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Membrane-associated redox activities in *Thermotoga neapolitana*

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Abstract Elemental sulfur reduction by the hyperthermophilic bacterium *Thermotoga neapolitana* provides an alternative to hydrogen evolution during fermentation. Electrons are transferred from reduced cofactors (ferredoxin and NADH) to sulfur by a series of unknown steps. One enzyme that may be involved is an NADH:methyl viologen oxidoreductase (NMOR), an activity that in other fermenting organisms is associated with NADH:ferredoxin oxidoreductase. We found that 83% of NMOR activity was contained in the pellet fraction of cell extracts subjected to ultracentrifugation. This pellet fraction, presumably containing cell membranes, was required for electron transfer to NAD^+ from ferredoxin-dependent pyruvate oxidation. However, the NMOR activity in this fraction used neither *Thermotoga* nor clostridial ferredoxins as substrates. NMOR activity was also detected in aerobically prepared vesicles. By comparison with ATPase activities, NMOR was found primarily on the cytoplasmic face of these vesicles. During these studies, an extracytoplasmic hydrogenase activity was discovered. In contrast to the soluble hydrogenase, this hydrogenase activity was completely inhibited when intact cells were treated with cupric chloride and was present on the extracytoplasmic face of vescides. In contrast to a soluble hydrogenase reported in *Thermotoga maritima*, this activity was air-stable and was inhibited by low concentrations of nitrite.

Key words Hyperthermophile · Hydrogenase · Oxidoreductase · Ferredoxin · Membrane

Introduction

The reduction of sulfur compounds is central to energy conservation among many hyperthermophiles. Elemental sulfur or polysulfides serve as the terminal electron acceptor for sulfur respiration among several crenarchaeotes [for a review, see Maier (1996)]. The growth of many heterotrophic hyperthermophiles including *Pyrococcus furiosus* and *Thermotoga maritima* is enhanced by the reduction of sulfanes, but the reason for this has been elusive. For the archaeon *P. furiosus*, growth yield measurements suggest that polysulfide may provide an alternative means for energy conservation (Schicho et al. 1993). However, sulfur reduction does not allow the bacterium *T. maritima* to use glucose more efficiently, nor are the relative amounts of its fermentation products altered (Schröder et al. 1994). This finding is consistent with the proposal that in *Thermotoga*, sulfur reduction provides an alternative to production of growth-limiting quantities of hydrogen and so indirectly stimulates growth (Huber et al. 1986).

We are studying the process of electron transfer to sulfur reduction in *Thermotoga neapolitana*, a close relative of *T. maritima* (Belkin et al. 1986). We have found that this organism uses an NADH:polysulfide oxidoreductase as its primary sulfur reductase (Childers and Noll 1994, 1995a) while its hydrogenase, which also catalyzes sulfur reduction (Ma et al. 1993), provides a relatively lower level of sulfur reductase activity. During the course of these studies, we have found other oxidoreductase activities including glycerol dehydrogenase, NADH oxidase, and an NADH:methyl viologen oxidoreductase (NMOR) [Childers and Noll (1995b) and Childers (1997) unpublished work]. The latter activity is usually ascribed to NADH:ferredoxin oxidoreductase in other organisms. If this is its function, then it is an important intermediate in providing electrons for sulfur reduction. Our interest in NMOR was further heightened by our finding that much of its activity is associated with the cell membrane fraction of cell extracts, as described here. We began exploring membrane-associated oxidoreductase activities and found hydrogenase activity there as well. These results raise new questions regarding the pathways of electron transfer in this organism.

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Materials and methods

Growth of bacterial cultures

T. neapolitana strain NS-E was routinely grown at 77°C in a marine medium with 0.4% (w/v) glucose and 0.1% (w/v) starch as carbon sources and 0.25% (w/v) sodium thiosulfate (Childers et al. 1992). *Clostridium pasteurianum* (ATCC 6013) was grown under nitrogen in a medium containing per 1,000 ml: 10 g tryptone, 10 g yeast extract, 15 g dextrose, and 2 g K_2HPO_4 adjusted to pH 7.0. For both organisms, unless otherwise indicated, cells were harvested under anoxic conditions. Cells were harvested by centrifugation, washed in buffer, and stored under nitrogen.

