Determination of the Electron Self-Exchange Rate of Azurin from *Pseudomonas aeruginosa* by a Combination of Fast-Flow/Rapid-Freeze Experiments and EPR

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Abstract: The electron self-exchange rate of *Pseudomonas aeruginosa* azurin has been measured by combined fast-flow/rapid-freeze and EPR experiments performed on mixtures of ^{63}Cu- and ^{65}Cu-labeled azurin. At 4 °C, the electron self-exchange rate constant amounts to $(6.1 \pm 2.6) \times 10^5$ M^{-1} s^{-1} at pH 5.0 and $(3.2 \pm 0.7) \times 10^5$ M^{-1} s^{-1} at pH 9.0; at room temperature it amounts to $(2.4 \pm 1.0) \times 10^6$ M^{-1} s^{-1}. The data demonstrate that of the two experimental values reported in the literature for the rate constant at low pH, viz., 1.4×10^4 M^{-1} s^{-1} (25 °C) (*Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2039–2043) and $(0.4–1.4) \times 10^6$ M^{-1} s^{-1} (25 °C) (*Eur. J. Biochem.* **1985**, *153*, 559–564), the latter value is the correct one. The observation that the self-exchange rate is not strongly affected by pH is at variance with the conclusions drawn from earlier studies on the cytochrome c_{551}/azurin redox kinetics, but agrees with previous observations on the kinetics of the self-exchange reaction of azurin.

This paper addresses the question of the experimental determination of the electron self-exchange rate constant of azurin from *Pseudomonas aeruginosa*. Electron exchange reactions in which this blue-copper protein participates have been investigated by a large number of groups.[1–4] Its three-dimensional structure has been reported at 2.7-Å resolution,[5,6] and the protein has been used in many studies of electron transfer reactions with coordination complexes of the 3d transition metals[7–9] and with redox proteins, mostly cytochromes.[10–16] By applying Marcus theory,[17] theoretical values of the electron self-exchange rate of this protein and various other blue-copper proteins were obtained.[14,18,19] Conspicuous is the large spread in rates reported for azurin as obtained from various experimental studies of the electron transfer reactions with transition metal compounds. More consistent results were obtained with proteins as redox partners, as illustrated by Wherland et al.,[14] who reported an electron self-exchange rate constant of 9.9×10^5 M^{-1} s^{-1} for *Pseudomonas aeruginosa* azurin, based on a Marcus analysis of the electron transfer reactions between *c*-type cytochromes and blue-copper proteins.

A logical extension of these studies is the *direct* experimental determination of the electron self-exchange rates of the blue-copper proteins. The theoretical interpretation of these experimentally determined rates may be easier, since reactions between identical partners are likely to be less complex compared to electron transfer reactions between different proteins. In this way one might hope to gain detailed information about the electron transfer mechanism. This is of special interest for *Pseudomonas aeruginosa* azurin, since this protein has been postulated to exist in a redox-active and a redox-inactive form on the basis of the observed kinetics of the electron transfer with cytochrome-c_{551}.[15] The two forms participate in a protonation/deprotonation equilibrium with pK_a values of 5.9 and 7.1 for the oxidized and reduced protein, respectively,[20] and purportedly differ in reactivity by at least two to three orders of magnitude.[15] Corin et al.,[20] however, recently questioned the customary interpretation of the azurin/cytochrome c_{551} experiments, and argued that the redox properties of the blue-copper protein need not vary drastically with pH to be compatible with the observed pH dependence of the azurin/cytochrome c_{551} electron transfer kinetics. As pointed out above, a determination of the electron self-exchange rate of azurin and its pH dependence would bear directly on this point.[15]

Previously,[21,22] some of us determined the electron self-exchange rate constant of azurin under various conditions, using ^{1}H NMR spectroscopy, by measuring the spin–spin relaxation time (T_2) of a ligand histidine signal as a function of the degree of oxidation. This study showed that the electron self-exchange rate of azurin is independent of pH (in the region $5.0 < pH < 9.0$), with an average exchange rate of 1.3×10^6 M^{-1} s^{-1} at 309 K. At about the same time, Ugurbil and Mitra (UM) reported a similar study,[23] in which the azurin electron self-exchange rate was determined on the basis of spin–lattice relaxation time (T_1) measurements of a His-35 resonance in the ^{1}H NMR spectrum of the azurin as a function of the degree of oxidation. This study also showed that the electron self-exchange rate constant of azurin is independent of pH, in agreement with our results,[21,22] but a two orders of magnitude lower exchange rate of about 10^4 M^{-1} s^{-1} was reported. The difference is too large to be ascribed to experimental

