180°, while the Cu−S^{δ}−C^{γ}−$H^{\gamma 2}$ angle is more varied ranging from ∼−50° to −70° (note: hydrogens were added to the crystal structures using the program Insight II). At the present time we are not able to find any direct correlation between these bond lengths and dihedral angles, and the δ_{Fc} values of the Met $C^{\gamma}H$ protons.

In summary the paramagnetic ^{1}H NMR spectra of all of the PCu(II)s studied herein are very similar. The structures of the copper sites and the spin density distribution onto the ligands in the different members of this subclass of the cupredoxins are therefore alike. The *D. crassirhizoma* and *U. pertusa* PCu(II)s are the first members of their subclasses (seedless vascular plant and green alga respectively) to be studied by paramagnetic NMR, and they both give spectra analogous to those of all of the other proteins. This is particularly noteworthy for the *D. crassirhizoma* PCu(II) which has UV/vis, electron paramagnetic resonance (EPR), and resonance Raman spectra slightly different from those of all other PCu(II)s.[37] This has been associated with the $\pi-\pi$ interaction of the imidazole ring of the His87 ligand with Phe12 (see Figure 1). It is therefore interesting to note that the His87 resonances of *D. crassirhizoma* PCu(II) exhibit δ_{obs} values similar to those of all of the other proteins. Therefore, the interaction between His87 and Phe12 does not affect the spin density distribution onto this ligand.

His87 Protonation in PCu(I). All PCu(I)s exhibit protonation of their His87 ligand, except for the protein from *D. crassirhizoma*.[54] The absence of His87 protonation in this PCu(I) has been attributed to the $\pi-\pi$ interaction of the imidazole ring of this ligand with the phenyl ring of Phe12 (see Figure 1).[37,54] Structural studies have demonstrated that the active-site protonation in the PCu(I)s results in a three-coordinate site,[23] which is a favored geometry for this oxidation state of copper,[72] and thus leads to a dramatic increase in the reduction potential.[54,56−58] The pK_a* for His87 is typically in the range of 4.8−5.0 for most PCu(I)s.[50,54] Herein we have determined a pK_a* of ∼5.3 for His87 in *Synechococcus* PCu(I) from NMR studies, which is slightly higher than that for the corresponding residue in the reduced cyanobacterial protein from *A. variabilis* (pK_a* 5.0). Parsley PCu(I) is unusual with its His87 possessing a pK_a* of 5.6.[50] In the *U. pertusa* protein His87 has an even higher pK_a* (6.0) which is the largest reported to date for a PCu(I). It has previously been noted that other green algal PCu's have quite high active-site pK_a*'s, with a value of 5.5 reported for the *Scenedesmus obliquus* protein.[73] It therefore appears that the PCu's which have deletions at positions 59 and 60 in their primary structures, and possess a diminished upper acidic patch, also have high active-site pK_a values. This is contrary to what would be expected on the basis of simple electrostatics. Protonation of one of the His ligands has been shown to occur in reduced pseudoazurin[60,61] and amicyanin[59,62] (both cupredoxins) with pK_a values of ∼5 and ∼7, respectively. The features which control the pK_a value of this copper ligand are

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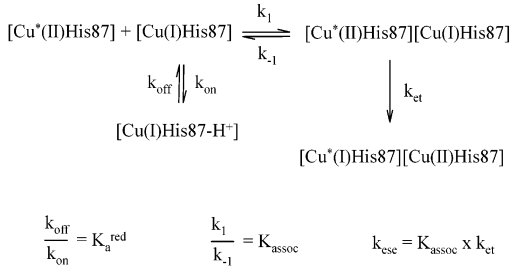
not yet completely understood [see ref 54 for a recent review of this subject].

ESE Reactivity of PCu's. We have used two approaches to determine the ESE rate constants of the PCu's. The first is a method (the standard method) which has been widely used previously[47,49,50,54,59,66,74] and relies on observing the enhanced relaxation of protons in conventional 1D spectra of PCu(I) resulting from the presence of small amounts of PCu(II), which satisfy the slow-exchange condition (those protons which are close to the active site, vide supra). This method gives precise results, providing that suitable protons are resolved in the spectrum of the reduced protein. In all of the present studies, except in the case of the *Synechococcus* PCu, all PCu(I) spectra possess sufficiently resolved resonances which we have shown belong to the slow-exchange regime. An alternative approach, which has also been applied herein, utilizes the enhanced relaxation of protons in partly oxidized samples to selectively observe a small number of resonances using the super-WEFT sequence.[52,53] The resonances which are selected by this approach are those which are situated close to the copper ion and thus are likely to belong to the slow-exchange limit. The broadening of these resonances caused by increasing the concentration of PCu(II) has been measured. However, it should be noted that, as with the standard method, signals still need to be sufficiently resolved to provide reliable data. Furthermore, the determination of peak widths (T_2 values) is prone to larger errors than the measurement of T_1 values, as the longitudinal relaxation rates (T_1^{-1}) of protons in proteins are usually significantly smaller than the transverse relaxation rates (T_2^{-1}). Therefore, a small change in the relaxation rates can be measured with higher precision for T_1^{-1} than for T_2^{-1}. Having said this, the super-WEFT approach does provide a very convenient way of determining the ESE rate constant in situations where resolved resonances cannot be found in the standard 1D spectrum of the reduced protein, such as in the case of the *Synechococcus* PCu studied herein. However, this method can only be used if the ESE rate is sufficiently fast, and thus in experiments with the *U. pertusa* protein the diamagnetic protons in the vicinity of the active site in PCu(I) do not experience sufficient relaxation enhancement to allow them to be selectively observed with this approach (see Figure 4).

