Effect of Divers Anions on the Electron-Transfer Reaction between Iron and Rusticyanin from *Thiobacillus ferrooxidans*†

Robert C. Blake II,* Kathy J. White, and Elizabeth A. Shute
Department of Biochemistry, Meharry Medical College, Nashville, Tennessee 37208
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ABSTRACT: Rusticyanin is a soluble blue copper protein found in abundance in the periplasmic space of *Thiobacillus ferrooxidans*, an acidophilic bacterium capable of growing chemolithotrophically on soluble ferrous sulfate. The one-electron-transfer reactions between soluble iron and purified rusticyanin were studied by stopped-flow spectrophotometry in acidic solutions containing each of 14 different anions. The second-order rate constants for both the Fe(II)-dependent reduction and the Fe(III)-dependent oxidation of the rusticyanin varied as a function of the identity of the principal anion in solution. Analogous electron-transfer reactions between soluble iron and bis(dipicolinato)cobaltate(III) or bis(dipicolinato)ferrate(II) were studied by stopped-flow spectrophotometry under solution conditions identical with those of the rusticyanin experiments. Similar anion-dependent reactivity patterns were obtained with soluble iron whether the other reaction partner was rusticyanin or either of the two organometallic complexes. The Marcus theory of outer-sphere electron transfer reactions was applied to this set of kinetic data to demonstrate that the rusticyanin may possess at least two electron-transfer pathways for liganded iron, one where the pattern of electron-transfer reactivity is controlled largely by protein-independent activation parameters and one where the protein exhibits an anion-dependent kinetic specificity. The exact role of rusticyanin in the iron-dependent respiratory electron transport chain of *T. ferrooxidans* remains unclear.

Thiobacillus ferrooxidans is an acidophilic, chemolithotrophic bacterium that can obtain all of its energy for the assimilation of carbon dioxide from the aerobic oxidation of ferrous ions. Metabolic energy is derived from oxidative phosphorylation coupled to respiratory electron transfer [see Ingledew (1982) for a review]. Rusticyanin is an acid-stable, type I copper protein with a molecular mass of 16 551 daltons (Ronk et al., 1991) found in abundance in the periplasmic space of this bacterium (Cox et al., 1978; Ingledew et al., 1977). Rusticyanin may constitute as much as 5% of the total soluble protein synthesized by cells of *T. ferrooxidans* that have been grown autotrophically on ferrous ions (Cox & Boxer, 1978). Furthermore, the synthesis of rusticyanin is repressed when *T. ferrooxidans* is grown solely on reduced sulfur compounds and induced when such sulfur-grown cells are subsequently exposed to soluble iron (Kulpa et al., 1986; Jedlicki et al., 1986). These observations are compelling evidence in support of the hypothesis that rusticyanin must be an important component of the iron respiratory chain.

The exact role that the rusticyanin plays in the iron-dependent electron-transport scheme is unclear. Previous kinetic studies on the electron-transfer reactions between sulfatoiron and purified rusticyanin indicated that the rates of reaction were far too slow to support the hypothesis that rusticyanin is the primary oxidant of ferrous ions in the iron-dependent respiratory chain (Blake & Shute, 1987; Lewis et al., 1983; Lappin et al., 1985). It is clear, however, that the role of rusticyanin as the primary oxidant of Fe(II) has not been entirely eliminated. Conditions experienced by the rusticyanin in the microenvironment of the periplasmic space may not have been duplicated in the in vitro kinetic experiments referenced above. Perhaps the kinetic behavior of the rusticyanin is influenced by complexation of the rusticyanin with other species in the periplasm, such as its physiological oxidant. Alternatively, the reductant of rusticyanin in the periplasm could be Fe(II) in a different form from the solvated sulfate complex. If the Fe(II) were to exchange coordination ligands upon entry to the periplasm, the presence of the different ligand could greatly alter the kinetic behavior of the ferrous complex.

The present paper describes a kinetic study of the transfer of electrons between iron and purified rusticyanin in the presence of different anions. These experiments were conducted, in part, to generate a broader picture of the electron-transfer reactivity between soluble iron and rusticyanin under a variety of solution conditions. The results presented below indicate that the rusticyanin may possess at least two distinct electron-transfer pathways for liganded iron. The first pathway was characterized by a protein-independent pattern of electron-transfer reactivity. For liganded iron in this category, the anion-dependent reactivity pattern could be quantitatively described solely by the physicochemical properties of the individual iron–anion complexes. The second pathway, utilized by citratoiron(II) and oxalacetatoiron(II), produced second-order rate constants for the liganded iron-dependent reduction of rusticyanin that were as much as 2 orders of magnitude greater than those anticipated on the basis of the physicochemical properties of the two complexes.

Experimental Procedures

Preparation of Reagents. Large-scale growth of *T. ferrooxidans* (ATCC 23270) for the purification of rusticyanin was achieved by batch culture at ambient temperatures in the acidic ferrous sulfate growth medium described previously (Tuovinen & Kelly, 1973) that had been supplemented with 1.6 mM cupric sulfate. Rusticyanin was subsequently purified to electrophoretic homogeneity from cell-free extracts of *T. ferrooxidans* according to a slight modification (Blake & Shute, 1987) of a previously published procedure (Cox & Boxer, 1978). The protein was isolated with its copper center

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* Author to whom correspondence should be addressed.

