Proton Nuclear Magnetic Resonance Assignments and the Electron Self-exchange Rate Constant for Pseudoazurin from *Achromobacter cycloclastes*†

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A partial assignment of proton resonances has been made for *Achromobacter cycloclastes* pseudoazurin. These include the imidazole ring resonances of the three histidine residues and also the $C^{\varepsilon}H_3$ resonances of methionines. Some of these assigned resonances have been used to determine the self-exchange rate constant of pseudoazurin by line-broadening measurements on 1:1 mixtures of oxidised and reduced protein. The self-exchange rate constant has also been determined using a Marcus analysis of the rate constant for the cross-reaction between pseudoazurin and azurin. Good agreement between the values determined by these two methods is found with a self-exchange rate constants of 2.9×10^3 M^{-1} s^{-1} from NMR and 2.7×10^3 M^{-1} s^{-1} from the cross-reaction with azurin, both values being at 25 °C, $I = 0.100$ M. The self-exchange rate constant for pseudoazurin is much smaller than those of most other type 1 blue copper proteins and is quite similar to those found for the higher plant plastocyanins. From the X-ray crystal structure of pseudoazurin it is known that the copper at the active site is co-ordinated by His, Cys, His and Met residues. On the assumption that exchange is *via* His-81 protruding at the surface of the protein it is likely that the neighbouring basic residues impede the approach of the two proteins in a suitable orientation for this to occur.

Pseudoazurin ($M_r \approx 14\,000$) is a single type 1 blue copper protein found in denitrifying bacteria and methylotrophs.[1-8] In denitrifying bacteria its function is to donate the electron necessary for the reduction of NO_2^- to NO by nitrite reductase under anaerobic conditions.[2,3,5] Nitrite reductase can be either a copper-containing protein consisting of both a type 1 and a type 2 copper site, approximately 12.5 Å apart,[9] or a cd_1 type cytochrome.[5,10] In the copper protein, nitrite binding is thought to take place at the type 2 site,[9,11] and the type 1 site is thought to act as the acceptor of the electron from pseudoazurin.

Pseudoazurins from three sources, namely *Alcaligenes faecalis* S-6,[3] *Achromobacter cycloclastes*[1,2] and *Pseudomonas* AM1[4] have been characterised. From their amino-acid sequences[4,12,13] it is known that *A. cycloclastes* pseudoazurin has an extra C-terminal residue giving 124 amino acids in total. The degree of homology is greatest between *A. cycloclastes* and *A. faecalis* S-6 pseudoazurins with 65% conservation of amino acid residues. If the *Pseudomonas* AM1 sequence is included this declines to 42% conservation of amino acid residues.

The crystal structure of oxidised pseudoazurin from *A. faecalis* S-6 has been determined.[14-16] The overall structure of the molecule is that of a β-barrel made up of two β-sheets. The folding pattern of pseudoazurin is very similar to those of azurin and plastocyanin, but there seems to be a greater similarity to the latter. Although there is only approximately 30% homology between their amino-acid sequences, the C^{α} positions of 74 of the residues of pseudoazurin can be aligned with the corresponding residues of poplar plastocyanin[17] with a root mean square deviation of 0.70 Å.[14]

The active site of pseudoazurin has the copper atom co-ordinated by His-40, Cys-78, His-81 and Met-86 in a distorted tetrahedral arrangement. The length of the Cu–Met-86 S^{δ} bond is 2.76 Å which is shorter than the corresponding bonds in plastocyanin from poplar[18] (2.82 Å), *Enteromorpha prolifera*[19] (2.92 Å) and in azurin from *Alcaligenes dentrificans*[20] (3.11 Å). As a result of the closer axial approach the copper atom lies further out of the plane of the three equatorial ligands.

The oxidised form of pseudoazurin has the characteristic spectral properties of a type 1 blue copper protein, with three absorption bands in its visible spectrum at approximately 450, 600 and 750 nm,[1-3,5,21] that at *ca.* 600 nm being weaker and that at *ca.* 450 nm being stronger than the corresponding bands in plastocyanin and azurin. The X-band EPR spectrum of oxidised pseudoazurin from *A. cycloclastes* is rhombic[21] and exhibits the narrow hyperfine coupling constant characteristic of blue copper proteins.

Resonance Raman studies[22] have shown that the two visible absorption bands at *ca.* 450 and 600 nm in pseudoazurin are both due to Cys ⟶ Cu^{II} charge-transfer transitions. The sums of the molar absorptivities (ε) of these two transitions are similar in most blue copper proteins and an increase in the absorbance at the lower wavelength is thought to be due to the metal being further out of the plane of the equatorial ligands and closer to the axial ligand in a geometry which is more tetrahedral. Rhombicity in the EPR spectrum of the type 1 blue copper proteins is also thought to be a characteristic of a copper site in which a closer axial approach lowers the symmetry.[22c,23]

Since little is known about the electron-transfer reactivity of pseudoazurin we have investigated this by determining the self-exchange rate constant of this metalloprotein. Self-exchange rate constants simplify the interpretation of kinetic data because of the absence of any driving force and because of the fact that the structure of only one protein has to be considered. In this paper we study pseudoazurin from *A. cycloclastes* and make ^1H NMR assignments of hydrogens mainly close to the copper site. Some of the assigned resonances are then used in the de-

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

termination of the self-exchange rate constant of pseudoazurin by relaxation rate measurements. Previously ^{1}H NMR spectroscopy has been used to determine the self-exchange rate constant of the blue copper proteins azurin,[24] plastocyanin[25] and amicyanin.[26] In the present work the approach is similar to that used for higher plant plastocyanins[25a,b] and also for the Met121Gln[23a] mutant of *Alcaligenes denitrificans* azurin. The self-exchange rate constant determined in this way is in agreement with that from a Marcus[27] analysis of results from a cross-reaction with azurin. This latter method of determining the self-exchange rate constant has previously[28] provided reliable data for *Anabaena variabilis* and parsley plastocyanins.