Preparation of cell extracts

T. neapolitana cells were broken by sonication (Branson Sonifier 450; 15 W) under a stream of nitrogen for 70 s, followed by cooling on ice for 2 min. This procedure was repeated three times. Inside an anaerobic chamber, the broken cells were transferred to centrifuge tubes and debris were removed by centrifugation at 17,500 × *g* for 45 min at 4°C. The resulting supernatant (crude extract) was subjected to ultracentrifugation at 100,000 × *g* for 1 h at 24°C. The pellet was resuspended in anoxic buffer. *C. pasteurianum* cell extracts were prepared by procedures similar to those used for *T. neapolitana* except that the extracts were kept on ice at all times and ultracentrifugation was performed at 4°C. Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Enzyme activity measurements

NMOR activity in *T. neapolitana* was determined at 60°C. The NADH-dependent reduction of methyl viologen ($\varepsilon = 8250\ M^{-1}cm^{-1}$) was measured at 600 nm. This and all spectrophotometric measurements were made with a Beckman DU-68 spectrophotometer equipped with a Kinetics Soft-Pac module or a Zeiss PM4 photometer, each with heated cuvette holders. The reactions were carried out in 2-ml glass cuvettes. The 1-ml reaction mixture contained 2 mM methyl viologen, 20 μM FAD, 0.25 mM NADH, and 1–3 μg protein in 50 mM Tris-HCl buffer (pH 8.7). Cuvettes were sealed with red rubber serum stoppers and were preheated for 10 min at 60°C in a water-jacketed cuvette holder. One unit of NMOR activity catalyzed the reduction of 1 μmol methyl viologen per min.

NADH:ferredoxin oxidoreductase activity was measured in cell extracts of *C. pasteurianum* at room temperature by following the reduction of metronidazole (Blusson et al. 1981). The 1-ml reaction mixture contained an NADH regenerating system (250 μM NADH, 30 μl 96% ethanol, 45 U yeast alcohol dehydrogenase, and 20 mM acetyl phosphate) and an acetyl-CoA regenerating system (20 mM acetyl phosphate, 2 U phosphotransacetylase, 30 mM ammonium sulfate, and 2 mM glutathione), 1 mM CoA, 10 μM FAD, 0.1 mM metronidazole, and 4.6 μM clostridial ferredoxin (Blusson et al. 1981). The assay was carried out under an atmosphere of carbon monoxide to avoid reoxidation of ferredoxin by hydrogenase.

NADH:polysulfide oxidoreductase (NPOR) assays were performed in 3-ml semimicro disposable cuvettes. The NADH-dependent reduction of polysulfide was measured by monitoring NADH oxidation at 340 nm ($\varepsilon = 6220\ M^{-1}cm^{-1}$). The 1-ml reaction mixture contained 0.7–1.0 mM polysulfide, 20 μM FAD, 0.25 mM NADH, and 20–80 μg protein in 50 mM Pipes buffer (pH 7.1). Sealed cuvettes were preheated as described above. One unit of NPOR activity catalyzed the oxidation of 1 μmol NADH per min.

Hydrogenase activity in cell extracts was measured in 2-ml glass cuvettes by monitoring the reduction of methyl viologen at 600 nm. The 1-ml reaction mixture contained 2 mM methyl viologen and 1–2 μg protein in 50 mM Epps (pH 9.3) or 40 mM Hepes (pH 7.5), each containing 2 mM dithiothreitol. Cuvettes were flushed with either nitrogen (control) or hydrogen for 30 s and were preheated for 10 min at 60°C for whole cells and extracts or at 70°C for vesicles. The reaction was initiated by the addition of extract by syringe. One unit of hydrogenase activity catalyzed the reduction of 1 μmol methyl viologen per min.