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(1) Wilson, M. T.; Greenwood, C.; Brunori, M.; Antonini, E. *Biochem. J.* **1975**, *145*, 449–457.
(2) Cummins, D.; Gray, H. B. *J. Am. Chem. Soc.* **1977**, *99*, 5158–5167.
(3) Rosen, P.; Pecht, I. *Biochemistry* **1976**, *15*, 775–786.
(4) Chapman, S. K.; Sanemasa, I.; Watson, A. D.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1983**, 1949–1953.
(5) Adman, E. T.; Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *J. Mol. Biol.* **1978**, *123*, 35–47.
(6) Adman, E. T.; Jensen, L. H. *Isr. J. Chem.* **1981**, *21*, 8–12.
(7) Rosenberg, R. C.; Wherland, S.; Holwerda; R. A.; Gray, H. B. *J. Am. Chem. Soc.* **1976**, *98*, 6364–6369.
(8) McArdle, J. V.; Coyle, C. L.; Gray, H. B.; Yoneda, G. S.; Holwerda, R. A. *J. Am. Chem. Soc.* **1977**, *99*, 2483–2489.
(9) Wherland, S.; Holwerda, R. A.; Rosenberg, R. C.; Gray, H. B. *J. Am. Chem. Soc.* **1975**, *97*, 5260–5262.
(10) Antonini, E.; Finazzi-Agro, A.; Avigliano, L.; Guerriere, P.; Rotilio, G.; Mondovi, B. *J. Biol. Chem.* **1970**, *245*, 4847–4849.
(11) Pecht, I.; Rosen, P. *Biochem. Biophys. Res. Commun.* **1973**, *50*, 853–858.
(12) Brunori, M.; Greenwood, C.; Wilson, M. T. *Biochem. J.* **1974**, *137*, 113–116.
(13) Parr, S. R.; Barber, D.; Greenwood, C.; Brunori, M. *Biochem. J.* **1977**, *167*, 447–455.
(14) Wherland, S.; Pecht, I. *Biochemistry* **1978**, *17*, 2585–2591.
(15) Silvestrini, M. C.; Brunori, M.; Wilson, M. T.; Darley-Usmar, V. *J. Inorg. Biochem.* **1981**, *14*, 327–338.
(16) Farver, O.; Blatt, Y.; Pecht, I. *Biochemistry* **1982**, *21*, 3556–3561.
(17) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322.
(18) Farver, O.; Pecht, I. *Metal Ions in Biology*. Vol. 3. *Copper Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1981; pp 151–192.
(19) Canters, G. W.; Groeneveld, C. M. *Frontiers in Bioinorganic Chemistry*; Xavier, A. V., Ed.; VCH Verlagsgesellschaft: Weinheim, BRD, 1986; pp 694–703.
(20) Corin, A. F.; Bersohn, R.; Cole, P. E. *Biochemistry* **1983**, *22*, 2032–2038.
(21) Groeneveld, C. M.; Canters, G. W. *Eur. J. Biochem.* **1985**, *153*, 559–564.
(22) Groeneveld, C. M.; Canters, G. W. *Rev. Port. Quim.* **1985**, *27*, 145.
(23) Ugurbil, K.; Mitra, S. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 2039–2043.

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uncertainties. It was decided, therefore, to perform fast-flow/rapid-freeze experiments in combination with EPR measurements of mixtures of isotopically labeled (^{63}Cu)azurin/(^{65}Cu)azurin at pH 5.0 and 9.0, in order to determine the electron self-exchange rate constant of azurin with an independent technique. This technique has been used earlier, with success, for the determination of the electron self-exchange rate of stellacyanin.[24] At 4 °C, the electron self-exchange rate constant of azurin measured with this new technique turns out to be $(6.1 \pm 2.6) \times 10^5$ M^{-1} s^{-1} at pH 5.0 and $(3.2 \pm 0.7) \times 10^5$ M^{-1} s^{-1} at pH 9.0. These rates correspond to a rate of about 10^6 M^{-1} s^{-1} at room temperature in accordance with our earlier results.[21,22]

Materials and Methods

Protein Preparation. Azurin was isolated from *Pseudomonas aeruginosa* bacterial paste and purified as described by Ambler[25] and Parr et al.[26] The ratio of the optical absorbance at 625 and 280 nm was used as a measure for the purity of the azurin,[25,26] and amounted to 0.56 after the last column purification.

