Determination of Self-Exchange Rate Constants for Rusticyanin from *Thiobacillus ferrooxidans* and a Comparison with Values for Other Type 1 Blue Copper Proteins

Panayotis Kyritsis,[†] Christopher Dennison,[†] W. John Ingledew,[‡] William McFarlane,*[,†] and A. Geoffrey Sykes*[,†]

Department of Chemistry, The University of Newcastle, Newcastle upon Tyne NE1 7RU, U.K., and Department of Biochemistry, The University of St. Andrews, St. Andrews KY16 7AL, U.K.

Received May 4, 1995[⊗]

Electron self-exchange rate constants, k_{ese} (25 °C), for the type 1 Cu protein rusticyanin from *Thiobacillus ferrooxidans* have been determined by ^{1}H NMR methods at pH 5.7 (1.0×10^4 M^{-1} s^{-1}) and pH 2.0 (1.7×10^4 M^{-1} s^{-1}), $I = 0.100$ M. The protein is unusual in that it is stable at pH 2.0, which is the physiological pH. The rate constants are compared to k_{ese} values obtained for five other type 1 Cu proteins, which lie in the range 10^3-10^6 M^{-1} s^{-1}. These rate constants are appraised collectively for the first time, when it is concluded that in all cases self-exchange occurs *via* the surface-exposed coordinated imidazole of the hydrophobic patch region adjacent to the Cu, with the spread in values accounted for by overall and localized charge on the protein molecule. In the case of rusticyanin the overall charge balance from the amino acid composition is +4 for RCu^I at pH 7 but at pH 2 may be as high as +17. The similarity of rate constants at the two pH's suggests that charge is localized on the more remote regions of the molecule away from the active site. From recent ^{1}H and ^{15}N NMR assignments the global fold of the protein has been determined, and a predominance of hydrophobic amino acids in the loops closest to the active site has been indicated. The hydrophobicity of the active site and shielding of the imidazole ring of His-143 may result in a longer distance for electron transfer and account for the mid-range k_{ese} values obtained. We also draw attention to the fact that from simple electrostatics the hydrophobic active site favors the lower charged Cu^I to the Cu^{II}, and that this may be a major contributing factor to the high value of the Cu^{II}/Cu^I reduction potential (680 mV).

Introduction

Rusticyanin is a single type 1 copper protein which is a component of the acidophilic bacterium *Thiobacillus ferrooxidans* (*T. ferrooxidans*).[1] The organism transports electrons from, e.g., insoluble iron(II) sulfide ores to molecular oxygen *via* an electron transport chain which includes a number of cytochromes.[2] In acidic media the iron(III) and sulfate so formed are both soluble. The solubilization process is used commercially in the extraction of copper and uranium from their sulfide ores.[2] Rusticyanin functions as a one electron-transfer component in the respiratory chain of the bacterium.[3] The protein has an isoelectric point of 9.1 and is unusual in being stable at pH 2, which is the pH for optimum growth of *T. ferrooxidans*.[4] Other type 1 (blue) copper proteins are not stable at pH < 4.0.[5] Another significant difference in the case of rusticyanin is the high reduction potential (680 mV *vs* nhe), whereas other members of this class of proteins have values in the range 180−420 mV.[5] Like other type 1 proteins in the Cu^{II} state, rusticyanin exhibits a characteristically strong absorbance band at 597 nm (ϵ = 2240 M^{-1} cm^{-1}),[6] and gives narrow hyperfine structure in its EPR spectrum.[7] Alignment of the 155 residue amino acid sequence (M_r 16 300) with other type 1 copper proteins indicates quite low homology, except in the C-terminus section containing three of the ligands to the Cu.[8] There is at present no X-ray crystal structure for rusticyanin, although crystals have been obtained which diffract to 2.0 Å resolution.[9] Structural information is however available from a detailed NMR investigation, and most of the ^{1}H and ^{15}N resonances of the reduced protein have been assigned.[10] The side chains of Cys-138, His-143, and Met-148 at the C-terminus end of the sequence coordinate the Cu, and the fourth ligand is most likely His-85. Consistent with this assignment the His85Ala mutant has been prepared,[10] when significant changes in the UV−vis spectrum are observed. The NMR studies have also demonstrated that the copper active site is surrounded by aromatic residues, resulting in a highly hydrophobic region which protects the Cu and enables it to remain functional at pH 2. Whereas in the Cu^I state plastocyanin, pseudoazurin, and amicyanin undergo reversible protonation of the C-terminus active site histidine, pK_a's in the range 4.8−6.9,[5,11] rusticyanin shows no similar tendency even at pH 2.

[†] The University of Newcastle upon Tyne.
[‡] The University of St. Andrews.
[⊗] Abstract published in *Advance ACS Abstracts,* September 1, 1995.

