ϵ_s^p in comparison with ϵ_s^w in eq C4, because the corresponding enthalpy and entropy contributions are in a crucial way determined by the temperature coefficients of both these quantities.

The entropy associated with eq C4 is

$$\Delta S_A^w = -\partial U_r^{us}/\partial T \approx \frac{1}{\epsilon_s^w}\left(\gamma_s^w + 2\frac{\epsilon_s^p}{\epsilon_s^w}\gamma_s^p\right)\left[\frac{1}{4}\frac{z^2e^2}{r_0} + \sum_i \frac{z_i}{r_i}\right] + \gamma_s^w \frac{1}{\epsilon_s^w} ze^2 \sum_i \frac{z_i}{r_i} \approx \gamma_s^w U_r^{us} + 2\frac{1}{\epsilon_s^w}\frac{\epsilon_s^p}{\epsilon_s^w}\gamma_s^p\left(\frac{1}{4}\frac{z^2e^2}{r_0} + \sum_i \frac{z_i}{r_i}\right) \quad (C5)$$

if $\epsilon_s^w \gg \epsilon_s^p$. In eq C5 $\gamma_s^w = d \ln \epsilon_s^w/dT$ and $\gamma_s^p = d \ln \epsilon_s^p/dT$. The enthalpy is

$$\Delta H_A^w = U_r^{us} + T\Delta S_A^w = (1 + T\gamma_s^w)\frac{ze^2}{\epsilon_s^w}\sum_i \frac{z_i}{r_i} + \frac{1}{\epsilon_s^w}\left(1 + T\gamma_s^w + 2\gamma_s^p T\frac{\epsilon_s^p}{\epsilon_s^w}\right)\left[\frac{1}{4}z^2e^2\frac{1}{r_0} + ze^2\sum_i \frac{z_i}{r_i}\right] = U_r^{us}(1 + T\gamma_s^p) + 2\frac{1}{\epsilon_s^w}T\gamma_s^p\frac{\epsilon_s^p}{\epsilon_s^w}\left[\frac{1}{4}z^2e^2\frac{1}{r_0} + ze^2\sum_i \frac{z_i}{r_i}\right] \quad (C6)$$

We notice that since $|T\gamma_s^w| > 1$ and $\gamma_s^w < 0$, the activation enthalpy contribution from the direct interaction with the protein surface charges is negative. The contribution from the second group of terms, i.e. the image terms, must therefore be positive and significant.

Registry No. cyt *c*, 9007-43-6; $[Co(phen)_3]^{3+}$, 18581-79-8; $[Co(phen\text{-}SO_3^-)_3]$, 99747-69-0; Cl^-, 16887-00-6; PO_4^{3-}, 14265-44-2.

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Kinetics of the Oxidation of High-Potential Iron-Sulfur Protein from *Chromatium* by Ferrocenium Derivatives

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Kinetic studies for the oxidation of high-potential iron-sulfur protein from *Chromatium vinosum* by five ferrocenium derivatives are reported. Second-order rate constants for protein oxidation by ferrocenium and its 1,1′-dimethyl, hydroxymethyl, chloromercurio, and phenyl derivatives are $(2.2 \pm 0.1) \times 10^6$ M^{-1} s^{-1}, $(0.35 \pm 0.03) \times 10^6$ M^{-1} s^{-1}, $(14 \pm 1) \times 10^6$ M^{-1} s^{-1}, $(5.1 \pm 0.8) \times 10^6$ M^{-1} s^{-1}, and $(13 \pm 1) \times 10^6$ M^{-1} s^{-1}, respectively, at pH 7.0 (phosphate), $\mu = 0.13$ (phosphate/NaCl), and 25 °C. The protein oxidation by ferrocenium was found to depend upon pH ($pK_a = 6.90 \pm 0.12$) with the protonated protein being about half as reactive as its conjugate base. No rate saturation is observed at high ferrocenium derivative concentration nor is inhibition by either highly positively or negatively charged redox-inactive complexes observed. Three of the reactions have been studied as a function of temperature, and the temperature dependence of the equilibrium constant has been obtained for two of these. These results are used to discuss the site of electron transfer on the protein's surface and to estimate the protein's self-exchange electron-transfer rate constant and activation enthalpy.

