Determination of the Self-exchange Rate Constant for Plastocyanin from *Anabaena variabilis* by Nuclear Magnetic Resonance Line Broadening†

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The rate constant (25 °C) for self exchange between the copper-(I) and -(II) forms of *Anabaena variabilis* plastocyanin has been determined by an NMR method. The procedure used depends upon the enhanced relaxation of protons near the paramagnetic Cu^{II} in the oxidised protein. At pH 7.5, I = 0.10 M the rate constant is 3.2×10^5 M^{-1} s^{-1} and provides confirmation of that obtained indirectly by application of the Marcus equation to the cross-reaction of azurin [ACu(I)] with *A. variabilis* plastocyanin [PCu(II)]. This gives 5.9×10^5 M^{-1} s^{-1} under the same conditions. The rate constant is substantially (at least 10^2 times) larger than those obtained for higher-plant plastocyanins, *e.g.* spinach, and this is attributable to the higher negative charge on the latter. Since the rate constant is comparable to that for azurin itself a similar mechanism for self exchange involving association of protein molecules *via* hydrophobic adjacent surfaces is suggested.

Plastocyanin ($M_r \approx 10\,500$) is a single type I blue copper protein located in the thylakoid of the chloroplast and is involved in photosynthetic electron transport in higher plants and algae. Its function is to transfer electrons from membrane-bound cytochrome f to P700 of photosystem I.[1,2] The characteristic features of plastocyanin (PCu) are its intense blue colour and narrow hyperfine coupling constant in the EPR spectrum of the oxidised protein.[3-6] Depending on its source, plastocyanin has approximately 100 amino acids. In some cases, *e.g.* parsley and green algae plastocyanins, there are deletions at or near positions 57 and 58[3,4] and in one case, that of the blue-green algae plastocyanin from *Anabaena variabilis*, there are additional residues giving 104 in total.[7] There are now 30 known full amino-acid sequences of plastocyanins.[8,9] In the 22 higher-plant sequences 45 of the 99 residues are invariant. With the inclusion of eight algal sequences this number reduces to 22. The invariant residues include His-37, Cys-84, His-87 and Met-92 which are the four co-ordinating to the copper at the active site.[10]

In all but two cases plastocyanins are acidic with the charge on the reduced protein estimated to be -9 ± 1 at pH ≈ 7. Reactions of plastocyanin have indicated the presence of two areas for interaction with redox partners; these are the remote (close to Tyr-83) and adjacent (close to His-87) sites.[11-14] Features of the former are the negatively charged 42–45 and 59–61 regions on two protruding sections of polypeptide on either side of the solvent-exposed Tyr-83.[10] The exceptions are *A. variabilis*[7] and *Anabaena* sp. PCC7937[15] plastocyanins. In *A. variabilis* plastocyanin the negative charge at the remote site is not retained and the only acidic residues in this area are Asp-42 and Glu-85. The presence of basic residues His-59, Lys-60 and Arg-88 (the side chain of which appears to bridge Asp-42 and Glu-85) further minimises negative charge in this area. As a result of its ratio of acidic to basic amino acids, reduced *A. variabilis* plastocyanin has a charge of +1 at pH 7.[7]

The rate constant for the self-exchange reaction of plastocyanin [PCu(I) + PCu(II) ⟶ PCu(II) + PCu(I)] provides information on electron transfer in this protein without the need to consider driving forces, it is an integral part of the Marcus equation,[16] and it is also useful because its interpretation needs the consideration of the structure of only one protein. It has been shown previously by NMR spectroscopy[17,18] and by cross-reactions[19] that the rate of electron self exchange in higher-plant plastocyanins is slow in comparison to those in other blue copper proteins. This has been explained by the electrostatic repulsion caused by the high negative charge on these species (at neutral pH). This reasoning has been further vindicated by the apparent catalysis of electron self exchange in spinach plastocyanin by redox-inert multivalent cations.[18]

