Electron Transport in the Dissimilatory Iron Reducer, GS-15

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Mechanisms for electron transport to Fe(III) were investigated in GS-15, a novel anaerobic microorganism which can obtain energy for growth by coupling the complete oxidation of organic acids or aromatic compounds to the reduction of Fe(III) to Fe(II). The results indicate that Fe(III) reduction proceeds through a type b cytochrome and a membrane-bound Fe(III) reductase which is distinct from the nitrate reductase.

GS-15 is a novel anaerobic organism which can obtain energy for growth by completely oxidizing several short-chain fatty acids and aromatic compounds to carbon dioxide with Fe(III) as the electron acceptor (10, 12, 14, 17). Reduction of nitrate to ammonia and reduction of Mn(IV) to Mn(II) can also serve as electron-accepting reactions in GS-15 (14). It has been proposed that the metabolism of GS-15 can be used as a model for the mechanism that is responsible for most of the organic matter oxidation coupled to Fe(III) reduction in such environments as aquatic sediments (9, 13–16), deep pristine aquifers (11), and aquifers impacted with organic contaminants (10, 12). The only other organism isolated in pure culture that is known to obtain energy to support growth by oxidizing organic compounds with Fe(III) as the electron acceptor is *Shewanella putrefaciens* (16, 20). *S. putrefaciens* has a very limited substrate range with Fe(III) as the electron acceptor, and formate is the only organic compound that *S. putrefaciens* is known to completely oxidize under such conditions (16). Thus, compared with GS-15 metabolism, the metabolism of *S. putrefaciens* may represent a less significant mechanism for organic matter oxidation in Fe(III)-reducing environments.

The finding that GS-15 obtains energy to support growth by completely oxidizing acetate with Fe(III) as the sole electron acceptor suggests that GS-15 must be able to conserve energy through electron transport to Fe(III) (14). However, electron transport to Fe(III) has not been investigated previously in cells which have been grown with Fe(III) as the electron accceptor. Aerobically grown cells of *S. putrefaciens* 200 were found to constitutively produce an Fe(III) reductase that is linked to an electron transport chain which may include type b, c, and d cytochromes (1–3, 22, 23). However, there was no evidence that the electron transport chain to Fe(III) that was produced in aerobically grown cells could function to conserve energy to support anaerobic growth. In fact, when aerobically grown cells were put under anaerobic conditions with Fe(III) as the potential electron acceptor, 95 to 98% of the cells were inactivated within 2 days and there was little or no subsequent growth (2). Studies with strain MR-1 of *S. putrefaciens* have suggested that anaerobic electron transport systems are not synthesized during growth under aerobic conditions (21).

In *S. putrefaciens* 200, another, more active Fe(III) reductase is induced when cells are grown in the presence of low concentrations of dissolved oxygen. However, it is believed that this Fe(III) reduction serves only as an electron sink and

is not involved in oxidative phosphorylation via proton translocation (1). In cell suspensions of strain MR-1 grown anaerobically with fumarate as the electron acceptor, there was a proton pulse when Fe(III) was provided as an electron acceptor (21). This is consistent with energy transduction via oxidative phosphorylation during dissimilatory Fe(III) reduction (21). However, other organisms, such as *Aquaspirillum magnetotacticum* and *Escherichia coli*, in which respiratory chains are not involved in Fe(III) reduction (24, 27), also translocate protons when Fe(III) is provided as an electron acceptor to cell suspensions (25).

In order to investigate mechanisms for Fe(III) reduction in GS-15, cells were grown in 1-liter vessels (4) in a previously described medium (pH 6.7) with acetate as the sole electron donor (14). Fe(III) or nitrate, supplied as Fe(III) citrate (ca. 50 mM) or sodium nitrate (20 mM), was used as the sole terminal electron acceptor. Ascorbic acid (0.04 g/liter) was added as a reducing agent to nitrate medium.