High-potential iron-sulfur protein (HiPIP) (*Chromatium vinosum*) is a small (M_R 9300) protein with an Fe_4S_4 active core capable of undergoing one-electron oxidation-reduction with a potential of 0.350 V at pH 7.[1] Its relatively high stability in both oxidation states, a redox potential close to that of many available redox agents, and the availability of the X-ray crystal structure[2] of both the oxidized ($HiPIP_o$) and reduced ($HiPIP_r$) protein have made it an attractive molecule for numerous physical and chemical studies. These studies have included the determination of its redox kinetics with small inorganic reagents[3-7] and with other metalloproteins.[7-10]

This paper reports our studies of the oxidation of $HiPIP_r$ by five ferrocenium ion derivatives. Of particular interest are the nature and location of the reactive site on $HiPIP_r$ for ferrocenium electron transfer, the effect that electrostatic and hydrophobic interactions have on the reaction rate, and the influence of $HiPIP_r$ protonation on its redox activity. In addition, these studies are used to estimate the self-exchange rate constant for HiPIP and its self-exchange enthalpy of activation.

Experimental Section

Laboratory distilled water was further purified by reverse osmosis and ion exchange (Sybron/Barnstead Nanopure). All chemicals were reagent grade or of the highest purity available. Argon gas, passed through two chromous or vanadous scrubbing towers to remove traces of molecular oxygen, was used for preparing anaerobic solutions.

Ferrocene, ferrocenium, their derivatives, and their solutions were prepared as described previously.[11,12] The ferrocenium ion solutions vary greatly in their stability at pH 7.0,[11,12] with (chloromercurio)ferrocenium and 1,1′-dimethylferrocenium solutions being stable for hours while phenylferrocenium undergoes significant decomposition in minutes. Experiments with phenylferrocenium were performed by preparing solutions of its hexafluorophosphate salt in dilute acid, in which phenylferrocenium is much more stable, and bringing these solutions to the desired pH at the time of mixing in the stopped-flow. Phenylferrocenium

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was the only complex in this study requiring this treatment. In all cases, ferrocenium solutions were prepared, filtered, and analyzed immediately before reaction. The ferrocene products of the reactions studied are stable but have very low water solubility. Potassium tetrakis(oxalato)-zirconate(IV), $K_4[Zr(C_2O_4)_4]\cdot 5H_2O$, ($\mu$-amido)bis[pentaamminecobalt-(III)] bromide, $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$, and potassium octacyanomolybdate(IV), $K_4[Mo(CN)_8]\cdot 2H_2O$, were prepared and purified by published methods.[13]

Chromatium vinosum (ATCC 17899) cells were grown anaerobically, and HiPIP was isolated from them by the method of Bartsch[14] with the following modifications. All of the following steps were conducted at 4 °C unless otherwise noted. A 100-g sample of frozen cell paste was thawed with stirring in ca. 250 mL of 0.02 M, pH 8.0, Tris buffer and sonicated for 5 min, and, if necessary, the pH was adjusted to 8.0. A 2-mg sample of DNAase was added, and the suspension was stirred for 15 min followed by centrifugation at 44000*g* for 60 min. The pellet was frozen and saved for an additional extraction as above. The supernatant was dialyzed against 1 mM Tris, pH 8.0, and adsorbed on a column of Whatman DEAE-52 anion-exchange resin equilibrated with 0.020 M Tris, pH 8.0. The HiPIP was rechromatographed on DEAE-52 as necessary to obtain protein with an absorbance (A) ratio at 283–388 nm of $A_{283}/A_{388} = 2.6 \pm 0.1$. Reduced protein solutions were prepared by reduction with a small excess of sodium dithionite or sodium ascorbate followed by extensive anaerobic dialysis. Solutions of fully oxidized protein were prepared, when needed, by oxidation with excess potassium ferricyanide followed by extensive dialysis. Stock solutions of HiPIP were stored frozen in reaction buffer. HiPIP solutions were analyzed spectrophotometrically based on $\epsilon = 1.61 \times 10^4$ M^{-1} cm^{-1} at 388 nm for the reduced protein.

Buffers used included Tris (tris(hydroxymethyl)aminomethane), MES (2-morpholinoethanesulfonic acid), and phosphate (NaH_2PO_4, Na_2HPO_4) and were prepared by adjusting the final pH with NaOH or HCl and the use of a Model 611 Orion pH meter.