Hydrogenase activity in whole cells was determined at 60°C by measuring methyl viologen reduction at 600 nm in 2-ml glass cuvettes. The 1-ml reaction mixture contained 5 mM methyl viologen, 10 nmol sodium dithionite, 10 μl whole cells (200–400 μg protein) in buffer [the mineral solution, NaCl, and Hepes components of *T. neapolitana* medium (pH 7.6)]. Cuvettes were sealed with red rubber serum stoppers and were preheated for 10 min at 60°C. Whole cells were preincubated in $CuCl_2$ (0.5 or 1 mM) for 10 min at room temperature. Reactions were started by adding the cells to the reaction mixtures. One unit of hydrogenase activity catalyzed the reduction of 1 μmol methyl viologen per min.

Phosphoglycerate kinase (PGK) activity was determined in 3-ml semimicro disposable cuvettes by monitoring the oxidation of NADH at 340 nm. The 1-ml reaction mixture contained 80 mM triethanolamine (pH 7.6), 5 mM 3-phosphoglyceric acid, 3 mM cysteine-HCl, 0.3 mM NADH, 2 units of glyceraldehyde 3-phosphate dehydrogenase, 1 mM ATP, 2 mM magnesium sulfate, and 16–20 μg protein. Cuvettes were preheated aerobically for 10 min at 60°C. One unit of PGK activity catalyzed the oxidation of 1 μmol NADH per min.

Pyruvate-driven reduction of NAD^+ was measured by monitoring NAD^+ reduction at 340 nm in 2-ml glass cuvettes. The 0.5-ml reaction mixture contained 0.2 mM CoA, 2 mM magnesium chloride, 20 μM FAD, 20 mM pyruvate, 0.5 mM ADP, 2 mM potassium phosphate, pyruvate:ferredoxin oxidoreductase (POR)-containing extract (70–100 μg protein), NMOR-containing extract (10–20 μg protein), 20 μM *T. maritima* ferredoxin, and 2 mM NAD^+ in 50 mM Tris-HCl (pH 8.7). The POR-containing extract was the supernatant fraction from ultracentrifugation stripped of low-molecular-weight cofactors by elution through a column of Sephadex G-25 in 100 mM Tris-HCl (pH 8.0). The reaction was performed at 60°C.

Ferredoxin purification

Ferredoxin was purified from 30 g (wet wt.) of *T. neapolitana* cells. The supernatant from ultracentrifugation of cell extract (100,000 × *g*) was applied to a Q-Sepharose column (1.6 × 23.4 cm) equilibrated with 50 mM Tris-HCl (pH 7.4) plus 10% glycerol at 1 ml/min inside an anoxic chamber at room temperature. The ferredoxin started to elute when 0.45 M NaCl was applied to the column. Fractions (4 ml) with an A_{390}/A_{280} ratio greater than 0.13 were pooled and concentrated by ultrafiltration using a YM 3 membrane (Amicon). The concentrated fractions were applied to a hydroxyapatite column (1 × 10 cm; BioRad) equilibrated with 20 mM Tris-HCl (pH 7.4) plus 10% glycerol at 0.5 ml/min under oxic conditions at room temperature. The ferredoxin was eluted with a linear gradient of potassium phosphate (50 ml, 0–0.2 M) in the same buffer. Fractions (1 ml) with an A_{390}/A_{280} ratio above 0.28 were pooled and concentrated as above. The concentrate was directly applied to a Sephadex G-50 column (0.6 × 39.5 cm) equilibrated with 20 mM Tris-HCl (pH 7.4) plus 10% glycerol at 0.6 ml/min under oxic conditions at room temperature. Fractions with an A_{390}/A_{280} ratio higher than 0.7 were pooled and concentrated using a 3 K centrifugal microconcentrator (Filtron).

T. maritima ferredoxin was purified from *Escherichia coli* containing the cloned gene kindly provided by R. Sterner (Darimont and Sterner 1994). The cloned gene was expressed in *E. coli* strain SG 200–50 as described (Darimont and Sterner 1994). Cells (3 g, wet weight) were resuspended in 3.5 ml 50 mM Tris-HCl (pH 7.4) and lysed by sonication. Cell debris were removed from the resulting homogenate by centrifugation, and the supernatant was heated for 10 min at 90°C and was chilled on ice. The 4 ml supernatant was split in 2-ml fractions that were loaded one after the other on a Sephadex G-25 column (1.4 × 30.2 cm) equilibrated with 50 mM Tris-HCl (pH 7.4). Fractions with the highest absorption at 390 nm

were pooled and concentrated by ultrafiltration. The yield was 1.07 mg *T. maritima* ferredoxin/l cell culture.

