Electron Self-Exchange in High-Potential Iron–Sulfur Proteins. Characterization of Protein I from *Ectothiorhodospira vacuolata*†

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ABSTRACT: During previous research on oxidized and reduced high-potential iron–sulfur proteins (HiPIP hereafter), qualitative different electron self-exchange rates were noticed. We have now investigated this phenomenon in detail for HiPIP I and II from *Ectothiorhodospira vacuolata*, which differ significantly in total charge and in which the sequence homology is the largest among all known HiPIPs. We have also characterized the electronic structure of HiPIP I through ^{1}H NMR and EPR spectroscopies to parallel the existing characterization of HiPIP II and other HiPIPs. This investigation has allowed us to propose a model, according to which the productive collisions for electron transfer occur through a hydrophobic patch near the cluster. The effects of total charge and redox potential are considered. The possible formation of dimers through the hydrophobic patch at liquid helium temperature is discussed in light of the EPR spectra.

High-potential iron–sulfur proteins (HiPIPs hereafter) are proteins containing four iron four sulfur clusters in a cubane arrangement (Berg & Holm, 1982; Thomson, 1985), with redox potentials (see Table I) ranging from +450 to +50 mV (Mizrahi et al., 1980; Przysiecki et al., 1985). Although their biological role is not certain, they can at least be investigated as electron-transfer models. The reduced forms contain four irons at the actual oxidation state +2.5, as shown by Mössbauer spectroscopy (Moss et al., 1968; Middleton et al., 1980) and consistent with NMR spectroscopy (Nettesheim et al., 1983; Krishnamoorthi et al., 1986; Sola et al., 1989; Cowan & Sola, 1990; Bertini et al., 1991, 1992a,b, 1993a; Gaillard et al., 1992; Banci et al., 1993a). The ground state is diamagnetic, paramagnetism being introduced by the population of the excited states. The oxidized forms apparently contain two iron(III) ions and two iron ions at the oxidation state +2.5. Again, this is consistent with Mössbauer and magnetic Mössbauer spectroscopy (Dickson et al., 1974; Middleton et al., 1980; Bertini et al., 1993b) and particularly with NMR spectroscopy (Banci et al., 1991a,b 1993a,b; Bertini et al., 1992a,b, 1993a; Nettesheim et al., 1992). The mixed-valence pair seems to have a larger subspin than the two irons at +3. The actual values are difficult to define because of the possible presence of low-symmetry components and of the simplicity of the model based on Heisenberg exchange theory (Blondin & Girerd, 1990). Reasonable numbers are $^9/_2$ and 4 or $^7/_2$ and 3 for the mixed-valence pair and the ferric pair, respectively (Noodleman, 1988; Rius & Lamotte, 1989; Blondin & Girerd, 1990; Mouesca et al., 1991).

Interest in these oxidized proteins stands on the localization of the mixed-valence pair within the protein frame, because this may be related to the overall potential and possibly to the mechanism of electron transfer. Recently it was proposed that the iron ions constituting the mixed-valence pair are bound to Cys 63 and 77 in *Ectothiorhodospira halophila* HiPIP II (*Cromatium vinosum* numbering) (Banci et al., 1993a) and that for other oxidized HiPIPs [*C. vinosum* (Bertini et al., 1992a; Nettesheim et al., 1992), *Rhodopseudomonas globiformis* (Bertini et al., 1993a), *Rhodocyclus gelatinosus* (Bertini et al., 1992b), and *E. vacuolata* HiPIP II (Banci et al., 1993a,b)] an equilibrium may exist between two species in which one iron at oxidation state +2.5 is interchanged with an iron at oxidation state +3, according to Scheme I.

Table I: Global Charges, Reduction Potentials, and Self-Exchange Rates for HiPIPs from Various Bacterial Sources

bacterial source	net charges[a]	no. of residues[b,c,d]	$E_{1/2}$ (mV)[c]	k_{ex} (M^{-1} s^{-1})[e]
E. halophila (iso II)	−13	78	+50	$\leq 5 \times 10^3$
E. vacuolata (iso II)	−6	72	+150	3×10^4
E. vacuolata (iso I)	−3	72	+260	1×10^5
C. vinosum	−3	85	+360	$\simeq 3 \times 10^5$
Rp. globiformis	0	54	+450	$\simeq 3 \times 10^5$
Rc. gelatinosus	+4	75	+330	3×10^{-5}–10^6

[a] Estimated from the known sequences assuming charges of +1 for Lys and Arg, −1 for Asp and Glu, and 0 for His. The primary sequence of HiPIP I and II from *E. vacuolata* and that from *Rp. globiformis* was kindly provided to us by R. P. Ambler (unpublished results). [b] Tedro et al., 1981. [c] Przysiecki et al., 1985. [d] R. P. Ambler, unpublished results. [e] This work.

