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Electron Transfer Reactions of High-Potential Iron–Sulfur Proteins and *c*-Type Cytochromes†

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ABSTRACT: Studies of electron transfer by biological oxidation–reduction proteins frequently focus on the interaction of a particular protein with nonphysiological oxidants and/or reductants. This approach, although valuable, is limited by the size and chemistry of the nonphysiological reactants. To further the understanding of biological electron transfer, we have investigated the interaction of two examples of high-potential iron–sulfur proteins (HIPIP's) with mitochondrial cytochrome *c* (horse heart) and bacterial cytochrome c_2 from *Rhodospirillum rubrum*, *Rhodopseudomonas palustris*, *Rhodopseudomonas capsulata*, and *Rhodopseudomonas sphaeroides*. On the basis of the kinetics of electron transfer between the various HIPIP's and cytochromes, it appears that the interactions are more complex than those observed with nonphysiological reactants. We conclude that (1) specific sites on both the HIPIP's and the cytochromes mediate electron transfer with the effect of ionic strength different from that expected on the basis of the interaction of the various proteins with the iron hexacyanides, (2) the interaction of HIPIP with some of the cytochromes investigated is heterogeneous, resulting from at least two possible orientations (cytochrome dependent) for interaction leading to electron transfer, and (3) no long-lived complexes between the HIPIP's and cytochromes are formed due to rapid equilibrium between the two proteins. This last conclusion suggests that the measured second-order rate constant is in fact the product of the association constant (for any HIPIP and a particular cytochrome) and a first-order rate constant reflecting the rate-limiting step leading to products.

In previous publications we reported on the interaction of a class of high-potential iron–sulfur proteins (HIPIP) with nonphysiological oxidants and reductants (Mizrahi et al., 1976, 1980). These studies indicate that electron transfer to the HIPIP iron–sulfur cluster takes place at a specific region on the protein surface and involves electrostatic interactions. Further, it appears that the iron–sulfur cluster charge is distributed on the protein surface in the region of the site of electron transfer (Mizrahi et al., 1980) and affects the kinetics of electron transfer.

The specific electron donor and acceptor that interact with HIPIP in vivo are unclear at the present time although a recent report suggests *Chromatium* HIPIP may serve as an electron acceptor for a thiosulfate oxidizing enzyme (Fukumori & Yamamaka, 1979). In a preliminary communication, we have reported on the interaction of HIPIP with several *c*-type cytochromes (Cusanovich & Mizrahi, 1979). Although no convincing evidence exists that *c*-type cytochromes naturally interact with HIPIP, this reaction provides a particularly useful model system in that substantial structural information concerning both *c*-type cytochromes and HIPIP is available (Carter et al., 1974, Freer et al., 1975; Salemme et al., 1973; Dickerson et al., 1971). Further, many *c*-type cytochromes have oxidation–reduction potentials similar to HIPIP, thus providing kinetic data on both oxidation and reduction. Our previous studies with *c*-type cytochromes suggested that neither the oxidation–reduction potential nor the structure of the cytochromes in the region of the exposed heme edge (the supposed site of electron transfer) played a measurable role in the reaction with HIPIP (Cusanovich & Mizrahi, 1979). In addition, contrary to the conclusions with nonphysiological reactants, electrostatic interactions were not thought to be of major importance over the ionic strength range studied. We report here more extensive studies on the interaction of HIPIP from two sources (*Chromatium vinosum* and *Rhodopseudomonas gelatinosa*) with horse heart cytochrome *c* and cytochrome c_2 from *Rhodospirillum rubrum*, *Rhodopseudomonas capsulata*, and *Rhodopseudomonas palustris*.