Preparation of vesicles

No precautions were taken to prevent air exposure after the cultures were cooled on ice. Cells in 2 l of an exponentially growing culture were cooled on ice, harvested by centrifugation, resuspended in cell washing buffer [300 mM KCl, 2 mM magnesium sulfate, and 40 mM potassium phosphate, (pH 7.0)], and centrifuged at $1,000 \times g$ to remove precipitated metals. The resulting supernatant was centrifuged at $6,000 \times g$ to pellet the cells, and these were washed again with cell washing buffer. The cell pellet was washed once with 30 ml spheroplasting buffer (cell washing buffer containing 350 mM sucrose), and the final cell pellet was resuspended in 5 ml spheroplasting buffer. Lysozyme and EDTA were added to 2 mg ml^{-1}, respectively, and the mixture was incubated at 37°C for 30 min. The preparation was examined microscopically to assure that over 90% of the cells were spherical. This spheroplast suspension was diluted rapidly into 1.08 l water. Cell lysis was evident by the release of nucleic acids. RNase and DNase (5 mg each) were added to the suspension, which was then gently mixed for 15 min at room temperature. Following centrifugation at $8,000 \times g$ for 30 min at 4°C, the resulting pellet was resuspended in 30 ml 40 mM potassium phosphate (pH 7.0) and centrifuged at 12 000 g for 15 min at 4°C. This pellet was washed once more in the same buffer. This pellet of right-side-out vesicles was resuspended in 40 mM potassium phosphate (pH 7.0) to a concentration of 2–5 mg protein ml^{-1} and was stored at 4°C.

Inside-out vesicles were prepared from spheroplasts prepared as above. Spheroplasts prepared from 1.5 l of culture were washed twice with cell washing buffer and resuspended in 10 ml of this buffer containing DNase. This suspension was passed twice through a French press at 7 MPa while keeping the suspension chilled. The lysate was centrifuged at $6,000 \times g$ for 20 min at 4°C to remove unlysed cells and spheroplasts. The supernatant was centrifuged at $131,200 \times g$ for 1 h at 4°C. The pellet was resuspended in 10 ml 40 mM potassium-phosphate buffer (pH 7.0) and centrifuged for 15 min at $33,000 \times g$ at 4°C. The pellet was washed in the same buffer, and this pellet was resuspened in 40 mM potassium-phosphate buffer (pH 7.0) to a concentration of 1–2 mg protein ml^{-1} and was stored at 4°C.

ATPase measurements

ATPase activity in vesicles was measured as the decrease in ATP as measured with firefly luciferase. A 0.5-ml reaction mixture contained 40 mM potassium Hepes (pH 7.5), 6 μM disodium ATP, 100 μM $MgSO_4$, and 10–30 μg protein. As indicated, Tween 20 was added to 1% (v/v). The reaction mixture without protein was heated for 5 min at 80°C, and the reaction was initiated by the addition of protein. At intervals of 20 or 30 s, 10-μl aliquots were removed and added to 0.6 ml luciferase assay buffer [5 mM Na_2HAsO_4, 4 mM $MgSO_4$, 20 mM glycylglycerin (pH 7.6)]. To measure the ATP content of the aliquots, 30 μl of firefly luciferase (Sigma) was added to each cuvette, and the resulting luminescence was measured with a Lumac Biocounter M1500. The background rate of hydrolysis of ATP at 80°C was negligible over the time course of these reactions.