Experimental

Isolation and Purification of Pseudoazurin from A. cycloclastes IAM 1013.—*A. cycloclastes* pseudoazurin (PACu) was isolated and purified according to the method of Iwasaki and Matsubara[1] with some modifications. The crude pseudoazurin-containing fraction was purified by first loading onto a CM-Sephadex C-50 column equilibrated in 20 mM phosphate buffer at pH 6.0. The blue band which formed on the top of the column was eluted using 100 mM phosphate buffer at the same pH and concentrated on a Sephadex G-100 gel column. Pseudoazurin was isolated from the gel column eluent by a chromatofocussing method using a pH gradient from 9.0 to 7.0. The protein obtained was applied to a Sephacryl S-200 gel column and final purification was through a DEAE-Sephadex A-50 column equilibrated in 20 mM Tris [tris(hydroxymethyl)aminomethane]–HCl buffer at pH 7.5. Pure pseudoazurin had a UV/VIS absorbance (A) peak ratio A_{277}/A_{593} of 1.4:1. Protein used for NMR and kinetic studies was repurified by loading onto a CM-52 column equilibrated in 5 mM phosphate buffer at pH 6.0. The protein was eluted using 100 mM phosphate buffer again at pH 6.0. Protein purified in this way eluted as a single band on fast protein liquid chromatography (FPLC) (Mono-S cation-exchange column). The concentration of protein samples was determined from the PACu(II) peak at 593 nm (ε = 3700 M^{-1} cm^{-1}).[21]

Isolation of Azurin.—Azurin was obtained from *Pseudomonas aeruginosa* as previously described.[29] Samples of ACu(II) were purified to give a A_{280}/A_{625} ratio of 1.67–1.72:1 by FPLC (Pharmacia) using a Mono-S cation-exchange column.

Visible Absorption Spectra of PACu(II) *and* ACu(II).—The differences between the absorption spectra of the oxidised forms of azurin and pseudoazurin (Fig. 1) are a consequence of structural differences at the active sites of the two proteins as was mentioned in the Introduction. These differences were utilised in the kinetic studies.

Kinetic Studies.—All the reactions were monitored on a Dionex D-110 stopped-flow spectrophotometer. The PACu(I) reductions of ACu(II) were at 25 °C in either Tris buffer (pH 7.1–8.6) or mes [2-(*N*-morpholino)ethanesulfonic acid] buffer (pH 6.2–7.0) all at I = 0.100 M (NaCl). The stopped-flow spectrophotometer was interfaced to an IBM computer PC/AT-X for data acquisitions using software from On Line Instruments Systems (Bogart, GA, USA). All the rate constants are an average of five determinations using the same solutions. Reactions were monitored at 640 nm where the decrease in absorbance shows the reduction of ACu(II), and at 452 nm where the concurrent oxidation of pseudoazurin could be followed.

NMR Sample Preparation.—For the acquisition of proton NMR spectra the protein was usually exchanged into 99.9% deuteriated 37.3 mM phosphate buffer at pH 7.50 (I = 0.100 M) by ultrafiltration using a small Amicon set up. Experiments were also carried out in 99.9% deuteriated 100 mM phosphate buffer pH 7.50 (I = 0.268 M) and 99.9% deuteriated 1 mM phosphate buffer pH 7.50 (I = 0.003 M). Protein samples were reduced by the addition of cooled aliquots of 0.1 M $Na_2S_2O_4$ in 99.9% D_2O (0.1 M NaOD). Any excess reductant was exchanged out of the samples which were to be used for the self-exchange rate determinations. Pseudoazurin was oxidised using a 0.1 M solution of $K_3[Fe(CN)_6]$ in 99.9% D_2O. Any excess oxidant was exchanged out of the protein using the appropriate buffer. Protein solutions of the correct concentration were transferred to an NMR tube which was flushed with argon and sealed. The pH of the NMR samples was measured using a narrow CMAWL/3.7/180 pH probe in combination with a Radiometer PHM62 pH meter which was calibrated using aqueous buffers. The pH of the samples was adjusted using NaOD or DCl (0.1 M) as necessary and no correction was made for the deuterium isotope effect.

