to be less valid. More rewarding is the appearance of a maximum in the k_{ED}^{calc} vs μ profile at $\mu \sim 0.3$, the same ionic strength at which the maximum occurs in the experimental data (cf. Table Ic). Thus, the admittedly oversimplified scheme (eq 9 with $k' \sim 0$) is adequate to account for most of the features of the experimental data. We conclude, on the basis of kinetic results obtained here, that the reaction of a multivalent anion with a drug–nucleic acid complex proceeds primarily via a pathway in which the drug molecule first dissociates from the DNA surface and is then attacked by the anion while free in solution.

Acknowledgment. We gratefully acknowledge financial support for this work from the National Science Foundation (Grant CHE 8613592), National Institutes of Health (Grant GM 34676), Monsanto Corp., and SmithKline Beckman Corp. We are pleased also to acknowledge useful conversations with Professor T. A. Stephenson of this department.

Registry No. [Pb(TMpyP)](ClO$_4$)$_4$, 103623-51-4; EDTA, 60-00-4.

Contribution from the Department of Chemistry,
The University, Newcastle upon Tyne NE1 7RU, U.K.

Interpretation of Effects of pH on Electron-Transfer Reactions of *Chromatium vinosum* HIPIP with $[Co(phen)_3]^{3+/2+}$ and $[Fe(CN)_6]^{3-/4-}$ as Redox Partners

R. Timothy Hartshorn, Meng-Chay Lim, and A. Geoffrey Sykes*

Received December 1, 1987

Effects of pH 5.5–9.0 on rate constants for the $[Co(phen)_3]^{3+}$ and $[Fe(CN)_6]^{3-}$ oxidations of $HIPIP_{red}$ and the $[Co(phen)_3]^{2+}$ and $[Fe(CN)_6]^{4-}$ reductions of $HIPIP_{ox}$ have been studied, with the inorganic reactant in large excess. The trends observed yield acid dissociation pK_a values that can be assigned to the only histidine residue (His42). This assignment has been confirmed by studies using diethyl pyrocarbonate (DEPC) His42-modified $HIPIP_{red}$ and $HIPIP_{ox}$. Protonation of His42, which via Cys43 is directly attached to the Fe_4S_4, affects the reduction potential of the HIPIP active site. All the rate constant effects observed can be explained by variations in the reduction potential, supplemented (or otherwise) by the effect that protonation of His42 has on the overall charge and the interaction with the redox partner charge. For such a 1+ charge to be as influential, a reaction site close to His42 may be implicated, but this is not firmly established at this time. From structural information the reduced cluster has elongated Fe–S bond lengths of 2.38 Å (reduced) as compared to 2.22 Å (oxidized). Protonation of His42 results in an increase in $E°$ (30 mV) corresponding to a relative stabilization of $HIPIP_{red}$, consistent with a less tightly wrapped peptide so that expansion of the Fe_4S_4 core can occur more readily. On DEPC modification $E°$ decreases by 15 mV at pH 8.5, and $E°$ is no longer dependent on pH.

Introduction

High-potential iron–sulfur protein (HIPIP) from *Chromatium vinosum* (M_r 9300) has been the subject of extensive characterization.[1,2] It contains a single cuboidal Fe_4S_4 cluster that is coordinated to the S atoms of four cysteines (residues 43, 45, 63, and 77) of a single polypeptide chain (85 amino acids).[3] It has a pI of 3.88, and estimated charge (pH $\sim$ 7) of −3 (two Arg and one His included as positive charges) in the oxidized state. A distinctive feature is the high reduction potential (+350 mV for HIPIP from *C. vinosum*),[4] which is in contrast to that of the structurally closely related ferredoxins (−400 mV).[5] This is directly attributable to different one-electron-redox changes, involving the $Fe_4S_4^{3+/2+}$ (HIPIP) and $Fe_4S_4^{2+/+}$ (ferredoxin) states.[6,7] It is only with very strong reductants, such as e^-_{aq} in pulse radiolysis experiments, that the $Fe_4S_4^+$ superreduced HIPIP form is generated.[8] X-ray crystal structure information has been obtained for the oxidized and reduced states of HIPIP.[9,10] Structural differences that have been proposed to explain the different behavior of Fe_4S_4 clusters in HIPIP and ferredoxin proteins include the buried nature of the cluster in HIPIP and the extent of H-bonding of core S^{2-} ligands.[6]

Kinetic studies on redox reactions of HIPIP with a number of inorganic reagents as well as with other metalloproteins have been reported.[11–20] In all these reactions, it is known that HIPIP serves as a single-electron donor or acceptor. As yet little information has been forthcoming concerning the identity of binding sites for redox partners on HIPIP.

