Kinetic Studies on Reactions of Iron–Sulfur Proteins. 4. Redox Reactions of *Chromatium vinosum* HIPIP with $Co(4,7\text{-DPSphen})_3^{3-}$, $Mn(CyDTA)(H_2O)^-$, and $Fe(CN)_6^{3-}$ (Oxidants) and $Fe(CN)_6^{4-}$ (Reductant)†

I. Kofi Adzamli, D. Martin Davies, Carmel S. Stanley, and A. Geoffrey Sykes*

Contribution from the Department of Inorganic Chemistry, The University, Newcastle upon Tyne, NE1 7RU, England. *Received July 7, 1980*

Abstract: Limiting kinetics are observed for the $Co(4,7\text{-DPSphen})_3^{3-}$ oxidation of HIPIP(r) to HIPIP(o), consistent with association, $K(25\ °C) = 3420\ M^{-1}$, $\Delta H° = -8.9$ kcal mol^{-1}, and $\Delta S° = -13.7$ cal K^{-1} mol^{-1}, prior to electron transfer, $k_{et} = 0.020\ s^{-1}$, $\Delta H^{\ddagger}_{et} = 15.9$ kcal mol^{-1}, and $\Delta S^{\ddagger}_{et} = -12.9$ cal K^{-1} mol^{-1}. The oxidation with $Mn(CyDTA)(H_2O)^-$ gives second-order rate constants $k\ (=Kk_{et}) = 1.26 \times 10^3\ M^{-1}\ s^{-1}$ at 25 °C, $\Delta H^{\ddagger} = 3.6$ kcal mol^{-1}, and $\Delta S^{\ddagger} = -27.7$ cal K^{-1} mol^{-1}. This reaction is remarkably slow for the high driving force ($\Delta E° = 410$ mV) consistent with high reorganization requirements for reduction of the Mn(III) complex. The reduction of HIPIP(o) with $Fe(CN)_6^{4-}$ has been investigated over a wide range of complex concentrations, second-order rate constant $k\ (=Kk_{et}) = 2.4 \times 10^2\ M^{-1}\ s^{-1}$, with no evidence for limiting kinetics ($K < 10\ M^{-1}$). Further studies on the oxidation of HIPIP(r) with $Fe(CN)_6^{3-}$ have also been carried out. Rate constants are independent of pH (6–9), whereas those for the oxidation with $Co(4,7\text{-DPSphen})_3^{3-}$ give a pK_a of 7.1, which suggests that a different site is being used. With $Mn(CyDTA)(H_2O)^-$ as oxidant a pK_a of 7.74 is obtained, which is assigned to acid dissociation of the Mn(III) coordinated H_2O. The oxidations with $Mn(CyDTA)(H_2O)^-$ and $Fe(CN)_6^{3-}$ both proceed unaffected by the presence of $Co(4,7\text{-DPSphen})_3^{3-}$, which for the concentrations used does not contribute to the oxidation but binds to >50% of the protein. It can be concluded therefore that $Co(4,7\text{-DPSphen})_3^{3-}$ associates at a different site on the protein to the other two complexes. Ionic strength effects on rate constants are also considered. Calculated self-exchange rate constants for HIPIP are relatively small for a protein, reflecting the buried (ca. 4.5 Å) nature of the Fe_4S_4 cluster.

High-potential iron–sulfur proteins (HIPIP) have been the subject of extensive characterization.[1,2] They contain a single cubane-like Fe_4S_4 cluster[3] coordinated to four cysteinyl sulfurs of a single peptide chain (M_r = ca. 9500) and structurally have similar features to the Fe_4S_4 clusters in ferredoxins. The HIPIP which has been most extensively investigated is that isolated from the photosynthetic purple sulfur bacteria *Chromatium vinosum*,[4] in which it has been implicated in light-induced electron transport.[5]

The amino-acid sequence[6] and X-ray crystal structure of oxidized and reduced *Chromatium vinosum* HIPIP have been reported.[7] A particularly distinctive property of HIPIP is the high reduction potential ($E°$ = 350 mV),[4] which is in contrast to that of the ferredoxins ($E°$ = ca. −400 mV).[8] It is now clear that HIPIP and the 4Fe–4S ferredoxins are involved in two different one-electron redox cycles. Thus there are three oxidation states available to the Fe_4S_4 clusters with HIPIP (reduced) and ferredoxins (oxidized) sharing the same spin-paired oxidation states.[9] It appears that it is the packing of the peptide about the Fe_4S_4 unit which is responsible for the different redox properties of the proteins. Thus the iron-sulfur cluster of HIPIP is decidedly more buried than those in 8Fe–8S[10] and 2Fe–2S proteins.[11] Also it has been noted that the number of N–H···S hydrogen bonds to the $Fe_4S_4(SR)_4^{n-}$ unit of the HIPIP are not as extensive as in the ferredoxins,[9,12] thus making it more difficult to operate over the n = 2−, 3− redox cycle applicable in the latter case.

Here we report studies on the redox reacticity of *Chromatium vinosum* HIPIP using inorganic complexes as redox partners. One aim is to obtain information concerning reaction sites on the protein. The complexes selected provide different ligand sets, thus giving maximum opportunity for the selection and utilization of different sites. The two relevant oxidation states of HIPIP are referred to as HIPIP(r) and HIPIP(o) for the reduced and oxidized forms, respectively.