Kinetic measurements were made by observing the formation of oxidized protein, $HiPIP_o$, at 480–490 nm where the largest absorbance changes occur. A Durrum model D-110 stopped-flow spectrophotometer interfaced to a Nicolet Model 1090 digital oscilloscope and an Apple II computer was used. Typically four traces from one drive syringe loading were collected per experiment. Each trace consisted of ca. 500–1000 digitized voltages (V) and times. These digitized data were treated by using a nonlinear least-squares program to fit for the observed pseudo-first-order rate constant. The initial and final voltages were fixed at the observed values in this treatment, and a weighting factor that assumed a constant absolute error in the measured voltage was used. In addition, the data acquisition programs provided for the display of $\ln |\Delta V|$ vs. time and these plots were linear for more than 3 half-lives. Kinetic measurements were made with the ferrocenium derivative in at least a 10-fold concentration excess of $HiPIP_r$. In some experiments with 1,1′-dimethylferrocenium as the oxidizing agent the reaction did not proceed to completion, and in runs proceeding to less than 95%, k_{obsd} was obtained from a rigorous, nonlinear least-squares treatment[15] of kinetic data, assuming opposing first- and second-order reactions using the equation derived by King.[16] This equation has the advantage of not being limited to any particular values for initial concentrations. The use of the equation does require knowledge of the equilibrium constant, obtained in these studies by spectrophotometric titration of $HiPIP_r$ at 480 nm by ferrocenium and 1,1′-dimethylferrocenium. Static spectrophotometric measurements were made with a Cary 14 or Hewlett-Packard 8451 spectrophotometer.

Results

The oxidation of $HiPIP_r$ by five ferrocenium derivatives (1,1′-dimethylferrocenium, $Fe(CpMe)_2^+$; ferrocenium, $FeCp_2^+$; (chloromercurio)ferrocenium, $FeCpCpHgCl^+$; (hydroxymethyl)ferrocenium, $FeCpCpCH_2OH^+$; phenylferrocenium, $FeCpCpPh^+$), eq 1, was observed spectrophotometrically at 480

$$HiPIP_r + FeCp_2^{+\prime} \rightarrow HiPIP_o + FeCp_2' \qquad (1)$$

nm where $HiPIP_r$ absorbs more strongly than does $HiPIP_o$. $FeCp_2^{+\prime}$ and $FeCp'_2$ represent the particular ferrocenium and ferrocene derivatives used, respectively. The reduction of the ferrocenium derivatives makes a very small contribution to the absorbance change at this wavelength. All five ferrocenium derivatives were observed to oxidize $HiPIP_r$ at pH 7.0 (0.02 M phosphate), $\mu = 0.13$ M (sodium phosphate/NaCl), with a second-order rate law (eq 2).

$$-\frac{d[HiPIP_r]}{dt} = k_{exptl}[HiPIP_r][FeCp_2^{+\prime}] \qquad (2)$$

Since all kinetic measurements were made with the ferrocenium derivative in a pseudo-first-order concentration excess of $HiPIP_r$, the first-order dependence of reaction rate upon $[HiPIP_r]$ is established by the linearity (greater than 3 half-lives) of the plots of ln $[HiPIP_r]$ vs. time. The first-order dependence upon ferrocenium derivative concentration is established by the linearity of the plots of the pseudo-first-order rate constants, k_{obsd}, vs. initial ferrocenium derivative concentrations as displayed for three of the reactions in Figure 1. No rate saturation is observed for any of the reactions studied. Kinetic data for all five reactions as a function of initial ferrocenium concentration, pH, and temperature are summarized in Table I. Second-order rate constants, k_{exptl}, obtained from least-squares analysis of data displayed in Figure 1 and Table I are summarized in Table II. The uncertainties listed represent 1 standard deviation.

The oxidation of $HiPIP_r$ by $FeCp_2^+$ at 25.0 °C, $\mu = 0.13$, was observed to depend markedly upon pH between pH 5 and 9 as shown in Figure 2. These data are found to conform to the following sequence of reactions, eq 3–5, where H^+P and P are

$$H^+P \overset{K_a}{\rightleftharpoons} P + H^+ \qquad (3)$$

$$H^+P + FeCp_2^+ \xrightarrow{k_{H^+}} \text{products} \qquad (4)$$

$$P + FeCp_2^+ \xrightarrow{k} \text{products} \qquad (5)$$

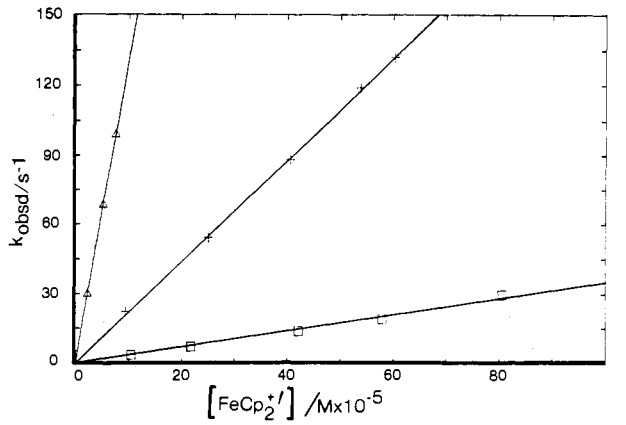

Figure 1. Observed first-order rate constants, k_{obsd}, for the oxidation of $HiPIP_r$ by $Fe(CpMe)_2^+$ (□), $FeCp_2^+$ (+), and $FeCpCpPh^+$ (△) vs. the ferrocenium ion derivative concentration.

