REACTION OF *C*-TYPE CYTOCHROMES WITH THE IRON HEXACYANIDES

MECHANISTIC IMPLICATIONS

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ABSTRACT The reaction of *c*-cytochromes with iron hexacyanides is similar in mechanism to the interaction of cytochromes with their physiological oxidants and reductants in that the formation of complexes precedes electron transfer. Analysis of the kinetics of oxidation and reduction of a number of *c*-cytochromes by solving the simultaneous differential equations defining the mechanism is possible, and allows assignment of all six rate constants describing a minimum three-step mechanism [cyto(Fe^{+3}) + Fe^{+2} $\rightleftharpoons$ cyto (Fe^{+3}) – Fe^{+2} $\rightleftharpoons$ cyto(Fe^{+2}) – Fe^{+3} $\rightleftharpoons$ cyto(Fe^{+2}) + Fe^{+3}]. We find that the usual steady-state approximations are not valid. Furthermore, the ratio of first-order rate constants for electron transfer was ~1.0, and no correlation was found between any of the six rate constants and the differences in oxidation-reduction potential of the iron-hexacyanides and different cytochromes *c*. However, it was found that the ratio of the rate constants for complex formation between ferricytochrome *c* and potassium ferrocyanide and ferrocytochrome *c* and potassium ferricyanide was proportional to the difference in oxidation-reduction potentials. Thus the minimum three-step mechanism given above accurately describes the observed kinetic data. However, this mechanism leads to a number of conceptual difficulties. Specifically, the mechanism requires that the collision complexes formed [cyto(Fe^{+3}) – $Fe(CN)_6^{-4}$ and cyto(Fe^{+2}) – $Fe(CN)_6^{-3}$] have very different equilibrium constants, and further requires that formation of the collision complexes be accompanied by "chemistry" to make the intermediates isoenergetic. A more complex five-step mechanism which requires that the reactants [$Fe(CN)_6^{-4}$ and ferricytochrome *c* or $Fe(CN)_6^{-3}$ and ferrocytochrome *c*] form a collision complex followed by a first-order process before electron transfer, was found to yield results similar to those of the three-step mechanism. However, describing the formation of the collision complex in terms of a rapid equilibrium circumvents conceptual difficulties and leads to a physically reasonable mechanism. In this mechanism the reactants are in rapid equilibrium with the collision complexes and the rate constants for complex formation are controlled by diffusion and accessibility. The collision complexes then rearrange, possibly through conformational changes and/or solvent reorganization, to yield isoenergetic intermediates that can undergo rapid reversible electron transfer. The five-step mechanism can be described by the same rate constants obtained from the three-step mechanism with the appropriate adjustments to account for rapid equilibrium. This more complex analysis associates the oxidation-reduction potential of a particular cytochrome with the relative magnitude of the first-order conversion of the oxidant and reductant collision complexes to their respective intermediates. Thus the cytochromes *c* control their oxidation-reduction potential by chemical and/or structural alterations. This mechanism appears to be general in that it is consistent with the observed kinetics of 11 different cytochromes *c* from a wide variety of sources with a range of oxidation-reduction potentials.

INTRODUCTION

Studies to elucidate the mechanism of action of cytochrome *c* have recently been the major focus of many research groups. Indeed, substantial progress has been made in the structural and chemical aspects of the oxidation and reduction of *c*-type cytochromes (1–4). It is now generally believed that electron transfer to and from *c*-type cytochromes takes place in the region of the exposed heme (upper front in the usual description, reference 5) with specific lysine residues playing an important role in the interaction of cytochromes *c* with their oxidase and reductase as well as with a wide variety of nonphysiological reactants (6–12). Nevertheless, a number of fundamental problems remain concerning the mechanism of action of *c*-cytochromes. These center on two primary questions: first, the nature and quantitative contribution of all chemical and structural interactions mediating each step in electron transfer and second, the molecular basis of the oxidation-reduction potential of a particular cytochrome.

It is well established that the reaction of mitochondrial cytochrome *c* with its oxidase and reductase involves at least two steps: complex formation, and at least one subsequent first-order process, possibly electron transfer (13, 14). The binding of mitochondrial cytochrome *c* to its oxidase has been well studied (13) although analysis of the kinetic mechanism is less detailed. Generally, steady-state kinetics are utilized which, while providing an overview of the mechanism of electron transfer, fail to directly address the molecular basis of each step in the reaction or to specify clearly the number of steps involved. Some studies on the transient kinetics of the interaction of cytochrome *c* with its oxidase have been reported; however, the findings are not detailed (15, 16) because very little information is available on the structure of cytochrome oxidase (or reductase), thus limiting analysis, and because the reactions are rapid at or near the limit of mixing methods. The general process of complex formation followed by at least one first-order process has also been reported for the reaction of *c*-cytochromes with reaction centers from photosynthetic bacteria in which the course of the reaction can be observed much earlier, as mixing methods are not required (17, 18). Nevertheless, these studies are limited by a lack of information concerning the structure of the reaction centers.

The reaction of *c*-cytochromes with iron hexacyanides represents a case in which complex formation followed by a first-order process is observed with a nonphysiological reactant. The binding of the iron hexacyanides by mitochondrial cytochrome *c* was first noted by Stellwagen and Shulman (19) using NMR, and subsequent kinetic studies were performed by Miller and Cusanovich (11). Further, the kinetics of the reaction of the iron hexacyanides with a variety of *c*-cytochromes have been reported (11, 12, 20, 21), as well as studies on iron hexacyanide binding (22, 23). Thus, the iron hexacyanide-cytochrome system represents a well-defined model system in terms of available structural information and is apparently similar in kinetic mechanism to the physiological system. In view of the foregoing, we are reporting here a detailed analysis of the kinetic mechanism for the reaction of cytochrome *c* with the iron hexacyanides. This analysis differs from those reported previously in that no a priori assumptions are made to simplify the kinetic equations. The kinetic data used were restricted to reactions at high ionic strengths (>0.1 M) to saturate ion binding sites and hence permit a comparative study of structurally homologous *c*-cytochromes without complications due to specific ion effects. The studies reported establish that use of the steady-state approximation is

not quantitatively correct and further suggest that the cytochrome oxidation-reduction potential is controlled by first-order processes but not by the rate constants for electron transfer.

METHODS

Horse heart cytochrome (type VI), bovine heart cytochrome *c* (type V), chicken heart cytochrome *c* (type X), tuna heart cytochrome *c* (type XI), and *Candida krusei* cytochrome *c* (type VII) were purchased from Sigma Chemical Co. (St. Louis, Mo.) and further purified by chromatography on Sephadex G-75 (4°C, 10 mM Tris-cacodylate 100 mM NaCl, pH 7.4). *Rhodomicrobium vannielii* cytochrome c_2 was purified as described previously (24). Data for other cytochromes c_2 and *Euglena* cytochrome *c*-552 were taken from previous publications (12, 20, 21). All kinetic data were collected using a Durrum-Gibson stopped-flow, (Dionex Corp., Sunnyvale, Calif.), 3-ms mixing time, interfaced to a Data General Nova II computer (Data General Corp., Westboro, Mass.). Stopped-flow experiments were conducted anaerobically at 20°C, at constant ionic strength in a buffer consisting of 20 mM Tris-cacodylate or 10 mM potassium phosphate, 100 mM NaCl, pH 7.0, with identical results. Mammalian cytochromes *c* were monitored at 415 nm ($\Delta E_{mM} \simeq 42$) and *R. vannielii* cytochrome c_2 at 550 nm ($\Delta E_{mM} = 19.9$). In all cases the heme concentration was ~5 μM. The value of the observed rate constant (k_{obs}) was obtained from a least-squares analysis of at least triplicate kinetic traces. For cytochromes *c* from horse heart, bovine heart, and tuna heart, the results are the average of three different sets of triplicate data. The average deviation among experiments was <5%. For the other cytochromes studied, a single triplicate set of data was used.