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in the oxidized state. The purified rusticyanin could be stored in 0.001 N sulfuric acid for at least 4 months at 4 °C without appreciable deleterious effects. For experiments involving oxidation of the protein, rusticyanin was reduced by reacting it with an excess of dithionite. The reduced protein was then dialyzed at 4 °C against an appropriate solution (pH 2.0) of the anion in question to remove the excess reducing agent. Reduced rusticyanin is remarkably stable to air oxidation. Samples of the reduced protein have been stored in 0.001 N sulfuric acid for up to 3 months at 4 °C before air-oxidized rusticyanin could be detected.

The synthesis and characterization of $Co(dipic)_2^-$[1] were accomplished as described elsewhere (Mauk et al., 1979), with the exception that $Co(SO_4)\cdot 7H_2O$ was substituted for $Co(NO_3)\cdot 6H_2O$. The absorbance properties of the $Co(dipic)_2^-$ complex at pH 2.0 were identical with those of the complex at neutral pH and were stable for at least a week in the acidic solutions; nonetheless, solutions of $Co(dipic)_2^-$ for kinetic experiments were prepared fresh daily. Solutions of $Fe(dipic)_2^{2-}$ were prepared anaerobically in a manner similar to that described previously. A nitrogen-purged aqueous solution of 100 μM ferrous sulfate in 0.01 N sulfuric acid was added to degassed 0.01 N sulfuric acid containing 300 μM 2,6-pyridinedicarboxylic acid. Portions of the stock reductant solution thus prepared were subsequently diluted for kinetic experiments with degassed solutions containing the appropriate anions. All solution transfers and dilutions were performed using Hamilton gas-tight syringes. The absorbances properties of the $Fe(dipic)_2^{2-}$ complex were independent of pH in the range of 2.0–7.0.

Stopped-Flow Measurements. Kinetic measurements were performed on the stopped-flow spectrophotometer described previously (Blake & Shute, 1987). The one-electron-transfer reactions examined in this investigation fell into one of three categories, depending on whether the reaction partner opposite the liganded iron was rusticyanin, $Co(dipic)_2^-$, or $Fe(dipic)_2^{2-}$. For kinetic experiments involving the rusticyanin, the purified protein and the ferric or ferrous ions were prepared in identical solutions of acidic anionic media and added to separate syringes of the stopped-flow spectrophotometer. Unless noted otherwise, all kinetic experiments were performed at a pH of 2.0. The temperature of the driving syringes was maintained at 25 ± 1 °C by circulating water. Room temperature solutions were allowed to equilibrate for 10 min in the driving syringes. Reactions were initiated by rapidly mixing 0.1 mL of the solution from each driving syringe. Spectral changes were linear to an absorbance of 1.8. The changes in the oxidation state of rusticyanin were monitored at 597 nm. A typical absorbance change (2-cm path length) of ±0.06 provided acceptable signal to noise characteristics at this wavelength.

Kinetic experiments involving the metal complexes of 2,6-pyridinedicarboxylic acid were conducted by substituting either $Co(dipic)_2^-$ or $Fe(dipic)_2^{2-}$ for rusticyanin in the experimental protocol described above. The reduction of $Co(dipic)_2^-$ and the oxidation of $Fe(dipic)_2^{2-}$ were monitored at 510 and 480 nm, respectively. In either case, typical absorbance changes of from 0.03 (for $Co(dipic)_2^-$) to 0.06 (for $Fe(dipic)_2^{2-}$) provided acceptable signal to noise characteristics.

When either rusticyanin, $Co(dipic)_2^-$, or $Fe(dipic)_2^{2-}$ was mixed in the stopped-flow spectrophotometer with a 10-fold or greater molar excess of liganded iron (pseudo-first-order conditions), each kinetic trace could be described mathematically as a single-exponential function of time, given by

$$\Delta A_t = \Delta A_\infty e^{-k_{obs}t}$$

where ΔA_t is the absorbance at the end of the reaction minus the absorbance at time t ($A_\infty - A_t$), ΔA_∞ is the total absorbance change observed ($A_\infty - A_0$), and k_{obs} is the observed pseudo-first-order rate constant for the absorbance change. Each kinetic trace consisted of 200 data points collected over a time interval that ranged from 200 ms to 1200 s, depending on the reactivity of the electron-transfer partners. Each electron-transfer reaction was permitted to proceed to between 95 and 98% completion. Accurate values of ΔA_∞ and k_{obs} were then obtained from each kinetic trace according to an iterative nonlinear regression analysis as described previously (Blake & Shute, 1987).

Materials. The ferrous and ferric salts of perchlorate, chloride, and bromide were obtained from Morton Thiokol, Inc. (Alfa Products), as were the sodium salts of selenate and perchlorate. Cobaltous sulfate and 2,6-pyridinedicarboxylic acid were obtained from Fisher Chemical Co. and Aldrich Chemical Co., respectively. Citric, isocitric, oxalacetic, malic, succinic, fumeric, lactic, pyruvic, and α-ketoglutaric acids were obtained from Sigma Chemical Co., as was 30% hydrogen peroxide. All other chemicals were reagent grade.