The ESE rate constants for the higher plant PCu from spinach, the green algal PCu from *U. pertusa*, and the cyanobacterial proteins from *A. variabilis* and *Synechococcus* have been determined. At a pH* value where His87 cannot influence the et reactivity of the proteins the observed rate constants (25 °C) are 2.5×10^3, 3.5×10^4, 2.4×10^5, and 3.5×10^5 M^{-1} s^{-1} for the spinach (pH* 8.0) *U. pertusa* (pH* 8.0), *A. variabilis* (pH* 6.2), and *Synechococcus* (pH* 8.0) proteins, respectively (all at $I = 0.10$ M). These compare with rate constants (again at 25 °C) of 5.0×10^4 and 3.4×10^3 M^{-1} s^{-1} for parsley (at pH* 7.5)[49] and *D. crassirhizoma* (at pH* 7.9)[54] PCu's, respectively (also at $I = 0.10$ M). Previous studies have reported ESE rate constants (25 °C) of $\sim 4 \times 10^3$ M^{-1} s^{-1} for spinach PCu at pH* 6.0 ($I = 0.10$ M)[75] and 1.5×10^5 M^{-1} s^{-1} for *A. variabilis* PCu at pH* 7.0 ($I = 0.05$ M)[67] in good agreement with our results. The ESE rate constants of the PCu's at moderate ionic strength span 2 orders of magnitude (as do the ESE rate constants of all cupredoxins[74]). The self-exchange process in PCu's (and all cupredoxins) is thought to involve the association of two protein molecules via the hydrophobic surfaces which surround the His87 ligand.[43,68,70,71,74] Given the comparable active-site structures of the Cu(II) proteins which we have demonstrated in the paramagnetic ^{1}H NMR studies and which, given the rigidity of the cupredoxin fold, also hold for the Cu(I) forms (at a pH value well above the pK_a of His87) we can anticipate that the reorganization energies of the various PCu's are similar. Additionally, we have shown that the spin density distributions onto the ligands are alike in all of the PCu(II)s, and thus variations in this feature will not influence et. This is perhaps most relevant for the His87 ligand which is in the proposed et pathway for self-exchange.[70,71] The observed range of ESE rate constants for the various proteins is therefore probably a consequence of different association constants (K_{assoc}, see Scheme 1) for two PCu molecules (it is also possible that the structures of the encounter complexes for the ESE process differ in the various PCu's and therefore et occurs over quite different distances, but this is less likely especially considering the results obtained at elevated ionic strength). The small ESE rate constants for spinach and *D. crassirhizoma* PCu's can thus be attributed to the large number of acidic residues which are present on the surfaces of these two proteins (see Figure 1). The smaller size of the upper acidic patch in parsley and *U. pertusa* PCu (see Figure 1) results in the approximately 10-fold increase in the ESE rate constant. The two cyanobacterial PCu's exhibit ESE rate constants which are ~100-times larger than those of the spinach and *D. crassirhizoma* proteins. This is due to the almost complete absence of an acidic patch in these proteins (see Figure 1). A basic arginine residue adjacent to the C-terminal His ligand in all cyanobacterial PCu's has been shown to be important for the interaction with both cytochrome *f* [20] and PSI[19] but does seem to hamper ESE (we are currently investigating this further by analyzing the influence of ionic strength on the ESE rate constants of cyanobacterial PCu's). The influence of the acidic patch on the ESE rate constant is further demonstrated by measurements at high ionic strength for spinach and *U. pertusa* PCu at pH* 8.0 (see Table 3 and Figure 6). As expected, for two such highly charged proteins, the ESE rate constants increase at elevated ionic strength. The approximately 36-fold and 5-fold increases, due to shielding of the negative charge on the proteins' surfaces, results in spinach and *U. pertusa* PCu's, respectively, having rate constants in line with those of the cyanobacterial proteins. To summarize, the ESE process in the cyanobacterial PCu's is governed mainly by hydrophobic interactions, while in the case of the green algal and higher plant proteins a combination of

