The Multicenter Aerobic Iron Respiratory Chain of
Acidithiobacillus ferrooxidans Functions as an Ensemble with
a Single Macroscopic Rate Constant*

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Background: The order of electron transfer in the respiratory chain of Acidithiobacillus ferrooxidans is unknown.
Results: The multicenter respiratory chain functioned as an ensemble with a single macroscopic rate constant.
Conclusion: The crowded respiratory proteins behaved as would a single protein with a common standard reduction potential of 650 mV.
Significance: In situ spectroscopy constitutes a new and effective means to study cellular respiration.

Electron transfer reactions among three prominent colored proteins in intact cells of Acidithiobacillus ferrooxidans were monitored using an integrating cavity absorption meter that permitted the acquisition of accurate absorbance data in suspensions of cells that scattered light. The concentrations of proteins in the periplasmic space were estimated to be 350 and 25 mg/ml for rusticyanin and cytochrome c, respectively; cytochrome a was present as one molecule for every 91 nm² in the cytoplasmic membrane. All three proteins were rapidly reduced to the same relative extent when suspensions of live bacteria were mixed with different concentrations of ferrous ions at pH 1.5. The subsequent molecular oxygen-dependent oxidation of the multicenter respiratory chain occurred with a single macroscopic rate constant, regardless of the proteins’ in vitro redox potentials or their putative positions in the aerobic iron respiratory chain. The crowded electron transport proteins in the periplasm of the organism constituted an electron conductive medium where the network of protein interactions functioned in a concerted fashion as a single ensemble with a standard reduction potential of 650 mV. The appearance of product ferric ions was correlated with the reduction levels of the periplasmic electron transfer proteins; the limiting first-order catalytic rate constant for aerobic respiration on iron was 7,400 s⁻¹. The ability to conduct direct spectrophotometric studies under noninvasive physiological conditions represents a new and powerful approach to examine the extent and rates of biological events in situ without disrupting the complexity of the live cellular environment.

Acidithiobacillus ferrooxidans is the most extensively characterized member of a group of chemolithotrophic bacteria that inhabit ore-bearing geological formations exposed to the atmosphere and obtain all of their energy for growth from the dissolution and oxidation of minerals within the ore. Energy is derived from oxidative phosphorylation coupled to aerobic respiratory electron transfer. A number of laboratories have described redox-active proteins expressed by At. ferrooxidans that might participate in the iron respiratory chain (1–13). Perhaps the most generally accepted model for the iron respiratory chain of At. ferrooxidans comes from a combination of classic reductionist studies (7, 10–14), bioinformatic analyses of genomic sequences (15–18), and proteomic and transcriptomic studies (19–23). The rux operon is hypothesized to encode all of the principal electron transport proteins that compose the iron respiratory chain (15). The primary oxidation of extracellular ferrous iron is thought to be accomplished by an outer membrane cytochrome c (7, 12) that passes the electrons to rusticyanin, a type I blue copper protein located in the periplasmic space of this Gram-negative organism (1, 24). These electrons are then transferred from the rusticyanin via a different periplasmic cytochrome c (14) to an aat₃-type terminal oxidase that spans the cytoplasmic membrane and transfers electrons to molecular oxygen (6, 13). A preparation of “respirasomes” that included all four proteins in a stable super-complex was obtained from cell-free extracts of At. ferrooxidans (25). This respirasome was shown to catalyze the iron-dependent reduction of molecular oxygen.

The colored prosthetic groups of most electron transport proteins include intrinsic spectrophotometric probes whereby transient changes in the oxidation-reduction state of the proteins may be monitored with great sensitivity. This laboratory exploited an integrating cavity absorption meter to study respiratory electron transfer reactions in situ in intact bacteria under physiological conditions (26). The observation chamber of this novel spectrophotometer included a reflecting cavity completely filled with the absorbing suspension. The premise was that accurate UV-visible spectroscopy of electron transfer reactions among colored proteins could be conducted in highly turbid suspensions if the live bacteria were irradiated in an isotropic homogeneous field of incident measuring light. Under those conditions, the absorbed radiant power should be independent of scattering effects (27–30). Equilibrium and kinetic studies

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were conducted on the Fe(II)-dependent reduction and O₂-dependent oxidation of cytochromes in intact Leptospirillum ferrooxidans at pH 1.7 (26). A cytochrome with a reduced spectral peak at 579 nm was shown to be an obligatory intermediate in the aerobic iron respiratory chain of L. ferrooxidans.

In this study, the same integrating cavity absorption meter was used to study aerobic respiration on soluble iron as catalyzed by intact At. ferrooxidans. First, the steady state kinetic parameters for the overall reaction were determined for whole cells under physiological solution conditions at pH 1.5. Second, the iron-dependent reductive half-reaction was exploited to identify and quantify the rusticyanin and cytochromes a and c that were reduced by extracellular iron. Third, the oxidative half-reaction was exploited to reveal that oxidation of the multicenter respiratory chain occurred with a single macroscopic rate constant. Finally, bacteria-monitored turnover studies were used to correlate changes in the redox levels of intracellular electron transport proteins with the appearance of product ferric ions. An unexpected outcome was the demonstration that, despite their different electrochemical potentials in vitro, the three principal types of redox-active proteins in the iron respiratory chain behaved as a single ensemble in situ.

