Oxidation–Reduction Properties of *Chromatium vinosum* High Potential Iron–Sulfur Protein†

Isaac A. Mizrahi, Fern E. Wood, and Michael A. Cusanovich*

ABSTRACT: The oxidation–reduction properties of the high potential iron–sulfur protein (HIPIP) from *Chromatium vinosum* have been investigated. Both equilibrium and kinetic measurements demonstrate electron transport by HIPIP is pH independent in the pH range 7–11. The kinetics of reduction (potassium ferrocyanide, SO_2^-, $S_2O_4^{2-}$, sodium ascorbate, and *Rhodospirillum rubrum* cytochrome c_2) and oxidation (potassium ferricyanide and *Rhodospirillum rubrum* cytochrome c_2) of HIPIP are reported. Based on the data obtained with different reactants and the influence of ionic strength, pH, and temperature on the kinetics of oxidation and reduction, a number of conclusions can be drawn. (1) HIPIP undergoes rapid outer-sphere electron transfer with no evidence of kinetic complexity and no indication of complex formation with various reactants. (2) The site of oxidation of reduced HIPIP has an apparent negative charge while the site of reduction of oxidized HIPIP is uncharged. (3) HIPIP appears to interact with a physiological reactant (*R. rubrum* cytochrome c_2) at the same site as nonphysiological oxidants or reductants suggesting single minimum energy pathways for the oxidation and reduction processes. (4) Based on a comparison of the rates of oxidation and reduction with different reactants, it appears that steric restrictions and differences in oxidation–reduction potential are less important than electrostatic attraction and/or repulsion in determining the absolute rate constants. (5) The thermodynamic activation parameters indicate that both oxidation and reduction by the iron hexacyanides are driven entropically with the enthalpic terms making no contribution to HIPIP oxidation and a small contribution to HIPIP reduction. Based on the data reported here and available structural and physical-chemical information, possible mechanisms of the oxidation and reduction of HIPIP are discussed and their relative merits analyzed. The more likely mechanisms include electron transfer via a tyrosine residue, electron transfer through a nonaqueous media to the iron–sulfur chromophore, and direct interaction between the iron–sulfur chromophore and the different oxidants and reductants.

Chromatium vinosum high potential iron–sulfur protein (HIPIP)[1] has been the subject of extensive characterization. This protein is a member of a class of small (molecular weight about 9000) soluble proteins containing a single four iron–sulfur cluster (Carter et al., 1971) associated with a single polypeptide chain. Characteristic of HIPIP is its high oxidation–reduction potential ($E_0' = 350$ mV). This is opposed to other classes of non-heme iron proteins such as rubredoxin and ferredoxins which are of low oxidation–reduction potential (<100 mV). Examples of HIPIP have been isolated from the photosynthetic purple sulfur bacteria *Chromatium vinosum* (Bartsch, 1963) and *Thiocapsa pfenigii* (Meyer, 1970) and the photosynthetic purple bacteria *Rhodopseudomonas gelatinosa* (DeKlerk and Kamen, 1966), *Rhodomicrobium vannielii,* and *Rhodospirillum tenue* (T. E. Meyer, personal communication). Moreover, HIPIP has been isolated from a denitrifying bacterium *Micrococcus species* (Hori, 1961). The biological function of HIPIP is presently unclear although it has been implicated in light-driven electron transport in *Chromatium vinosum* (Kennel et al., 1972; Dutton and Leigh, 1973; Evans et al., 1974). This latter observation does not explain the presence of HIPIP in the denitrifying bacteria and, for the present, leaves the question of function open.

Considerable physical-chemical work has been completed on *Chromatium vinosum* HIPIP, with the amino acid sequence (Dus et al., 1973) and three-dimensional structure (Carter et al., 1974a,b) determined. From the structural studies the iron–sulfur cubane has been characterized and compared to the almost identical cluster in *Peptococcus aerogenes* ferredoxin (Carter et al., 1972). From this work it has been proposed that three oxidation states are available to the iron–sulfur cluster, with HIPIP (reduced) and ferrodoxin (oxidized) sharing the same "spin-paired" oxidation state. Thus, it is clear that it is the packing of the protein about the iron–sulfur cluster not the cluster itself that is responsible for the oxidation–reduction potentials of HIPIP and ferredoxin.

Carter et al. (1974a,b) have discussed the structure of HIPIP in detail and only the general features will be repeated here. In particular, the iron–sulfur cluster is surrounded by nonpolar amino acid side chains including the aromatics Tyr-19, Phe-48, Phe-66, Trp-80, and Trp-76. The closest point of approach of the cluster to the solvent appears to be where two of the inorganic sulfur atoms are about 4.5 Å from the surface (Carter et al., 1974b). However, from space-filling models Carter et al. (1974b) feel nonpolar residues pack so as to prevent direct contact of the iron–sulfur cluster with the solvent. Carter et al. (1974b) propose that an electrostatic charge–dipole interaction exists between the cluster and Tyr-19 which makes the tyrosine hydroxyl group available for hydrogen bonding, possibly stabilizing a phenoxy radical transition state.