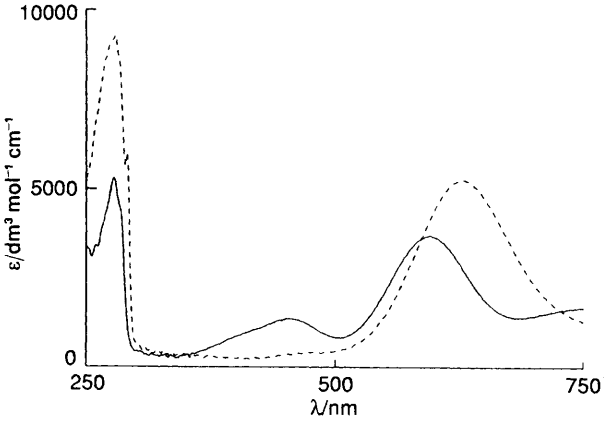

Fig. 1 UV/VIS absorption spectra of azurin (- - - -) and pseudoazurin (——)

NMR Spectra.—All proton NMR spectra were acquired at 500.14 MHz on a Bruker AMX500 spectrometer, mostly at 25 °C using samples in 5 mm o.d. borosilicate glass tubes. For one-dimensional spectra 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 3.74.

One-dimensional spectra were also acquired using Hahn Spin-Echo (HSE) [$90°_x$–t–$180°_y$–t–] (t = 60 ms) and Carr–Purcell–Meiboom–Gill (CPMG) [$90°_x$–t–$(180°_y$–$2t)_n$–$180°_y$–t] (n = 59, t = 1 ms) pulse sequences. Combinations of spectra from these two pulse sequences were used to identify singlets, doublets and triplets in the aromatic region and to identify singlets in the aliphatic region of one-dimensional proton spectra.[30] The HSE pulse sequence was also used to determine T_2 (spin–spin) relaxation times of certain resonances. The intensity (I_t) of a resonance obtained with a total delay of $2t$ is given in equation (1) where I_0 is the intensity for t = 0. Plots of

$$I_t = I_0 \exp(-2t/T_2) \quad (1)$$

ln (I_t) against $2t$ gave straight lines of slope $-T_2^{-1}$.

A delay-correlation (COSY)[31] two-dimensional spectrum was also obtained to reveal the long-range couplings (*ca.* 2 Hz) between the C^δ and C^ε protons of the imidazole ring of histidine residues. The experiment uses a conventional COSY pulse sequence but includes two extra delays, each of length τ = 40 ms: (T_W–$90°$–τ–t_1–$90°$–τ–t_2–)$_n$.

A nuclear Overhauser (NOESY) spectrum was also obtained with a mixing time of 300 ms. In these experiments 128 free induction decays were acquired into 2 K data points for 512 values of t_1. Spectra were transformed into a 1 K × 1 K data matrix using resolution-enhancing weighting functions in both dimensions.

Results

Assignment of Proton Resonances.—In general, sharp singlets in the aromatic region of ^{1}H NMR spectra of proteins arise from either the imidazole ring protons of histidines or the $C^{\delta}H$ of tryptophan residues. In spectra obtained with the HSE pulse sequence peaks can be identified on the basis of their multiplicity. With the total delay period ($2t$) equal to $1/J$ doublets are inverted and singlets and triplets are of normal phase. The coupling constants for the doublets and triplets of the aromatic spin systems of tyrosine and phenylalanine residues are *ca.* 8–9 Hz and so this is achieved with $t = 60$ ms which also allows for selection on the basis of T_2 relaxation rates. With $t = 30$ ms (*i.e.* $2t = 1/2J$) doublets are partially nulled, singlets and the central components of triplets have normal phase, and the outer components of triplets are inverted. Spectra obtained with the CPMG pulse sequence have all resonances of normal phase irrespective of n for short values of t, and in those with a total delay $[2(n + 1)t]$ of 120 ms the resonances of protons with short values of T_2 are removed or significantly attenuated. Such resonances will include NH signals but not those from histidine or tryptophan ring protons.

It is known from its crystal structure that *A. faecalis* pseudoazurin contains three histidines, two of which (His-40 and His-81) co-ordinate to the copper at the active site and the third at position 6 is free. Because of the high sequence homology between *A. faecalis* and *A. cycloclastes* pseudoazurins, in particular the conservation of the positions of the histidines, the same can also be assumed for the latter protein. Since pseudoazurin does not contain any tryptophan residues it follows that any sharp singlets in the aromatic region of the spectrum must arise from histidines (the coupling between the C^{δ} and the C^{ε} protons of a histidine residue is too small to be resolved). In Fig. 2(*a*) the aromatic region of the ^{1}H NMR spectrum of reduced pseudoazurin is shown. Its complexity is reduced in Fig. 2(*b*) obtained using the CPMG sequence with a total delay of 120 ms to attenuate broader resonances. Fig. 2(*c*) obtained using the HSE pulse sequence ($t = 60$ ms) has singlets and triplets of normal phase and doublets inverted and Fig. 2(*d*) [the sum of Fig. 2(*b*) and 2(*c*)] thus has doublets eliminated. The triplets in Fig. 2(*d*) can be distinguished from the singlets by their greater widths and by comparison with Fig. 2(*e*) obtained using the HSE pulse sequence ($t = 30$ ms) in which their outer components are inverted. Furthermore, by using resolution enhancing weighting functions prior to transformation of the free induction decay the fine structure of the triplets in Fig. 2(*b*) could be partially resolved. These considerations lead to the identification of six singlets of δ 7.92, 7.54, 7.20, 7.04, 7.01 and 6.84 which must all be histidine resonances.