Here we report studies of *C. vinosum* HIPIP with two inorganic redox couples $[Co(phen)_3]^{3+/2+}$ ($E°$ = 370 mV) and $[Fe(CN)_6]^{3-/4-}$ ($E°$ = 410 mV)

$$HIPIP_{red} + [Co(phen)_3]^{3+} \rightleftharpoons HIPIP_{ox} + [Co(phen)_3]^{2+} \quad (1)$$

$$HIPIP_{red} + [Fe(CN)_6]^{3-} \rightleftharpoons HIPIP_{ox} + [Fe(CN)_6]^{4-} \quad (2)$$

in order to gain further insight into the mechanism of reaction and factors affecting reactivity. With the two inorganic oxidants

(1) Averill, B. A.; Orme-Johnson, W. H. *Met. Ions Biol. Syst.* **1978**, *7*, 127.
(2) Yasunobu, K. T.; Tanaka, M. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic: New York, 1973; Vol. 2, pp 29–130.
(3) (a) Dus, K.; Tedro, S.; Bartsch, R. G. *J. Biol. Chem.* **1973**, *248*, 7318. (b) Tedro, S. M.; Meyer, T. E.; Bartsch, R. G.; Kamen, M. D. *J. Biol. Chem.* **1981**, *256*, 731.
(4) Bartsch, R. G. *Methods Enzymol.* **1971**, *23*, 644.
(5) Stombaugh, N. A.; Sundquist, J. E.; Burris, R. M.; Orme-Johnson, W. H. *Biochemistry* **1976**, *15*, 2633.
(6) Carter, C. W. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic: New York, 1977; Vol. 3, pp 157–204.
(7) Carter, C. W., Jr.; Kraut, J.; Freer, S. T.; Alden, R. A.; Sieker, L. C.; Adman, E. T.; Jensen, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3526.
(8) Butler, J.; Sykes, A. G.; Buxton, G. V.; Harrington, P. C.; Wilkins, R. G. *Biochem. J.* **1980**, *189*, 641.
(9) Carter, C. W., Jr.; Kraut, J.; Freer, S. T.; Xuong, N. F.; Alden, R. A. *J. Biol. Chem.* **1974**, *249*, 4212.
(10) Carter, C. W., Jr.; Kraut, J.; Freer, S. T.; Alden, R. A. *J. Biol. Chem.* **1974**, *249*, 6339.
(11) Adzamli, I. K.; Davies, D. M.; Stanley, C. S.; Sykes, A. G. *J. Am. Chem. Soc.* **1981**, *103*, 5543.
(12) Rawlings, J.; Wherland, S.; Gray, H. B. *J. Am. Chem. Soc.* **1976**, *98*, 2177.
(13) Mizrahi, I. A.; Wood, F. E.; Cusanovich, M. A. *Biochemistry* **1976**, *15*, 343.
(14) Nettesheim, D. G.; Johnson, W. V.; Feinberg, B. A. *Biochim. Biophys. Acta* **1980**, *593*, 371.
(15) Przysiecki, C. T.; Cheddar, G.; Meyer, T. E.; Tollin, G.; Cusanovich, M. A. *Biochemistry* **1985**, *24*, 5647.
(16) Feinberg, B. A.; Johnson, W. V. *Biochem. Biophys. Res. Commun.* **1980**, *93*, 100.
(17) Bennett, L. E. In *Iron-Sulfur Proteins*; Lovenberg, W., Ed.; Academic: New York, 1971; Vol. 3, pp 331–380.
(18) Chapman, S. K.; Knox, C. V.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1984**, 2775.
(19) Augustin, M. A.; Chapman, S. K.; Davies, M. D.; Watson, A. D.; Sykes, A. G. *J. Inorg. Biochem.* **1984**, *20*, 281.
(20) Pladziewicz, J. R.; Abrahamson, A. J.; Davis, R. A.; Likar, M. D. *Inorg. Chem.* **1987**, *26*, 2058.

