# Effect of pH on the Self-Exchange Reactivity of the Plant Plastocyanin from Parsley†

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The self-exchange rate constant (25 °C) for parsley plastocyanin is $5.0 \times 10^4$ $M^{-1}$ $s^{-1}$ at pH* 7.5 ($I = 0.10$ M). This value is quite large for a higher plant plastocyanin and can be attributed to a diminished upper acidic patch in this protein. The self-exchange rate constant is almost independent of pH* in the range 7.5−5.6, with a value (25 °C) of $5.6 \times 10^4$ $M^{-1}$ $s^{-1}$ at pH* 5.6 ($I = 0.10$ M). At this pH*, the ligand His87 is protonated in ∼50% of the reduced protein molecules (p$K_a$* 5.6), and this would be expected to hinder electron transfer between the two oxidation states. However, this effect is counterbalanced by the enhanced association of two parsley plastocyanins at lower pH* due to the partial protonation of the acidic patch.

## Introduction

Plastocyanin (PCu) is a type 1 blue copper protein (cupredoxin) found in the thylakoid of the chloroplast, where it is involved in electron transport from cytochrome *f* of the $b_6f$ complex to $P700^+$ of PSI.[1,2] Most higher plant and green algal PCu's comprise 97−99 amino acid residues, but if the cyanobacterial proteins are included, this number can vary from 91 to 105.[3−5] Parsley PCu is unusual when compared to the other higher plant proteins in that it has only 97 residues, with deletions at positions 57 and 58.[6] The corresponding deletions are also found in the green algal PCu's.[5,7,8]

Crystal structures of a number of PCu's from various sources have been published.[3−5,9−18] In all cases, the protein comprises eight β-strands, which constitute two β-sheets, giving the molecule an overall topology known as a β-sandwich (see Figure 1). The copper ion is located approximately 6 Å from the protein surface and has a distorted tetrahedral geometry. Three amino acid side chains form strong bonds to the copper, namely the thiolate sulfur of Cys84 and the $N^\delta$ atoms of His37 and His87. The copper ion is slightly displaced from the plane of these three equatorial ligands toward the weakly coordinated axial Met92 (see Figure 1). NMR spectroscopy has also been used to determine the solution structure of $PCu^I$ from various sources, including parsley.[6,19−22]

The structure of PCu reveals two surface areas as potential binding sites for redox partners.[10] The first is formed by a cluster of nonpolar side chains, known as the hydrophobic patch, which surround the solvent-exposed His87 ligand (see Figure 1). The second consists of a group of Asp and Glu residues, which surround the solvent-exposed Tyr83, and has become known as the acidic patch (see Figure 1). The latter surface can be divided into upper (E59, E60, and E61) and lower (D42, E43, D44, and E45) acidic patches in higher plant plastocyanins (see spinach structure in Figure 1). In parsley plastocyanin, the upper acidic patch consists of a single glutamate and is thus almost nonexistent (see Figure 1).[6] A diminished upper acidic patch is

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† Abbreviations: PCu, plastocyanin; UV/vis, ultraviolet/visible; NMR, nuclear magnetic resonance; pH*, pH-meter reading uncorrected for the deuterium isotope effect; MES, 2-morpholinoethanesulfonic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; DE-52, (diethylamino)ethyl cellulose; 1D, one-dimensional; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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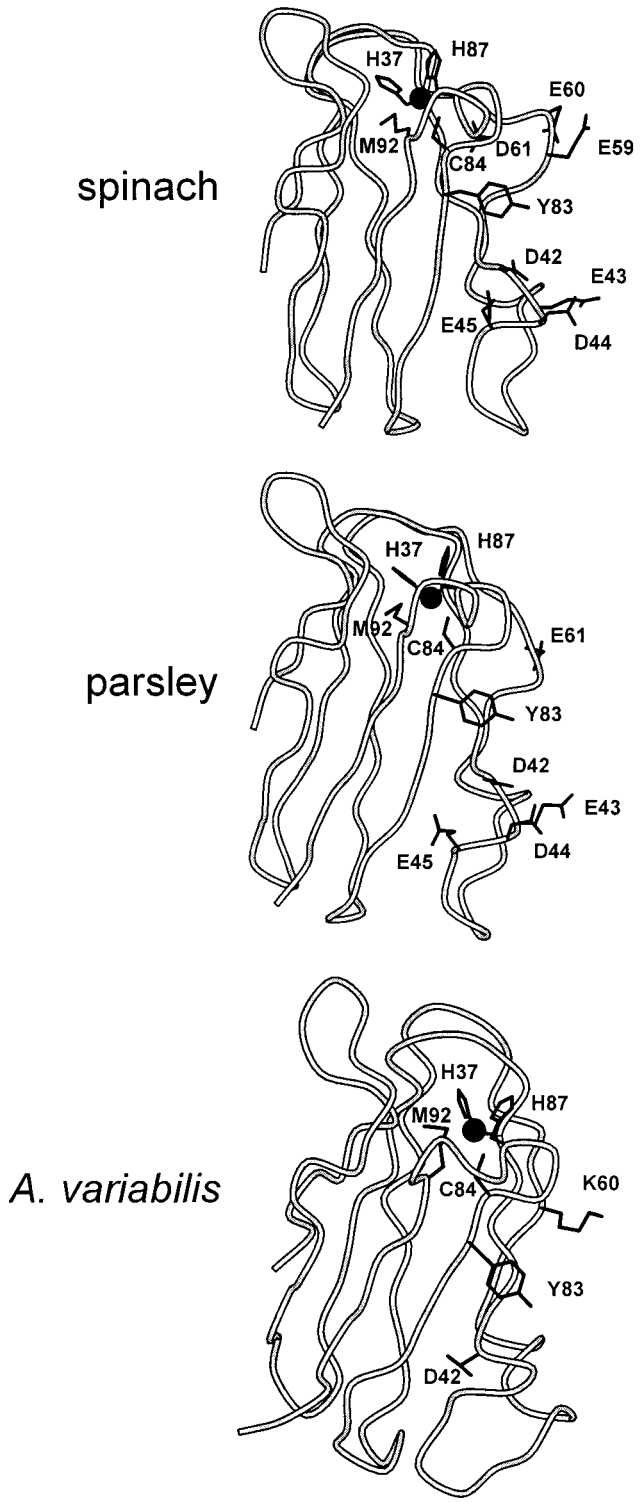