Materials and Methods

HIPIP's from *C. vinosum* and *Rps. gelatinosa* were prepared as described previously (Cusanovich, 1967; Bartsch,

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Table I: Second-Order Rate Constants for the Reaction of *Chromatium* and *Rps. gelatinosa* HIPIP with *c*-Type Cytochromes[a]

	$k \times 10^{-5}$ (M^{-1} s^{-1})		product of charges
	fast	slow	
Chromatium HIPIP ($E_{m,7}$ = 350 mV) Reduction			
reductant			
Rsp. rubrum cytochrome c_2 ($E_{m,7}$ = 332 mV)	2.1 ± 0.3		0
horse heart cytochrome *c* ($E_{m,7}$ = 260 mV)	0.87 ± 0.11		0
Rps. capsulata cytochrome c_2 ($E_{m,7}$ = 343 mV; μ = 0.068)	1.5	0.10	minus/minus
Rps. sphaerodies cytochrome c_2 ($E_{m,7}$ = 350 mV)	2.0 ± 0.2	0.11 ± 0.03	0
Rps. palustris cytochrome c_2 ($E_{m,7}$ = 368 mV; μ = 0.068)	1.2	0.19	minus/minus
Rps. palustris cytochrome c_2 ($E_{m,7}$ = 351 mV; pH 8.0; μ = 0.068)	1.5	0.17	ND
Rps. gelatinosa HIPIP ($E_{m,7}$ = 330 mV) Reduction			
reductant			
horse heart cytochrome *c*	0.26 ± 0.03		0
Rps. capsulata cytochrome c_2	2.4 ± 0.4	0.11 ± 0.01	0
Chromatium HIPIP Oxidation			
oxidant			
Rsp. rubrum cytochrome c_2 (μ = 0.055)	0.65		plus
Rps. palustris cytochrome c_2	2.0 ± 0.5	0.36 ± 0.11	0
Rps. palustris cytochrome c^2 (pH 8.0; μ = 0.068)	1.3	0.26	ND

[a] All data are reported for 20 °C, pH 7.0, unless otherwise noted. The product of charges is from the simplified Debye–Huckel relation; see the text. Standard deviations given were obtained as described in the text. ND is defined as not determined. In those cases where two products of charges are given, they are for fast/slow.

1978). Horse heart cytochrome *c* was from Sigma Chemical Co. (Type VI) and was further purified by fractionation on Sephadex G-75 to remove dimers. *Rps. rubrum* ATH 1.1.1, *Rps. capsulata* ATH 2.3.1, *Rps. sphaeroides* ATH 2.4.1, and *Rps. palustris* ATH 2.1.37 cytochromes c_2 were purified by standard methods (Bartsch, 1971).

The kinetic measurements were conducted in a Durrum-Gibson stopped-flow spectrophotometer, and kinetic data were analyzed as described previously (Mizrahi et al., 1980). All experiments were performed at 20 °C with a buffer of 10 mM potassium phosphate supplemented with NaCl to give the indicated ionic strength unless otherwise noted. The reduced forms of the various proteins were obtained by the addition of a slight excess of sodium dithionite, followed by gel filtration over Sephadex G-25 equilibrated with the desired buffer. In all cases, identical results were obtained whether the reaction was monitored at 415, 418, 480, 506, or ~550 nm. The wavelengths 415, 418, and 550 nm have absorbance changes due primarily to cytochrome (76–92% of the absorbance change), while 480 and 506 are primarily HIPIP wavelengths (80–100% of the absorbance change). For the experiments presented, the cytochrome was the fixed reactant (concentration typically 1–2 μM) and HIPIP was the variable reactant (concentration range 3–30 μM). All kinetic data reported are means of two or more experiments.