Scheme 1



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hydrophobic and electrostatic interactions are important. A similar conclusion is emerging from studies of the interaction of PCu's from these various sources with their physiological redox partners.[7−20]

Another family of et metalloproteins, the cytochromes, have had their ESE reactivity studied in some detail. Cytochrome *c* from both mammalian and bacterial sources has a ring of lysine residues around the exposed heme edge.[76,77] This feature is responsible for a small ESE rate constant of $\sim 10^3$ M^{-1} s^{-1} at $I = 0.10$ M which increases to $\sim 10^5$ M^{-1} s^{-1} at high ionic strength.[78−80] Cytochrome b_5, which possesses a number of acidic residues around the periphery of its exposed heme edge,[81] has an ESE rate constant also of $\sim 10^3$ M^{-1} s^{-1} at lower ionic strength which rises to $\sim 10^5$ M^{-1} s^{-1} as the NaCl concentration is increased.[78,79,82] Cytochrome c_{551}, which lacks charged residues around its exposed heme edge,[83] has an ESE rate constant of $\sim 10^7$ M^{-1} s^{-1}, which is independent of ionic strength.[78,84] In the case of Fe−S proteins a more limited number of investigations of ESE reactivity have been carried out. High-potential iron−sulfur proteins (HiPIPs) have ESE rate constants of $\sim 10^5$ M^{-1} s^{-1} which are independent of ionic strength (over the relatively narrow range studied).[85−87] The rubredoxins, which are the simplest of the Fe−S proteins, have an ESE rate constant of $\sim 10^5$ M^{-1} s^{-1} at lower ionic strength, which increases to $\sim 10^6$ M^{-1} s^{-1} at elevated ionic strength.[88,89] It therefore appears that all three families of redox metalloproteins (cupredoxins as represented by the PCu's, cytochromes and Fe−S proteins) possess similar ESE rate constants ($\sim 10^5$−10^6 M^{-1} s^{-1}) at elevated ionic strength, i.e. when the influence of surface features of the protein are minimized. This magnitude of ESE rate constant (which is influenced by the reorganization energy, the distance for et, and the protein−protein association constant) therefore appears to be a consequence of a structure that has evolved to be an efficient redox metalloprotein.

The ESE rate constant of *U. pertusa* PCu is almost independent of pH* in the range 8.0−5.0 (at $I = 0.10$ M, see Table 3 and Figure 6). This is surprising, considering that the pK_a* of His87 is 6.0 in the reduced protein and so at pH* 5.0, ∼90% of the PCu(I) molecules will possess a three-coordinate active site. This form of PCu(I) is known to be redox-inactive,[56,59,90,91] and a smaller ESE rate constant would be expected (see Scheme 1 where [Cu(I)His87-H^+] is the form of PCu(I) in which His87 is protonated and is the redox inactive species). We have previously found that the ESE rate constant of parsley PCu is also almost unaffected by pH* in the range 7.5−5.6 (see Figure 6), with the low end of this range corresponding to the pK_a* of His87 in this reduced PCu. The absence of any pH* dependence of the ESE reactivity in higher plant and green algal PCu's can be attributed to compensation effects caused by the presence of an acidic patch. At lower pH values the acidic residues start to become protonated, thus neutralizing the charge in this region of the protein, leading to a larger K_{assoc} (see Scheme 1). This argument is confirmed by the influence of pH* on the ESE rate constant of *A. variabilis* PCu (see Figure 6) which lacks an acidic patch (see Figure 1). In this case the ESE rate constant is ∼3 times smaller at pH* 4.8 (the pK_a* of His87 is 5.0) compared to the situation at pH* 6.2. Also, the ESE rate constant of *D. crassirhizoma* PCu actually increases at lower pH* values. This is due to the absence of His87 protonation in this PCu(I) plus the neutralization of the arc of acidic residues around the hydrophobic patch of this protein (see Figure 1) at lower pH*, which enhances protein−protein association. Furthermore, we have also studied the influence of pH* on the ESE rate constant of *U. pertusa* PCu at high ionic strength ($I \approx 0.50$ M, see Table 3 and Figure 6). Under these conditions, where the influence of the acidic patch on the association of two PCu molecules is diminished, the ESE rate constant is ∼4.5 times smaller at pH* 5.3 (3.4×10^4 M^{-1} s^{-1}) as compared to pH* 8.0 (1.5×10^5 M^{-1} s^{-1}). This confirms that the protonation of His87 diminishes the ESE reactivity of PCu. Thus, as the pH is lowered, more of the reduced protein exists as [Cu(I)His87-H^+] (see Scheme 1) which is redox inactive. It would perhaps be expected that the ESE process would be more dramatically affected at low pH. The fact that a decrease in ESE rate constants are observed at, and below, pH values which correspond to the pK_a of His87 in the various PCu(I)s but that the proteins are still reasonably competent at ESE suggests that the process has now become gated (by the activation barrier for the transition from three-coordinate [Cu(I)His87-H^+] to four-coordinate [Cu(II)His87]). From Scheme 1 it is apparent that k_{off}, the rate of deprotonation of [Cu(I)His87-H^+] could gate the ESE reaction at low pH. Values of k_{off} have been measured for amicyanin by electrochemistry and are very temperature-dependent (35 s^{-1} at 273 K and >750 s^{-1} at 295 K).[91] We are currently investigating the effect of temperature on the ESE reactivity of various PCu's at low pH, and at high ionic strength, to shed more light on this matter.

