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Energy Transduction by Anaerobic Ferric Iron Respiration in *Thiobacillus ferrooxidans*

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Formate-grown cells of the obligately chemolithoautotrophic acidophile *Thiobacillus ferrooxidans* were capable of formate- and elemental sulfur-dependent reduction of ferric iron under anaerobic conditions. Under aerobic conditions, both oxygen and ferric iron could be simultaneously used as electron acceptors. To investigate whether anaerobic ferric iron respiration by *T. ferrooxidans* is an energy-transducing process, uptake of amino acids was studied. Glycine uptake by starved cells did not occur in the absence of an electron donor, neither under aerobic conditions nor under anaerobic conditions. Uptake of glycine could be driven by formate- and ferrous iron-dependent oxygen uptake. Under anaerobic conditions, ferric iron respiration with the electron donors formate and elemental sulfur could energize glycine uptake. Glycine uptake was inhibited by the uncoupler 2,4-dinitrophenol. The results indicate that anaerobic ferric iron respiration can contribute to the energy budget of *T. ferrooxidans*.

Thiobacillus ferrooxidans is an obligately autotrophic, acidophilic bacterium. Energy for autotrophic growth can be derived from the oxidation of ferrous iron and various inorganic sulfur compounds, including metal sulfides (9). Molecular hydrogen (4) and formate (17) can also be used as energy sources for autotrophic growth.

T. ferrooxidans is generally considered to be an obligately aerobic organism (9). However, under anaerobic conditions, the organism can use ferric iron as an alternative electron acceptor for the oxidation of elemental sulfur (2). At present, there is no published experimental evidence that ferric iron respiration by *T. ferrooxidans* is an energy-transducing process.

T. ferrooxidans is an important organism for the biological leaching of metal ores (15). Oxidation of metal sulfides may occur by a direct biological oxidation of the sulfur moiety of the minerals. Alternatively, ferric iron formed by the bacteria during the oxidation of ferrous iron may act as a chemical oxidant (23). In particular, during large-scale, in situ bioleaching operations, *T. ferrooxidans* may often encounter environments which contain low dissolved oxygen concentrations and high ferric iron concentrations. If ferric iron respiration is an energy-transducing process, this may increase the stability of *T. ferrooxidans* populations in leaching operations. Furthermore, ferric iron-dependent oxidation of sulfur-containing minerals by the bacteria may contribute to the rate of bioleaching under such conditions.

In spite of the potential significance of the process for the industrial application of *T. ferrooxidans*, little is known about the mechanism and physiological function of ferric iron respiration by this organism. So far, ferric iron respiration by *T. ferrooxidans* has only been reported with elemental sulfur as an electron donor. We have recently found that under aerobic conditions, *T. ferrooxidans* can use formic acid as an energy source for autotrophic growth. It was therefore of interest to investigate whether formate oxidation can be coupled to the reduction of ferric iron under anaerobic conditions. The main aim of the present study was to investigate whether ferric iron reduction by *T. ferrooxidans* is an energy-transducing process.

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MATERIALS AND METHODS

Microorganism and maintenance. *T. ferrooxidans* LMD 81.69 (ATCC 21834) was obtained from the culture collection of the Laboratory of Microbiology and Enzymology, Delft, the Netherlands. The organism was maintained in ferrous iron-grown shake flask cultures. Cultures were regularly checked for purity by immunofluorescence microscopy. Antisera against *T. ferrooxidans* were obtained as described previously (14).

