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Hydrogen formation from glycolate driven by reversed electron transport in membrane vesicles of a syntrophic glycolate-oxidizing bacterium

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Oxidation of glycolate to 2 CO_2 and 3 H_2 ($\Delta G^{\circ\prime}$ = +36 kJ/mol glycolate) by the proton-reducing, glycolate-fermenting partner bacterium of a syntrophic coculture (strain FlGlyM) depends on a low hydrogen partial pressure (p_{H2}). The first reaction, glycolate oxidation to glyoxylate ($E^{\circ\prime}$ = −92 mV) with protons as electron acceptors ($E^{\circ\prime}$ = −414 mV), is in equilibrium only at a p_{H_2} of 1 μPa which cannot be maintained by the syntrophic partner bacterium *Methanospirillum hungatei*; energy therefore needs to be spent to drive this reaction. Glycolate dehydrogenase activity (0.3−0.96 U · mg protein^{-1}) was detected which reduced various artificial electron acceptors such as benzyl viologen, methylene blue, dichloroindophenol, $K_3[Fe(CN)_6]$, and water-soluble quinones. Fractionation of crude cell extract of the glycolate-fermenting bacterium revealed that glycolate dehydrogenase, hydrogenase, and proton-translocating ATPase were membrane-bound. Menaquinones were found as potential electron carriers. Everted membrane vesicles of the glycolate-fermenting bacterium catalyzed ATP-dependent H_2 formation from glycolate (30−307 nmol H_2 · min^{-1} · mg protein^{-1}). Protonophores, inhibitors of proton-translocating ATPase, and the quinone analog antimycin A inhibited H_2 formation from glycolate, indicating the involvement of proton-motive force to drive the endergonic oxidation of glycolate to glyoxylate with concomitant H_2 release. This is the first demonstration of a reversed electron transport in syntrophic interspecies hydrogen transfer.

The syntrophic oxidation of longer-chain fatty acids, ethanol, acetate, and certain aromatic compounds during methanogenic degradation is carried out by a highly specialized group of anaerobic bacteria, the obligately proton-reducing bacteria (Wolin, 1982; McInerney, 1986; Dolfing, 1988; Schink, 1991). Substrate degradation by these bacteria depends on a very low p_{H_2} which is maintained by hydrogen-consuming partner organisms, e.g. methanogenic bacteria.

Recently, a new type of syntrophic oxidation was discovered: the syntrophic oxidation of glycolate by a Gram-positive spore-forming rod in coculture with *Methanospirillum hungatei* (strain FlGlyM, Friedrich et al., 1991):

$$4\ \text{Glycolate}^- + 4\ H^+ \rightarrow 3\ CH_4 + 5\ CO_2 + 2\ H_2O \qquad (1)$$
$$\Delta G^{\circ\prime} = -68.4\ \text{kJ/mol glycolate.}$$

This syntrophic association is based on interspecies hydrogen transfer as indicated by hydrogen formation at levels of 100−10 Pa p_{H_2}, and lack of formate formation. In crude extracts of the glycolate-fermenting bacterium, all enzymes necessary for the metabolism of glycolate could be demonstrated, with the exception of the initial reaction (Fig. 1; Friedrich et al., 1991). Glycolate is oxidized to glyoxylate by a recently discovered enzyme activity. Glyoxylate condenses with acetyl-CoA to malyl-CoA. ATP formation via substrate level phosphorylation is coupled to malyl-CoA conversion to L-malate. An $NADP^+$-dependent malic enzyme activity oxidizes malate to pyruvate. Pyruvate synthase activity finally converts pyruvate to acetyl-CoA and CO_2, thus regenerating the acceptor for a new condensation with glyoxylate. The complete oxidation of one mole glycolate yields one mole ATP. Electrons released in oxidation reactions (Fig. 1) are liberated as H_2 which is consumed by the methanogenic partner bacterium. Whereas the redox potentials of the malic enzyme ($E^{\circ\prime}$ = −331 mV; calculated with $CO_{2(g)}$) and the pyruvate synthase reaction ($E^{\circ\prime}$ = −470 mV; calculated after Thauer et al., 1977) are low enough to allow hydrogen release at p_{H_2} = 10 Pa, glycolate oxidation to glyoxylate would require a p_{H_2} of <1 μPa for direct proton reduction.

This paper reports on measurement of the glycolate-oxidizing enzyme activity, on subcellular distribution of enzymes involved in the energetically critical step of glycolate oxidation to glyoxylate with protons as electron acceptors, and on direct H_2 formation from glycolate with everted membrane vesicle preparations. This is, to our knowledge, the first demonstration of reversed electron transport in syntrophic interspecies hydrogen transfer.

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Abbreviations. CCCP, carbonylcyanide *m*-chlorophenylhydrazone; $(cHxN)_2C$, *N,N'*-dicyclohexylcarbodiimide; Cl_2Ind, 2,6-dichloroindophenol; ETH 157, *N,N'*-dibenzyl-*N,N'*-diphenyl-1,2-phenylenedioxydiacetamide; MK, menaquinone; p_{H2}, hydrogen partial pressure; PMS, phenazine methosulfate; SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalonitrile.

Enzymes. ATPase or ATP synthase, H^+-translocating (EC 3.6.1.34); glycolate:acceptor oxidoreductase (EC 1.1.99.14); glyoxylate reductase (EC 1.1.1.26); hydrogenase or hydrogen:acceptor oxidoreductase (EC 1.12.99.−); malic enzyme (EC 1.1.1.40).