**Figure 1.** Representations of the structures of Cu(II) spinach,[15] Cu(I) parsley,[6] and Cu(I) *A. variabilis*[21] PCu's. The copper ion is shown as a black sphere in all cases, and the side chains of the coordinating amino acids are included. Also shown are the charged residues in the region that surrounds the solvent-exposed Tyr83 residue.

also found in the green algal plastocyanins[5,12,13] whereas, in the cyanobacterial protein from *Anabaena variabilis*, the only acidic residue is Asp42 (see Figure 1).[21]

At low pH, in $PCu^I$, the ligand His87 becomes protonated.[23−26] This results in the copper ion becoming three-coordinate[11] and leads to a dramatic increase in the protein's reduction potential.[23,27,28] The large difference in coordination geometry between the three-coordinate Cu(I) site and the distorted tetrahedral Cu(II) center will also lead to a large increase in the reorganization energy of the redox center. In most cases, His87 has a p$K_a$ of ~5 in $PCu^I$; however, previous studies on parsley $PCu^I$ have indicated a higher value of 5.7.[29]

The electron-self-exchange reaction is an intrinsic property of all redox systems.[30] In the case of redox metalloproteins, it is an extremely useful reaction to study because it is necessary to know the structure of only one protein when the rate constants are interpreted. 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. A final reason for determining self-exchange rate constants is their fundamental importance to the Marcus theory. NMR spectroscopy is the only routine method 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 $10^6$ $M^{-1}$ $s^{-1}$.[31−39] In the case of the PCu's, the only precisely determined self-exchange rate constant is that for the protein from the cyanobacterium *A. variabilis* ($3.2 \times 10^5$ $M^{-1}$ $s^{-1}$).[36] A value of $\sim 4 \times 10^3$ $M^{-1}$ $s^{-1}$ has been reported for spinach plastocyanin,[32] while an upper limit of $2 \times 10^4$ $M^{-1}$ $s^{-1}$ has been quoted for the protein from French bean.[31] Herein we report the first precise determination of the self-exchange rate constant for a higher plant PCu. Furthermore, we study the effect of pH on the active site of this protein and investigate, for the first time, the effect the protonation of His87 has on the self-exchange rate constant of a PCu.

## Experimental Section

**Isolation and Purification of Parsley Plastocyanin.** Parsley PCu was purified by using the method of Plesnicar and Bendall[40] with modifications. Destalked leaves (~15 kg) were homogenized in 1 kg batches in 900 g of crushed ice, 100 mL of 1 M Tris buffer (pH 7.5), and 1 L of chilled acetone (−20 °C) for 60 s. The resulting mixture was filtered through cheesecloth and then muslin. The filtrate was centrifuged at 5000 rpm for 15 min at 4 °C, and the supernatant was collected, treated with 1.2 volumes of chilled acetone, and allowed to stand overnight at 4 °C to produce a precipitate.

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The supernatant was removed by decanting and centrifugation (as above), and the pellet was suspended in degassed Tris buffer (pH 7.5) and dialyzed overnight against the same buffer (degassed). Degassed buffer was used to ensure the stability of 2Fe-2S ferredoxin which was extracted along with PCu. The dialysate was centrifuged to remove insoluble impurities, and the supernatant was loaded onto a DE-52 column equilibrated with 60 mM Tris buffer, pH 7.5. PCu was then eluted using the same buffer containing 0.2 M NaCl. PCu was detected by the addition of small aliquots of a 20 mM solution of $K_3Fe(CN)_6$. The 2Fe-2S ferredoxin was eluted using the same buffer containing 0.5 M NaCl. The remainder of the procedure did not require the use of degassed solutions.