For the oxidised protein the same approach leads to the identification of two histidine resonances (δ 7.70 and 7.01) in the aromatic region. These cannot arise from either of the co-ordinated histidines (His-40 and His-81) as these protons are well within 10 Å of the paramagnetic Cu^{II} and their resonances will therefore be broadened beyond detection.[24c,32] These two signals therefore arise from His-6. In the reduced protein the same resonances can be identified by the pH dependence of their chemical shifts and occur at δ 7.92 and 7.04 at pH 7.50, the former being assigned to the $C^{\varepsilon}H$ owing to the greater sensitivity of its chemical shift to pH.[33] The fact that these protons have different chemical shifts in the two oxidation states at the same pH indicates that the pK_a of His-6 is different in the two oxidation states of the protein. The remaining four histidine resonances in the reduced protein are assigned to the two ligand histidines.

These assignments were confirmed by means of a delay-COSY experiment which also revealed the coupling patterns. In Fig. 3 part of the aromatic region of the delay-COSY spectrum of reduced pseudoazurin is shown. The relevant correlations are δ 7.92/7.04 (His-6), 7.54/6.84 (co-ordinated His A) and 7.21/7.01 (co-ordinated His B).

In the aromatic region of the NOESY spectrum of reduced pseudoazurin the only significant NOE correlation between histidine resonances is between those at δ 7.54 and 7.20. Consideration of the distances in the crystal structure of *A. faecalis* pseudoazurin[15] shows that this NOE could only arise between the $C^{\varepsilon}H$ protons of the two co-ordinated histidines. Hence the C^{δ} and C^{ε} protons of His A give rise to resonances at δ 6.84 and 7.54 respectively whilst the corresponding protons of His B occur at δ 7.01 and 7.20 respectively.

Methionine $C^{\varepsilon}H_3$ resonances can be identified as singlets in the aliphatic region of the sum of spectra obtained with the HSE and CPMG pulse sequences. In the spectrum of the reduced protein four such resonances can easily be identified at δ 2.18, 2.07, 1.80 and 1.67 which correspond to the $C^{\varepsilon}H_3$ resonances of four of the five methionine residues in *A. cycloclastes* pseudoazurin. In the oxidised protein only one such resonance is clearly identifiable (δ 2.09). In the crystal structure of pseudoazurin[15] the distances from the Cu^{II} of the C^{ε} of the four methionine residues Met-7, Met-16, Met-84 and Met-86 are all less than 10 Å (7.35, 6.28, 8.16 and 3.52 Å respectively). How-

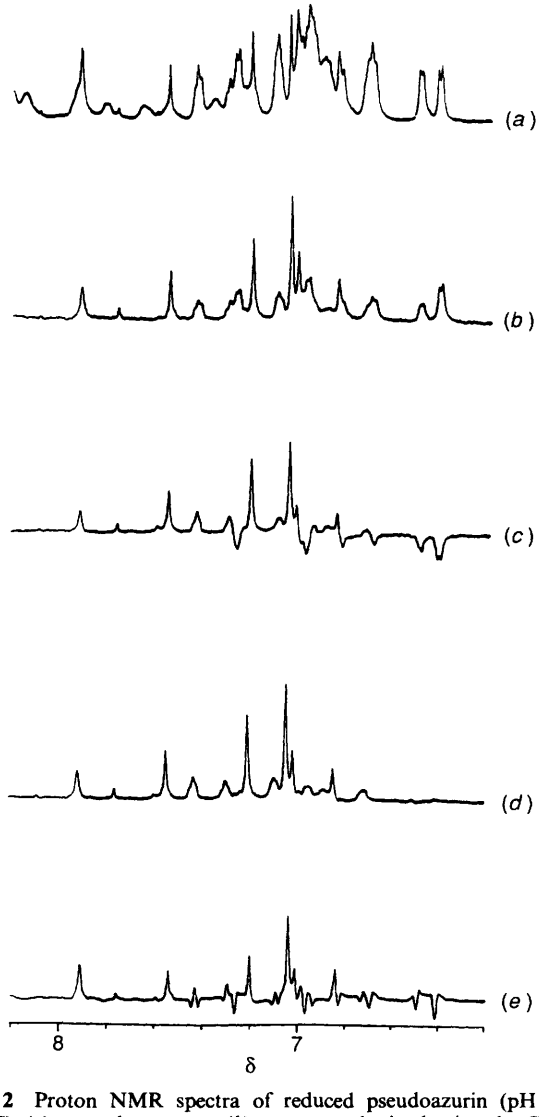

Fig. 2 Proton NMR spectra of reduced pseudoazurin (pH 7.50, 25 °C); (*a*) normal spectrum, (*b*) spectrum obtained using the CPMG pulse sequence ($t = 1$ ms, $n = 59$) in which the broader components are attenuated, (*c*) spectrum obtained using the HSE pulse sequence ($t = 60$ ms) in which the broader components are attenuated and doublets are inverted, (*d*) sum of (*b*) and (*c*), (*e*) spectrum obtained using the HSE pulse sequence ($t = 30$ ms) in which the broader resonances and doublets are attenuated and the outer components of triplets are inverted

