Respiratory Enzymes of *Thiobacillus ferrooxidans*. Kinetic Properties of an Acid-Stable Iron:Rusticyanin Oxidoreductase†

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ABSTRACT: Rusticyanin is an acid-stable, soluble blue copper protein found in abundance in the periplasmic space of *Thiobacillus ferrooxidans*, an acidophilic bacterium capable of growing autotrophically on soluble ferrous sulfate. An acid-stable iron:rusticyanin oxidoreductase activity was partially purified from cell-free extracts of *T. ferrooxidans*. The enzyme-catalyzed, iron-dependent reduction of the rusticyanin exhibited three kinetic properties characteristic of aerobic iron oxidation by whole cells. (i) A survey of 14 different anions indicated that catalysis by the oxidoreductase occurred only in the presence of sulfate or selenate, an anion specificity identical to that of whole cells. (ii) Saturation with both sulfatoiron(II) and the catalyst produced a concentration-independent rate constant of 3 s^{-1} for the reduction of the rusticyanin, which is an electron transfer reaction sufficiently rapid to account for the flux of electrons through the iron respiratory chain. (iii) Values for the enzyme-catalyzed pseudo-first-order rate constants for the reduction of the rusticyanin showed a hyperbolic dependence on the concentration of sulfatoiron(II) with a half-maximal effect at 300 μM, a value similar to the apparent K_M for iron shown by whole cells. On the basis of these favorable comparisons between the behavior patterns of isolated biomolecules and those of whole cells, this iron:rusticyanin oxidoreductase is postulated to be the primary cellular oxidant of ferrous ions in the iron respiratory electron transport chain of *T. ferrooxidans*.

Thiobacillus ferrooxidans is the most extensively characterized member of a group of chemolithotrophic bacteria that inhabit ore-bearing geological formations exposed to the atmosphere and that obtain all of their energy for growth from the dissolution and oxidation of minerals within the ore. A principal activity of this Gram-negative, obligately acidophilic bacterium is autotrophic growth by aerobic respiration on soluble ferrous ions. Metabolic energy is derived from oxidative phosphorylation coupled to respiratory electron transfer (Ingledew, 1982). Electron transport components that have been implicated in the respiratory oxidation of Fe(II) include a blue copper protein called rusticyanin (Cobley & Haddock, 1975; Cox & Boxer, 1978), two or more *c*-type cytochromes (Ingledew & Cobley, 1980; Tikhonova et al., 1967; Vernon et al., 1960; Mansch & Sand, 1992), two *a*-type cytochromes, one or more iron–sulfur proteins (Fry et al., 1986; Fukumori et al., 1988), a porin (Kulpa et al., 1986), and a putative polynuclear Fe(III)–sulfate chelate around the outer cell wall of this Gram-negative bacterium (Ingledew, 1986).

Rusticyanin is an acid-stable, type I copper protein with a molecular mass of 16 551 Da (Ronk et al., 1991) that is found in abundance in the periplasmic space of *T. ferrooxidans*. This blue copper protein 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). 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 (Jedlicki et al., 1986). The standard reduction potential of the purified protein, 680 mV (Ingledew & Cobley, 1980; Blake et al., 1991), is compatible with a role in a respiratory chain where the physiological electron donor, sulfatoiron(II), has a potential no lower than 650 mV. Although these observations support 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.

The present paper describes the discovery, partial purification, and relevant kinetic properties of an acid-stable iron:rusticyanin oxidoreductase from cell-free extracts of *T. ferrooxidans* ATCC 23270. Three important kinetic properties of the enzyme-catalyzed, Fe(II)-dependent reduction of the rusticyanin are described: (i) the reaction was sufficiently rapid to be of physiological significance; (ii) the rate of the reaction exhibited a hyperbolic concentration dependence that was similar to that observed with the intact bacterium; and (iii) the reaction exhibited an anion specificity identical to that exhibited by the intact organism for the Fe(II)-dependent reduction of molecular oxygen. The probable participation and role of this oxidoreductase in the iron respiratory electron transport chain of *T. ferrooxidans* are discussed.

EXPERIMENTAL PROCEDURES

Stopped-Flow Measurements. Rapid-scan kinetic measurements were performed on a stopped-flow spectrophotometer that consisted of an absorbance mixing chamber equipped with an OLIS-RSM data acquisition and analysis system (On Line Instrument Systems, Inc., Bogart, GA). The optical system of this dual-beam apparatus consisted of a xenon source powered by an OLIS XL75 adjustable power supply and a unique form of subtractive double monochromator capable of achieving 1000 spectral scans/s. Interchangeable grating monochromators (400 lines blazed at 550 nm) permitted the acquisition of repeated kinetic scans of the contents of the observation cell (1.7-cm path length) over a 230-nm span in the visible region. The signals from the sample and reference phototubes were amplified and fed to a Gateway 2000 (North Sioux City, SD) 486DX2-66V computer that

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permitted the collection and analysis of absorbance data as a function of both time and wavelength. The samples of cell-free extract and ferrous ions were prepared in identical solutions of 0.01 N sulfuric acid and added to separate syringes of the stopped-flow spectrophotometer. 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 0.14 mL of the solutions from each driving syringe. An operational bandwidth of 1.0 nm provided acceptable signal to noise characteristics between 400 and 700 nm.

Kinetic measurements at a single wavelength were performed on the stopped-flow spectrophotometer described previously (Blake & Shute, 1987). The reactants to be rapidly mixed 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 solutions from each driving syringe. Spectral changes were linear to an absorbance of 1.8. The changes in the oxidation state of the blue protein 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.

Assay of Iron:Rusticyanin Oxidoreductase. The routine assay was performed in the stopped-flow spectrophotometer at 597 nm and quantified the ability of the preparation to stimulate the Fe(II)-dependent reduction of the rusticyanin. Standard reaction conditions after mixing were 15 μM purified rusticyanin and 5.0 mM Fe(II) in 0.2 M sulfate, pH 2.0, at 25 °C. Purified rusticyanin was introduced into one syringe of the stopped-flow spectrophotometer. A solution containing the Fe(II) and the sample to be analyzed was introduced into the other syringe. Control experiments indicated that the sample could be introduced into either of the two solutions; the order of mixing did not affect the outcome of the experiment. Under these experimental conditions, the uncatalyzed iron-dependent reduction of the rusticyanin occurred with an apparent pseudo-first-order rate constant of 1.0×10^{-2} s^{-1}. One unit of iron:rusticyanin oxidoreductase was arbitrarily defined as the amount of catalyst that would double the apparent pseudo-first-order rate constant for the Fe(II)-dependent reduction of the rusticyanin under the above experimental conditions.