Materials

Chemicals were of reagent grade and were purchased from Sigma (St. Louis, Mo., USA) or Fisher Scientific (Springfield, N.J., USA). Glyceraldehyde 3-phosphate dehydrogenase was obtained from Sigma. Gases obtained from Connecticut Airgas (New Haven, Conn., USA) were prepurified and passed through heated copper to remove traces of oxygen. Ultra VU 3-ml semimicro disposable cuvettes and cuvette caps were from Baxter Scientific (Bedford, Mass., USA), and glass semimicro cuvettes were from Starna Cells (Atascadero, Calif., USA).

Results and discussion

The NMOR is membrane-associated

NMOR activity has been reported in extracts of two *Thermotoga* species (Janssen and Morgan 1992; Schröder et al. 1994). Since viologen dyes can often substitute for ferredoxin, it was assumed that this activity was due to an NADH:ferredoxin oxidoreductase. We have shown previously that NMOR activity is detectable in cell extracts of *T. neapolitana* (Childers and Noll 1995b). To determine the location of the NMOR activity in the cell, cell extracts were subjected to ultracentrifugation, and enzyme activities in the supernatant and pellet fractions were measured. Since no *Thermotoga* cytoplasmic membrane enzymes that could serve as markers for the cytoplasmic membrane fraction are known, the activity of cytoplasmic enzymes was measured for comparison. Over 93% of the total phosphoglycerate kinase activity was recovered following ultracentrifugation, and 96% of this activity was in the supernatant (Table 1). Similarly, 98% of the NPOR activity was in the supernatant as we had shown previously (Childers and Noll 1995a). We also found that 80–96% of the hydrogenase activity as measured by hydrogen-dependent reduction of methyl viologen was present in the soluble fraction. By contrast, 83% of the NMOR activity was in the pellet fraction. We examined the possibility that the NMOR activity remained in the pellet fraction because the enzyme adhered to lipids after the cells had been

Table 1 Localization of NADH:methyl viologen oxidoreductase and hydrogenase activities in cell extracts (*NMOR* NADH:methyl viologen oxidoreductase, *NPOR* NADH:polysulfide oxidoreductase, *PGK* phosphoglycerate kinase, and *CpNFOR* NADH: ferredoxin oxidoreductase in *Clostridium pasteurianum* cell extract

Enzyme	Cell extract		Ultracentrifugation fractions					
			Pellet			Supernatant		
	Units	U/mg protein	Units (%)		U/mg protein	Units (%)		U/mg protein	% Recovery
NMOR	432.0	4.8	306.2	(83)	37.8	61.9	(17)	1.0	85.2
NPOR	18.0	0.2	0.8	(2)	0.1	37.1	(98)	0.6	210.6
Hydrogenase	4050.9	45.0	115.8	(4)	8.1	2581.2	(96)	41.7	67.0
PGK	234.0	2.6	8.1	(4)	1.0	210.5	(96)	3.4	93.4
CpNFOR	0.81	0.02	0.03	(4.8)	0.007	0.63	(95)	0.03	81.5

cooled to room temperature during harvesting. We heated this pellet fraction at 77°C for 10 min and then immediately centrifuged it at 100,000 × *g* 1 h. All the activity remained in the pellet following this treatment, suggesting that NMOR cannot be irreversibly released by heat treatment.

The location of NMOR activity in the membrane fraction was unexpected. Although this activity has been studied in other fermenting strict anaerobes, its location in those organisms has not been examined. Since NADH:ferredoxin oxidoreductase activity in *C. pasteurianum* has been extensively investigated (Jungermann et al. 1973; Petitdemange et al. 1976), we examined its location in cell extracts. We subjected cell extracts of *C. pasteurianum* to ultracentrifugation and found that, in contrast to *T. neapolitana*, NADH:ferredoxin oxidoreductase activity in this organism is located primarily in the cytoplasm (Table 1). Although the level of NADH:ferredoxin oxidoreductase activity that we observed in *C. pasteurianum* is much lower than NMOR activity in *T. neapolitana*, it is similar to that reported previously for *C. pasteurianum* and other clostridia (Petitdemange et al. 1976).