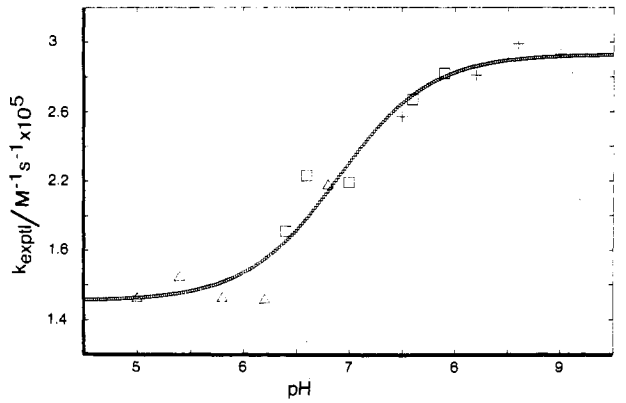

Figure 2. Experimental rate constants, k_{exptl}, for the $FeCp_2^+$ oxidation of $HiPIP_r$, vs. pH buffered with MES (△), phos (□), and Tris (+).

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Table I. Dependence of Observed Rate Constants for the Oxidation of $HiPIP_r$ by Ferrocenium Derivative on Oxidant Concentration, Temperature, and pH[a]

ferrocenium derivative	pH (buffer)[b]	T, °C	$10^5[FeCp_2^+]$, M	k_{obsd}, s^{-1}
$FeCp_2^+$	7.0 (phos)	25.0	9.5	22.2
			25.1	54.2
			40.6	88.3
			53.8	119
			60.3	132
	5.0 (MES)		44.3	67.8
	5.4		40.1	66.2
	5.8		24.2	37.0
	6.2		25.3	38.5
	6.4 (phos)		35.4	67.6
	6.6		35.7	79.6
	6.8 (MES)		36.8	80.2
	7.5 (Tris)		18.3	47.0
	7.6 (phos)		28.1	75.0
	7.9		22.1	62.3
	8.2 (Tris)		35.0	98.4
	8.6		46.1	138
	9.0		53.2	156.0
	7.0 (phos)	2.0	23.2	17.3
			39.0	31.8
			53.4	46.8
		14.5	18.3	24.0
			37.6	51.1
			45.6	60.4
			75.0	94.2
		37.0	14.2	50.4
			23.4	88.7
			35.0	146.0
			47.7	206.0
$Fe(CpMe)_2^+$	7.0 (phos)	25.0	10.5	3.5
			21.8	7.2
			42.2	13.9
			58.0	19.2
			80.6	29.7
		2.0	55.3	6.8
			82.5	9.9
			125.0	16.9
		14.0	44.1	8.3
			76.2	15.4
			107.0	21.7
		37.0	10.1	7.4
			23.9	15.2
			36.9	21.3
			43.1	26.8
$FeCpCpPh^+$	7.0 (phos)	25.0	2.3	30.4
			5.3	68.8
			7.6	99.6
		2.0	3.3	19.7
			5.2	28.1
			8.2	45.2
		14.0	1.53	11.9
			3.62	30.9
			7.74	63.4
			10.3	84.6
$FeCpCpHgCl^+$	7.0 (phos)	25.0	5.85	37.5
			18.1	96.1
			33.9	178
			43.8	253
			54.6	249
$FeCpCpCH_2OH^+$	7.0 (phos)	25.0	2.97	42.8
			5.75	68.9
			7.00	137
			11.7	188
			11.9	179
			16.5	192

[a] All data at μ = 0.13 (0.020 M Buffer, NaCl). [b] Buffers used are sodium dihydrogenphosphate (phos), tris(hydroxymethyl)aminomethane (Tris), 2-morpholinoethanesulfonic acid (MES).

protonated and deprotonated forms of $HiPIP_r$, respectively. $FeCp_2^+$ does not undergo protonation over this pH range. From eq 3–5, eq 6 can be obtained for the pH dependence of k_{exptl}. The

$$k_{exptl} = \frac{k_{H^+}[H^+] + kK_a}{[H^+] + K_a} \quad (6)$$

data displayed in Figure 2 have been fit by least-squares regression to eq 6, yielding $k_{H^+} = (1.51 \pm 0.08) \times 10^5\ M^{-1}\ s^{-1}$, $k = (2.93 \pm 0.07) \times 10^5\ M^{-1}\ s^{-1}$, and $K_a = (1.27 \pm 0.34) \times 10^{-7}$ M ($pK_a = 6.90 \pm 0.12$).