Cells were harvested by centrifugation (5,000 × g, 15 min, 4°C) and washed three times in N_2-sparged buffer A (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 10 mM $MgCl_2$, pH 7.4). Fe(III) reductase activity was assayed as previously described (5). The reaction mixture, which contained 10 mM Tris acetate buffer (buffer B, pH 8.1), 0.8 mM β-NADH, 0.5 mM ferrozine, 0.2 mM Fe(III) citrate, and 10 μM flavin mononucleotide, was placed in the main body of a Thunberg cuvette, and the cells (0.3 to 0.4 mg of protein, as determined by the assay of Lowry et al. [18]) were place in the side arm. The reaction cuvette was flushed with N_2 for 5 min, and then the reaction was initiated by mixing the assay solution with the contents of the side-arm. Complexation of reduced iron and ferrozine (ε_{562}, 28,000 · M^{-1} · cm^{-1}) was monitored over time at 562 nm with a recording, double-beam spectrophotometer with reaction mixture without cells in the reference beam. To assay nitrate reductase activity, cells were transferred with a syringe to stoppered serum bottles containing growth media (20 ml) supplemented with 2 or 20 mM sodium nitrate. The loss of nitrate over time was followed by analyzing samples by ion chromatography.

Fe(III) reductase. Fe(III)-grown cells had twice as much Fe(III) reductase specific activity [210 ± 14 nmol of Fe(II) produced per min per mg of protein; $n = 3$] as nitrate-grown cells [98 ± 8 nmol of Fe(II) produced per min per mg of protein; $n = 3$]. Fe(III) reduction was not detected when cell preparations were omitted or heated to 100°C for 5 min. Nitrate reductase activity (110 nmol of nitrate consumed per min per mg of protein) was detected only in cells cultured with nitrate as the sole terminal electron acceptor.

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TABLE 1. Effects of respiratory inhibitors on Fe(III) and NO_3^- reduction

Inhibitor	Site of inhibition	Concn $(M)^a$	Solvent	% Inhibition of:	
				Fe(III) reduction	NO_3^- reduction
HOQNO	Cytochrome b to cytochrome c	10^{-5}	Ethanol	0	0
NaCN	Cytochrome oxidase	10^{-3}	H_2O	0	100
NaN_3	Cytochrome oxidase	10^{-3}	H_2O	0	100
Rotenone	NADH dehydrogenase	10^{-4}	Acetone	30	0
O_2	Terminal reductase			100	100

^{a} Respiratory inhibitors (10 μl) were added to Fe(III) reductase or nitrate reductase assays from anaerobic concentrated stock solutions.

Exposure of cells to air for 5 min irreversibly inhibited Fe(III) and nitrate reductase activities (Table 1). Of the various potential metabolic inhibitors tested, only rotenone inhibited Fe(III) reduction. Sodium cyanide and sodium azide completely inhibited nitrate reduction but not Fe(III) reduction. This finding and the fact that nitrate reduction was expressed only in nitrate-grown cells indicate that the Fe(III) reductase is a different enzyme than the nitrate reductase.

In order to localize the site for Fe(III) reduction, washed cells were broken by three passes through a French pressure cell at 18,000 lb/in² and collected under N_2. All subsequent manipulations were made under an N_2 atmosphere. The suspension was centrifuged (5,000 × g, 30 min, 4°C) to remove unbroken cells. The supernatant was removed and centrifuged (200,000 × g, 2 h, 4°C). The resultant supernatant, which contained the soluble proteins, had no Fe(III) reductase activity when it was assayed as described above for whole cells. The pelleted membranes were washed in 200 volumes of buffer A, centrifuged (200,000 × g, 1 h, 4°C), and resuspended in 5 volumes of buffer A. This membrane fraction actively reduced Fe(III) [280 nmol of Fe(II) produced per min per mg of protein]. Association of the Fe(III) reductase with a semipermeable membrane is consistent with its role in energy transduction. In previous studies with organisms which reduce Fe(III) for assimilatory purposes, the Fe(III) reductases have been found to be either membrane bound (5, 7, 19) or soluble (6, 24, 27).