The final step in the purification of $PCu^{II}$ was performed on a DE−52 column equilibrated with 10 mM MES, pH 5.8, and eluted with a 0−0.4 M NaCl gradient in the same buffer. All fractions with a final peak ratio of $A_{278}/A_{597} \leq 1.68$, which gave a single band on an SDS-PAGE gel, were pooled. Fractions with higher ratios underwent further purification.

**NMR Sample Preparation.** For p$K_a$ determinations, the protein was exchanged into 99.9% deuterated 20 mM phosphate buffer using ultrafiltration (Amicon 5000 MWCO membrane) and reduced by the addition of 0.1 M $Na_2S_2O_4$ in 0.1 M NaOD. The pH of a sample was determined using a narrow pH probe (Russell CMAWL/3.7/180) with an in-house pH meter calibrated using aqueous buffers. No correction was made for the deuterium isotope effect, and pH values measured in deuterated solutions are designated by pH*. The titration was started at pH* 7.6, and the pH* was adjusted by the addition of small aliquots of 1.0 M DCl or 1.0 M NaOD. For self-exchange rate constant measurements, samples were exchanged into 39 mM phosphate at pH* 7.5, 51 mM phosphate at pH* 6.8, 73 mM phosphate at pH* 6.2, and 90 mM phosphate at pH* 5.6 (all with $I = 0.10$ M). $PCu^{I}$ was produced as above, with the excess reductant exchanged out by ultrafiltration. The reduced sample was placed in an NMR tube, which was then 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 concentrations of the oxidized protein in the samples were determined by transferring the mixed samples to a 2 mm UV/vis cuvette and measuring the absorbance at 597 nm ($\epsilon = 4500\ M^{-1}\ cm^{-1}$). Readings were taken before and after the acquisition of NMR spectra, with an average of the two readings used for all subsequent calculations.

**$^1$H NMR Spectroscopy.** All proton NMR spectra were acquired at 500.16 MHz on a JEOL Lambda 500 spectrometer at 25 °C. Free induction decays were accumulated into 16K data points and zero-filled to give 32K data points for transformation. The residual HDO signal was suppressed by presaturation at its resonant frequency, and all chemical shifts are quoted in parts per million (ppm) relative to water at $\delta$ 4.80.[41] Spin−lattice relaxation times ($T_1$) were determined using a standard inversion recovery sequence ($d$−180°−$\tau_D$−90°−acq). The values of $\tau_D$ ranged from 10 ms to 10 s, with the total relaxation delay ($d$ plus acq) always greater than 5 times the $T_1$ of the resonances being analyzed. The solvent peak was irradiated during $d$ and $\tau_D$. An exponential fit of a plot of peak intensity against $\tau_D$ for a particular proton yielded its $T_1$ value. Spin−spin relaxation times ($T_2$) were derived from peak widths at half-height using the relation $\nu_{1/2} = (\pi T_2)^{-1}$.

**UV/Vis Spectroscopy.** UV/vis spectra were recorded at 25 °C on either a Shimadzu UV-2101PC or a Philips PU8740 spectrophotometer.

## Results

**Protonation of His87 Measured by $^1$H NMR Spectroscopy.** In a previous study, a p$K_a$ value of 5.7 for His87 in parsley $PCu^{I}$ has been quoted at 40 °C[29] (note: the residues in parsley PCu are numbered as if the deletions at positions 57 and 58 are included in the amino acid count). In view of the importance to our subsequent studies, we repeated this experiment at 25 °C. In Figure 2, part of the aromatic region of the $^1$H NMR spectrum of parsley $PCu^{I}$ at pH* 7.5 is shown. The resolved singlet at 7.62 ppm has been assigned to the $C^{\epsilon 1}H$ proton of His87.[6] Upon lowering of the pH*, this resonance shifts in a downfield direction and is found at 8.48 ppm at pH* 4.9. This clearly indicates that His87 is becoming protonated at the $N^{\delta}$ atom and is no longer coordinated to the copper and that exchange between the deprotonated and protonated forms of the histidine is fast on the NMR time scale. The dependence of the chemical shift of this resonance on pH* is shown in Figure 3, and a fit (three parameters, nonlinear least squares) of the data to eq 1, where $\delta_H$ and $\delta_L$ are the chemical shifts at high and low pHs, respectively, gives a p$K_a$* of 5.6 at 25 °C. The $C^{\delta 2}H$ resonance of His87 ($\delta$ 7.04 ppm) is situated in a crowded region of the spectrum (see Figure 2), and the pH dependence of its chemical shift cannot be followed. The chemical shifts of a number of other resolved resonances in the 1D $^1$H NMR spectrum of parsley $PCu^{I}$ are also dependent on pH, in particular those of the $C^{\delta 2}H$, $C^{\epsilon 1}H$, and $N^{\epsilon 1}H$ of His37, the $C^{\delta}H$ of Leu12, the $C^{\epsilon}H$ of Phe29, the $C^{\xi}H$ of Phe35, the $C^{\epsilon}H$ of Phe41, and