Absorption spectra were taken on a Cary 118 recording spectrophotometer (Cary Instruments, Monrovia, Calif.). Oxidation-reduction potentials were determined by the method of mixtures as previously described (20). Carbon-14-labeled sodium ferrohexacyanide (33.9 mCi/mol) was purchased from ICN Pharmaceuticals (ICN Pharmaceuticals, Inc., Covina, Calif.), with iron hexacyanide binding measured as described by Stellwagen and Cass (23). Potassium ferrocyanide and potassium ferricyanide were reagent grade and used without further purification.

THEORY

The minimum mechanism for the reaction of cytochrome *c* and c_2 with iron hexacyanides can be given by Eq. 1.

$$\text{cyto}\,(Fe^{+3}) + Fe(CN)_6^{-4} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C_2 \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} \text{cyto}\,(Fe^{+2}) + Fe(CN)_6^{-3} \qquad (1)$$

where C_1 and C_2 are cyto (Fe^{+3}) – $Fe(CN)_6^{-4}$ and cyto (Fe^{+2}) – $Fe(CN)_6^{-3}$ complexes, respectively. In Eq. 1, K_1 (k_{12}/k_{21}) and K_3 (k_{34}/k_{43}) represent complex formation and K_2 (k_{23}/k_{32}) electron transfer, thus $K_{eq} = K_1 \cdot K_2 \cdot K_3$. However, in terms of oxidation-reduction titrations, assuming C_1 and cyto (Fe^{+3}), and C_2 and cyto (Fe^{+2}) are spectrally identical, K_{app} (apparent) is given by Eq. 2.

$$K_{app} = \frac{([\text{cyto}\,(Fe^{+2})] + [C_2])\,([Fe\,(CN)_6^{-3}])}{([\text{cyto}\,(Fe^{+3})] + [C_1])\,([Fe\,(CN)_6^{-4}])}. \qquad (2)$$

The assumption noted above appears to be valid, since only a single spectral species has been observed at any wavelength (this work and references 11 and 20). It is important to note that if $[C_1]$ and $[C_2]$ are small, $K_{eq} \simeq K_{app}$. In a previous analysis of kinetic data for the reaction of potassium ferrocyanide with a variety of *c*-cytochromes, it was assumed that k_{43} $[Fe(CN)_6^{-3}]$ was small and k_{23} was rate limiting; therefore, a steady-state assumption was utilized for graphical analysis (11, 12, 20, 21). This approach, although yielding a reasonable description of experimental data, became increasingly suspicious on more detailed analysis and suggested that analysis with no a priori assumptions was required. To conduct this

analysis, we have reanalyzed available data using fourth-order Runge-Kutta numerical integration of systems of simultaneous differential equations which describe Eq. 1 (25).

$$-\frac{d[\text{cyto}\,(Fe^{+3})]}{dt} = k_{12}\,[Fe(CN)_6^{-4}] \times [\text{cyto}\,(Fe^{+3})] - k_{21}\,[C_1]. \tag{3}$$

$$\frac{d[C_1]}{dt} = k_{12}[Fe(CN)_6^{-4}] \times [\text{cyto}\,(Fe^{+3})] + k_{32}[C_2] - [C_1](k_{21} + k_{23}). \tag{4}$$

$$\frac{d[C_2]}{dt} = k_{23}[C_1] + k_{43}[\text{cyto}\,(Fe^{+2})] \times [Fe(CN)_6^{-3}] - [C_2](k_{34} + k_{23}). \tag{5}$$

$$\frac{d[\text{cyto}(Fe^{+2})]}{dt} = k_{34}[C_2] - k_{43}[\text{cyto}(Fe^{+2})] \times [Fe(CN)_6^{-3}]. \tag{6}$$

For the analysis conducted, there are six unknowns (rate constants) and, in the best cases, two sets of data, k_{obs} vs. $[Fe(CN)_6^{-4}]$ and k_{obs} vs. $[Fe(CN)_6^{-3}]$. However, in those cases where plots of k_{obs} vs. [iron hexacyanide] become nonlinear at high iron hexacyanide concentrations (for example, *Rhodospirillum rubrum* cytochrome c_2, reference 20), effectively four rate constants are specified. Furthermore, in all cases K_{app} is known (K_{eq} if the iron hexacyanide concentration is low or the concentrations of C_1 and C_2 are small), further constraining the data. However, a more important constraint is that for consecutive first- and second-order reactions, many conditions result in fits that are not exponential (26). That is, plots of ln (ΔA) vs. time at a particular iron hexacyanide concentration are not linear. For none of the data used in this analysis was this the case, as ln (ΔA) vs. time plots were always found to be accurately first-order for at least four half-lives. This situation severely restricts the possible solutions for the differental equations and effectively reduces the number of independent variables.

For the analysis of any particular cytochrome, the following minimum data base was available: k_{obs} vs. $[Fe(CN)_6^{-4}]$, ln ΔA vs. t at each $[Fe(CN)_6^{-4}]$ and K_{app} and/or K_{eq}. Further, in the case of horse heart cytochrome *c*, *Euglena* cytochrome *c-552*, and cytochrome c_2 from *Rhodospirillam rubrum*, *Rhodopseudomonas sphaeroides*, *Rps. capsulata* and *Rps. palustris*, k_{obs} $[Fe(CN)_6^{-3}]$ data were also available.

The strategy used to analyze the mechanism was to find the minimum values of the six rate constants which fit available data. In general it was found that k_{12}, k_{21}, k_{34}, and k_{43} were severely constrained. However k_{23} and k_{32} could take on a wide range of values, and, except when otherwise noted, the smallest values that reasonably describe the data will be reported.