In view of the structural information available we have investigated the oxidation–reduction properties of *Chroma-*

† From the Department of Chemistry, University of Arizona, Tucson, Arizona 85721. *Received July 8, 1975.* One of us, I.A.M., was supported in part by funds from a training agent (GM 01982) from the U.S. Public Health Service. This work was supported by the U.S. Public Health Service Research Grant GM 21277.

[1] Abbreviations used are: HIPIP, high potential iron–sulfur protein; TAGP buffer, Tris–sodium acetate–glycine–potassium phosphate buffer.

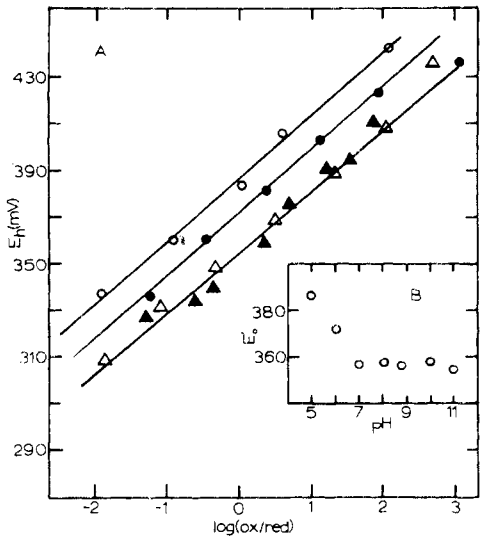

FIGURE 1: (A) Oxidation-reduction titrations of HIPIP. Buffer, 25 m*M* TAGP-1 m*M* potassium ferricyanide. Temperature, 25 ± 1°, HIPIP concentration, 50 μM, reductant sodium dithionite. (○) pH 5.0; (●) pH 6.0; (▲) pH 8.0; (△) pH 7.0. (B) Plot of measured oxidation-reduction midpoint potentials vs. pH.

tium vinosum HIPIP. These studies include the effect of pH on the oxidation-reduction potential and the kinetics of oxidation and reduction using potassium ferro- and ferricyanide, sodium ascorbate, sodium dithionite, and ferro- and ferricytochrome c_2 (from *Rhodospirillum rubrum*). The goals of the studies reported here are threefold: (1) to obtain information on the mechanism of electron transfer to and from the iron-sulfur cluster including the participation, if any, of specific amino acid side chains; (2) to compare and contrast the oxidation-reduction properties of HIPIP to those of the *c*-type cytochromes to determine if any major mechanistic differences exist; (3) to investigate the interaction of two well-defined proteins (HIPIP and cytochrome c_2) and relate these data to those obtained with nonphysiological oxidants and reductants.

Materials and Methods

Chromatium vinosum HIPIP (Bartsch, 1963) and *Rhodospirillum rubrum* cytochrome c_2 (Bartsch et al., 1971) were isolated and purified as described in the indicated references. Stock solutions of the oxidized or reduced proteins were prepared by the addition of a slight excess of potassium ferricyanide or sodium dithionite (Hardman and Holden Limited) and the excess reagent was removed by gel filtration on Sephadex G-25. Studies over a range of pH values were performed in Tris-sodium acetate-glycine-potassium phosphate buffer (TAGP buffer). All chemicals were reagent grade and were used without further purification.

All kinetic studies were conducted in a Durrum-Gibson stopped-flow spectrophotometer with a mixing time of 3.5 msec. All reactions were maintained at 20 ± 0.2°C unless otherwise noted. The reaction of oxidized HIPIP with potassium ferrocyanide was followed at 480 nm. The reaction of reduced HIPIP with potassium ferricyanide was monitored at 500 nm to prevent any interference by the ferricyanide absorbance. Anaerobic studies were performed as previously described (Miller and Cusanovich, 1975).

Oxidation-reduction titrations were performed anaerobically as described by Velick and Strittmatter (1956). For equilibrium constant calculations, the midpoint potentials for ferri-ferrocyanide given by O'Reilly (1973) were used. All absorption spectra were obtained on a Cary 118 recording spectrophotometer. The equilibrium constant for the dissociation of $S_2O_4^{2-}$ to SO_2^- was measured by electron spin resonance as described by Miller and Cusanovich (1975).