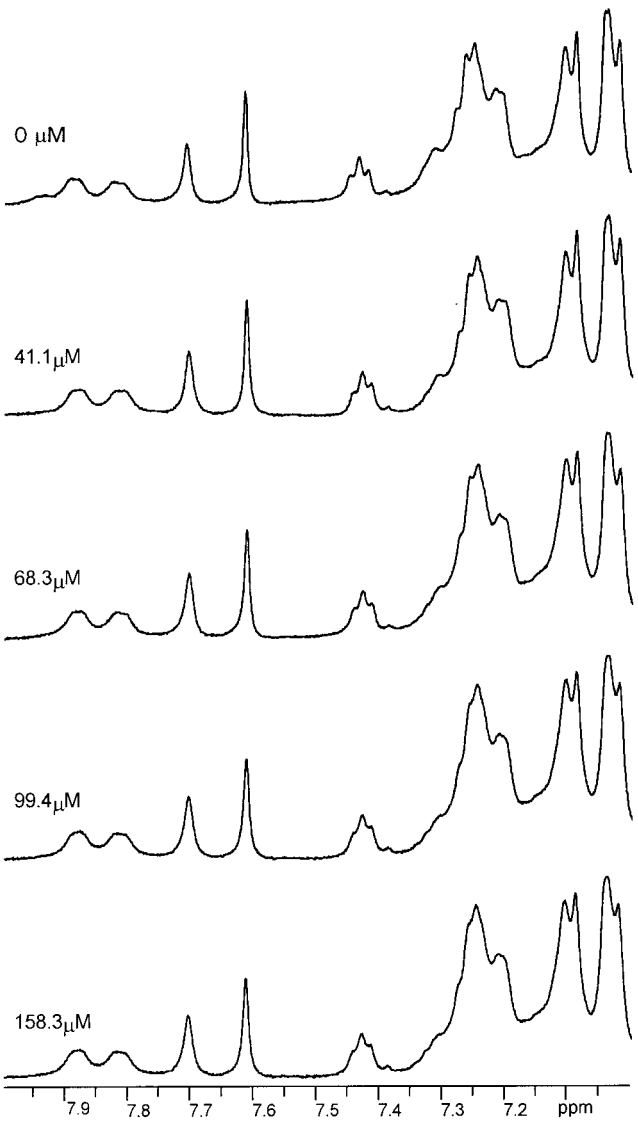


**Figure 2.** $^1$H NMR spectra (25 °C) of $PCu^{I}$ (∼1.7 mM) at pH* 7.5 showing the effect of increasing $[PCu^{II}]$ on the His87 $C^{\epsilon 1}H$ ($\delta$ 7.62 ppm) and the His37 $C^{\delta 2}H$ ($\delta$ 7.72 ppm) resonances.

$$\delta = (K_a\delta_H + [H^+]\delta_L)/(K_a + [H^+]) \quad (1)$$

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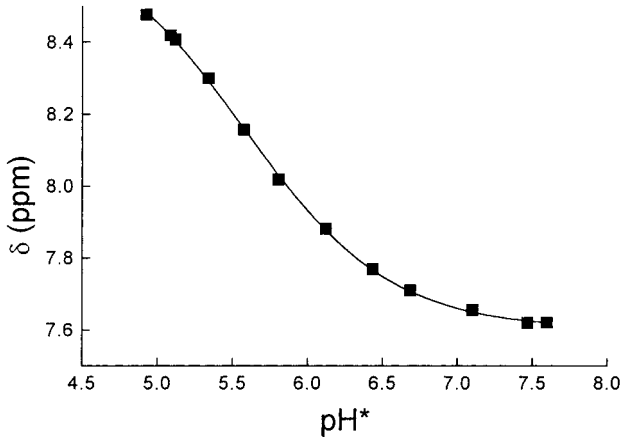


**Figure 3.** Variation of chemical shift with pH* of the His87 $C^{\epsilon 1}H$ resonance. The line shown is the fit of the data to eq 1.

the $C^{\epsilon}H_3$ of Met92. None of these peaks shift as much as the His87 resonance upon lowering of the pH* (overall shifts of 0.04−0.51 ppm), and all give p$K_a$* values of 5.6−5.7. The width of the $C^{\epsilon 1}H$ proton signal of His87 increases as the pH is lowered. This broadening of the protonated ligating His resonance has been observed in previous $^1$H NMR studies on plastocyanin[25] and amicyanin[35] and has been attributed to the presence of two forms of the protonated histidine that are exchanging at an intermediate rate on the NMR time scale. This aspect of the pH* dependence of the spectrum of parsley $PCu^I$ will be the subject of further studies.

**Determination of the Electron-Self-Exchange Rate Constant by NMR Spectroscopy.** In a mixture of $PCu^I$ and $PCu^{II}$, the slow-exchange condition[33,36,42,43] applies to protons that obey the following relationship:

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

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. In these circumstances, for dilute solutions containing only a small (<10%) proportion of the oxidized form of the protein, it can be shown that expression 3 applies,[33,36,42,43]

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

where $T_i$ is the observed relaxation time of the resonance for 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 straight line of slope $k$.