Results

The data obtained in our studies of the reaction of various *c*-type cytochromes are summarized in Tables I and II. In general, two situations were encountered. The reactions of *Chromatium* HIPIP with ferri- and ferrocytochrome c_2 from *Rsp. rubrum* and with horse heart ferrocytochrome *c* were found to yield linear ln (ΔA) vs. time plots for at least three half-lives, consistent with a simple pseudo-first-order process. Similarly, the reduction of *Rps. gelatinosa* HIPIP by horse heart ferrocytochrome *c* was accurately first order at any HIPIP concentration. Typical data are presented in Figure 1A in the form of ln (ΔA) vs. time plots for the reaction of oxidized *Chromatium* HIPIP with reduced horse heart cytochrome *c*. Figure 1B presents second-order plots (k_{obsd} vs. [HIPIP]) for the data given in Figure 1A. In all cases, second-order plots were linear over the concentration ranges

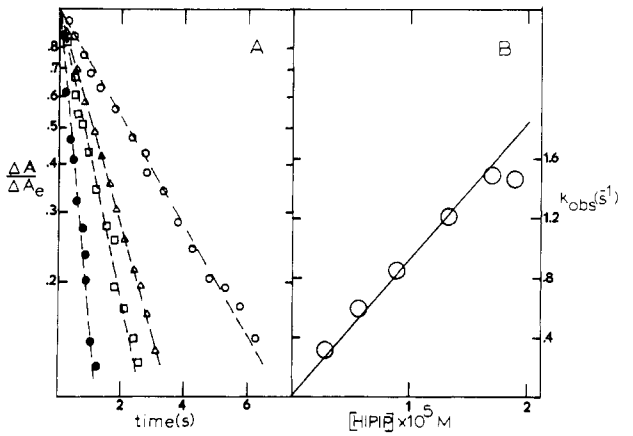

FIGURE 1: Reaction of oxidized *C. vinosum* HIPIP with reduced horse heart cytochrome *c*. (A) Ln ($\Delta A/\Delta A_e$) vs. time plots. $\Delta A/\Delta A_e$ represents the observed absorbance change at any time *t* divided by the total absorbance change observed for the particular reaction. Buffer: 20 mM potassium phosphate and 20 mM NaCl, pH 7.0, 20 °C. The reaction was monitored at 550 nm, and the horse heart cytochrome *c* concentration was 1.5 μM. [HIPIP]: (○) 3; (△) 5.7; (□) 8.7; (●) 19 μM. (B) Second-order plot of the data in (A). Conditions were for (A); observed rate constants determined as described previously (Mizrahi et al., 1980) are plotted against [HIPIP].

studied, and the apparent second-order rate constants are summarized in Table I. Each second-order rate constant was determined at four or more ionic strengths, and only in the case of the oxidation of *Chromatium* HIPIP by *Rsp. rubrum* cytochrome c_2 was an ionic strength dependence noted. The standard deviations given in Table I were derived from the rate constants measured at various ionic strengths when no ionic strength effect was observed (μ = 18–130 mM). When an ionic strength dependence was noted, the sign of the apparent product of charges is reported (–, when k_{obsd} decreased with increasing μ; +, when k_{obsd} increased with increasing μ) for the ionic strength range studied. If no ionic strength dependence was observed, the product of charges is reported as zero.

A second pattern was observed when either *Chromatium* or *Rps. gelatinosa* HIPIP was reacted with cytochrome c_2 from *Rps. capsulata*, *Rps. sphaeroides*, or *Rps. palustris*. In these cases the ln (ΔA) vs. time plots were clearly biphasic, indicating the presence of two kinetic species. A typical ln (ΔA)

Table II: Phase Distribution for the Reaction of *Chromatium* and *Rps. gelatinosa* HIPIP with *c*-Type Cytochromes[a]