Chemostat cultivation. *T. ferrooxidans* LMD 81.69 was grown in formate-limited chemostat cultures (D = 0.01 h^{-1}, pH 1.8, 30°C, S_R [substrate concentration in reservoir medium] = 100 to 500 mM formic acid). Formate-limited steady-state cultures were obtained by carefully increasing the formic acid concentration in the reservoir medium of ferrous iron-grown cultures (17). The mineral medium contained the following per liter of demineralized water: $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; Na_2SO_4, 1.0 g; $FeSO_4 \cdot 7H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 1 mg; $CuSO_4 \cdot 5H_2O$, 2 mg; $MnSO_4 \cdot H_2O$, 1 mg; $NaMoO_4 \cdot 2H_2O$, 0.5 mg; $CoCl_2 \cdot 6H_2O$, 0.5 mg; $Na_2SeO_4 \cdot 10H_2O$, 1 mg; $NiCl_2 \cdot 6H_2O$; 1 mg. The mineral medium was sterilized at 120°C. Formic acid solutions were sterilized separately at 110°C. Continuous cultivation was performed in Applikon laboratory fermentors with a working volume of 1.5 liters. The cultures were continuously sparged with water-saturated air (1.5 liters · min^{-1}) and stirred at 800 rpm. The dissolved oxygen concentration in the cultures was monitored with a steam-sterilizable Clark-type electrode. Chemostat cultures were grown at a dissolved oxygen concentration of more than 75% of air saturation.

Oxygen uptake experiments. Cells were harvested by centrifugation (10,000 × g, 15 min) and resuspended in 50 mM K_2SO_4–50 mM Na_2SO_4 (pH 3.0). Substrate-dependent oxygen uptake was measured with a Clark-type oxygen electrode. Oxygen uptake rates were calculated assuming an oxygen concentration of 236 μM in air-saturated cell suspen-

sions and were corrected for the (low) endogenous respiration rates. Elemental sulfur was added as stock solutions in acetone. The acetone concentration in the assays did not exceed 1% (vol/vol).

Ferric iron reduction assays. Cells were harvested by centrifugation (10,000 × *g*, 15 min) and resuspended in 50 mM K_2SO_4–50 mM Na_2SO_4 (pH 3.0). Cell suspension (4 ml) was added to a reaction chamber kept at a constant 30°C. After the addition of 0.5 mM $Fe_2(SO_4)_3$, the suspension was made anaerobic by flushing (15 min) with water-saturated argon. Norprene tubing (Cole Parmer Industries, Chicago, Ill.) was used to minimize oxygen diffusion. After the addition of substrate to the reaction chamber, 100-μl samples were taken with a gas-tight Hamilton syringe. Ferrous iron was assayed by a modification of the phenanthroline method (19). The samples were added to 900 μl of an Fe^{2+} assay mixture containing the following: 12.5% (wt/vol) ammonium acetate in 25% (vol/vol) acetic acid anhydride, 50 μl; 0.1% *o*-phenanthroline in 10% (vol/vol) ethanol, 50 μl; and demineralized water, 800 μl. After a 5-min incubation, the mixture was centrifuged (15,000 × *g*, 5 min). The absorbance of the supernatant at 510 nm was measured with a Vitatron spectrophotometer. The ferrous iron assay was calibrated with standard solutions of $FeSO_4 \cdot 7H_2O$ in 0.05 M H_2SO_4. Rates of ferric iron reduction in the absence of an electron donor were negligible.

Amino acid uptake experiments. Cells were harvested by centrifugation (10,000 × *g*, 15 min) and resuspended in 50 mM K_2SO_4,–50 mM Na_2SO_4 (pH 3.0). To deplete endogenous energy sources, the suspension was sparged with water-saturated air for 3 h at room temperature. A cell suspension (0.5 ml) was added to a 1-ml reaction chamber kept at a constant 30°C which was made anaerobic by a continuous flow of water-saturated argon. Ferric iron [0.5 mM $Fe_2(SO_4)_3$] was added to the cell suspension 15 min prior to the addition of an electron donor. ^{14}C-labeled amino acids (20 μM; 7.77 TBq · mol^{-1}) were added 2 min prior to the addition of an electron donor. Samples were withdrawn with a gas-tight Hamilton syringe, diluted in 2 ml of 0.1 M LiCl (4°C), and immediately filtered over 0.45-μm-pore-size membrane filters (Schleicher and Schüll, Dassel, Federal Republic of Germany). After being washed with 2 ml of 0.1 M LiCl, the filters were transferred to glass scintillation vials containing 5 ml of scintillation fluid (Emulsifier Scintillator 299, Packard). Radioactivity was measured in a Beckman LS 3801 benchtop scintillation counter.