During the investigation of these proteins through NMR, it was realized that electron-transfer self-exchange in partially oxidized proteins is slow on the NMR time scale, *i.e.*, it is slow with respect with the differences in chemical shifts between oxidized and reduced forms at magnetic fields between 200 and 600 MHz. The differences in shifts are of the order of some tens of parts per million. In this paper we report ^{1}H NMR measurements of self-exchange electron transfer in HiPIPs I and II from *E. vacuolata* at different ionic strengths. We compare our data with the values obtained for other proteins whose biological function as electron carriers is ascertained. We have also analyzed in some detail the ^{1}H NMR spectrum of HiPIP I, whose spectra has already been reported but without assignment (Krishnamoorthi et al., 1986).

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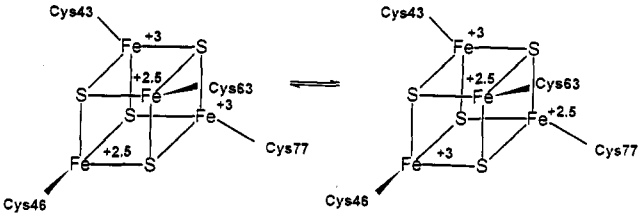
Scheme I



MATERIALS AND METHODS

All chemicals used were of the best quality available. Oxidized HiPIPs I and II were isolated according to the procedure reported for *E. shaposhnikovii* HiPIP (Kusche & Truper, 1984). The 14-T NMR spectra were collected on a Bruker AMX 600 spectrometer, operating at the proton Larmor frequency of 600.14 MHz. NOESY experiments (Macura & Ernst, 1980) were performed using a $2K \times 1K$ data points matrix. Mixing and relaxation delays of 30 and 400 ms, respectively, were used. The same pulse sequence was used to perform an EXSY experiment over a spectral region of 80 ppm (Santos et al., 1984). Mixing and relaxation delays of 6 and 150 ms, respectively, were used. COSY (Bax et al., 1981) and TOCSY (Davis & Bax, 1985) experiments were also performed on both oxidized and reduced species. $2K \times 1K$ data points matrices were collected with use of relaxation delays of 400 ms. A spin-locking period of 30 ms was used for TOCSY experiments. All the saturation-transfer experiments were performed by saturating a signal for 80 ms. To avoid artifacts due to the effects of the selective irradiation, spectra were recorded with selective irradiation in off-resonance positions symmetrical with respect to the saturated signal and subtracted from the spectrum obtained upon saturation of the signal of interest (Ramaprasad et al., 1984; Banci et al., 1989). The resulting difference spectra make it possible to quantify the fractional variation of intensity of the signal experiencing saturation-transfer effects.

Relaxation times were measured by using the inversion recovery method (Vold et al., 1968).

The EPR spectrum of HiPIPs from *E. vacuolata* at 9.55 GHz was collected at 2 K on a Bruker ER 420 EPR spectrometer equipped with an Oxford cryostat. The field calibration was obtained through an NMR gaussmeter. Field modulation was 100 kHz, and the power values ranged from 5 to 20 dB.

Methodological Approach. The self-exchange electron-transfer measurements were performed on partially oxidized samples at pH 6.3. The ionic strength was varied from 2.5 $\times 10^{-2}$ to 0.4 with NaCl. The ^{1}H NMR spectra showed evidence of both oxidized and reduced species. By saturating one signal, *e.g.*, that of the oxidized form, a change in intensity of the corresponding signal of the reduced species was observed. The fractional variation η of signal intensity is related to the pseudo-first-order exchange rate constant k' by the following equation

$$\eta = k'/(R + k') \quad (1)$$

where R is the longitudinal relaxation rate of the signal of the reduced form, of which k' can be regarded as the reciprocal lifetime. In turn, k', in the assumption that the electron-exchange reaction takes place through the second-order kinetic process

$$ox_1 + red_2 \rightleftharpoons red_1 + ox_2 \quad (2)$$

is related to the second-order-rate constant k by the relation

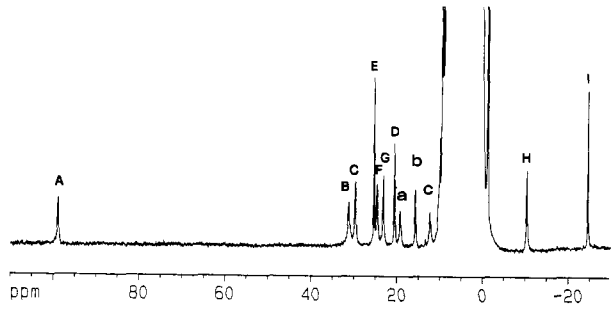


FIGURE 1: ^{1}H NMR spectrum of partially oxidized HiPIP I from *E. vacuolata*, 600 MHz, 300 K, pH 6.3. Signals labeled with uppercase letters arise from the oxidized form; those labeled with lowercase letters arise from the reduced form.

$$k' = k[ox] \quad (3)$$

where [ox] is the concentration of the oxidized species. This assumption is verified, in the present case, by performing the measurements at different protein concentrations in a concentration range spanning a factor of 4 (see below).