RESULTS

Horse Heart Cytochrome c

Because a large body of data exists for horse heart cytochrome *c*, the reaction of this protein with iron hexacyanides will be discussed in some detail to fully illustrate our approach. Fig. 1 *A* presents a k_{obs} vs. $[Fe(CN)_6^{-4}]$ plot for horse heart cytochrome *c*. The data presented demonstrate that a nonbinding buffer (Tris-cacodylate) and a binding buffer (potassium phosphate) give identical results in the presence of ~100 mM NaCl and further establish our experimental reproducibility. Fig. 1 *B* illustrates the oxidation of ferrocytochrome *c* by $[Fe(CN)_6^{-3}]$, using the data of Creutz and Sutin (27). Substantially fewer data are available on the oxidation of cytochrome *c* by $Fe(CN)_6^{-3}$ than for reduction by $Fe(CN)_6^{-4}$ due to the rapid rate constants for oxidation. Nevertheless, the Creutz and Sutin data are similar to those reported by others (28, 29). The solid and dashed lines in Fig. 1 are curves derived as described in the Theory section. Curve *a* represents what we consider the best fit, and the corresponding rate constants are given in Table I. Curves *b-f* represent alterations in specific

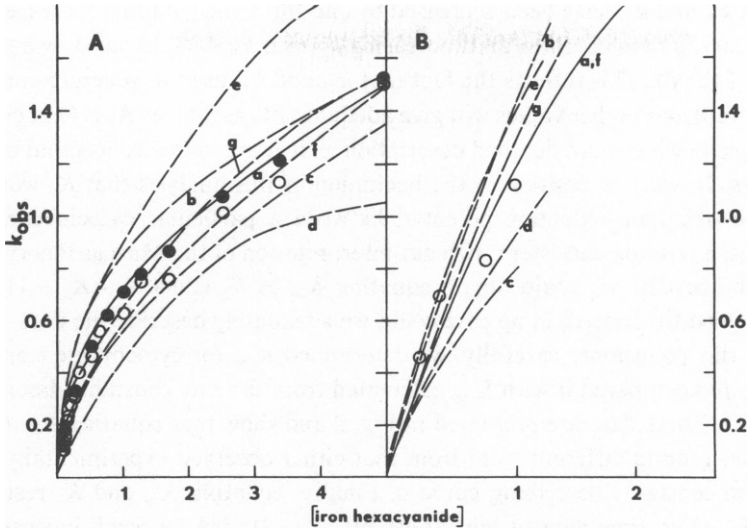

FIGURE 1 Oxidation and reduction of horse heart cytochrome *c*. (*A*) Reduction of ferricytochrome *c* by potassium ferrocyanide. ○, 10 mM potassium phosphate and NaCl to give an ionic strength of 0.118 M, pH 7.0; ●, 20 mM Tris-cacodylate and NaCl to give an ionic strength of 0.116 M, pH 7.0, k_{obs}, 10^2 s^{-1}. Abscissa potassium ferrocyanide, 10^{-3} M, reaction monitored at 415 nm 20°C. Curve *a* calculated from parameters given in Table I, other curves calculated from parameters in Table I except as noted; *b*, k_{12} and k_{21} increased twofold; *c*, k_{12} and k_{21} decreased two fold; *d*, k_{34} and k_{43} decreased by 1/3; *e*, k_{34} and k_{43} increased by 1/3; *f*, k_{23} and k_{32} increased 10-fold; *g*, $k_{12} = 2.8 \times 10^5$ M^{-1} s^{-1}, $k_{21} = 2{,}000$ s^{-1}, $k_{23} = 250$ s^{-1}, $k_{32} = 7 \times 10^4$ s^{-1}, $k_{43} = 5.4 \times 10^7$ M^{-1} s^{-1}, $k_{34} = 1.9 \times 10^5$ s^{-1}. (*B*) Oxidation of ferrocytochrome *c* by potassium ferricyanide. Data from Creutz and Sutin (27). Abscissa, potassium ferricyanide, 10^{-5} M, k_{obs}, 10^2 s^{-1}. Theoretical curves *a*–*g* defined as described in *A* above.

rate constants and are presented to give the reader an appreciation of the limits of the analysis. As K_{eq} must be kept constant for any experimental condition, any rate constant that is altered must be accompanied by a similar alteration of at least one other rate constant. In curve *b*, k_{12} and k_{21} have been increased twofold and, in curve *c*, each have been decreased twofold. In

TABLE I
KINETIC ANALYSIS OF THE THREE-STEP MECHANISM

Cytochrome source	$k_{12} \times 10^{-5}$	$k_{21} \times 10^{-2}$	$k_{23} \times 10^{-3}$	$k_{32} \times 10^{-3}$	$k_{34} \times 10^{-2}$	$k_{43} \times 10^{-6}$
	M^{-1} s^{-1}	s^{-1}	s^{-1}	s^{-1}	s^{-1}	M^{-1} s^{-1}
Horse, bovine	1.00	7.1	2.6	2.5	2.4	20.0
Tuna	0.72	5.1	3.7	3.5	1.9	16.0
Chicken	0.90	6.4	2.6	2.5	2.1	18.0
C. krusei	1.90	6.0	4.6	5.6	5.2	74.0
Rsp. rubrum	1.20	14.0	1.4	1.5	14.0	6.3
R. vannielli	0.25	20.0	2.0	2.0	16.0	0.4
Rps. sphaeroides	13.00	17.0	1.2	1.6	9.5	7.0
Rps. palustris	5.40	7.0	0.8	1.0	5.0	2.0
Rps. capsulata	4.00	8.1	1.2	2.1	15.0	2.4
Euglena	0.12	6.4	2.0	2.0	4.0	0.08

Buffer either 20 mM Tris-cacodylate-100 mM NaCl or 10 mM potassium phosphate-100 mM NaCl, pH 7.0. Temperature 20°C.

curve *d* both k_{34} and k_{43} have been decreased by one-third and similarly increased by one-third in curve *e*. Curve *f* demonstrates that increasing k_{23} and k_{32} 10-fold has only a slight effect on the quality of the fit. This reflects the fact that k_{23} and k_{32} used to generate curve *a* represent minimum values and higher values will give adequate fits as long as K_2 is kept constant. Curve *g* requires a somewhat more detailed description as it represents a conceptual departure from the other fits. It was our position at the beginning of this analysis that K_2 would reflect the difference in oxidation-reduction potential between a particular cytochrome and the iron hexacyanides, a position consistent with our interpretation of the Marcus theory (30). Curve *g* represents the best fit we could obtain equating K_{eq} to K_2 (thus $K_1 \cdot K_3 = 1$), and is not a particularly good fit. Indeed, in no case could we adequately describe the data with $K_{eq} = K_2$. To examine this point more carefully, we determined K_{app} for cytochrome *c* as a function of $[Fe(CN)_6^{-4}]$ and compared it with K_{app} calculated from the rate constants describing curves *a* and *g* (Fig. 1). These data are presented in Fig. 2 and show that equating K_{eq} and K_2 yields a curve that has a quite different form from that either observed experimentally or calculated from the rate constants describing curve *a*. Finally, equating K_{eq} and K_2 results in slightly biphasic ln (ΔA) vs. time plots at high $[Fe(CN)_6^{-4}]$ ($>10^{-3}$M), a result inconsistent with the observed data.