It is therefore imperative, when determining the self-exchange rate constant of a cupredoxin by $^1$H NMR spectroscopy, that the protons used be in the slow-exchange regime. In all of these studies, we used the His37 $C^{\delta 2}H$ and His87 $C^{\epsilon 1}H$ resonances. Most experiments were carried out at $[PCu]_T$ values of ~2 mM, and the $k$ values are all $\sim 5 \times 10^4$ $M^{-1}$ $s^{-1}$ (vide infra). The $T_{1,red}^{-1}$ values for the imidazole ring resonances used are in the range 0.5−0.7 $s^{-1}$, while the $T_{1,ox}^{-1}$ values for the His37 $C^{\delta 2}H$ and His87 $C^{\epsilon 1}H$ resonances of spinach $PCu^{II}$ are 370 and >1000 $s^{-1}$, respectively.[44] The $T_{2,red}^{-1}$ values for these resonances range from 10 to 50 $s^{-1}$, while the $T_{2,ox}^{-1}$ values are $>10^4$ $s^{-1}$.[44] Therefore, inequality 2 applies to both the $T_1$ and $T_2$ data for all the experiments described herein, and this is especially so for the data derived from the His87 $C^{\epsilon 1}H$ resonance. Verification that the protons used herein 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.[33,36] Furthermore, the observed $T_i$ values are independent of $[PCu]_T$.[36] In this study, we noticed that eq 3 remains valid when a much higher proportion of $PCu^{II}$ is present (up to 50%). However, most of the data quoted are for studies carried out with $[PCu^{II}] \ll [PCu]_T$.

In Figure 2 is shown the effect of increasing $[PCu^{II}]$ on part of the aromatic region of the $^1$H NMR spectrum of $PCu^I$ at pH* 7.5. The $C^{\epsilon 1}H$ resonance of His87 (7.62 ppm) and the $C^{\delta 2}H$ signal of His37 (7.72 ppm) both broaden as $[PCu^{II}]$ is increased. However, the amount of broadening is quite small, severly curtailing the precision of a determination of the self-exchange rate constant from $T_2$ measurements. In Figure 4 is shown the effect of increased $[PCu^{II}]$ on the $T_1$ relaxation times of these two protons at pH* 7.5. The contrast between the two inversion recovery experiments carried out at $[PCu^{II}]$ of 41 and 158 $\mu$M is much more significant than the corresponding change in line broadening, and thus the measurement of $T_1$ values provides a more precise method of determining the self-exchange rate constant (the estimated error in the self-exchange rate constants determined from $T_1$ measurements is ±10%, while it is ±20% for those obtained from $T_2$ measurements). Plots of $T_i^{-1}$ against $[PCu^{II}]$ for the His37 $C^{\delta 2}H$ resonance at pH* 6.2 and 5.6 are shown in Figure 5, and the slopes of these plots ($k$ values) are listed in Table 1 along with the values determined at other pH* values for this resonance and at all the pH* values for the His87 $C^{\epsilon 1}H$ resonance. From the values shown, it is clear that the $k_2/k_1$ ratios (where $k_1$ and $k_2$ are the slopes from the plots of $T_1^{-1}$ and $T_2^{-1}$, respectively, against $[PCu^{II}]$) are all very close to 1, highlighting that the resonances used are indeed in the slow-exchange regime. As stated previously, the $k_1$ values provide the more precise value of the self-exchange rate constant.

## Discussion

It is significant that the p$K_a$* of 5.6 obtained for His87 in parsley $PCu^I$ is higher than that determined for the corresponding residue in other plant and cyanobacterial plastocyanins (4.8−5.0).[29] The green algal PCu's, which have the corresponding deletions at positions 57 and 58 in their primary structures and have a diminished upper acidic patch, also have high active-site p$K_a$* values.[45,46] Protonation of one of the active-site histidines has also been observed in the cupredoxins amicyanin[35] and pseudoazurin,[47] which have p$K_a$* values of 6.8 and 4.8, respectively. Loop-directed mutants of amicyanin have been produced[48,49] which possess the active-site loops of plastocyanin, azurin, and pseudoazurin. Interestingly, the plastocyanin loop mutant of amicyanin has an active-site p$K_a$* of 5.7. This observation may just be coincidental, or it could indicate similarities between this variant of amicyanin and the plastocyanins with quite high active-site p$K_a$* values. The cause of