Results

Reduction by Liganded Fe(II). The initial object of these experiments was to obtain detailed kinetic data on the one-electron reduction of rusticyanin by Fe(II) in the presence of different anions. Accordingly, stopped-flow spectrophotometric experiments on the reduction of purified rusticyanin were performed with excess Fe(II) in the presence of each of 14 different anions. Two types of anions were investigated in this study: inorganic anions that were soluble in acidic solutions (pH 2.0) of iron, and small organic anions representing principal metabolites of intermediary carbon metabolism. Each anion employed in this study bound more tightly to the Fe(III) than it did to the Fe(II). Consequently, each anion made the complexed Fe(II) more reducing than the uncomplexed hexoaquoiron(II); that is, the standard reduction potential of the ferric/ferrous couple was always lower than 770 mV in the presence of an excess of the anionic ligand. The Fe(II)-dependent reduction of rusticyanin was investigated under experimental conditions where the concentration of each anion was in sufficient excess to ensure that the principal reducing agent in every instance was a complex of Fe(II) with one or more anion molecules.

The visible absorbance spectrum of oxidized rusticyanin exhibits a prominent peak at around 600 nm (Blake & Shute, 1987), a peak that disappears upon the one-electron reduction of the protein. The blue absorbance of the copper center in oxidized rusticyanin thus constitutes an intrinsic spectrophotometric probe whereby transient changes in the redox state of the molecule may be monitored with great sensitivity. When oxidized rusticyanin was mixed in a stopped-flow spectrophotometer with a 10-fold or greater molar excess of liganded Fe(II), each kinetic trace of the loss in absorbance at 597 nm could be described mathematically as a single-exponential function of time (primary data not shown). As long as pseudo-first-order conditions were maintained, protein concentrations from 5.0 to 45 μM affected only the amplitudes of the observed spectral changes, not the values of k_{obs}. The amplitudes were directly proportional to the concentration of the rusticyanin.

Examples of the dependence of the pseudo-first-order rate constants for the reduction of rusticyanin upon the total

[1] Abbreviations: $Co(dipic)_2^-$, bis(dipicolinato)cobaltate(III); $Fe(dipic)_2^{2-}$, bis(dipicolinato)ferrate(II).

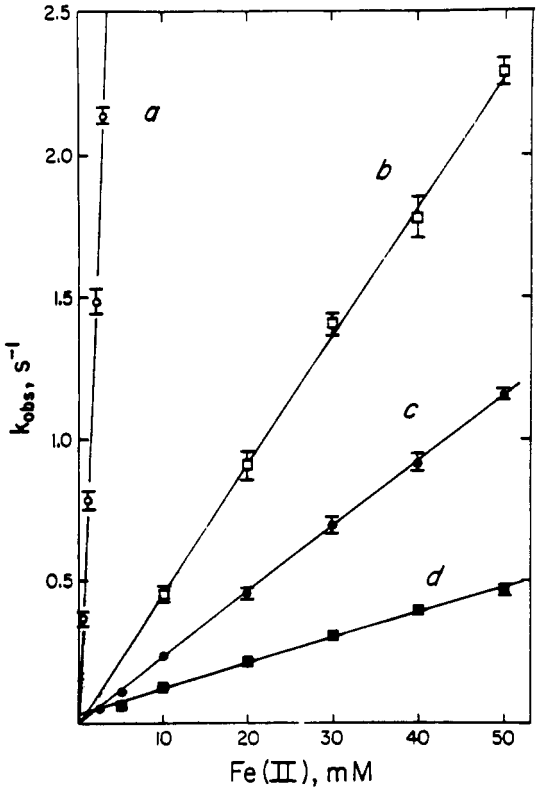

FIGURE 1: Dependence of the pseudo-first-order rate constant for the reduction of rusticyanin upon the final concentrations of total Fe(II) in the presence of citrate (a), pyrophosphate (b), chloride (c), and selenate (d). The values of k_{obs} obtained in the presence of selenate were multiplied by 10 to facilitate comparison of the curves. Final concentrations after mixing: rusticyanin, 15 μM; citrate, pyrophosphate, chloride, and selenate, 0.1, 1.0, 2.0, and 0.5 M in experiments a, b, c, and d, respectively. Each error bar represents the standard deviation of at least four replicate determinations. The slope of each line was determined by linear regression analysis.

concentration of Fe(II) in the presence of selected anions are shown in Figure 1. A typical kinetic study for each anion employed at least five different iron concentrations spanning a 5–50-fold range in concentrations. Regardless of the identity of the principal anion present, the pseudo-first-order rate constants for the reduction of the rusticyanin were directly proportional to the concentration of the liganded Fe(II) in all cases; no convincing evidence for rate saturation was obtained in any of these studies.