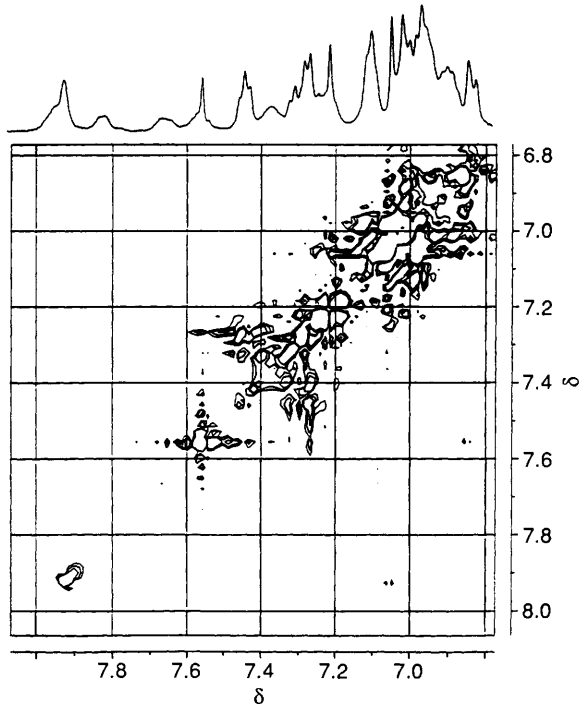

Fig. 3 Part of the aromatic region of the delay–COSY ^{1}H NMR spectrum of reduced pseudoazurin (pH 7.50, 25 °C)

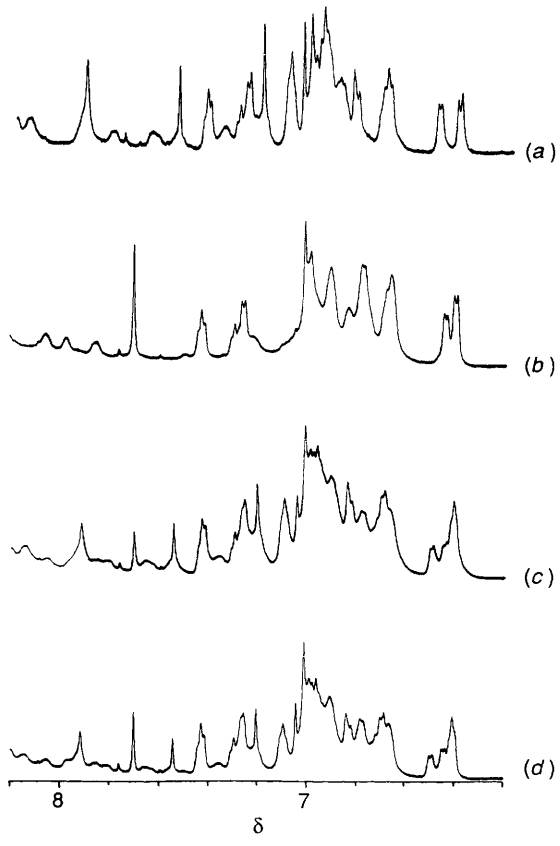

Fig. 4 Proton NMR spectra of pseudoazurin (pH 7.50, 25 °C); (*a*) reduced protein, (*b*) oxidised protein, (*c*) 1 : 1 mixture of oxidised and reduced protein, (*d*) sum of spectra (*a*) and (*b*)

ever the C^{ε} of Met-48 is 16.9 Å from the active site. Therefore the $C^{\varepsilon}H_3$ resonance present in the oxidised protein is assigned to Met-48.

Determination of the Electron Self-exchange Rate Constant of Pseudoazurin by NMR Spectroscopy.—At a pH of 7.50 the His-6 $C^{\varepsilon}H$ gives a sharp signal at δ 7.92 in the reduced protein and a sharp signal at δ 7.70 in the oxidised protein [Fig. 4(*a*), 4(*b*)]. In a 1 : 1 mixture of the two forms at a concentration of 0.5 mM both peaks are present in the spectrum [Fig. 4(*c*)] thus showing the rate of self exchange at this concentration to be relatively slow on an NMR time-scale. This conclusion is confirmed by the observation that in mixtures of the two forms there is very little broadening of resonances from the reduced form that have no observable counterparts in the spectrum of the oxidised protein. A careful comparison of the spectrum of the 1 : 1 mixture [Fig. 4(*c*)] and the sum of the spectra of the oxidised and reduced forms [Fig. 4(*d*)] shows a close similarity, but with a small amount of broadening of several resonances in the former. This broadening is due to self exchange [PACu(I) + PACu(II) $\rightleftharpoons$ PACu(II) + PACu(I)] for which equation (2) is

$$\text{Rate} = k_{ex}[\text{PACu(I)}][\text{PACu(II)}] \quad (2)$$

applicable, where k_{ex} is the self-exchange rate constant and [PACu(I)] and [PACu(II)] are the concentrations of reduced and oxidised pseudoazurin respectively. Since [PACu(I)] is equal to [PACu(II)] equation (3) follows. The broadening

$$\text{Rate} = k_{obs}[\text{PACu(I)}] \quad (3)$$

produced by self exchange ($\pi\Delta\nu_{\frac{1}{2}}$) is equal to the reciprocal of the exchange lifetime of the resonances (τ^{-1}) which in turn is equal to k_{obs}. Using equation (4), k_{ex} can be determined from

$$k_{ex} = k_{obs}/[\text{PACu(II)}] \quad (4)$$

the broadening of certain resonances in the 1 : 1 mixture of the two proteins.