Experimental Section

Protein. The HIPIP from *Chromatium vinosum* strain D (CAMR Microbial Products, Porton) was isolated by using a modification of the procedure described by Bartsch.[13] The dark red frozen cells (100 g), stored at −17 °C, were suspended in 400 mL of 0.2 M Tris–HCl, pH 7.3 at ca. 4 °C. The suspension was homogenized for ca. 2 min in a Waring blender and then sonicated in 100-mL portions for ca. 5 min by using a Rapidis Soniprobe to rupture the cell membranes. During sonication the beaker containing the mixture was immersed in ice. The sonicated mixture was centrifuged at 30 000 g for 10 min (4 °C) to remove cell debris. The supernatant liquid was collected and the precipitate washed with ca. 200 mL of 0.2 M Tris–HCl, pH 7.3, and recentrifuged. The combined supernatant solutions were then treated with solid ammonium sulfate (B.D.H. Reagent Grade) to achieve 60% saturation (37.4 g/100 mL) and stirred for 20 min to aid coagulation. After centrifugation at 30 000 g for ca. 20 min the precipitate containing protein contaminants and further cell debris was discarded. The supernatant was again treated with ammonium sulfate to raise the saturation to 90% (25.1 g/100 mL). After ca. 20 min equilibration the centrifugation procedure was repeated, the straw yellow colored supernatant was discarded, and the solid HIPIP was dissolved in a minimum volume of 1 mM Tris–HCl, pH 8.0, and a small amount (a few grains) of dithiothreitol or dithionite added to reduce any oxidized HIPIP.

Reduced HIPIP reacts slowly with O_2, and all subsequent procedures were carried out under O_2-free conditions. Temperatures ca. 0 °C were maintained whenever possible. The protein was dialyzed (Sigma sacks 250-74) against 1.0 mM Tris–HCl, pH 8, for a minimum of 12 h, with

† No reprints available.

(1) E.g. Averill, B. A.; Orme-Johnson, W. H. "*Met. Ions Biol. Syst.* **1978**, *7*, 127.
(2) E.g.: Yasunobu, K. T.; Tanaka, M. *Iron-Sulphur Proteins* **1977**, *3*.
(3) Carter, C. W.; Freer, S. T.; Yuong, Ng. H.; Akden, R. A.; Kraut, J. *Cold Spring Harbor Symp. Quant. Biol.* **1971**, *36*, 381.
(4) Bartsch, R. G. "Bacterial *Photosynthesis*"; San Pietro, A., Gest, H., Vernon, L. P., Eds.; Antioch Press: Yellow Springs, OH, 1963; p 315.
(5) E.g.: Evans, M. C. W.; Lord, A. V.; Reeves, S. G. *Biochem. J.* **1974**, *138*, 177.
(6) Dus, K.; Fedro, S.; Bartsch, R. G. *J. Biol. Chem.* **1973**, *248*, 7318.
(7) Carter, C. W.; Kraut, J.; Freer, S. T.; Alden, R. A. *J. Biol. Chem.* 1974, *249*, 4212. Carter, C. W.; Kraut, J.; Freer, S. T.; Yuong. Ng. H.; Alden, R. A.; Bartsch, R. G. *Ibid.* **1974**, *249*, 6339.
(8) Stombaugh, N. A.; Sundquist, J. E.; Burris, R. M.; Orme-Johnson, W. H. *Biochemistry* **1976**, *15*, 2633.
(9) Carter, C. W. *Iron-Sulphur Proteins* **1977**, *3*, 158.
(10) Adam, E. T.; Sieker, L. C.; Jensen, L. H. *J. Biol. Chem.* **1973**, *248*, 3989; **1976**, *251*, 3801.
(11) Tsukihara, T.; Fukuyama, K.; Tahara, H.; Katsube, Y.; Matsuura, Y.; Tanaka, N.; Kakudo, M.; Wada, K.; Matsubara, H. *J. Biochem.* **1978**, *84*, 1645.
(12) Adman, E. T.; Watenpaugh, K. D.; Jensen, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 4854.
(13) Bartsch, R. G. *Methods Enzymol.* **1971**, *23*, 644.

0002-7863/81/1503-5543$01.25/0

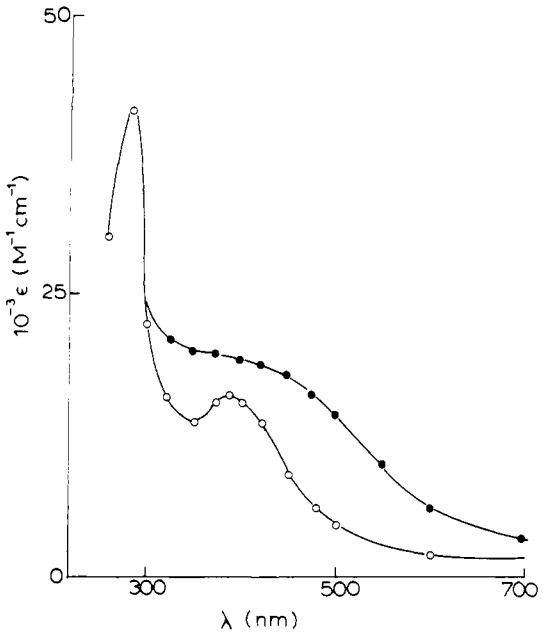

Figure 1. Spectra of *Chromatium vinosum* HIPIP in the reduced (○) and oxidized (●) forms at pH 7.0 (10^{-2} M phosphate) and I = 0.10 M (NaCl).

at least three changes of buffer. After adsorption on a DEAE-23 cellulose column (20 cm × 2.5 cm diameter) equilibrated with 1 mM Tris–HCl at pH 8, the column was washed with ca. 200 mL of loading buffer. Almost all the column appeared pink due to cell debris, but this was not eluted and did not interfere with the purification procedure. The column was washed with ca. 100 mL of 30 mM NaCl in 20 mM Tris–HCl, followed by ca. 100 mL of 40 mM NaCl in 20 mM Tris–HCl, which effectively eluted all of the green reduced protein.