0020-1669/88/1327-4603$01.50/0

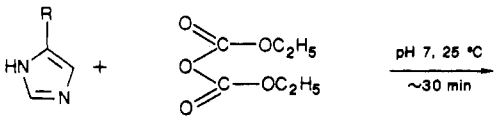
there is a favorable thermodynamic driving force. However by having the inorganic complex in suitable excess, the reverse reactions can also be studied in separate experiments.

Experimental Section

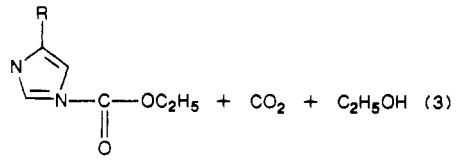
Protein. The HIPIP from *Chromatium vinosum* strain D (NCIB 10441) from the PHLS Centre, Porton, U.K., was isolated and purified as described previously.[4,11] The extracted protein was stored in the reduced form under nitrogen. Concentrations of reduced protein were determined spectrophotometrically by using the absorbance peak at 388 nm ($\epsilon = 1.61 \times 10^4$ M^{-1} cm^{-1}).[4]

The oxidized form of the protein, $HIPIP_{ox}$, was prepared from $HIPIP_{red}$ by oxidizing with a slight excess of $[Fe(CN)_6]^{3-}$. The product was then diafiltered by using an Amicon cell containing a YM5 membrane with at least five changes of buffer. The concentration of $HIPIP_{ox}$ was determined spectrophotometrically at 348 nm ($\epsilon = 2.0 \times 10^4$ M^{-1} cm^{-1}).[4,11] UV–vis spectra of both states have been reported previously.[11]

Modified Protein. To a 4×10^{-5} M solution of oxidized or reduced HIPIP (1 mL) (as required) in 100 mM phosphate at pH 7, a 40-fold excess of diethyl pyrocarbonate (DEPC) (Sigma Chemicals) in ethanol (e.g. 18 μL of 98 mM DEPC) was added.[21] Absorbance changes at 220–320 nm were monitored against a control solution with no DEPC present until no further absorbance changes (peak at 238 nm) were observed (typically ~30 min).[22] The protein was then diafiltered into 2 mM buffer, pH 7.5, $I = 0.10$ M (NaCl), and used in kinetic studies. Degassed buffer was used in the case of the reduced HIPIP. The modification occurring is as in (3). The stability of the DEPC stock protein was checked on completion of the kinetic runs.



diethyl pyrocarbonate (DEPC)



Complexes. Tris(phenanthroline)cobalt(III) trichloride, $[Co(phen)_3]Cl_3 \cdot 7H_2O$, was prepared according to the published procedure.[23] The crude product obtained was recrystallized from a mixture of acetone and 95% ethanol. Tris(phenanthroline)cobalt(II) was prepared in situ by diluting 1 mL of a solution of $CoCl_2 \cdot 6H_2O$ (BDH, AnalaR) (25 mM) to 25 mL with buffer, $I = 0.10$ M (NaCl) and 1,10-phenanthroline. A ratio of phen:Co(II) of 6 was used to ensure that formation of the tris complex was essentially complete. Potassium hexacyanoferrate(III), $K_3[Fe(CN)_6]$ (BDH, AnalaR), peak at 420 nm ($\epsilon = 1010$ M^{-1} cm^{-1}), and potassium hexacyanoferrate(II), $K_4[Fe(CN)_6] \cdot 3H_2O$ (Hopkin and Williams, AnalaR), peak at 320 nm ($\epsilon = 320$ M^{-1} cm^{-1}), were used without further purification.

Kinetics. All kinetic runs were at 25 °C with ionic strength adjusted to 0.10 M with sodium chloride (BDH, AnalaR). A pH-jump method was employed in which the protein solution, at pH 7.5 with 2.0×10^{-3} M buffer, was mixed on the stopped-flow apparatus with a solution of complex containing 2.0×10^{-2} M buffer at the required pH. In this way protein was conserved. The pH of solutions was determined by using a Radiometer pH-meter Model PHM62 complete with a combination electrode. Buffers used were Mes/NaOH where Mes is 2-morpholinoethanesulfonic acid, pH 5.5–6.5; Hepes/NaOH where Hepes is *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid, pH 6.5–7.5; and Tris/HCl where Tris is tris(hydroxymethyl)aminoethane, pH >7.2 (all from Sigma Chemicals).