(1) (a) Blake, R. C., II; Shute, E. A.; Greenwood, M. M.; Spencer, G. H.; Ingledew, W. J. *FEMS Microbiol. Rev.* **1993**, *11*, 9. (b) Ingledew, W. J.; Boxer, D. H., *Methods Enzymol.* **1994**, *243*, 387.
(2) (a) Ingledew, W. J. *Biochim. Biophys. Acta.* **1982**, *683*, 89. (b) Blake, R. C., II; Shute, E. A.; Howard, G. T. *Appl. Environ. Microbiol.* **1994**, *60*, 3349. (c) Leduc, L. G.; Ferroni, G. D. *FEMS Microbiol. Rev.* **1994**, *14*, 103.
(3) Blake, R. C., II; Shute, E. A. *Biochemistry* **1994**, *31*, 9221.
(4) Blake, R. C., II; Shute, E. A. J. *Biol. Chem.* **1987**, *262*, 14983.
(5) Sykes, A. G. *Struct. Bond.* **1990**, *75*, 175.
(6) Cox, J. C.; Boxer, D. H. *Biochem. J.* **1978**, *174*, 497.
(7) Cox, J. C.; Aasa, R.; Malmström, B. G. *FEBS Lett.* **1978**, *93*, 157.
(8) (a) Ronk, M.; Shively, J. E.; Shute, E. A.; Blake, R. C., II. *Biochemistry* **1991**, *30*, 9435. (b) Yano, T.; Fukumori, Y.; Yamanaka, T. *FEBS Lett.* **1991**, *288*, 159. (c) Nunzi, F.; Woudstra, M.; Campese, D.; Bonicel, T.; Morin, D.; Bruschi, M. *Biochem. Biophys. Acta* **1993**, *1162*, 28.
(9) Djebli, A.; Proctor, P.; Blake, R. C., II; Shoham, M. J. *Mol. Biol.* **1992**, *227*, 581.
(10) (a) Hunt, A. H.; Toy-Palmer, A.; Assa-Munt, N.; Cavanagh, J.; Blake, R. C., II; Dyson, H. J. *J. Mol. Biol.* **1994**, *244*, 370. (b) Toy-Palmer, A.; Prytulla, S.; Dyson, H. J. *FEBS Lett.*, in press. Dyson, J. Personal communication.
(11) (a) Dennison, C.; Kohzuma, T.; McFarlane, W.; Suzuki, S.; Sykes, A. G. *J. Chem. Soc., Chem. Commun.* **1994**, 581. (b) Dennison, C.; Kohzuma, T.; McFarlane, W.; Suzuki, S.; Sykes, A. G. *Inorg. Chem.* **1994**, *33*, 3299. (c) Kyritsis, P.; Dennison, C.; Kalverda, A. P.; Canters, G. W.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1994**, 3017.

0020-1669/95/1334-5370$09.00/0 © 1995 American Chemical Society

It was of interest to see what effect these different properties of rusticyanin might have on the electron self-exchange (ese) rate constant (k_{ese}), here determined by NMR techniques. Such rate constants are a measure of the inherent electron-transfer reactivity of the protein molecule in a process which has no thermodynamic driving force.[12] Electron self-exchange rate constants have been obtained for five other type 1 copper proteins,[13] and a comparison of these values, including consideration of the k_{ese} reactivity of rusticyanin, is the subject of the present paper. Earlier studies concerning the redox reactivity of rusticyanin with inorganic complexes have been reported.[14]

Experimental Section

Isolation and Purification of Rusticyanin from *T. ferrooxidans*. Relevant procedures have been described in previous work.[1b,14b] Rusticyanin was purified by fast protein liquid chromatography (FPLC) using a Mono-S (HR 5/5) cation-exchange column (Pharmacia) and 20 mM acetate buffer at pH 4.5. Elution of the protein was achieved using an ionic strength gradient created by 1 M NaCl. Protein fractions used for the preparation of NMR samples had absorbance ratios A_{278}/A_{597} between 5.5 and 6.5 and A_{450}/A_{410} (which is sensitive to cytochrome impurities) > 1.5.

Preparation of NMR Samples. For the acquisition of ^{1}H NMR spectra rusticyanin was exchanged by Amicon ultrafiltration into 99.9% deuterated 85.5 mM phosphate buffer at pH 5.7, which gives $I = 0.100$ M. Studies were also carried out at pH 3.4 and pH 2.0 in 1 mM H_2SO_4/D_2O titrated with 1 M NaOD and at ionic strength 0.100 M adjusted with NaCl. Protein samples were reduced by the addition of cooled aliquots of 0.1 M sodium dithionite, $Na_2S_2O_4$ (Fluka), in 99.9% D_2O. Any excess reductant was exchanged out of the samples which were to be used for NMR studies. Rusticyanin was oxidized by addition of small amounts of $K_2[IrCl_6]\cdot 6H_2O$ (Johnson Matthey). Again, excess oxidant was exchanged out by Amicon ultrafiltration using appropriate buffer solutions.

Half-oxidized mixtures of rusticyanin were prepared in the NMR tube by adding oxidized protein to reduced protein (both at approximately the same concentration). Total protein concentrations used were in the range 0.27−1.17mM. The concentration of oxidized protein was determined by transferring the protein solution to a 2-mm-path length UV/vis spectrophotometer cell and monitoring the absorbance at 597 nm. The pH of the NMR samples was measured using a Radiometer PHM62 pH meter in combination with a narrow CMAWL/3.7/180 pH electrode. Values of pH were uncorrected for the deuterium isotope effect.