To our knowledge, this activity has not been detected in membranes of fermentative organisms. In the aerobic methylotroph *Methylosinus trichosporium* OB3b, a ferredoxin:NAD^+ oxidoreductase has been purified from the soluble fraction of cell extracts (Chen and Yoch 1989). In plants and cyanobacteria, a similar activity, ferredoxin:NADP reductase, is bound to membranes by an anchor protein or an N-terminal polypeptide extension of the ferredoxin:NADP reductase (Forti et al. 1983; Schluchter and Bryant 1992). Ferredoxin:NADP oxidoreductase from *Pyrococcus furiosus* is a soluble enzyme with sulfide dehydrogenase activity (Ma et al. 1994). Although *T. neapolitana* also has a similar sulfur reductase activity, it is an enzyme distinct from the NMOR (Table 1) (Childers and Noll 1995b).

Ferredoxin is not a substrate for NMOR

We attempted to determine whether ferredoxin could serve as a substrate for the NMOR. Dithionite is commonly used to reduce ferredoxin, but it also chemically reduces NAD^+. Therefore ferredoxin was reduced by the oxidative decarboxylation of pyruvate via the endogenous pyruvate:ferredoxin oxidoreductase (POR) (Blamey and Adams 1994). POR activity in the supernatant fraction stripped of low-molecular-weight cofactors reduced NAD^+ only if the lipid fraction was provided (Table 2). Both *C. pasteurianum* (not shown) and partially purified recombinant *T. maritima* ferredoxins stimulated NAD^+ reduction. These data provide additional evidence that a membrane-associated activity is required to couple electron transfer from pyruvate oxidative decarboxylation to NAD^+ reduction.

Since the above experiment cannot directly show that ferredoxin is a substrate of NMOR, we attempted to measure the opposite reaction, reduction of ferredoxin by NADH, by observing the chemical reduction of the dye metronidazole by reduced ferredoxin (Blusson et al. 1981). However, we were unable to detect reduction of the dye using NADH as an electron donor with the membrane fraction. The dye could be reduced by the reduced ferredoxin generated in a POR assay (data not shown), so the dye is stable at high temperatures.

Table 2 The lipid fraction and ferredoxin are required to couplepyruvate:ferredoxin oxidoreductase (POR) activity with NAD^+ reduction. (NAD^+ reduction was measured at 340 nm at 60°C as described in Materials and methods. Where indicated, 20 mM pyruvate, 6 μg lipid fraction protein, or 20 μM partially purified *Thermatoga maritima* ferredoxin was added

Lipid fraction	Ferredoxin	Pyruvate	NAD^+ reduction (nmol min^{-1})
–	+	+	0.8
+	–	+	0.8
+	+	–	0.0
+	+	+	9.8

These observations suggest that although ferredoxin is required for electron flow from pyruvate to NAD^+, it is not a substrate of the NMOR. It is interesting to note that the purified cytoplasmic hydrogenase from *T. maritima* could not be shown to use *Thermotoga* ferredoxin as an electron donor either (Juszczak et al. 1991).

Extracytoplasmic hydrogenase activity can be detected

The location of NMOR activity prompted us to ask whether other oxidoreductase activities are also membrane-associated. Unexpectedly, we found that hydroge-

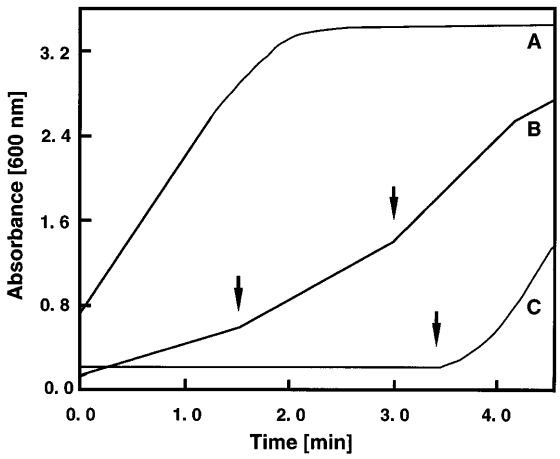

Fig. 1 Hydrogenase activity in whole cells and the effect of cupric chloride on this activity. Hydrogenase activity was measured as the reduction of methyl viologen under a hydrogen atmosphere. *A* Hydrogenase activity was measured using crude cell extract. *B* Hydrogenase activity was measured using a suspension of whole cells as described in Materials and methods. At the time points indicated by *arrows*, 10 μl Triton X-100 was added to the assay mixture. *C* Whole cells were treated with 0.5 mM cupric chloride under anoxic conditions as described in Materials and methods. At the time point indicated by the *arrow*, 10 μl Triton X-100 was added to the assay mixture