One-equivalent interconversions of $HIPIP_{red}$ and $HIPIP_{ox}$ were observed, consistent with 1:1 stoichiometries. Reactions were monitored on a Dionex stopped-flow spectrophotometer at 480 nm, which gives the largest difference in absorbance between $HIPIP_{red}$ and $HIPIP_{ox}$. A large excess (>10-fold) of complex was used in all runs with protein concentrations of $(0.78–2.1) \times 10^{-5}$ M. Plots of absorbance (A) changes, e.g. ln $(A_t - A_\infty)$ against time, were linear to 3–4 half-lives and gave first-order rate constants k_{obs}. Experimental data were acquired by means of a transient recorder, and on-line analysis was performed with the aid of a PET Commodore computer. Fitting procedures in programs from On Line Instrument Systems (Jefferson, GA), run on an IBM PC/AT-X computer, were also used.

Table I. Summary of Rate Constants (25 °C) for the Reactions of *Chromatium vinosum* HIPIP with $[Co(phen)_3]^{3+/2+}$ and $[Fe(CN)_6]^{3-/4-}$ as Redox Partners, $I = 0.10$ M (NaCl)

	(i) $HIPIP_{red} + [Co(phen)_3]^{3+}$ [a]
pH	5.34, 5.78, 6.10, 6.50, 7.00, 7.51, 7.99, 8.51
$10^{-3}k/M^{-1}$ s^{-1}	1.8, 1.95, 2.20, 2.42, 3.04, 3.52, 3.99, 4.21
	$HIPIP_{red}(mod) + [Co(phen)_3]^{3+}$
pH	5.47, 6.51, 7.05, 7.48, 7.91, 8.45
$10^{-3}k/M^{-1}$ s^{-1}	6.9, 6.8, 6.8, 6.8, 6.8, 6.9
	(ii) $HIPIP + [Fe(CN)_6]^{3-}$ [b]
pH	5.50, 5.50, 6.00, 6.50, 7.00, 7.25, 7.50, 7.75, 8.00, 8.25, 8.45, 9.00
$10^{-3}k/M^{-1}$ s^{-1}	2.41, 2.24, 2.02, 2.06, 2.09, 2.10, 2.15, 2.20, 2.20, 2.31, 6.9, 2.34
	$HIPIP_{red}(mod) + [Fe(CN)_6]^{3-}$
pH	5.61, 6.41, 7.00, 7.60, 7.96, 8.54
$10^{-3}k/M^{-1}$ s^{-1}	3.00, 2.84, 2.88, 3.00, 2.91, 2.93
	(iii) $[Co(phen)_3]^{2+} + HIPIP_{ox}$ [c]
pH	5.50, 6.00, 6.50, 7.00, 7.50, 8.50
$10^{-3}k/M^{-1}$ s^{-1}	1.91, 1.70, 1.60, 1.45, 1.29, 1.26
	$[Co(phen)_3]^{2+} + HIPIP_{ox}(mod)$
pH	5.50, 6.03, 6.51, 7.53, 8.45
$10^{-3}k/M^{-1}$ s^{-1}	1.10, 1.15, 1.15, 1.13, 1.11
	(iv) $[Fe(CN)_6]^{4-} + HIPIP_{ox}$ [d]
pH	5.50, 6.00, 6.50, 7.00, 7.50, 8.00, 8.50, 9.00
$10^{-2}k/M^{-1}$ s^{-1}	4.20, 3.41, 2.78, 2.07, 1.73, 1.50, 1.46, 1.37
	$[Fe(CN)_6]^{4-} + HIPIP_{ox}(mod)$
pH	5.56, 6.08, 6.54, 7.52, 8.80
$10^{-2}k/M^{-1}$ s^{-1}	1.03, 1.04, 0.99, 1.06, 1.09

[a] $[Co(phen)_3^{3+}]$ in range $(2.2–3.0) \times 10^{-4}$M; [protein] = 1.5×10^{-5}M. [b] $[Fe(CN)_6^{3-}]$ in range $(4.9–6.2) \times 10^{-4}$M; [protein] = 2.1×10^{-5}M. [c] $[Co(phen)_3^{2+}] = 5.0 \times 10^{-4}$M; [protein] = 1.6×10^{-5}M. [d] $[Fe(CN)_6^{4-}]$ in range $(1.08–1.30) \times 10^{-3}$; [protein] = 1.6×10^{-5}M.