It is evident from Figure 1 that the identity of the anion in solution had a significant influence on the rate of the electron transfer from Fe(II) to the rusticyanin. The value of the slope of each line in Figure 1 represents an apparent second-order rate constant for the electron-transfer reaction observed in the presence of that anion. Values of the second-order rate constants for the Fe(II)-dependent reduction of the rusticyanin obtained in the presence of each of 14 different anions are tabulated in the first column of Table I. Previous studies have indicated that other oxidation–reduction reactions involving ferrous ions are equally dependent upon the identity of the anions present. For example, the rate of the Fe(II)-dependent reduction of molecular oxygen in acid solution was reported by other laboratories to increase in the order perchlorate (George, 1954) < sulfate (Lamb & Elder, 1931) < chloride (Posner, 1953) ~ phosphate (Cher & Davidson, 1955) < pyrophosphate (Huffman & Davidson, 1956), which is exactly how one would arrange the corresponding rate constants for the Fe(II)-dependent reduction of rusticyanin. To investigate whether the anion-dependent differences in rate observed

Table I: Second-Order Rate Constants for the Fe(II)-Dependent Reduction of Rusticyanin or $Co(dipic)_2^-$ in the Presence of Different Anions

anionic species	rate constant for RCu(II) → RCu(I) (M^{-1} S^{-1})	(SD[a])	rate constant for $Co(dipic)_2^-$ → $Co(dipic)_2^{2-}$ (M^{-1} s^{-1})	(SD)
perchlorate	0.022	0.0022	7.9	0.9
bromide	0.74	0.08	352	38
selenate	1.08	0.14	188	21
sulfate	2.11	0.08	286	17
α-ketoglutarate	4.56	0.33	719	31
pyruvate	13.4	0.9	752	39
isocitrate	15.3	1.7	1010	55
malate	20.2	1.9	893	65
phosphate	21.7	2.8	2540	180
chloride	23.5	1.5	3550	240
lactate	39.4	4.1	1360	90
pyrophosphate	70	11	6100	480
citrate	710	81	1370	90
oxalacetate	820	178	1590	240

[a] SD = standard deviation.

herein were due to some specific property of the rusticyanin, or merely to differences in the intrinsic electron-transfer reactivity of each unique iron–anion complex or to a combination of both effects, it was of interest to investigate the Fe(II)-dependent reduction of an alternative oxidant under the same experimental conditions as those employed in the rusticyanin study.

The bis(dipicolinate) complex of Co(III) was selected as the alternative oxidant for these studies. $Co(dipic)_2^-$ has been used extensively in characterizing the electron-transfer reactivity of metalloproteins, particularly other type I blue copper proteins (Mauk et al., 1979, 1982). The object of the present experiments was to obtain detailed kinetic data on the one-electron reduction of $Co(dipic)_2^-$ by Fe(II) in the presence of different anions. The visible absorbance spectrum of $Co(dipic)_2^-$ exhibits a peak at around 510 nm that disappears upon the one-electron reduction of the complex. When $Co(dipic)_2^-$ was mixed in a stopped-flow spectrophotometer with a 10-fold or greater molar excess of Fe(II), each kinetic trace of the loss in absorbance at 510 nm could be described mathematically as a single-exponential function of time (primary data not shown). The values of the pseudo-first-order rate constants for the reduction of $Co(dipic)_2^-$ were directly proportional to the concentration of the total Fe(II) in the presence of each of the 14 anions investigated. The values of the corresponding second-order rate constants for the Fe(II)-dependent reduction of $Co(dipic)_2^-$ are tabulated in the third column of Table I.

It was evident that the values of the second-order rate constants in the two sets of kinetic data were related; in general, ligation of the ferrous ion by a particular anion influenced the rates of reduction of both the protein copper center and the small, organocobalt compound in a similar manner. The similar tendencies between the two series of rate constants are illustrated in Figure 2, where the values of the second-order rate constants for the liganded Fe(II)-dependent reduction of the rusticyanin are plotted *versus* the corresponding values for the reduction of the $Co(dipic)_2^-$. Although there is considerable scatter about the linear regression line drawn through the data points in Figure 2, the line nonetheless serves to emphasize that a rough correlation exists between the two sets of rate constants. There would be considerably less scatter among the remaining points if data points labeled l (citrate) and m (oxalacetate) were omitted from the linear regression calculation. Subsequent analysis of these and ad-

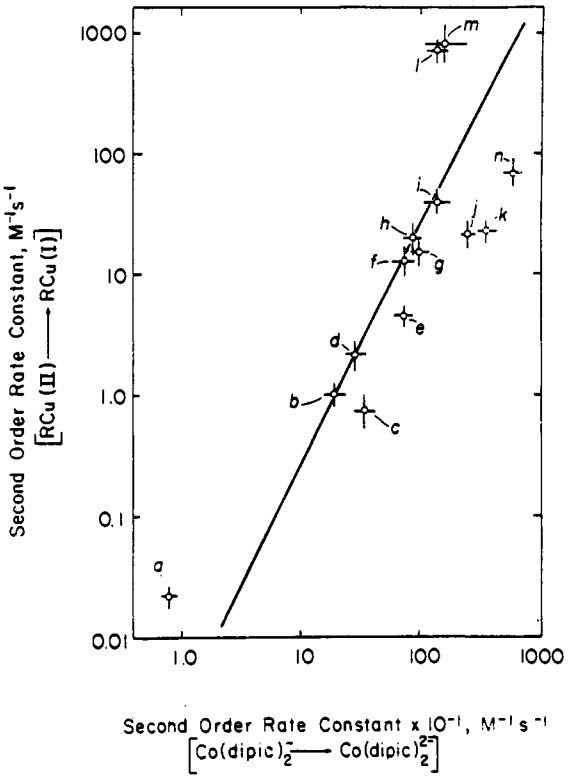

FIGURE 2: Dependence of the second-order rate constant for the Fe(II)-dependent reduction of rusticyanin upon the corresponding rate constant for the reduction of $Co(dipic)_2^-$ in the presence of different anions. Values for the second-order rate constants were obtained from Table I. Anions: a, perchlorate; b, selenate; c, bromide; d, sulfate; e, α-ketoglutarate; f, pyruvate; g, isocitrate; h, malate; i, lactate; j, phosphate; k, chloride; l, citrate; m, oxalacetate; and n, pyrophosphate. The line was determined by a linear regression analysis using all of the data points. The correlation coefficient was 0.83.