Iron Hexacyanide Binding

To extend our studies and confirm the results of Stellwagen and Cass (23), we studied the binding of the iron hexacyanides to horse heart cytochrome *c* using equilibrium dialysis and ^{14}C-labeled iron hexacyanide in 10 mM potassium phosphate, 100 mM NaCl, pH 7.0, 25°C. Three situations were investigated: cyto(Fe^{+2}) – $Fe(CN)_6^{-4}$, cyto(Fe^{+3}) – $Fe(CN)_6^{-4}$, and cyto(Fe^{+3}) – $Fe(CN)_6^{-3}$. In all three cases, we obtained a binding constant of ~400 M^{-1} per ligand bound, with a stoichiometry of two iron hexacyanides bound per cytochrome. These

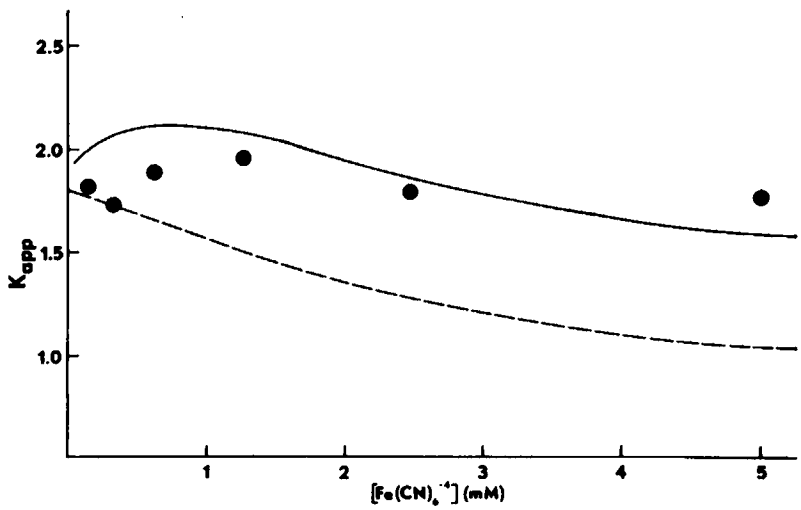

FIGURE 2 Effect of potassium ferrocyanide concentration on K_{app}. •, experimental data in 20 mM Tris-cacodylate and NaCl to give an ionic strength of 0.116 M, 20°C, $K_{app} \times 10^3$. —, theoretical curve using parameters given in Table 1 for horse heart cytochrome *c*. – –, theoretical curve using parameters given for curve *g*, see legend for Fig. 1.

results are in reasonable agreement with those of Stellwagen and Cass (23). However, we found that determining the binding constant with high accuracy was not possible due primarily to the strong quenching shown by the iron hexacyanides and cytochrome during counting. Hence, the binding constants determined here are only accurate to within a factor of 2–3.

C. krusei Cytochrome c

Although reliable rate constants for the oxidation of *C. krusei* cytochrome *c* were not available because of the very large rate constant, the reduction of this cytochrome by $Fe(CN)_6^{-4}$ was analyzed as described above. The rate constants used to fit the data given in Fig. 3 are presented in Table I. As with horse heart cytochrome *c*, we measured K_{app} for *C. krusei* cytochrome *c* and compared it with K_{app} calculated from the rate constants given in Table I and from the best fit equating K_2 and K_{eq}. As can be seen in Fig. 3 *B*, equating K_2 and K_{eq} results in a quite different curve from that observed experimentally.

Mammalian Cytochromes c

Bovine cytochrome *c* was found to yield rate constants identical to those for horse heart cytochrome *c*. However, tuna and chicken heart cytochrome *c* yielded somewhat different results. The experimental data are given in Fig. 4 together with the fitted curve obtained from the rate constants presented in Table I. For both the tuna and chicken cytochromes, only data for the reduction by $Fe(CN)_6^{-4}$ were fitted, but with the constraint that K_2 be the best value near that found for horse heart cytochrome *c* (assuming structural and kinetic homology).

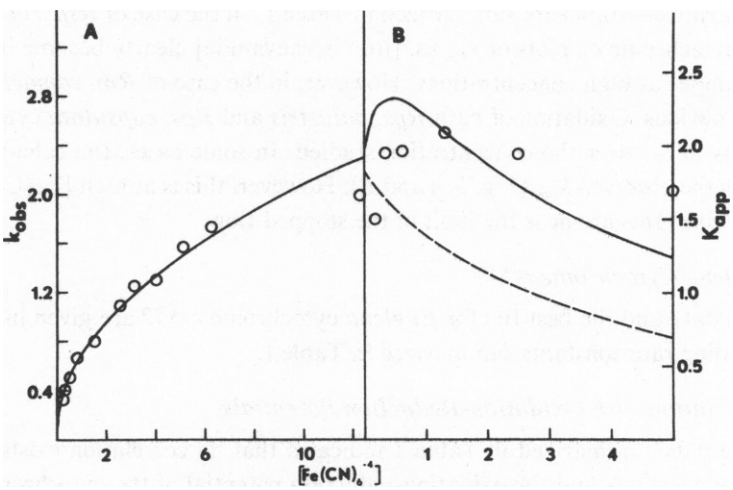

FIGURE 3 Reduction of *C. krusei* cytochrome *c*. (*A*) Reduction of oxidized *C. krusei* cytochrome *c* by potassium ferrocyanide, abscissa, 10^{-4} M. Buffer, 20 mM Tris-cacodylate and NaCl to give an ionic strength of 0.116 M, pH 7.0, 20°C, k_{obs}, 10^2 s^{-1}. —, theoretical curve using parameters given in Table I. (*B*) Effect of potassium ferrocyanide concentration on K_{app}. Conditions as in *A*, $K_{app} \times 10^3$. —, theoretical curve using parameters given in Table I, abscissa, 10^{-3} M; ---, theoretical curve for best fit with $K_2 = K_{eq}$, $k_{12} = 5.4 \times 10^5$ M^{-1} s^{-1}, $k_{21} = 1{,}700$ s^{-1}, $k_{23} = 420$ s^{-1}, $k_{32} = 1.05 \times 10^5$ s^{-1}, $k_{34} = 1.6 \times 10^5$ s^{-1}, $k_{43} = 1.1 \times 10^8$ M^{-1} s^{-1}.

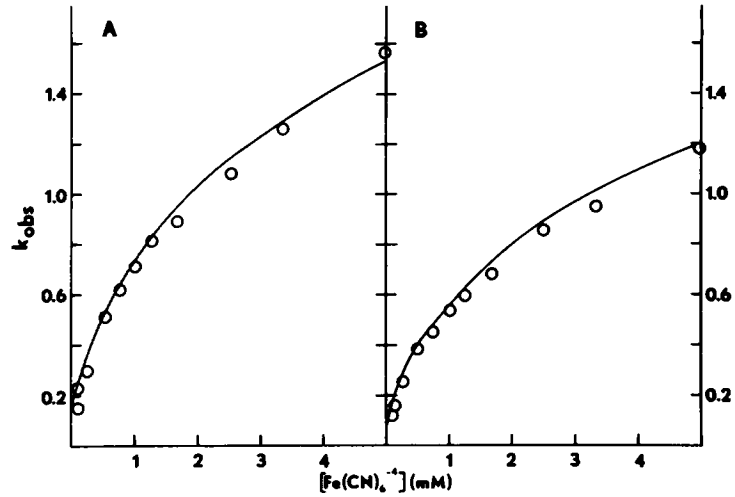

FIGURE 4 Reduction of chicken and tuna heart cytochrome *c*. (*A*) Reduction of chicken heart cytochrome *c* by potassium ferrocyanide, 20 mM Tris-cacodylate and NaCl to give an ionic strength of 0.116 M, pH 7.0, 20°C, k_{obs}, 10^2 s^{-1}. —, theoretical curve for parameters given in Table I. (*B*) Reduction of tuna heart cytochrome *c* by potassium ferrocyanide. Conditions as given in *A*. —, theoretical curve for parameters given in Table I.