	fraction of slow phase ($\Delta A_s^0/\Delta A_e$) at μ (M) of			
	0.018	0.038	0.068	0.135
	Chromatium HIPIP Reduction			
reductant				
Rsp. rubrum cytochrome c_2	0	0	0	0
horse heart cytochrome *c*	0	0	0	0
Rps. capsulata cytochrome c_2	0.65 ± 0.08	0.73 ± 0.11	0.65 ± 0.04	0.77 ± 0.05
Rps. sphaeroides cytochrome c_2	0.80 ± 0.05	0.84 ± 0.03	0.82 ± 0.04	0.86 ± 0.09
Rps. palustris cytochrome c_2	0.47 ± 0.04	0.43 ± 0.06	0.47 ± 0.11	0.47 ± 0.05
Rps. palustris cytochrome c_2 (pH 8.0)	ND	ND	0.38 ± 0.10	ND
	Rps. gelatinosa HIPIP Reduction			
reductant				
horse heart cytochrome *c*	0	0	0	0
Rps. capsulata cytochrome c_2	ND	ND	0.69 ± 0.07	0.74 ± 0.09
	Chromatium HIPIP Oxidation			
oxidant				
Rps. rubrum cytochrome c_2	0	0	0	0
Rps. palustris cytochrome c_2	0.47 ± 0.02	0.43 ± 0.08	0.51 ± 0.03	0.52 ± 0.05
Rps. palustris cytochrome c_2 (pH 8.0)	ND	ND	0.44 ± 0.11	ND

[a] All data are reported for 20 °C, pH 7.0, unless otherwise noted. Zero entered in the table indicates that no kinetic heterogeneity was observed.

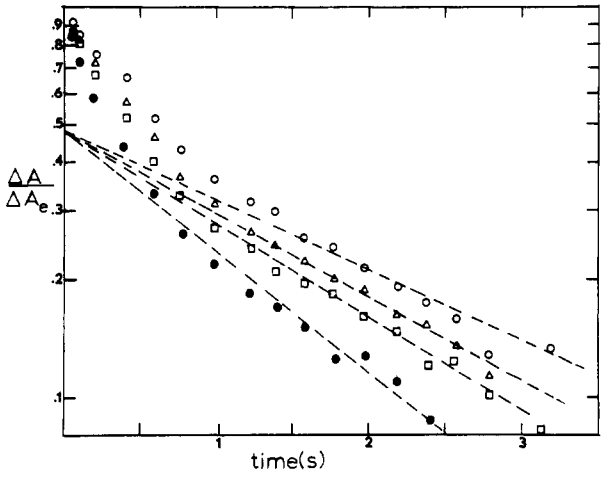

FIGURE 2: Reaction of oxidized *C. vinosum* HIPIP with reduced *Rps. palustris* cytochrome c_2. Ln ($\Delta A/\Delta A_e$) vs. time plots. ΔA and ΔA_e are defined in the legend to Figure 1A. Buffer: 20 mM potassium phosphate and 100 mM NaCl, pH 7.0, 20 °C. The reaction was monitored at 552 nm, and the *Rps. palustris* cytochrome c_2 concentration was 3.5 μM. [HIPIP]: (○) 7.2; (△) 11.2; (□) 17.7; (●) 31.0 μM.

vs. time plot is presented in Figure 2. The biphasic data were analyzed by utilizing eq 1:

$$\Delta A_t = (\Delta A_e - \Delta A_s^0)e^{-k_f t} + \Delta A_s^0 e^{-k_s t} \quad (1)$$

where ΔA_t is the absorbance change at any time t, ΔA_s^0 is the total contribution of the slow kinetic species to the absorbance change, ΔA_e is the the total absorbance change observed in the experiment, and k_f and k_s are the pseudo-first-order rate constants for the fast and slow kinetic species, respectively. The experimental data (ΔA_t, ΔA_e, and t) were fitted by means of a computer program which used a steepest descents procedure to optimize the three variables (k_f, k_s, and ΔA_s^0), that is, to obtain a minimum least-squares error. Subsequently, both k_s and k_f were plotted against the HIPIP concentration to obtain apparent second-order rate constants. Typical second-order plots for the reaction of oxidized *Chromatium* HIPIP with ferrocytochrome c_2 from *Rps. palustris* are given in Figure 3. In all cases studied, within experimental error, the second-order plots were linear and yielded the second-order