Since the rate of self exchange is so slow it is difficult to measure the increased linewidths accurately and an alternative way to determine the exchange-induced broadening of resonances is to compare the T_2 values from spin-echo experiments on fully oxidised or fully reduced protein with those for the corresponding resonances in the 1 : 1 mixture. The results obtained by measuring peak widths are in Table 1 whilst those from the spin-echo pulse sequence are shown in Table 2. Plots of ln (I_t) against $2t$ for the $C^{\varepsilon}H_3$ methionine resonance at δ 2.18 in the fully reduced protein and in the 1 : 1 mixture of oxidised and reduced proteins are shown in Fig. 5. This method of determining the self-exchange rate results in errors of *ca.* ± 20% compared with significantly larger errors when direct measurements of linewidths are used.

In order to confirm that the observed line-broadenings did indeed arise from an exchange process spectra were obtained of a 1 : 1 mixture of oxidised and reduced pseudoazurin at temperatures between 25 and 50 °C (Fig. 6). These spectra show the increase in broadening at higher temperatures resulting from faster exchange. Effects arising from altered molecular tumbling rates would lead to sharper lines at higher temperatures. The self-exchange rate constant was determined from the spectra at 50 °C and these data are shown in Table 3.

The self-exchange rate constant of pseudoazurin was also determined in 100 mM phosphate buffer (I = 0.268 M) at pH 7.50 and was found to be the same as under I = 0.100 M conditions. In 1 mM phosphate buffer at pH 7.50 (I = 0.003 M) the rate of self exchange increased. Data calculated under these conditions are in Tables 4 and 5 respectively.

Determination of the Self-exchange Rate Constant of Pseudoazurin from the Cross-reaction with Azurin.—In Fig. 1 the UV/VIS spectra of oxidised pseudoazurin and azurin are shown. The most intense visible absorption band in azurin is at a higher wavelength than in pseudoazurin and plastocyanin. This has been attributed[20] to the existence of two hydrogen

Table 1 Self-exchange rate constants (25 °C, I = 0.100 M) for *A. cycloclastes* pseudoazurin determined from linewidth measurements

	[PACu(I)] = [PACu(II)]		
	6.1 × 10^{-4} M	5.2 × 10^{-4} M	3.3 × 10^{-4} M
Resonance	$10^{-3}k$/M^{-1} s^{-1}	$10^{-3}k$/M^{-1} s^{-1}	$10^{-3}k$/M^{-1} s^{-1}
His-6 $C^{\varepsilon}H$	3.4	4.1	2.1
His-6 $C^{\varepsilon}H$ *	3.0	4.1	3.3
His A $C^{\varepsilon}H$	3.3	3.8	3.1
His B $C^{\varepsilon}H$	3.5	3.7	2.7
Met $C^{\varepsilon}H_3$ (δ 2.18)	5.5	5.2	4.4

* His-6 $C^{\varepsilon}H$ resonance in the oxidised protein.

Table 2 Self-exchange rate constants for *A. cycloclastes* pseudoazurin (25 °C, I = 0.100 M) calculated from T_2 determinations using a spin-echo pulse sequence; [PACu(I)] = [PACu(II)] = 5.2 × 10^{-4} M

Resonance	$10^{-3}k$/M^{-1} s^{-1}
His-6 $C^{\varepsilon}H$	3.5
His-6 $C^{\varepsilon}H$ *	2.8
His A $C^{\varepsilon}H$	2.5
His B $C^{\varepsilon}H$	2.6
Met $C^{\varepsilon}H_3$ (δ 2.18)	3.3

* His-6 $C^{\varepsilon}H$ resonance in the oxidised protein.

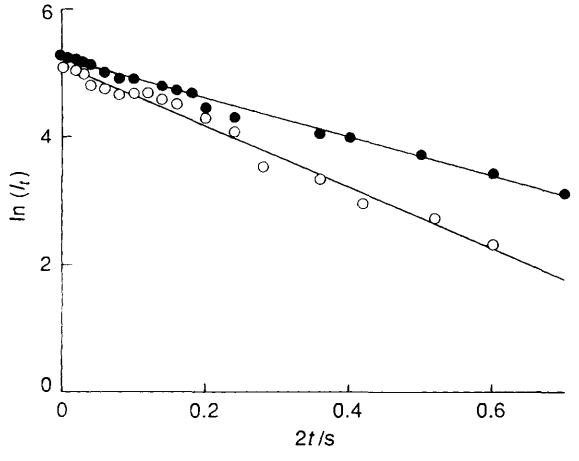

Fig. 5 Plots of ln (I_t) against 2t for the Met $C^{\varepsilon}H_3$ of pseudoazurin at δ 2.18 (from HSE pulse sequence); (●) PACu(I), (○) 1:1 mixture of oxidised and reduced protein

bonds to the sulfur of the co-ordinating cysteine ligand compared with only one in pseudoazurin[16] and plastocyanin.[17] Rate constants, k_{12}, for the PACu(I) reduction of ACu(II), equation (5), were determined from the absorbance changes at

$$\text{PACu(I)} + \text{ACu(II)} \longrightarrow \text{PACu(II)} + \text{ACu(I)} \quad (5)$$