Purification of Iron:Rusticyanin Oxidoreductase. T. ferrooxidans ATCC 23270 was grown on a large scale by batch culture at ambient temperatures in an acidic ferrous sulfate growth medium (Tuovinen & Kelly, 1973) supplemented with 1.6 mM cupric sulfate (Blake & Shute, 1987). Bacterial cells at stationary phase were concentrated by tangential filtration in a Millipore Pellicon and harvested by centrifugation of the retentate in a Sorvall Refrigerated Superspeed centrifuge at 10000*g* for 15 min. Typical yields of this obligate autotroph ranged from 50 to 200 mg of wet cell paste/L. Cells were routinely washed three times with 0.001 N sulfuric acid and subsequently frozen in 0.001 N sulfuric acid (1:4, g of wet cell paste:mL) and stored at −20 °C.

All protein purification activities were conducted at 4 °C. Frozen cells were thawed, and the cell suspension was subjected to sonic oscillation for 1 min/g of wet weight cells at a power output of 125 W using an ultrasonic processor manufactured by Heat System-Ultrasonics, Inc. Care was taken to maintain the temperature of the solution below 7 °C. Centrifugation of the sonicate at 20000*g* for 120 min yielded a dark tan pellet and a cloudy blue supernatant. The supernatant, which was designated as the crude cell-free extract, was then subjected to ammonium sulfate precipitation. The bulk of the iron:rusticyanin oxidoreductase activity precipitated between 30% and 45% saturated ammonium sulfate. A red pellet was obtained by centrifugation of the 45% saturated solution for 20 min at 20000*g*. The pellet was suspended in and dialyzed against 0.001 N sulfuric acid. The cloudy, suspended preparation was applied to a column of SP-Sephadex C-25 equilibrated and developed with 0.001 N sulfuric acid. All of the enzymatic activity eluted from the column in the void volume.

The column eluate was then collected and made 1% (w/v) in Zwittergent 3-12 by the addition of the solid detergent. Dissolution of the detergent greatly clarified the cloudy solution and stimulated the catalytic activity of the preparation. The detergent-containing preparation was then reapplied to a column of SP-Sephadex C-25 equilibrated with 0.001 N sulfuric acid containing 1% (w/v) Zwittergent 3-12. No iron:rusticyanin oxidoreductase activity was eluted in the void volume. After the column was washed with approximately two column volumes of the sulfate–Zwittergent buffer, about 10% of the iron:rusticyanin oxidoreductase activity eluted when the resin was washed with the sulfate–Zwittergent buffer containing 300 mM NaCl. The specific activity of this partially purified preparation was around 14 units/mg of protein, representing a 10-fold increase over that of the material in the 30–45% ammonium sulfate pellet. Although further column development with sulfate–Zwittergent buffer containing 400 and 500 mM NaCl eluted another 20% of the enzyme activity initially applied to the resin, these latter fractions contained much more contaminating protein than that eluted with the lower salt concentration. The iron:rusticyanin oxidoreductase eluted with 300 mM NaCl was therefore the preparation used in all of the kinetic studies detailed in this paper. The dilute iron:rusticyanin oxidoreductase was concentrated to approximately 30 mg of protein/mL by ultrafiltration through an Amicon Centriprep-30 membrane concentrator (significant losses of both protein and enzyme activity were suffered using the Amicon PM membrane series) and stored at 4 °C in 0.001 N sulfuric acid containing 1% Zwittergent.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions was routinely performed on a Pharmacia PhastSystem using PhastGel gradient 10-15 polyacrylamide gels and PhastGel SDS buffer strips. Protein was quantified using the copper–bicinchoninic acid assay (Smith et al., 1985).

Purification of Rusticyanin. The rusticyanin substrate for the assay was purified to electrophoretic homogeneity from cell-free extracts of *T. ferrooxidans* using a slight modification (Blake & Shute, 1987) of a previously published procedure (Cox & Boxer, 1978). Rusticyanin was isolated with its copper center in the oxidized state. The oxidized, purified rusticyanin was 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, the rusticyanin was reduced by reacting it with an excess of sodium dithionite. The reduced protein was then dialyzed at 4 °C against 0.001 N sulfuric acid to remove excess reducing agent. Reduced rusticyanin was remarkably stable to air oxidation. Samples of the reduced protein were stored in 0.001 N sulfuric

acid for up to 3 months at 4 °C before air-oxidized rusticyanin was detected.

Purification of Soluble Cytochrome c_{552}. Soluble cytochrome c_{552} was obtained as a byproduct during the purification of the rusticyanin (Blake et al., 1993b). Following elution of the purified rusticyanin from a column of CM-Sephadex C-25 with 0.01 M sodium acetate, pH 5.5, containing 200 mM NaCl, the reddish-brown cytochrome that remained bound to the ion exchange column was then eluted with the same acetate buffer amended with 300 mM NaCl. This eluate comprised the highly purified cytochrome c_{552}. A trace of reddish-brown cytochrome remained bound to the resin material.

Steady State Absorbance Measurements. Absorbance spectra were obtained on a Cary 14 dual-beam spectrophotometer rebuilt and modified by On Line Instrument Systems, Inc. (Bogart, GA). Instrument control and data analysis were accomplished *via* a Compac 386SX computer interfaced to the rebuilt spectrophotometer.

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. Citric, isocitric, oxalacetic, malic, succinic, fumeric, lactic, pyruvic, and α-ketoglutaric acids were obtained from Sigma Chemical Co. The Zwittergent detergents were obtained from Calbiochem. The BCA protein assay reagents were obtained from Pierce Chemical Co. All other chemicals were reagent grade.

RESULTS

The Fe(II)-dependent reduction of electrophoretically homogeneous rusticyanin was at least 2 orders of magnitude too slow to account for the facile iron-dependent reduction of oxygen in the intact organism (Blake & Shute, 1987). Yet the Fe(II)-dependent reduction of rusticyanin in crude cell-free extracts was rapid. Figure 1A shows a series of kinetic scans acquired when Fe(II) and a cell-free extract of *T. ferrooxidans* were mixed rapidly in a stopped-flow spectrophotometer equipped with a rapid-scan module. Cell-free extracts of *T. ferrooxidans* are characterized by the presence of conspicuous quantities of two types of colored redox-active biomolecules: rusticyanin and cytochrome *c* (Blake et al., 1993a,b). The oxidized forms of both colored species are evident in the initial kinetic scans in Figure 1A. The absorbance due to oxidized rusticyanin bleached rapidly when the crude extract was mixed with Fe(II). Figure 1B shows the spectra calculated from a multiwavelength global fit of the data set in Figure 1A to a single-exponential function of time:

$$\text{absorbance} = Ae^{-kt} + B$$

where A (the dashed line in the figure) represents the absorbance of the species that changed with time, B (solid line) represents the absorbance at infinite time, and k is the pseudo-first-order rate constant for the absorbance change. The spectrum at the end of the reaction was that of a reduced *c*-type cyochrome, with maxima at 424, 523, and 550 nm. The spectrum that changed with time is compared to that of oxidized, purified rusticyanin in Figure 1C. The similarities between the two spectra were sufficient to conclude that rusticyanin was the principal colored species that was electrochemically reduced when the cell-free extract was exposed to Fe(II).

