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EXPERIMENTAL ARTICLES

Characterization of Membrane-Bound Fe(III)–EDTA Reductase Activities of the Thermophilic Gram-Positive Dissimilatory Iron-Reducing Bacterium *Thermoterrabacterium ferrireducens**

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Abstract—Whole-cell suspensions of *T. ferrireducens* reduced Fe(III) citrate, Fe(III)–EDTA, and ferrihydrite with glycerol as an electron donor. After cell disruption, the highest activity was registered with Fe(III)–EDTA as the electron acceptor and NADH or NADPH as electron donors. About 80% of the NAD(P)H-dependent Fe(III)–EDTA reductase activities were associated with the membrane fraction of the cells. Treatment of the membranes with lauryl maltoside led to complete solubilization of the NADH-dependent and 70% solubilization of the NADPH-dependent Fe(III)–EDTA reductase activities. After purification by ion-exchange chromatography, the NADH-dependent activity was concentrated 8-fold, and the NADPH-dependent activity was concentrated 11-fold, with a yield of about 10% for both activities. The Fe(III)–EDTA-reducing enzyme complex included *c*-type cytochromes and a protein with a molecular mass of ca. 115 kDa, consisting of two polypeptides. This is the first description of membrane-bound Fe(III)-reducing oxidoreductase activities from a gram-positive dissimilatory Fe(III)-reducing bacterium.

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Key words: dissimilatory Fe(III) reduction, thermophilic microorganisms, gram-positive bacteria, Fe(III) reductase, *Thermoterrabacterium ferrireducens.*

Bacteria and archaea of different phylogenetic and physiological groups [1, 2] are capable of dissimilatory Fe(III) reduction. In spite of recent progress in understanding the electron transport pathways involved in Fe(III) reduction by dissimilatory iron reducers [3, 4], none of the proteins investigated so far has been proved to function as a terminal Fe(III) reductase in vivo. The biochemistry of dissimilatory Fe(III) reduction has been intensively investigated for representatives of two genera with a gram-negative cell wall type: *Shewanella* and *Geobacter.* The electron transfer chain of these microorganisms consists of various components, primarily *c*-type cytochromes (for a review, see [1]). The majority of the cytochromes described can reduce Fe(III) in vitro; however, it is unlikely that all of them function as terminal iron reductases in whole cells. Membrane-bound NADH-dependent enzyme complexes able to reduce Fe(III) have been characterized only for *G. sulfurreducens* [5, 6]. It should be noted that not all of the microorganisms capable of dissimilatory Fe(III) reduction obligatorily possess cytochromes.

Thermoterrabacterium ferrireducens is a thermophilic bacterium performing dissimilatory reduction of Fe(III); phylogenetically, it belongs to *Firmicutes* [7]. The microorganism is capable of organotrophic and lithoautotrophic growth with insoluble and soluble Fe(III) and uranium(VI) compounds, has a gram-positive cell wall surrounded by an S-layer, and contains *c*-type cytochromes [8, 9]. Since *T. ferrireducens* has no periplasm, the mechanism of Fe(III) reduction in this bacterium is expected to be different from that in gram-negative organisms. Presented in the current paper are data on various Fe(III)-reducing activities of *T. ferrireducens* and characterization of partially purified membrane-bound Fe(III)–EDTA reductase activities of the microorganism.

MATERIALS AND METHODS

Organism, cultivation conditions, and growth determination. *Thermoterrabacterium ferrireducens* strain DSM 11255^T was obtained from the German Collection of Microorganisms (Deutsche Sammlung von Mikroorganismen und Zellkulturen), Braunschweig, Germany. The organism was cultured in anaerobic medium [7] with glycerol (30 mM) as the carbon

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source and electron donor and Na fumarate (20 mM), ferric citrate (20 mM), or ferrihydrite (90 mM Fe(III)) as electron acceptors, or without acceptors. Ferrihydrite (low-crystalline Fe(III) oxide) was prepared by titrating a $FeCl_3 \cdot 6H_2O$ solution with 10% NaOH to pH 8.0. When cultivating with fumarate or without an acceptor, the medium was prereduced by the addition of 0.5 g/l of $Na_2S \cdot 9H_2O$. Growth was assessed by direct cell count under a MIKMED-1 phase-contrast-equipped light microscope (LOMO, Russia). In case of ferrihydrite-grown cells, the iron-containing precipitate was dissolved with a mixture of ammonium oxalate and oxalic acid [8].