Table I: The Effect of Ionic Strength or the Reaction of HIPIP with Iron Hexacyanides.[a]

Ionic Strength	k_{ox} (M^{-1} sec^{-1})	k_{red} (M^{-1} sec^{-1})
0.008	1152	149
0.033	1470	138
0.058	1947	173
0.108	2525	152
0.158	3013	136
0.208	3818	154

[a] Reactions were conducted in 10 m*M* Tris-Cl (pH 7.3) supplemented with varying amounts of NaCl, temperature 20°, HIPIP concentration 20–50 μM. Oxidation was monitored at 500 nm, reduction at 480 nm.

Results

Oxidation-Reduction Potentials. Oxidation-reduction potentials were measured in 25 m*M* TAGP buffer adjusted to the appropriate pH. Figure 1A presents the effect of pH on the oxidation-reduction titrations over the pH range 5–8. The solid lines in Figure 1A are calculated for a N = 1.0 reaction (from the Nernst equation) at the indicated midpoint potentials. Below pH 5 HIPIP was found to be unstable for the time period of the titrations with a progressive loss of visible absorbance with time. Titrations were also conducted at pH 9 and 11 and yielded midpoint potentials identical with those obtained at pH 7 and 8. However, at pH 11 the protein was somewhat unstable and the curves obtained were not linear at the extremes. Figure 1B summarizes the midpoint potentials obtained as a function of pH. At pH 7.0 our midpoint potential (356 mV) is in excellent agreement with that reported by Bartsch (1963) (350 mV).

Reaction of HIPIP with Iron Hexacyanides. The reaction of oxidized HIPIP with potassium ferrocyanide and reduced HIPIP with potassium ferricyanide was investigated under pseudo-first-order conditions. In all cases to be reported, the reactions were found to be accurately first-order for at least 4 half-lives. Further, second-order plots (k_{obsd} vs. [reactant]) for the reaction of HIPIP and iron hexacyanides were found to be linear as long as constant ionic strength was maintained.

The effect of ionic strength on the reaction of HIPIP with iron hexacyanides is summarized in Table I. No effect of specific ions was noted: the buffers Tris-Cl, potassium phosphate, and TAGP and the salts KCl and NaCl gave identical results at a particular ionic strength. Ferrocyanide reduction was found to be independent of ionic strength yielding a second-order rate constant of 150 ± 13 M^{-1} sec^{-1}. In contrast ferricyanide oxidation was clearly dependent on ionic strength. From Debye-Hückel plots (Frost and Pearson, 1961) we obtained a value of 890 M^{-1} sec^{-1} for ferricyanide oxidation at infinite dilution and a slope of +1.4. Thus, from the charge on ferricyanide (−3), we calculated a charge of −0.47 on the HIPIP molecule at the site of oxidation. As polyvalent ions are involved, the apparent

Table II: Thermodynamic Parameters for the Reaction of HIPIP with Iron Hexacyanides.[a]

	E_{act} (kcal/mol)	$H^{\ddagger}$ (kcal/mol)	$S^{\ddagger}$ (eu)	$TS^{\ddagger}$[b] (kcal/mol)	$G^{\ddagger}$[b] (kcal/mol)
HIPIP reduction	6.0	4.2 ± 1.0	−34.8 ± 3.60	−10.2	14.4
HIPIP oxidation	0.0	0.0 ± 1.3	−45.1 ± 4.2	−13.2	13.2

[a] Buffer, 10 m*M* Tris-Cl (pH 7.3); HIPIP concentration, 50 μM. [b] Temperature 20°.

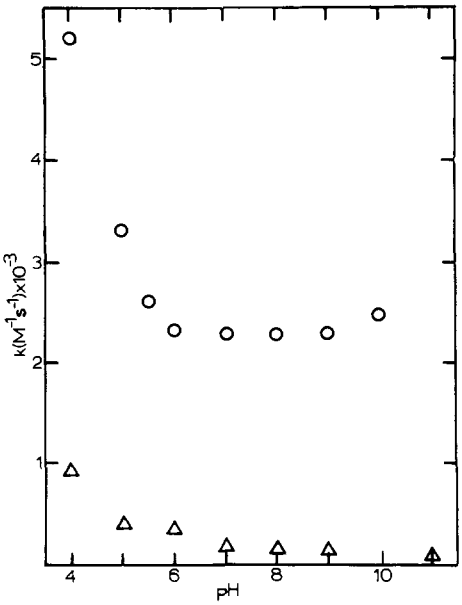

FIGURE 2: Influence of pH on the reaction of HIPIP with iron hexacyanides. Buffer 25 m*M* TAGP adjusted to the appropriate pH. Temperature 20°; HIPIP concentration, 50 μM. (○) Ferricyanide oxidation; (△) ferrocyanide reduction.

charge and rate at infinite dilution can only be considered estimates.