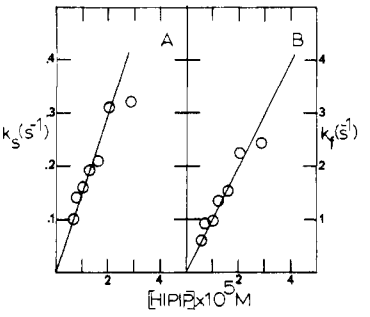

FIGURE 3: Second-order plots for the reaction of oxidized *C. vinosum* HIPIP with reduced *Rps. palustris* cytochrome c_2. Conditions were as given in the legend to Figure 2. The various rate constants were obtained by deconvolution of the kinetic data as described under Materials and Methods and previouly (Mizrahi et al., 1980). (A) Plot of the observed rate constant for the slow kinetic species (k_s) against [HIPIP]. (B) Plot of the observed rate constant for the fast kinetic species (k_f) against [HIPIP].

rate constants given in Table I. As with the monophasic reactions discussed above, the effect of ionic strength was investigated and is summarized in Table I. Only in the reactions involving oxidized *Chromatium* HIPIP with reduced cytochrome c_2 from *Rps. capsulata* and *Rps. palustris* were ionic strength dependencies noted. For the biphasic reactions, it was found that the fraction of slow phase ($\Delta A_s^0/\Delta A_e$) was independent of HIPIP concentration. Thus Table II presents the fraction of slow phase at various ionic strengths plus the standard deviations calculated from the fraction of slow phase at each HIPIP concentration (generally five to seven different HIPIP concentrations were used). As the reaction of *Chromatium* HIPIP with *Rps. palutris* cytochrome c_2 gave an approximately 50:50 distribution in terms of the two kinetic phases, this reaction was investigated at a second pH (pH 8.0) to determine if pH influenced the relative amounts of the two kinetic species. No pH effect was noted. The rate constants and phase distributions are summarized in Tables I and II.

The biphasic kinetics described above suggest a heterogeneity associated with cytochromes c_2 from *Rps. capsulata*, *Rps. sphaeroides*, and *Rps. palustris*. Previous studies indicated that reduction of these cytochromes c_2 by ferrocyanide was kinetically homogeneous (Wood et al., 1977). However,

Table III: HIPIP–Cytochrome Electrostatic Interactions

reaction	sign of product[a] of charges (isoelectric points)	sign of product[b] of charges (iron hexacyanides)	sign of product[c] of charges (obsd)
reduced *Chromatium* HIPIP			
+*Rsp. rubrum* cytochrome c_2	+	−	−
+*Rps. palustris* cytochrome c_2	−	−	0
oxidized *Chromatium* HIPIP			
+*Rsp. rubrum* cytochrome c_2	+	0	0
+horse heart cytochrome *c*	−	0	0
+*Rps. capsulata* cytochrome c_2	0	0	−
+*Rps. sphaeroides* cytochrome c_2	+	0	0
+*Tps. palustris* cytochrome c_2	−	0	−
oxidized *Rps. gelatinosa* HIPIP			
+horse heart cytochrome *c*	+	+	0
+*Rps. capsulata* cytochrome c_2	0	+	0

[a] The sign on the product of charges was estimated from the isoelectric point of the proteins and the fact that the experimental pH was 7.0. Isoelectric points used were as follows: *Chromatium* HIPIP, 3.7; *Rps. gelatinosa* HIPIP, 9.5; horse heart cytochrome *c*, 10.4; *Rsp. rubrum* cytochrome c_2, 6.2; *Rps. capsulata* cytochrome c_2, 7.1; *Rps. sphaeroides* cytochrome c_2, 5.5; *Rps. palustris* cytochrome c_2, 9.7. [b] The sign on the product of charges was estimated from the apparent charge at the site of electron transfer deduced from studies on the effect of ionic strength on the interaction of the iron hexacyanides with the various proteins (Mizrahi et al., 1976, 1980; Wood & Cusanovich, 1975; Miller & Cusanovich, 1975; Wood et al., 1977). [c] Data from Table I.