NMR Spectra. All proton NMR spectra were acquired at 500.14 MHz on a Bruker AMX500 spectrometer using samples in 5-mm-od borosilicate glass tubes. 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 in parts per million (ppm), relative to the internal standard dioxane at δ 3.74 ppm.

Results

NMR spectra of 1:1 mixtures of oxidized and reduced forms of rusticyanin show close similarities to the sum of the individual spectra from the two forms. A significant difference is the small amount of extra broadening of the resonance at 2.14 ppm which is observed for the mixtures, Figure 1. This peak has been assigned as a methionine C$^\epsilon$H peak on the basis of the following evidence. Using Hahn spin-echo (HSE) [$90°_x-t-180°_y-t-$] (t = 60 ms)[15] and Carr−Purcell−Meiboom−Gill (CPMG)

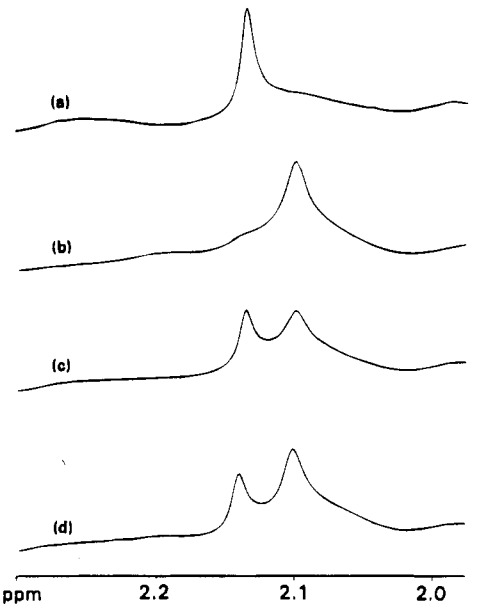

Figure 1. Showing the C$^\epsilon$H methionine resonance (δ 2.14 ppm) for (a) reduced rusticyanin, (b) oxidized rusticyanin, (c) sum of a and b, and (d) 1:1 mixture of reduced and oxidized rusticyanin, [RCu^{II}] = 4.9×10^{-4} M.

[$90°_x-t-(180°_y-2t)_n-180°_y-t$] ($n$ = 59, t = 1 ms)[16] pulse sequences, it was possible to identify the singlets in the aliphatic region of the one-dimentional proton spectra. The peak at 2.14 ppm is a singlet, and integration with respect to the C$^\epsilon$H proton of His-39 (8.60 ppm)[10] gives an intensity ratio of 3.06:1. It is not possible at this stage to identify the actual methionine residue from which this resonance arises. Rusticyanin contains Met-18, Met-99, and Met-148, where the last of these is one of the ligands to the Cu. The C$^\epsilon$H methionine resonances were not assigned in the previous work of Dyson and colleagues.[10a,47] The extra broadening in the mixture is due to the electron self-exchange process (1).

$$RCu^I + RCu^{II} \rightleftharpoons RCu^{II} + RCu^I \quad (1)$$

Equation 2 relates the extent of broadening ($\nu_{1/2}$) at half peak height, and to the self-exchange rate constant k_{ese}, where [RCu^{II}] is the concentration of oxidized protein in the mixture, and $\Delta\nu_{1/2} = \nu_{1/2mix} - \nu_{1/2red}$.

$$k_{ese} = \pi\Delta\nu_{1/2}/[RCu^{II}] = k_{obs}/[RCu^{II}] \quad (2)$$

As the amount of actual broadening was small, it was difficult to determine k_{obs} (and hence k_{ese}) very precisely, and therefore k_{obs} was also estimated in one case using comparative measurements of the transverse relaxation times, T_2, from spin-echo experiments on corresponding signals in the reduced protein and the 1:1 mixture where $k_{ese} = T_2^{-1}/[RCu^{II}]$. The HSE pulse sequence[15] was used to determine the T_2 (spin−spin) relaxation time of the methionine C$^\epsilon$H resonance at 2.14 ppm. The intensity (I_t) of the resonance obtained with a total delay of $2t$ is given in eq 3, where I_0 is the intensity for t = 0.

$$I_t = I_0 \exp(-2t/T_2) \quad (3)$$

Plots of ln I_t against $2t$ give straight lines of slope $-T_2^{-1}$, Figure 2. The results obtained from measurements of peak

(12) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta.* **1985**, *811*, 265.
(13) Kyritsis, P.; Dennison, C.; Sykes, A. G. In *Bioinorganic Chemistry NATO Advanced Study Institute*, Kessissoglou, D. P., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1995; pp 67−76.
(14) (a) Lappin, A. G.; Lewis, C. A.; Ingledew, W. J. *Inorg. Chem.* **1985**, *24*, 1446. (b) McGinnis, J.; Ingledew, W. J.; Sykes, A. G. *Inorg.Chem.* **1986**, *25*, 3730. (c) Blake, R. C., II; White, K. J.; Shute, E. A. *J. Biol. Chem.* **1991**, *266*, 19203.
(15) Carr, H. Y.; Purcell, E. M. *Phys. Rev.* **1954**, *94*, 630.
(16) Campbell, I. D.; Dobson, C. M.; Williams, R. J. P.; Wright, P. E. *FEBS Lett.* **1975**, *57*, 96.