Experimental Procedures

Cell Culture—At. ferrooxidans ATCC 23270 was cultured autotrophically on soluble ferrous ions at 30 °C in the medium described by Tuovinen and Kelly (31), adjusted to pH 1.5, and amended with 44 g/liter of FeSO₄·7H₂O and 0.4 g per liter of CuSO₄·5H₂O. Previous studies demonstrated that higher concentrations of holorusticyanin were produced by this strain when the medium was supplemented with additional soluble copper (2). All of the salts and acids used herein were reagent grade. Cells grown to stationary phase were harvested by centrifugation, washed four times with 0.02N H₂SO₄, and resuspended in 0.02N H₂SO₄ adjusted to pH 1.5. Absolute numbers of A. ferrooxidans cells were determined by electrical impedance measurements in a Multisizer 4 particle counter (Beckman Coulter, Inc., Brea, CA) fitted with a 30-μm aperture. The instrument was programmed to siphon 50 μl of sample that contained Isoton II as the electrolyte. The current applied across the aperture was 600 μA. Voltage pulses attendant with impedance changes as particles passed through the aperture were monitored with an instrument gain of four.

Relative numbers of A. ferrooxidans cells were determined by photon correlation scattering spectroscopy with a DelsaNano C particle size analyzer, also from Beckman Coulter, Inc. Cell densities were adjusted to ~2 × 10⁷ cells/ml in 0.02 N sulfuric acid to produce an optimum photon count/s of 8,000–10,000. Determination of the relative numbers of light scattering species as a function of particle diameter was accomplished by the time domain method with operating and analysis software provided by Beckman Coulter, Inc.

Absorbance Measurements with Cell Suspensions—Absorbance measurements on intact cells in suspension were conducted in an OLIS CLARiTY 1000A spectrophotometer (On Line Instrument Systems, Inc., Bogart, GA) that employed a novel integrating cavity absorption meter. The sample and reference observation cells of this dual beam spectrophotometer were each composed of a 9.3-ml spherical cavity fused with a 6-mm inner diameter quartz tube. Each quartz chamber was surrounded by a tightly packed proprietary white powder that served to maximize diffuse reflectance of light on the exterior walls of the spherical flask. The apertures in the reflecting sphere through which the measuring light entered and the transmitted/scattered light exited to the photomultiplier tube were positioned at a 90° angle such that the light had to undergo many reflections and cell transversals before it was quantified using the photomultiplier tube. A white Teflon plug with a 6-mm outer diameter was inserted into the quartz tube to minimize the loss of light out of the neck. A 1.0-cm white stir bar was included in the sample chamber to facilitate sample mixing and suspension of any particulate matter.

In a typical measurement, identical 8-ml solutions that contained ferrous sulfate in sulfuric acid, pH 1.5, were added to both the sample and reference observation cavities of the spectrophotometer. The contents of both observation cavities were maintained at 30 °C using resistors with variable duty cycles embedded in the structures of the observation cavities. A stable baseline from 365 to 555 nm was recorded when the focus of the observation was the production of ferric ions or the iron- and oxygen-dependent changes in the Soret region of heme proteins within the bacterium. A baseline from 470 to 660 nm was recorded when the focus was the iron- and oxygen-dependent changes in the longer wavelength regions of the colored electron transport proteins in the intact cells. In either case, a small volume was withdrawn from the sample cavity and replaced with an equal volume of suspended L. ferrooxidans to initiate subsequent reactions and absorbance changes within the cavity. Raw absorbance spectra, typically 6.2/s, were then collected until any visible absorbance changes had ceased.

Previous studies demonstrated that raw absorbance spectra obtained with integrating cavity absorption meters appeared distorted compared with the corresponding spectra of the same materials obtained using a conventional linear spectrophotometer (27–30). Unlike single path length spectrophotometers where the Beer-Lambert law governs nonlinearity in the measured light intensity as a function of analyte concentration, the measuring light in the CLARiTY makes multiple random passes with different path lengths that exacerbate the apparent nonlinearity with analyte concentrations. Raw absorbance values obtained in the CLARiTY were converted to equivalent absorbance values/cm using Fry’s method (32) with analysis software provided by OLIS, Inc. Global fits of absorbance changes as a function of both time and wavelength were accomplished by the singular value decomposition method (33) using analysis software also provided by OLIS, Inc. The output of applying the singular value decomposition method to each three-dimensional data set of absorbances as a function of time
and wavelength included three products as follows: a matrix of spectral eigenvectors that represented the changes in absorbance for the principal absorbing species; a matrix of kinetic eigenvectors that represented the mechanism(s) that accounted for the time dependences of the principal absorbance changes; and a diagonal matrix of eigenvalues that minimized the differences between the calculated and the observed data.