Determination of enzyme activities. For the experiments with washed whole-cell suspensions, 2-l cultures in the late exponential phase of growth were harvested by microfiltration (membrane pore size, 0.2 μm) under a 100% N_2 atmosphere. The cells were washed anaerobically with 20 mM MES buffer (pH 6.5) and resuspended in a fresh cultivation medium devoid of organic compounds, electron donors, and acceptors to a final cell density of about 10^{10} cells/ml. Aliquots (0.10–0.15 ml) of the cell suspensions were added to 1 ml of a reaction mixture consisting of anaerobic medium with 30 mM glycerol as an electron donor and Fe(III)–EDTA (10 mM) or Fe(III) citrate (20 mM) or ferrihydrite (90 mM Fe(III)) as an electron acceptor. The reaction mixture was incubated for 48 h at 65°C, and the kinetics of Fe(III) reduction was followed by determination of the Fe(II) concentration in subsamples by dipyridyl assay [7]. Reaction mixtures lacking cells or an electron donor were used as negative controls. Activity with NAD(P)H as electron donors was analyzed in the presence of ferrozine by a modified assay [5]. The reaction mixture contained 0.2 mM NADH or NADPH as an electron donor, 1 mM ferrozine, and 0.5 mM Fe(III)–EDTA, 0.5 mM Fe(III) citrate, or 0.9 mM Fe(III) in the form of ferrihydrite as an electron acceptor in an N_2-saturated 100 mM HEPES buffer (pH 7.0) in an anaerobic (100% N_2) cuvette. The assay was started by adding a protein sample (0.005–0.5 mg of protein in volume of 0.1 ml). The production of Fe(II) over time at 65°C was spectrophotometrically monitored by changes in A_{562}. Reaction mixtures without protein samples or electron donors were used as negative controls. The reaction mixture for flavin reductase activity determination contained 0.2 mM NADH or NADPH as an electron donor, 0.15 mM FAD or FMN, and 0.1 mM $MgCl_2$ in an N_2-saturated 50 mM Tris–HCl buffer (pH 7.0). The assay, carried out anaerobically, was started by adding the protein sample; the oxidation of NAD(P)H and the reduction of flavins were monitored at 65°C by the decrease in A_{340} and A_{447}, respectively. Reaction mixtures without protein samples, with protein samples but without NAD(P)H, and with protein samples and NAD(P)H but without flavins were used as negative controls. In all activity evaluations, milliunits (mU) of activity were used, defined as the amount of enzyme that catalyses the reduction of 1 nmol of Fe(II) (or FAD or FMN) per min at 65°C. The specific activity (mU/mg) was determined as the total activity (in mU) of a preparation divided by its total protein content (in mg). Protein concentration was determined by the Lowry method [10].

Spectrophotometric determination of the oxidoreductase activity. This assay was based on rapid scanning of UV-visible absorbance spectra of the sample, which allows simultaneous determination of the redox state of cytochromes and of the amount of NAD(P)H oxidized upon the addition of Fe(III) to the reaction mixture. The reaction mixture in an anaerobic (N_2) cuvette contained 120 μl of a protein sample in 20 mM MES buffer, pH 6.8; 0.15 mM NADH or NADPH; and 0.1 mM Fe(III)–EDTA, 0.1 mM Fe(III) citrate, or 0.09 mM Fe(III) in the form of ferrihydrite as an electron acceptor. The redox state of the cytochromes was determined from the characteristic absorbance peaks at 420, 526 and 552 nm (reduced form) and at 410 nm (oxidized form). After the addition of NAD(P)H, the reaction mixture was incubated for 4–6 min, during which the cytochromes in the sample became completely reduced. Then, Fe(III) was added and the oxidation of cytochromes was judged from changes in the difference spectra of reduced versus oxidized cytochromes. Simultaneously, the NAD(P)H oxidation rate was determined from the decrease in the absorbance at 340 nm. All the activity measurements were made on UV-visible spectrophotometers (HP8453, Hewlett Packard, United States and Specord UV VIS, Carl Zeiss, Germany), equipped with temperature-controlled cuvette holders, at 65°C. The described spectrophotometric assay was also used for determination of reducing activity towards fumarate and sodium 9,10-anthraquinone-2,6-disulfonate (AQDS) in membrane and soluble fractions of *T. ferrireducens* cells. Fumarate or AQDS were added instead of Fe(III) to the reaction mixture to a final concentration of 0.5 mM, and the reduction of these compounds was judged from reoxidation of prereduced cytochromes.