The reaction rates for three of the reactions were also studied as a function of temperature (Table I). The activation enthalpies and entropies obtained from least-squares regression of these data to the Eyring equation for $FeCp_2^+$, $Fe(CpMe)_2^+$, and $FeCpCpPh^+$ oxidations of $HiPIP_r$, respectively, are 7.2 ± 0.3, 7.3 ± 0.3, and 5.4 ± 0.3 kcal/mol and −9.9 ± 0.9, −13.1 ± 1.1, and −12.5 ± 1.0 cal/(mol K) (Table III).

The equilibrium constants, K, for the $FeCp_2^+$ and $Fe(CpMe)_2^+$ oxidations of $HiPIP_r$ have been determined by spectrophotometric titration of $HiPIP_r$ at three temperatures. With $Fe(CpMe)_2^+$ as the oxidizing agent, K is 0.0282 ± 0.0038 at 11.0 °C, 0.0435 ± 0.0016 at 25.0 °C, and 0.0578 ± 0.0063 at 37.0 °C, while for the $FeCp_2^+$ oxidation of $HiPIP_r$, K is 1.65 ± 0.10 at 11.0 °C, 1.94 ± 0.14 at 25.0 °C, and 2.32 ± 0.14 at 37.0 °C. From these data $\Delta H°$ values of 4.7 ± 0.7 kcal/mol for the $Fe(CpMe)_2^+$ reaction and 2.1 ± 0.6 kcal/mol for the $FeCp_2^+$ reaction are obtained.

A limited number of kinetic measurements were made on the $FeCp_2^+$ oxidation of $HiPIP_r$ at pH 7.0, 25.0 °C, and at μ = 0.04 and 0.27 in addition to the measurements already described at μ = 0.13. Derived experimental rate constants were found to be independent of ionic strength over this interval within experimental error.

Some experiments for the $FeCp_2^+$ oxidation of $HiPIP_r$ at pH 7.0, 25.0 °C, and μ = 0.13 were also done in the presence of added $[(NH_3)_5CoNH_2Co(NH_3)_5]Br_5$, $K_4[Zr(C_2O_4)_4]\cdot 5H_2O$, or $K_4[Mo(CN)_8]\cdot 2H_2O$. The +5- and −4-charged ions produced by these salts are redox-inactive under the conditions of these experiments. No effect of the added +5-charged cobalt or −4-charged molybdenum complexes on reaction rate was observed in these studies even with the complex in greater than 100-fold concentration excess of $HiPIP_r$. In the presence of the −4-charged zirconium complex *apparent* inhibition of the rate of electron transfer was observed; however, it was subsequently determined that this complex induces the precipitation of $HiPIP_r$ under the conditions of the experiment and this observation is equivocal.

Discussion

A considerable amount of work has appeared[3-6] detailing the modest but important effects of ionic strength on the redox reactions of HiPIP, especially for redox partners of relatively high charge (ca. ±3). Considering the single positive charge on the reactants used here, the small negative charge (estimated to be −3 at pH 7.0) on $HiPIP_r$,[17] and the absence of regions of charge concentration on the HiPIP surface,[2] it was expected that electrostatic effects would play at most a small role in these reactions. The independence of the experimental rate constant on ionic strength observed for the $FeCp_2^+$ oxidation of $HiPIP_r$ for the limited ionic strength range studied here supports this expectation and suggests that features other than charge are controlling these redox processes.

The observed pH dependence of the $FeCp_2^+$ oxidation of $HiPIP_r$ is attributed to the decreased reactivity of protonated $HiPIP_r$ since $FeCp_2^+$ does not undergo protonation over the pH range of this study. The observed pK_a (6.90 ± 0.12) of this protonation is close to that expected for a histidine residue, of which this peptide has one,[17] His-42.