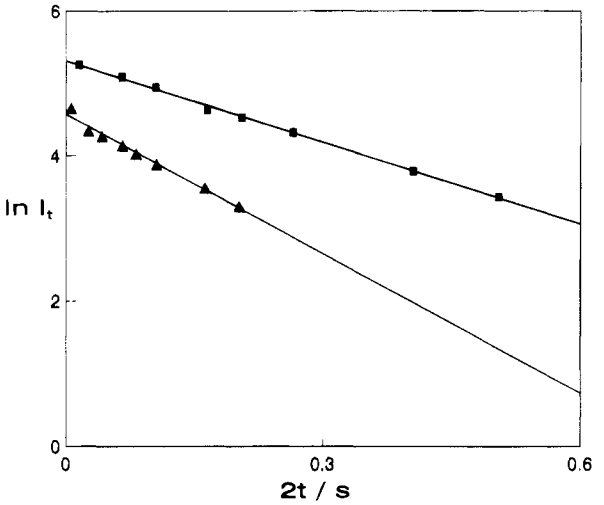

Figure 2. Plot of ln (I_t) against $2t$ in the spin-echo experiment (25 °C) for the C^eH methionine resonance (δ 2.14 ppm) in RCu^I (■) and in a 1:1 mixture with RCu^{II} (▲), $[RCu^{II}] = 4.9 \times 10^{-4}$ M, pH 5.7, I = 0.100 M.

Table 1. Self-Exchange Rate Constants (25 °C) for Rusticyanin Obtained from NMR Peak Width Measurements and from T_2 Values Determined by Spin-Echo Experiments for the C^eH Methionine Resonance at 2.14 ppm

pH	10^4-[RCu^{II}]/M	$10^{-4}k_{ese}$/(M^{-1} s^{-1})	pH	10^4-[RCu^{II}]/M	$10^{-4}k_{ese}$/(M^{-1} s^{-1})
2.0	2.70	1.7[a]	5.7	4.90	1.2[a]
5.7	3.70	0.9[a]	5.7	4.90	0.6[b]
5.7	4.00	0.8[a]	5.7	11.7	1.6[a]
5.7	4.09	1.0[a]			

[a] Peak-width measurement. [b] Spin-echo experiment.

widths and from the T_2 values from spin-echo experiments are given in Table 1.

Support for these values was provided by observations of the signal assigned to C^eH of a histidine (most probably His-128),[10] which occurs at 8.46 ppm in the reduced form of the protein and at 8.42 ppm in the oxidized protein. The small chemical shift difference (ca. 20 Hz at our field strength) provides the opportunity to observe exchange-induced coalescence in mixtures, and indeed at $[RCu^{II}] = 1.17 \times 10^{-3}$ M a single peak was observed from this proton in a 1:1 mixture of the two forms. At a concentration of 4.0×10^{-4} M the resonance in the 1:1 mixture appeared as two peaks in accord with the expected reduction in exchange rate at the lower total concentration of protein. Relevant spectra are shown in Figure 3. This method can only give a range of values for the self-exchange rate constant because of the uncertainty in establishing the precise conditions under which the coalescance of the two peaks occurs. Thus a range $(1.9-3.8) \times 10^4$ M^{-1} s^{-1} of self-exchange rate constants for rusticyanin was evaluated in this way. While the range of values reinforces the magnitude of the self-exchange rate constants, the method is not sufficiently accurate to include them alongside other entries in Table 1. The accuracy of the spin-echo result in Table 1 is *ca.* ±20% as compared to significantly larger errors when the direct measurement of line widths is used. In the case of k_{ese} values from Marcus cross reaction studies, Table 2, errors are estimated to be in the range 5−20% based on the accuracy of measured rate constants.

Discussion

Self-exchange rate constants k_{ese} for rusticyanin at two pH's are included alongside those for other type 1 copper proteins