Results

Quantification of Bacteria—The data shown in Fig. 1 illustrate how the intact cells of \( \text{At. ferrooxidans} \) were quantified and characterized. Fig. 1A shows representative examples of the absolute number of cells as a function of cell size determined with different suspensions of \( \text{At. ferrooxidans} \) in the Multisizer 4. The Multisizer counted the number of intact bacteria suspended in an electrically conductive liquid by aspirating a measured volume of the suspension through a small aperture with an immersed electrode on either side. A current passing through the aperture between the two electrodes enabled the bacteria to be detected by the momentary changes in the electrical impedance as they passed through the aperture, because each bacterium displaced its own volume of electrolyte solution within the aperture. Narrow ranges of these small volumes were converted to mean spherical equivalents using analysis software provided by the manufacturer and represented by the corresponding spherical diameters on the abscissa of Fig. 1A.

The inset in Fig. 1A shows the dependence of the absolute number of cells on the volume of a diluted cell suspension added to the electrolyte solution in the Multisizer. The number of cells was not directly proportional to the volume of the cell suspension added to the electrolyte solution. Rather, the curve drawn through the data points in the inset of Fig. 1A was defined using nonlinear regression analyses as \( \frac{y}{H_1} = 100^{0.84}x^{1.27} \). Bacterial suspensions larger than 200 \( \mu l \) produced bacterial counts that exhibited even larger standard deviations than those shown in the inset of Fig. 1A. The origins of the unexpected behavior are unknown. Because of the uncertainties associated with larger volumes of the bacterial suspension, only those data derived from analyses of bacterial suspensions less than or equal to 100 \( \mu l \) were used to quantify the intact bacteria for the spectroscopic experiments described below.

Bacteria with spherical equivalent diameters less than 0.6 \( \mu m \) were under-represented by the electrical impedance measurements, by which observations were limited to particles with effective diameters between 2 and 60% of the 30-\( \mu m \) aperture employed. Conversely, light diffraction measurements were capable of resolving particles with effective diameters smaller than 0.1 nm. The solid curve in Fig. 1B shows the mean number of counts in each narrow size range for over 220 Multisizer assays of suspended \( \text{At. ferrooxidans} \). The dashed curve in Fig. 1B shows the relative number of \( \text{At. ferrooxidans} \) cells as a function of spherical equivalent diameters as determined by the laser light diffraction method. Close correspondence between the electrical impedance and the light diffraction curves was observed down to 0.6 \( \mu m \), indicating that the two instruments were monitoring the same population of particles. The area

![FIGURE 1. Quantification and characterization of intact At. ferrooxidans. A, electrical impedance measurements of the absolute number of bacteria as a function of cell diameter. Curves a, b, and c represent 50, 100, and 200 \( \mu l \), respectively, of a dilution of a stock suspension of bacteria. Inset, the number of cells as a function of the microliters of cell suspension. Each datum represents the mean and standard deviation of at least 20 determinations. B, comparison of cell suspensions by electrical impedance (solid curve, left ordinate) and static light diffraction (dashed curve, right ordinate) methods. The scale of each ordinate was chosen to facilitate comparison of the curves. The area under the electrical impedance curve was 66% that under the light diffraction curve. C, dependences of the volume and surface area of \( \text{At. ferrooxidans} \) as function of the number of cells. The lines drawn through the data points were determined by linear regression analyses.](image-url)
under the electrical impedance curve was 66% that under the light diffraction curve. Consequently, the absolute counts of *At. ferrooxidans* cells obtained by the electrical impedance method were multiplied by 1.34 to correct for the percentage of bacterial counts that were below the limit of detection of the instrument.

Fig. 1C shows the dependences of the volume and the surface area of intact *At. ferrooxidans* on the number of cells derived from over 220 assays using electrical impedance measurements. Each value of the volume represents the sum of the individual volumes for all of the cells measured in that particular assay. The volumetric measurements conducted in the MultiSizer are independent of the shape of the particles in the suspension. Each value of the surface area is the sum of individual surface areas calculated from the corresponding volumetric data assuming that each bacterial particle is a perfect sphere. *At. ferrooxidans* is actually rod-shaped with an aspect ratio of ~1:4. Consequently, the values of the surface areas calculated using the spherical model represent a conservative estimate of the true surface area; a more accurate estimate of the surface area of the spherical model represent a conservative estimate of the true surface area; a more accurate estimate of the surface area of the rod is obtained from the slopes of the two lines in Fig. 1C.