Analytical procedures. Formic acid in reservoir medium was assayed by the method of Lang and Lang (11). Dry weights of cell suspensions were determined by filtrating aliquots over nitrocellulose filters (0.45-μm pore diameter; Schleicher and Schüll). The cells were washed three times with demineralized water and dried to constant weight at 70°C.

Chemicals. [U-^{14}C]glycine (210 Ci · mol^{-1}, 7.77 TBq · mol^{-1}) was obtained from Amersham International PLC. All other chemicals were reagent grade and were obtained from commercial sources.

RESULTS

Ferric iron respiration by formate-grown *T. ferrooxidans*. Formate-grown cells of *T. ferrooxidans* exhibited substrate-dependent oxygen uptake with ferrous iron and elemental sulfur (Table 1). Even after prolonged formate-limited cultivation (over 20 volume exchanges in the chemostat), no loss of ferrous iron- and sulfur-oxidizing activity was observed (17). Under anaerobic conditions, formate-grown cells could oxidize formate and elemental sulfur with ferric iron as an electron acceptor (Table 1). The rates of sulfur- and formate-dependent ferric iron reduction under anaerobic conditions were approximately 50% lower than the corresponding oxidation rates with oxygen as the electron acceptor (Table 1). As shown in Fig. 1, the observed stoichiometries of electron donor and ferric iron consumption were as expected from the complete oxidation of elemental sulfur and formate according to the following equations:

$$S + 6Fe^{3+} + 4H_2O \rightarrow SO_4^{2-} + 6Fe^{2+} + 8H^+$$
$$HCOOH + 2Fe^{3+} \rightarrow CO_2 + 2Fe^{2+} + 2H^+$$

To investigate whether the electron acceptors oxygen and ferric iron can be simultaneously utilized by *T. ferrooxidans*, formate-dependent reduction of ferric iron was studied in aerobic cell suspensions. Indeed, a transient accumulation of ferrous iron under aerobic conditions was also observed

TABLE 1. Rates of substrate-dependent reduction of oxygen and ferric iron by formate-grown cells of *T. ferrooxidans*[a]

Electron donor	Rate of electron acceptor reduction (nmol of electrons · min^{-1} · mg^{-1})	
	O_2	Fe^{3+}
None	<5	<5
50 μM elemental sulfur	200	94
100 μM formate	360	205
100 μM ferrous iron	2,960	

[a] Cells were harvested by centrifugation and resuspended in 50 mM Na_2SO_4–50 mM K_2SO_4 (pH 3.0). O_2 consumption was assayed with a Clark-type electrode. Time-dependent reduction of ferric iron under anaerobic conditions was followed by direct assay of ferrous iron (see Materials and Methods).

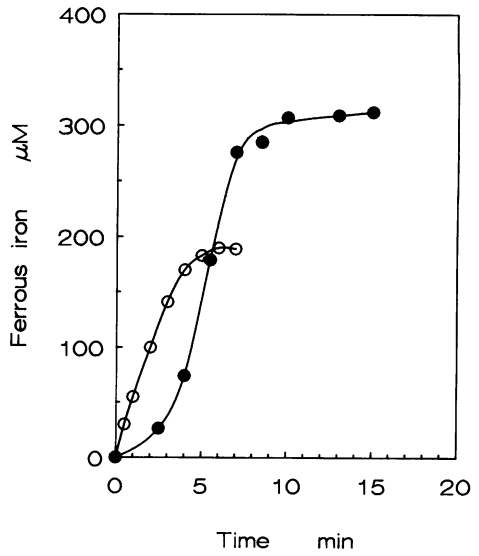

FIG. 1. Formate- and elemental sulfur-dependent reduction of ferric iron (1 mM) under anaerobic conditions. *T. ferrooxidans* was pregrown in an aerobic formate-limited chemostat culture. Symbols indicate the addition of 100 μM formate (cell density, 0.27 mg [dry weight] · ml^{-1}) (○) or 50 μM elemental sulfur (cell density, 0.72 mg [dry weight] · ml^{-1}) (●).