From the rates at infinite dilution (pH 7.3) an equilibrium constant (k_{red}/k_{ox}) of 0.168 can be calculated. This value compares favorably with an equilibrium constant of 0.125 calculated from the midpoint potentials of HIPIP (356 mV) and the iron hexacyanides (400 mV, estimated from the data of O'Reilly, 1973). The excellent agreement between the kinetic and equilibrium measurements suggests a simple reversible reaction as given by

$$\text{ferrocyanide} + \text{oxidized HIPIP} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{ferricyanide} + \text{reduced HIPIP} \quad (1)$$

The influence of pH on the reaction of HIPIP and iron hexacyanides is given in Figure 2. In terms of eq 1, k_{21} is independent of pH between pH 6 and 10, with the rate of oxidation increasing as the pH is lowered below 6. Within experimental error, k_{12} is independent of pH between pH 7 and 9 with the rate of reduction increasing at pH values below 7. It is observed that k_{12} decreases somewhat (~40%) between pH 9 and 11. However, as HIPIP is somewhat unstable at alkaline pH (>10), the significance of this cannot be assessed. In addition, the values of k_{12} and k_{21} at pH 4 are less defined than those at higher pH values due to the instability of the protein at this pH.

The effect of temperature on the reaction of HIPIP with iron hexacyanides is summarized in Table II. Thermodynamic parameters were obtained by least-squares analysis of plots of $1/T$ vs. ln k_{12} or ln k_{21}. We found k_{21} is independent of temperature while k_{12} has both an entropic and enthalpic contribution. Summing the $\Delta G^{\ddagger}$ values for the reaction as given by eq 1 yields a net $\Delta G^{\ddagger}$ of 1.2 kcal/mol. This value is in excellent agreement with ΔG obtained from oxidation-reduction potentials for the buffer used in this experiment (ΔG = 1.36 kcal/mol, 10 m*M* Tris-Cl (pH 7.3)), further supporting the application of eq 1.

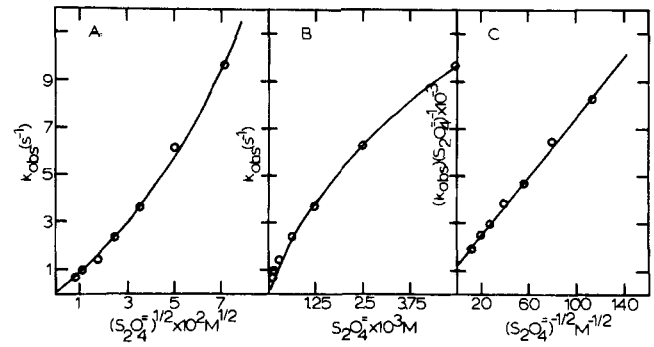

FIGURE 3: (A) Plot of pseudo-first-order rate constants vs. $[S_2O_4^{2-}]^{1/2}$. (B) Plot of pseudo-first-order rate constants vs. sodium dithionite concentration. (C) Plot of $k_{obsd}[S_2O_4^{2-}]^{-1}$ vs. $[S_2O_4^{2-}]^{-1/2}$. Conditions as given in the legend of Table I.

Anionic Reductants. For the purpose of comparison to other oxidation-reduction proteins (see Discussion) and to ascertain if the observations with ferrocyanide were unique we have investigated the reaction of HIPIP with sodium ascorbate and sodium dithionite. The reactions were conducted in 10 m*M* Tris-Cl (pH 7.3) supplemented with varying amounts of NaCl to vary the ionic strength. Reduction by sodium ascorbate was found to be accurately first-order for at least 4 half-lives with second-order plots linear over the ascorbate concentration range 50-0.1 m*M*. For the ionic strength range 0.01-0.11 (4 ionic strengths) we found a second-order rate constant of 3.9 ±1.4 M^{-1} sec^{-1} with no dependency on ionic strength noted.

The reaction of oxidized HIPIP with sodium dithionite was pseudo-first-order at any given dithionite concentration (5–0.15 m*M*) but second-order plots were nonlinear (Figure 3B) becoming independent of $S_2O_4^{2-}$ concentration. Such behavior has been noted for dithionite reduction of myoglobin and horse heart cytochrome *c* (Lambeth and Palmer, 1973; Miller and Cusanovich, 1975). Two possibilities are likely in this situation as $S_2O_4^{2-}$ is in equilibrium with the anion radical SO_2^- ($S_2O_4^{2-} \rightleftharpoons 2SO_2^-$). (a) SO_2^- is the sole reductant; hence a plot of k_{obsd} *vs.* $[S_2O_4^{2-}]^{1/2}$ should be linear. As shown in Figure 3A, this is clearly not the case. (b) Both SO_2^- and $S_2O_4^{2-}$ act as reductants; then complex kinetic plots would be expected. Indeed, if $k_{obsd}/[S_2O_4^{2-}]$ is plotted against $(S_2O_4^{2-}]^{-1/2}$, a straight line should be obtained with a slope of $k_{SO_2^-}$ (K_{eq}') and intercept of $k_{S_2O_4^{2-}}$ (Lambeth and Palmer, 1973). In this case, K_{eq}' is the equilibrium constant for the conversion of $S_2O_4^{2-}$ to SO_2^- and can be determined by EPR (Lambeth and Palmer, 1973). Typical data are given in Figure 3C and

Table III: Effect of Ionic Strength on the Reaction of Cytochrome c_2 with HIPIP.