**Initial Velocity Kinetic Studies**—The formation of product ferric ions was evident as soon as intact cells of *At. ferrooxidans* were introduced into an aerobic solution of ferrous ions at pH 1.5. The inset in Fig. 2A shows four representative time courses for the increases in absorbance at 380 nm obtained when 1.44 × 10⁸ cells of *At. ferrooxidans* were mixed with different concentrations of ferrous ions and monitored over time at 30 °C. Ferric ion and its first hydrolysis product (FeOH₂⁺) have broad absorbance bands with peaks at 240 and 300 nm, respectively (34). For the purposes of these experiments, we determined an absorption coefficient of 68 m⁻¹ cm⁻¹ at 380 nm for oxidized iron in sulfuric acid, pH 1.5 (data not shown). Initial velocities of the changes in ferric concentration as a function of time were obtained from primary data such as those shown in the inset of Fig. 2A, and the resulting secondary plot of initial velocity as a function of starting ferrous ion concentration is shown in the main panel. The parameters for the rectangular hyperbola drawn through the data points in Fig. 2A were derived from a nonlinear least squares fit of the Michaelis-Menten equation to the initial velocity data. Values for *V*ₘₐₓ and *Kₘ* of 630 ± 20 nmol/min and 0.22 ± 0.03 mM, respectively, were obtained from the analysis. That value of *V*ₘₐₓ corresponds to 550 ± 18 fmol/min/cell.

The observations that aerobic respiration on soluble iron could be modeled using the Michaelis-Menten equation were consistent with the minimal kinetic mechanism shown in Fig. 2B. Briefly, the iron-dependent reduction of electron transport proteins in the bacterium is depicted as a relatively rapid reaction, which is consistent with observations that reduction of the intact cells is complete within the 0.5-s dead time of the mixing even when the concentration of soluble ferrous ions is only 50 μM (see below). The bacterium with its reduced cellular components is then shown as reacting with molecular oxygen to regenerate the oxidized bacterium in a slower reaction that constitutes the rate-limiting catalytic step. The Michaelis complex in this model is perhaps more accurately portrayed as the concentration of available respiratory electrons within the intact bacterium, because the catalyst in this case is not a single enzyme but an entire organism that contains numerous catalytic centers. The exchange of electrons between the bacterium and soluble iron is depicted as a reversible reaction until or if experimental observations indicate otherwise.

**Reduction of the Bacterium**—Washed cell pellets of *At. ferrooxidans* were a tan color with just a trace of bluish tint. The dashed curve in Fig. 3A shows the absorbance spectrum of air-oxidized *At. ferrooxidans* that was obtained in the CLARITY spectrophotometer in sulfuric acid, pH 1.5. Even though the cell suspension contained 5.8 × 10⁸ cells/ml and was roughly as turbid as nonfat milk, the resulting absorbance spectrum contained no evidence of the light scattering artifacts that one would observe by conducting the same absorbance measurements using a conventional linear spectrophotometer. The oxidized spectrum in Fig. 3A showed a clearly defined Soret peak at
411 nm and a broad absorbance at higher wavelengths that extended well beyond 600 nm.

The goal of the initial spectroscopic experiments was to determine whether the principal electron transport proteins in the cells changed color when the cells were suspended in sulfuric acid and subsequently exposed to excess concentrations of soluble ferrous ions under physiological conditions. The solid curve in Fig. 3A shows the absorbance spectrum that was obtained within 0.5 s when the cells of oxidized *At. ferrooxidans* were exposed to 300 μM ferrous sulfate at pH 1.5. Exposure to soluble iron caused the Soret peak to shift to 414 nm and assume a shoulder at around 440 nm. In addition, several prominent peaks and troughs appeared in the absorbance spectrum at longer wavelengths.

A more detailed presentation of the iron-dependent absorbance changes in the intact bacterium is offered by the reduced minus oxidized difference spectrum shown in the inset of Fig. 3A. It was evident that all three types of electron transfer proteins hypothesized to participate in the aerobic iron respiratory chain of *At. ferrooxidans* were represented in the difference spectrum. The participation of c-type cytochromes was indicated by the peaks at 417, 520, and 551 nm, whereas the participation of a-type cytochromes was indicated by the peaks at 441 and 598 nm. Finally, the broad trough in the difference spectrum from 500 to 650 nm was consistent with the hypothesis that large quantities of rusticyanin were also reduced by soluble iron.

The data shown in Fig. 3B illustrate the approach taken to quantify the visible electron transport proteins in intact *At. ferrooxidans* that were transiently reduced by soluble iron. Curve a in Fig. 3B is a reduced minus oxidized difference spectrum for 13.4 nm cytochrome a that was calculated from spectra published using the electrophoretically homogeneous cytochrome oxidase purified from cell-free extracts of *At. ferrooxidans* (13). Similarly, curve c of Fig. 3B is a difference spectrum for 20.6 nm cytochrome c that was calculated from published spectra of cytochrome c also purified from the same organism (10). Curve b of Fig. 3B is a difference spectrum for 311 nm rusticyanin that was calculated from the spectra of rusticyanin purified by this laboratory (2) and others (1). Curve d of Fig. 3B is the sum of curves a, b, and c. The data points in Fig. 3B represent the difference spectrum shown in the inset of Fig. 3A. The close correspondence between the calculated and the observed spectra indicated the following: (i) these three components were the principal electron transport proteins that were reduced by extracellular iron, and (ii) we had the means to quantify the concentration of each type of reduced protein.