Saturation-transfer experiments were performed by irradiating signals of the oxidized species and observing the variation of intensity of the reduced species and vice-versa. The obtained values were properly averaged and multiplied by the concentration of the appropriate species to yield the second-order rate constant values. For each single saturation-transfer experiment, a reference spectrum was recorded and the intensity of each signal measured by integrating peak intensities. The relative amounts of oxidized and reduced species were calculated for each experiment by integrating one or more signals for each of the two species. When far-shifted signals were taken into consideration, their fractional reference intensities due to weaker transmitter power were taken into account. This is done simply by measuring the actual intensities.

The fractional variation of intensity of a signal of, for instance, the reduced species upon saturation of a signal of the oxidized species, η, is obtained on the basis of the following relation

$$\eta = \left[\frac{I_{0red} - I_{red}}{I_{0red}}\right] = \left[\frac{I_{0red} - I_{red}}{I_{0ox}}\right]\left[\frac{I_{0ox}}{I_{0red}}\right] \quad (4)$$

where $(I_{0red} - I_{red})$ and I_{0ox} are the experimental values as obtained from the 1D difference spectra (Ramaprasad et al., 1984), and I_{0ox}/I_{0red} is obtained by integrating peaks in a reference spectrum, as explained before. As the concentrations of oxidized and reduced species may not remain perfectly constant during a saturation-transfer experiment, the reference spectrum used to estimate the amount of oxidized and reduced species is the average between two reference spectra; the first being recorded prior to the saturation-transfer experiment and the second being recorded after the difference spectrum was collected. This procedure was repeated for each of the single saturation-transfer experiments performed.

RESULTS

Signal Assignment of HiPIP I from E. vacuolata. The ^{1}H NMR spectrum of *E. vacuolata* HiPIP I, at pH 6.3, is shown in Figure 1. Hyperfine-shifted signals arising from both the oxidized and the reduced forms of the protein are shown. The exchange connectivities between oxidized and reduced forms as well as the dipolar connectivities among signals of the oxidized forms are observed in the EXSY (NOESY) experiment reported in Figure 2A. As both exchange connectivities

