Enzymatic reduction of iron oxides by anaerobic bacteria

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Dissimilatory microbial iron(III)-reduction has been proposed as an important chemical change that takes place in the development of certain anaerobic soils and sediments. Several studies have demonstrated that iron(III)-reduction accounts for the oxidation of 35 to 65% of organic matter in anaerobic sediments (Lovley, 1991). Microbial oxidation coupled to iron(III)-reduction is an important natural mechanism for contaminant removal from groundwater or shallow aquifers polluted with landfill leachates (Albrechtsen and Christensen, 1994). Futhermore, iron(III)-reduction can influence the biogeochemical cycles of important nutrients such as nitrogen, carbon, sulphur and phosphorous (Lovley, 1991). Up to now, numerous iron(III)-reducing bacteria have been described in the literature (Lonergan *et al.*, 1996). These bacteria are versatile towards electron donors and acceptors which can be used for growth. Although iron(III)-reducing bacteria are studied for many years, little is known about the biochemistry and the mechanism of iron(III)-reduction. Iron(III)-reducing bacteria are able to utilize Fe(III) either chelated or present in minerals such as ferrihydrite, goethite and others. Important minerals such as magnetite, siderite and vivianite are formed during the activity of those bacteria. The question arises whether Fe(III) is taken up and reduced in the periplasm or cytoplasm or whether electrons are transferred to Fe(III) of the minerals. Evidence has been presented that direct contact between the cells and the metal oxide is necessary for generation of energy (Arnold *et al.*, 1988). Our experimental data obtained with *Geobacter sulfurreducens* indicate that a peripheral *c*-type cytochrome localized on the outside of the outer membrane is the terminal reductase of the iron oxide respiration.

Methods

Geobacter sulfurreducens was cultivated with Fe(III)-citrate (50 mM) and acetate (30 mM) in a medium composed as described (Lovley and Phillips, 1993). Cell harvest and preparation of subcellular fractions were carried out under anaerobic condi-

TABLE 1. Iron(III) reductase activity in different fractions of *Geobacter sulfurreducens*

Starting material	Treatment	Fraction	Protein (mg)	Iron(III)-reductase activity [a] specific activity (mU/mg)	total activity (mU)	%
Crude extract	ultracentrifugation	soluble	8 ± 1	10 ± 1	73 ± 5	17
		membranes	60 ± 1	6 ± 1	362 ± 6	83
Membranes	none	membranes	6 ± 1	6 ± 1	36 ± 6	100
	KCl + ultracentr.	soluble	4 ± 1	1.4 ± 1	5 ± 1	35
		membranes	2 ± 1	4.5 ± 1	10 ± 1	65
Whole cells	centrifugation	soluble	2 ± 1	0	0	0
		cells	2 ± 1	17 ± 1	37 ± 2	100
	KCl + centrifug.	soluble	4 ± 1	2 ± 1	8 ± 1	34
		cells	3 ± 1	6 ± 1	15 ± 2	66

[a] Reactions were performed in HEPES buffer 50 mM, pH 7, containing 0.3 mM Fe(III)-NTA, 0.5 mM Ferrozine, and 1 mM NADH. 1 mU corresponds to 1nmol Fe(II) formed per minute.

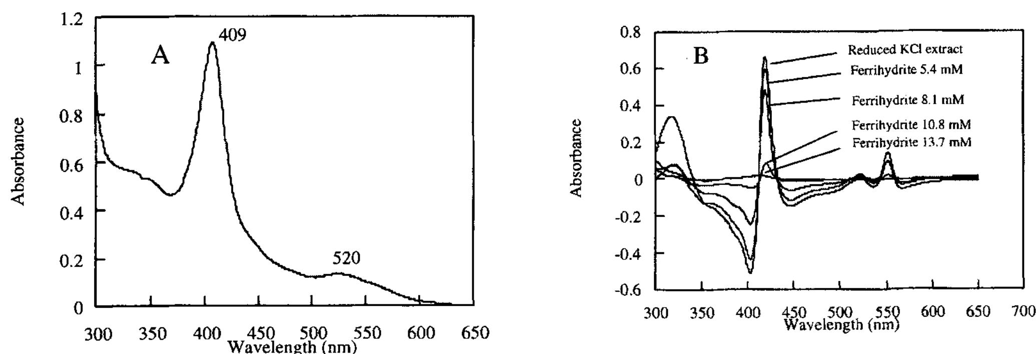

FIG. 1. UV-visible spectrum of KCl extract obtained from whole cells of *Geobacter sulfurreducens*. 1A: oxidized KCL extract. 1B: disappearence of redox difference spectrum after addition of increasing amounts of ferrihydrite.

tions. To prepare cellular fractions a protocol adapted from a method described by Myers and Myers (1992) was used. Iron(III)-reductase activity was assayed anaerobically in 1-cm cuvettes by photometric monitoring the appearance of Fe(II) over time in the presence of ferrozine. The iron(III)-reductase was extracted by stirring a cell suspension for one hour in a buffer containing 0.5 M KCl, Tris 100 mM, pH 7.6. UV-visible spectra of KCl extracted proteins were recorded on a U-2000 spectrophotomter (Hitachi, Tokyo, Japan) in a 1 cm quartz cuvette containing 0.1 to 0.3 mg protein/ml. To record redox difference spectra of KCl extract, the protein solution was reduced with sodium dithionite and subsequently reoxidized by adding increasing amount of ferrihydrite to the reaction mixture.

Results and discussion

Table 1 presents the distribution of the iron(III)-reductase in subcellular fractions of *Geobacter sulfurreducens*. 83 % of the iron(III)-reductase was found in the membrane fraction. The enzyme could be solubilized by KCl treatment of the membrane fraction of *Geobacter sulfurreducens*. Indications for a localization of iron(III)-reductase in the outer membrane were obtained with the solubilization of iron(III)-reductase activity from whole cells by the KCl treatment. This treatment allows the detachment of peripheral outer membrane proteins and not the detachment or release of proteins from the cytoplasm. Microscopic examination of cells shows that they were not lysed after this treatment. The UV-visible spectrum of the KCl extract obtained from whole cells exhibited a strong peak at 409 nm and shoulders centered at 520 nm. These features are characteristic for hemoproteins (Fig. 1A). Redox difference spectra of this sample exhibited a Soret band at 420 nm and β and α bands at 522 and 552 nm, respectively, all which are characteristic for *c*-type cytochromes. Addition of increasing amount of ferrihydrite to the cuvette containing the dithionite reduced cytochrome led to the disappearance of this spectrum (Fig. 1B).

Geobacter sulfurreducens is able to grow on ferrihydrite to give magnetite within 6 days. The fact that ferrihydrite was able to reoxidize the *c*-type cytochrome obtained by KCl extraction of whole cells suggest that this cytochrome is involve in the reduction of iron oxides and is probably even the terminal reductase.

Conclusions

The results obtained indicate that the terminal iron(III) reductase of *Geobacter sulfurreducens* is a *c*-type cytochrome localized on the outside of the outer membrane as a peripheral enzyme. This finding is in agreement with the use of insoluble iron oxides as electron acceptor by Fe(III)-reducing bacteria.

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