Ionic Strength[a]	k_{12} (M^{-1} sec^{-1})	k_{21} (M^{-1} sec^{-1})
0.035	2.4×10^5	7.5×10^4
0.055		6.5×10^4
0.085		5.0×10^4
0.135	1.8×10^5	3.3×10^4

[a] Buffer 20 mM potassium phosphate (pH 7.0) supplemented with varying concentrations of NaCl to give the indicated ionic strengths. Cytochrome c_2 concentration 5 μM, 20°, the reaction was monitored at 418 nm.

are consistent with reduction by both $S_2O_4^{2-}$ and SO_2^-. For the ionic strength range 0.01–0.11 (4 ionic strengths), we found a second-order rate constant of $2.1 \pm 0.9 \times 10^6$ M^{-1} sec^{-1} for SO_2^- reduction and $1.2 \pm 0.3 \times 10^3$ M^{-1} sec^{-1} for $S_2O_4^{2-}$ reduction. In both cases, the reaction was found to be independent of ionic strength.

Reaction of HIPIP and Cytochrome c_2. As studies described above utilized small nonphysiological reactants which are not likely to be structurally representative of natural reactants, we have investigated the reaction of HIPIP with *Rhodospirillum rubrum* cytochrome c_2. The studies reported in this section have been restricted by the concentration range of the reactants due to the large absorbances and limited quantities of the proteins.

The reaction of HIPIP and cytochrome c_2 (oxidation and reduction) was investigated at the wavelengths 418, 450, 504, and 550 nm. In all cases, the reaction was pseudo-first-order for at least 3 half-lives with no differences due to wavelength noted. Based on these observations, subsequent reactions were monitored at 418 nm to obtain maximum absorbance changes and the HIPIP concentration was varied relative to a fixed cytochrome c_2 concentration. Within experimental error, second-order plots were linear and yielded second-order rate constants of $3.1 \times 10^4 \pm 0.3$ M^{-1} sec^{-1} for oxidation of reduced HIPIP and $1.5 \times 10^5 \pm 0.7$ M^{-1} sec^{-1} for the reduction of oxidized HIPIP (20 mM potassium phosphate–100 mM NaCl (pH 7.0)). In terms of eq 1 this yielded an equilibrium constant (k_{12}/k_{21}) of 4.8 as compared with a value of 4.0 calculated from the respective midpoint potentials (E_m (cytochrome c_2) = 320 mV, E_m (HIPIP) = 356 mV).

The effect of ionic strength on the interaction of cytochrome c_2 with HIPIP was investigated and the results are tabulated in Table III. The reduction of oxidized HIPIP was investigated at only two ionic strengths as within experimental error the results were the same. In contrast, the oxidation of reduced HIPIP was found to steadily decrease with increasing ionic strength. This type of behavior is consistent with cation–anion interaction and extrapolation to infinite dilution yielded an approximate rate constant of 1.4×10^6 M^{-1} sec^{-1}. It must be stressed that due to the large experimental error and the uncertainties involved in describing the interactions of polyvalent ions, this rate constant is only an estimate. Nevertheless, the trend is observed and is consistent with an ionic interaction.

Discussion

The results presented here demonstrate that *Chromatium vinosum* HIPIP undergoes oxidation–reduction in what appears to be a kinetically simple fashion. At pH 7.0 where these studies were primarily focussed the reaction of HIPIP with a variety of oxidants and reductants was found to obey pseudo-first-order kinetics at any given reactant concentration and to be second-order as a function of oxidant and reductant concentration. Over the time span available to us we found no evidence for a complex between HIPIP and the reactants used. This indicates that collisions leading to electron transport by HIPIP must form complexes with lifetimes of less than 5–10 msec.