Figure 1D shows a comparison of the time course for the Fe(II)-dependent reduction of rusticyanin in the cell-free extract with that of purified rusticyanin. Data for the former kinetic trace were extracted from the data set in Figure 1A, while those for the latter were obtained on a stopped-flow spectrophotometer operated with standard monochromatic light. The pseudo-first-order rate constant for the reduction of the rusticyanin in the crude extract was 2.1 s^{-1}, which is some 900-fold faster than that for the purified protein under identical solution conditions. Additional kinetic experiments showed that the rate of the Fe(II)-dependent reduction of purified rusticyanin was a linear function of the amount of cell-free extract present (data not shown). On the basis of these observations, a standard protocol for the assay of an iron:rusticyanin oxidoreductase was devised. The routine assay was performed in the stopped-flow spectrophotometer and quantified the ability of the preparation to catalyze the Fe(II)-dependent reduction of the rusticyanin.

Efforts to fractionate and purify the cellular component(s) responsible for stimulating the Fe(II)-dependent reduction of the purified rusticyanin by standard biochemical separation techniques were severely hampered by both the lability of the activity and the strong tendency of the proteins in the preparation to form large aggregates. Although the catalytic activity of the crude cell-free preparation was reasonably stable at pH 2.0 (<10% loss/week at 4 °C), even brief exposures to pH values greater than 3.5 caused extensive irreversible losses in iron:rusticyanin oxidoreductase activity. For all practical purposes, the inability to venture beyond pH 3.5 limited the application of powerful ion exchange techniques to chromatography on SP-Sephadex. Even more importantly, the strong tendency toward aggregation and the intractable behavior of the aggregates prevented the application of other purification techniques. Thus, the aggregates, with their associated enzymatic activity, were eluted in the void volume when column chromatography was attempted under strongly acidic conditions on SP-Sephadex, phenyl- or octyl-Sepharose, aminoalkyl- or alkyl-agarose, immobilized dye ligands of various structures, hydroxylapatite, immobilized iminodiacetic acid complexed with Fe(III), Cu(II), Ni(II), or Co(II), Sephadex G-200 *p*-(hydroxymercuri)benzoate-agarose, and thiopropyl-agarose. The narrow acidic pH range and the aggregation problem also limited and thwarted attempts to purify the protein by nondenaturing gel electrophoresis. Finally, efforts to covalently couple rusticyanin to an insoluble support to provide for an affinity-based separation led to the base-dependent denaturation of the blue protein.

Some purification of the aggregate-associated enzymatic activity was achieved by column chromatography on SP-Sephadex after the aggregate had been partially dissociated by treatment with Zwittergent 3-12. A sodium dodecyl sulfate–polyacrylamide gel electrophoresis pattern of the partially purified iron:rusticyanin oxidoreductase is shown in Figure 2. A major band was noted at 19 000 Da, along with several less intense bands elsewhere in the gel. Other ionic and nonionic detergents (other Zwittergent detergents with different alkyl chain lengths, Triton X-100, CHAPSO, dioctyl sulfosuccinate, alkyl β-D-glucopyranoside detergents with different alkyl chain lengths, etc.) were either less effective than Zwittergent 3-12 or had no discernible effect on the physical properties of the aggregates whatsoever. The addition of the Zwittergent produced a higher specific activity in the preparation, perhaps indicating that partial disaggregation of the proteins served to make the oxidoreductase more accessible to its water-soluble substrates. An equivalent concentration