The protein was diluted three times and loaded onto a smaller DEAE-23 column (4 cm × 1.5 cm diameter) equilibrated with 20 mM Tris–HCl, pH 8. Elution was with 0.5 M NaCl in 20 mM Tris–HCl, pH 7.3. After dilution 25 times with water (O °C), the protein was loaded on a DEAE-23 column (12.5 cm × 2 cm diameter) previously equilibrated with 20 mM Tris–HCl, pH 7.3. The column was rinsed with 200 mL of loading buffer, ca. 200 mL of 0.03 M NaCl/20 mM in Tris–HCl, and finally 0.04 M NaCl/20 mM Tris–HCl. The protein was collected in 25-mL fractions and those with absorbance ratios A_{283}/A_{388} = 2.50 ± 0.05 were combined and concentrated on a small DEAE-23 column as above. For kinetic runs the pure protein was dialyzed for ca. 72 h against the required buffer (usually 0.01 M phosphate) with frequent (ca. 6 h) changes of buffer. Solutions of HIPIP(r) were stored under N_2 and standardized spectrophotometrically from the known spectrum (Figure 1) with peak at 388 nm (ϵ 16.1 × 10^3 M^{-1} cm^{-1}).[13]

The protein HIPIP(o) was prepared by oxidation with $Fe(CN)_6^{3-}$ solution. An extra drop of $Fe(CN)_6^{3-}$ was added to ensure complete green to red color change, monitored by scan spectra at 350–550 nm. Complexes then removed by repeating the dialysis procedure, the bulk solution being changed ca. 12 times/48 h. Solutions of HIPIP(o) were standarized spectrophotometrically (Figure 1) from the absorbance λ = 348 nm (ϵ = 20 × 10^3 M^{-1} cm^{-1}).[13] Isoelectric points for the protein are at pH 3.88 (oxidized) and 3.68 (reduced).[13] The protein is reported to be unstable below pH 5 and above pH 11.[14]

Complexes. Potassium hexacyanoiron(II), $K_4[Fe(CH)_6]\cdot 3H_2O$ (Hopkin and Williams, Analar), λ_{max} = 337 nm (ϵ = 350 M^{-1} cm^{-1})) and potassium hexacyanoiron(III), $K_3[Fe(CN)_6]$ (B.D.H., Analar), λ_{max} = 420 nm (ϵ = 1010 M^{-1} cm^{-1}), were used without further purification. The purple complex (1,2-diaminocyclohexane)tetrakis(acetato)manganate(III) ($K[MnCyDTA]\cdot 2.5H_2O$) was prepared by a literature procedure.[15] The high-spin complex has a structure $Mn(CyDTA)(H_2O)^-$ where the CyDTA is either pentadentate or hexadentate with H_2O occupying a further coordination position. The absorption spectrum is pH dependent. At pHs in the range 2–6 a single broad peak at λ_{max} = 510 nm (ϵ = 345 M^{-1} cm^{-1}) is observed. The deep red to straw yellow color change at pHs in the range 7–9 corresponds to acid dissociation (pK_a = 8.1) of the coordinated H_2O.[15] A formal reduction potential of 760 ± 10 mV (vs. NHE) was obtained by cyclic voltammetry for the $Mn(CyDTA)(H_2O)^{-,2-}$ couple at 25 °C, pH 7 (10^{-2} M phosphate), and I = 0.10 M (NaCl). This compares satisfactory with the previously reported value of 814 mV, determined at pH 4 and I = 0.2 M.[15] Tris(4,7-bis(phenyl 4-sulfonate)-1,10-phenanthroline)cobaltate(III) was prepared by reacting the ligand, known commercially as bathophenanthroline sulfonate (Sigma Chemicals), with chloropentaamminecobalt(III) ($[Co(NH_3)_5Cl]Cl_2$) by the procedure described.[16,17] The sample gave λ_{max} = 293 nm (ϵ = 1.21 × 10^5 M^{-1} cm^{-1}) and λ_{min} = 258 nm (ϵ = 3.7 × 10^4 M^{-1} cm^{-1}) on the basis of the formula $Na_3[Co(4,7\text{-DPSphen})_3]$, in good agreement with previous values. The complex was shown to obey Beer's Law.

Table I. Reduction Potentials for Relevant Couples

couple	$E°$, mV	ref
HIPIP(o)/HIPIP(r)	350	4
$Co(4,7\text{-DPSphen})_3^{3-,4-}$	340	*a*
$Mn(CyDTA)(H_2O)^{-,2-}$	760	this work
$Fe(CN)_6^{3-,4-}$	420(410)	*c*

[a] McArdle, J. V.; Coyle, C. L.; Gray, H. B.; Yoneda, G. S.; Holwerda, R. A. *J. Am. Chem. Soc.* 1977, *99*, 2483. [b] A value of 814 mV is reported in ref 15. [c] Butler, J.; Davies, D. M.; Sykes, A. G. *J. Inorg. Biochem.*, in press. Kolthoff, I. M.; Tomsicek, W. J. *J. Phys. Chem.* 1935, 39, *945*.

Kinetics. The ionic strength was adjusted to 0.103 ± 0.003 M (except as stated) by using Analar NaCl. Solutions were buffered to pH 7.0 with 1.0 × 10^{-2} M phosphate (Na_2HPO_4/NaH_2PO_4), except for the pH variations when the "pH jump" method was employed (i.e., the protein solution at pH 7.0 with 1.0 × 10^{-3} M buffer was mixed with a solution of complex containing 2.0 × 10^{-2} M buffer to give the required pH). The pH of solutions was checked by using a Radiometer standard pH meter Model PHM62. To enable a wide pH range to be investigated, we also used other buffers (1.0 × 10^{-2} M), citrate (pK_a = 6.4), cacodylate (6.2), and borate (9.27). Solutions of HIPIP(r) were retained under air-free conditions. Air oxidation is relatively slow, and it was not necessary therefore to make up solutions of complex air-free, with the exception of $Fe(CN)_6^{4-}$ which is itself air sensitive over periods of > ca. 10 min. Solutions of $Co(4,7\text{-DPSphen})_3^{3-}$ did not give satisfactory reproducibility after being stored overnight.