Bacterial Cytochrome c_2

The reaction of the iron hexacyanides with five different cytochromes c_2 was analyzed as described above. The experimental data and best fits are given in Fig. 5 and 6 and the corresponding rate constants are summarized in Table 1. In the case of *Rsp. rubrum* and *Rps. sphaeroides* cytochrome c_2, plots of k_{obs} vs. [iron hexacyanide] clearly become independent of [iron hexacyanide] at high concentrations. However, in the case of *Rm. vannielli* cytochrome c_2, this is less obvious. Oxidation of both *Rps. palustris* and *Rps. capsulata* cytochrome c_2 by $Fe(CN)_6^{-3}$ was linear over the concentration studied. In some cases, the calculated k_{obs} does not agree with the observed k_{obs} (Fig. 5 *A* and *B*). However, this is at high $[Fe(CN)_6^{-4}]$ and the observed rate constants are near the limit of the stopped-flow.

Euglena Cytochrome c-552

Experimental data and the best fits for *Euglena* cytochrome *c-552* are given in Fig. 6 *C* with the corresponding rate constants summarized in Table I.

Correlation with Oxidation-Reduction Potentials

Perusal of the data summarized in Table 1 indicates that no correlation exists between any individual rate constants and the oxidation-reduction potential of the cytochrome (Table II). Further, no correlation exists between the oxidation-reduction potential and the individual equilibrium constants (K_1, K_2, K_3, see Table II). However, a correlation is found between the ratio of k_{43} and k_{12} and the oxidation-reduction potential. This is illustrated in Fig. 7 where the log k_{43}/k_{12} is plotted against the difference in oxidation-reduction potential of the iron hexacyanides and a particular cytochrome (ΔE (mV) = 0.058 log $[K_{eq}^{-1}]$. Thus, a straight line is obtained with an intercept $\Delta E = 0$, that is, at log $(k_{43}/K_{12}) = 1$, and a slope of 0.069. To

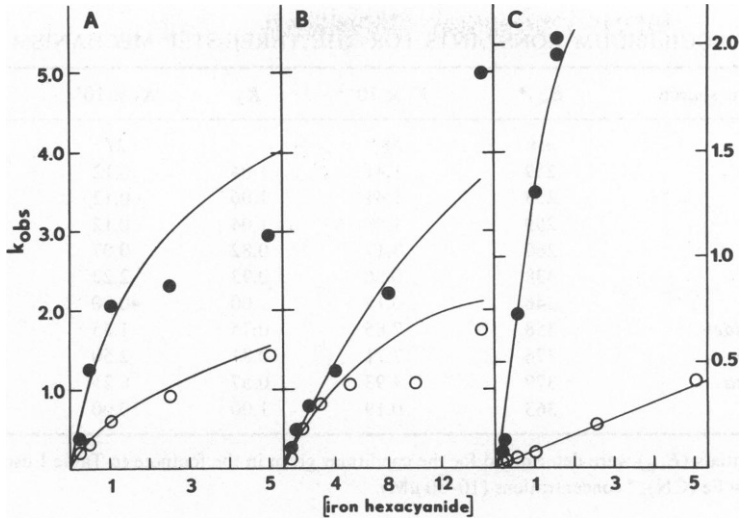

FIGURE 5 Oxidation and reduction of bacterial cytochromes c_2. Buffer, 10 mM potassium phosphate and NaCl to give an ionic strength of 0.118 M, k_{obs}, 10^2 s^{-1}. o, reduction by potassium ferrocyanide; •, oxidation by potassium ferricyanide. —, theoretical curves calculated from parameters given in Table 1. (*A*) *Rsp. rubrum* cytochrome c_2, abscissa, 10^{-4} M-reduction; 10^{-5} M-oxidation. (*B*) *Rps. sphaeroides* cytochrome c_2, abscissa, 10^{-4} M-reduction; 10^{-5} M-oxidation. (*C*) *R. vannielii* cytochrome c_2, abscissa 10^{-3} M.

extend the analysis we have taken kinetic data for *Rsp. rubrum* cytochrome c_2 oxidation and reduction obtained as a function of pH and ionic strength (12, 20) and plotted k_{43}/k_{12} against ΔE. These data are presented in Fig. 8 and yield a straight line with an intercept of $\Delta E = 0$ and a slope of 0.068 for the pH data (Fig. 8 *A*), and a slope of 0.054 for the ionic strength data

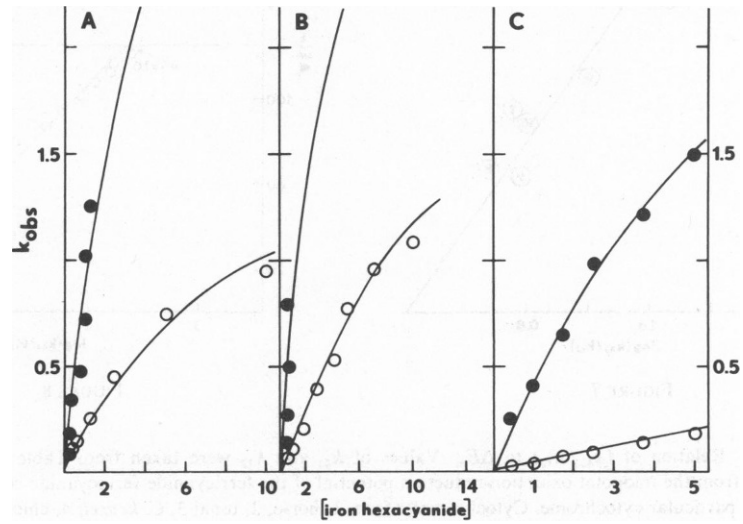

FIGURE 6 Oxidation and reduction of bacterial cytochromes c_2 and *Euglena* cytochrome *c-552*. Conditions as described in the legend to Fig. 5. (*A*) *Rps. palustris* cytochrome c_2, abscissa 10^{-4} M. (*B*) *Rps. capsulata* cytochrome c_2, abscissa 10^{-4} M. (*C*) *Euglena* cytochrome *c-552*, abscissa, 10^{-3} M.

TABLE II
EQUILIBRIUM CONSTANTS FOR THE THREE-STEP MECHANISM

Cytochrome source	$E_{m,7}$*	$K_1 \times 10^{-2}$	K_2	$K_3 \times 10^4$	$K_{eq} \times 10^3$
	mV	M^{-1}		*M*	
Horse, bovine	259	1.41	1.04	0.12	1.8
Tuna	259	1.41	1.06	0.12	1.8
Chicken	259	1.40	1.04	0.12	1.7
C. krusei	260	3.17	0.82	0.07	1.8
Rsp. rubrum	338	0.86	0.93	2.22	17.6
R. vannielii	346	0.12	1.00	40.00	48.2
Rps. sphaeroides	358	7.65	0.75	1.35	77.5
Rps. palustris	376	7.71	0.81	2.50	156.0
Rps. capsulata	379	4.93	0.57	6.25	176.0
Euglena	363	0.19	1.00	50.00	95.0

*Midpoint potentials ($E_{m,7}$) were determined for the conditions given in the footnote to Table I using the method of mixtures and low Fe $(CN)_6^{-4}$ concentrations (10–50 μM).