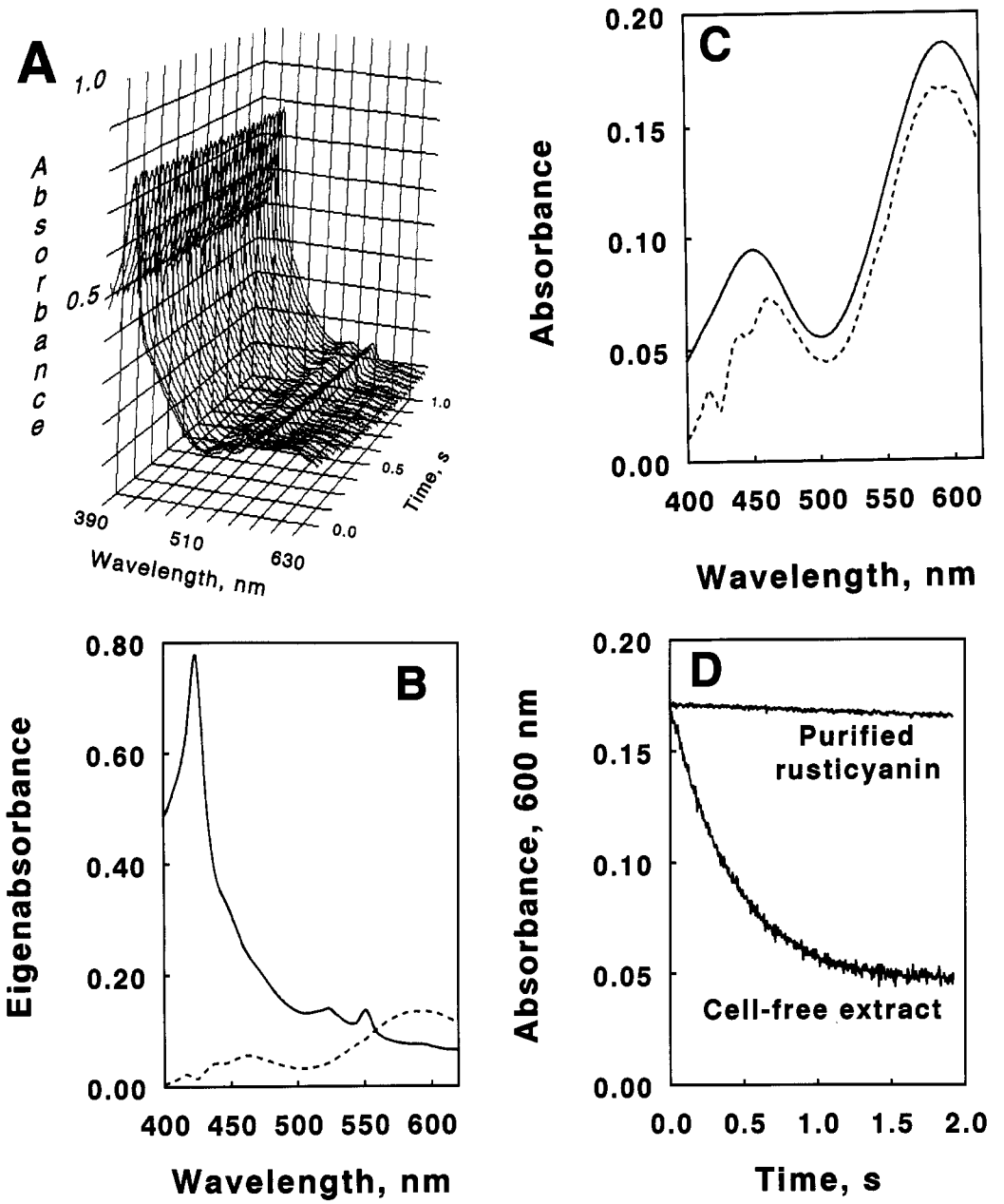

FIGURE 1: Demonstration of rapid reduction of rusticyanin by Fe(II) in cell-free extracts of *T. ferrooxidans*. (A) Kinetic scans from 400 to 630 nm when a cell-free extract of *T. ferrooxidans* was mixed rapidly with soluble ferrous ions. The reaction was monitored at 25 °C in a dual-beam stopped-flow spectrophotometer equipped with a rapid-scan module. A 230-nm scan was taken every millisecond for 2 s; every 20th scan is presented. The final concentrations in the observation cell after mixing were as follows: cell-free extract, 0.3 mg of protein/mL; ferrous ion, 1.0 mM; sulfuric acid, 0.01 N. The reference solution contained all components except the cell-free extract. (B) Eigenspectra of the principal light-absorbing species present in the cell-free extract of *T. ferrooxidans*. Spectra were computed from a global fit of the data set represented in panel A to the equation, absorbance = $Ae^{-kt} + B$, assuming the existence of two major species. The spectrum represented by the dashed line was completely bleached over the time course of the observation. The spectrum represented by the solid line was that remaining after the absorbance changes were complete. (C) Comparison of the absorbance spectrum of purified rusticyanin (solid line) with that of the computed species that bleached when ferrous ions were mixed rapidly with the cell-free extract from *T. ferrooxidans* (dashed line). The absorbance spectrum of 1.4 mg/mL oxidized rusticyanin was determined in 0.01 N sulfuric acid at 25 °C. (D) Comparison of the Fe(II)-dependent reduction of purified rusticyanin with that of the rusticyanin present in cell-free extracts of *T. ferrooxidans*. The time course of the reduction of purified rusticyanin was monitored at 600 nm in the stopped-flow spectrophotometer described previously (Blake & Shute, 1987). The concentrations after mixing were as follows: rusticyanin, 46 μM; ferrous ions, 1.0 mM; sulfuric acid, 0.01 N. The temperature was 25 °C. The time course of the reduction of the rusticyanin present in cell-free extracts of *T. ferrooxidans* was extracted from the set of kinetic data represented in A.

of Zwittergent alone had no discernible effect on the Fe(II)-dependent reduction of the rusticyanin (data not shown). The detergent-solubilized, partially purified enzyme activity did not bind to any of the chromatography materials listed earlier other than SP-Sephadex, nor did exposure to those materials achieve any further purification. The considerable effort that was expended to further purify this enzyme activity was justified on the basis of the interesting kinetic properties of the catalyst. This detergent-containing, partially purified preparation of iron:rusticyanin oxidoreductase was subsequently utilized in all of the kinetic experiments described in this paper.

Cytochrome c. Absorbance spectra of the partially purified iron:rusticyanin oxidoreductase are shown in Figure 3. The only conspicuous component in the visible region was a *c*-type cytochrome with a Soret peak at 414 nm in the oxidized state

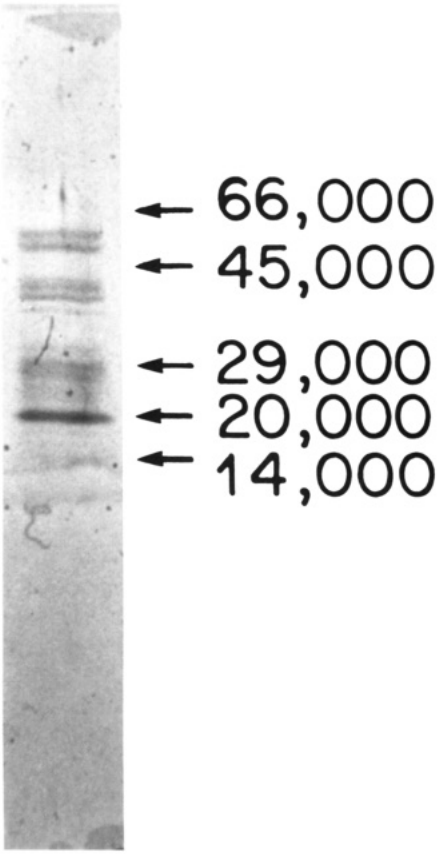

FIGURE 2: SDS–polyacrylamide gel electrophoresis analysis of the partially purified iron:rusticyanin oxidoreductase. Electrophoresis under reducing conditions was performed as described in the Experimental Procedures. Protein bands were visualized by staining with Coomassie Blue. The molecular mass standards (from high to low) were bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lactalbumin.

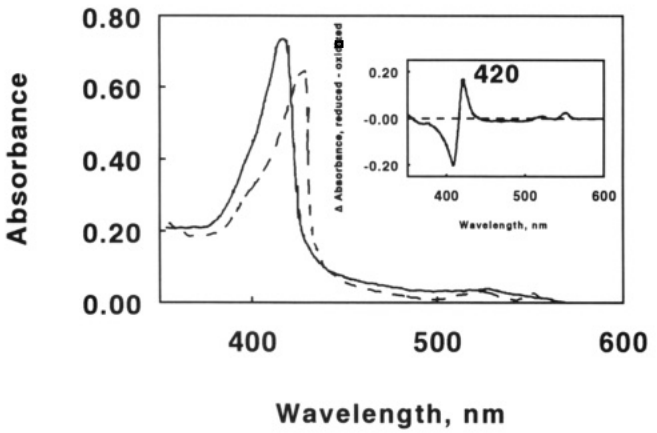

FIGURE 3: Absorbance spectra of oxidized (solid line) and reduced (dashed line) cytochrome *c*. Both spectra were determined in 0.01 N sulfuric acid at 25 °C. The absorbance spectrum of reduced cytochrome *c* was determined 10 min after mixing the sample of oxidized cytochrome *c* with an excess of ferrous pyrophosphate. Inset: A difference spectrum representing the absolute spectrum of the reduced cytochrome *c* minus that of the oxidized cytochrome.

and peaks at 419, 525, and 550 nm in the Fe(II)-reduced form. The inset in Figure 3 shows that the maximum of the difference spectrum between the reduced minus the oxidized cytochromes was at 420 nm.