Cytochrome b. In order to determine their cytochrome content, intact cells were washed and suspended to a cell density of about 10^9 cells per ml in buffer A. With both Fe(III)-grown cells (Fig. 1, spectrum 1) and nitrate-grown cells (Fig. 2, spectrum 1) the difference spectra, which were generated by placing aerated cell suspensions in the reference beam and dithionite-reduced samples in the sample beam of the double-beam spectrophotometer, had absorption maxima at 558, 530, and 430 nm. Low-temperature (77 K) spectra gave the same absorption maxima (data not shown). These absorption maxima are characteristic of type b cytochromes in other prokaryotes (26).

The type b cytochrome in GS-15 was reduced when cell suspensions were placed under H_2. To investigate the possible reoxidation of cytochromes by Fe(III) or nitrate, 500 μl of a 4 mM solution of Fe(III) citrate or sodium nitrate was placed in the sidearm of a Thunberg cuvette containing intact cells in the lower chamber. The control cuvette contained intact cells and 500 μl of buffer A in the sidearm. After the cuvettes were sparged with H_2, the contents of the sidearms were mixed with the cells, and the cuvettes were immediately placed in the spectrophotometer with the control cuvette in the reference beam. When Fe(III) was added to Fe(III)-grown cells, absorption minima developed at the wavelengths characteristic of the type b cytochrome (Fig. 1,

spectrum 2). This suggested that the Fe(III) reductase could accept electrons from the type b cytochrome. Addition of Fe(III) also oxidized the type b cytochrome in nitrate-grown cells (Fig. 2, spectrum 2), which was consistent with significant Fe(III) reductase activity in these cells. Nitrate oxidized the type b cytochrome in nitrate-grown cells (Fig. 2 spectrum 3) but not in Fe(III)-grown cells, which lacked nitrate reductase activity (Fig. 1, spectrum 3). Inhibitors of nitrate reduction (sodium azide and sodium cyanide) prevented oxidation of the type b cytochrome by nitrate (Fig. 2, spectrum 4) but had no effect on Fe(III) oxidation of the cytochrome (Fig. 1, spectrum 4). Brief exposure of the cell suspension to air completely inhibited Fe(III) and nitrate
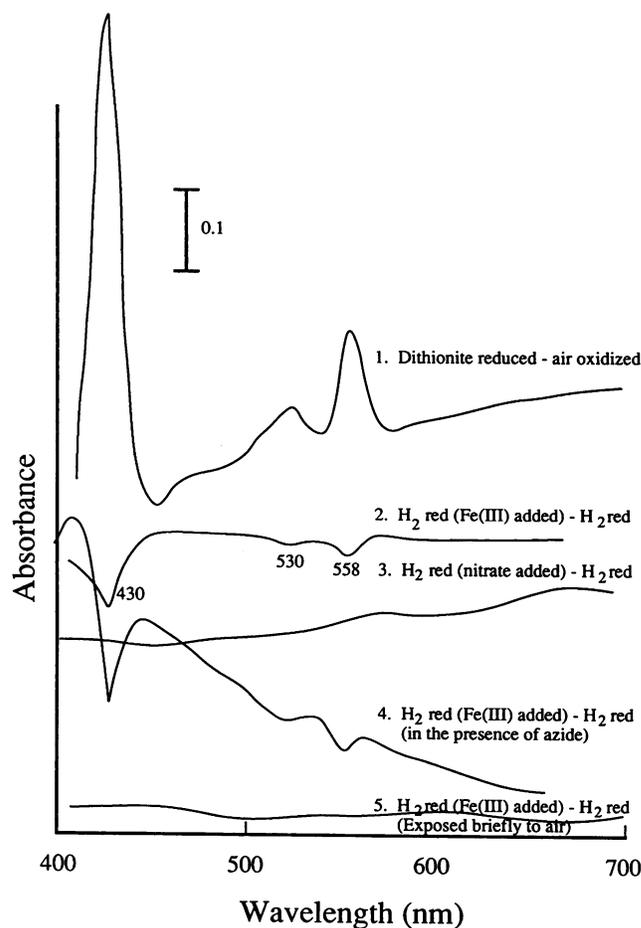