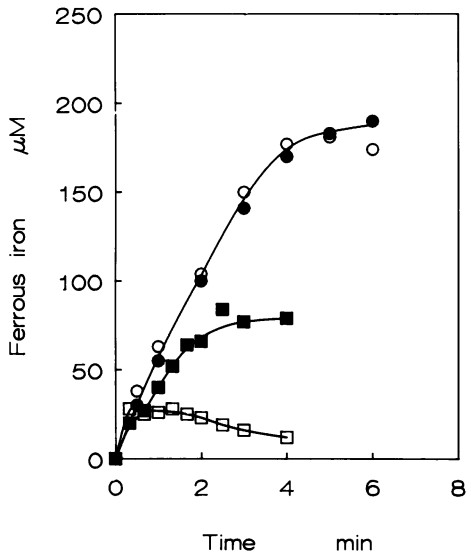

FIG. 2. Effect of azide on formate-dependent reduction of ferric iron by *T. ferrooxidans* (0.27 mg [dry weight] · ml^{-1}) under aerobic and anaerobic conditions. *T. ferrooxidans* was pregrown in an aerobic formate-limited chemostat culture. The initial formate and ferric iron concentrations in the assays were 100 μM and 1 mM, respectively. Open symbols: no azide; closed symbols: 10 μM azide. Circles denote anaerobic experiments; squares denote aerobic experiments.

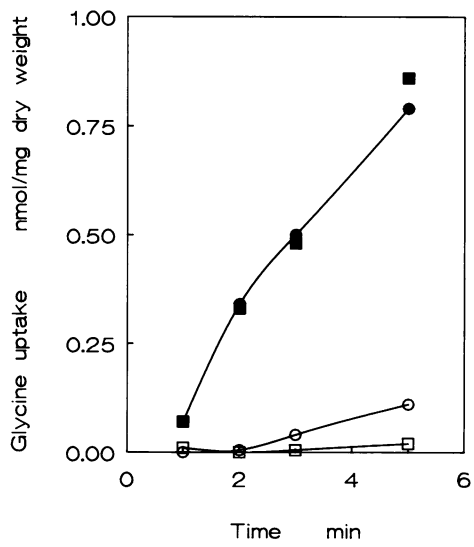

FIG. 3. Aerobic glycine uptake by energy-starved cell suspensions of *T. ferrooxidans* (0.28 mg [dry weight] · ml^{-1}). *T. ferrooxidans* was pregrown in an aerobic formate-limited chemostat culture. The initial [^{14}C]glycine concentration in the assays was 22 μM. Symbols indicate the additions of 100 μM formate (●), 1 mM $FeSO_4$ (■), or 1 mM $FeSO_4$ and 100 μM 2,4-dinitrophenol (□) or no addition (○).

(Fig. 2). At first sight this seems peculiar, since the maximum rates of ferrous iron-dependent oxygen uptake by the cells were much higher than the rate of formate-dependent ferric iron reduction (Table 1). However, the observed low rates of ferrous iron reoxidation can be explained by the relatively high K_s of the cells for ferrous iron, which is approximately 1 mM (data not shown).

Low concentrations of azide almost completely inhibited ferrous iron-dependent oxygen uptake but only slightly affected the aerobic oxidation of formate and elemental sulfur (Table 2). In contrast, the rates of sulfur- and formate-dependent ferric iron reduction (under anaerobic conditions) were not reduced by azide (Table 2; Fig. 2). The addition of azide to aerobic, formate-respiring cell suspensions which contained ferric iron led to an increased, nontransient accumulation of ferrous iron (Fig. 2). In effect, azide acts as a ferrous iron-trapping agent by preventing its reoxidation by the bacteria. However, since the exact mechanism of azide inhibition in *T. ferrooxidans* is not known, the amount of ferrous iron formed in the presence of azide cannot be used to quantify the significance of ferric iron respiration under aerobic conditions.