Different concentrations of each electron transport protein were reduced within 0.5 s after the intact bacterium was exposed to different concentrations of soluble iron. The five reduced minus oxidized difference spectra shown in Fig. 3C were obtained when *At. ferrooxidans* was mixed with soluble ferrous sulfate at concentrations ranging from 50 to 400 μM. The greater the concentration of soluble ferrous ions, the
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FIGURE 4. Oxidation kinetics of iron-reduced At. ferrooxidans. A, kinetic scans from 500 to 650 nm obtained when 4.6 × 10⁹ cells of At. ferrooxidans were mixed with 200 μM ferrous sulfate, pH 1.5. The reaction was monitored at 30 °C in a dual-beam CLARiTY spectrophotometer equipped with a rapid-scan module. Six scans were taken every second for 220 s; every 50th scan is presented. B, spectral eigenvectors of the principal light-absorbing species present in the intact bacteria. Spectra were computed from a global fit of the data set represented in A to a single exponential function of time assuming the existence of two major species. Spectrum a was identical to that obtained 0.5 s after the cells were mixed with ferrous ions; spectrum b was identical to that obtained after the absorbance changes were complete. C, comparison of the time-dependent oxidation of the bacteria monitored at 580 nm (a) with the kinetic eigenvector obtained from the global fit kinetic analysis (b). The curve drawn through the data in b represents a single exponential function of time with an apparent first-order rate constant of 0.021 s⁻¹. Inset, a residual plot of the differences between the kinetic eigenvector and the computed single exponential function of time. D, dependence of the apparent rate constants for the oxidation of reduced At. ferrooxidans on the initial concentrations of ferrous iron. Each datum represents the mean and standard deviation of at least four determinations.

greater was the extent of reduction of each type of protein. The approach used to quantify the reduced proteins that is illustrated in Fig. 3B was applied to each of the difference spectra shown in Fig. 3C. The data in Fig. 3D show the dependences of the concentrations of reduced rusticyanin and cytochromes a and c on the concentration of extracellular Fe(II). The expected stoichiometry for aerobic respiration on soluble iron dictates that four ferrous ions are oxidized for each molecular oxygen that is reduced to two water molecules. Consequently, all of the spectra shown in Fig. 3C were obtained under conditions where the electron accepting capacity for the total molecular oxygen, greater than 800 μM, exceeded the electron donating capacity of the limiting soluble ferrous ions, less than or equal to 400 μM. When the soluble ferrous concentrations were 2–4 mM and far exceeded the limited electron-accepting capacity of the soluble molecular oxygen, the maximum concentrations of reduced proteins in the organism were observed to be 510, 22, and 36 nM for rusticyanin and cytochromes a and c, respectively.

The data in Fig. 3D can also be used to estimate the concentrations of the respiratory components in the periplasm of the bacterium. For example, the maximum rusticyanin concentration was determined spectrophotometrically as 510 nM, which corresponds to 4.08 nmol in the 8-ml volume of the observation cuvette. At 0.31 μM³ per cell, the total cellular volume of the 4.6 × 10⁹ cells in the cuvette was 1.43 × 10⁸ μM³. If 10–20% of that total cellular volume were periplasmic space (35, 36), then the concentration of rusticyanin in the periplasm was between 14.3 and 28.5 nM or 240 and 470 mg/ml. Similarly, the concentration of cytochrome c in the periplasmic space was between 17 and 33 mg/ml.

It must be noted that direct observations on electron transport reactions in intact bacteria also have their limitations if one or more of the colored components has a small absorption coefficient or is present in considerably lower concentrations than those of the other components. A case in point is the b-type cytochrome that we expect to be present to participate in the “uphill” transfer of reducing power from soluble ferrous iron to NAD(P)⁺ (16). We found no evidence for b-type cytochromes in the spectral data shown herein, nor have we (2, 7) or others (2, 4, 6, 8, 10–14) reported its existence in cell-free extracts of this bacterium.

Oxidation of the Bacterium—The iron-dependent reduction of electron transport proteins within At. ferrooxidans was always complete within the practical mixing time of 0.5 s. Under solution conditions where the initial concentrations of ferrous ions were less than or equal to 400 μM, the absorbance spectrum of the cell suspension gradually changed from its partially reduced form back to its original air-oxidized form. Fig. 4A shows a series of rapid kinetic scans obtained when a cell suspension was mixed with 200 μM ferrous sulfate at pH 1.5. The first scan shows the spectrum of the partially reduced form of the cell suspension that was observed immediately, with peaks at 520, 551, and 598 nm and a broad trough of lower absorbance over the entire region. Over the subsequent 200 s,
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The absorbance spectrum returned to that observed originally in the air-oxidized state.