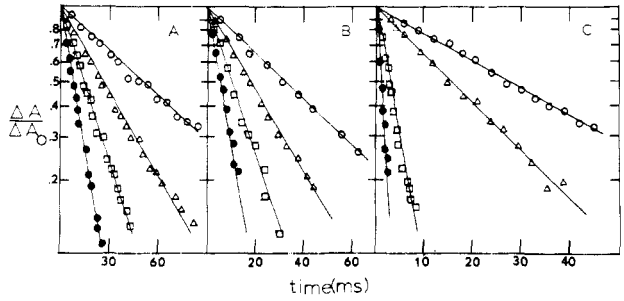

FIGURE 4: Reaction of cytochrome c_2 with potassium ferricyanide. Buffer: 20 mM potassium phosphate and 100 mM NaCl, pH 7.0, 20 °C. The reaction was monitored at the wavelength of the reduced α peak for each cytochrome. Plotted is $\Delta A/\Delta A_0$ where ΔA is the absorbance at any time t and ΔA_0 is the total observed absorbance change for the particular reaction. The cytochrome c_2 concentration was 2–3 μM. (A) Oxidation of *Rps. capsulata* ferrocytochrome c_2. [Ferricyanide]: (○) 28; (△) 37.5; (□) 75; (●) 120 μM. (B) Oxidation of *Rps. palustris* ferrocytochrome c_2. [Ferricyanide]: (○) 25; (△) 50; (□) 90; (●) 150 μM. (C) Oxidation of *Rps. sphaeroides* ferrocytochrome c_2. [Ferricyanide]: (○) 10; (△) 25; (□) 75; (●) 150 μM.

to investigate this point further, we examined the oxidation of the three cytochromes c_2 by potassium ferricyanide at pH 7.0 (μ = 0.135 M) and give the ln (ΔA) vs. time plots in Figure 4. Clearly, all the reactions were accurately first order (plots of k_{obsd} vs. [ferricyanide] were linear) and yielded second-order rate constants of 7.7×10^5, 7.9×10^5, and 3.3×10^6 M^{-1} s^{-1} for *Rps. capsulata*, *Rps. palustris*, and *Rps. sphaeroides* cytochromes c_2, respectively.

Discussion

The data reported here indicate that the reaction between *c*-type cytochromes and HIPIP's is second order as we found no evidence for any change in the rate-limiting step. However, this interpretation is limited by the fact that only a narrow range of concentration of the excess protein was investigated due to the strong absorbance of the reactants. Thus, it is possible that complexes may form, followed by one or more first-order processes. It is important to note that in the absence of strong electrostatic interactions, only transient complexes are likely to form between the reacting proteins. If this is the case, then the apparent second-order rate constants reported (Table I) would actually be the product of the association constant (K) and the limiting first-order rate constant (k) (Cusanovich, 1978). Unfortunately, we cannot pursue this point experimentally at present due to the limited protein concentration ranges available. However, if any first-order processes are present, we can put a lower limit on their rate constants. As the maximum pseudo-first-order rate constants observed were only on the order of 10 s^{-1}, any true first-order processes must be greater than 30–50 s^{-1}. This lower limit is not particularly large and does not exclude the possibility that conformational changes and/or electron transfer (first-order processes) following complex formation take place (Cusanovich, 1978).