Purification of Fe(III)–EDTA reductase activities. For purification of Fe(III)–EDTA reductase activities, a fumarate-grown culture from a fermentor was used. The volume of the medium was 200 l, and the volume of inoculum was 10 l. Fermentation was carried out in a batch mode. Cell growth was monitored by measuring optical density of the culture. Anaerobic conditions were controlled by automatic determination of dissolved oxygen concentration. Late-exponential-phase cells were harvested by centrifugation. The cell paste (150 g of wet biomass) was stored in a frozen state at –80°C until use for enzyme purification. All further manipulations with the biomass were carried out aerobically at room temperature. Cells were slowly

thawed and suspended in 20 mM MES buffer (pH 6.8) to a concentration of 0.1 g wet cells per ml of buffer. The suspension was centrifuged at 11 180 *g* for 10 min, and the pellet was resuspended in 100 mM Tris–HCl buffer (pH 7.8) to a cell density of 0.3 g wet cells per ml of buffer. To disrupt the cells, lysozyme was added to the suspension to a concentration of 1.5 mg/ml, and the mixture was incubated for 1.5 h with stirring at room temperature. The obtained suspension was homogenized and passed through a continuous high-pressure Cell Disruptor at 1 kbar. The cell extract, checked microscopically for the absence of whole cells, was centrifuged for 5 min at 450 *g* to discard coarse cell debris and mineral particles. The supernatant was then centrifuged at 30000 *g* for 15 min, yielding a pellet consisting of a red-brownish and an uncolored fraction. The supernatant and the uncolored pellet were discarded and the membrane-containing red-brownish pellet was resuspended in 20 mM MES buffer (pH 6.8) to a protein content of approximately 10 mg/ml. Membrane proteins were extracted from this suspension with lauryl maltoside. The detergent (from a 10% aqueous stock solution), solid KCl, and DNase were slowly added under stirring to concentrations of 1%, 0.5 M and 0.03 mg/ml respectively, and the extraction was carried out for 30 min at room temperature. Insoluble material was pelleted by ultracentrifugation for 30 min at 150 000 *g*. The supernatant (lauryl maltoside extract) was assayed for Fe(III)-reducing activity and total protein content and then desalted by concentrating it fivefold with a Centriprep YM-10 centrifugal filter device (Millipore Co.) and subsequently diluting it sixfold with 10 mM potassium phosphate buffer (pH 7.0) containing 0.05% lauryl maltoside. The desalted lauryl maltoside extract was filtered through a syringe filter with a 0.2-μm pore size and loaded onto a POROS HQ anionic exchange chromatography column (Amersham Biosciences) equilibrated with 10 mM potassium phosphate buffer (pH 7.0). Unbound proteins were collected as one fraction. Bound proteins were eluted with a linear gradient of NaCl (0 to 0.5 M). Fractions (1–2 ml) were collected automatically every 30 s to be assayed for Fe(III) reductase activity. Two more columns were used for further purification of the Fe(III) reductase activity obtained after chromatography on POROS HQ. The column with ceramic hydroxyapatite CHT 20 (BioRad) was equilibrated with 10 mM potassium phosphate buffer (pH 6.8); bound proteins were eluted with a 0.01–1 M potassium phosphate linear gradient. The column with DEAE-Sepharose (BioRad) was equilibrated with 50 mM Tris–HCl buffer (pH 8.0), and bound proteins were eluted with a linear gradient of 0 to 0.5 M NaCl. All column buffers contained 0.05 % (w/v) lauryl maltoside.