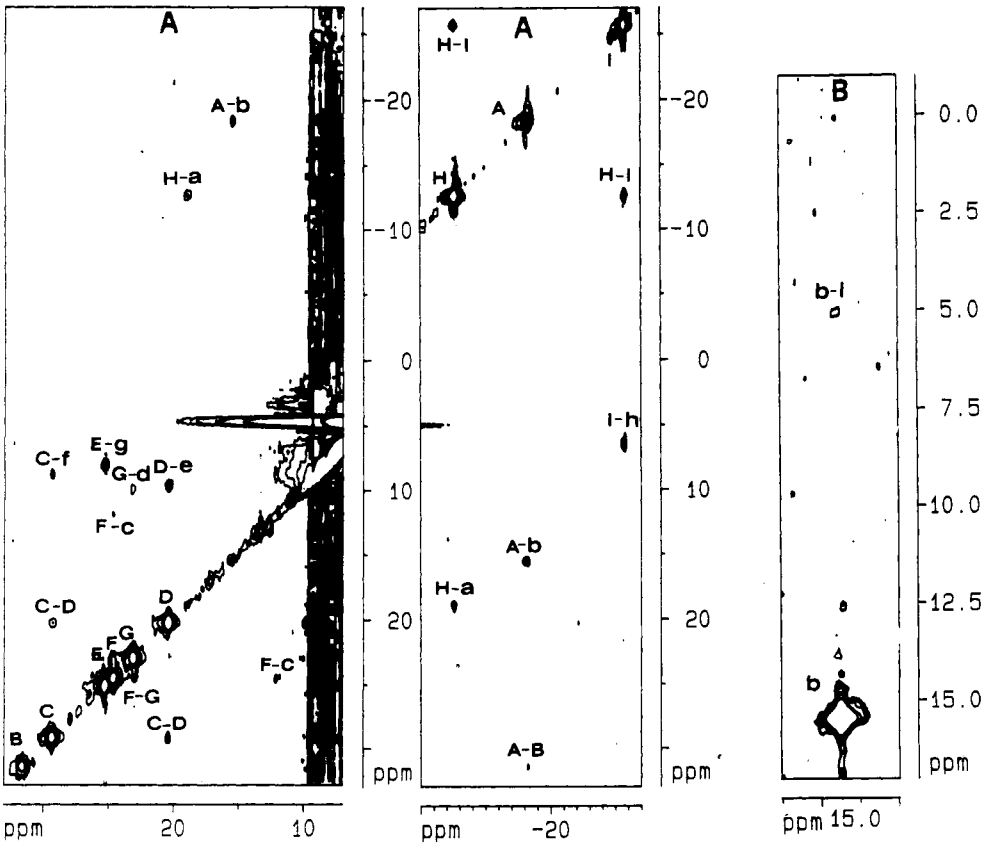


FIGURE 2: (A) ^{1}H NMR EXSY spectrum of ca. 80% oxidized HiPIP I from *E. vacuolata*, 600 MHz, 295 K, pH 6.3. Connectivities indicate the pairwise assignment of the β-CH_2 cysteine protons of the oxidized form (uppercase letters) and the correspondence for each signal from oxidized to reduced species (lowercase letters). Conditions: mixing time 6 ms, relaxation delay 150 ms. 1024 × 240 experiments were acquired, 704 scans each. Squared cosine bell weighting functions were applied in both dimensions prior to zero-filling (1024 × 1024) and Fourier transformation. A spectral window of 44 kHz (73.1 ppm) was used in both dimensions. Under the chosen conditions, the far downfield-shifted signal A is folded into the investigated spectral region at −18.14 ppm. This allows the detection of both scalar and exchange connectivities of signal A. (B) NOESY spectrum of fully reduced HiPIP I from *E. vacuolata*, 600 MHz, 298 K, pH 6.3. The reported region allows us to observe the connectivity between signals b and i of the reduced species. Conditions: mixing time 5 ms, relaxation delay 80 ms. 1024 × 256 experiments were acquired. Squared cosine bell weighting functions were applied in both dimensions prior to Fourier transformation.

and dipolar connectivities are revealed by the same pulse sequence (Ernst et al., 1987), the appropriate choice of the experimental parameters allowed us to observe with a single experiment both types of connectivities. The spectrum of Figure 2 shows that signals A/B, C/D, F/G, and H/I display strong NOE effects within each pair, thus providing evidence that they belong to geminal β-CH_2 protons of iron-coordinated cysteines, as in the case of the corresponding HiPIP II from the same source (Banci et al., unpublished). Signal E is dipolarly connected with C and D, as can be seen by monodimensional NOE experiments (data not shown). Owing to its larger T_1 value (Table II), signal E is assigned to the α-CH of the same cysteine to which signals C and D belong. All of the hyperfine-shifted signals except signal B have an exchange connectivity with the corresponding cysteine proton in the reduced state; this makes straightforward the pairwise assignment of the β-CH_2 protons in the reduced state. A NOESY experiment on the fully reduced derivative, reported in Figure 2B, allows the detection of the dipolar connectivity between signal b (corresponding to signal A in the oxidized form) and signal i, unobserved through EXSY. The T_1 values for the hyperfine-shifted signals in both the oxidized and the reduced states, which have been used for the electron-transfer measurements, are reported in Table II.

Chemical shifts, T_1 values, and line widths of *E. vacuolata* HiPIP I are very close to those of *E. vacuolata* HiPIP II (Banci et al., unpublished), for which the sequence-specific assignments of the cysteine protons have been obtained. Also,

Table II: Chemical Shifts, T_1 Values, and Signal Assignment for Hyperfine-Shifted Signals of the ^{1}H NMR Spectrum of Oxidized and Reduced Forms of HiPIP I from *E. vacuolata* at 295 K[a]

oxidized			reduced			
signal	δ (ppm)	T_1 (ms)	signal	δ (ppm)	T_1 (ms)	assignment[b]
H	−12.96	14	a	19.56	3	cysteine 43 Hβ1
I	−25.88	45	h	6.42	*c*	cysteine 43 Hβ2
G	23.02	10	d	10.05	*c*	cysteine 46 Hβ1
F	24.58	8	c	12.13	3	cysteine 46 Hβ2
B	31.72	4	i	5.05	*c*	cysteine 63 Hβ1
A	99.50	7	b	15.56	5	cysteine 63 Hβ2
D	20.43	19	e	9.82	*c*	cysteine 77 Hβ1
C	29.40	10	f	8.90	*c*	cysteine 77 Hβ2
E	25.33	52	g	8.20	*c*	cysteine 77 Hα

[a] Reported T_1 values were measured at 300 K. Signals are labeled according to previously reported HiPIP II from the same bacterial source. [b] *C. vinosum* HiPIP numbering. [c] Not measured because of overlap in the diamagnetic region.

the patterns of the 1D NOEs from each β-CH_2 protons to signals in the diamagnetic part of the spectrum are very similar to those of the HiPIP II. On the basis of these compelling evidences, we propose that the assignment is the same as that proposed for HiPIP II from *E. vacuolata*; the assignment is reported in Table II.