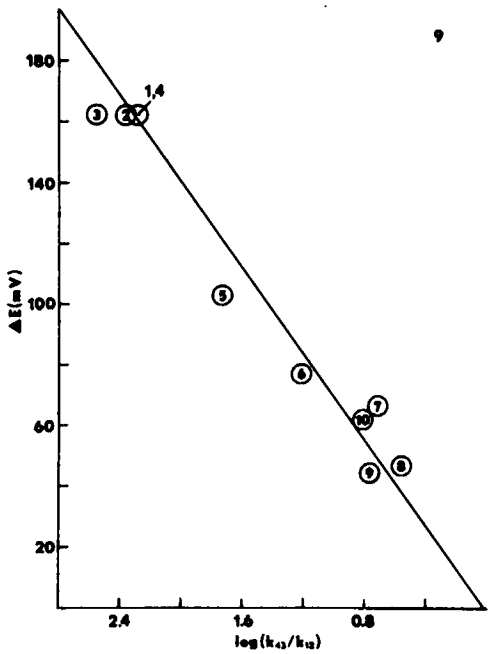

FIGURE 7

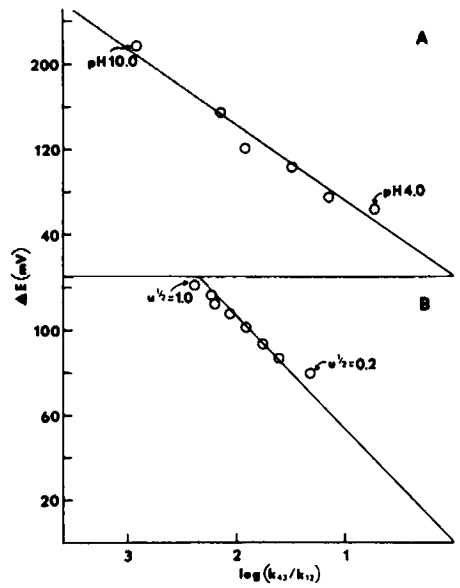

FIGURE 8

FIGURE 7 Relation of (k_{43}/k_{12}) to ΔE_m. Values of k_{43} and k_{12} were taken from Table I and ΔE_m calculated from the midpoint oxidation-reduction potential of the ferricyanide-ferrocyanide couple minus that of the particular cytochrome. Cytochromes *c* from 1, horse; 2, tuna; 3, *C. krusei;* 4, chicken; 5, *Rsp. rubrum;* 6, *R. vannielii;* 7, *Rps. sphaeroides;* 8, *Rps. palustris;* 9, *Rps. capsulata;* 10, *Euglena.*

FIGURE 8 Effect of pH and ionic strength on *Rsp. rubrum* cytochrome c_2 oxidation-reduction. Plotted as $\ln k_{43}/k_{12}$ vs. ΔE_m with ΔE_m obtained as described in Fig. 7. Data taken from Post et al. (23). (*A*) Effect of ionic strength (0.04-1 M). (*B*) Effect of pH (4 to 10).

(Fig. 8 *B*). Thus, three independent sets of data yield an essentially identical relationship between oxidation-reduction potential and the ratio of the rate constants for complex formation (k_{43}/k_{12}) as expected if $K_{eq}^{-1} = k_{43}/k_{12}$.

DISCUSSION

Analysis of kinetic data for the reaction of cytochrome *c* with the iron hexacyanides clearly requires use of the simultaneous differential equations without any a priori assumptions. This is demonstrated by the fact that by use of the usual "steady-state" approximations, values for k_{23} are typically 100–500 s^{-1} (11, 12, 20), but using the general solution gives a minimum value of 1,000–3,000 s^{-1}. The discrepancy arises because the usual assumption that the reverse reactions are negligible in the presence of high concentrations of oxidant or reductant is not valid. Thus, the limiting rate constants (k_{23} and k_{32}) are substantially underestimated. To illustrate this point, in Table III k_{12} and k_{23}, determined from a steady-state analysis and a complete solution, are compared for several cytochromes *c*. As can be seen, k_{12} is somewhat different with the two analyses; however, k_{23} is substantially different. Although our analysis can only specify the minimum values of k_{23} and k_{32} for a particular cytochrome, it does establish that their ratio (K_2) is near to 1.0 (1.04–0.57), Table II). This last observation has important ramifications as it has been the explicit assumption or proposal of many previous studies that the ratio of k_{23} to k_{32} is related to the overall equilibrium constant for the reaction (19, 30, 31). To our knowledge, the only experimental results in conflict with the interpretation presented here is the work of Ilan and Schafferman (31) who report a value of 40,000 s^{-1} for k_{32} and 300 s^{-1} for k_{23}. Although the value of k_{32} reported by Ilan and Schafferman is not in conflict with our results as we are reporting minimum values of k_{32}, their analysis of k_{23} must be in error as they did not consider the reverse reaction when analyzing the reduction of cytochrome *c* by $Fe(CN)_6^{-4}$.

Based on our experience in analyzing the kinetic data for the various cytochromes *c*, we estimate that the values of k_{12}, k_{23}, k_{34}, and k_{43} reported here are within 20–30% of the correct values (see Fig. 1 *A* and *B*); k_{23} and k_{32} are minimum values as discussed previously. It is striking that the relatively complex three-step mechanism is so constrained in terms of allowable values for the individual rate constants. This results from the fact that oxidation and reduction reactions are found to be accurately pseudo-first-order over a wide range of reactant concentrations. Thus, solutions involving two exponentials are excluded, severely restricting

TABLE III
COMPARISON OF RATE CONSTANTS OBTAINED BY "STEADY-STATE" AND EXPLICIT SOLUTION OF THE THREE-STEP MECHANISM

Cytochrome source	Steady state/explicit	
	K_{12}	k_{23}
Horse heart	0.800	0.058
Rsp. rubrum	0.550	0.180
Rsp. sphaeroides	0.300	0.181
Rps. palustris	0.296	0.139
Rps. capsulata	0.375	0.416
R. vannielii	0.760	0.018

the values for the individual rate constants. In addition, the apparent change in the rate limiting step observed in second-order plots (k_{obs} vs. [reactant]) and knowledge of K_{eq} (or K_{app}, depending on the conditions) further confines the values of the individual rate constants to a narrow range.