Observations of the redox state of the cytochrome *c* during the Fe(II)-dependent reduction of rusticyanin were consistent with the cytochrome as a mediator of electron flow. Curve a in Figure 4 shows the time course of the complex absorbance

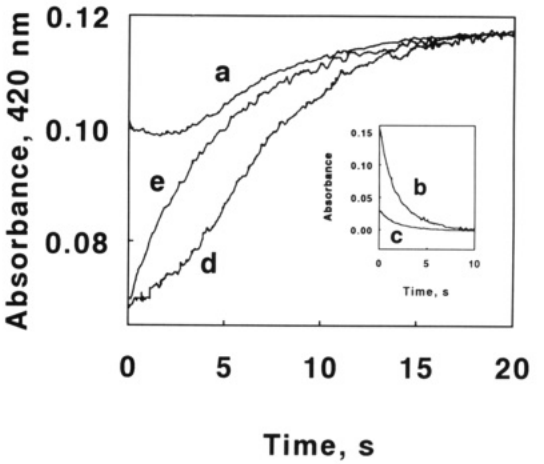

FIGURE 4: Time course of the absorbance changes at 420 and 597 nm when oxidized cytochrome *c* was mixed with ferrous sulfate in the presence and absence of oxidized rusticyanin. The concentrations after mixing were as follows: cytochrome *c*, 1.9 μM; ferrous ion, 25 mM; sulfate, 0.2 M; rusticyanin (when present), 38 μM. The pH was 2.0. (a) Absorbance at 420 nm due to the reduction of both rusticyanin and cytochrome *c*; (b) absorbance at 597 nm due to the reduction of rusticyanin; (c) calculated absorbance change at 420 nm solely due to the reduction of rusticyanin; (d) absorbance at 420 nm solely due to reduction of cytochrome *c* in the presence of rusticyanin (representing kinetic trace a minus that of c); (e) absorbance change at 420 nm due to the reduction of cytochrome *c* in the absence of rusticyanin.

changes at 420 nm when the cytochrome was mixed with ferrous sulfate in the presence of an excess of rusticyanin. Redox-dependent absorbance changes of both the cytochrome and the excess rusticyanin contributed to the overall absorbance change observed at 420 nm. Curve b in the inset shows the time course of the reduction of rusticyanin at 597 nm, a wavelength where cytochrome *c* exhibited a negligible redox-dependent absorbance change. Curve c represents the calculated contribution of reduction of the rusticyanin to the absorbance changes at 420 nm. Curve d, the time course of the increase in absorbance at 420 nm solely due to the cytochrome in the presence of rusticyanin, was obtained by subtracting curve c from curve a. Curve e shows the time course of the absorbance increase at 420 nm due to the iron-dependent reduction of the cytochrome in the absence of rusticyanin. The Fe(II)-dependent reduction of the cytochrome thus showed a pronounced lag in the presence of rusticyanin not observed in its absence. The existence and duration of this rusticyanin-dependent lag were consistent with the hypothesis that cytochrome *c* acted to transfer electrons from Fe(II) to rusticyanin. When an excess of oxidized rusticyanin was mixed with the reduced cytochrome, the cytochrome was oxidized rapidly (data not shown).

An acid-soluble, acid-stable cytochrome c_{552} was readily purified from cell-free extracts of *T. ferrooxidans*. Unlike the highly aggregated cytochrome described above, this soluble cytochrome bound tightly to cation exchange resins in the absence of Zwittergent. The molecular weight of the electrophoretically homogeneous cytochrome was estimated to be around 15 000 Da by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). The purified cytochrome exhibited a Soret peak at 411 nm in the oxidized state and peaks at 417, 523, and 552 nm in the dithionite-reduced form. Electron transfer between the purified cytochrome and rusticyanin was not observed. Further, all efforts to prepare a cell-free extract that served to catalyze the Fe(II)-dependent reduction of this cytochrome c_{552} were unsuccessful.

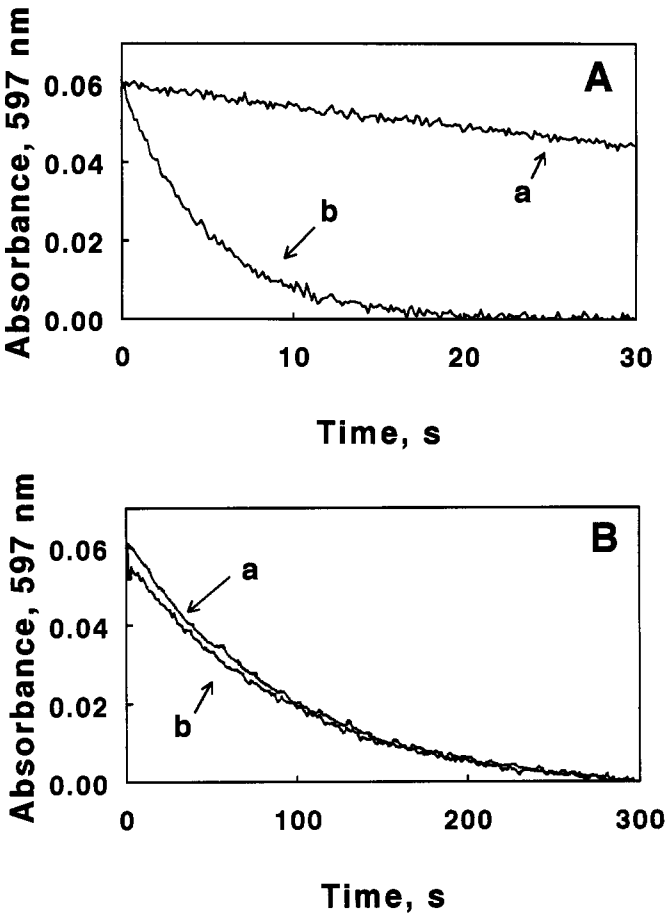

FIGURE 5: Representative kinetic traces illustrating the effects of anions on the efficacy of the iron:rusticyanin oxidoreductase. (A) Time course of the absorbance changes at 597 nm when oxidized rusticyanin was mixed with acidic ferrous selenate in the absence (a) or presence (b) of the enzyme. Final concentrations after mixing were as follows: rusticyanin, 15 μM; ferrous ions, 50 mM; selenate, 0.5 M; enzyme, when present, 5.0 units/mL. (B) Time course of the absorbance changes at 597 nm when oxidized rusticyanin was mixed with acidic ferrous perchlorate in the absence (a) or presence (b) of the enzyme. Final concentrations after mixing were as follows: rusticyanin, 15 μM; ferrous ions, 50 mM; perchlorate, 1.0 M; enzyme, when present, 5.0 units/mL.