Gel electrophoresis. Purified proteins were analyzed by routine methods of nondenaturing and denaturing polyacrylamide gel electrophoresis (ND-PAGE and SDS-PAGE) using Amersham Biosciences Phast system equipment. 12.5% homogeneous and 4–15% and 8–25% gradient gels were used. Samples were preconcentrated 20- to 50-fold with Microcon YM-10 centrifugal filter devices (Millipore Co.), and 4 μl of the concentrate, containing up to 20 μg protein, was applied per lane. High-molecular-weight native PAGE markers (Pharmacia Biosciences) were used as standards. ND-PAGE gels were stained in situ, first for the presence of Fe(III) reductase activity and then for protein with Coomassie Brilliant Blue (according to the standard Coomassie Blue protocol for Phast system by Amersham Biosciences). The method of in-gel staining for iron-reductase activity was modified from [11]. Staining was performed at 65°C in an N_2 atmosphere; all the buffers and stock solutions were presaturated with N_2. The active bands were excised from the native gel and extracted for ca. 12 h with 100 mM HEPES buffer (pH 7.0) containing 0.05% lauryl maltoside. The extracts were concentrated 20-fold with Microcon YM-10 and applied onto 12.5% SDS gels. The same gels were used for hemoprotein staining by the method described in [12]. Horse heart cytochrome *c* and cytochrome *cd*1 from *Paracoccus denitrificans* (4 μg of protein per gel lane) were used as positive controls. After performing heme-staining, the gels were stained with Coomassie Brilliant Blue.

Table 1. Fe(III)–EDTA, Fe(III) citrate, and ferrihydrite-reducing activity (mU/mg protein) of suspensions of *T. ferrireducens* whole cells grown with different electron acceptors and glycerol as the electron donor

Fe(III) form	Cultivation conditions				
	without acceptor	fumarate	ferric citrate	Fe(III)–EDTA	ferrihydrite
Ferrihydrite	37	60	33	19	86
Ferric citrate	4	2	3	0	0
Fe(III)–EDTA	3	2	5	1	0

Note: Protein and activity determinations were performed in triplicate. Mean values are presented.

RESULTS

Fe(III)-reducing activity of whole cells. Washed cell suspensions of *T. ferrireducens* grown with a range of electron acceptors reduced soluble ferric citrate, Fe(III)–EDTA, and insoluble ferrihydrite at different rates (Table 1). With glycerol as the electron donor, all the cell suspensions exhibited the highest Fe(III) reducing activity with ferrihydrite; the activity with soluble Fe(III) forms was significantly lower. Not only cells grown with Fe(III), but also cells grown with fumarate as an electron acceptor and cells grown without any electron acceptor possessed iron-reducing activity.

Table 2. Distribution of Fe(III)–EDTA reductase activity among cellular fractions of *T. ferrireducens*

Fraction	Protein, mg	NADH-dependent activity			NADPH-dependent activity		
		specific, mU/mg protein	total, mU	%	specific, mU/mg protein	total, mU	%
Whole vs. disintegrated cells							
Whole cells	4060	0.0	0	0	0.0	0	0
Disintegrated cells[a]	4172	32.92	137336	100	52.07	217205	100
Soluble vs. membrane fraction							
Soluble fraction	310	43.07	13361	21	27.27	8460	12
Membrane fraction	1942	53.67	49734	79	71.18	64309	88
Soluble vs. insoluble fraction of lauryl maltoside extract of membranes							
Soluble extract fraction	250	20.33	5079	100	19.93	4977	70
Insoluble extract fraction	100	0.0	0	0	21.8	2180	30

Note: Protein and activity determinations were performed in triplicate. Mean values are presented.
[a] After consecutive lysozyme and high pressure treatments of the cells.

Table 3. Purification of Fe(III)–EDTA reductase activities from the soluble fraction of lauryl maltoside extract of *T. ferrireducens* cell membranes

Preparation	Protein, mg	NADH-dependent activity			NADPH-dependent activity		
		specific[a], mU/mg	total, mU	yield, %	specific[a], mU/mg	total, mU	yield, %
Lauryl maltoside extract	148.3	20.5	3037.6	100	16.7	2468.9	100
POROS HQ	81.6	63.2	1923.7	63	36.7	1368.5	55
Hydroxyapatite CHT and DEAE-Sepharose[b]	7.6	162.5	317.7	11	181.4	228.1	9

Note: Protein and activity determinations were performed in triplicate. Mean values are presented.
[a] Specific activity is given for the fractions with the highest NADH- or NADPH-dependent activities.
[b] Values presented were obtained after the last chromatography column, with DEAE-Sepharose.