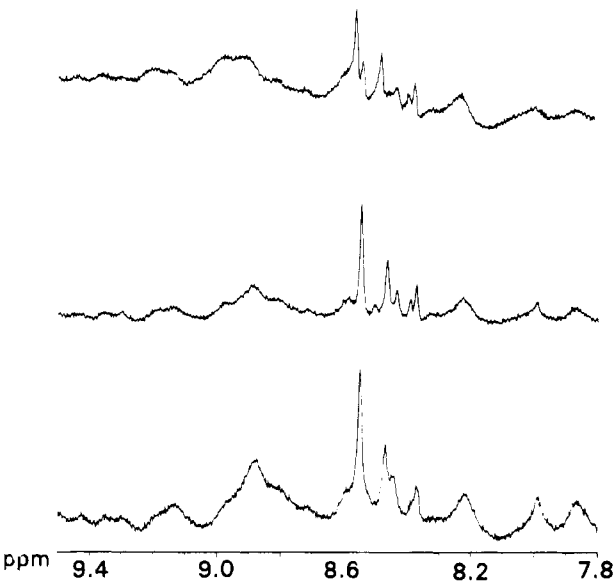

Figure 3. Proton NMR spectra for rusticyanin (pH 5.7, 25 °C, I = 0.100 M). The spectra are for (a) the sum of spectra of reduced and oxidized protein, (b) the 1:1 mixture of oxidized and reduced protein, $[RCu^{II}] = 4.0 \times 10^{-4}$ M, and (c) the 1:1 mixture of oxidized and reduced protein, $[RCu^{II}] = 1.17 \times 10^{-3}$ M.

Table 2. Electron Self-Exchange Rate Constants k_{ese} (25 °C), for Different Type 1 Copper Proteins, I = 0.100 M, Except As Indicated

protein	k_{ese}/(M^{-1} s^{-1})	pH	method	charge balance[a]	ref
azurin (*P.a.*)	9.6×10^5	4.5[b]	NMR	−1	17
	7.0×10^5	9.0[b]	NMR		17
	2.4×10^6	5.0[c]	EPR		18
azurin (*A.d.*)	4.0×10^5	6.7[b]	NMR		19
plastocyanin	3.2×10^5	7.5	NMR	+1	20
(*A.v.*)	5.9×10^5	7.5	Marcus		21
plastocyanin (parsley)	3.3×10^3	7.5	Marcus	−8	21
plastocyanin (spinach)	$\sim 4 \times 10^3$	6.0[d]	NMR	−9	22
plastocyanin (french bean)	$\ll 2 \times 10^4$	7.4[e]	NMR	9	23
stellacyanin	1.2×10^5	7.0[f]	EPR	+7	24
amicyanin (*T.v.*)	1.3×10^5	8.6[g]	NMR	−4	25
	1.2×10^5	8.6	Marcus		26
pseudoazurin	2.9×10^3	7.5	NMR	+1	27
(*A.c.*)	2.7×10^3	7.5	Marcus		27
rusticyanin	1.7×10^4	2.0	NMR	+4	this work
	1.0×10^4	5.7	NMR		

[a] For Cu^I from a summation of charges on Asp/Glu (−1) and Arg/Lys (+1) with uncoordinated His as +1. [b] I not indicated, 20 mM phosphate buffer. [c] 20 °C, 20 mM Mes buffer. [d] 10 mM cacodylate buffer, 0.1 M KCl. [e] 50 °C, 10 mM phosphate buffer. [f] 20 °C, 0.1 M phosphate buffer. [g] I = 0.05 M, similar value was also obtained at I = 0.40 M.

in Table 2.[17-27] It is noted that the k_{ese} values vary from 2.8×10^3 M^{-1} s^{-1} for pseudoazurin to $\sim 1.0 \times 10^6$ M^{-1} s^{-1} for the

(17) Groeneveld, C. M.; Canters, G. W. *J. Biol. Chem.* **1988**, *263*, 167.
(18) Groeneveld, C. M.; Dahlin, S.; Reinhammer, B.; Canters, G. W. *J. Am. Chem. Soc.* **1987**, *109*, 3247.
(19) Groeneveld, C. M.; Ouwerling, M. C.; Erkelens, C.; Canters, G. W. *J. Mol. Biol.* **1988**, *200*, 189.
(20) Dennison, C.; Kyritsis, P.; McFarlane, W.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1993**, 1959.
(21) de Silva, D. G. A. H.; Beoku-Betts, D.; Kyritsis, P.; Govindaraju, K.; Powls, R.; Tomkinson, N.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1992**, 2145.
(22) Armstrong, F. A.; Driscoll, D. C.; Hill, H. A. O. *FEBS Lett.* **1985**, *190*, 942.

azurins. X-ray crystal structures are now available for members of the plastocyanin, azurin, pseudoazurin, and amicyanin series but not as yet for rusticyanin or stellacyanin.[28-31] Similar Cu active site and β-sandwich structures have been found in all cases.[10] The agreement of k_{ese} values obtained from NMR with those from Marcus cross-reaction studies is good, fully supporting the strategy used of selecting azurin as an essentially electroneutral protein as the redox partner for the cross-reaction studies,[29] thereby minimizing the effect of work terms. In order to measure rate constants by the stopped-flow method, it is essential that the driving force for the reaction is not too high. In the case of rusticyanin we have not so far been able to identify protein redox partners for cross-reaction studies.