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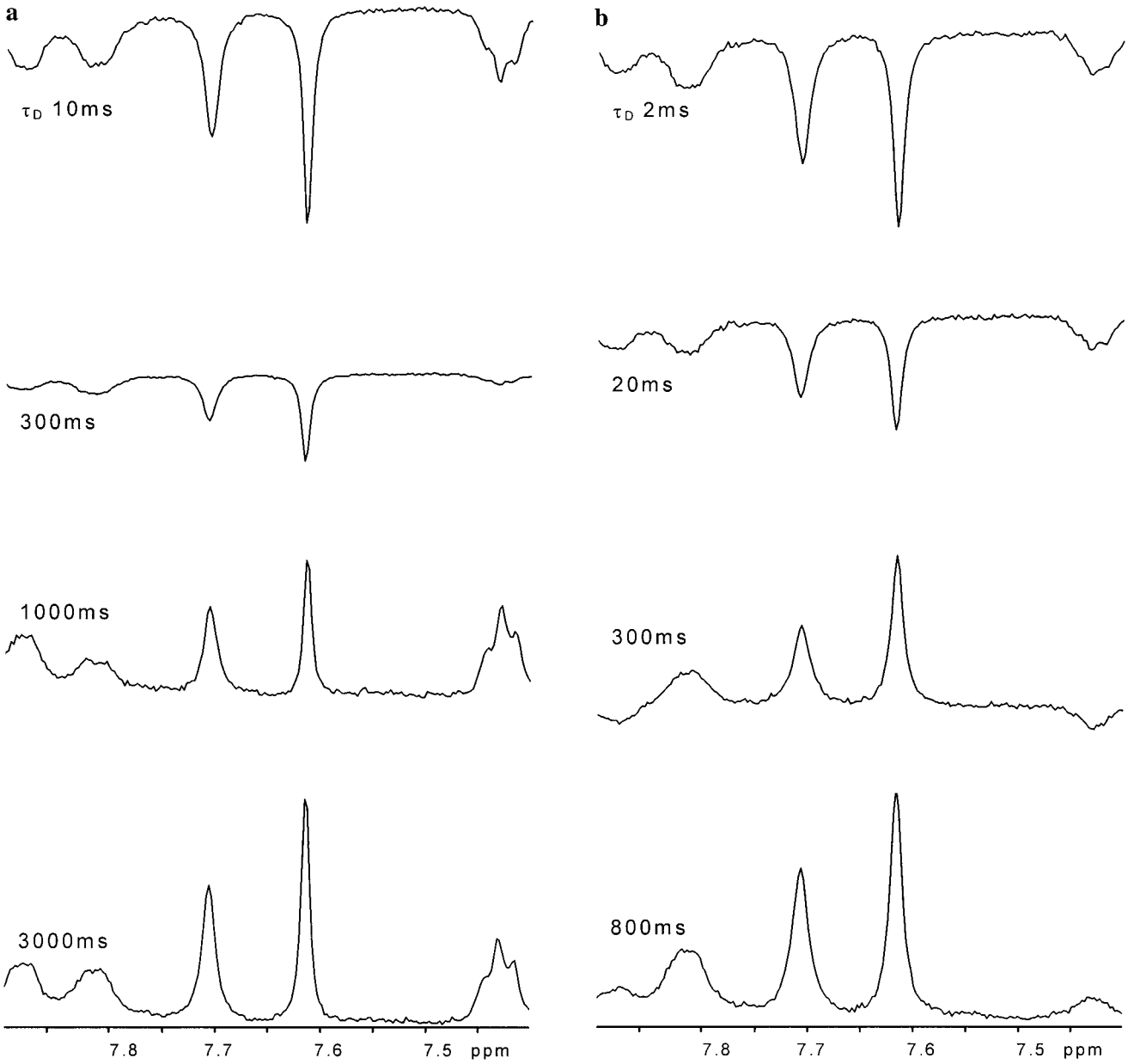


**Figure 4.** Selected NMR spectra from the inversion recovery experiments with (a) $[PCu^{II}] = 41\ \mu M$ and (b) $[PCu^{II}] = 158\ \mu M$, showing the His37 $C^{\delta 2}H$ resonance at $\delta$ 7.72 ppm and the His87 $C^{\epsilon 2}H$ peak at $\delta$ 7.62 ppm at pH* 7.5.

the different $pK_a$* values in the different cupredoxins, and also in the plastocyanins from various sources, is not known. It may be that the solvent exposure of the His ligand which protonates is an important factor. A more solvent-exposed His would lead to a weakening of Coulombic interactions because of a higher effective dielectric constant, and this may weaken the interaction between the Cu and the His. Consequently, those proteins which show higher $pK_a$* values would have a more exposed His ligand. Other important factors include the pattern and number of hydrogen bonds at the active site and the influence of electrostatic interactions in the different proteins.[49] It is also interesting to note that the protonation of His87 in parsley $PCu^I$, which causes a structural change at the Cu(I) site,[11] affects the chemical shift of a number of other NMR resonances as well as those belonging to the protonating residue. This includes protons associated with the other ligands and also with residues in the hydrophobic patch which must undergo a subtle structural change as a consequence of protonation at His87.