Owing to its relative lack of charge (at neutral pH), *A. variabilis* plastocyanin should differ from all other known plastocyanins in having a self-exchange rate constant more in accord with those of other blue copper proteins (10^5–10^6 M^{-1} s^{-1}). Support for this view is provided by cross-reactions with azurin when a self-exchange rate constant of 5.9×10^5 M^{-1} s^{-1} was obtained.[19] Owing to the scope for error in rate constants determined by indirect methods it is desirable to have an independent measurement of this value, and in this work the self-exchange rate constant of *A. variabilis* plastocyanin has been directly determined using an NMR technique in which the enhanced relaxation of certain proton resonances caused by the paramagnetic Cu^{II} of the oxidised protein has been utilised. This is the first direct and accurate determination of a self-exchange rate constant for a plastocyanin.

Experimental

Isolation of A. variabilis *Plastocyanin.*—*A. variabilis* PCu was obtained as previously described.[20] Final purification was to a UV/VIS absorbance (A) peak ratio A_{278}/A_{597} of 1.15:1. The final purification step was carried out on a G-50 gel column. Protein concentrations were determined from the PCu(II) peak at 597 nm (ε = 4500 M^{-1} cm^{-1}).

NMR Sample Preparation.—For the acquisition of proton NMR spectra the protein was exchanged into 99.9% deuteriated 73 mM phosphate buffer at pH 6.20 (I = 0.10 M) using a small Amicon set-up. The protein was reduced under appropriate air-free conditions by the addition of cooled small aliquots of 0.1 M $Na_2S_2O_4$ in 99.9% D_2O (0.1 M NaOD). Any excess of $Na_2S_2O_4$ present was exchanged out and the protein was transferred to an NMR tube which had been flushed with argon to prevent air oxidation and which was then sealed. The pH of the sample was

† *Non-SI unit employed:* M = mol dm^{-3}.

measured using a narrow Russell CMAWL/3.7/180 pH probe in combination with a Radiometer-PHM62 pH meter which was calibrated using aqueous buffers. All samples were at pH 6.20, with this value adjusted using NaOD or DCl (0.1 M) as necessary; no correction was made for the deuterium isotope effect. An oxidised protein solution was made in the same way as above except that a 0.1 M solution of $[Fe(CN)_6]^{3-}$ was used to oxidise the protein and no air-free techniques were employed.

Solutions containing the desired proportions of oxidised and reduced PCu were prepared by adding small portions of the oxidised sample to the reduced protein. The concentration of oxidised protein present in the NMR sample was then confirmed by transferring the protein mixture to a 2 mm UV/VIS spectrophotometer cell under air-free conditions and monitoring the absorbance (A) at 597 nm. Good agreement was found between A_{597} readings taken immediately before and after the NMR measurements, and the values used in the calculations are averages of the two.

NMR Spectra.—The calculation of the self-exchange rate constant by this method depends on determining the effect of the paramagnetic oxidised form of PCu on the relaxation behaviour of certain proton resonances of the reduced protein. Thus the relaxation times of specific protons had to be determined in solutions containing different proportions of the two forms of the protein. Values of T_1 were obtained by a standard inversion-recovery method (180°–τ_D–90° acquire) with *ca.* 2 s between scans, whilst T_2 values were derived from peak widths at half-height using the relation $\nu_{\frac{1}{2}} = (\pi T_2)^{-1}$. Allowance was made for the *ca.* 2 Hz contribution of ^4J(HH) to the line shape of the histidine proton resonances.

All proton NMR spectra were acquired at 500.14 MHz on a Bruker AMX500 spectrometer at 298 K. Typically, 512 free induction decays were accumulated into 16 K data points and transformed into 32 K data points after zero-filling. The residual HDO resonance was suppressed by presaturation at its resonant frequency. All chemical shifts are cited in parts per million (ppm) relative to internal dioxane at δ 3.741.