Apoprotein was prepared by reducing 1 mL of a 20 mM Tris/HCl solution (pH 8.3) containing approximately 25 mg of azurin with a slight excess of sodium dithionite. The resulting colorless solution was dialyzed overnight at 4 °C against 0.1 M KCN in 0.15 MTris/HCl buffer, pH 8.3.[27] Excess KCN was removed from the dialysis bag by a 4-h dialysis against 50 mM Tris/HCl buffer, at room temperature, followed by dialysis overnight against 50 mM sodium acetate buffer, pH 6.1, at 4 °C.

Small quantities (7–8 mg) of the pure isotopes ^{63}Cu and ^{65}Cu, commercially available as metal from Intersales Holland B.V., were dissolved in 0.3 M HNO_3. The pH's of the copper-isotope solutions (Cu(II form) were adjusted to 5.5–6.0, by adding small amounts of 0.1 M NaOH. The azurin was reconstituted by the addition of small amounts of the pure copper-isotope solution to the apoprotein solution. The reconstitution was followed by monitoring the increase of the optical absorption at 625 nm on a Cary-219 spectrophotometer, and the addition of copper solution was stopped after the absorbance at 625 nm had become constant. The ratio of the absorbances at 625 and 280 nm after reconstitution amounted to 0.55 for both isotope–azurin solutions, indicating that no apoprotein was present after the reconstitution. The removal of the unbound copper(II) ions was accomplished by ultrafiltration with an Amicon cell.

Rapid-Freeze Experiments. The concentrations of both isotopic forms of azurin (in 20 mM MES buffer (MES = 2-[*N*-morpholino]ethanesulfonic acid), pH 5.0, or in 20 mM borate buffer, pH 9.0) were spectrophotometrically measured by determining the absorbance of the protein solution at 625 nm ($\epsilon_{625} = 5700$ M^{-1} s^{-1} [28]). Solutions were prepared so that the concentrations were adjusted to the same value. Samples of (^{63}Cu)azurin (1–2 mL) were made anaerobic by alternately decreasing the pressure and flushing with argon gas. The protein was then anaerobically reduced by addition of aliquots (2–3 μL) of a dithionite solution (0.25–1 M in 0.1 M NaOH). The titration was stopped when a very small amount of azurin remained oxidized (~1%), showing that no excess of reductant was introduced.

Reduced (^{63}Cu)azurin was mixed with oxidized (^{65}Cu)azurin by fast-flow methods, and the mixture was rapidly frozen after a fixed delay as described earlier.[24] The total quenching time of the reaction was corrected for the freezing time according to Ballou.[29]

EPR Measurements of the Azurin Mixtures. The EPR spectra of the rapidly frozen samples were recorded on a Bruker ER 200D-SRC EPR spectrometer, equipped with a low-temperature system consisting of an ESR 9 continuous-flow cryostat and a Precision temperature controller manufactured by Oxford Instruments. The latter instrument was calibrated against carbon resistors placed in the spectrometer cavity. The spectrometer was interfaced to an ASPECT-2000 computer, which was used to collect and manipulate the data under control of the Bruker EPR program, and to store the spectra on disk. The data were accumulated in 8K, with a sweep width of 20 mT, yielding a resolution of 2.4 μT per point. To record the EPR spectra of samples with a low protein concentration (50 and 100 μM), the average of 128 scans per spectrum was stored, while for the EPR samples with a higher protein concentration (350 μM) 16 scans per sample were accumulated.