The upfield signals H and I correspond to the β-CH_2 protons of a cysteine that is bound to an iron which is always in the +3 oxidation state; signals A and B arise from a cysteine bound to an iron which is always in the +2.5 oxidation state;

Table III: Rates of Electron Self-Exchange for *E. vacuolata* HiPIPs at 295 K

[NaCl] (mM)	HiPIP I k_{ex} (M^{-1} s^{-1})	HiPIP II k_{ex} (M^{-1} s^{-1})
	$(8.6 \pm 0.7) \times 10^4$	$(2.9 \pm 1.0) \times 10^4$
50	$(1.4 \pm 0.3) \times 10^5$	
100	$(1.0 \pm 0.3) \times 10^5$	$(3.4 \pm 0.9) \times 10^4$
200	$(9.1 \pm 0.3) \times 10^4$	$(2.6 \pm 0.3) \times 10^4$

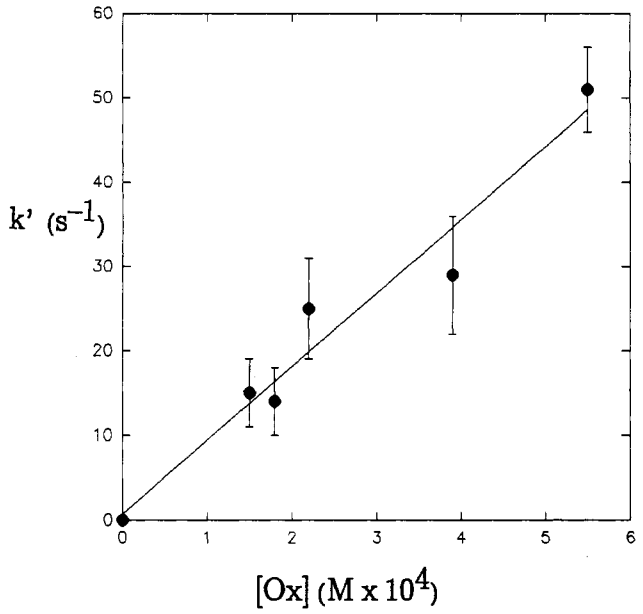


FIGURE 3: Observed values of the pseudo-first-order electron self-exchange rate constant k' in HiPIP I from *E. vacuolata* as a function of the concentration of the oxidized species.

the signals C/D and F/G belong to cysteines bound to iron ions which experience the equilibrium depicted in Scheme I. An estimated amount of cysteine 77 (signals C,D) bound to iron +2.5 is about 60%; this value is similar to that proposed for HiPIP II from *E. vacuolata* (65%) (Banci et al., unpublished). The temperature dependence of the shifts is consistent with the above interpretation. Also, the chemical shift values of all the β-CH_2 protons of the coordinated cysteines in the reduced form are very similar to those of all the other proteins investigated so far (Bertini et al., 1992a,b, 1993a; Nettesheim et al., 1992; Banci et al., 1993a,b).

Electron Self-Exchange. The rate constants for the electron self-exchange for the two HiPIPs are reported in Table III. They are the first values reported for proteins of this class. The second-order rate constant obtained from eqs 1 and 3 remained constant with the concentrations of the whole protein and of oxidized and reduced species, thus justifying the assumptions. Figure 3 shows the pseudo-first-order rate constant k' as a function of the concentration of the oxidized species. Although the single measurements are affected by a sizable experimental error, the linear dependence of k' expected on the basis of eq 3 is clear and unambiguous. The data reported in Table III derive from several measurements. The experimental errors reported arise from (i) uncertainty in the integrals of the signals, (ii) uncertainty in both ratios and absolute values of concentrations, and (iii) uncertainty in the relaxation times. Within the above experimental errors, the second-order rate constants for both proteins are independent of ionic strength. Typical variations of rate constants in cases where ionic strength dependence was observed are around 1 order of magnitude, in particular over this range of ionic strength values (Gupta et al., 1972).

It can also be noted that the two proteins exhibit electron self-exchange rate constants differing by about a factor of 3, again well beyond the experimental error.

The saturation-transfer technique is the only technique capable of measuring interconversion rates of this magnitude.

DISCUSSION

Electron self-exchange reactions of cytochromes *c*, c_{551}, and b_5 (Gupta et al., 1972; Gupta, 1973; Concar et al., 1986, 1991; Dixon et al., 1989, 1990; Timkovich et al., 1988) and copper proteins azurin (Groeneveld & Canters, 1985; Groeneveld et al., 1988; Ugurbil & Mitra, 1984), plastocyanin (Beattie et al., 1975; Armstrong et al., 1985), and amicyanin (Lommen et al., 1988) have been investigated to clarify the influence of structural and/or external factors (pH, ionic strength) on the rates of electron-transfer reactions. In particular, the dependence of self-exchange rate constants k on ionic strength has been the subject of experimental and theoretical studies (van Leeuwen, 1983; Marcus & Sutin, 1985). Cytochromes *c* and b_5 are characterized by exchange rate constants which increase markedly with ionic strength, whereas cytochrome c_{551} and azurin rate constants are independent of ionic strength. Various factors, most of them dependent on the structures of the proteins, have been put forward to rationalize these differences: size and global charge of the protein, hydrophobic character of the areas of the protein which are assumed to interact during the electron transfer, solvent-exposure of the redox active center, and electrostatic monopole–monopole and monopole–dipole interactions between the protein molecules. Values of k for the electron self-exchange rate of the above systems range from about 1×10^3 M^{-1} s^{-1}, observed for cytochromes *c* and b_5 (at low ionic strength) (Concar et al., 1986; Dixon et al., 1989), to about 4×10^7 M^{-1} s^{-1}, observed for cytochrome c_{551} from *Pseudomonas* (Timkovic et al., 1988). Values of 1.3×10^6, 8.9×10^4, and 3×10^3 M^{-1} s^{-1} are observed for azurin (Groeneveld & Canters, 1985), amicyanin (Lommen et al., 1988), and plastocyanin (Armstrong et al., 1985), respectively. The above-mentioned values compare well with the values reported in Table III. When the data are extrapolated at infinite ionic strength, cytochrome b_5 and cytochrome *c* display k values of about 5×10^5 M^{-1} s^{-1}, while azurin and cytochrome c_{551} are unaffected by ionic strength.