Results

In this paper we determine the self-exchange rate constant of a plastocyanin by an NMR method which uses the theory developed by McLaughlin and Leigh[21] and which has been previously applied to the blue copper proteins azurin[22–25] and amicyanin.[26,27] The appearance of the resonance given by a particular proton in a plastocyanin that is in reversible equilibrium between the paramagnetic (oxidised) and reduced forms depends upon three main factors: first, the rate of exchange between the two forms, secondly the frequency separation of the resonances in the two environments and thirdly the nuclear relaxation times in the two environments. The second and third of these factors are, in general, different for different protons in the same protein and in practice it is only protons that are in close proximity to the paramagnetic centre that can provide the data needed to determine the exchange rate. Such protons will experience in the oxidised form (not necessarily measurable) paramagnetic shifts and will also have very short relaxation times owing to the paramagnetism of the proximate unpaired electron. Consequently these protons may not give an observable resonance. In general it is not possible to solve the equations of McLaughlin and Leigh for the rate constant unless the values of T_1 and T_2 are known for both forms of the protein, and these are not usually available. However, it can be shown that the equations can be solved in two extreme situations as follows.

(*a*) The slow-exchange regime requires that relation (1)

$$k[PCu]_T \ll 1/T_{i,ox} - 1/T_{i,r} \quad (1)$$

applies, where k is the second-order rate constant for the electron self-exchange reaction, $[PCu]_T$ is the total plastocyanin concentration and $T_{i,ox}$ and $T_{i,r}$ (i = 1 or 2) are the relaxation times in the oxidised and reduced forms respectively. In these circumstances for dilute solutions containing only a small proportion of the oxidised form of the protein it can be shown for the observable signals that expression (2) applies, where

$$1/T_i = (1/T_{i,r}) + k[PCu(II)] \quad (2)$$

[PCu(II)] is the concentration of oxidised plastocyanin. Thus a plot of $1/T_i$ against [PCu(II)] will be a straight line of slope k.

(*b*) The 'fast-exchange' regime for which $1/T_i$ is independent of k.

It is crucial to establish that the inequality (1) applies to the protons which are to be used for the determination of the rate constant, although it is not usually possible to do this from *a priori* considerations. However the different exchange regimes have features that enable them to be characterised experimentally.

The slow-exchange regime has the following properties. (*i*) Identical values of k derived from both the T_1 and T_2 data: in practice deviations of up to a factor of *ca.* 2 can be accommodated and since, in general, $T_1 > T_2$ it follows from equation (1) that the T_2 data should provide the more reliable estimate of k in cases where there is a discrepancy. (*ii*) The rate of self exchange in blue copper proteins is markedly temperature-dependent and thus in the slow-exchange regime the observed T_i values should decrease significantly with temperature. By contrast, in the fast regime a variation of observed T_i would only arise as a result of a change in $T_{i,r}$ which itself should be relatively insensitive to temperature variation and in fact should show a slight increase with increasing temperature as a result of more rapid molecular tumbling of these large molecules. (*iii*) In the 'slow-exchange' regime equation (2) shows that the observed T_i should be unaffected by the total plastocyanin concentration ($[PCu]_T$) whereas for the 'fast-exchange' limit T_i should increase with increasing $[PCu]_T$.

In the present work it was found (see below) that the slow-exchange regime applied to the four protons (C^ε and C^δ) of the two histidine (37 and 87) ligands. The resonances of these protons have been assigned[28,29] and are at δ 7.59 (His-87, C^εH), 7.52 (His-37, C^δH), 7.14 (His-87, C^δH) and 7.05 (His-37, C^εH). At 500 MHz and pH 6.20 these resonances were sufficiently resolved to be used for individual determinations of the self-exchange rate constant.