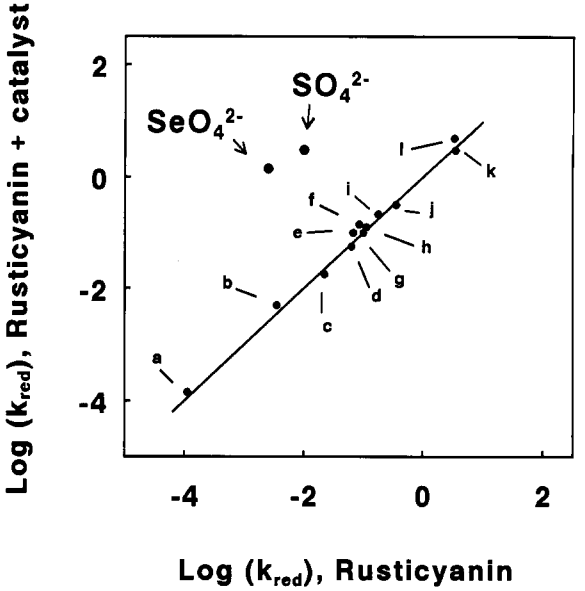

FIGURE 6: Anion specificity of the iron:rusticyanin oxidoreductase. The pseudo-first-order rate constants for the Fe(II)-dependent reduction of the rusticyanin in the presence of the iron:rusticyanin oxidoreductase are plotted as a function of the corresponding rate constants in the absence of enzyme for diverse anions. Final concentrations after mixing in all experiments were as follows: rusticyanin, 15 μM; ferrous ions, 50 mM; iron:rusticyanin oxidoreductase, 5.0 units/mL. Anions: a, perchlorate, b, bromide; c, α-ketoglutarate; d, pyruvate; e, isocitrate; f, malate; q, phosphate; h, chloride; i, lactate; j, pyrophosphate; k, citrate; l, oxalacetate.

Anion Specificity. A prominent but poorly understood feature of autotrophic iron oxidation by *T. ferrooxidans* is its requirement for sulfate ions. Sulfate is the principal anion in the bacterium's natural environment. Laboratory experiments with numerous nonphysiological inorganic and organic anions revealed that only selenate could substitute for sulfate in supporting Fe(II) oxidation by resting cell suspensions (Lazaroff, 1977). A specific requirement for sulfate or selenate was also reported for the iron-dependent reduction of oxygen catalyzed by cell-free electron transport particles (Ingledew, 1982). It was of interest to investigate whether a similar anion-dependent behavior pattern could be discerned in the electron transfer reaction catalyzed by the iron:rusticyanin oxidoreductase.

The object of these experiments was to obtain kinetic data on the enzyme-catalyzed, one-electron reduction of purified rusticyanin by Fe(II) in the presence of different anions. Figure 5 shows representative kinetic traces that illustrate the experiments performed to determine the anion specificity of the catalyzed reaction. Figure 5A shows the loss in absorbance at 597 nm as a function of time when the rusticyanin was mixed with Fe(II) in the absence (experiment a) or presence (experiment b) of the iron:rusticyanin oxidoreductase, with selenate as the principal anion present. The conclusion drawn from the data in Figure 5A was the same as that drawn from the data in Figure 1D where sulfate was the principal anion present: the enzyme catalyzed the Fe(II)-dependent reduction of rusticyanin. Figure 5B shows the loss in absorbance as a function of time when the rusticyanin was mixed with Fe(II) in the absence (experiment a) or presence (experiment b) of the iron:rusticyanin oxidoreductase, with perchlorate as the principal anion present. The two experimental traces were superimposable, indicating that the enzyme did not catalyze the Fe(II)-dependent reduction of rusticyanin in the presence of perchlorate.

Experiments such as those illustrated in Figure 5 were performed with each of 14 different anions, and the results are summarized in Figure 6. The anions utilized in this study ranged from those that coordinate weakly with soluble iron (perchlorate, chloride) to those that coordinate very tightly (citrate, oxalacetate). With the exception of sulfate, none of these anions are found at significant levels in the bacterium's natural habitat. In the absence of the catalyst, the values of the individual rate constants obtained in the presence of different anions varied over some 5 orders of magnitude. Previous studies with the same collection of anions showed that the reduction of rusticyanin was controlled largely by the inherent kinetic and thermodynamic reactivity of the anion-liganded iron reagent and was independent of any protein-mediated recognition or specificity (Blake et al., 1991). Thus, the rapid rate constant observed in the presence of a nonphysiological anion such as 2.0 M chloride, for example, was solely due to the electron transfer reactivity of the trichloroferrate(II) complex. Catalysis of electron transfer from Fe(II) to rusticyanin did not occur in the presence of 12 of the 14 anions surveyed. The iron:rusticyanin oxidoreductase only stimulated the electron transfer reaction in the presence of sulfate or selenate. This is precisely the anion specificity reported for whole cells of *T. ferrooxidans* for the overall

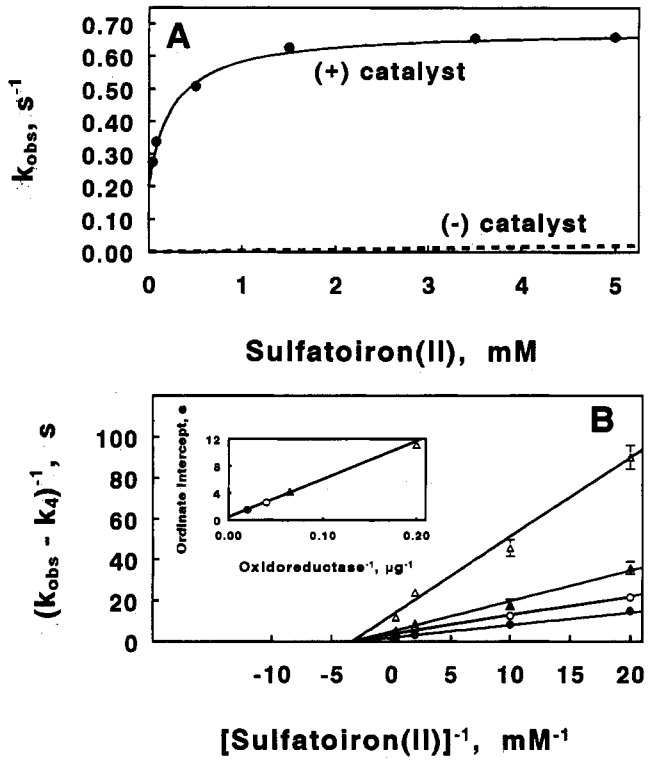

FIGURE 7: Kinetic properties of iron:rusticyanin oxidoreductase. (A) Dependence of the pseudo-first-order rate constant for the reduction of rusticyanin on the concentration of sulfatoiron(II) in the presence ((+) catalyst) and absence ((–) catalyst) of the iron:rusticyanin oxidoreductase. Final concentrations after mixing were as follows: rusticyanin, 15 μM; sulfate 0.2 M; iron:rusticyanin oxidoreductase, 25 units/mL. The curve drawn through the data points in the presence of the catalyst was determined by nonlinear regression analysis. (B) Secondary kinetic plots of the pseudo-first-order rate constants for the iron:rusticyanin oxidoreductase-catalyzed, sulfatoiron(II)-dependent reduction of the rusticyanin. Inset: The dependence of the ordinate intercepts of the secondary plots on the reciprocal of the enzyme concentration.

respiratory process, the Fe(II)-dependent reduction of molecular oxygen (Lazaroff, 1963; Schnaitman et al., 1969; Tuovinen et al., 1971).