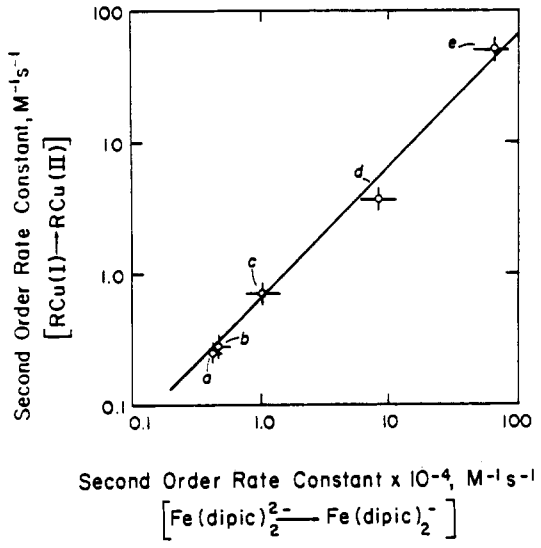

FIGURE 3: Dependence of the second-order rate constant for the Fe(III)-dependent oxidation of rusticyanin upon the corresponding rate constant for the oxidation of $Fe(dipic)_2^{2-}$ in the presence of different anions. Values for the second-order rate constants were obtained from Table II. Anions: a, sulfate; b, selenate; c, perchlorate; d, bromide; and e, chloride. The line was determined by a linear regression analysis. The correlation coefficient was 0.996.

ditional data (Figure 4) suggests that, indeed, there may be good reason to omit these two points.

Oxidation by Liganded Fe(III). The object of these experiments was to obtain detailed kinetic data on the one-electron oxidation of reduced rusticyanin by Fe(III) in the

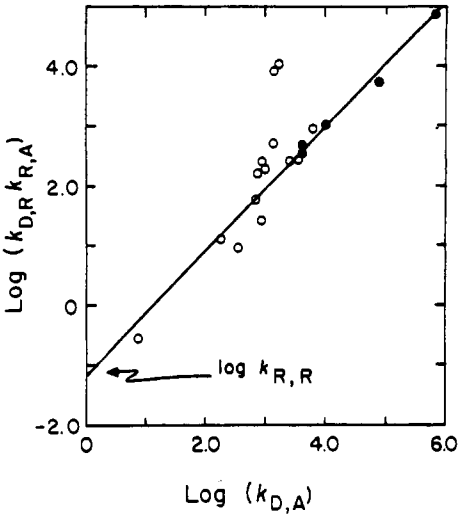

FIGURE 4: Correlation of the second-order rate constants for electron transfer using relative Marcus theory. The second-order rate constants $k_{D,R}$, $k_{R,A}$, $k_{D,A}$, and $k_{R,R}$ represent the rates of electron transfer from a donor to rusticyanin, from rusticyanin to an acceptor, from a donor directly to an acceptor, and from one molecule of rusticyanin to another, respectively. The open circles represent electron transfer from Fe(II) (the donor) to $Co(dipic)_2^-$ (the acceptor) in the presence of different anions, while the closed circles represent electron transfer from $Fe(dipic)_2^{2-}$ (the donor) to Fe(III) (the acceptor) in the presence of different anions. The slope and ordinate intercept of the line were determined by a linear regression analysis that omitted the two outlying points, representing data obtained in the presence of citrate and oxalacetate, from the calculation. The correlation coefficient was 0.97.

Table II: Second-Order Rate Constants for the Fe(III)-Dependent Oxidation of Rusticyanin or $Fe(dipic)_2^{2-}$ in the Presence of Different Anions

	rate constant for RCu(I) → RCu(II)		rate constant for $Fe(dipic)_2^{2-} \rightarrow Fe(dipic)_2^-$	
anionic species	(M^{-1} s^{-1})	(SD[a])	(M^{-1} s^{-1})	(SD)
sulfate	0.26	0.02	4200	400
selenate	0.28	0.03	4600	600
perchlorate	0.73	0.16	10000	800
bromide	3.8	0.3	82000	9000
chloride	52	6.0	640000	70000

[a] SD = standard deviation.

presence of different anions. Accordingly, stopped-flow spectrophotometric experiments on the oxidation of rusticyanin were performed with excess Fe(III) in the presence of each of five different anions. The concentration of each anion in these protein oxidation studies was identical with the concentration of that anion employed in the corresponding protein reduction studies. Pseudo-first-order kinetic behavior was observed in all circumstances, and values for the second-order rate constants for the liganded Fe(III)-dependent oxidation of the reduced rusticyanin are given in the first column of Table II. Like the Fe(II)-dependent reduction of rusticyanin, the rate of the Fe(III)-dependent oxidation of rusticyanin was clearly dependent upon the identity of the anion in solution. To investigate the possible origins of this anion dependency, the Fe(III)-dependent oxidation of an alternative reductant was investigated under the same experimental conditions as those employed in the rusticyanin study.