Fig. 4B shows the spectral eigenvectors calculated from a multichannel global fit of the data set in Fig. 4A to a single exponential function of time (t) as shown in Equation 1,

\[ \text{absorbance} = (a - b)e^{-kt} + b \]  

(Eq. 1)

where \(a\) represents the spectrum of the reduced form of the suspension that was observed 0.5 s after mixing the cells with the soluble iron; \(b\) represents the spectrum of the final oxidized form of the suspension present at the end of the reaction, and \(k\) is the single pseudo-first-order rate constant for all of the absorbance changes. The kinetic eigenvector obtained from the global fit analysis is represented by curve \(b\) in Fig. 4C. The smooth curve in Fig. 4C drawn through the data represents a single exponential function of time with a rate constant of 0.021 s\(^{-1}\). Curve \(a\) in Fig. 4C shows the actual absorbance changes at 580 nm that were extracted from the data set in Fig. 4A. The close correspondence between the actual absorbance at 580 nm and the computed eigenvector absorbance is evident. The increase in absorbance at 580 nm is due predominantly to the oxidation of reduced rusticyanin. Favorable comparisons such as that shown in Fig. 4D were also obtained with data extracted at 550 and 598 nm, representing absorbance changes due primarily to cytochromes \(c\) and \(a\), respectively (data not shown). It was evident that all of the absorbance changes in the data set shown in Fig. 4A could be adequately accommodated with a single exponential function of time, despite the fact that three different electron transport proteins with quite different spectral and electrochemical properties all contributed to the overall absorbance changes.

Data sets analogous to that shown in Fig. 4A were also generated when intact \(A. \text{ferrooxidans}\) was mixed with different concentrations of ferrous sulfate. Regardless of the starting concentration of ferrous sulfate, the resulting body of multiwavelength absorbance changes could be adequately described by a single exponential function of time. Fig. 4D shows the dependence of the pseudo-first-order rate constants obtained from these global fit analyses on the concentration of ferrous ions. The higher the initial concentration of reduced iron, the slower were the rates of oxidation of the reduced forms of the cellular electron transport proteins back to their original oxidized states.

**Bacteria-monitored Turnover Studies**—The kinetic mechanism shown in Fig. 2B predicts that the rate of appearance of product ferric ions should be directly proportional to the concentration of the Michaelis complex, which in this instance is represented by the concentration of available respiratory electrons in the partially reduced bacterium. The close correspondence between these two quantities is illustrated with the two kinetic traces shown in Fig. 5A. Curve \(a\) in Fig. 5A shows the increase in absorbance at 580 nm that accompanied the oxidation of the partially reduced bacterium that was formed within 0.5 s after \(A. \text{ferrooxidans}\) was mixed with 100 \(\mu M\) ferrous sulfate. The data discussed above in Fig. 4 indicated that the
change in absorbance at 580 nm could serve as a surrogate for the overall bacterial oxidation process because all of the visible respiratory absorbance changes behaved as a single unit. The curve in Fig. 5A shows the increase in absorbance at 380 nm that accompanied the formation of product ferric ions. When the respective ordinates were arranged on Fig. 5 to facilitate a comparison of the two curves, it was evident that the two processes occurred concomitantly. A more quantitative expression of this relationship is shown in Equation 2,

\[
\frac{d[Fe(III)]}{dt} = k_{cat}[\text{respiratory electrons}] \tag{Eq. 2}
\]

where the concentration of “respiratory electrons” represents the sum of the concentrations of reduced rusticyanin and cytochromes a and c. The inset in Fig. 5A shows a plot, according to Equation 2, derived from the tangents to curve b as a function of the corresponding concentrations of available respiratory electrons. The slope of the line yielded a value for \(k_{cat}\) of 7500 ± 300 s\(^{-1}\). Conversion of the value for \(V_{max}\) determined above in the initial velocity studies to a turnover number/mol of available respiratory electrons yielded the comparable value for \(k_{cat}\) of 7,300 ± 230 s\(^{-1}\).

A second means of relating product formation to spectral changes in the intact bacterium involves a variation of the enzyme-monitored kinetic method used to study the kinetic mechanisms of enzymes that utilize colored prosthetic groups (37, 38). In this variation, the total concentration of substrate or product is equated to the area swept out by the absorbing electron transport proteins during aerobic respiration. A more formal expression of this relationship is obtained by integrating Equation 2 to yield Equation 3,

\[
[Fe(III)]_{total} = [Fe(II)]_{total} = k_{cat} \int [\text{respiratory electrons}] \, dt \tag{Eq. 3}
\]

Fig. 5B shows a series of difference kinetic traces (reduced minus oxidized) at 580 nm for the oxidation of the reduced bacterium that was formed in the presence of different concentrations of ferrous sulfate. In this case, the area swept out by the absorbing species refers to the area bounded by the baseline in the difference spectrum and the negative difference values. It was evident that the areas in question increased with increasing total concentrations of the reducing substrate because both the amplitudes and the half-times for oxidation increased as the iron increased. The inset in Fig. 5B shows a plot according to Equation 4, which is a rearranged form of to produce Equation 3,

\[
\int [\text{respiratory electrons}] \, dt = \frac{[Fe(II)]_{total}}{k_{cat}} \tag{Eq. 4}
\]

where the value of \(1/k_{cat}\) extracted from the slope of the plot yielded a value for \(k_{cat}\) of 7,300 ± 330 s\(^{-1}\).