The binding of the iron hexacyanides to horse ferri- and ferrocytochrome *c* presents an interesting problem. From our studies and those of Stellwagen and Cass (23) and Ilan and Shafferman (31), it appears that ferrocyanide binds to ferri- and ferrocytochrome with approximately equal affinity (within a factor of 2–3). Further, ferricyanide binds to ferricytochrome *c* with an affinity of the same order of magnitude as ferrocyanide (23). In the case of ferrocyanide binding to ferricytochrome *c*, the apparent affinity constant (K_1) is in reasonable agreement when the kinetic and equilibrium dialysis results are compared (140 M^{-1}, Table II vs. 400–1,000 M^{-1}). However, our kinetic analysis yields a value of ~8.3×10^4 M^{-1} for K_3^{-1}, a binding constant much greater than that observed for the binding of ferrocyanide to ferri- and ferrocytochrome *c* or ferricyanide to ferricytochrome *c*. The difference in the affinity of ferricyanide for ferro- and ferricytochrome *c* (8×10^4 M^{-1} vs. ~400 M^{-1}) can be explained by arguing that oxidized and reduced cytochrome *c* have different solution structures. Such a conclusion is not inconsistent with a large body of data including hydrogen-deuterium exchange kinetics (32) and solvent perturbation (33), although it is inconsistent with structural data (3). Explaining the difference in the binding constant of ferro- and ferricyanide to ferro-cytochrome *c* (~400 M^{-1} vs. 8×10^4 M^{-1}) is a more difficult problem. Inspection of the data in Table I indicates that the difference in the binding constant of ferrocyanide to ferricytochrome *c* as compared with the binding of ferricyanide to ferrocytochrome *c* resides largely in the binding rate constants (k_{12} and k_{43}) and not in the rate constants for dissociation (k_{21} and k_{34}) which are of the same order of magnitude. However, we have no data on rate constants for the binding of ferrocyanide to ferrocytochrome *c* and hence we cannot state whether one or both rate constants (association and dissociation) are affected. In the extremes, if the association rate constant for ferrocyanide binding to ferrocytochrome *c* is substantially less than that for ferricyanide binding, then steric differences would have to be invoked. This is unreasonable as the radii of ferri- and ferrocyanide are similar. On the other hand, if the difference arises in the dissociation rate constants, then factors other than electrostatic interactions would have to be considered since ferrocyanide should dissociate less readily for electrostatic reasons. One possibility is that the iron hexacyanides have to approach the exposed heme edge of ferrocytochrome *c*, which has substantial π donor character and thus would destabilize complexes with the more negatively charged ferrocyanide relative to ferricyanide. It should be remembered that the difference in free energy in ferrocyanide binding (−3.6 kcal/mol) and ferricyanide binding (−6.8 kcal/mol) is only 3.2 kcal/mol less than one hydrogen bond. Along these lines it can be noted that Gray and co-workers (34) have presented rather compelling evidence that inorganic complexes with π-conducting ligands are able to approach the heme much more closely than ionic complexes such as the iron hexacyanides.

The discussion presented above, although plausible, is not convincing (see below). The most important conclusion derived is that the limiting first-order processes (k_{23}, k_{32}) do not reflect the difference in oxidation-reduction potential between the iron hexacyanides and *c*-type cytochromes. Indeed, K_2 is close to 1.0 (1.0–0.57). This observation suggests that once a

complex is formed (K_1 and K_3^{-1}), electron transfer takes place with essentially equal rate constants. It follows that C_1 and C_2 (Eq. 1) can be viewed as intermediates and that they are isoenergetic, and thus pass through the same transition state with equal probability. This conclusion suggests that C_1 and C_2 are not the collision complexes but have undergone reorganization to bring them to the same energy levels. Available theoretical description of electron transfer mechanism, both outer-sphere electron transfer and electron tunneling models, are generally formulated in terms of a single-step reaction mechanism. Thus, these are not directly applicable to the three-step mechanism presented here. Some efforts have been made to apply the "Marcus-Hush" theory for outer-sphere electron transfer (35) to more complex mechanisms (30, 36). However, these approaches are not sufficiently refined to apply to the three-step (or five-step, see below) mechanism at this time.

The three-step mechanism described above, although mathematically consistent with all available data, presents serious conceptual problems. First, since the ratio k_{12}/k_{43} controls the oxidation-reduction potential of a particular cytochrome (Figs. 7 and 8), the rate constants for the formation of the complexes C_1 and C_2 must be influenced by the factors controlling the oxidation-reduction potential. Thus C_1 and C_2 cannot be collision complexes whose formation is dictated by only the electrostatic and steric factors involved. Second, as described earlier, although a plausible explanation can be made for the large observed difference K_1 and K_3^{-1}, it is not particularly convincing in terms of the known structure of ferri- and ferrocytochrome *c*. In view of the problems inherent in the three-step mechanism, a five-step mechanism given by Eq. 7 can be considered.

$$\text{cyto}(Fe^{+3}) + Fe(CN)_6^{-4} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} C_1 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} C_1' \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} C_2' \underset{k_r'}{\overset{k_f'}{\rightleftharpoons}} C_2 \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} \text{cyto}\,(Fe^{+2}) + Fe(CN)_6^{-3} \qquad (1)$$

In Eq. 7, C_1 and C_2 represent collision complexes and C_1' and C_2' intermediates formed following some "chemistry" with $K_1' = k_f/k_r$, $K_3' = k_f'/k_r'$. Analysis of the five-step mechanism generally yields solutions similar in form to those found for a three-step mechanism. $K_1 \ll K_3^{-1}$ and $K_1' K_2 K_3' \simeq 1$. However, one special case exists that does lead to a more physically reasonable model. If a rapid equilibrium exists between $Fe(CN)_6^{-4}$ and C_1, that is $k_{12}\,[Fe(CN)_6^{-4}]$, $k_{21} \gg k_f$ (Eq. 7), then the apparent second-order rate constant (k_{12} in the three-step mechanism) will be $K_1 k_f$ in the five-step mechanism (30). In this case, k_{23} and k_{32} would be unchanged from the values obtained in the three-step mechanism. In addition, the products $K_1 K_1'$ and $K_3 K_3'$ would reflect binding of ferro- and ferricyanide and k_r and k_f' would reflect the rate-limiting steps for breakdown of the intermediates (k_{21} and k_{34} in the three-step mechanism). To quantitate a five-step rapid equilibrium mechanism, assumptions have to be made. For reduction it can be assumed that k_{12} is diffusion controlled ($\sim 1.67 \times 10^{10}$ $M^{-1}\,s^{-1}$), but the diffusion coefficient must be corrected for the fractional area of the surface available for formation of productive complexes (ignoring electrostatic effects). Since the exposed heme edge represents ~3% of the surface of cytochrome *c* (37), we can estimate that k_{12} is on the order of 5×10^8 $M^{-1}\,s^{-1}$. Since k_{23} is rate limiting (2,600 s^{-1}) a value of k_f equal to or greater than k_{23} is required. In fact simulation of the five-step rapid equilibrium mechanism indicates that a minimum value of 10,000 s^{-1} for k_f will describe the horse heart cytochrome *c* data (larger values will also work). Thus taking k_f as 10,000 s^{-1} and k_{12} as 5×10^8 $M^{-1}\,s^{-1}$, a value of 5×10^7 s^{-1} for k_{21} is obtained, with k_{12} (three-step) = $K_1 k_f$ (five-

step) = $1 \times 10^5\ M^{-1}\ s^{-1}$. Taking $K_1 \simeq K_3^{-1}$, it follows that $k_r' \simeq 2 \times 10^6\ s^{-1}$. The various rate constants for a five-step rapid equilibrium derived for horse heart cytochrome *c* as described above are summarized in Table IV.