The alternative reductant chosen for these studies was the bis(dipicolinate) complex of Fe(II). The visible absorbance spectrum of $Fe(dipic)_2^{2-}$ exhibits a peak at around 480 nm that disappears upon the one-electron oxidation of the complex. When $Fe(dipic)_2^{2-}$ was mixed in a stopped-flow spectropho-

tometer with a 10-fold or greater molar excess of Fe(III), each kinetic trace of the loss in absorbance at 480 nm could be described mathematically as a single-exponential function of time (primary data not shown). The values of the pseudo-first-order rate constants for the oxidation of $Fe(dipic)_2^{2-}$ were directly proportional to the concentration of the total Fe(III) in the presence of each of the five anions investigated. Values of the second-order rate constants for the Fe(III)-dependent oxidation of $Fe(dipic)_2^{2-}$ are given in the third column of Table II.

Figure 3 shows the correlation between the second-order rate constants for the liganded Fe(III)-dependent oxidation of the rusticyanin and the corresponding rate constants for the oxidation of $Fe(dipic)_2^{2-}$. It is evident that ligation of the ferric ions by a particular anion influenced the rates of oxidation of both the protein copper center and the small, organoiron compound in a similar manner.

Marcus Theory. It was of interest to estimate how much of the variation in the second-order rate constants in Figures 2 and 3 was due to a ligand-dependent influence on the intrinsic electron-transfer reactivity of the complexed iron and how much was due to protein-dependent factors. Certainly, ligation of the soluble iron by a particular anion would be expected to alter both the thermodynamic driving force for electron transfer and the inherent reactivity of the liganded iron reagent. Concurrently, changes in the charge, size, and ligand structure of the complexed iron might be expected to influence the accessibility of the small reagent to the rusticyanin copper center. It should be noted that other comparably sized blue copper proteins show remarkable kinetic variability in their reactions with small organometallic complexes. For example, the second-order rate constants for the electron-transfer reactions of *Pseudomonas aeroginosa* azurin with $Fe(EDTA)^{2-}$ or tris(oxalato)cobaltate(III) differ by nearly 5 orders of magnitude (Holwerda et al., 1980; Wherland et al., 1975). Extensive kinetic data is available on the electron-transfer reactions of selected inorganic and small organometallic complexes with isolated electron-transport proteins, especially the cytochromes, blue copper proteins, and various iron–sulfur proteins (Wherland & Gray, 1977; Holwerda et al., 1976; Marcus & Sutin, 1985). In particular, the Marcus theory of outer-sphere electron-transfer reactions has been widely employed to deduce, from the relevant kinetic and thermodynamic data, the factors governing the rates of outer-sphere electron-transfer reactions between small organometallic complexes and metalloproteins (Marcus, 1964; Sutin, 1973). It was therefore of interest to process the set of kinetic data obtained here by using relative Marcus theory to compensate for the variation in driving force and inherent reactivity of the reagents.

Extensive experimental data on a variety of known outer-sphere electron-transfer reactions have established that the kinetic and thermodynamic properties of the reaction partners may frequently be correlated by the "cross-reaction" equation developed by Marcus, given by

$$k_{X,Y} = \sqrt{k_{X,X}k_{Y,Y}K_{X,Y}} \quad (1)$$

where $k_{X,Y}$ is the second-order rate constant for the transfer of an electron from X to Y, $k_{X,X}$ and $k_{Y,Y}$ are the two self-exchange rate constants (e.g., for the transfer of an electron from one molecule of X to another molecule of X), and $K_{X,Y}$ is the equilibrium constant for the electron transfer. Each self-exchange rate constant is a measure of the intrinsic reactivity of each reactant and is related to the energy barrier created by the internal and solvent nuclear rearrangements that must occur immediately prior to actual electron transfer. Equation 1 is a much simplified version of the theoretical treatment developed by Marcus for outer-sphere electron-transfer reactions, but it has nonetheless been shown to correlate a large body of kinetic and thermodynamic data, particularly in those electron-transfer reactions of an acceptor with a series of related donors (or vice versa).

The transfer of an electron from liganded iron to $Co(dipic)_2^-$ may occur either directly as given by

$$Fe(II)X + Co(dipic)_2^- \xrightarrow{k_{D,A}} Fe(III)X + Co(dipic)_2^{2-} \quad (2)$$

or indirectly via rusticyanin as an intermediary electron carrier as given by

$$Fe(II)X + RCu(II) \xrightarrow{k_{D,R}} Fe(III)X + RCu(I) \quad (3)$$

$$RCu(I) + Co(dipic)_2^- \xrightarrow{k_{R,A}} RCu(II) + Co(dipic)_2^{2-} \quad (4)$$

where Fe(II)X and Fe(III)X represent complexes between iron and an anion, X, of unspecified stoichiometry and electronic state. If reactions 2–4 occur by outer-sphere mechanisms, then it follows from eq 1 that

$$k_{D,A} = \sqrt{k_{D,D}k_{A,A}K_{D,A}} \quad (5)$$

$$k_{D,R} = \sqrt{k_{D,D}k_{R,R}K_{D,R}} \quad (6)$$

$$k_{R,A} = \sqrt{k_{R,R}k_{A,A}K_{R,A}} \quad (7)$$

By recognizing that

$$K_{D,A} = K_{D,R}K_{R,A} \quad (8)$$

expressions for each of the three equilibrium constants may be obtained from eqs 5–7 and substituted into eq 8 to yield, after rearrangement