Reduction of HIPIP is essentially independent of ionic strength with ionic reductants (ferrocyanide, SO_2^-, $S_2O_4^{2-}$, ascorbate, and cytochrome c_2). Moreover, ferricyanide oxidation is only slightly ionic strength dependent, with an apparent net charge on the HIPIP molecule of −0.5 as compared to −3 or −4 for the net protein charge at pH 7.0 as estimated from the amino acid sequence (Dus et al., 1973) and the charge on the iron cluster (Herskovitz et al., 1972; Averill et al., 1973). Thus, electron transfer clearly does not result from random collisions and suggests specific sites of oxidation and reduction. The noted discrepancy between apparent charge at the site of oxidation and reduction leaves open the possibility that oxidation and reduction take place at different sites on the molecule. However, this discrepancy could be accounted for by reorientation about a single site of electron transport on oxidation and reduction. Structural studies (Carter et al., 1974b) indicate no substantial changes in conformation on oxidation or reduction; however, it is not clear that this approach would detect reorientations of surface residues in solution. Alternatively, the greater negative charge of reduced HIPIP at the iron–sulfur cluster could account for the observed charge differences.

The effects of pH on the reaction of HIPIP with the iron hexacyanides and on the oxidation–reduction potential indicate there are no or very slight effects of pH-induced ionizations in the pH range 7–11 on electron transfer. At pH values below 7 both oxidation and reduction appear to be influenced by an ionization with a pK_a of less than 6. The exact value of this ionization cannot be determined due to the instability of HIPIP at pH values of less than 5. However, the observed increase in the rate of ferricyanide oxidation at low pH values is consistent with the protonation of an anionic group, hence facilitating the reaction with the anionic oxidant. The apparent increase in the rate of reduction of HIPIP by ferrocyanide could result from two possibilities: (1) a general unfolding of the protein (also a possible explanation for the increase in the rate of ferricyanide oxidation) or (2) protonation of an anionic group which is in close proximity to a cationic group. Hence at pH 7 the net charge at the site of electron transfer (for reduction) is zero and it becomes positively charged at pH values below 7.

Table IV summarizes the rate constants obtained for the reaction of HIPIP with both physiological and nonphysiological reactants. The individual rate constants for the nonphysiological reactants and the HIPIP are all substantially less (1–4 orders of magnitude) than the analogous reactions with horse heart cytochrome c (Miller and Cusanovich, 1975) and *Rhodospirillum rubrum* cytochrome c_2 (Wood and Cusanovich, 1975). In general, it would appear that the major factor is electrostatic. Both cytochrome c_2 and cytochrome c have been shown to have positively charged sites of electron transport (Miller and Cusanovich, 1975; Wood and Cusanovich, 1975) and hence react rapidly with the various anionic reactants used here. The driving force for

most of the reactions studied should be more favorable for HIPIP as it has a higher oxidation-reduction potential than the cytochromes. Moreover, the reaction of HIPIP with cytochrome c_2 is rapid. In this latter case the size of the interacting molecules would rule out steric hindrance as a major factor in the studies reported here. Thus it appears that driving force and steric factors take on lesser importance than electrostatic attraction or lack of repulsion. Further, the reaction of cytochrome c_2 with HIPIP shows interactions (plus-minus, neutral) as predicted from the reaction of the respective macromolecules with the various nonphysiological reactants. This result suggests that physiological and nonphysiological reactants interact in a similar fashion and are consistent with single minimum energy pathways for oxidation and reduction.

The iron hexacyanides are outer-sphere electron transfer agents and their rapid reaction with HIPIP is consistent with an outer-sphere reaction for HIPIP. The other reactants used here (SO_2^-, $S_2O_4^{2-}$, cytochrome c_2, and ascorbate) are less well defined in mechanistic terms. However, the effect of ionic strength on all reductants used is consistent with the same site of reduction and suggestive of outer-sphere electron transfer in all cases. Recently the reactions of a number of cationic reductants with the non-heme iron protein, rubredoxin, were reported (Jacks et al., 1974). These studies were, in general terms, consistent with those reported here. Jacks et al. (1974) proposed outer-sphere electron transfer for reduction of rubredoxin by $Ru(NH_3)_6^{2+}$, $V(H_2O)_6^{2+}$, and $Cr(H_2O)_6^{2+}$ with rate constants in the range 9.5×10^4 to 1.2×10^3 M^{-1} sec^{-1}. Further, they found that the reactions were driven entropically (−31 to −41 eu) with little or no enthalpic term (0–1.4 kcal/mol). Jacks et al. (1974) attributed the small activation enthalpies to a favorable electrostatic interaction between the reactants as they found a negative charge at the site of reduction of rubredoxin. The larger activation energy (6.0 kcal/mol) reported here for the reduction of HIPIP by ferrocyanide could in a similar vein be attributed to the lack of electrostatic attraction. However, this argument fails to explain the negligible activation enthalpy for ferricyanide oxidation of HIPIP and leaves open the origin of the enthalpies of activation.