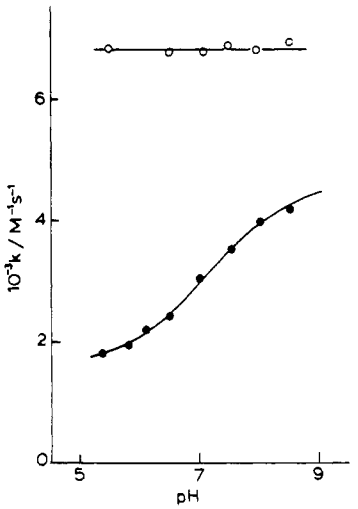


Figure 1. Variation of rate constants (25 °C) with pH for the $[Co(phen)_3]^{3+}$ oxidation of *C. vinosum* $HIPIP_{red}$ (●) and DEPC-modified $HIPIP_{red}$ (○) at $I = 0.10$ M (NaCl).

Results

Second-order rate constants k were obtained from experimental first-order k_{obs} values in accordance with rate law 4. It has

$$\text{rate} = k_{obs}[\text{protein}] = k[\text{complex}][\text{protein}] \quad (4)$$

previously been demonstrated that there is no saturation kinetics for the reactions with $[Co(phen)_3]^{3+}$ and $[Fe(Cp)_2]^+$.[12,20] Table I lists rate constants k for the $[Co(phen)_3]^{3+}$ and $[Fe(CN)_6]^{3-}$ oxidation of $HIPIP_{red}$, and $[Co(phen)_3]^{2+}$ and $[Fe(CN)_6]^{4-}$ reduction of $HIPIP_{ox}$.

(21) Miles, E. W. *Methods Enzymol.* **1977**, *47*, 431.
(22) Jackman, M. P.; Lim, M.-C.; Osvath, P.; de Silva, D. G. A. H.; Sykes, A. G., submitted for publication.
(23) E.g.: Segal, M. G.; Sykes, A. G. *J. Am. Chem. Soc.* **1978**, *100*, 4585.

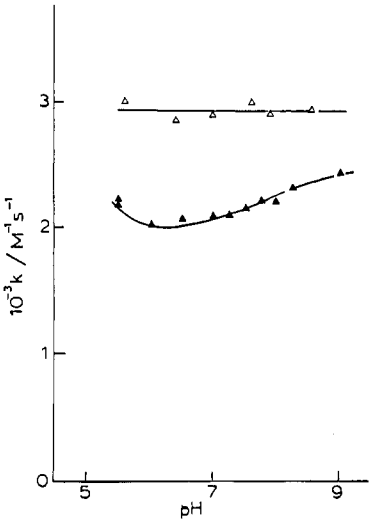


Figure 2. Variation of rate constants (25 °C) with pH for the $[Fe(CN)_6]^{3-}$ oxidation of *C. vinosum* $HIPIP_{red}$ (▲) and DEPC-modified $HIPIP_{red}$ (△) at I = 0.10 M (NaCl).

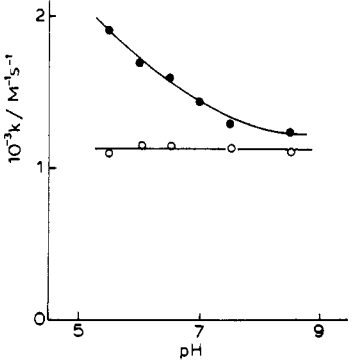


Figure 3. Variation of rate constants (25 °C) with pH for the $[Co(phen)_3]^{2+}$ reduction of *C. vinosum* $HIPIP_{ox}$ (●) and DEPC-modified $HIPIP_{ox}$ (○) at I = 0.10 M (NaCl).

With $[Co(phen)_3]^{3+}$ as oxidant a well-defined effect of pH is observed (Figure 1). The dependence can be fitted to expression 5, which is derived from the reaction sequence (6)–(8), where P

$$k = \frac{k_1[H^+] + k_2K_a}{K_a + [H^+]} \quad (5)$$

$$H^+P \rightleftharpoons H^+ + P \quad (6)$$

$$H^+P + C \xrightarrow{k_1} \text{products} \quad (7)$$

$$P + C \xrightarrow{k_2} \text{products} \quad (8)$$

and C represent the protein and complex, respectively. The fit gives $k_1 = (1.84 \pm 0.06) \times 10^3$ M^{-1} s^{-1}, $k_2 = (4.22 \pm 0.08) \times 10^3$ M^{-1} s^{-1}, and $K_a = (1.0 \pm 0.2) \times 10^{-7}$ M, corresponding to $pK_a = 7.0 \pm 0.1$.