We have concentrated our analysis of the self-exchange rates on HiPIPs I and II from *E. vacuolata* because, among all the possible HiPIPs, they have the largest homology, whereas their total charge varies by a factor of 2.

In the case of the two HiPIPs from *E. vacuolata*, the absence of dependence of k on ionic strength can hardly be explained by the global charge present on the two proteins: HiPIP II, which at neutral pH bears a total charge of –6, should show a larger dependence of its k on the ionic strength than HiPIP I, for which the total charge is smaller (–3), but this is not the case. A possible explanation is that electron self-exchange occurs through a hydrophobic patch close to the iron–sulfur cluster. The probable tertiary structure of both HiPIPs from *E. vacuolata* can be reasonable deduced from the X-ray structural data reported in the literature for the HiPIPs of *C. vinosum* (Carter et al., 1974; Freer et al., 1975) and for *E. halophila* HiPIP I (Breiter et al., 1991). The two proteins exhibit similar three-dimensional structures, particularly in the vicinity of the iron–sulfur cluster. Taking into account the even higher sequence homology of *E. vacuolata* and *C. vinosum* HiPIPs (R. P. Ambler, personal communication; Przysiecki et al., 1985), one can safely conclude that the tertiary

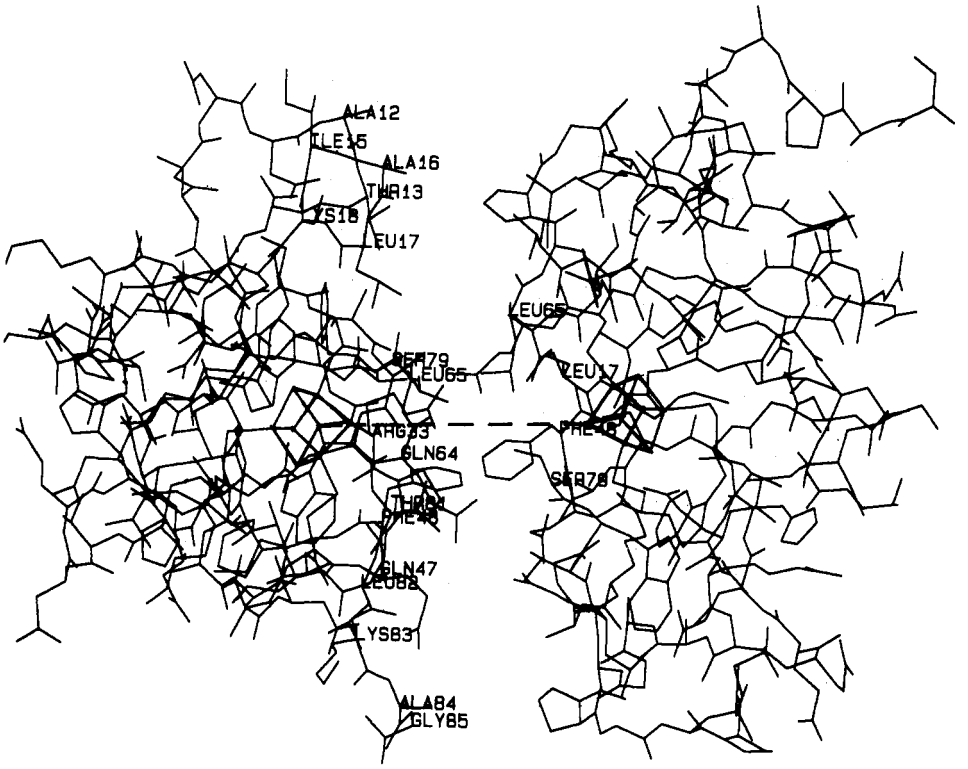


FIGURE 4: Possible approach of two HiPIP molecules. The X-ray structure of *C. vinosum* HiPIP (Carter et al., 1974) has been used in Figure. The distance between the two most exposed sulfide ions of the two clusters is ca. 10.5 Å. In the molecule on the left side are labeled all the residues consituting the hydrophobic patch; in the molecule on the right side are labeled the four residues, lying on the surface of the protein, which are closest to the cluster.

structures of the latter proteins are quite similar. Inspection of the molecular model of *E. vacuolata* HiPIPs obtained by simple substitution of amino acids from the *C. vinosum* HiPIP structure reveals in the immediate vicinity of the iron–sulfur cluster an extended (ca. 13-Å diameter) flat-shaped, hydrophobic patch which appears to be a good candidate as the area of interaction of the protein molecules during electron self-exchange. If this assumption is correct, one expects that the affinity constant for the formation of the dimer is largely determined by hydrophobic interactions. Therefore, the exchange rate would be expected to be unaffected by variations in ionic strength, as long as the total charge is small and the charged amino acid residues are well removed from the interaction area. A similar explanation was put forward (Groeneveld et al., 1988) to explain why the azurin self-exchange rate does not vary with the ionic strength.