640 and 452 nm with PACu(I) present in large (> 10 fold) excess, Table 6. The data were analysed within the framework of the Marcus equation[27] [(6) and (7)], where k_{11} and k_{22} are

$$k_{12}^2 = k_{11}k_{22}K_{12}f \quad (6)$$

$$\log f = \frac{(\log K_{12})^2}{4\log(k_{11}k_{22}/Z^2)} \quad (7)$$

the self-exchange rate constants for azurin and pseudoazurin respectively, k_{12} is the rate constant for the cross-reaction which has an equilibrium constant K_{12} and Z is the collision frequency (≈10^{11} M^{-1} s^{-1}). As $\Delta E^{\circ} \longrightarrow 0$ so $f \longrightarrow 1$; in these studies f was found to be > 0.95 and was consequently ignored.

For accurate application of the Marcus equation it is important to know the driving force of the reaction involved. The variation in E° with pH for *P. aeruginosa* azurin is well

Table 3 Self-exchange rate constants for *A. cycloclastes* pseudoazurin (25 °C, I = 0.268 M); [PACu(I)] = [PACu(II)] = 1.8 × 10^{-4} M

Resonance	$10^{-3}k$/M^{-1} s^{-1}
His-6 $C^{\varepsilon}H$	4.5
His-6 $C^{\varepsilon}H$ *	3.1
His A $C^{\varepsilon}H$	3.0
His B $C^{\varepsilon}H$	3.0
Met $C^{\varepsilon}H_3$ (δ 2.18)	6.3

* His-6 $C^{\varepsilon}H$ resonance in the oxidised protein.

Table 4 Self-exchange rate constants for *A. cycloclastes* pseudoazurin (25 °C, I = 0.003 M); [PACu(I)] = [PACu(II)] = 6.3 × 10^{-4} M

Resonance	$10^{-4}k$/M^{-1} s^{-1}
His A $C^{\varepsilon}H$	3.7
His B $C^{\varepsilon}H$	3.3
Met $C^{\varepsilon}H_3$ (δ 2.18)	2.8

Table 5 Self-exchange rate constants for *A. cycloclastes* pseudoazurin (50 °C, I = 0.100 M); [PACu(I)] = [PACu(II)] = 6.4 × 10^{-4} M

Resonance	$10^{-4}k$/M^{-1} s^{-1}
His-6 $C^{\varepsilon}H$ *	1.6
His A $C^{\varepsilon}H$	1.6
His B $C^{\varepsilon}H$	2.8
Met $C^{\varepsilon}H_3$ (δ 2.18)	2.0

* His-6 $C^{\varepsilon}H$ resonance in the oxidised protein.

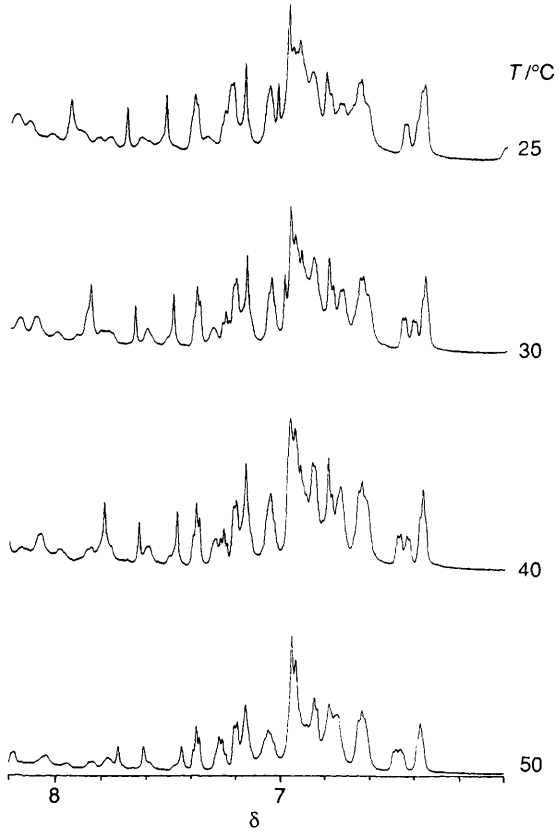

Fig. 6 Proton NMR spectra of reduced pseudoazurin (pH 7.50) at different temperatures

Table 6 Rate constants (25 °C), $I = 0.100$ M (NaCl) for the PACu(I) (reactant in > 10 fold excess) reduction of ACu(II) ($\approx 7 \times 10^{-6}$ M)

pH	$10^{-5}k/M^{-1}\ s^{-1}$ (640 nm)	$10^{-5}k/M^{-1}\ s^{-1}$ (452 nm)
6.25	1.16	1.05
6.60	1.03	0.97
7.14	1.11	0.96
7.57	1.21	1.00
8.16	1.29	1.02
8.58	1.36	1.21

characterised.[29b,34] At pH 7.5 a value of 305 mV [*vs.* normal hydrogen electrode (NHE)] was used. The reduction potential of *A. cycloclastes* pseudoazurin has been shown to be pH-dependent in the range 5–11 and to have a value of 280 mV (*vs.* NHE) at pH 6.[35] More recent studies have also indicated a pH dependence of the reduction potential of pseudoazurin with a value of 259 mV (*vs.* NHE) at pH 7.0[36] and a value of 254 mV at pH 7.5.