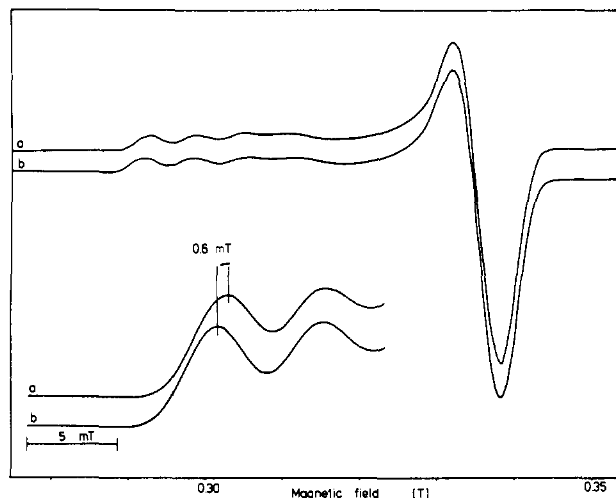

Figure 1. X-band EPR spectrum of a 350 μM solution of (a) oxidized (^{63}Cu)azurin and (b) oxidized (^{65}Cu)azurin. Conditions: pH 5.0, 20 mM MES buffer, other conditions as described in the Materials and Methods section. Inset: amplified part of the low-field region of the EPR spectrum.

The following conditions for measuring the EPR spectra were employed: temperature, 60.0 ± 0.5 K; modulation amplitude, 3.2 mT; microwave power, 31 mW; and microwave frequency, 9.46 GHz. The spectra were recorded between 285 and 305 mT, with a sweep rate of 1 mT/s and a time constant of 50 ms.

Analysis of the Spectra. Before the analysis was started, all EPR spectra were corrected for small differences in the microwave frequency by aligning them along the field axis with an accuracy of 2.4 μT. The analysis proceeded by determining the field position of the first hyperfine line of the EPR spectra by a derivative method. The position of the top depends on the oxidized (^{63}Cu)azurin/(^{65}Cu)azurin ratio (vide infra) and can therefore be used for an experimental determination of this ratio.

Results

The electron self-exchange reaction of the azurin can be monitored by EPR, because of the small but significant difference between the EPR spectra of oxidized (^{65}Cu)azurin and oxidized (^{63}Cu)azurin, as is shown in Figure 1. The top of the first hyperfine line of oxidized (^{63}Cu)azurin occurs at a 0.6 mT higher magnetic field than the first hyperfine line of oxidized (^{65}Cu)-azurin. The experiment consists of mixing equal amounts of reduced (^{63}Cu)azurin and oxidized (^{65}Cu)azurin. As the electron-exchange reaction proceeds, the concentration of oxidized (^{65}Cu)azurin decreases and that of oxidized (^{63}Cu)azurin increases, and the first hyperfine line moves from the "pure oxidized (^{65}Cu)azurin position" to a position corresponding to the average of the two isotopic forms of azurin.

We have carefully checked that the shift of the first hyperfine line is solely due to the electron transfer event. In a similar study on stellacyanin, for instance, the EPR spectrum of rapidly reoxidized stellacyanin was not completely identical with that of the resting oxidized protein.[24] In the present study, therefore, rapid-freeze experiments were performed in which reduced native azurin was mixed with $Co^{III}(phen)_3^{3+}$ (phen = 1,10-phenanthroline) as an oxidant. The EPR spectra of the mixtures with different quenching times, ranging from 25 ms to 4 min, do not show differences between resting oxidized azurin and rapidly reoxidized azurin. We therefore conclude that complications as observed in the experiments on stellacyanin are absent for azurin.

Provided the concentrations of both reactants are equal, the increase in the concentration of oxidized (^{63}Cu)azurin is given by

$$c = A(1 - e^{-k_{app}t}) \tag{1}$$

where c is the concentration of oxidized (^{63}Cu)azurin at time t, A is the concentration of oxidized (^{63}Cu)azurin at equilibrium (large t), and $k_{app} = k[Az]_{tot}$ with k the second-order rate constant.

Three sets of experiments were performed, one at high and two at low $[Az]_{tot}$. For the former experiment an amount of $[Az]_{tot}$

(24) Dahlin, S.; Reinhammar, B.; Wilson, M. T. *Biochem. J.* **1984**, *218*, 609–614.
(25) Ambler, R. P. *Biochem. J.* **1963**, *89*, 341–349.
(26) Parr, S. R.; Barber, D.; Greenwood, C.; Phillips, B. W.; Melling, J. *Biochem. J.* **1976**, *157*, 423–430.
(27) McMillan, D. R.; Rosenberg, R. C.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *71*, 4760–4762.
(28) Goldberg, M.; Pecht, I. *Biochemistry* **1976**, *15*, 4197–4208.
(29) Ballou, D. P. *Ph.D.* Thesis, 1971, University of Michigan.