Comparison of Self-Exchange Rate Constants among Various HiPIPs. During the investigations of other oxidized and reduced HiPIPs, estimates of the self-exchange rate constants were obtained, although the meaning of such values was not the aim of the research. It was found that significant variations of k occur for the various HiPIPs (Table I). For *E. halophila* HiPIP II, we have an upper limit of ca. 5×10^3 M^{-1} s^{-1}, estimated from the absence of detectable saturation transfer under experimental conditions similar to those of the present work. The other extreme is represented by the HiPIP from *Rc. gelatinosus*. In this case, large saturation transfer effects have been observed through EXSY experiments, which give an estimate of k of about 3×10^5–10^6 M^{-1} s^{-1} (Table I) (estimated from a single measurement).

We will attempt to explain these variations on the basis of two structural factors: the total charge of the protein and the more or less hydrophobic character of the solvent-exposed area in close proximity to the iron–sulfur cluster site.

Considering first the sum of electric charges on the protein, k is expected to increase with a decrease in charge. In agreement with this assumption, Gupta (Gupta et al., 1972; Gupta, 1973) observed that the self-exchange rate of horse heart cytochrome *c*, which bears a +7 charge at pH 7.0, shows a ca. 10-fold increase when going from pH 7 to pH 10, which is the isoelectric point for the protein. On the other hand, azurin, with a −1 global charge, has a k value which is nearly independent of pH in the pH range 5–9 and of ionic strength (Groeneveld & Canters, 1985; Groeneveld et al., 1988). The charges present on HiPIPs vary from −13 to +4: the HiPIP from *E. halophila* II, which has a global charge of −13, indeed has the slowest self-exchange rate, whereas faster exchange rates are observed with the proteins from *Rp. globiformis*, *C. vinosum*, and *Rc. gelatinosus*, which bear charges between 0 and |4|. However, this approximate relationship between the self-exchange rates of HiPIPs and their global charges does not explain why k for the protein from *Rc. gelatinosus* is the largest and why the proteins from *C. vinosum* and *Rp. globiformis* have the same k but different charges. Within this class of proteins, we may consider that the hydrophobic interactions are dominant in productive collisions. Figure 4 shows a possible scheme for the interaction through the hydrophobic part of HiPIPs.

The structural similarities existing within this family of proteins are deduced from the existing X-ray structural studies on *C. vinosum* (Carter et al., 1974; Freer et al., 1975), *E. halophila* HiPIP I (Breiter et al., 1991), and *Rc. tenuis* (Rayment et al., 1992) and from the close resemblance of their NMR spectra (Nettesheim et al., 1983; Krishnamoorthi et al., 1986; Bertini et al., 1991, 1992a, 1993a; Banci et al., 1993a,b). No structural information is available for the HiPIP from *Rp. globiformis*. The amino acids of interest are listed in Table IV. In the HiPIPs from *C. vinosum*, *E. vacuolata*

Table IV: Amino Acid Residues Occurring at the Hydrophobic Patch in the Vicinity of the Cluster[a]

bacterial source	12	13	15	16	17	18	33	47	48	64	65	66	67	79	81	82	83	84	85
C. vinosum	Ala	Thr	Ile	Ala	Leu	Lys	Arg	Gln	Phe	Gln	Leu			Ser	Thr	Leu	Lys	Ala	Gly
E. vacuolata I	Pro	Glu	Leu	Ala	Leu	Asn		Leu	Leu	Ala	Val			Ala	Val	Ala	Arg	Gly	
E. vacuolata II	Ala	Ala	Gln	Ala	Leu	Glu		Leu	Leu	Ser	Val			Ala	Val	Ala	Arg		
R. gelatinosus	Pro	Gln	Val	Ala	Leu	Gly	Lys	Ala	Leu	Pro	Leu			Ala	Ala	Lys	Lys	Ala	
E. halophila II					His		Arg		Phe	Gln	His	Pro	Asp	Val	Ala	Pro	Ala		

[a] This region of the surface is proposed to be the area of interaction of the protein molecules during the self-exchange reaction. Charged residues are underlined. Histidine residues have been considered in this table as charged residues. Residues are labeled according to the *C. vinosum* numbering. Alignment follows the already proposed alignment of the primary sequences (Przysiecki et al., 1985; Breiter et al., 1991; R. P. Ambler, personal communication).