The analysis presented in the preceding discussion, although requiring some assumptions, does yield a physically reasonable model. Thus, a collision complex is formed at or near the diffusion-controlled limit which rapidly breaks down, or which can be interconverted to intermediates C_1' C_2' which are isoenergetic. The model just described is shown diagrammatically in Fig. 9. For the five-step mechanism, the ratio k_f/k_r' (Eq. 7) is primarily responsible for the value of the oxidation-reduction potential. The equilibrium constants K_2' and K_3' are controlled by the chemistry of the cytochrome and reactants. These reactions can be viewed as solvent reorganization, adjustment of bond lengths, and/or other transient fluctuations from equilibrium resulting in the intermediates C_1' and C_2' being isoenergetic, which permits rapid reversible electron transfer. Thus, the five-step mechanism circumvents the inherent problems of the three-step mechanism, with the binding constants for the formation of the collision complexes approximately the same for both ferri- and ferrocyanide, and the oxidation-reduction potential controlled by a first-order process in which chemical and/or structural alterations can take place. The five-step mechanism presented here, although complex relative to the usual descriptions of outer-sphere electron transfer, is in fact consistent with what would be expected for a protein-mediated process. Enzymes typically function via multiple steps in their reaction mechanisms, with the protein moiety mediating one or more first-order process following the formation of the enzyme-substrate complex. Thus, the five-step mechanism requires that oxidation and reduction of *c*-type cytochromes is conceptually similar to enzymatic catalysis.

There is no difficulty in extending the analysis described to the other *c*-type cytochromes

TABLE IV
KINETIC PARAMETERS FOR A FIVE-STEP MECHANISM AS APPLIED TO HORSE HEART CYTOCHROME *C**

Parameter	Value
k_{12}	$5 \times 10^8\ M^{-1}\ s^{-1}$
k_{21}	$5.0 \times 10^7\ s^{-1}$
K_1	$10.0\ M^{-1}$
k_f	$1 \times 10^4\ s^{-1}$
k_r	$710\ s^{-1}$
$K_1' K_1$	$140\ M^{-1}$
k_{23}	$2{,}600\ s^{-1}$
k_{32}	$2{,}500\ s^{-1}$
K_2	1.04
k_f'	$240\ s^{-1}$
k_r'	$2 \times 10^6\ s^{-1}$
K_3'	1.2×10^{-4}
k_{34}	$5 \times 10^7\ s^{-1}$
k_{43}	$5 \times 10^8\ M^{-1}\ s^{-1}$
$K_3 K_3'$	$1.2 \times 10^{-5}\ M$
$K_1 K_2 K_3$	1.04
$K_1' K_3'$	1.69×10^{-3}

*Parameters taken from Eq. 7 and values assigned are as described in the text.

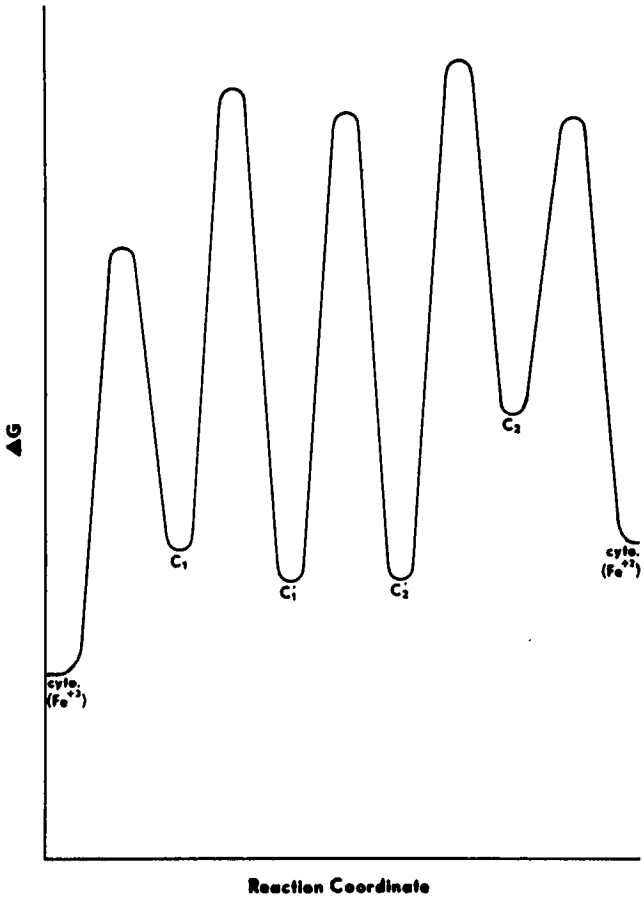

FIGURE 9 Free energy profile for horse heart cytochrome *c*—five step mechanism. ΔG values are arbitrary, although on a relative basis they are correct. The profile was calculated assuming both $Fe(CN)_6^{-4}$ and $Fe(CN)_6^{-3}$ were 100 μM at 20°C.

studied. The rate constants k_r, k_{23}, k_{32}, and k_f' would be identical to those given by k_{21}, k_{23}, k_{32}, and k_{34} in Table I. Furthermore, k_{12}, k_{21}, k_{34}, and k_{43} would have values approximating those that describe the rapid equilibrium for horse heart cytochrome *c* (Table IV), and the ratio k_f/k_r' would be equal to the ratio k_{12}/k_{43} derived from the data given in Table I. Realistically, the absolute value of k_f and k_r' (Table IV) represents lower limits, with their ratio the important point. Thus application of the five-step rapid equilibrium mechanism is general and does not require altering the values of the first-order rate constants derived from the three-step mechanism. It should be noted that the mechanism presented here is applicable to reactants other than the iron hexacyanides. Thus, in those cases where no change in rate-limiting step is observed, for example, in ferrous EDTA or ascorbate reduction of cytochrome *c*, the five-step mechanism would predict $K_1 k_f \ll k_{23}$; hence, the reaction would appear second-order over the entire accessible concentration range. On the other hand, reactions such as the reduction of photosynthetic reaction centers by reduced cytochromes which show an apparent change in rate-limiting step (but are irreversible) are controlled by

k_{23} at high reactant concentrations: $k_{23} \gg K_1 k_f$. Interestingly, the reaction of a variety of *c*-type cytochromes with oxidized *R. rubrum* reaction centers have limiting rate constants (k_{23}) on the order of 2,000–7,000 s^{-1}, values of the same order of magnitude as those observed with the iron hexacyanides (17).

In summary, we have shown that the steady-state approximation used to facilitate analysis of the kinetics of the interaction of the iron hexacyanides and *c*-type cytochromes yields misleading results. However, by solving the simultaneous differential equations describing the reaction mechanism, sufficient constraints exist to allow specifying the individual rate constants for a three-step mechanism. Further, analysis of a wide variety of *c*-type cytochromes indicates that the mechanistic details are similar in all cases with the apparent differences due to the particular protein moiety involved. Although mathematically adequate, the three-step mechanism does not yield a reasonable physical description of the iron hexacyanide reaction which is compatible with the expected oxidation-reduction properties. However, use of a five-step mechanism with a rapid equilibrium between reactants and their respective collision complexes does lead to a reasonable physical model and suggests that the protein moiety of a particular cytochrome controls the oxidation-reduction potential by making the intermediates that undergo rapid adiabatic electron transfer isoenergetic.

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