The self-exchange rate constant (25 °C) of parsley PCu at pH* 7.5 ($I = 0.10$ M) is $5.0 \times 10^4\ M^{-1}\ s^{-1}$ (an average of the $k_1$ values determined for the His37 $C^{\delta 2}H$ and the His87 $C^{\epsilon 1}H$ resonances). This is an order of magnitude larger than the approximate value reported for spinach PCu ($\sim 4 \times 10^3\ M^{-1}\ s^{-1}$ at $I \sim 0.1$ M).[32] The value for parsley PCu is also an order of magnitude larger than that calculated from the cross-reaction of this protein with azurin using the Marcus equation,[50] which depends on a number of assumptions. The self-exchange process in cupredoxins is thought to involve the association of two protein molecules via the hydrophobic surfaces which surround the exposed C-terminal His ligand[51−54] (the hydrophobic patch is a conserved feature of all structurally characterized cupredoxins[55]). This encounter complex implicates the exposed histidine ligand in the pathway for electron transfer.[56] The histidine ligand that protrudes through the hydrophobic patch is also thought to be involved in the reaction of various

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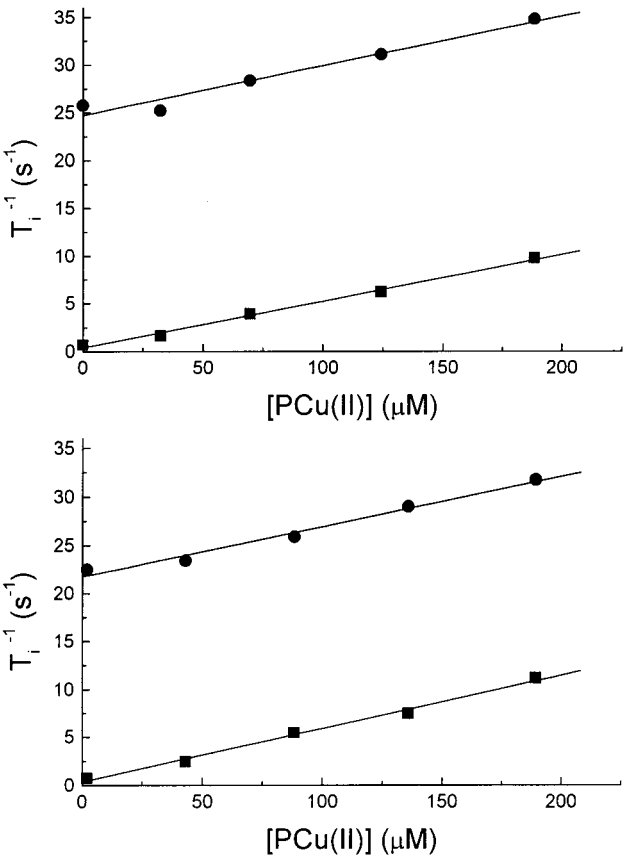


**Figure 5.** Plots of $T_1^{-1}$ (■) and $T_2^{-1}$ (●) against [$PCu^{II}$] for the His37 $C^{\delta}H$ resonance at pH* 5.6 (upper) and 6.2 (lower).

cupredoxins with their physiological redox partners.[57,58] Plastocyanin has two prominent recognition surfaces, and site-directed mutagenesis studies have indicated that both the acidic and the hydrophobic patches are important for the interaction with PSI.[59−69] However, it has been found that the lower acidic patch is more influential in this process.[62,66] The interaction of cytochrome *f* of the $b_6f$ complex with PCu also involves both of the surface patches of the copper protein.[70−73] In this case, both the lower and the upper acidic patches are important but only half of the hydrophobic patch appears to be involved.[73] It should be noted that, in its reaction with both cytochrome *f* and PSI, PCu uses His87 as the pathway for electron transfer. It therefore appears that, for the physiological interactions of PCu, the two surface patches can be split in half and that these are used in different combinations. Interestingly, in the nonphysiological reaction between PCu and cytochrome *c*, it has been shown that the initial docking of the two proteins involves both the upper and the lower acidic patches[74−76] but the subsequent rearrangement, which occurs prior to electron transfer, involves only the upper acidic cluster.[74,75] The relatively small self-exchange rate constant for spinach PCu (and also French bean PCu) most likely arises from the presence of the large acidic patch around Tyr83, which hinders protein−protein association. The larger self-exchange rate constant for parsley PCu can then be attributed to the diminished nature of the upper acidic patch in this protein (the upper acidic patch is quite close to the hydrophobic patch and therefore would be expected to have a more significant effect on the association of two PCu molecules). It should be noted that parsley PCu has the acidic residue Glu85 which is situated close to the upper acidic patch. It has been suggested[6] that this residue may compensate for the absence of two of the three acidic residues usually found in this area in plant PCu's. The residues Glu53 and Glu95 are also thought to bolster the acidic patch in parsley PCu, but these two residues are situated close to the lower acidic patch. Nevertheless, it would appear that two parsley PCu molecules can associate more readily than other plant PCu's, resulting in the protein having a much larger self-exchange rate constant. If this is due to the removal of charge at the upper acidic patch, then differences in

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

| | His37 $C^{\delta 2}H$ | | | His87 $C^{\epsilon 1}H$ | | |
|---|---|---|---|---|---|---|
| pH* | $k_1/M^{-1}\,s^{-1}$ | $k_2/M^{-1}\,s^{-1}$ | $k_2/k_1$ | $k_1/M^{-1}\,s^{-1}$ | $k_2/M^{-1}\,s^{-1}$ | $k_2/k_1$ |
| 7.5 | $4.4 \times 10^4$ | $5.0 \times 10^4$ | 1.1 | $5.5 \times 10^4$ | $4.1 \times 10^4$ | 0.8 |
| 6.8 | nd[b] | $5.2 \times 10^4$ [c] | | nd[b] | $5.7 \times 10^4$ | |
| 6.2 | $4.9 \times 10^4$ | $5.2 \times 10^4$ | 1.1 | $6.0 \times 10^4$ | nd[d] | |
| 5.6 | $5.5 \times 10^4$ | $5.2 \times 10^4$ | 0.9 | $5.6 \times 10^4$ | nd[d] | |