Since it is difficult to separate cells grown with Fe(III) from insoluble iron compounds which are adsorbed by the cell surface, fumarate-grown cells were used for enzyme purification.

Distribution of the Fe(III)-reducing activities among cell fractions. Crude cell extracts obtained after cell disruption were unable to reduce ferric citrate, Fe(III)–EDTA, or ferrihydrite with glycerol as an electron donor. When NADH or NADPH was used as the donor, the highest rate of Fe(III) reduction was recorded with Fe(III)–EDTA (52 mU/mg). With ferric citrate, the iron-reducing activity was 7 mU/mg. About 80% of the NAD(P)H-dependent Fe(III)–EDTA reductase activity was associated with the membrane fraction of the cells (Table 2). Although we did detect a low NADH-dependent ferrihydrite-reducing activity (ca. 3.5 mU/mg) in the membrane fraction of the cells, we were unsuccessful in developing a reliable method for determination of Fe(III)-reducing activity with NAD(P)H and ferrihydrite due to the high light absorbance of this insoluble compound, interfering with colorimetric monitoring of NAD(P)H concentration, and changes in the reaction conditions caused by the formation of chelated Fe(III) and Fe(II) compounds upon the addition of Fe(II)-capture reagents (ferrozine and *ortho*-phenanthroline) to the reaction mixture, as well as due to the high rate of the reaction, precluding accurate estimation of its kinetic parameters by determination of Fe(II) in subsamples with dipyridyl.

Solubilization and purification of membrane-bound NADH- and NADPH-dependent Fe(III)–EDTA reductase activities. Treatment of the membranes with the nonionic detergent lauryl maltoside led to complete solubilization of the NADH-dependent