TABLE 2. Effects of azide (10 μM) on oxidation processes catalyzed by *T. ferrooxidans*[a]

Electron donor	Electron acceptor	% Inhibition
Elemental sulfur	O_2	18
Elemental sulfur	Fe^{3+}	0
Formate	O_2	28
Formate	Fe^{3+}	0
Fe^{2+}	O_2	95

[a] Activities observed in the absence of inhibitors are given in Table 1. *T. ferrooxidans* was pregrown in aerobic formate-limited chemostat cultures.

During aerobic incubation of *T. ferrooxidans* with ferric iron and elemental sulfur, the ferrous iron concentration did not exceed approximately 10 μM (data not shown). This can be explained by the rate of ferric iron-dependent sulfur oxidation, which was lower than the rate of anaerobic formate oxidation (Table 1).

Corbett and Ingledew (3) demonstrated that the aerobic and anaerobic oxidation of elemental sulfur with ferric iron as an electron acceptor by *T. ferrooxidans* can be inhibited to a significant extent by 2-heptyl-4-hydroxyquinoline-*N*-oxide (HOQNO). Also, both the aerobic and anaerobic oxidations of formate were inhibited by HOQNO (over 80% inhibition by 100 μM HOQNO). As already indicated by Corbett and Ingledew (3), this observation strongly suggests that the *bc*1 segment of the respiratory chain is involved in electron transport from sulfur to ferric iron. The same apparently holds for (an)aerobic formate oxidation. Since bacterial *bc*1 complexes are generally assumed to be proton translocating, it seemed plausible that ferric iron respiration by *T. ferrooxidans* is an energy-transducing process. This hypothesis was tested by studying active uptake of amino acids.

Amino acid uptake studies. A preliminary study revealed that under aerobic conditions, formate-grown *T. ferrooxidans* cells exhibited uptake of various radiolabeled amino acids (data not shown). The highest uptake rates were observed with glycine. This substrate was therefore used for further uptake studies.

Immediately after sampling from the chemostat cultures, aerobic glycine uptake was only slightly stimulated by the addition of electron donors (data not shown). However, after 3 h of preincubation at room temperature, uptake of glycine in the absence of added electron donors was negligible (Fig. 3). Under aerobic conditions, glycine uptake could be ener-

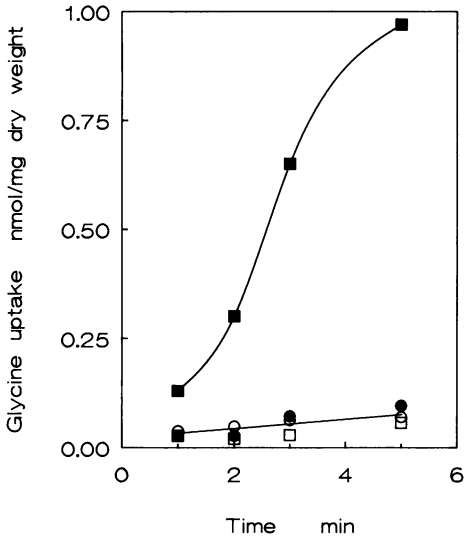

FIG. 4. Anaerobic glycine uptake by energy-starved *T. ferrooxidans* (0.63 mg [dry weight] · ml^{-1}). *T. ferrooxidans* was pregrown in an aerobic formate-limited chemostat culture. The initial [^{14}C] glycine concentration in the assays was 18 μM. Symbols indicate no addition or the addition of (○) 100 μM formate (●), 1 mM Fe^{3+} (□), or 100 μM formate and 1 mM Fe^{3+} (■).