$$\log (k_{D,R}\, k_{R,A}) = \log (k_{D,A}) + \log (k_{R,R}) \quad (9)$$

where all of the rate constants except $k_{R,R}$, the self-exchange rate constant for the rusticyanin, are readily determined by direct experimentation. The second-order rate constant, $k_{R,A}$, for the transfer of an electron from reduced rusticyanin to $Co(dipic)_2^-$ was determined to be 12.4 M^{-1} s^{-1} in stopped-flow spectrophotometric experiments using five different concentrations of an excess of organometallic reagent (data not shown). This value compares favorably with that of 11.4 M^{-1} s^{-1} obtained elsewhere for the same reaction (McGinnis et al., 1986). A plot according to eq 9 was constructed by use of $k_{R,A}$ and values for $k_{D,R}$ and $k_{D,A}$ taken from Table I; the resulting plot is illustrated by the open circles in Figure 4.

The transfer of an electron from $Fe(dipic)_2^{2-}$ to liganded iron may be treated in a manner similar to that outlined above to yield the equivalent of eq 9 where $k_{D,R}$, $k_{R,A}$, and $k_{D,A}$ represent the cross-reaction rate constants for electron transfer from $Fe(dipic)_2^{2-}$ to rusticyanin, from rusticyanin to liganded iron, and from $Fe(dipic)_2^{2-}$ to liganded iron, respectively. The value of $k_{D,R}$ was determined to be 1380 M^{-1} s^{-1} in independent stopped-flow spectrophotometric experiments involving $Fe(dipic)_2^{2-}$ and oxidized rusticyanin (data not shown). A plot according to eq 9 was then constructed by use of this value of $k_{D,R}$ and values for $k_{R,A}$ and $k_{D,A}$ taken from Table II; the resulting plot is illustrated by the closed circles in Figure 4.

Recognizing that each datum in Figure 4 was derived from three individual experimental observations, each with its own level of experimental error, the close correlation among 17 of the 19 points is exceptional. One can conclude that 17 of the liganded iron complexes have equal kinetic access to the redox

Table III: Kinetic and Thermodynamic Constants for Selected Iron Complexes Calculated with Use of Relative Marcus Theory

redox-active complex	self-exchange rate constant (M^{-1} s^{-1})	reduction potential (mV)
hexoaquoiron	0.25	770
sulfatoiron	8.7	630
selenatoiron	4.4	650
chloroiron	19000	700
bromoiron	45	720

center of the rusticyanin and utilize the same reaction pathway for electron transfer. The value of the abscissa intercept in Figure 4 yielded a value of 0.063 M^{-1} s^{-1} for the rusticyanin self-exchange rate constant, $k_{R,R}$. One interpretation of this very low self-exchange rate constant is that the redox-active copper center in the rusticyanin is substantially buried in the hydrophobic interior of the protein and is relatively inaccessible to small hydrophilic electron-transfer reagents. Once the value of $k_{R,R}$ was known, it was possible to calculate the operational self-exchange rate constants for iron in the presence of five of the anions examined here. If one considers the reverse of reaction 3 given by

$$\text{Fe(III)X} + \text{RCu(I)} \xrightarrow{k_{R,D}} \text{Fe(II)X} + \text{RCu(II)} \quad (10)$$

it is evident from eq 1 that

$$k_{R,D} = \sqrt{k_{R,R}k_{D,D}K_{R,D}} \quad (11)$$

Expressions for each of the two equilibrium constants in eqs 6 and 11 may be obtained and substituted into the relationship $K_{D,R} = 1/K_{R,D}$ to yield, after rearrangement, the equation

$$k_{D,D} = k_{D,R}k_{R,D}/k_{R,R} \quad (12)$$

where only experimentally determined rate constants are on the right side of the equation. It was thus possible to calculate a value of $k_{D,D}$ for iron in the presence of each of the five anions in Table II by use of values of $k_{R,D}$ from Table II, corresponding values of $k_{D,R}$ from Table I, and the value of $k_{R,R}$ obtained from Figure 4. The values of these five calculated self-exchange rate constants are given in Table III. One could now use either eq 6 or 11 to calculate individual equilibrium constants for each of the five electron-transfer reactions where values of $k_{D,D}$ were available. Choosing 770 mV as the reduction potential for the Fe(III)/Fe(II) couple in aqueous perchlorate as a reference point, a value of 683 ± 10 mV was calculated for the reduction potential of the rusticyanin, a value identical with that determined independently by potentiometric experiments (Ingledew & Cobley, 1980; Lappin et al., 1985). This reduction potential for the rusticyanin was then used to calculate the other four reduction potentials listed in Table III for the Fe(III)/Fe(II) couple in the presence of the different anions. Although independently determined reduction potentials for the latter four redox couples in Table III are not available for the exact ionic strengths and solution conditions employed in the present study, reasonable estimates for the values of these potentials based on data acquired from solutions of similar composition are available (Sillen & Martell, 1964) and are in reasonable agreement with those of the calculated potentials.