In the following discussion the relative merits of what we consider to be the three most likely mechanisms for the oxidation and reduction of HIPIP will be evaluated in terms of available data. Although no final decision can be made at this time, it is our view that the discussion is useful in developing a conceptual approach to biological electron transport and bears directly on the design of future studies. From the proposal of Carter et al. (1974), the iron-sulfur cluster can be envisioned as buried in the interior of the proteins a small distance (3–5 Å) from the plane of the aromatic residue tyrosine-19, with both oxidation and reduction mediated by a phenoxy radical transition state. To be consistent with our results, this mechanism would require the presence of a negatively charged amino acid side chain in the vicinity (4–10 Å) of Tyr-19 for reduced HIPIP with this side chain reoriented upon oxidation so as not to affect subsequent reduction. A phenoxy radical transition state appears unlikely to us as this would require a substantial energy of activation for formation since the reduction potential of phenols has been estimated to be on the order of −2 V (Castro, 1975). Further, it has been proposed (Carter et al., 1974b) that a water molecule serves to stabilize the transition state by accepting the tyrosine hydroxyl proton. This proposal should predict that at alkaline pH values a substantial change in rate and oxidation-reduction potential would be observed. This was not found in the studies reported here. A second possibility would have the iron-sulfur cluster insulated from the solvent near the site of electron transfer by the packing of hydrophobic groups to provide a "dielectric barrier". Carter et al. (1974b) pointed out that the S*2 and S*4 inorganic sulfur atoms are only 4.5 Å from the surface of the molecule but insulated by the side chains of Leu-17, Phe-48, Leu-65, Phe-66, and Ser-79. It can be proposed that a reactant approaches the region of S*4 and S*2 and the electron "hops" the 4–5 Å through a nonaqueous media to or from the chromophore. In regards to charge, Asp-45 has its carboxyl oxygen 8.4 Å from S*2 (8.0 Å from Fe1 which is bonded to S*2; distances were calculated from available coordinates, Carter et al., 1974a); hence it could provide a negative charge near the site of oxidation of reduced HIPIP. This negative charge would have to reorient on oxidation so that the net charge would be zero for oxidized HIPIP or the site of reduction is different from that for oxidation.

Table IV: Summary of Rate Constants for the Oxidation and Reduction of HIPIP.

	k ($M^{-1}sec^{-1}$)	Apparent Charge Interactions
Oxidants		
Ferricyanide	890[a]	Minus–minus
Ferricytochrome c_2	1.4×10^6[a]	Plus–minus
Reductants		
Ferrocyanide	150	Minus–neutral
Ferrocytochrome c_2	2.2×10^5	Plus(?)–neutral
Sodium ascorbate	3.9	Minus–neutral
SO_2^-	2.1×10^6	Minus–neutral
$S_2O_4^{2-}$	1.2×10^3	Minus–neutral

[a] These values are estimates from extrapolation to infinite dilution.

Finally, it can be proposed that a portion of the iron-sulfur cluster is actually solvent exposed and electron transport takes place on collision with the oxidant or reductant. Although possibly the most attractive in terms of a simple interaction of two redox chromophores with a minimum energy barrier, this proposal requires that the solution and crystal structure of HIPIP be different. This is a difficult point to address with available data. However, it is clear from the size of interacting molecules (cytochrome c_2-HIPIP) that electron transfer is mediated largely by surface interactions. Hence, the question can be reduced to whether or not the surface structure is the same in the crystal as in the solution. It would seem that distortions in the crystal are possible (or even likely) as the packing into a crystal lattice of similarly charged molecules could be expected to lead to perturbations which facilitate lattice formation. If the iron-sulfur cluster is solvent accessible, the negative charge at the site of oxidation of reduced HIPIP reported here could be in part or whole provided by the iron-sulfur cluster itself.

In summary, although the exact route(s) of electron transfer to and from the iron-sulfur cluster of HIPIP cannot be specified it is clear that the redox reactions are kinetically simple and suggestive of outer-sphere electron transfer. It appears that a wide variety of oxidants and reductants interact with HIPIP by the same general mechanism. The oxidation and reduction of HIPIP appear to be primarily controlled by electrostatic interactions and not differ-

ences in oxidation–reduction potentials or steric restrictions.

References

Averill, B. A., Herskovitz, T., Holm, R. H., and Ibers, J. A. (1973), *J. Am. Chem. Soc. 95,* 3523.

Bartsch, R. G. (1963), in Bacterial Photosynthesis, San Pietro, A., Gest, H., and Vernon, L. P., Ed., Yellow Springs, Ohio, Antioch Press, p 315.

Bartsch, R. G., Kakuno, T., Horio, T., and Kamen, M. D. (1971), *J. Biol. Chem. 246,* 4489.

Carter, C. W., Jr., Freer, S. T., Yuong, Ng. H., Alden, R. A., and Kraut, J. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 381.

Carter, C. W., Jr., Kraut, J., Freer, S. T., and Alden, R. A. (1974b), *J. Biol. Chem. 249,* 6339.