Kinetic Properties. The kinetic properties of the iron:rusticyanin oxidoreductase were investigated to determine whether the enzyme-catalyzed reaction was sufficiently rapid to be of physiological significance. In the absence of a catalyst, the reduction of the rusticyanin by excess sulfatoiron(II) obeyed second-order kinetic behavior over a 100-fold range of sulfatoiron(II) concentrations (Blake & Shute, 1987). This second-order kinetic behavior is represented by the (–) catalyst curve in Figure 7A. The curve labeled (+) catalyst in Figure 7A shows the corresponding dependence of the iron:rusticyanin oxidoreductase-catalyzed reduction of the rusticyanin upon the concentration of excess sulfatoiron(II). The value of k_{obs} was a rectangular hyperbolic function of the sulfatoiron(II) concentration, with a positive ordinate intercept. The latter kinetic behavior is consistent with at least two kinetic mechanisms (Strickland et al., 1975):

$$c(\mathrm{III})\mathrm{RCu(II)} + \mathrm{FeSO_4} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} c(\mathrm{II})\mathrm{RCu(II)FeSO_4}^+ \underset{k_4}{\overset{k_3}{\rightleftharpoons}} c(\mathrm{III})\mathrm{RCu(I)FeSO_4}^+ \quad (1)$$

or

$$c(\mathrm{III}) + \mathrm{FeSO_4} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} c(\mathrm{II}) + \mathrm{FeSO_4}^+$$

$$c(\mathrm{II}) + \mathrm{RCu(II)} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} c(\mathrm{III}) + \mathrm{RCu(I)} \quad (2)$$

where c and RCu represent cytochrome c and rusticyanin, respectively. In either event, application of the steady state assumption to the reduced cytochrome gives the following expression for k_{obs} for the reduction of rusticyanin as a function of the soluble iron concentration and the individual rate constants:

$$k_{obs} = k_1k_3[\mathrm{FeSO_4}]/(k_1[\mathrm{FeSO_4}] + k_2) + k_4 \quad (3)$$

A value of $k_4 = 0.2\ s^{-1}$ was thus obtained from the ordinate intercept of the (+) catalyst curve in Figure 7A. With this value of k_4, a double-reciprocal plot of $(k_{obs} - k_4)^{-1}$ *versus* $[\mathrm{Fe(II)}]^{-1}$ generated a linear plot, from which values of $k_3 = 0.46\ s^{-1}$ and $k_2/k_1 = 300\ \mu$M were calculated.

Figure 7B shows double-reciprocal plots with different fixed concentrations of the iron:rusticyanin oxidoreductase. Extrapolation of the enzyme concentration to infinitely high values, as illustrated in the inset of Figure 7B, demonstrated that (i) the value of the pseudo-first-order rate constant for the Fe(II)-dependent, enzyme-catalyzed reduction of rusticyanin was saturable with regard to the enzyme, and (ii) the concentration-independent limiting first-order rate constant approached 3 s^{-1}. The former observation lent support to the kinetic mechanisms represented schematically in eqs 1 and 2, where the maximum rates of rusticyanin reduction must be saturable with the catalyst, as well as with sulfatoiron(II). The latter observation addressed the kinetic competency of the reaction under consideration to serve in the overall process of respiration on iron in the intact organism.

Ferrous ion-dependent oxygen consumption is very rapid in intact cells of *T. ferrooxidans*. Kinetic studies on intact cells in acidic ferrous sulfate yielded maximal rates of oxygen consumption ranging from 0.4 to 2.0 mL of molecular oxygen/h/mg of total cellular protein (Kulpa et al., 1986; Schnaitman et al., 1969; Lacey & Lawson, 1970; Bodo & Lundgren, 1974). If 5% of the total cellular protein is assumed to be rusticyanin, then the range of apparent turnover numbers for the transfer of electrons from Fe(II) to molecular oxygen by whole cells must be between 2 and 10 s^{-1} (Blake & Shute, 1987). In order for the electron transfer reaction studied here to be physiologically significant in the iron-dependent respiratory chain, the apparent rate constant for the Fe(II)-dependent, iron:rusticyanin oxidoreductase-catalyzed reduction of rusticyanin must be greater than or equal to the overall turnover number for the entire process, 2–10 s^{-1}. The data in Figure 7B indicate that such is the case at high concentrations of the oxidoreductase, concentrations of this acid-stable activity that might well be achieved in the microenvironment of the periplasmic space of this bacterium. Although such numerical comparisons are subject to unavoidable ambiguities and errors, they are nonetheless a necessary prerequisite to establishing the kinetic competency of a given reaction to participate within the larger series of reactions that constitutes the biological function.

Each plot in Figure 7B crossed the abscissa at the same point, indicating a half-maximal effect at around 300 μM sulfatoiron(II). Values for the apparent K_M for Fe(II) in whole cells range from 1.0 to 5.0 mM in the various acidic sulfate media employed. If one adjusts the values of each of

these latter kinetic constants to include the weak binding interaction between soluble Fe(II) and sulfate (McAndrew et al., 1975), then the apparent mean value for the K_M for sulfatoiron(II) exhibited by whole cells may be estimated to be around 250 μM.

DISCUSSION

A basic tenet in studies such as these is that the physicochemical basis of biological specificity ought to be exhibited in its utmost simplicity in the interactions of proteins with small ligands. The demonstration that the enzyme-catalyzed transfer of an electron from Fe(II) to rusticyanin exhibited an anion specificity identical to that of whole cells actively respiring on iron is a strong indication that the reaction in question is an integral component of the overall process. In poorly studied organisms such as *T. ferrooxidans*, where useful molecular biological and genetic tools are just beginning to emerge (Kusano et al., 1992a; Rawlings & Kusano, 1993), such favorable comparisons between the behavior of isolated biomolecules and that of intact organisms is a principal means of identifying reactions of probable physiological significance. Additional evidence that the reaction studied here is relevant to biological function was supplied by the observation that the kinetic properties of the oxidoreductase-catalyzed reaction (the values of the K_M for Fe(II) and the concentration-independent limiting first-order rate constant for electron transfer) mirrored those of the whole cell-dependent process of respiration on iron.