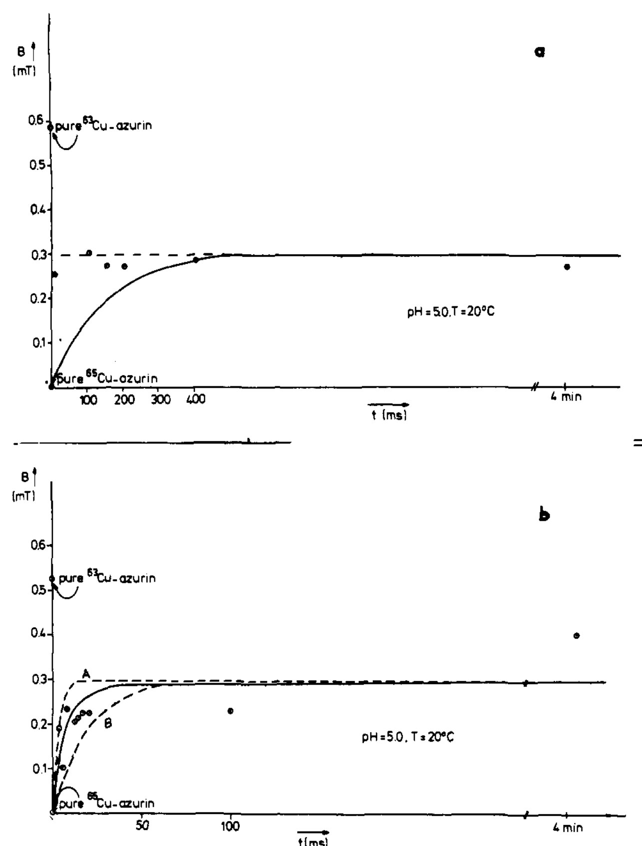

Figure 2. (a) Result of the rapid-freeze/EPR experiments performed at pH 5.0 at a mixing temperature of 20 °C with a total azurin concentration of 350 μM. The relative position (in mT) of the first hyperfine line is shown as a function of the quenching time (in ms) of the reaction. The position of the first hyperfine line in the pure (^{63}Cu) and (^{65}Cu)-azurin EPR spectra are indicated by arrows in the figure. The solid line is a theoretical curve calculated (see eq 1) with $k = 2 \times 10^4$ M^{-1} s^{-1}. (b) Same as Figure 2a but total azurin concentration of 50 μM. The solid line is a least-squares fit of the data points to eq 1 with $k = 2.4 \times 10^6$ M^{-1} s^{-1}. Dotted lines are theoretical curves calculated on the basis of $k = 6.0 \times 10^6$ M^{-1} s^{-1} (A) and $k = 1.0 \times 10^6$ M^{-1} s^{-1} (B).

= 350 μM was employed. The mixing of the isotopic forms of azurin (at pH 5) was performed at room temperature, and the results of this set of rapid-freeze experiments are shown in Figure 2a, where the position of the first hyperfine line is displayed as a function of the quenching time. The points in Figure 2a are scattered around a position corresponding to the average of the two pure isotopic forms of azurin. This indicates that the reaction is already completed for all quenching times employed here. Especially the measurement with a quenching time of 10 ms demonstrates that the electron self-exchange rate of azurin must be much higher than 10^4 M^{-1} s^{-1}. This is also evident from a comparison of the experimental points with the decay curve calculated on the basis of $k = 2 \times 10^4$ M^{-1} s^{-1} (solid curve, Figure 2a).

The next set of rapid-freeze experiments was performed with a low protein concentration (50 μM) at room temperature, yielding the results presented in Figure 2b. Values of A and k (see eq 1) were extracted from the data with the help of an iterative non-linear Gauss–Newton least-squares procedure.[30] This yielded a value of $(2.4 \pm 1.0) \times 10^6$ M^{-1} s^{-1} for the electron self-exchange rate constant of azurin at pH 5.0 and room temperature. The corresponding fit of the data points is represented by the solid curve in Figure 2b. The quoted uncertainty (40%) is a statistical estimate of the standard deviation. The statistical significance of the analysis is illustrated by the two dotted curves in Figure 2b, which correspond to reaction kinetics 2.5 times faster or slower,

(30) Fraser, R. D. B.; Suzuki, E. *Physical Principles and Techniques of Protein Chemistry*, Part C (Leach, S. J., Ed.); Academic Press: New York, 1973; pp 301–355.