Reactions were monitored on a Durrum-Gibson stopped-flow spectrophotometer at 480 nm, which gives the biggest absorbance differences between HIPIP(r) and HIPIP(o) forms (Figure 1). Stoichiometries defined as protein to complex consumed were assumed to be 1:1 in all cases. A large (>10-fold) excess of complex was used in all runs with the protein generally ca. 5 × 10^{-6} M. For runs proceeding to completion plots of absorbance (A) changes, $\ln|A - A_\infty|$ against time, were linear for > 3 half-lives, and first-order rate constants k_{obsd} were obtained from the slopes. At least two traces were analyzed for each run (agreement ±5%). For the slower runs with $Co(4,7\text{-DPSphen})_3^{3-}$ as oxidant when, because of the slowness of the reaction, A_∞ was not as well-defined, Guggenheim plots were also carried out.[18] Reaction with $Fe(CN)_6^{4-}$ as reductant did not always proceed to completion, and for those proceeding <95% of rate constants k_{obsd} for the forward reaction were obtained by a rigorous equilibration kinetic treatment requiring (1), where C_o, C_t, and

$$\ln\left(\frac{C_o^2 - C_eC_t}{(C_t - C_e)C_o}\right) = k_{obsd}\left(\frac{C_o + C_e}{C_0 - C_e}\right)t \qquad (1)$$

C_e are the initial, time t, and equilibrium concentrations of HIPIP(o) respectively.[19] The equilibrium constant for (2) was determined from

$$Fe(CN)_6^{4-} + HIPIP(o) \rightleftharpoons Fe(CN)_6^{3-} + HIPIP(r) \qquad (2)$$

a series of experiments in which C_o and C_e were first measured by conventional Perkin-Elmer 554 spectrophotometry, and then C_∞ corresponding to 100% conversion to HIPIP(r) was determined by adding crystals of sodium dithionite. The value obtained at 25 °C was 0.070 ± 0.020 in accordance with the value from reduction potentials (Table I).

The $Mn^{III}CyDTA$ complex is more resistant to decomposition than the corresponding EDTA complex.[15,20] Acid solutions of Mn(Cy-

(14) Mizrahi, I. A.; Wood, F. E.; Cusanovich, M. A. *Biochemistry* **1976**, *15*, 343.
(15) Hamm, R. E.; Suwyn, M. A. *Inorg. Chem.* **1967**, *6*, 139.
(16) McArdle, J. V.; Yocum, K.; Gray, H. B. *J. Am. Chem. Soc.* 1977, *99*, 4141.
(17) Lappin, A. G.; Segal, M. G.; Weatherburn, D. C.; Sykes, A. G. *J. Am. Chem. Soc.* **1979**, *101*, 2297.
(18) Frost, A. A.; Pearson, R. G. "Kinetics and Mechanisms", 2nd ed.; Wiley: New York, 1961; p 49.
(19) Reference 18, p 187.
(20) Schroeder, K. A.; Hamm, R. E. *Inorg. Chem.* **1964**, *3*, 391.

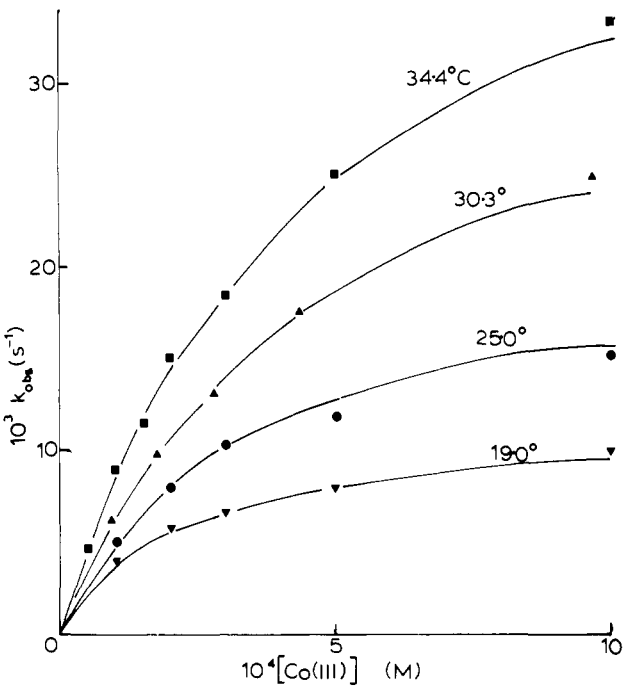

Figure 2. The oxidation of *Chromatium vinosum* HIPIP(r) with Co(4,7-DPSphen)$_3^{3-}$. The dependence of first-order rate constants k_{obsd} on [Co(III)] (pH 7.0 (10^{-2} M phosphate), I = 0.10 M (NaCl)). The curves drawn correspond to the best fit.

DTA)$(H_2O)^-$ are reported to decompose with a rate constant of 6.8 × 10^{-6} s^{-1} at 25 °C.[15] At pH 6.95 (10^{-2} M phosphate) with [Mn(CyDTA)$(H_2O)]^-$ = 2.3 × 10^{-3} M (I = 0.10 M), a rate constant of 8.6 × 10^{-5}) s^{-1} was obtained. All such Mn(III) solutions were used therefore within 10 min of preparation.

A nonlinear least-squares program,[21] with weighting $1/k^2_{obsd}$, was used to analyze data.