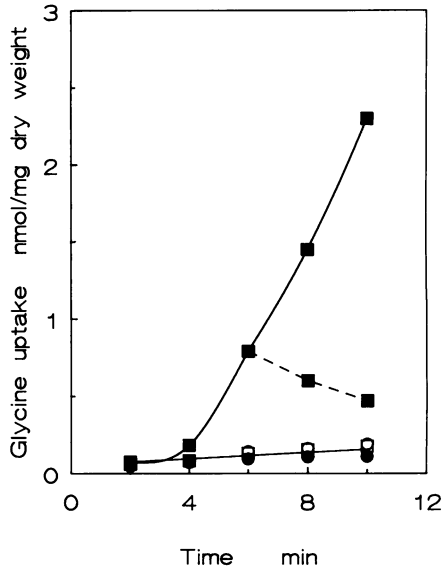

FIG. 5. Anaerobic glycine uptake by energy-starved *T. ferrooxidans* (0.72 mg [dry weight] · ml^{-1}). *T. ferrooxidans* was pregrown in an aerobic formate-limited chemostat culture. The initial [^{14}C] glycine concentration in the assays was 18 μM. Symbols indicate no addition (○) or the addition of 50 μM elemental sulfur (●), 1 mM Fe^{3+} (□), or 50 μM elemental sulfur and 1 mM Fe^{3+} (■). The dashed line indicates a parallel experiment in which 100 μM 2,4-dinitrophenol was added after 6 min.

gized by the electron donors formate and ferrous iron (Fig. 3). Uptake of glycine in the presence of ferrous iron could be completely inhibited by the uncoupler 2,4-dinitrophenol (Fig. 3). This inhibitor did not affect ferrous iron-dependent oxygen uptake by *T. ferrooxidans*, in accordance with earlier observations (8).

Solute uptake studies with intact microbial cells inevitably involve a combination of solute transport and metabolism. The possibility that during the course of the uptake experiments glycine is incorporated into protein cannot be excluded. However, the uncoupler sensitivity of glycine uptake and the dependence of the process on respiration clearly indicate that glycine uptake is an energy-dependent process. Therefore, glycine uptake studies could be used to assess whether energy transduction also occurs during anaerobic ferric iron respiration by *T. ferrooxidans*.

Under anaerobic conditions, uptake of glycine was also negligible when no energy sources were added (Fig. 4 and 5). In the absence of oxygen, the addition of the electron donors formate (Fig. 4), elemental sulfur (Fig. 5), or ferrous iron (data not shown) could not supply the energy required for glycine uptake. However, when formate or elemental sulfur was added in combination with ferric iron, uptake of glycine did occur (Fig. 4 and 5). Glycine uptake energized by ferric iron-dependent formate oxidation was comparable to that observed under aerobic conditions (Fig. 3 and 4). The addition of ferric iron without an electron donor did not significantly stimulate amino acid uptake (Fig. 4 and 5).

When the uncoupler 2,4-dinitrophenol was added during the course of an anaerobic glycine uptake experiment, a net efflux of radioactivity was observed (Fig. 5). This observation suggests that at least part of the glycine had been accumulated in the cells without having been incorporated into proteins.

DISCUSSION

The ability of *T. ferrooxidans* to use ferric iron as an electron acceptor for the oxidation of elemental sulfur has been known for more than a decade (2). However, the mechanism and physiological function of the process have so far remained unclear. We have tried to summarize the experimental data discussed in this paper in a simple scheme (Fig. 6). In this scheme, we have assumed that the oxidation of both elemental sulfur and ferrous iron occur in the periplasm (7, 8). The near-neutral optimum pH of formate dehydrogenase in cell extracts (17) suggests that formate oxidation by *T. ferrooxidans* occurs in the cytoplasm. This would imply that the site of coupling to the electron transport chain is located at the internal side of the cytoplasmic membrane. The similar azide and HOQNO sensitivities of

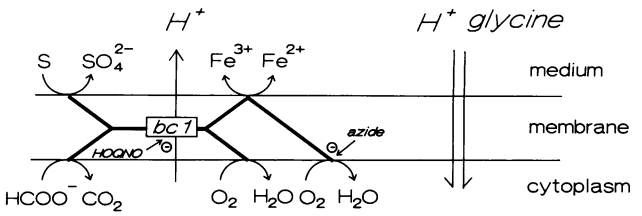

FIG. 6. Hypothetical scheme: aerobic and anaerobic oxidation processes catalyzed by *T. ferrooxidans*. Electron transport is indicated by solid lines. In addition to the *bc*1 complex, charge separation and/or proton pumping occurs during electron transfer from this complex to oxygen and during ferrous iron-dependent oxygen consumption (not shown). Coupling of glycine uptake to proton influx may either be direct or involve ATP as an intermediate.

formate and elemental sulfur oxidation suggest that the pathways of electron transport employed for the oxidation of these two compounds are largely identical (Fig. 6).