[a] The estimated error in the $k_1$ values is ±10%, and that for the $k_2$ values is ±20%. [b] Not determined. [c] This value was derived from measurements made at $PCu^{II}$ concentrations up to 466 μM (29% oxidized sample). [d] Not determined owing to the very broad nature of the His87 $C^{\epsilon 1}H$ resonance at these pH* values.


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the interactions of parsley PCu, as compared to other plant PCu's, with its physiological partners must exist.

The large self-exchange rate constant of parsley PCu at neutral pH* along with the relatively high p$K_a$* of His87 in parsley $PCu^I$ has allowed us to investigate the effect of pH on the self-exchange reactivity of a plastocyanin for the first time. The self-exchange rate constant of parsley PCu is almost independent of pH (see Table 1). The value of $5.6 \times 10^4$ $M^{-1}$ $s^{-1}$ for the self-exchange rate constant at pH* 5.6 (at which ~50% of the $PCu^I$ molecules will be protonated at His87 and will thus be three-coordinate) is a surprising outcome of this study. To understand this fully, the self-exchange process needs to be examined in more detail.

The self-exchange rate constant, like the second-order rate constant for any intermolecular electron-transfer process, is a function of the association constant of the two molecules, $K_{assoc}$, and of the first-order rate constant for electron transfer, $k_{et}$. As His87 becomes protonated in $PCu^I$, the reorganization energy of the site will increase and hence $k_{et}$ will be smaller.[77] Additionally, movement of the His87 ligand away from the copper ion will affect the supposed pathway for electron transfer.[55] Notwithstanding this, the self-exchange rate constant at pH* 5.6 is actually slightly larger than that at pH* 7.6, and this indicates that lowering the pH* facilitates protein−protein association and thus leads to a larger value of $K_{assoc}$, which compensates for the expected decrease in $k_{et}$ (it should be noted that the movement of His87 away from the copper ion may make protein−protein association more difficult). Previous studies have shown that the negative charge at the acidic patch of parsley PCu is considerably diminished at pH 5.6, which will lead to an increase in $K_{assoc}$.[29] Previous studies of the pH dependence of the self-exchange rate constant of amicyanin, a cupredoxin which does not possess an acidic patch but which exhibits an active-site protonation, have been carried out. In this case, the self-exchange rate constant decreases by a factor of 2.5 at the pH corresponding to the p$K_a$ of the active-site His in the reduced protein compared to the value at higher pH.[35]

**Table 2.** Electron-Self-Exchange Rate Constants ($k_{ese}$) of Plastocyanins from Various Sources Determined by NMR Spectroscopy

| source | $k_{ese}/M^{-1}$ $s^{-1}$ | pH | temp/°C | ref |
|---|---|---|---|---|
| spinach (*Spinacia oleracea*) | $\sim 4 \times 10^3$ | 6.0 | 25 | 32 |
| French bean (*Phaseolus vulgaris*) | $\ll 2 \times 10^4$ | 7.4 | 50 | 31 |
| *Anabaena variablis* | $3.2 \times 10^5$ | 7.5 | 25 | 36 |
| parsley (*Petroselinium crispum*) | $5.0 \times 10^4$ | 7.5 | 25 | this work |

In Table 2 are listed the self-exchange rate constants of plastocyanins from different sources. The values range from $\sim 4 \times 10^3$ $M^{-1}$ $s^{-1}$ for the protein from spinach[32] to $3.2 \times 10^5$ $M^{-1}$ $s^{-1}$ for the cyanobacterial PCu from *A. variabilis*.[36] The value for parsley PCu is midway between these two values. These observations, along with the surface topologies of the proteins (see Figure 1), indicate that the major factor in controlling the self-exchange rate constants of the PCu's is the size of the acidic patch. This observation is further supported by the effect of pH on the self-exchange rate constant of parsley PCu. Interestingly, the range of self-exchange rate constants found for the PCu's is similar to that found for the whole cupredoxin family (from $4 \times 10^3$ $M^{-1}$ $s^{-1}$ for pseudoazurin from *Achromobacter cycloclastes*[39] to $1 \times 10^6$ $M^{-1}$ $s^{-1}$ for azurin from *Pseudomonas aeruginosa*).[33] It is also thought that the major factor which modulates the observed self-exchange rate constant for a particular cupredoxin is the distribution of charged residues close to the hydrophobic patch (i.e., differences in $K_{assoc}$). Variations in the reorganization energy and the distance for electron transfer (i.e., differences in $k_{et}$) will also influence the observed self-exchange rate constant.

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