Results

Oxidant Co(4,7-DPSphen)$_3^{3-}$. At pH 7.0 first-order rate constants k_{obsd} (Table II)[22] for the oxidation of HIPIP(r) give a nonlinear dependence on [Co(III)] (Figure 2). Plots of $(k_{obsd})^{-1}$ against $[Co(III)]^{-1}$ are linear, consistent with the sequence (3) – (4)and the derived relationship (5). From a nonlinear least-

$$\text{HIPIP(r)} + \text{Co(III)} \overset{K}{\rightleftharpoons} \text{HIPIP(r), Co(III)} \quad (3)$$

$$\text{HIPIP(r), Co(III)} \overset{k_{et}}{\rightleftharpoons} \text{HIPIP(o)} + \text{Co(II)} \quad (4)$$

$$k_{obsd} = \frac{Kk_{et}[\text{Co(III)}]}{1 + K[\text{Co(III)}]} \quad (5)$$

squares treatment of data at temperatures 19.0–34.3 °C the following values were obtained: K(25 °C) = 3420 ± 130 M^{-1}, $\Delta H°$ = −8.9 ± 1.7 kcal mol^{-1}, $\Delta S°$ = −13.7 ± 5.8 cal K^{-1} mol^{-1}, k_{et}(25 °C) = 0.020 ± 0.0015 s^{-1}, $\Delta H^{\ddagger}_{et}$ = 15.9 ± 1.0 kcal mol^{-1}, and $\Delta S^{\ddagger}_{et}$ = −12.9 ± 3.2 cal K^{-1} mol^{-1}. The dependence of k_{obsd} on pH was investigated (Table III).[22] Two acid dissociation pK_a's are apparent (Figure 3). The second of these at the higher pH's (which is better defined) give pK_a = ca. 7.1 by procedures outlined.[23,24] Rate constants $10^3 k_{obsd}$ (s^{-1}) at pH 7.0 vary with ionic strength (M) as follows: 10.1 (0.05); 11.2 (0.10); 11.7 (0.20); 12.1 (0.47). The effective protein charge deduced from the Debye–Hückel plot[25] of log Kk_{et} (k_{et} may be assumed not to vary with I) against $I^{1/2}$ is −0.15.

Oxidant Mn(CyDTA)$(H_2O)^-$: at pH 6.95 first-order rate constants k_{obsd} (Table IV[22]) give a linear dependence on oxidant

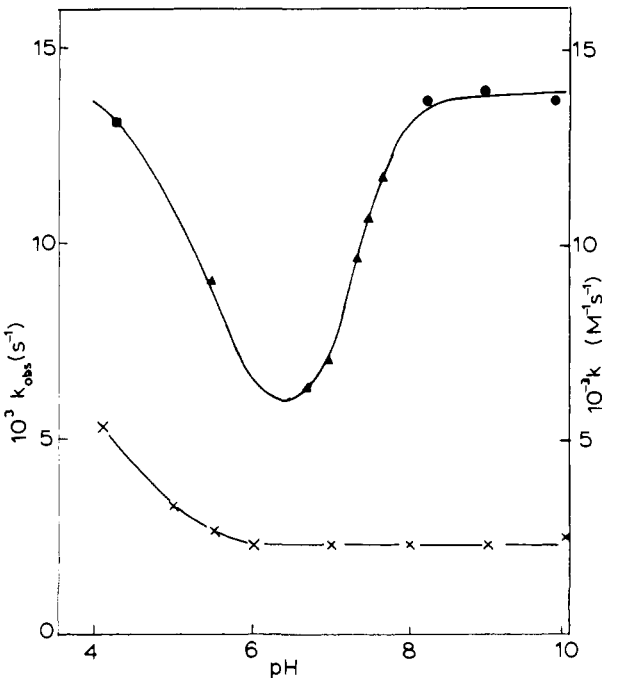

Figure 3. The oxidation of *Chromatium vinosum* HIPIP(r) with Co(4,7-DPSphen)$_3^{3-}$ (1.75 × 10^{-4} M). The dependence of first-order rate constants k_{obsd} on pH (I = 0.10 M (NaCl)), using buffers (10^{-2} M) acetate (■), phosphate (▲), and borate (●). The lower line (right-hand side scale) is the pH dependence reported previously[14] for the reaction of HIPIP(r) with $Fe(CN)_6^{3-}$.

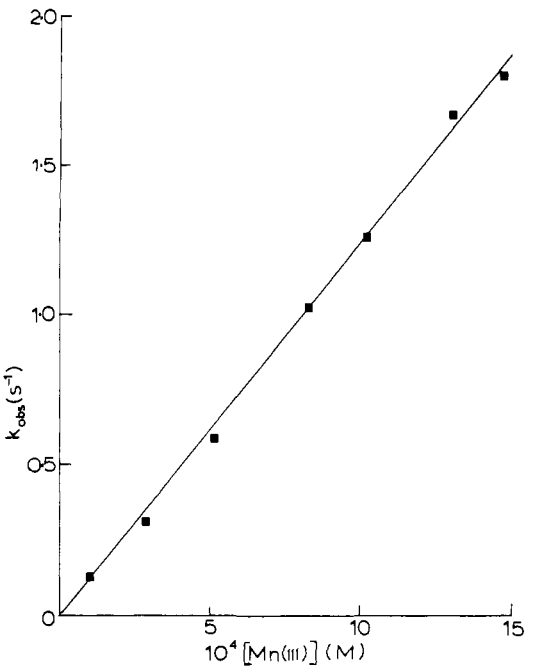

Figure 4. The oxidation of *Chromatium vinosum* HIPIP(r) with Mn(CyDTA)$(H_2O)^-$. The dependence of first-order rate constants k_{obsd} on [Mn(III)] at 25 °C, pH 7.0 (10^{-2} M phosphate), and I = 0.10 M (NaCl).

[Mn(III)] (Figure 4). From the slope the second-order rate constant k(25 °C) = 1.26 × 10^3 M^{-1} s^{-1}, and the temperature dependence of k (Table V[22]) gives $\Delta H^{\ddagger}$ = 3.6 ± 0.3 kcal mol^{-1} and $\Delta S^{\ddagger}$ = −32.4 ± 1.1 cal K^{-1} mol^{-1}. When the pH is varied (Table VI[22]), two pK_a's are again apparent (Figure 5). The second of these (at the higher pH's), which is the more fully defined, gives pK_a = 7.74 ± 0.08.