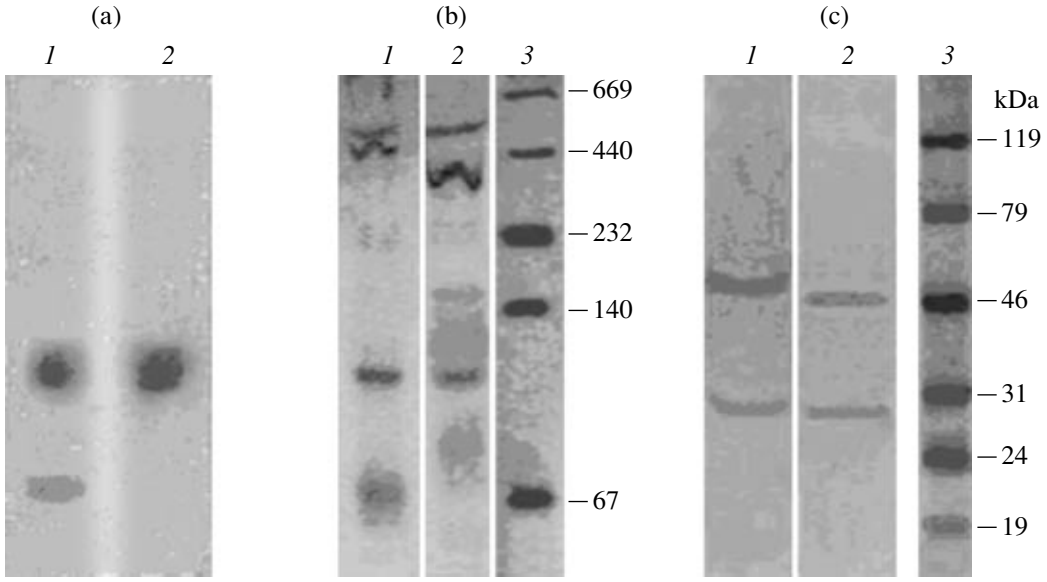

Fig. 1. Analysis of purified Fe(III)–EDTA reductase activities by gel electrophoresis. (a, b) Nondenaturing PAGE of purified Fe(III)–EDTA reductase activities. The gels were consecutively stained (a) with ferrozine for Fe(III) reductase activity and (b) with Coomassie Blue for protein. (c) 12.5% SDS-PAGE of activity bands (with a molecular mass of ca. 115 kDa) from the native gel. For all the gels, lane *1* is the fraction with a high NADPH-dependent and low NADH-dependent activity, lane *2* is the fraction with a high NADH-dependent and low NADPH-dependent activity, and lane *3* represents molecular weight markers for nondenaturing or SDS-PAGE. The molecular masses are indicated in kDa.

Fe(III)–EDTA reductase activity and 70% solubilization of NADPH-dependent Fe(III)–EDTA reductase activity (Table 2). The obtained preparations with Fe(III)–EDTA reductase activities were subsequently purified using POROS HQ, CHT hydroxyapatite, and DEAE-Sepharose ion exchange chromatography (Table 3). Most of the collected chromatographic fractions possessed both NADH- and NADPH-dependent Fe(III)–EDTA reductase activities but in varying ratios. Some of the fractions eluted after DEAE-Sepharose chromatography possessed exclusively NADH-dependent activity (elution at 121–134 mM NaCl) or exclusively NADPH-dependent activity (elution at 150–177 mM NaCl). After the last purification step, the NADH-dependent Fe(III)–EDTA reductase activity was enriched 8-fold, and the NADPH-dependent Fe(III) reductase activity was enriched 11-fold (compared to the activity of the lauryl maltoside extract of membranes), with a yield of ca. 10% for both activities.

ND- and SDS-PAGE. Native PAGE of the purified fractions possessing Fe(III)–EDTA reductase activities revealed the presence of several proteins (Fig.1). In-gel staining for Fe(III)–EDTA reductase activity showed a single activity band with a molecular mass of about 115 kDa for NADH-dependent Fe(III) reductase activity and two activity bands (ca. 70 and 115 kDa) for NADPH-dependent Fe(III) reductase activity. No detectable activity was observed in gel assays when Fe(III) citrate was used as the electron acceptor. Staining of the gels for hemoproteins was negative presumably owing to the low concentration of cytochromes. SDS-PAGE analysis of the high molecular weight activity bands of the native gel (115 kDa) revealed that each of them was represented by at least two polypeptides with molecular masses of 30 and 50 kDa for the NADH-dependent activity and 30 and 53 kDa for the NADPH-dependent activity (Fig. 1). Our attempts to analyze the 70 kDa band from the native gel in the same way were unsuccessful, presumably due to the low protein concentration in this fraction or to its higher sensitivity to denaturation.

Biochemical properties of the purified Fe(III)–EDTA reductase activities. Fractions with the highest NADH- or NADPH-dependent Fe(III)–EDTA reductase activities (160–180 mU/mg), obtained after the last purification step, possessed NADH- and NADPH-dependent ferric citrate reductase (41 and 73 mU/mg) and flavin reductase (81 and 60 mU/mg) activities. The addition of FMN and horse heart cytochrome *c* was required for developing NAD(P)H-dependent Fe(III)–EDTA reductase activities in native gels. Fe(III)–EDTA reductase activities of intact purified fractions were detected without the addition of these cofactors. The temperature optimum of both NADH- and NADPH-dependent activities was about 70°C. At temperatures above 85°C, thermal inactivation occurred, and below 40°C, the activity was low (Fig. 2).

Spectrophotometric studies of NAD(P)H-dependent Fe(III)–EDTA reductase activities. The studies of UV-visible absorbance spectra revealed the presence of cytochromes in all of the chromatographic fractions exhibiting Fe(III)–EDTA reductase activity. The spec-