Our working hypothesis for the iron respiratory chain of *T. ferrooxidans* is summarized by the scheme represented in Figure 8A. The cytochrome *c*-containing iron:rusticyanin oxidoreductase described here is chosen as the primary initial electron acceptor from soluble Fe(II). The observed acid stability places this activity outside the plasma membrane in this obligately acidophilic organism. Electrons are then passed to the acid-stable rusticyanin and from there to molecular oxygen by an as yet uncharacterized series of reactions. In the absence of further investigations to identify the physiological oxidant for rusticyanin, the cellular mediator for the subsequent electron transfer from rusticyanin to molecular oxygen is depicted as a cytochrome *a*-containing terminal oxidase. It may be noted that the rate of electron transfer from rusticyanin to a purified cytochrome *a*-containing oxidase was determined elsewhere to be 38 s^{-1} (Yamanaka et al., 1991), a rate of sufficient rapidity to be of physiological significance.

The scheme represented in Figure 8B is adapted from Yamanaka et al. (1991). This hypothesis provides no significant role for rusticyanin. Instead, the electron transport chain from Fe(II) to molecular oxygen is seen to be composed of, in the order of electron flow, an iron–sulfur protein, an acid-soluble *c*-type cytochrome, and an *a*-type cytochrome oxidase. All three electron transfer components depicted in Figure 8B have been purified to electrophoretic homogeneity from cell-free extracts of *T. ferrooxidans* (Fukumori et al., 1988; Yamanaka et al., 1991; Sato et al., 1989). The gene for the iron–sulfur protein was cloned and the primary structure of the 6400-Da protein deduced from the DNA sequence of the open reading frame in the cloned DNA (Kusano et al., 1992b). Several anomalous features of the iron–sulfur protein-catalyzed, Fe(II)-dependent reduction of the soluble cytochrome *c* were noted. In particular, the ferrocytochrome *c* obtained upon the addition of iron–sulfur protein (purified and stored at pH 6.0) to a reaction mixture containing ferricytochrome *c* and Fe(II) (final pH 3–3.5) was observed to reoxidize rapidly, even in the presence of a large excess of

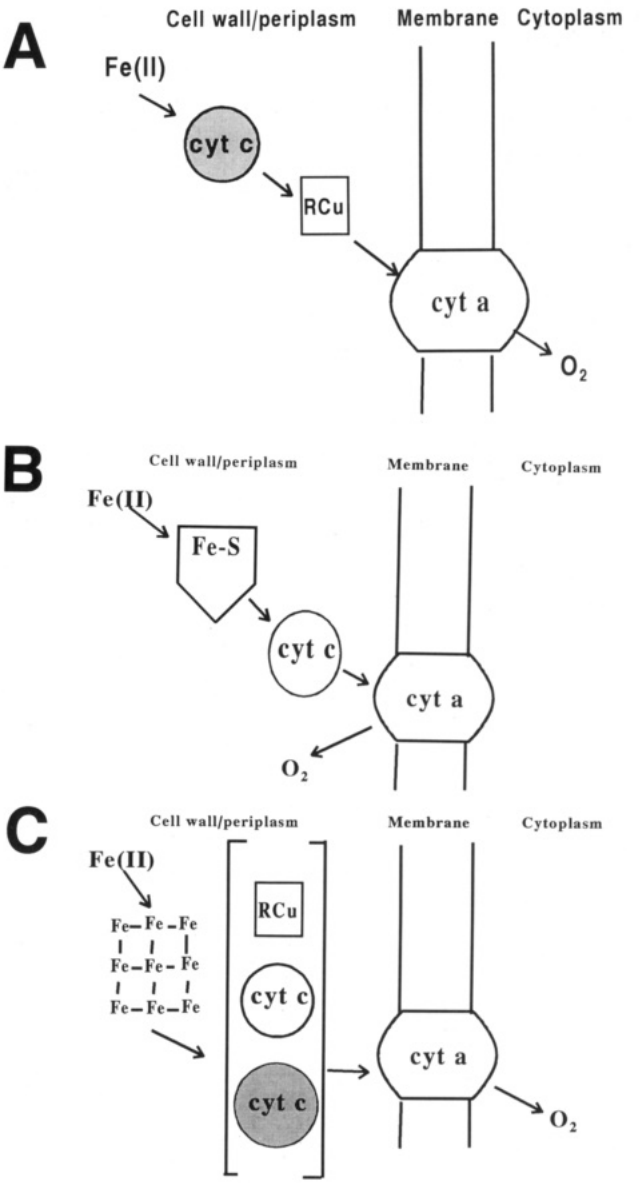

FIGURE 8: Three schemes that depict the aerobic iron respiratory chain of *Thiobacillus ferrooxidans*. Symbols: RCu, rusticyanin; unshaded cyt c, water-soluble *c*-type cytochrome, shaded cyt c, detergent-soluble *c*-type cytochrome; Fe–S, iron–sulfur protein; cyt a, *a*-type cytochrome; Fe, polynuclear iron coat.

Fe(II). Furthermore, when Fe(II) was added to the iron–sulfur protein preincubated with ferricytochrome *c* at pH 3.5, the enzyme-catalyzed reduction of the cytochrome was not observed. No other information regarding the pH stability of the iron–sulfur protein was given. As noted elsewhere (Ehrlich et al., 1991), one interpretation of these observations is that the iron–sulfur protein is actually a cytoplasmic protein that is rapidly destroyed at acidic pH and that a transient intermediate of the denatured protein triggers the autoxidation of Fe(II), with the concomitant reduction of the soluble cytochrome *c*.

The scheme represented in Figure 8C is adapted from Ingledew et al. (1977). Electrons derived from the oxidation of soluble Fe(II) are thought to be conducted through an extracellular polynuclear iron complex to an acceptor, either cytochrome *c* or rusticyanin, in the periplasmic space. The order of subsequent electron transfer among the soluble or membrane-associated periplasmic components is unresolved (Ingledew & Cobley, 1980) and is represented in Figure 8C by placing the possible redox-active components within

brackets. Both the rusticyanin and several species of cytochrome *c* (resolved by potentiometric and spectral studies) were shown to be periplasmic by electron paramagnetic probe techniques (Ingledew & Houston, 1986) and by the slow release of components following spheroplast formation. Oxygen reduction is depicted on the cytoplasmic face of the plasma membrane, since acidification of the cytoplasm with small proton-conducting organic acids led to the electrochemical reduction of all visible electron transfer components, including the *a*-type cytochromes, in cells exposed to Fe(II) (Alexander et al., 1987).

The different working hypotheses noted above may reflect investigator-dependent differences in data interpretation that arise in any hypothesis-driven investigation or they may reflect actual differences in the respective biological systems. Comparative studies have revealed large differences in rusticyanin expression in mesophilic iron-oxidizing bacteria that also express conspicuous quantities of *c*-type cytochromes (Blake & McGinness, 1993). Expression levels for rusticyanin range from 5% of the total soluble protein in *T. ferooxidans* ATCC 23270 to levels insufficient for detection in *T. prosperus*. Given such a diversity of rusticyanin expression in physiologically and morphologically similar bacteria (bacteria that, nonetheless, exhibit little or no homology in heterologous DNA hybridization experiments), it would not be surprising to learn that slightly different electron transfer mechanisms and pathways exist within these bacteria.

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