The protonation of the C-terminal His ligand in PCu(I) and other reduced cupredoxins may play an important physiological function. For example, it is thought that His87 protonation could provide a metabolic feedback mechanism whereby the drop in pH resulting from a high rate of photosynthesis would result in a decrease in et activity of PCu. In this context it is interesting to note that in the case of amicyanin the His ligand does not protonate when the protein is complexed to its physiological electron donor (methylamine dehydrogenase).[62] In the case of PCu(I) it has recently been shown that the protonation of His87 has little effect on the interaction with cytochrome *f*.[17] Free PCu(I) in the inner thylakoid of the chloroplast will be mainly in the protonated (redox-inactive) form when the system is illuminated (under these conditions the pH of the inner thylakoid

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can drop below 5). The fact that the reduction of $P700^+$ by PCu(I) is hardly affected at low pH[92] therefore seems a little puzzling. It may be that the approach of a hydrophobic surface of PSI to the hydrophobic patch of [Cu(I)His87-H^+] destabilizes the positive charge on His87 and thus [Cu(I)His87], the redox active species, would be rapidly re-formed (see Scheme 1). Therefore, His87 protonation may provide a way of ensuring specificity of photosynthetic et as the reaction with other oxidants would be expected to be much more significantly gated.

Conclusions

In this study we have assigned the paramagnetic ^{1}H NMR spectra of the PCu(II) from *D. crassirhizoma* (fern) and also the protein from *U. pertusa* (green alga). Even though the spectroscopic properties, and active-site structure, of the *D. crassirhizoma* PCu(II) are slightly unusual, the paramagnetic ^{1}H NMR spectrum is very similar to those of other PCu(II)s. Paramagnetic ^{1}H NMR spectra have now been assigned for PCu(II)s from higher plants, a seedless vascular plant, a green alga, and cyanobacteria, and in all cases they are similar. This indicates that the copper-site structures and the spin density distribution onto the ligands of this family of et proteins, which play a key role in photosynthesis, has changed very little throughout evolution. This is in contrast to the surface properties of the PCu's which have been altered quite dramatically throughout the evolutionary process (see Figure 1).

The ESE rate constants of *Synechococcus* and *U. pertusa* PCu's have been determined, with the latter being the first to be published for a green algal PCu. There are now ESE rate constants available for a range of PCu's with very different surface properties. At moderate ionic strength ($I = 0.10$ M) the ESE rate constant of a PCu is dictated by the presence of acidic residues on the proteins' surfaces. The rate constants range from $\sim 3 \times 10^3$ M^{-1} s^{-1} in the case of the *D. crassirhizoma* protein to $\sim 4 \times 10^5$ M^{-1} s^{-1} in the cyanobacterial proteins. The influence of charged residues on protein−protein association is further demonstrated by the larger ESE rate constant of spinach and *U. pertusa* PCu's at elevated ionic strength. Under conditions where the influence of surface charges on protein−protein association is minimized, PCu's all possess ESE rate constants of $\sim 10^5 - 10^6$ M^{-1} s^{-1}. A similar conclusion can be drawn from corresponding data for other families of redox metalloproteins. It therefore appears that this magnitude of ESE rate constant is a consequence of a protein-encapsulated metal center which is a competent redox catalyst. The influence of pH on the ESE rate constant demonstrates that His87 protonation in PCu(I) has an inhibiting effect on this reaction. Thus, we can conclude that ligand protonation may provide a mechanism for ensuring specificity in photosynthetic et.

Acknowledgment. We thank Dr. A. Lawler for carrying out the electrochemistry experiments on *U. pertusa* PCu. We are grateful for financial support from Newcastle University, the Royal Society, CVCP (for an ORS award to K.S.), and EPSRC (for a grant to purchase the NMR spectrometer). We thank Professor A. Harriman and A. Amini (University of Newcastle upon Tyne) for access to and assistance with Insight II, and Professor P. Schürmann (Université de Neuchâtel, Switzerland) for providing the spinach PCu gene.

Supporting Information Available: Tables with results of saturation transfer experiments and a figure showing variation with pH of the reduction potential of *U. pertusa* PCu (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA021005U

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