Proton NMR,[18] kinetic,[3a] and UV–visible spectral studies[3b] of Feinberg and co-workers also indicate that His-42 in $HiPIP_r$ from *C. vinosum* is protonated with a pK_a of 6.9–7.3 and that this

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Table II. Experimental and Calculated Rate Constants for Ferrocenium Derivative HiPIP$_r$ Reactions

ferrocenium derivative	E°,[a] V	$10^{-6}k_{22}$,[b] M^{-1} s^{-1}	ΔE°,[a] V[c]	$10^{-5}k_{exptl}$,[d] M^{-1} s^{-1}	$10^{-3}k_{11}$-(calcd),[e] M^{-1} s^{-1}
$Fe(CpMe)_2^+$	0.30	8.3	−0.05	0.35 ± 0.03	1.1 (3.6)[f]
$FeCp_2^+$	0.38	5.7	0.03	2.2 ± 0.1	2.7 (4.4)[f]
$FeCpCpHgCl^+$	0.39	5.3	0.04	5.1 ± 0.8	11.0
$FeCpCpCH_2OH^+$	0.39	4.2	0.04	14.0 ± 1.0	100.0
$FeCpCpPh^+$	0.44	18.0	0.09	13.0 ± 1.0	3.2

[a] E° values of ferrocenium obtained from ref 11. [b] From ref 21 and 22. [c] Calculated from a value of 0.350 V for HiPIP,[23] and the values in column 2. [d] All data at 25 °C, pH 7.0 (phos), μ = 0.13 (phos, NaCl). [e] Calculated by using eq 8. [f] These values are calculated on the basis of the equilibrium constants measured spectrometrically in this work rather than the E° values given in column 2 and ref 23.

Table III. Activation Parameters for Reactions of Ferrocenium Ion Derivatives with HiPIP$_r$, Plastocyanin, Cytochrome *c*, and Ferrocene Derivatives

ferrocenium derivative	reductant	$\Delta H^\ddagger$, kcal/mol	$\Delta S^\ddagger$, cal/(mol K)
$FeCp_2^+$	HiPIP[a]	7.2 ± 0.3	−9.9 ± 0.9
	plastocyanin[b]	5.5 ± 0.3	−12.6 ± 0.8
	cytochrome *c*[c]	5.0 ± 0.1	−10.6 ± 0.3
	ferrocene[d]	5.6 ± 0.6	−8.9 ± 2.0
	1,1′-dimethylferrocene[e]	3.0 ± 1.0	−15.0 ± 3.0
$Fe(CpMe)_2^+$	HiPIP[a]	7.3 ± 0.3	−13.1 ± 1.1
	plastocyanin[b]	6.7 ± 0.4	−11.7 ± 1.2
	1,1′-dimethylferrocene[d]	5.5 ± 0.2	−8.2 ± 0.7
$FeCpCpHgCl^+$	plastocyanin[b]	5.0 ± 0.4	−13.1 ± 1.5
	cytochrome *c*[c]	4.3 ± 0.2	−11.6 ± 0.8
$FeCpCpPh^+$	HiPIP[a]	5.4 ± 0.3	−12.5 ± 1.0
	plastocyanin[b]	6.2 ± 0.5	−5.9 ± 1.7
	cytochrome *c*[c]	5.5 ± 0.1	−5.7 ± 0.3
	ferrocene[e]	3.0 ± 0.6	−14.4 ± 2.1

[a] This work. [b] Reference 11. [c] Reference 12. [d] Recalculated from data in ref 22. [e] Reference 21.

protonation increases the HiPIP reduction potential by ca. 25–30 mV,[3b,4] thus making oxidation of protonated HiPIP$_r$ less favorable. In addition, Adzamli et al.[5b] have studied the reactions of several complexes with HiPIP$_r$ and find a similar pH dependence (pK_a = 7.1) for the oxidation of HiPIP$_r$ by $Co(4,7\text{-DPSphen})_3^{3-}$ (DPSphen = bis(4-sulfonatophenyl)-1,10-phenanthroline), although in this study at least two reactive sites on HiPIP$_r$ are apparently involved. Using a protein self-exchange rate constant derived from our observed second-order rate constant (1.51×10^5 M^{-1} s^{-1}) for the oxidation of protonated HiPIP$_r$ by ferrocenium, the redox potentials and ferrocenium self-exchange rate constant given in Table II, the relationship of Marcus, eq 7, and an assumed potential for deprotonated HiPIP$_r$ oxidation that is 30 mV higher than that for protonated HiPIP$_r$, we calculated a value of 2.7×10^5 M^{-1} s^{-1} for the second-order rate constant for oxidation of deprotonated HiPIP$_r$ by $FeCp_2^+$. The experimental value for the oxidation of deprotonated HiPIP$_r$ by ferrocenium is 2.9×10^5 M^{-1} s^{-1}. This calculation shows that the observed differences in redox reactivity of the protonated and deprotonated HiPIP$_r$ can be approximately accounted for solely by the presumed effect of His-42 protonation on the HiPIP reduction potential. One difficulty with this view is the observation that the rate of oxidation of HiPIP$_r$ by $[Fe(CN)_6]^{3-}$ at constant ionic strength is independent of pH[4] (between pH 6 and 8) since one expects all oxidations of HiPIP$_r$ to be influenced by pH if this view is correct. This question is addressed in part by the determination[3a] of an apparent pH dependence (pK_a = 7) for this reaction if rate constants are corrected for electrostatic effects, resulting from changes in protein charge, which necessarily accompany changes in pH.