Most striking is the observation that all of the second-order rate constants for the fast phase (Table I) are very similar [typically $(1-2) \times 10^{-5}$ M^{-1} s^{-1}]. This observation excludes differences in oxidation–reduction potential of the reactants as a factor controlling the kinetics since no correlation exists between rate constants and oxidation–reduction potential (Table I). This conclusion, coupled with an apparent lack of correlation of the interaction with expected electrostatics (see below), suggests that some common underlying factor(s) must be controlling the electron transfer reaction. Evidence bearing on the factors involved in the interaction of HIPIP and the cytochromes is provided by the biphasic kinetic data. Examination of Table II shows that the apparent heterogeneity is cytochrome dependent, not HIPIP dependent. Both *Chromatium* and *Rps. gelatinosa* HIPIP's yield monophasic kinetics when reacted with horse heart cytochrome *c*. However, both have biphasic kinetics when reacted with *Rps. capsulata* cytochrome c_2 with the amount of slow phase approximately the same (~70%) for the interaction of both HIPIP's with *Rps. capsulata* cytochrome c_2. It is important to note that both kinetic phases observed with *Rps. capsulata*, *Rps. palustris*, and *Rps. sphaeroides* cytochromes c_2 are second order and that the relative contribution of the two phases is (1) independent of HIPIP concentration (at any ionic strength), (2) independent of ionic strength, (3) independent of redox state, and (4) in the one case examined, independent of pH. These observations establish that the two kinetic species of cytochrome c_2 observed are not readily interconvertible. Further, all of the proteins (HIPIP's and cytochromes) studied were kinetically homogeneous with respect to their reaction with the iron hexacyanides. The observations noted above suggest that those cytochromes which have biphasic kinetics in their interaction with HIPIP must have two possible orientations for their interaction with HIPIP. Thus, it follows

that in the cases of horse heart cytochrome *c* and *Rsp. rubrum* cytochrome c_2, only one orientation is allowed. On the basis of the data and arguments presented here, we propose that a given cytochrome and HIPIP can form one or two weak complexes (or rapid equilibria), depending on the cytochrome structure which orients the respective chromophores for optimal electron transfer. The subsequent rate of electron transfer would be determined by either the distance and/or relative orientation of the respective chromophores. The data and arguments presented would exclude long-range electron tunneling and require that reasonably stringent orientation factors control electron transfer between HIPIP and *c*-type cytochromes. The analysis presented has not incorporated any contribution of nonpolar interactions as we do not have any specific data bearing on this point. However, it is certainly feasible that nonpolar interactions play an important role in defining the specific orientations and distance between chromophores.

The ionic strength effects reported here are difficult to analyze quantitatively. In only two out of nine examples studied was the observed sign on the product of charges the same as that expected on the basis of isoelectric points (Table III). The expected sign of the product of charges can be predicted from previous studies of the reaction of cytochromes and HIPIP's with the iron hexacyanides (Mizrahi et al., 1976, 1980; Wood & Cusanovich, 1975; Miller & Cusanovich, 1975; Wood et al., 1977) over the ionic strength range used herein (Table III). In only four out of nine examples studied are the observed and expected ionic strengths dependencies in agreement. However, as pointed out recently by Koppenol (1980), protein–protein interactions can have ionic strength dependencies much different from those expected from a simple interaction of monopoles. This results from the dipole moment of the reactants and the orientation of the respective dipole moments as well as the charge on the reactants. Thus, the results obtained are consistent with the view that specific regions on the interacting molecules are involved, and these regions are defined by the dipole moments and their relative orientation. It is clear that treatment of the data presented here without regard for the distribution of charge on the reactants or the specific orientations in the transition state is inadequate.

Quantitative analysis of the structures of the interacting molecules in terms of specific mechanisms is difficult at this time due to the large number of interactions possible. Nevertheless, future studies will have to investigate the possible orientation(s) of HIPIP and cytochromes *c* and c_2 in an attempt to quantitate the optimum interactions. The studies presented here do establish that HIPIP and cytochromes interact in a relatively complex fashion in terms of the type of interactions involved. Further, the primary structure of the cytochrome influences the kinetic homogeneity of electron transfer between HIPIP and *c*-type cytochromes.

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