Calculation of the Self-exchange Rate Constant.—Selected traces from the standard inversion-recovery experiments used to determine T_1 for each of the four histidine protons at different concentrations of the oxidised protein and with $[PCu]_T$ = 2 mM are shown in Figs. 1 and 2. The enhanced rate of relaxation at the higher concentration of PCu(II) is immediately apparent. The effect of the presence of oxidised (paramagnetic) protein on the T_2 relaxation times of the same protons can be seen in Fig. 3. Fig. 4 shows plots of T_1^{-1} and T_2^{-1} against [PCu(II)] for the C^δH of His-87 (δ 7.14) and similar plots were obtained for the other three histidine protons. From the slopes of these plots the rate constant data of Table 1 were calculated. Values of T_2^{-1} were also obtained by a similar titration with $[PCu]_T$ = 1 mM.

Table 1 Self-exchange rate constants k_1 and k_2 derived from T_1 and T_2 data respectively with $[PCu]_T$ = 2 mM

Resonance (δ)	$10^{-5}k_1/M^{-1}\,s^{-1}$	$10^{-5}k_2/M^{-1}\,s^{-1}$	k_2/k_1
His-37 C^εH (7.05)	1.4	3.4	2.4
His-37 C^δH (7.52)	1.5	2.9	1.9
His-87 C^εH (7.59)	3.2	2.4	0.8
His-87 C^δH (7.14)	2.2	2.9	1.3

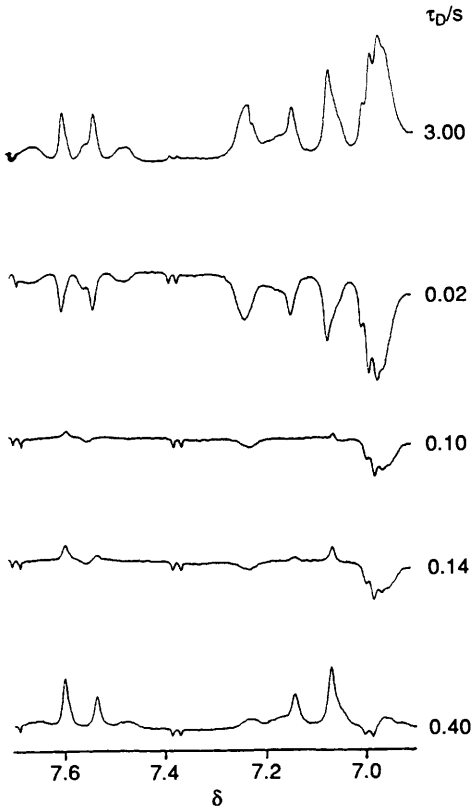

Fig. 1 Selected NMR spectra from the inversion-recovery experiment with [PCu(II)] = 27 μM showing four ligand histidine proton resonances (His-37 $C^{\varepsilon}H$ at δ 7.05 ppm, His-87 $C^{\delta}H$ at δ 7.14, His-37 $C^{\delta}H$ at δ 7.52 and His-87 $C^{\varepsilon}H$ at δ 7.59)

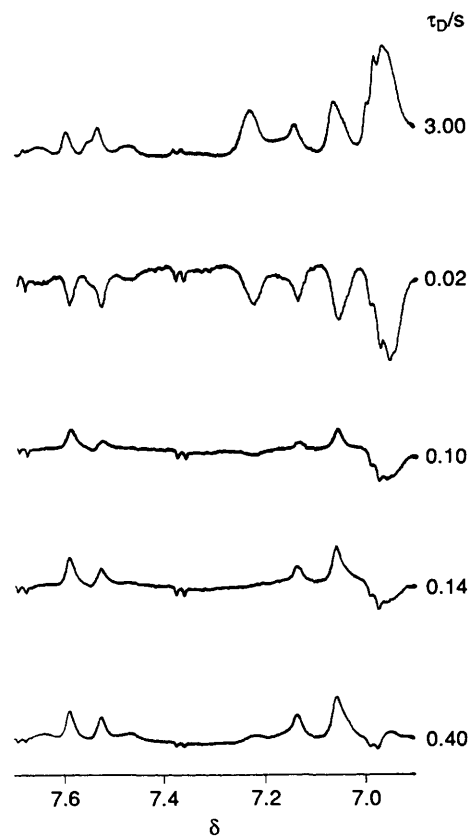

Fig. 2 Selected NMR spectra from the inversion-recovery experiment with [PCu(II)] = 47 μM showing the four ligand histidine proton resonances