With $[Fe(CN)_6]^{3-}$ as oxidant all rate constants pH 5.5–9.0 lie within a band ±8% (Figure 2). The much milder trend with pH 6.5–9.0 can be fitted to (5), which gives $k_1 = (2.00 \pm 0.08) \times 10^3$ M^{-1} s^{-1}, $k_2 = (2.38 \pm 0.08) \times 10^3$ M^{-1} s^{-1}, and $K_a = (1.66 \pm 1.0) \times 10^{-8}$ M, corresponding to a pK_a of 7.8 ± 0.3. Acceptable fits are obtained with a pK_a of 7.0, the pK_a obtained with $[Co(phen)_3]^{3+}$.

Rate constants for the $[Co(phen)_3]^{2+}$ and $[Fe(CN)_6]^{4-}$ reductions of $HIPIP_{ox}$ increase with decreasing pH; see Figures 3 and 4, respectively. Due to protein instability at pH <5 a lower pH limit of 5.5 was adopted, and for this reason pK_a values are less well defined. However the pK_a is clearly less than 7 in each case, with values in the range 6–7 likely.

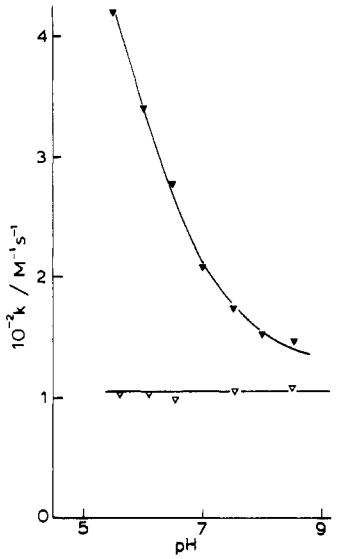


Figure 4. Variation of rate constants (25 °C) with pH for the $[Fe(CN)_6]^{4-}$ reduction of *C. vinosum* $HIPIP_{ox}$ (▼) and DEPC-modified $HIPIP_{ox}$ (▽) at I = 0.10 M (NaCl).

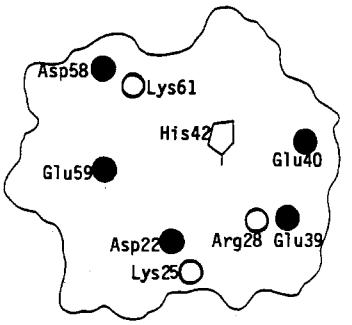


Figure 5. Proximity of acidic and basic residues to the His42 of *C. vinosum* HIPIP, from X-ray structural information by Carter and colleagues.[9,10]

Rate constants for DEPC-modified $HIPIP_{red}$ and $HIPIP_{ox}$ are also listed in Table I. In all cases there is now no dependence on pH over the range 5.5–8.5 (Figures 1–4). Since protonation of His42 can no longer occur, this confirms that protonation at His42 is relevant in the studies on native protein. With $HIPIP_{ox}$ it was found that a 20-fold excess of DEPC gave only ~80% modification of $HIPIP_{ox}$.

Discussion

The dependence of rate constants on pH in the range 5.5–8.5 for the $[Co(phen)_3]^{3+}$ oxidation of native $HIPIP_{red}$ gives a pK_a of 7.0. Such a value implicates the only histidine present, His42, which is the most likely amino acid to have a pK_a in this region. Proton NMR, kinetic and UV–vis spectrophotometric pK_a values of 6.9–7.3 have been obtained previously for this histidine on $HIPIP_{red}$.[14,16,24] Likewise rate constants for the ferrocenium ($[Fe(Cp)_2]^+$) oxidation of $HIPIP_{red}$ yield a pK_a of 6.9.[20] Protonated His42 on $HIPIP_{red}$ is about half as reactive as the unprotonated form with both $[Co(phen)_3]^{3+}$ and $[Fe(Cp)_2]^+$ as oxidants. Both trends are consistent with the electrostatics. The possibility that charge as well as aromatic character of the ligands on both complexes is determining a specific site selectivity close to His42 has been considered. No rate saturation behavior is observed with either $[Co(phen)_3]^{3+}$ or $[Fe(Cp)_2]^+$, and in the latter case it has been demonstrated that there is no inhibition by the highly charged redox-inactive complex $[(NH_3)_5CoNH_2Co(NH_3)_2]^{5+}$.[20] There is therefore no evidence for extensive asso-

(24) Nettesheim, D. G.; Meyer, T. E.; Feinberg, B. A.; Otvos, J. D. *J. Biol. Chem.* **1983**, *258*, 8235.