Reduction of ferric iron by *T. ferrooxidans* may be catalyzed by the same oxidoreductase enzyme involved in the aerobic oxidation of ferrous iron (Fig. 6). This hypothesis is supported by the observation that both ferrous iron-dependent oxygen uptake and elemental sulfur-dependent reduction of ferric iron are repressed during the growth of *T. ferrooxidans* LMD 81.68 in thiosulfate-limited chemostat cultures (5).

At low concentrations, azide is a specific inhibitor of ferrous iron-dependent oxygen uptake by *T. ferrooxidans* (1) (Table 2). At first sight, the azide insensitivity of anaerobic sulfur- and formate-dependent ferric iron reduction suggests that ferric iron reduction is not catalyzed by the same enzyme involved in the aerobic oxidation of ferrous iron. Alternatively, the site of azide inhibition may be a terminal oxidase rather than the ferrous iron oxidoreductase. Following this line of reasoning, the azide insensitivity of formate- and elemental sulfur-dependent oxygen uptake could then be interpreted as an indication that different terminal oxidases are involved in oxygen consumption by *T. ferrooxidans* (Fig. 6).

Sugio et al. (21) proposed that ferric iron is also the electron acceptor for sulfur oxidation under aerobic conditions. Indeed, the same group reported the purification of a periplasmic sulfur-ferric iron oxidoreductase (22). Our experiments indicate that ferric iron and oxygen can be corespired (Fig. 1). However, various considerations indicate that it is very unlikely that the mechanism proposed by Sugio and coworkers plays a quantitatively significant role in aerobic sulfur oxidation by *T. ferrooxidans* (3, 18). For example, the growth yields of *T. ferrooxidans* on reduced sulfur compounds are higher than the growth yields on ferrous iron (6). This would clearly not be expected if electrons derived from sulfur enter the electron transport chain at the same level as those derived from ferrous iron. The observation that formate can also be used as an electron donor for ferric iron respiration strongly suggests that substrate oxidation and ferric iron reduction occur at different sites of the electron transport chain. This seems difficult to reconcile with the involvement of a single periplasmic sulfur-ferric iron oxidoreductase enzyme.

Active uptake of amino acids by obligately autotrophic bacteria has been reported previously (13). It has been demonstrated that in various obligate chemolithoautotrophs, including thiobacilli, added organic compounds may be used to provide a limited amount of the cell carbon (10, 12).

The anaerobic amino acid uptake studies (Fig. 4 and 5) clearly indicate that in *T. ferrooxidans*, ferric iron respiration can be an energy-transducing process. In this respect, *T. ferrooxidans* resembles neutrophilic iron-reducing bacteria, in which ferric iron-dependent proton translocation has been demonstrated (20).

We have tried to confirm the results from the amino acid uptake studies by ATP assays, using a luciferin-luciferase assay. However, assays were hindered by the inhibition of luciferase by ferric iron, and we failed to obtain reproducible results.

The ability of *T. ferrooxidans* to oxidize formate with ferric iron under anaerobic conditions may be of ecological significance. Small amounts of formate may be produced in the natural habitat of *T. ferrooxidans*, resulting either from chemical reactions or from the activity of (presently unknown) acidophilic fermentative microorganisms. If formate is present, formate oxidation can provide metabolic energy. At the same time, concentrations of formate above 100 μM are toxic for *T. ferrooxidans* (16). The ability of *T. ferrooxidans* to oxidize and detoxify formate may increase the chances of survival during spells of anaerobiosis.

The question of whether ferric iron respiration can support autotrophic growth of *T. ferrooxidans* under anaerobic conditions will be addressed in a forthcoming paper.

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