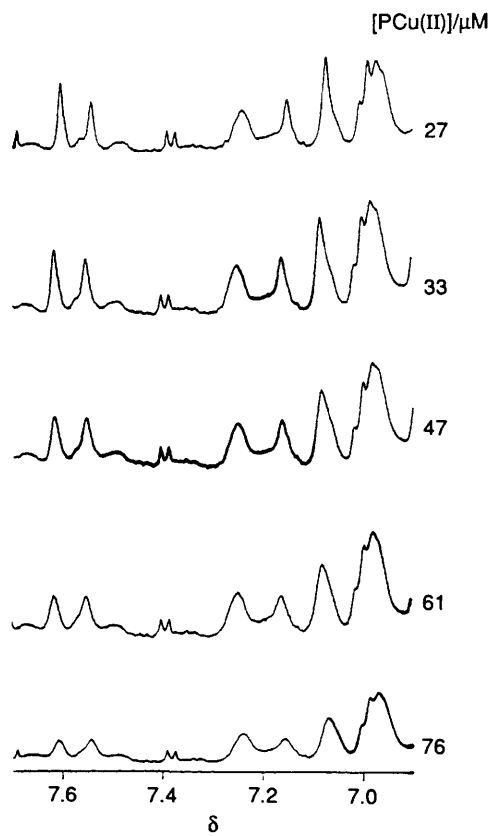

Fig. 3 Selected NMR spectra showing the effect of [PCu(II)] on the linewidths of the four ligand histidine proton resonances

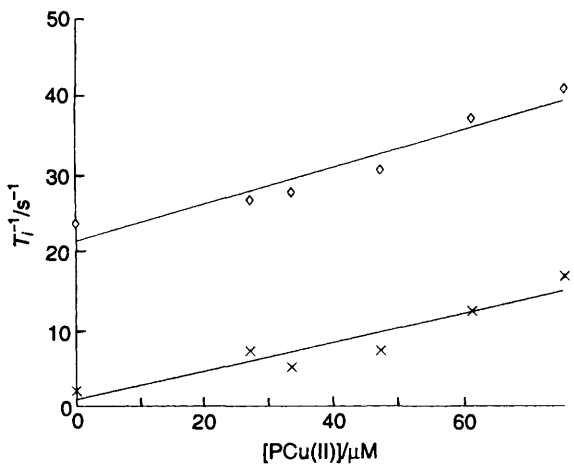

Fig. 4 Plots of T_1^{-1} (◇) and T_2^{-1} (×) against [PCu(II)] for the $C^{\delta}H$ of His-87 (δ 7.14)

Table 2 Self-exchange rate constants derived from T_2 data with $[PCu]_T$ = 1 mM

Resonance (δ)	$10^{-5}k_2/M^{-1}\ s^{-1}$
His-37 $C^{\varepsilon}H$ (7.05)	1.6
His-37 $C^{\delta}H$ (7.52)	2.1
His-87 $C^{\varepsilon}H$ (7.59)	1.9
His-87 $C^{\delta}H$ (7.14)	1.7

The self-exchange rate constants obtained at this total protein concentration are as shown in Table 2. These measurements were carried out at pH 6.20 and a correction factor of 1.1 {determined from the reaction[19] of *A. variabilis* PCu(I) with $[Fe(CN)_6]^{3-}$} was used to obtain a rate constant at pH 7.5.