Reductant $Fe(CN)_6^{4-}$. This reaction was investigated over a wide range of concentrations, (0.39–50.0) × 10^{-4} M.[25] At the lower $Fe(CN)_6^{4-}$ concentrations the reaction is incomplete and an equilibration kinetic treatment was required (See Experimental

(21) Moore, R. H.; Zeigler, R. K. Los Alamos Report LA 2367, 1959, and Addenda.
(22) See paragraph at end of paper regarding supplementary material.
(23) Alberty, R. A.; Massey, V. *Biochim. Biophys. Acta* **1975**, *13*, 347.
(24) Dixon, H. B. F.; Tipton, K. F. *Biochem. J.* **1973**, *133*, 837.
(25) Reference 18, p 150.

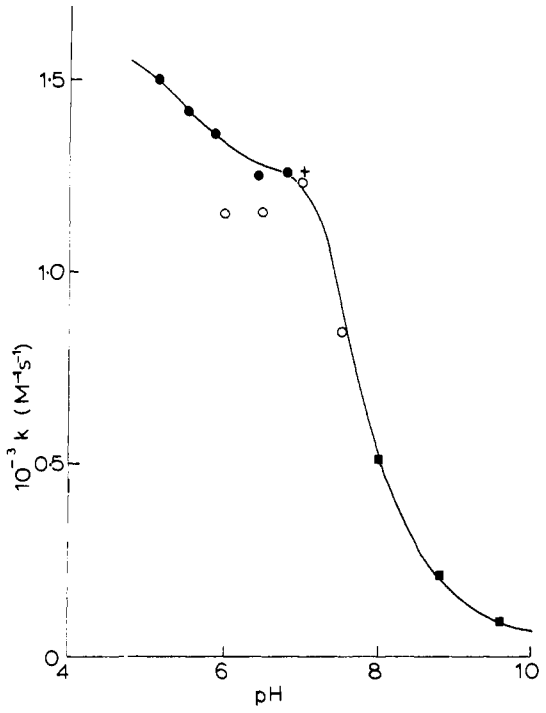

Figure 5. The dependence of second-order rate constants for the oxidation of *Chromatium vinosum* HIPIP(r) with the $Mn^{III}CyDTA$ complex on pH using buffers (10^{-2} M) cacodylate (●), citrate (○), phosphate (+), and borate (■) (I = 0.10 M NaCl)).

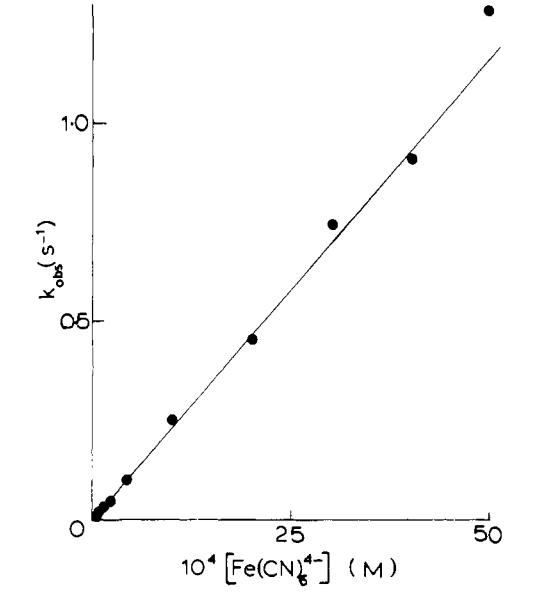

Figure 6. The reduction of *Chromatium vinosum* HIPIP(o) with $Fe(CN)_6^{4-}$. The dependence of first-order rate constants k_{obsd} on [$Fe(CN)_6^{4-}$] at 25 °C, pH 7.0 (10^{-2} M phosphate), and I = 0.10 M (NaCl).

Section). First-order rate constants, k_{obsd} (listed in Table VII[22]), are in all cases for the forward reaction and give a linear dependence on reductant (Figure 6). It is estimated that K is <10 M^{-1} for association prior to electron transfer.

Competition Studies. The oxidant $Co(4,7\text{-DPSphen})_3^{3-}$ associates strongly with the protein (K = 3420 M^{-1}) but is a sluggish overall redox partner (Kk_{et} = 68 M^{-1} s^{-1}). This property can be made use of in competition studies. Thus negatively charged oxidants, either $Fe(CN)_6^{3-}$ or $Mn(CyDTA)(H_2O)^-$, were mixed with $Co(4,7\text{-DPSphen})_3^{3-}$ in one solution and then reacted with the protein HIPIP(r). For the highest Co(III) concentrations used (Table VIII[22]), it can be calculated that >50% of the protein is associated with $Co(4,7\text{-DPSphen})_3^{3-}$. No effect on rate constants arising from the presence of the Co(III) was observed. From the separate independent kinetic studies it can be concluded that $Co(4,7\text{-DPSphen})_3^{3-}$ makes a negligible contribution as oxidant.

Table IX. A Comparison of Association Constants (K), Rate Constants (k_{et}), and Related Enthalpy and Entropy Terms for Reactions with $Co(4,7\text{-DPS phen})_3^{3-}$ as Oxidant at 25 °C, pH 7.0, and I = 0.10 M (NaCl)

	K, M^{-1}	ΔH°, kcal mol^{-1}	ΔS°, cal K^{-1} mol^{-1}
PCu(I)[a]	4600	−4.2	2.7
ACu(I)[b]	2750	−3.7	3.3
HIPIP(r)[c]	3420	−8.9	−13.7
	k_{et}, s^{-1}	$\Delta H^\ddagger$, kcal mol^{-1}	$\Delta S^\ddagger$, cal K^{-1} mol^{-1}
PCu(I)[a]	0.041	13.2	−20.6
ACu(I)[b]	0.21	10.7	−25.6
HIPIP(r)[c]	0.020	15.9	−12.9

[a] Parsley plastocyanin. [b] *Pseudomonas aeruginosa* azurin.[26] [c] This work.