The self-exchange rate constants for rusticyanin fall in the middle of the region spanned for different type 1 Cu proteins. At pH $\sim$ 7 the overall charge balance on rusticyanin in the Cu^I state is +4, but at pH $\sim$ 2 this may be as high as +17, assuming that the free histidine and carboxylate residues are all protonated. The structural integrity of the protein at pH 2, the high reduction potential which increases by only $\sim$45 mV between pH 5 and pH 2,[32] and the lack of amino acid homology with other type 1 copper proteins are notable features. The small effect of the additional positive charge on the $E^{o\prime}$ of rusticyanin at pH 2.0 is consistent with the retention of *in vitro* redox reactivity.[14] It is surprising that the higher charge at pH $\sim$ 2 does not have an inhibitory effect on the self-exchange process as in the case of plant as opposed to *Anabaena variabilis* (*A. variabilis*) plastocyanins. This suggests that the charged residues are well away from the adjacent hydrophobic patch region. The protein is bigger than other type 1 Cu proteins (155 amino acids),[5] which may help disperse the cationic charge. The global fold of rusticyanin from NMR studies indicates a highly protected hydrophobic surround for the Cu active site.[10] The exposed imidazole of His-143 which is a possible reaction site is coordinated to and connects the Cu to the outside.

It appears that the imidazole of His-143 is not as exposed as in other cases, and there may therefore be a greater separation of the two Cu's when electron exchange occurs. More detailed structural information is needed in order to assess further this possibility.

A reasonably good understanding of k_{ese} rate constants for type 1 copper proteins is now possible, and it is important to consider k_{ese} for rusticyanin in this context. Thus, in the case of azurin electron transfer is *via* the adjacent (to the Cu) hydrophobic sites of Cu^I and Cu^{II} molecules. More precisely the reaction is believed to involve electron transfer between the two Cu's *via* the Cu-coordinated (and -exposed) imidazole of His-117.[33] Evidence in support of this has come from the determination of self-exchange rate constants for the Met44Lys and Met64Glu mutants.[33] The above pathway represents the closest Cu–Cu approach for electron transfer and is $\sim$14 Å. A second major factor which enhances the electron self-exchange reactivity of azurin is the charge balance of acidic (Asp and Glu) and basic (Lys, Arg, and His) residues at pH $\sim$ 7, which is -1 for ACu^I, and the lack of any localized charged regions on the protein surface.[29]

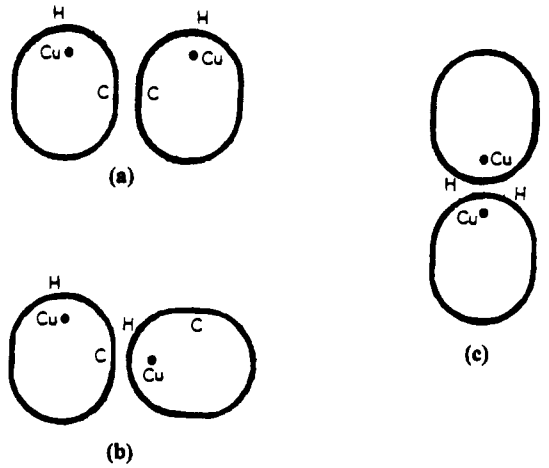

Figure 4. Possible orientations of the two plastocyanin molecules at the time of electron self-exchange. The diagrams show (a) remote–remote, (b) remote–adjacent, and (c) adjacent–adjacent modes of interactions. The letters H and C indicate the adjacent hydrophobic and charged (acidic patch) regions, respectively.

The higher plant plastocyanins are believed to undergo electron self-exchange by a similar mechanism involving the imidazole of His-87, but the high negative charge balance of -9 ± 1 for PCu^I at pH 7.0 and proximity of the acidic patch to the Cu active site retard the self-exchange process.[20,21] *A. variabilis* plastocyanin (charge balance +1) is unusual in that it lacks the conserved acidic patch region of other plastocyanins and behaves more like azurin with a k_{ese} value of 3.2×10^5 M^{-1} s^{-1}.[20] From extensive studies on the higher plant and green algal plastocyanins, as well as intramolecular electron-transfer processes involving the type 1 domain of ascorbate oxidase and nitrite reductase,[34,35] the remote acidic patch is used extensively for electron transfer.[5] However, for self-exchange, electron transfer *via* two adjacent (to the Cu) hydrophobic sites is favored, as illustrated in Figure 4. In this schematic representation possible encounter complexes are considered. Case a is