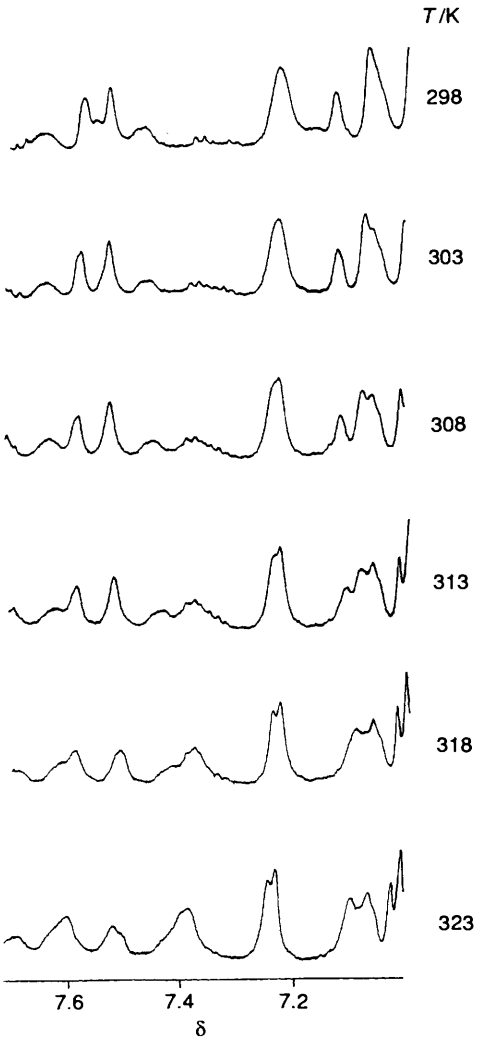

Fig. 5 Proton NMR spectra showing the effect of changing temperature on the self-exchange rate of a 2 mM reduced protein solution which was 1.5% oxidised

Thus from an average of the T_2 data of the four histidine protons (at $[PCu]_T$ = 2 mM) and using the factor of 1.1 a self-exchange rate constant of 3.2×10^5 M^{-1} s^{-1} is calculated.

It is important to recognise that this determination of the self-exchange rate constant does not depend upon assigning the observed resonances to particular protons, although this is desirable. However it is crucially dependent upon the correctness of the assignment of the behaviour of each of the four protons used to the slow-exchange regime. For these protons the k_2/k_1 ratios range from 2.4 to 0.8:1 and this fact alone provides compelling evidence that the 'slow-exchange' regime does indeed apply to all four protons, and more especially in respect of the T_2 data. Ratios k_2/k_1 in excess of 5–10:1 are to be expected for protons in the fast-exchange regime[25] and a value of 8:1 has been obtained for the $C^\varepsilon H$ of His-59 which is some 12 Å away from the copper. This analysis was confirmed by studying the effect of changing temperature upon a protein solution which was 2 mM in total protein and approximately 1.5% oxidised. As can be seen in Fig. 5 the resonances of all four histidine protons used in these calculations broaden (corresponding to reducing T_2) as the temperature is increased in accordance with criterion (*ii*) above. Resonances from protons not close to the copper sharpen somewhat with increasing temperature. It was also found that the linewidths and hence values of T_2 for the two ligand histidine $C^\delta H$ protons were similar in 0.5, 1 and 2 mM solutions of reduced plastocyanin

Table 3 Values of T_2^{-1}/s^{-1} for the four histidine protons at the same $[PCu_{ox}]$ but different $[PCu]_T$

	T_2^{-1}/s^{-1}		
Resonance (δ)	$[PCu]_T$ = 0.5	1.0	2.0
His-37 $C^\varepsilon H$ (7.05)	46.4	47.8	33.4
His-37 $C^\delta H$ (7.52)	27.3	27.9	25.4
His-87 $C^\varepsilon H$ (7.59)	32.5	31.1	23.1
His-87 $C^\delta H$ (7.14)	25.2	26.6	27.1

containing equal (small) concentrations of the oxidised protein (see Table 3). However from the data in Table 3 the two $C^\varepsilon H$ protons give narrower lines at a total protein concentration of 2 mM. This may be due to the fact that the determinations at 0.5 and 1.0 mM total protein were made on samples that had been exchanged to the same extent, whilst the spectra obtained at 2 mM total protein had been exchanged to a different degree. There are amide peaks which overlap with those of the $C^\varepsilon H$ of the two histidine ligands and their contribution to the apparent linewidth depends on how much the protein has been exchanged. Obviously the values obtained at a total protein concentration of 2 mM are more accurate owing to the protein being more thoroughly exchanged into deuteriated buffer. These facts along with the data in Table 2 show that criterion (*iii*) is satisfied.