Discussion

The oxidation of HIPIP(r) with $Co(4,7\text{-DPSphen})_3^{3-}$ like those of plastocyanin, PCu(I),[17] and azurin, ACu(I),[26] give limiting kinetics. Results have been interpreted in terms of 3–5, where K, k_{et}, and related parameters are shown in Table IX. An alternative mechanism involving conversion of $Co(4,7\text{-DPSphen})_3^{3-}$ to a more reactive form, Co(III) ⇌ *Co(III), is not applicable, since in reactions of this complex with two other proteins, stellacyanin, SCu^I,[27] and cytochrome c(II),[28] limiting kinetics are not observed. It has been demonstrated in the reactions of HIPIP(r), PCu(I), and ACu(I) that $Co(4,7\text{-DPSphen})_3^{3-}$ associates at a different site on the protein to that used by the oxidant $Fe(CN)_6^{3-}$. The basis of these studies is that $Co(4,7\text{-DPSphen})_3^{3-}$ associates strongly with the protein but as an oxidant reacts sluggishly. Since the Co(III) complex does not impede the reaction of $Fe(CN)_6^{3-}$, a different site must be used.

Similar competition studies lead to the conclusion that the oxidant $Mn(CyDTA)(H_2O)^-$ also uses a different site to $Co(4,7\text{-DPSphen})_3^{3-}$. In all such studies the presence of $Co(4,7\text{-DPSphen})_3^{3-}$ might have been expected to have some effect on the protein reactivity. None is observed. This is consistent with high-field ^1H NMR studies on PCu(I) in the presence of complexes $Cr(CN)_6^{3-}$ and $Cr(phen)_3^{3+}$. The complexes associate at two different sites (one in each case) and bring about a broadening of lines from amino acids in close proximity. No long-range effects corresponding to conformational changes have been reported however, and NMR lines from other amino acids remain unaffected by the association.[29,30] Such results are also able to rule out the dead-end mechanism as an alternative explanation for limiting kinetics, and there is a need to extend the NMR studies using the redox inactive Cr(III) analogue to $Co(4,7\text{-DPSphen})_3^{3-}$.

The effect of ionic strength has been investigated previously for the $Fe(CN)_6^{3-}$ and $Ru(NH_3)_5(py)^{3+}$ oxidations of HIPIP(r).[14,31] With the Debye–Hückel relationship (rigorous application of which is certainly not expected for reactions involving proteins), effective protein charges of −0.47 and −0.40, respectively, have been reported. At pH 7 HIPIP(r) is known to carry a negative charge which is probably ca. −3.[6] With $Co(4,7\text{-DPSphen})_3^{3-}$ the charge is diffuse due to the size of the ligands, and rate constants exhibit less dependence on ionic strength (effective charge −0.15). Since the $Co(4,7\text{-DiPSphen})_3^{3-}$ reaction does not respond strongly

(26) Lappin, A. G.; Segal, M. G.; Weatherburn, D. C.; Henderson, R. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1979**, *101*, 2302.
(27) Adzamli, I. K.; Segal, M. G.; Stanley, C. S.; Sykes, A. G., unpublished work.
(28) Chapman, S. K.; Davies, D. M.; Sykes, A. G., unpublished work.
(29) Cookson, D. J.; Hayes, M. T.; Wright, P. E., *Nature (London)* **1980**, *283*; *Biochim. Biophys. Acta.* **1980**, *591*.
(30) Handford, P. M.; Hill, H. A. O.; Lee, R. W.-K.; Henderson, R. A.; Sykes, A. G. *J. Inorg. Biochem.* **1980**, *13*, 83.
(31) Cummins, D.; Gray, H. B. *J. Am. Chem. Soc.* **1977**, *99*, 5158.

Table X. Comparison of Kinetic Parameters for the Overall Reactions of *Chromatium* HIPIP with Inorganic Complexes (I = 0.10 M (NaCl), T = 25 °C, pH 7.0 (phosphate) Except as Stated)

complex	k, M^{-1} s^{-1}	$\Delta H^{\ddagger}$, kcal mol^{-1}	$\Delta S^{\ddagger}$, cal K^{-1} mol^{-1}	ref
$Co(phen)_3^{3+}$	2.8×10^3	14.9	7.0	38
$Ru(NH_3)_5(py)^{3+}$ [a]	1.1×10^3	9.4	−13	33
$Co(4,7\text{-DPSphen})_3^{3-}$	68	7.0	−26.9	this work
$Mn(CyDTA)^-$	1.26×10^3	3.6	−27.7	this work
$Fe(CN)_6^{3-}$	2.0×10^3	−0.4	−45.0	14
	2.5×10^3	0.0	−45.1	38
$Fe(CN)_6^{4-}$	2.4×10^2			this work
	2.8×10^2	4.2	−34.8	14
$Fe(EDTA)^{2-}$	1.6×10^3	0.8	−41.0	38

[a] At pH 6.5 (phosphate buffer), I = 0.50 M (Na_2SO_4). Rate constant = 2.5×10^3 M^{-1} s^{-1} at I = 0.10 M. (Na_2SO_4). Values of the reduction potential for $Ru(NH_3)_5(py)^{3+,2+}$ are reported to be 273 and 253 mV.[27]

to changes in ionic strength, it follows that the association constant K is not strongly dependent on the electrostatics. A consideration of electrostatics alone does not seem to be a satisfactory explanation of the magnitude of $\Delta H°$ and $\Delta S°$ parameters for K in this and the corresponding reactions of PCu(I) and ACu(I).[17,26] Instead the favorable association probably stems in large part from the high aromaticity of the $Co(4,7\text{-DPSphen})_3^{3-}$ complex.