The bacteria-monitored turnover studies also presented the opportunity to investigate the apparent in situ redox potentials of the three principal iron-responsive electron-transfer proteins in the periplasm of *At. ferrooxidans*. The data shown in Fig. 5C are consistent with the hypothesis that the three electron-transfer proteins have similar reduction potentials in situ. The abscissa in Fig. 5C represents the half-cell Nernst potential for the reduction of ferrous iron, \(RT/nF\log([Fe(II)]/[Fe(III)])\), where \(R\) is the gas constant; \(T\) is the temperature; \(F\) is Faraday’s constant, and \(n\) is 1, the number of electrons in this case. The iron reaction quotient was calculated from the kinetic data in Fig. 5A using an absorption coefficient of 68 M\(^{-1}\) s\(^{-1}\) for the concentration of ferric iron produced at 380 nm and the difference between the ferric concentration and 100 µM as the concentration of ferrous iron that remained in the solution. The ordinate in Fig. 5C represents the half-cell Nernst potential for the reduction of each of the three principal in situ electron transfer proteins of interest. The reaction quotients for each protein were calculated by first collecting difference spectra on intact cells, such as that shown in Fig. 3B, at each iron concentration indicated in Fig. 5C. The corresponding concentrations of reduced rusticyanin and cytochromes a and c were then obtained from each difference spectrum as described above. Finally, the concentrations of the respective oxidized proteins were determined by the differences between the concentrations of the reduced proteins and the total concentrations of each protein.

If one combines the results from all three proteins, the resulting data set constitutes a redox titration where the three proteins exhibit a single common standard reduction potential of 650 ± 11 mV. One could also make a case from the data for assigning a slightly lower reduction potential to the cytochromes c (~640 mV) compared with the slightly higher reduction potentials for the other two proteins (~655 mV). However, given the uncertainties in the data, we have elected to report the more conservative estimate of a single broad reduction potential for all three redox-active proteins.

**Discussion**

There is no better means to establish physiological relevance in a metabolic function than to directly observe it as it occurs in situ in the intact organism. The observations described above were consistent with the hypothesis that rusticyanin and cytochromes a and c were the principal participants in the aerobic iron respiratory chain of *At. ferrooxidans*. Our initial hope was that we could observe the sequential reduction of these electron transport proteins in their hypothesized linear array. This was impossible because the reductive half-reaction was complete within the effective dead time of the observation. Consequently, our next hope was that we could observe the sequential oxidation of these electron transport proteins in their linear array under conditions where the electron-accepting capacity of the molecular oxygen exceeded the electron-donating capacity of the limiting soluble iron. This was also impossible because the oxidation of the reduced cellular proteins occurred simultaneously with a single macroscopic rate constant. If one assumed that the rate-limiting step in the respiratory process was the reduction of oxygen catalyzed by the terminal aa₃ oxidase, then the next hypothesis was that the prior electron transfer reactions leading up to the terminal oxidase were sufficiently relatively rapid so as to create a rapid equilibrium kinetic mechanism. Under rapid equilibrium conditions, the electrons within the respiratory chain would rapidly distribute among the
visible electron transport proteins according to their respective redox potentials. Fig. 6A shows a perspective of the relevant redox-active proteins from *At. ferrooxidans* where the standard reduction potentials of individual proteins were determined either *in vitro* using dilute purified protein preparations or *in situ* in liquid nitrogen. Based on their relative affinities for electrons, one would expect a greater percentage of cytochromes *a* to be reduced than either of the other two types of proteins in the presence of subsaturating concentrations of respiratory electrons. Instead, the relative ratios of the three principal redox-active proteins monitored in these *in situ* observations did not vary appreciably with the initial iron concentration, nor did they vary during the time courses of the subsequent oxidation reactions. The unavoidable conclusion was that the visible components of the iron respiratory chain functioned in a concerted fashion as a single ensemble with a single functional standard reduction potential of 650 mV.

A hypothesis to account for the ensemble-like behavior of the electron transport proteins in the respiratory chain is that the functional behavior of these proteins is influenced by specific and nonspecific interactions due to the macromolecular crowding in the periplasmic space. We have been aware for many years that rusticyanin must be present at high concentrations in the periplasm. We hypothesize that the periplasmic rusticyanin constituted as much as 5% of the total soluble protein expressed by the bacterium (24)! Values for the contribution of the periplasmic space to the total volume of a Gram-negative bacterium can vary from 10 to 20%, depending on whether one uses transmission electron microscopic measurements (35) or compartment-specific radioactive tracers (36), respectively, to estimate the fraction. Taking 15% as a compromise value, our data yield a periplasmic concentration for rusticyanin of 21.4 mM or 350 mg/ml. The latter value compares favorably with those of 330 mg/ml for hemoglobin in red blood cells (42) and 300–400 mg/ml for macromolecules in the interior of *Escherichia coli* (43). The volume of a single rusticyanin molecule is ~20 nm³, as determined using either the value of 0.73 cm³/g for the average partial specific volume of a globular protein (44) or the actual dimensions of the purified rusticyanin obtained from structural studies by x-ray crystallographic (45) or multidimensional NMR means (46). Consequently, the rusticyanin protein at 350 mg/ml occupies 4.6 × 10¹⁶ nm³ or 21% of the total volume in the periplasmic space. Depending on how much of the 25 mg/ml cytochrome *c* that we measured is actually in the periplasm and how much is embedded in the outer membrane, we can estimate that this respiratory cytochrome could readily occupy another 2% of the periplasmic space. What emerges from these calculations is the image represented in Fig. 6B, where the concentrations of respiratory proteins in the periplasm are orders of magnitude higher than any encountered in published *in vitro* functional studies. Studies conducted in dilute solutions have shown the following: (i) rusticyanin forms a complex with the periplasmic cytochrome *c* that lowers the standard reduction potential of the blue protein (14), and (ii) the transfer of electrons from soluble Fe(II) to rusticyanin is catalyzed by the outer membrane cytochrome *c* via a transient encounter-Michaelis complex (7, 12). It is not a stretch of the imagination to hypothesize that additional interactions that serve to further influence their function and reactivity occur among the respiratory proteins at these high concentrations in the periplasm. We hypothesize that these respiratory proteins have evolved to function as an ensemble in this crowded environment and that the crowded periplasmic space has a structure and order that we have not heretofore appreciated or characterized.