In the present work variations in pH had no apparent effect on k_{12}. At pH 7.57, k_{12} is 1.21×10^5 M^{-1} s^{-1} (640 nm) and 1.00×10^5 M^{-1} s^{-1} (452 nm). The self-exchange rate constant for *Pseudomonas aeruginosa* azurin has been shown[24d] to be 9.6×10^5 M^{-1} s^{-1} at pH 4.5 and 7.0×10^5 M^{-1} s^{-1} at pH 9.0 by an NMR line-broadening method and a value of 7.5×10^5 M^{-1} s^{-1} has been used[28] at pH 7.5. Use of the Marcus equation gives k_{22} values of 2.7×10^3 M^{-1} s^{-1} (640 nm) and 1.8×10^3 M^{-1} s^{-1} (452 nm) the former being more accurate due to the larger monitorable absorption change at 640 nm.

Discussion

The agreement between the NMR determination of the self-exchange rate constant (2.9×10^3 M^{-1} s^{-1} from the average of the values in Table 2) and that determined indirectly (2.7×10^3 M^{-1} s^{-1}) provides encouraging confirmation of the use of a Marcus analysis of the cross-reaction with azurin to determine the self-exchange rate constant of a redox protein although its precision is perhaps fortuitous. The electron self-exchange rate constant for pseudoazurin is much smaller than those for most other type 1 blue copper proteins (typically $\approx 10^5$ M^{-1} s^{-1}). In fact the value found is very similar to the self-exchange rate constants of higher plant plastocyanins, of which the self-exchange characteristics have been attributed to the high negative charge on these molecules at neutral pH. This high charge is thought to hinder electrostatically the association of two plastocyanin molecules. In the case of the cross-reaction with azurin such effects should be relatively unimportant owing to the low overall charge and the absence of regions of high charge density on azurin.

Pseudoazurin from *A. cycloclastes* is a basic protein (isoelectric point 8.4)[37] with an estimated charge of +1 for the reduced form at pH 7.0. It might therefore be expected that pseudoazurin would have a relatively large self-exchange rate constant, in contrast to experimental observation. This can be understood by more detailed consideration of the mechanism of the self-exchange process. The crucial factor is that for efficient self exchange an oxidised and reduced pseudoazurin molecule must not merely be able to associate, they must associate in the correct relative orientations. An unsymmetrical distribution of charge on the surface will seriously affect the relative stabilities of different modes of association even though the overall charge is close to zero.

It has been shown that in azurin the hydrophobic patch adjacent to the exposed histidine ligand (117) is the route for electron self exchange.[38] The hydrophobic patch of plastocyanin has also been postulated as the site of association prior to self exchange.[25c,28] If self exchange occurs in pseudoazurin by a similar mechanism (*i.e. via* the exposed His-81 ligand) then the presence of the conserved basic residues on the surface of pseudoazurin in this area (Lys-38, -46, -57 and -77) could hinder modes of association favourable to exchange. From the crystal structure of *A. faecalis* pseudoazurin[15] the basic residue Lys-38 is seen to be adjacent to the protruding histidine copper ligand (His-81 is the more prominent, but His-40 is also visible). It is interesting that the inclusion of a single basic residue in the hydrophobic patch of azurin causes a decrease in the self-exchange rate constant by two orders of magnitude.[38]

An alternative explanation of the small self-exchange rate constant for pseudoazurin could be that the pathway for self-exchange is different or that the reorganisation energy of this protein is relatively large. However, a small self-exchange rate constant should not be interpreted as an indication that the protein itself is inherently less effective in electron transfer. The electrostatic effects which hinder self exchange may in other circumstances facilitate association with a physiological redox partner and also create a degree of specificity. Thus the rate constant for the reaction of *A. cycloclastes* pseudoazurin with nitrite reductase (*A. cycloclastes* nitrite reductase is an acidic[36] protein) of 7.3×10^5 M^{-1} s^{-1} determined electrochemically[37] is unexceptional.

The effect of ionic strength on the self-exchange rate constant also indicates that self exchange is electrostatically hindered. In spinach plastocyanin[25b] the rate constant for self-exchange is relatively low ($\approx 4 \times 10^3$ M^{-1} s^{-1}) and increases as the ionic strength increases which has been interpreted as being due to the overall neutralisation of repulsive forces. In *Thiobacillus versutus* amicyanin[26b] and *P. aeruginosa* azurin[24b] the self-exchange rate constants are relatively high $(1–10) \times 10^5$ M^{-1} s^{-1} and are independent of ionic strength. The present work shows that pseudoazurin displays a third kind of behaviour namely a relatively low self-exchange rate constant which is independent of ionic strength at $I > 0.100$ M. We believe that this confirms that whilst there is no overall repulsive force between pseudoazurin molecules the sites of association are themselves incompatible.

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