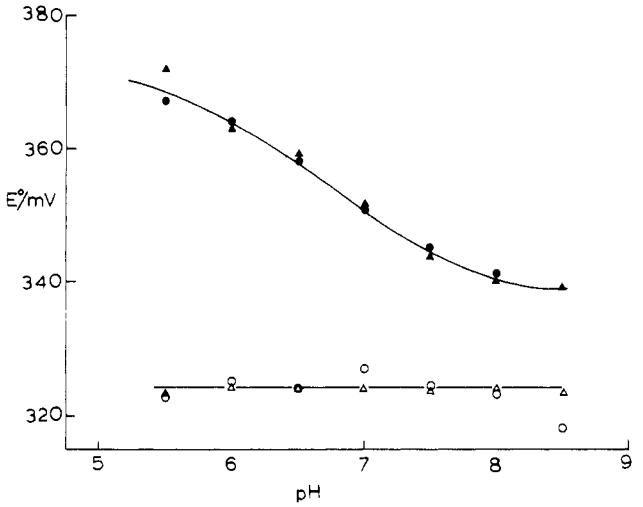


Figure 6. variation of reduction potential $E°$ (25 °C) for *C. vinosum* HIPIP (upper curve) and DEPC-modified HIPIP (lower curve) from rate constants with $[Co(phen)_3]^{3+/2+}$ (▲, △) and $[Fe(CN)_6]^{3-/4-}$ (●, ○) as redox partners, I = 0.10 M (NaCl).

ciation of the 5+ ion with the protein at the $[Fe(Cp)_2]^+$ reaction site, and it can be concluded that this site is not of high (negative) charge density. From a computer graphics space-filling model of $HIPIP_{ox}$,[9,10] we note that although His42 is circled by negatively charged residues, these are somewhat remote (Figure 5), with distances of α-carbon atoms from the imidazole of His42 as follows: Glu40, 6.8 Å; Glu39, 8.4 Å; Asp22, 10.4 Å; Glu59, 11.1 Å; Asp58, 13.0 Å. There are moreover positively charged Lys61, Arg28 and Lys25 residues in this same region.

It is now clear that rate constants for the $[Fe(CN)_6]^{3-}$ oxidation of $HIPIP_{red}$ exhibit a similar but much smaller dependence on pH (Figure 2). The trend between pH 6.5 and 9 gives pK_a ~7.8, with a satisfactory fit also for pK_a 7.0, clearly making His42 involvement a strong possibility. The behavior observed suggests an interpretation in which the active site reduction potential ($E°$), determined by protonation of His42 and favoring reaction at the higher pHs, is supplemented (or otherwise affected) by charge interactions from the same protonation. Thus for $HIPIP_{red}$ with $[Fe(CN)_6]^{3-}$ an overall charge of 5– becomes 4– on protonation of His42, and $E°$ and the electrostatic effects are in opposite directions, while with $[Co(phen)_3]^{3+}$ the two reinforce each other.

For this interpretation to be acceptable it must also be able to rationalize the behavior observed for the corresponding $[Co(phen)_3]^{2+}$ and $[Fe(CN)_6]^{4-}$ reductions of $HIPIP_{ox}$. From NMR studies His42 on $HIPIP_{ox}$ has a pK_a of 6.7,[24] which is clearly acceptable as far as the rate constant profiles in Figures 3 and 4 are concerned. Also the amplitude of the response with $[Fe(CN)_6]^{4-}$ is greater than that for $[Co(phen)_3]^{2+}$, an effect which electrostatic interactions is able to explain.

Consistent with the above interpretation, on DEPC modification of HIPIP, rate constants in all cases become independent of pH (Figure 1–4). There is therefore no doubt that protonation of His42 is responsible for the pH effects observed. An interesting point to note is the separation of rate constants for native and DEPC-modified $HIPIP_{red}$ at high pH (Figures 1 and 2), suggesting an effect of modification at the active site of $HIPIP_{red}$.