Carter, C. W., Jr., Kraut, J., Freer, S. T., Alden, R. A., Sieker, L. C., Adman, E., and Jensen, L. H. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3526.

Carter, C. W., Jr., Kraut, J., Freer, S. T., Yuong, Ng. H., Alden, R. A., and Bartsch, R. G. (1974a), *J. Biol. Chem. 249,* 4212.

Castro, C. E. (1975), in The Porphyrins, Dolphin, D., Ed. (in press).

DeKlerk, H., and Kamen, M. D. (1966), *Biochim. Biophys. Acta 112,* 175.

Dus, K., Tedro, S., and Bartsch, R. G. (1973), *J. Biol. Chem. 248,* 7318.

Dutton, D. L., and Leigh, J. S. (1973), *Biochim. Biophys. Acta 314,* 178.

Evans, M. C. W., Lord, A. V., and Reeves, S. G. (1974), *Biochem. J. 138,* 177.

Frost, A. A., and Pearson, R. G. (1961), Kinetics and Mechanism, New York, N.Y., Wiley.

Herskovitz, T., Averill, B. A., Holm, R. H., Ibers, J. A., Phillips, W. D., and Weiher, J. F. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2437.

Hori, K. (1961), *J. Biochem. 50,* 481.

Jacks, C. A., Bennett, L. E., Raymond, W. N., and Lovenberg, W. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1118.

Kennel, S. J., Bartsch, R. G., and Kamen, M. D. (1972), *Biophys. J.* 12, 882.

Lambeth, D. O., and Palmer, G. (1973), *J. Biol. Chem. 248,* 6095.

Meyer, T. E. (1970), Ph.D. Thesis, University of California at San Diego.

Miller, W. G., and Cusanovich, M. A. (1975), *Biophys. Struct. Mech. 1,* 97.

O'Reilly, J. E. (1973), *Biochim. Biophys. Acta 292,* 509.

Velick, S. F., and Strittmatter, G. (1956), *J. Biol. Chem. 221,* 265.

Wood, F. E., and Cusanovich, M. A. (1975), *Bioinorg. Chem. 4,* 337.

Phenylalanyl-tRNA Synthetases of Rat Liver: Differential Effects of Thyroid Hormone†

Jennifer B. Nielsen and Audrey E. V. Haschemeyer*

ABSTRACT: Thyroxine and analogues inhibit rat liver aminoacyl-tRNA synthetase activity for phenylalanine and tyrosine. A high yield purification of the major cytoplasmic form of phenylalanyl-tRNA synthetase (C_1) and its characterization is reported. Polyribosome-bound and other sedimentable forms are found to be indistinguishable from soluble enzyme by immunoprecipitation. Mitochondrial phenylalanyl-tRNA synthetase (M) and cytoplasmic activity (C_2) resistant to anti-C_1 antibody have been partially purified and characterized. Tissue levels of the three forms are estimated at 22, 1.8, and 4.1 units/g of liver for C_1, C_2, and M, respectively [1 unit = 1 nmol of Phe-tRNA/min, 30°C]. Charging capability toward rat liver and yeast tRNA, kinetic parameters, and physical properties are compared. Only enzyme C_1 is hormone inhibited [$K_I = 4 \times 10^{-6}$ M for triiodothyronine]. The data indicate that C_2 and M are not structurally related to C_1; C_2 may represent an independent cytoplasmic pool of M. Implications of C_1 inhibition in relation to effects on liver protein synthesis are discussed.

The thyroid hormones thyroxine and 3,3′,5-triiodothyronine are recognized to play a significant role in protein metabolism, although the tissue-specific and dose-dependent interplay of anabolic and catabolic effects have obscured the primary mechanism of action of the hormone. Recently, by use of rapid kinetic methods, it has been possible to show a correlation between thyroid hormone level and the rate of polypeptide chain assembly and release during protein synthesis in rat liver in vivo (Mathews et al., 1973). A 40% depression in elongation rate was found in surgically thyroidectomized animals compared to euthyroid controls; rates were restored to normal by triiodothyronine injections. A small elevation in synthetic rate occurred in normal animals given similar hormone injections. Data for other systems, however, indicate opposite effects in more acute hyperthyroidism (Kivirikko et al., 1967; Nadkarni and Samuel, 1973; Griffin and Miller, 1973), suggesting the possibility of multiple effects on the protein synthetic pathway particu-

† From the Department of Biological Sciences, Hunter College of the City University of New York, New York, New York 10021. *Received August 7, 1975.* This research has been supported by Grant HD 04670 from the U.S. Public Health Service. A fellowship from the Helen Hay Whitney Foundation (to A.E.V.H.) in the early stages of this work is gratefully acknowledged.