nase activity, as measured by methyl viologen reduction, could be detected using whole cells (Fig. 1). Membranes have been shown to be impermeable to methyl viologen; therefore, activity in whole cells is often used as supportive evidence for extracytoplasmic hydrogenases (Cypionka and Dilling 1986; Jones and Garland 1977; Schink and Friedrich 1994). When Triton X-100 was added to cells, hydrogenase activity increased approximately threefold (Fig. 1). Soluble hydrogenase activity in cell extracts is not stimulated by Triton X-100 (unpublished results), thus ruling out the possibility that a small amount of soluble hydrogenase released from leaking cells and stimulated by Triton X-100 could account for the stimulations of activity observed with whole cells. Triton X-100 lysed whole cells under these conditions as evidenced by the fact that the optical density at 600 nm of a cell suspension treated in the same manner decreased from 0.119 to 0.09 upon addition of 10 µl of Triton X-100, and further decreased to 0.023 with the addition of another 10 µl. By comparison, the same quantity of cells lysed by sonication have an optical density of 0.052 when 10 µl of Triton X-100 is added to the extract. Thus, the increase in hydrogenase activity seen when detergent was added to whole cells resulted from the release of a soluble hydrogenase.

Sensitivity to cupric chloride is also used to determine the location of hydrogenases (Schink and Friedrich 1994; Härtel and Buckel 1996). We treated whole cells with 0.5 mM cupric chloride for 10 min and tested hydrogenase activity in an aliquot of these cells. As shown in Fig. 1, activity was inhibited over 97% by this treatment. When Triton X-100 was added to pretreated cells, significant hydrogenase activity was detected (Fig. 1). We determined that the amount of cupric chloride introduced into the hydrogenase assay as a carry-over from the pretreated cells was insufficient to chemically reoxidize reduced methyl viologen. This rules out the possibility that rapid chemical reoxidation of methyl viologen reduced by hydrogenase may have accounted for the apparent inhibition of hydrogenase activity.

Because *T. neapolitana* is a marine organism and the buffer used for the hydrogenase assay is not iso-osmotic with marine growth media, we also looked for evidence that soluble proteins may be released by lysed cells during the hydrogenase assay. We could detect no NPOR activity when whole cells were suspended in buffer and heated to 60°C, but activity was detected when these cells were lysed by addition of Triton X-100 (0.2 U/mg protein). Combined with the observed changes in optical density discussed above, these results demonstrate that cells remain intact during the hydrogenase assay.

Enzyme activities in vesicles

Membrane vesicles were prepared to determine the location of hydrogenase and NMOR activities determined based upon their activities with the membrane-impermeable substrate methyl viologen. The orientation of the vesicles was indicated by the relative activities of membrane-bound ATPase in the presence and absence of detergent (Futai and Kanazawa 1983). As shown in Table 3, both right-side-out and inside-out vesicles were obtained. In spite of the fact that both types of vesicles were prepared under oxic conditions, both NMOR and hydrogenase activities were detectable with good activities. The hydrogenase activity in right-side-out vesicles was similar regardless of the presence of detergent, which is consistent with its location on the exterior face of the cytoplasmic membrane. This specific activity is similar to that detected using whole cells [0.617 U (mg protein)$^{-1}$ vs 1.3 U (mg protein)$^{-1}$ for whole cells]. In some measurements (e.g., those in Table 3), Tween 20 appeared to inhibit hydrogenase activity, but in others it did not. Inside-out vesicles had much less hydrogenase activity, so it is difficult to draw conclusions from those data. Since these vesicles were prepared by French press treatment, it is conceivable that the hydrogenase was dislodged during preparation.