Discussion

Rusticyanin appeared to possess at least two distinct electron-transfer pathways in its reactions with soluble iron complexed with hydrophilic ligands. One electron-transfer pathway was distinguished by an apparent lack of anion-specific protein activation requirements. Seventeen of the 19 experimental points in Figure 4 conformed closely to eq 9, indicating that the Marcus theory provided a means of quantitatively correlating a large number of electron-transfer reactions involving the rusticyanin, regardless of whether the protein was accepting or donating the electron. The existence of this correlation further suggested that each of those 17 electron-transfer reactions adopted the same reaction pathway into and out of the protein. The pattern of electron-transfer reactivity for those 17 reactions was thus controlled largely by the inherent reactivity of the liganded iron reagent and was independent of any anion-specific protein activation requirements.

The other electron-transfer pathway was distinguished by a dependence on the identity of the hydrophilic anion liganded to the iron. The two data points in Figure 4 that deviated significantly from the linear regression line drawn through the other 17 points were obtained in the presence of citrate and oxalacetate. The values of the second-order rate constants for the citratoiron(II)- and the oxalacetatoiron(II)-dependent reduction of the rusticyanin were some 1 to 2 orders of magnitude greater than those anticipated from the corresponding reactivity of these two iron complexes with Co(dipic)$_2^-$. These two positive deviations suggested that one or more separate reaction pathways might exist for the electron-transfer reactions between rusticyanin and specific species of liganded iron. Anion-dependent kinetic specificity could arise from any of several sources. For example, the rusticyanin could display a binding preference for a particular iron–anion complex, thus lowering the free energy necessary to bring the two reaction partners together in an encounter complex for electron transfer to occur. Alternatively, favorable protein–reagent interactions could occur with specific liganded species in the encounter complex, leading to a lower activation barrier for electron transfer with that species.

The inorganic anions featured in this survey were of interest in probing the origins of the apparent specificity for sulfate or selenate exhibited by whole cells actively respiring on soluble iron (Lazaroff, 1963, 1977; Schnaitman et al., 1969). Although a specific requirement for sulfate could conceivably be coupled in the intact organism to a separate metabolic process that merely influenced the rate of iron-dependent respiration, a specific requirement for sulfate or selenate has also been reported for the iron-dependent reduction of oxygen catalyzed by cell-free electron-transport particles (Ingledew, 1982). No kinetic evidence was obtained here to indicate that the rusticyanin exhibited any kinetic specificity for either sulfate or selenate. Instead, sulfate and selenate were grouped along with 15 other anions in the reaction category distinguished by an apparent lack of anion-specific protein activation requirements.

The small organic anions featured in this survey represented principal metabolites of intermediary carbon metabolism and were of interest due to the likelihood that each organic acid is compelled by its physicochemical properties to spontaneously partition between the external environment at acidic pH and the cytoplasm of the bacterial cell at neutral pH (Tuttle et al., 1979; Alexander et al., 1987). Indeed, small organic acids like pyruvate have been identified in the spent media of stationary phase acidophilic thiobacilli (Schnaitman & Lundgren, 1965; Borichewski, 1967). Each such organic acid could be present in varying concentrations in the periplasmic space and perhaps enhance the reactivity of any Fe(II) present. In order for rusticyanin to serve as the primary initial electron acceptor in the iron-dependent respiratory chain, the apparent rate constant for the Fe(II)-dependent reduction of rusticyanin must be greater than or equal to the overall turnover number for the entire process, which has been calculated to be around

10 s^{-1} (Blake & Shute, 1987). If, for instance, an oxalacetatoiron(II) complex were hypothesized to be the principal reducing agent for rusticyanin in the periplasm, then the concentration of total oxalacetatoiron(II) outside of the plasma membrane would have to be on the order of 10 mM to achieve an apparent rate constant approaching a value of 10 s^{-1}. That is a great deal of oxalacetate for a chemolithotroph to excrete; nonetheless, the effective local anion concentration in the microenvironment of the periplasmic space could be quite high.

As an alternative to the hypothesis that periplasmic citrate and/or oxalacetate are principal components in the iron-dependent respiratory electron-transport chain of *T. ferrooxidans*, the kinetic specificity exhibited by the rusticyanin may merely indicate that the protein recognizes some common structural feature(s) of the citratoiron(II) or oxalacetatoiron(II) complexes. Perhaps the physiological reductant of the rusticyanin is iron coordinated to a periplasmic macromolecule that bears a ligand structure similar to that of iron complexed with citrate or oxalacetate. Other investigators have suggested that a massive iron–sulfate complex forms a polynuclear layer around the outer cell wall of this Gram-negative organism and serves as the initial electron acceptor from Fe(II) in the bulk solution (Ingledew, 1986). Reducing equivalents derived from solution Fe(II) could move rapidly through the polynuclear iron coat until they reached the physiological reductant of the rusticyanin at the periplasmic surface. Rusticyanin could then serve to shuttle electrons from the outer cell layer across the periplasmic space to the cytoplasmic membrane. This is a teleologically attractive proposal for an organism that must extract electrons from insoluble, semiconducting mineral sulfides in its native habitat.

The principal conclusion is that anion-specific interactions between the rusticyanin and selected liganded iron species do occur. Whether these anion-specific interactions have any bearing on the physiological role of the rusticyanin in the iron respiratory electron-transport chain of *T. ferrooxidans* remains under investigation in this laboratory.

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