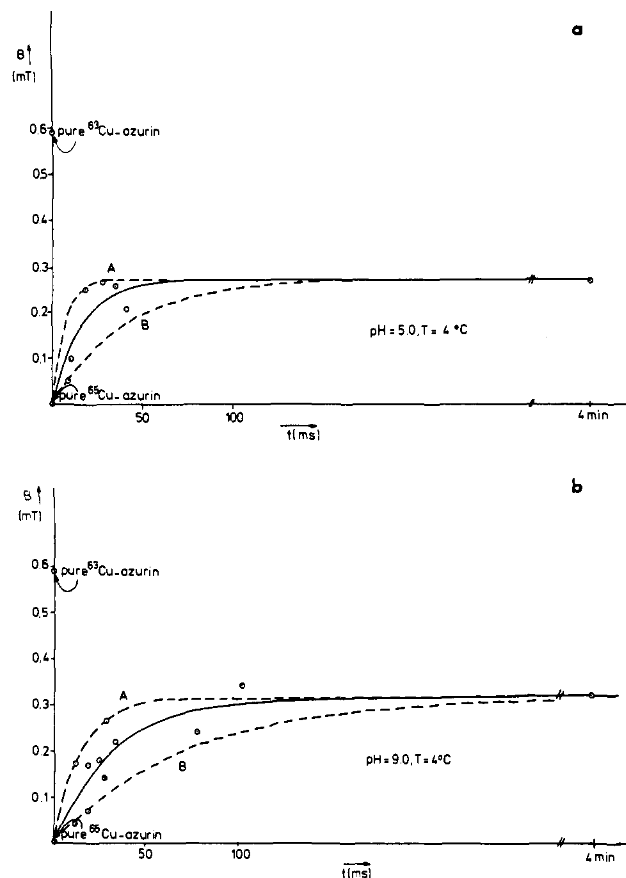

Figure 3. Same as Figure 2, but mixing temperature amounts to 4 °C and pH 5.0 (a) or pH 9.0 (b). Total azurin concentration amounts to 100 μM in both experiments. Dotted lines in (a) are theoretical curves calculated with $k = 15.0 \times 10^5$ M^{-1} s^{-1} (A) and $k = 2.5 \times 10^5$ M^{-1} s^{-1} (B); those in (b) correspond to k values of 6.4×10^5 (A) and 1.4×10^5 M^{-1} s^{-1} (B).

Table I. Electron Self-Exchange Rates of *Pseudomonas aeruginosa* Azurin at Room Temperature (pH 5.0) and 4 °C (pH 5.0 and 9.0) Obtained from the Present EPR Study and Previous NMR Studies[a]

		$k \times 10^{-6}$, M^{-1} s^{-1}		
pH	T, °C	b	c	d
5	25		0.4–1.4	0.014
	20	2.4 (1.0)		
5	4	0.61 (0.26)	0.05–0.8	
9	4	0.32 (0.07)	0.2	

[a] Values in parentheses denote the statistical estimate of the standard deviation obtained from the curve fitting according to eq 1. [b] Present study. [c] Reference 21 and 22. [d] Reference 23, measured at pH 4.5 in unbuffered solution.

respectively, than the value of k quoted above. The experimental points in the early ($t < 50$ ms) time domain (which is the significant part for the determination of k) are nicely bracketed by these curves. Because of the low $[Az]_{tot}$ concentration that had to be employed in this experiment, the EPR spectrometer was used at the limits of its sensitivity, resulting in a relatively imprecise value of k. A more precise value was obtained by lowering the temperature to 4 °C, which lowered k and allowed for larger values of $[Az]_{tot}$ (100 μM) to be employed. The results of both the low- and high-pH experiments at this temperature are presented in Figure 3. The data points were analyzed as above, yielding electron self-exchange rates for azurin at 4 °C of $(6.1 \pm 2.6) \times 10^5$ and $(3.2 \pm 0.7) \times 10^5$ M^{-1} s^{-1} at pH 5.0 and 9.0, respectively. Again, bracketing curves calculated as above have been drawn in Figure 3 to check the significance of the data analysis.