Table 3 Enzyme activities in vesicles. Hydrogenase activity was measured as hydrogen-dependent reduction of methyl viologen. NMOR activity was measured as NADH-dependent reduction of methyl viologen (*RSO* right-side-out, *ISO*, inside-out, *NMOR* NADH:methyl viologen oxidoreductase

Cell component	Specific activities [mmol min^{-1} (mg protein)$^{-1}$]		
	ATPase	Hydrogenase	NMOR
RSO vesicles	0.64	0.62	1.47
RSO vesicles + Tween 20	2.18	0.26	11.55
ISO vesicles	5.27	0.04	5.06
ISO vesicles + Tween 20	5.46	0.02	4.21

The activity of the NMOR in vesicles was consistent with its location on the inner face of the cytoplasmic membrane. In contrast to the hydrogenase, its activity was stimulated eightfold in right-side-out vesicles upon addition of Tween 20. The activity in inside-out vesicles was slightly less when treated with Tween 20.

As a control for cytoplasmic enzyme contamination, phosphoglycerate kinase activity was measured in a right-side-out preparation. An activity of 0.2 U (mg protein)$^{-1}$ was measured. This is less than 8% of that detected in cell extracts (Table 1) and compares favorably with the levels of cytoplasmic enzyme activities found in *Streptococcus mutans* vesicles (5–12%; Buckley and Hamilton 1994).

A soluble, iron-containing hydrogenase has been purified from *T. maritima* (Juszczak et al. 1991). Over 95% of the activity of that enzyme was found in the supernatant fraction of cell extracts following centrifugation for 1 h at 50,000 × *g*. No other hydrogenase activities were reported. That hydrogenase was found to be highly oxygen-labile, with more than 80% of the activity of the purified enzyme being lost after a 15-s exposure to air. Additionally, the activity in extracts was lost it had not been stabilized with reducing agents and glycerol. Similar to other iron hydrogenases, the soluble *T. maritima* hydrogenase is inhibited by a low concentration of carbon monoxide (K_i =

0.2 μM) but is uniquely insensitive to nitrite (K_i >25 mM). The hydrogenase activity that we detected in vesicles of *T. neapolitana* is also inhibited by carbon monoxide, but it is sensitive to sodium nitrite. Methyl-viologen-reducing activity was inhibited 50% by 50 μM sodium nitrite.

Implications of observed membrane-associated oxidoreductase activities

Membrane-associated hydrogenases are typically associated with hydrogen-uptake electron transport systems (Adams 1990; Przybyla et al. 1992). *Thermotoga* species are not known to grow by oxidation of hydrogen, although this has not been exhaustively examined. Typically, fermentative growth is inhibited by hydrogen accumulation unless a source of sulfane sulfur is provided or hydrogen is flushed from the culture (Belkin et al. 1986). In the presence of sulfur, fermentation can proceed under a 100% hydrogen atmosphere (Childers et al. 1992). We have attempted cultivation of *T. neapolitana* on a nonfermentable substrate (acetate) under a hydrogen atmosphere with nitrate, fumarate or sulfur as electron acceptors. We have not observed sustained growth under any of these conditions. In addition, we have not observed NAD^+ reduction under a hydrogen atmosphere catalyzed by a membrane fraction of cell extracts. If this hydrogenase functions in vivo as an uptake hydrogenase, we have yet to discover the conditions under which it does so. A second, extracytoplasmic hydrogenase is found in *C. pasteurianum* (Chen and Blanchard 1978). Its physiological role is unknown, but a role in nitrogen fixation has been postulated. Although no data have been presented, nitrogen fixation by *Thermotoga* has been reported (Huber and Stetter 1992).

Our results suggest the presence of membrane-associated hydrogenase activity in *T. neapolitana*. This activity possesses properties distinct from that reported for the hydrogenase purified from *T. maritima*: membrane association, oxygen stability, sensitivity to nitrite, and in vitro stability in cell extracts. Purification of the enzyme responsible for this membrane-associated hydrogenase activity is necessary for further comparisons with the soluble enzyme, and further physiological studies are needed in order to understand its role in cell metabolism.

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