(23) Beattie, J. K.; Fenson, D. J.; Freeman, H. C.; Woodcock, E.; Hill, H. A. O.; Stokes, A. M. *Biochim. Biophys. Acta* **1975**, *405*, 109.
(24) Dahlin, S.; Reinhammer, B.; Wilson, M. T. *Biochem. J.* **1984**, *218*, 609.
(25) Lommen, A.; Canters, G. W. *J. Biol. Chem.* **1990**, *265*, 2768.
(26) Kyritsis, P.; Kohzuma, T.; Kalverda, A. P.; Canters, G. W.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.*, in press.
(27) Dennison, C.; Kohzuma, T.; McFarlane, W.; Suzuki, S.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1994**, 437.
(28) (a) Guss, J. M.; Freeman, H. C. *J. Mol. Biol.* **1983**, *169*, 521. (b) Guss, J. M.; Harrowell, P. R.; Murata, M.; Norris, V. A.; Freeman, H. C. *J. Mol. Biol.* **1986**, *192*, 361. (c) Collyer, C. A.; Guss, J. M.; Sugimura, Y.; Yoshizaki, F.; Freeman, H. C. *J. Mol. Biol.* **1990**, *211*, 617.
(29) Baker, E. N. *J. Mol. Biol.* **1988**, *203*, 1071.
(30) (a) Adman, E. T.; Turley, S.; Branson, R.; Petratos, K.; Banner, D.; Tsernoglou, D.; Beppu, T.; Watanabe, H. *J. Biol. Chem.* **1989**, *264*, 87. (b) Petratos, K.; Banner, D. W.; Beppu, T.; Wilson, K. S.; Tsernoglou, D. *FEBS Lett.* **1987**, *218*, 209. (c) Petratos, K.; Dauter, Z.; Wilson, K. S. *Acta Crystallogr.* **1988**, *B44*, 628.
(31) (a) Durley, R.; Chen, L.; Lim, L. W.; Mathews, F. S.; Davidson, V. L. *Protein Sci.* **1993**, *2*, 739. (b) Romero, A.; Nar, H.; Messerschmidt, A.; Kalverda, P.; Canters, G. W.; Durley, R.; Mathews, F. S. *J. Mol. Biol.* **1994**, *236*, 1196. (c) Chen, L.; Durley, R.; Poliks, B. J.; Hamada, K.; Chen, Z.; Mathews, F. S.; Davidson, D. L.; Satow, Y.; Huizinga, E.; Vellieux, F. M. D.; Hol, W. G. J. *Biochemistry* **1992**, *31*, 4959.
(32) Haladjian, J.; Nunzi, F.; Bianco, P.; Bruschi, M. *J. Electroanal. Chem.* **1993**, *352*, 329.
(33) (a) van de Kamp, M.; Floris, R.; Hali, F. C.; Canters, G. W. *J. Am. Chem. Soc.* **1990**, *112*, 902. (b) van de Kamp, M.; Canters, G. W.; Andrew, C. R.; Sanders-Loehr, J.; Bender, C. J.; Peisach, J. *Eur. J. Biochem.* **1993**, *218*, 229. (c) Van Pouderoyen, G.; Mazumdar, S.; Hunt, N. I.; Hill, H. A. O.; Canters, G. W. *Eur. J. Biochem.* **1994**, *222*, 583.
(34) (a) Kyritsis, P.; Messerschmidt, A.; Huber, R.; Salmon, G. A.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1993**, 731. (b) Suzuki, S.; Kohzuma, T.; Deligeer; Yamaguchi, K.; Nakamura, N.; Shidara, S.; Kobayashi, K.; Tagawa, S. *J. Am. Chem. Soc.* **1994**, *116*, 11145.
(35) (a) Messerschmidt, A.; Ladenstein, R.; Bolognesi, M.; Avigliano, L.; Petruzzelli, R.; Rossi, A.; Finazzi-Agro, A. *J. Mol. Biol.* **1992**, *224*, 179. (b) Godden, J. W.; Turley, S.; Teller, D. C.; Adman, E. T.; Liu, M. Y.; Payne, W. J.; LeGall, J. *Science* **1991**, *258*, 438.
(36) (a) Nar, H.; Messerschmidt, A.; Huber, R.; van de Kamp, M.; Canters, G. W. *J. Mol. Biol.* **1991**, *218*, 427. (b) Nar, H.; Messerschmidt, A.; Huber, R.; van de Kamp, M.; Canters, G. W. *J. Mol. Biol.* **1991**, *221*, 765.

unfavorable because of repulsion between the two acidic patches and case b is unfavorable because the energetics do not allow interaction of hydrophobic and acidic (hydrophilic) regions, which leaves case c as the only acceptable possibility. More generally it can be argued that hydrophobic interactions, free from electrostatic repulsive forces, will be more favorable for self-exchange. The exposed imidazole provides a pathway for electron transfer which is the same for each self-exchange process.

In the case of pseudoazurin there is a low overall charge balance (+1 at pH 7.0), and the k_{ese} of 2.9×10^3 M^{-1} s^{-1} is small. This is attributed to the presence of a Lys residue at position 38 close to the exposed imidazole at the adjacent hydrophobic site.[27] The Met44Lys azurin mutant has a lysine at a position very similar to that of Lys38 in pseudoazurin and shows similar self-exchange reactivity ($k_{ese} < 1.0 \times 10^3$ M^{-1} s^{-1}). Owing to its high overall charge balance (+7 at pH 7.0) stellacyanin would have been expected to have a lower electron self-exchange rate constant than 1.2×10^5 M^{-1} s^{-1}, and this is at present the most difficult result to explain. The high value may be due to the carbohydrate content (40%), which could decrease the repulsive effect of charged areas at the protein surface during the self-exchange process.[24] In the case of amicyanin (charge balance −4), the corresponding surface-exposed residue is His-96. Here, there are positively charged residues close by which account for the order of magnitude smaller reactivity than in the case of azurin.[25] To summarize, a similar mechanism is believed to be effective in all these electron self-exchange processes involving the adjacent hydrophobic surface, and more specifically the exposed imidazole which is coordinated to the Cu. Variations in k_{ese} are largely explained by overall and/or localized effects of charge.