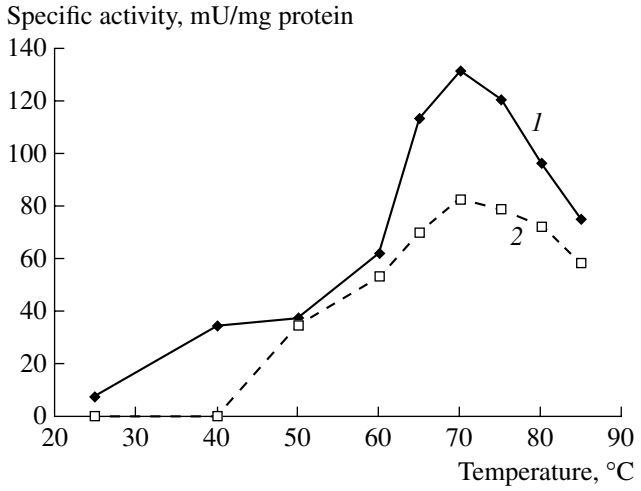

Fig. 2. Effect of temperature on (*1*) NADH-dependent and (*2*) NADPH-dependent Fe(III)–EDTA reductase activities. Presented are data for the preparations obtained after ion exchange chromatography on POROS HQ, hydroxyapatite CHT, and DEAE-Sepharose. Activity determinations were performed in triplicate. Mean values are presented.

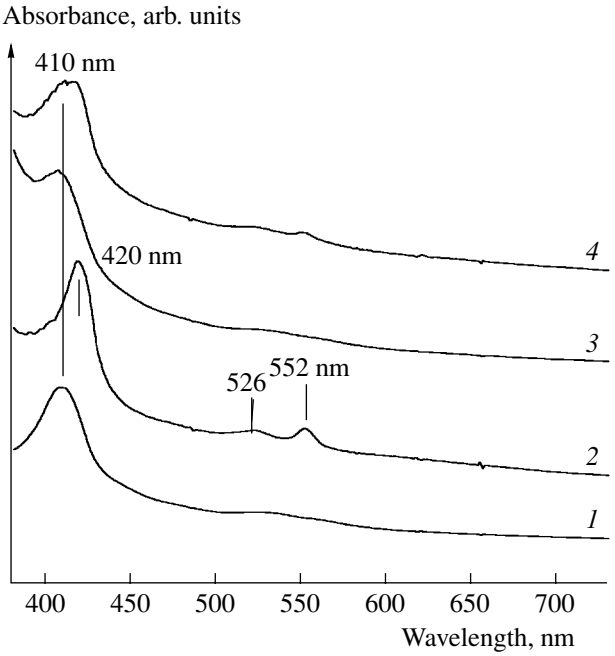

Fig. 3. Spectrophotometric characterization of the Fe(III)–EDTA reductase activity. Shown are spectra for one of the active membrane-associated fractions obtained after POROS HQ chromatography. Spectra (*1*) of oxidized (as it was isolated) fraction with a NADH-dependent iron-reductase activity of 63.2 mU/mg protein and a NADPH-dependent activity of 36.7 mU/mg protein and of the same sample (*2*) incubated anaerobically for 7 min at 65°C with 0.15 mM NADH and then (*3*) for 20 s at 65°C with 0.1 mM Fe(III)–EDTA and (*4*) of the fraction sample incubated anaerobically for 7 min at 65°C with 0.15 mM NADH and then for 5 min at 65°C with ferrihydrite containing 0.09 mM Fe(III). Essentially the same results were obtained with NADPH as a reductant instead of NADH.

tra, measured in different fractions obtained after POROS HQ ion exchange chromatography, exhibited a strong absorbance peak at 410 nm and broad bands at 525 and 565 nm. After reduction with dithionite, NADH, or NADPH, the absorption maxima were observed at 420, 526 and 552 nm, which corresponded to the γ-, β-, and α-bands of *c*-type cytochromes. The addition of either Fe(III)–EDTA or ferrihydrite to the NADH- or NADPH-reduced samples led to the oxidation of cytochromes in them (Fig. 3). With Fe(III)–EDTA, complete oxidation was observed, whereas with ferrihydrite, the cytochromes remained 30% reduced. Spectrophotometric analysis of NAD(P)H-reduced membrane fractions showed no cytochrome oxidation with fumarate or AQDS (synthetic analogue of humic acids) as potential electron acceptors; on the contrary, the addition of fumarate or AQDS to soluble fractions of *T. ferrireducens* cells led to rapid and complete oxidation of cytochromes (data not shown).