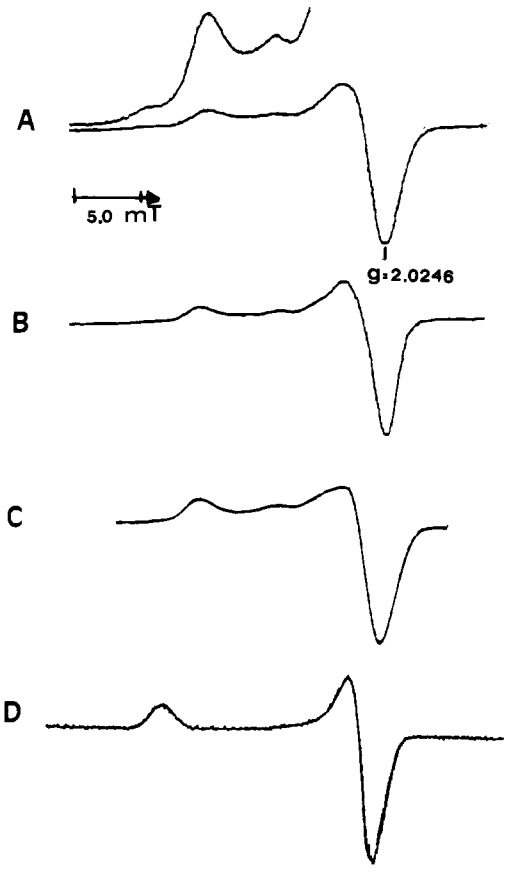


FIGURE 5: X-band EPR spectra of ca. 10^{-4} M solutions of (A) HiPIP I from *E. vacuolata*, (B) HiPIP II from *E. vacuolata*, (C) HiPIP from *C. vinosum*, and (D) HiPIP II from *E. halophila*. Spectra A and B were recorded at 2 K (9.55 GHz), spectrum C was recorded at 16 K (9.20 GHz), and spectrum D was recorded at 10 K (9.42 GHz). Spectrum C was adapted from Dunham et al. (1991) and is reported for comparison purposes.

(I and II), and *Rc. gelatinosus*, the charged residues (*e.g.*, Lys 18, Arg 33, and Lys 83 for the *C. vinosum* HiPIP (Figure 4)) lie at a distance ≥7 Å from the cluster (7.5 Å for Arg 33, 12 Å for Lys 18, 10 Å for Lys 83). Residues 17, 48, 65, and 79 are closest to the cluster and are all (except for Ser 79 in *C. vinosum*) hydrophobic residues (see Table IV). One cluster sulfide (the one bound to the iron ions which are coordinated to Cys 46, Cys 63, and Cys 77, respectively) is at van der Waals contact with at least one hydrophobic residue which, in turn, is capable of van der Waals contact with the same residue at the surface of the other molecule. This could be a reasonable electron-transfer pathway. The X-ray structure of HiPIP from *Rc. tenuis* (Rayment et al., 1992), which has recently appeared, shows two molecules per asymmetric unit oriented approximately as described here. Other residues are shown in Figure 4 and are reported in Table IV for completeness of information but are not discussed.

The HiPIP II of *E. halophila* exhibits a different pattern of amino acids in the area around the iron–sulfur cluster, as shown by the X-ray structure of *E. halophila* HiPIP I (Breiter et al., 1991), which has a large homology with HiPIP II from the same source, as confirmed by molecular dynamics simulations (Banci et al., 1993a). Compared to the previously described proteins, the main differences here concern the presence of two His residues at position-17 and position-65 and an Asp residue at position-67 (again *C. vinosum* numbering). The area is certainly much less hydrophobic, especially as far as the immediate proximity of the iron–sulfur cluster is concerned. Indeed, we have already pointed out that residues 17 and 65 are among the residues which are closer to the cluster. Furthermore, the minimal intercluster distance results in being larger, owing to the presence of bulky groups on the surface. This contributes to the lowering of the self-exchange rate in a similar fashion to that caused by the high overall charge.

It is interesting to note that a protein–protein interaction of the same type as that described here was proposed when the EPR spectra of oxidized HiPIP from *C. vinosum* were interpreted (Dunham et al., 1991). This spectrum (Antanaitis & Moss, 1975), which is similar to those reported for HiPIP II from *E. vacuolata* (Banci et al., 1993b) and for HiPIP I from the same source (Figure 5), has been recently reinterpreted (Dunham et al., 1991) as due to two forms: one in large excess with $g_{\parallel} = 2.11$ and $g_{\perp} = 2.03$ and a second with $g_1 = 2.04$, $g_2 = 2.07$, and $g_3 = 2.13$. The species in excess splits at concentrations of NaCl larger than 1 M. This particular feature was ascribed to the existence of a monomer–dimer equilibrium, the latter species appearing at large NaCl concentration and, of course, at low temperature. Dimerization would occur on the same basis on which we account for self-exchange. The absence of heterogeneity in the EPR spectrum of *E. halophila* HiPIP II would be consistent with the unability of the latter protein to form stable dimers.

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