From these studies it would appear that effects of DEPC modification as well as protonation of His42 are transmitted to the active site of HIPIP. This is not perhaps surprising in view of the direct connection of His42 via Cys43 to the Fe_4S_4 cluster, which is an unusual feature of this protein. Variations in reduction potential are able to influence reactivity whether or not the redox partners use the same reaction site, and whether or not this site is near to His42. The ratio of rate constants for the $[Co(phen)_3]^{3+,2+}$ and $[Fe(CN)_6]^{3-,4-}$ reactions respectively at different pHs can be converted into $\Delta E°$ values. Since the inorganic reagents do not exhibit protonation in the range pH 5–9 these variations are directly attributable to variations in protein $E°$ (Figure 6). Protonation increases the HIPIP reduction potential by 30 mV in agreement with previous studies.[14,25] The reactivity of both the unmodified $HIPIP_{red}$ and $HIPIP_{ox}$ states is affected. Superimposed on this, as already indicated, there are electrostatic effects as His42 protonates or deprotonates. A change in charge of 1+ would not normally be expected to be as influential as here observed unless His42 were located close to the site of the reaction. The distance separating the imidazole N^{ϵ} of His42 and the nearest μ_3-S of the Fe_4S_4 core is ~8 Å,[26] which is a reasonable distance for electron transfer. However in their studies on $[Fe(Cp)_2]^+$ Pladziewicz and colleagues[20] have expressed a preference for reaction at the hydrophobic patch close to Cys46 (which is also coordinated to the Fe_4S_4). This residue is separated from solvent by Phe48 and Thr81, and a μ_3-S of Fe_4S_4 is shielded by Ile 65. The distance to the surface is only 4 Å, representing the closest approach possible for the two reaction centers.[20]

Also relevant to this discussion, it has been shown in previous studies that in the oxidation of $HIPIP_{red}$ the complex $[Co(4,7\text{-}DPSphen)_3]^{3-}$ (4,7-DPSphen is 4,7-bis(4-sulfonatophenyl)-1,10-phenanthroline), which binds strongly to the protein (K = 3420 M^{-1}), does not interfere with the faster reaction with $[Fe(CN)_6]^{3-}$ as oxidant.[11] Therefore these two reactants do not appear to use the same reaction site. Interestingly rate constants for the $[Co(4,7\text{-}DPSphen)_3]^{3-}$ oxidation of $HIPIP_{red}$ vary significantly with pH (pK_a 7.1) and are a factor of ~3 greater at the higher pHs[11], which is comparable to rate constants of $[Co(phen)_3]^{3+}$ rather than $[Fe(CN)_6]^{3-}$. This suggests that the six remote sulfonatophenyl groups retain in large part the negative charge, allowing the remaining $Co(phen)_3^{3+}$ part of the complex to behave as a 3+ moiety.[27] The association constant of 3420 M^{-1} between complex and protein is (presumably) attributable to the high aromatic character of the complex.

To summarize, there is at this time no firm evidence regarding the precise location of reaction sites for inorganic redox partners on HIPIP. Over the pH ranges investigated, reduction potential ($E°$) and electrostatic effects determined by the overall charge are influential. Effects of protonation and DEPC modifications of His42 are manifest at the active site and consistent with relative stabilization of $HIPIP_{red}$ (increase in $E°$) and $HIPIP_{ox}$ (decrease in $E°$) respectively.

Acknowledgment. We thank Dr R. Hubbard at the University of York for help with the computer graphics, the University of Malaya, Kuala Lumpur, for a leave of absence to M.-C.L., and the U.K. Science Research Council for a studentship to R.T.H.

(25) Mizrahi, I. A.; Meyer, T. E.; Cusanovich, M. A. *Biochemistry* **1980**, *19*, 4727; **1976**, *15*, 343.
(26) See, e.g.: Jackman, M. P.; Lim, M.-C.; Salmon, G. A.; Sykes, A. G. *J. Chem. Soc., Chem. Commun.* **1987**, 179.
(27) Anomalous effects are sometimes observed with this oxidant: Chapman, S. K.; Davies, D. M.; Watson, A. D.; Sykes, A. G. *ACS Symp. Ser.* **1983**, *No. 211*, 184.