If an active core redox potential shift is the origin of the observed pH dependence, it is notable that protonation of the buried and somewhat remote His-42 can modulate the thermodynamic and kinetic behavior of the Fe_4S_4 redox site in HiPIP in this manner. An explanation,[3b] based on the interpretation of crystallographic[2] and other data, holds that protonation of His-42 results in a slightly less tightly wrapped peptide in the region of the Fe_4S_4 core stabilizing the reduced cluster, which has slightly elongated Fe–S bonds (2.22 Å oxidized; 2.38 Å reduced).[2]

The Marcus equation[19] for outer-sphere electron transfer, eq 7, has frequently been applied to the redox reaction of metalloproteins with small inorganic redox agents.[19a] In eq 7, k_{11} and

$$k_{12} = (k_{11}k_{22}K_{12}f)^{1/2} \quad (7)$$

$$\ln f = (\ln K_{12})^2/(4 \ln (k_{11}k_{22}/Z^2))$$

k_{22} are the electron-transfer self-exchange rate constants, k_{12} is the cross-reaction electron-transfer rate constant, K_{12} is the equilibrium constant, and Z is the collision frequency (10^{11} M^{-1} s^{-1} in these calculations). Serious difficulties can arise in this application due in part to the large size and irregular shape of the protein and to the uneven distribution of charge on its surface. Moreover, if more than one site on a protein's surface is used for electron transfer by a reagent, or by a group of reagents being compared, then the protein will display more than one self-exchange rate constant. In spite of these limitations, considerable insight to metalloprotein electron transfer has been achieved by evaluating the *apparent* protein self-exchange rate constant using eq 7. In particular, Gray and co-workers[6,20] have demonstrated that the *apparent* protein self-exchange rate constants derived from cross-reaction studies depend both on the proximity of the protein's metal to its surface, and to the hydrophobicity and bonding of the ligands in the inorganic partner. In general, hydrophobic ligands that are π-bonded to the metal are more effective at mediating electron transfer to proteins than are hydrophilic, σ-bonded ligands, and the most pronounced dependence on ligand type is found for proteins with buried metal sites. By these criteria, and considering their low charge (+1), the ferrocenium derivatives should be especially effective at accessing the more buried metals in metalloproteins.[11,12] To assess this, the *apparent* HiPIP self-exchange rate constant, k_{11}, is calculated from eq 8 (which is

$$\ln k_{11} = (\ln k_{12} - \tfrac{1}{2}(\ln K_{12}) + \ln Z) - \ln k_{22} - [(\ln Z - \ln k_{12})^2 + (\ln K_{12})(\ln Z - \ln k_{12})]^{1/2} \quad (8)$$

obtained by rearranging eq 7)[21] for each reaction studied. The ferrocenium derivative self-exchange rate constants,[21,22] HiPIP redox potential,[4,23] and ferrocenium derivative potentials,[11] corrected for solvent differences, are available. In addition, K_{12} has been directly measured in this work for two of the reactions. Usually these rate constants would also be corrected to infinite ionic strength so that comparisons with other systems can more readily be made.[20] However, the low charges of the reactants, the observed insensitivity of the $FeCp_2^+$ oxidation of HiPIP$_r$ to changes in ionic strength, and the approximate nature of this calculation make this correction unnecessary. The calculated *apparent* k_{11} values are listed in Table II. With the exception of k_{11} derived from the $FeCpCpCH_2OH^+$ reaction, which gives a k_{11} 10–100-fold higher than the other derivatives, the values are similar, with a mean value of ca. 5×10^3 M^{-1} s^{-1}. This compares with self-exchange rate constants for HiPIP calculated in a similar fashion from other HiPIP cross-reactions of 1 M^{-1} s^{-1}, 10^4 M^{-1} s^{-1}, and 10^{-2} M^{-1} s^{-1} from the reactions of HiPIP with $Fe(CN)_6^{3-}$, $Co(phen)_3^{3+}$, and $Fe(EDTA)^{2-}$, respectively.[5b,20] The value derived here is most similar with that obtained from the $Co(phen)_3^{3+}$ reaction. This, we believe, reaffirms the importance

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of the hydrophobic nature of the ligands and the relative unimportance of charge in accessing the hydrophobic redox center in HiPIP.