Discussion

The self-exchange rate constant for *A. variabilis* plastocyanin (3.2×10^5 M^{-1} s^{-1}) provides encouraging confirmation of the value of 5.9×10^5 M^{-1} s^{-1} obtained from cross-reactions with azurin.[19] Our present results thus confirm the validity in this case of the protein–protein cross-reaction method which depends on a Marcus analysis and the fact that azurin has charge pairing on its surface (work times are minimised).

The self-exchange rate constant for *A. variabilis* plastocyanin is much greater than that for parsley plastocyanin and very probably than those for all other higher-plant plastocyanins. As a consequence of their slow rates of self exchange it has proved impossible to measure the rate constant of any other plastocyanin accurately by the method used in this work. However, an upper limit for the rate of self exchange in French bean plastocyanin[17] of 2×10^4 M^{-1} s^{-1} at 50 °C and a pH of 7.0 and an approximate value for spinach plastocycnin[18] of 4×10^3 M^{-1} s^{-1} at 25 °C and a pH of 6.0 have been quoted. By cross-reactions with azurin a self-exchange rate constant of 3.3×10^3 M^{-1} s^{-1} at 25 °C and a pH of 7.50 (I = 0.100 M) has also been found for parsley plastocyanin.[19] The enhanced rate of self exchange in *A. variabilis* plastocyanin is presumably a consequence of the small overall charge on this protein making self exchange less hindered electrostatically.

The self-exchange rate constant for *A. variabilis* plastocyanin is, in fact, more in line with those of other type I blue copper proteins as can be seen in Table 4. In the case of azurin it has been shown, by site-directed mutagenesis, that the single mutation Met-44 ⟶ Lys which introduces a 1+ charge into the hydrophobic patch of this protein results in a decrease in the self-exchange rate constant.[32] Thus it has been postulated that the mechanism of self exchange in azurin is *via* hydrophobic patches involving a Cu • • • Cu separation of *ca.* 14 Å.

The similarity of the self-exchange rate constants for *A. variabilis* plastocyanin and azurin allied to the absence of any acidic patch in either suggests that self exchange occurs *via* similar mechanisms in each case. Thus the hydrophobic northern surfaces of *A. variabilis* plastocyanin are believed to interact giving a Cu • • • Cu distance, calculated from crystal structure information,[10] closer to 12 Å.

Table 4 Self-exchange rate constants (25 °C, except as indicated) for the reactions between the copper-(I) and -(II) forms of different blue copper proteins

Source	$k/M^{-1}\ s^{-1}$ (pH)	Technique	Ref.
Azurin (*Pseudomonas aeruginosa*)	9.6×10^5 (4.5)[a]	NMR	25
	7.0×10^5 (9.0)		
	2.4×10^6 (5.0)[b]	EPR	30
Azurin (*Alcaligenes denitrificans*)	4.0×10^5 (6.7)	NMR	24
Plastocyanin (*Anabaena variabilis*)	5.9×10^5 (7.5)	Calc.	19
Plastocyanin (*Anabaena variabilis*)	3.2×10^5 (7.5)	NMR	This work
Plastocyanin (parsley)	3.3×10^3 (7.5)	Calc.	19
Amicyanin (*Thiobacillus versutus*)	1.3×10^5 (8.6)[c]	NMR	27
Stellacyanin (*Rhus vernicifera*)	1.2×10^5 (7.0)[d]	EPR	31

[a] I not indicated. [b] ±40%, 22 °C. [c] $I = 0.05$ M. [d] $I = 0.22$ M, 20 °C.

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