Growth with Fe(III)–EDTA. *T. ferrireducens* grew on a medium containing 5 mM Fe(III)–EDTA and glycerol as a substrate in at least three consecutive 5% transfers. During the growth, Fe(III) chelated by EDTA was reduced to Fe(II), as observed, in particular, by the disappearance of the yellow coloration of the medium. The amount of Fe(II) formed was about 2 mmol/l, as detected with dipyridyl or ferrozine. Determination of higher Fe(II) concentrations by these methods in the described system is problematic, probably because of the high initial EDTA concentration, leading to the competition between the ligands for Fe(II). Presence of Fe(III)–EDTA did not increase the growth rate and the cell yield of *T. ferrireducens* as compared to its growth in medium lacking this Fe(III) compound (data not shown).

DISCUSSION

As a result of the investigations performed, we have elucidated the following facts. (i) Whole cell suspensions of *T. ferrireducens* reduce soluble ferric citrate, Fe(III)–EDTA, and insoluble ferrihydrite with glycerol as the electron donor. The maximum Fe(III) reduction rate is observed with ferrihydrite. (ii) Upon cell disruption, Fe(III)-reducing activity vanishes. When NADH or NADPH are used as the electron donors, the highest iron-reducing activity in disrupted cells is registered with Fe(III)–EDTA. (iii) About 80% of the NADH- and NADPH-dependent Fe(III)–EDTA reductase activities are associated with the membrane fraction of the cells. These activities can be solubilized and purified. (iv) The Fe(III)–EDTA-reducing enzyme complex includes *c*-type cytochromes and a protein with a molecular mass of ca. 115 kDa, consisting of two polypeptides.

Data available on the biochemical mechanisms of dissimilatory iron reduction by microorganisms possessing a cell wall different from the gram-negative type are so far scarce. There is a single report on NAD(P)H-dependent reduction of soluble Fe(III) by a membrane fraction of the gram-positive *Clostridium beijerinckii*; however, attempts to solubilize this enzymatic activity were unsuccessful [13]. Information on the biochemistry of Fe(III) reduction by thermophilic prokaryotes is limited to the extensive characterization of the iron reductase of the archaeon *Archaeoglobus fuldigus*, belonging to NAD(P)H:FMN oxidoreductases, the function of which, assimilatory or dissimilatory, remains unclear [14], and to preliminary characterization of the dissimilatory iron-reductase activity of the archaeon *Pyrobaculum islandicum*, not related to *c*-type cytochromes [15]. Like mesophilic gram-negative dissimilatory iron-reducers of the genera *Shewanella* and *Geobacter, T. ferrireducens* also employs *c*-type cytochromes to transfer electrons for Fe(III) reduction. The differences between the Fe(III) reductase activities of *T. ferrireducens* and the previously described membrane-bound iron reductase activities of *G. sulfurreducens* [5, 6] consist in preferential utilization of Fe(III)–EDTA as the electron acceptor by the former and in the presence in *T. ferrireducens* of two activities, NADH- and NADPH-dependent. The requirement for horse heart cytochrome *c* and FMN addition for in-gel determination of the iron-reductase activity shows that the components of the *T. ferrireducens* Fe(III)-reducing complex are not tightly bound to each other and can dissociate during electrophoresis.

The physiological role of the membrane-bound NAD(P)H-dependent Fe(III)–EDTA–reductase activities, characterized in the present work, in the metabolism of *T. ferrireducens* remains unclear. Our experiments on the cultivation of *T. ferrireducens* with Fe(III)–EDTA as the terminal electron acceptor demonstrated that the reduction of this compound does not stimulate the growth of the microorganism. In vivo, the membrane-bound Fe(III)-reducing oxidoreductases that we purified are likely to be, rather than terminal reductases, a part of the electron transfer chain operating during Fe(III) reduction. It is also possible that, inside the cell, they interact with soluble Fe(III) that is chelated with some ligands similar in their chemical properties to EDTA.

Thus, the results of our study show that thermophilic gram-positive bacteria possess membrane-bound Fe(III)-reducing oxidoreductase activities, which can be solubilized and partially purified. The investigation of such stable soluble enzyme complexes could facilitate further understanding of Fe(III) reduction mechanisms in dissimilatory iron-reducers.

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