Rusticyanin lacks the conserved Asn residue which follows the NH_2-terminal active site His in other type 1 Cu proteins. Instead[8] the residue after His-85 is Ser (umecyanin is also an exception with the corresponding Asn replaced by Asp[37]). It has been noted that the Asn residue forms H-bonds which are crucial in maintaining structural features at the Cu site.[38] The absence of this residue in rusticyanin could result in a different structural motif for the Cu site and help explain some features of the protein. Thus, with regard to reduction potentials, it has been observed that when the Asn-47 of *Alcaligens denitrificans* azurin is replaced by Leu, there is a 110 mV increase in $E^{\circ\prime}$.[39] Also in the double mutant (Asn47Leu + Met121Leu) of *Pseudomonas aeruginosa* azurin, the effect is even bigger ($E^{\circ\prime}$ is 510 mV).[40] Replacement of Met-121 alone results in only a 70 mV increase in the reduction potential.[41]

Out of 36 amino acids close to the Cu, 4 are ligands to the Cu, 7 are glycines, and no fewer than 20 are reported to be hydrophobic including 6 prolines and 7 aromatic residues. The hydrophobicity of the Cu active site accounts for the unusual stability of rusticyanin at low pH.[10] So far unexplained is the high reduction potential of rusticyanin (680 mV), although various factors including a shorter Cu−S(Met) bond[42] and a more buried copper active site may contribute.[43] Also, the long Cu−thioether axial bond present in blue copper proteins destabilizes the oxidized state and is therefore a key determining factor for the high reduction potentials generally observed.[44] However, in the rusticyanin one factor not so far taken into account is the hydrophobicity of the active site, which is more conducive to Cu^I than Cu^{II} and translates into a higher oxidizing ability of the Cu^{II} site. A high reduction potential (785 mV) has also been determined in the case of the type 1 Cu site of the multicopper laccase from the fungal source *Polyporus versicolor*.[45] However, in the latter the Cu site is lacking a methionine, and from sequence alignments appears to be replaced by a leucine,[46] which might imply a trigonal coordination of the Cu^I site. Such a geometry would stabilize the reduced form and result in a high reduction potential, much as is observed for the Cu^I form of plastocyanin at low pH when the His-87 protonates and dissociates from the Cu.[5]

Finally as far as studies on the reactivity of various cyano and substituted phenantholine Fe(II) complexes with rusticyanin are concerned,[10,14c] it may well be that the complexes with bulky hydrophobic phenanthroline ligands react more rapidly due to a favorable interaction at the adjacent hydrophobic region on rusticyanin. However, some caution is required, because the self-exchange rate constants for low-spin phenanthroline complexes are much greater than those for aqua Fe^{II} and Fe^{III} reactants.

In conclusion, current structural information on rusticyanin indicating a high degree of hydrophobicity and protection at the Cu site can explain observations concerning (i) the unusual stability at pH ~ 2.0, (ii) the high reduction potential, and (iii) a 2 order of magnitude smaller electron self-exchange rate constant k_{ese} as compared to azurin.

Acknowledgment. We thank the State Scholarship Foundation of Greece and the University of Newcastle for financial support (P.K.), EPSRC for a studentship (C.D.), and the Leverhulme Trust for a Fellowship (W.McF.).

IC9505413

(37) van Driessche, G.; Dennison, C.; Sykes, A. G.; van Beeumen, J. *Protein Sci.* **1995**, *4*, 209.
(38) Adman, E. T. *Adv. Protein Chem.* **1992**, *42*, 145.
(39) Hoitink, C. W. G.; Canters, G. W. *J. Biol. Chem.* **1992**, *267*, 13836.
(40) Pascher, T.; Karlsson, B. G.; Nordling, M.; Malmström, B. G.; Vänngård, T. *Eur. J. Biochem.* **1993**, *212*, 289.
(41) Karlsson, B. G.; Aasa, R.; Malmström, B. G.; Lundberg, L. G. *FEBS Lett.* **1989**, *253*, 99.
(42) Gray, H. B.; Malmström, B. G. *Comments Inorg. Chem.* **1983**, *2*, 203.
(43) Nunzi, F.; Guerlesquin, F.; Shepard, W.; Guigliarelli, B.; Bruschi, M. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 1655.
(44) Guckert, J. A.; Lowery, M. D.; Solomon, E. I. *J. Am. Chem. Soc.* **1995**, *117*, 2817.
(45) Reinhammar, B. *Biochim. Biophys. Acta* **1972**, *275*, 245.
(46) Messerschmidt, A. *Adv. Inorg. Chem.* **1991**, *40*, 121.
(47) **Note Added in Press:** The $C^{\epsilon}H$ resonance at δ2.14 has now been assigned by Dyson[10b] to Met-99. The corresponding resonances from Met-18 and Met-148 are at δ0.93 and 0.63; this is a crowded region of the spectrum, and hence the peaks are not suitable for detailed line-broadening studies.