The passage of electrons from extracellular soluble iron across the periplasmic space to the terminal oxidase and molecular oxygen is rapid. If the value of *V*ₘₐₓ obtained from the initial velocity studies in Fig. 2 is divided by the total numbers of respiratory electrons in the intact cells present in the assay (4.08, 0.176, and 0.288 nmol of rusticyanin and cytochromes *a* and *c*, respectively), the result of *k*ₘₐₓ equals 7,400 ± 240 s⁻¹, a value that is acceptably close to those calculated from the bacteria-monitored turnover studies in Fig. 5. The ratio of *k*ₘₐₓ/*V*ₘₐₓ

**FIGURE 6. Schematic summaries of aerobic respiration on soluble iron by *At. ferrooxidans*.** A, electrochemical perspective of the principal redox-active components of the respiratory chain. The approximate standard reduction potential of each component as determined individually is indicated by the line drawn to the vertical scale. Conditions and references are as follows: *a*, periplasmic diheme cytochrome *c*, pH 4.8 (10); *b*, rusticyanin in the presence of saturating cytochrome *c*, pH 4.8 (14); *c*, outer membrane cytochrome *c*, pH 4.8 (39); *d*, rusticyanin in the absence of saturating cytochrome *c*, pH 4.8 (14); *e*, *in situ* cytochrome *a* at liquid nitrogen temperatures (40); *f*, soluble iron in the presence of saturating sulfate, pH 2.0 (2); *g*, free rusticyanin, pH 2.0 (1); *h*, soluble aqueous iron (41); and *i*, oxygen at pH 2.0 (41). The approximate standard reduction potential of the combination of three components determined *in situ* is indicated by the bold arrow. B, representation of the principal electron transfer proteins in the periplasmic space of *At. ferrooxidans*, illustrating the macromolecular crowding and the relative quantities of the three types of components.
is thus $3.4 \times 10^7 \text{ m}^{-1} \text{s}^{-1}$, a value that approaches that of diffusion control for the encounter of a small mobile atom, Fe(II), with the surface of a much slower moving partner, the intact bacterial cell. Others have proposed that a respirasome composed of a stable multiprotein complex bridges the periplasmic gap and provides a conduit to conduct electrons from the outer membrane to the terminal oxidase in the cytoplasmic membrane (25). However, this hypothesis does not provide a role for the excess rusticyanin present in the periplasm, where the concentration of the small copper protein is 10-fold higher than those of the cytochromes $a$ and $c$ combined. Furthermore, this hypothesis also does not present a rationale for the observations described herein, where the entire pool of available respiratory electrons reduces and oxidizes concomitantly as an ensemble with a single functional reduction potential. Rather, one might expect the individual reduction potentials measured in vitro to be maintained in situ to provide the individual thermodynamic driving forces for rapid intermolecular electron transfer reactions down the hypothetical linear electron transport respirasome.

We propose an alternative hypothesis that the crowded, concentrated electron transport proteins in the periplasm of *At. ferrooxidans* constitute a network of proteins where inter-protein electron transfer interactions across the periplasmic space function effectively without a requirement for the defined linear series of electron transfer reactions set forth in the aforementioned respirasome. Furthermore, we posit that the frequent transient encounters among these crowded proteins favor protein conformations that create the concerted kinetic behavior and the single functional macroscopic reduction potential of 650 mV observed in situ. We speculate that the in situ electron transfer behavior described herein may be the rule, rather than the exception, in those chemolithotrophic bacteria rather than the exception, in those chemolithotrophic bacteria — cytochromes, in contrast with the view that electron transport occurs via weak, ill-defined interactions among a pool of cytochromes (50–53). Similarly, respiratory mic spaces of lar substrates (47–49). We note that transient interactions that respire either aerobically or anaerobically with extracellular, insoluble iron oxides (50–53). Molecular cloning of the gene encoding *Thiobacillus ferrooxidans* Fe(II) oxidase. *J. Biol. Chem.* 267, 11242–11247.

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