An important feature of HiPIP redox is the surface location(s) for electron transfer. The hydrophobic patch where Cys-46 (which is bonded to the Fe_4S_4 cluster) is separated from solvent by Phe-48 and Thr-81 and an Fe_4S_4 sulfur is separated from solvent by Ile-65 is the region of closest proximity (about 4 Å) of the cluster to the protein's surface[2,24] and would seem to be an especially attractive site for the ferrocenium reactions. The absence of rate-limiting kinetics at high ferrocenium derivative concentrations, the absence of an ionic strength dependence, and the ineffectiveness of either negatively or positively charged redox inhibitors[25] also argue against strong association of the ferrocenium derivatives (especially at or near a charged site) as a prerequisite to electron transfer. Weak association at the uncharged, hydrophobic patch would be possible.

The measurement of the equilibrium constants for the $Fe(CpMe)_2^+$ and $FeCp_2^+$ reactions at several temperatures makes possible the comparison of enthalpy changes accompanying these reactions. The more positive $\Delta H°$ value for the $Fe(CpMe)_2^+$ oxidation of $HiPIP_r$ (4.7 kcal/mol) compared with that for the oxidation by $FeCp_2^+$ (2.1 kcal/mol) probably reflects the greater loss in solvation of the more hydrophobic $Fe(CpMe)_2^+$ as it is reduced to $Fe(CpMe)_2$ as compared with the reduction of $FeCp_2^+$ to $FeCp_2$.

The activation parameters obtained are compared with those for other ferrocenium–metalloprotein and ferrocenium–ferrocene reactions in Table III. The similarities of the enthalpies and entropies of activation are remarkable. However, a meaningful comparison can only be made if the enthalpic contributions from the self-exchange rates and reaction driving force are known. Ignoring work terms, a relationship between the activation enthalpies and reaction enthalpy is given approximately by eq 9,[19b]

$$\Delta H_{12}^{\ddagger} = (\Delta H_{11}^{\ddagger} + \Delta H_{22}^{\ddagger})/2 + \Delta H°_{12}/2 \qquad (9)$$

where $\Delta H_{12}^{\ddagger}$ is the cross-reaction enthalpy of activation, $\Delta H_{11}^{\ddagger}$ and $\Delta H_{22}^{\ddagger}$ are the self-exchange activation enthalpies, and $\Delta H°_{12}$ is the enthalpy change of the cross-reaction. Since the reaction enthalpy changes, cross-reaction enthalpies of activation, and self-exchange enthalpies of activation of the ferroceniums are available for the $FeCp_2^+$ and $Fe(CpMe)_2^+$ reactions, the *apparent* HiPIP self-exchange enthalpy of activation can be estimated to be 6.7 kcal/mol and 4.4 kcal/mol, respectively, by using eq 9. This interpretation assumes a single reactive site on HiPIP for the ferrocenium. Similarly, the *apparent* self-exchange enthalpy of activation for spinach plastocyanin based on its reaction with $Fe(CpMe)_2^+$ is estimated to be 4.9 kcal/mol. This similarity of $\Delta H_{11}^{\ddagger}$ for HiPIP and plastocyanin derived from their reactions with $Fe(CpMe)_2^+$ may reflect the ability of $Fe(CpMe)_2^+$ to access hydrophobic sites on both proteins. By a similar analysis using data in Table III and the equilibrium constants and their temperature dependencies reported here and in ref 11, we estimate the protein self-exchange entropies of activation to be ca. −25 cal/(mol K) for plastocyanin and −23 and −28 cal/(mol K) for HiPIP on the basis of its reactions with $FeCp_2^+$ and $Fe(CpMe)_2^+$, respectively. One must be careful in interpreting activation parameters for plastocyanin electron transfer with positively charged reagents since multiple redox sites are known to be involved.[25,26] However, for complexes of low positive charge and high ligand hydrophobicity, or of negative charge, reaction is believed to occur exclusively or nearly so at a hydrophobic site where copper is closest to solvent.[26]

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