FIG. 1. Difference spectra for cells grown with Fe(III) as the electron acceptor. Abbreviation: red, reduced.
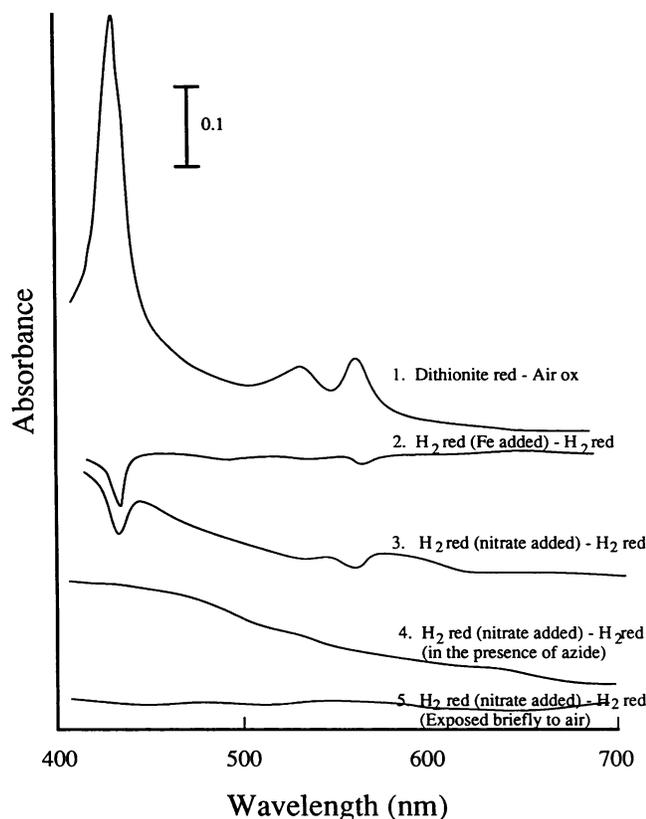
FIG. 2. Difference spectra for cells grown with nitrate as the electron acceptor. Abbreviations: red, reduced; ox, oxidized.

Spectrum labels:
1. Dithionite red - Air ox
2. H$_2$ red (Fe added) - H$_2$ red
3. H$_2$ red (nitrate added) - H$_2$ red
4. H$_2$ red (nitrate added) - H$_2$ red (in the presence of azide)
5. H$_2$ red (nitrate added) - H$_2$ red (Exposed briefly to air)

oxidation of the type b cytochrome (Fig. 1, spectrum 5, and Fig. 2, spectrum 5), which is consistent with the inhibition of Fe(III) reductase and nitrate reductase activities by air exposure. Rotenone, which partially inhibited Fe(III) reduction, precipitated at a concentration of 10^{-3} M and consequently interfered with cytochrome analyses.

No type c cytochromes were involved in Fe(III) or nitrate reduction, as shown by the lack of type c cytochrome spectra and by the lack of inhibition of Fe(III) reduction by 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO) (Table 1), which blocks electron transport between cytochrome b and cytochrome c (8). This is in contrast to HOQNO inhibition of Fe(III) reduction in aerobically grown cells of *S. putrefaciens* 200, which in addition to a cytochrome b contains type c and d cytochromes (1, 2, 22, 23).

These results indicate that electron transport in the dissimilatory Fe(III)-reducing microorganism GS-15 is mediated by an electron transport chain which includes a type b cytochrome and a membrane-bound Fe(III) reductase that is distinct from the dissimilatory nitrate reductase. This is the first description of a mechanism for electron transport to Fe(III) in cells known to obtain energy from Fe(III) reduction.

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