Discussion

The values of the electron self-exchange rates of azurin measured in the present study are collected in Table I, together with

the data from previous studies by Ugurbil and Mitra[23] and Groeneveld and Canters.[21,22]

When comparing the present results with the results of the latter two studies, there is agreement in that no clear effect of pH is observed on the magnitude of the electron self-exchange rate. This is in contrast to the effect of pH on the kinetics of the redox reaction between azurin and cytochrome c_{551}, on the basis of which the existence of a redox-active and a redox-inactive form of azurin was postulated.[15] For a detailed discussion of this pH effect the reader is referred to the literature.[18,21-23,31,32]

Regarding the magnitude of the electron self-exchange rate constant there is less agreement between the data in Table I. The room-temperature value of the electron self-exchange rate constant reported in the present study, although less accurate than the data at 4 °C, is clearly much larger (about two orders of magnitude) than the value reported by Ugurbil and Mitra (UM).[23] Even the value of the electron self-exchange rate constant at 4 °C, which has a better precision, is still more than one order of magnitude larger than the UM room-temperature value. On the other hand, the room temperature value of $k = 2.4 \times 10^6$ M^{-1} s^{-1} at pH 5.0 determined with the EPR/rapid-freeze method is in agreement with the values reported by Groeneveld and Canters (GC)[21,22]; under the same conditions of pH and temperature they found values of the electron self-exchange rate constant in the range of $(0.4-1.4) \times 10^6$ M^{-1} s^{-1}, depending on the details of buffer and ionic strength. It is also of interest to compare the more accurate 4 °C data of the present study with the results of GC. For that purpose, the latter data have to be extrapolated to 4 °C. This provides a value of $k = 2 \times 10^5$ M^{-1} s^{-1} at 4 °C (pH 9), which compares favorably with the value of $(3.2 \pm 0.7) \times 10^5$ M^{-1} s^{-1} found in the present study. For the low pH measurements, extrapolation of the GC data only provides a *range* of values at 4 °C of $(0.5-8) \times 10^5$ M^{-1} s^{-1}. Again the value reported in the present study of $(6.1 \pm 2.6) \times 10^5$ M^{-1} s^{-1} falls well within this range. The conclusion to be drawn from the data reported in Table I, therefore, must be that the present study corroborates the data reported earlier by GC.[21,22]

The question that remains is how NMR studies of the same protein can lead to a difference of two orders of magnitude in the experimental electron self-exchange rate. In this respect, it is worthwhile to note that UM based their conclusions on the behavior of the T_1 relaxation time of a His-35 proton, while GC studied the T_2 relaxation times of a proton from a copper–ligand histidine. Further work to clarify this point is in progress.

An intriguing aspect of the electron self-exchange rate of azurin is that it is relatively large even though the copper ion is buried inside the protein. The minimal distance over which the electron must travel from the outside to reach the copper ion is 7 Å. This obtains when the electron enters the protein at the so-called hydrophobic patch, which consists of a cluster of hydrophobic residues grouped around ligand histidine-117. This site is thought to be used in the electron self-exchange reaction of azurin, on the basis of three observations.

1. Electrostatic interactions do not have a conspicuous effect on the electron self-exchange rate.[21,22] This observation is compatible with electron transfer in an encounter complex, in which the azurin molecules associate along their hydrophobic patches.

2. The relatively high entropy of activation observed for the electron self-exchange reaction of azurin can be rationalized on the basis of the same assumption about the structure of the association complex.[21,22]

3. The high self-exchange rate appears compatible with current theories of electron transfer over long distances,[17] when an encounter complex is considered of the type described above. Although the copper–copper distance in such a complex amounts to about 14 Å, the effective electron transfer distance is lowered to 6 Å as a result of the favorable mutual orientation of the His-117 copper ligands, which partly protrude through the hydrophobic patch.[21] The high electron self-exchange rate constant of azurin comes out when a value of 6 Å is inserted for the effective electron transfer distance in the theoretical expressions for the electron transfer rate.[21]

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(31) Adman, E. T.; Canters, G. W.; Hill, H. A. O.; Kitchen, N. A. *FEBS Lett.* **1982**, *143*, 287–292; *Inorg. Chim. Acta* **1983**, *79*, 127–128.

(32) Adman, E. T. *Topics in Molecular and Structural Biology*. Part 1. *Metalloproteins*; Harrison, P., Ed; VCH Verlagsgesellschaft: Weinheim, BRD, 1984; pp 1–42.