The pH profile (Figure 5) for the $Mn(CyDTA)(H_2O)^-$ oxidation of HIPIP(r) is readily understood in terms of acid dissociation of the coordinated H_2O. The pK_a obtain from the data at pH >7.0, (Figure 5) gives a value 7.74 which compares with the independently measured value of 8.11.[15] The pH profile (Figure 3) with $Co(4,7\text{-DPSphen})_3^{3-}$ as oxidant gives a pK_a of 7.1 from data at pH >6.74. Such a value suggests involvement of the only histidine present (His-42) which is the most likely amino acid to have a pK_a in this region. Histidine-42 is located close to the Fe_4S_4 cluster. Thus electron transfer is in this instance more rapid when His-42 is deprotonated. Mizrahi et al.[14] have reported that the reaction with $Fe(CN)_6^{3-}$ as oxidant is independent of pH in the range 6–9 (Figure 3). However Feinberg and Johnson[32] have found that electrostatically corrected rate constants do in fact give a pH profile and pK_a ca. 7.0 with the HIPIP(r) having unprotonated His-42 again the more reactive. Interestingly the reaction of $Fe(CN)_6^{3-}$ with ACu(I) responds to pH (without electrostatic corrections), giving a pK_a of 7.1, which is believed to correspond to involvement of one of the two uncoordinated histidines (His-35).[26] All three oxidants $Co(4,7\text{-DPSphen})_3^{3-}$, $Mn(CyDTA)(H_2O)^-$, and $Fe(CN)_6^{3-}$ exhibit an increase in rate constants at pH <6 due presumably to some other protonation process or processes.

The $Fe(CN)_6^{4-}$ reductions of HIPIP(o) was studied over a wide range of $[Fe(CN)_6^{4-}]$. No limiting kinetics were observed, and it can be concluded that K for association prior to electron transfer is <10 M^{-1}. It has been found that the reaction is independent of ionic strength and certainly electrostatics do not apear to have an influence on this reaction.[14] As far as the application of Marcus theory[33] is concerned therefore work terms electrostatic in origin may be assumed to be unimportant. Since the self-exchange rate constant for $Fe(CN)_6^{3-,4-}$ is known (5×10^3 M^{-1} s^{-1}),[34,35] and $K_{12}(10)$ can be obtained from relevant $E°$ vaues in Table I, this approach gives HIPIP(o)/HIPIP(r) self-exchange rate constants as ca. 1.1 M^{-1} s^{-1}. Similar calculations with $Co(phen)_3^{3+}$ as redox partner give a protein self-exchange rate constant of 10^4 M^{-1} s^{-1} (which require correction for work terms) and with $Fe(EDTA)^{2-}$ as reductant a value of 10^{-2} M^{-1} s^{-1} (which seems anomalously low as in a similar calculation for data from the $Fe(EDTA)^{2-}$ reduction of cytochrome c).[36] The slow rate constant for the Mn(III) oxidation of HIPIP(r) (which has a very favorable driving force of 410 mV (Table I)) is most likely the result of a slow $Mn(CyDTA)(H_2O)^{-,2-}$ self-exchange. This can be rationalized in terms of the geometry change (if seven-coordinate) or distortion of the Mn(III) (d^4) coordination sphere (if six-cooridnate) and reorganization attendant on electron transfer. It can be further concluded that the conjugate base $Mn(CyDTA)(OH)^{2-}$ present at the higher pH's (Figure 5) is much less redox active than $Mn(CyDTA)(H_2O)^-$.

A feature of the reactions of HIPIP(r) with oxidants $Co(phen)_3^{3+}$, $Mn(CyDTA)(H_2O)^-$, $Fe(CN)_6^{3-}$, and $Ru(NH_3)_5(py)^{3+}$, having a wide range of ligands (as well as $E°$ values), is that rate constants at 25 °C are very similar in magnitude (Table X). A single rate-determining step is not a possible explanation since $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values show considerable variation. An isokinetic correlation of $\Delta H^{\ddagger}$ is to be noted.

Reactions of parsley 2-Fe ferredoxin, which has a more exposed cluster (cf. the X-ray structure details for *Spirulina platensis* 2-Fe ferredoxin[11]), with inorganic complexes have recently been studied. The protein has a much higher negative charge (ca. −18), and reactions with positively charged complexes appear to respond to this.[37] Thus limiting kinetics and high K values for association prior to electron transfer (k_{et}) are observed. However overall second-order rate constants (Kk_{et}) for a wide range of charges, 5+ to 3−, do not show as wide a variation as would have been expected; i.e., a favorable K seems to be counterbalanced by smaller k_{et}, in a way which is not fully understood. Again this behavior is reflected in an isokinetic correlation of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values obtained from Kk_{et}.[37]

The closest approach which a redox partner can make to the Fe_4S_4 cluster of HIPIP is the hydrophobic patch where Cys-46 is separated from the solvent by Thr-81 and Phe-48 and an inorganic sulfur is separated from solvent by Ile-65.[38] It appears fairly certain now that $Fe(CN)_6^{3-}$ uses a hydrophobic patch on PCu(I)[29] and it will be of interest therefore whether $Fe(CN)_6^{3-}$ uses a similar locality on HIPIP.

Acknowledgment. We thank the Science Research Council, London, for post-doctoral (I.K.A. and D.M.D.) and post-graduate (C.S.S.) support and the Royal Society for an equipment grant for the purchase of an M.S.E. hi-spin-21 centrifuge. This work was carried out at the University of Leeds.

Supplementary Material Available: Tables of rate constants (Tables II–VIII) (7 pages). Ordering information is given in any current masthead page.

(32) Feinberg, B. A.; Johnson, W. V. *Biochem. Biophys. Res. Commun.* **1980**, *93*, 100.
(33) Marcus, R. A. *J. Phys. Chem.* **1963**, *67*, 853.
(34) Campion, R. J.; Deck, C. F.; King, P.; Wahl, A. C. *Inorg. Chem.*, **1967**, *6*, 672 and comments in Table VI of ref 33.
(35) Hrepic, N. V.; Malin, J. M. *Inorg. Chem.* **1979**, *18*, 409.
(36) Rawlings, J.; Wherland, S.; Gray, H. B. *J. Am. Chem. Soc.*, **1976**, *98*, 2177.
(37) Armstrong, F. A.; Henderson, R. A.; Sykes, A. G. *J. Am. Chem. Soc.* **1979**, *101*, 6912.
(38) Adman, E. T. *Biochim. Biophys. Acta* **1979**, *549*, 107.