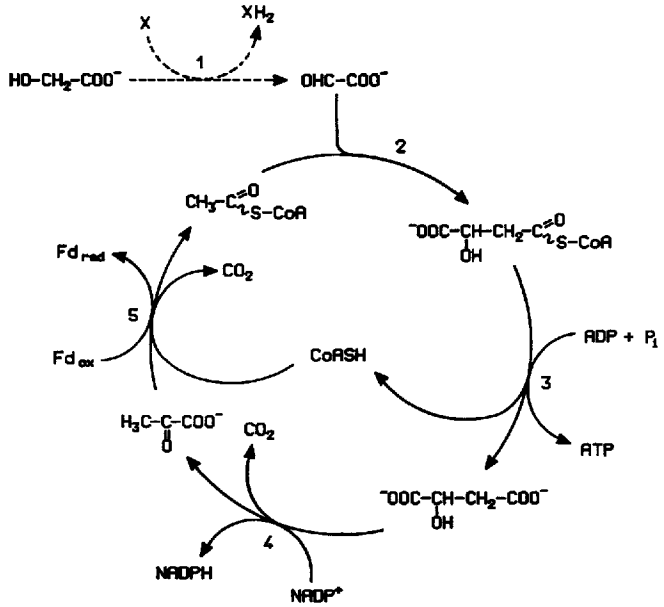

Fig. 1. Pathway of glycolate degradation by the fermenting bacterium in the coculture FlGlyM. The figure was modified from Friedrich et al., 1991. Numbers refer to the following enzyme activities measured in cell-free extracts: (1) unidentified glycolate-oxidizing activity, (2) malyl-CoA lyase, (3) malate:CoA ligase, (4) malic enzyme, (5) pyruvate synthase. $Fd_{ox/red}$ = oxidized/reduced ferredoxin; X = an unknown electron acceptor.

MATERIALS AND METHODS

Source of materials

3,5-Di-*tert*-butyl-4-hydroxybenzylidenemalonitrile (SF 6847) was from Wako-Chemie. 3,5,4′,5′-Tetrachlorosalicylanilide was purchased from Eastman Kodak Co. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was from Serva. Amiloride, antimycin, *N,N′*-dicyclohexylcarbodiimide [(cHxN)$_2$C], 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and rotenone were obtained from Sigma Chemie GmbH. All other chemicals were of analytical grade or of the highest commercially available purity, and were obtained from Fluka or Merck. Gases (>99.999% pure) were purchased from Messer Griesheim or from Sauerstoffwerke Friedrichshafen. Strains FlGlyM (DSM 6945; Friedrich et al., 1991) and FlGlyR were from our own culture collection.

Growth of bacteria

Strain FlGlyM was grown anaerobically in 1.2-l infusion bottles containing 1 l mineral salts medium as described earlier (Widdel and Pfennig, 1981; Schink and Pfennig, 1982; Friedrich et al., 1991) but with 2 mM cysteine as reducing agent instead of sodium sulfide. Sodium glycolate was added as sole growth substrate in portions of 10 mM during growth, up to a final concentration of 30 mM. 2 mM sodium acetate was added to the medium to meet assimilatory requirements of the syntrophic partner bacterium *Methanospirillum hungatei* strain SK. Strain FlGlyR was grown in pure culture with 30 mM sodium glyoxylate, fed in portions of 5−10 mM, in cysteine-reduced mineral medium without acetate.

Preparation of crude extract and membrane vesicles

Cells were harvested anoxically under an atmosphere of N_2/H_2 (95/5) in the late logarithmic growth phase. Cell suspensions (about 95 mg dry cell matter/l) were transferred into screw-capped polypropylene bottles incubated for at least two days under an oxygen-free atmosphere in an anaerobic glove box (McCoy) and centrifuged at 13700×*g* at +5°C. Cells were washed twice with 125 mM Tris/HCl pH 8.0, reduced with 2.5 mM dithioerythritol, and once with 1 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, reduced with 1 mM dithioerythritol. Cells of the fermenting bacterium of the coculture (strain FlGlyM) were selectively lysed by addition of 2 ml lysis buffer containing 1 mg/ml lysozyme (21500 U/mg) and 25 µg/ml DNase I (2000 Kunitz units/mg) in 1 mM Tris/HCl pH 8.0, 10 mM $MgCl_2$, reduced with 1 mM dithioerythritol, for 30 min at 35°C. Spheroplasts of the glycolate-fermenting bacterium were broken by passing the supernatant four times through a French pressure cell at 43 MPa pressure, to generate everted vesicles. *M. hungatei* was separated from crude extract of the glycolate-fermenting bacterium by centrifugation at 3300×*g* in N_2-gassed, butyl-rubber-stoppered 5-ml serum vials. Crude extracts of strain FlGlyR were prepared similarly. Subcellular fractionation was achieved by centrifugation at 417000×*g* (30 min). Cell-free extracts and subcellular fractions were stored at 0−4°C under N_2 until use.

Protein determination

Protein was quantified by a micro assay (Bradford, 1976) with bovine serum albumin as standard using a modified Bradford reagent (Bio-Rad Laboratories GmbH).

Hydrogen determination

Hydrogen was analyzed in either a GC 6000 Vega series 2 gas chromatograph (Carlo Erba Strumentazione) equipped with a HWD 430 thermal conductivity detector and a steel column (2 m×5 mm) filled with Carbosieve SII, 100/120 mesh, with N_2 as carrier gas (16 ml/min, injector/detector at 130°C, column at 120°C) or, if samples contained less than 0.2 nmol hydrogen, with a RGD2 reduction gas detector (Techmation) fitted to a steel column (1.8 m×2 mm) filled with molecular sieve (0.5 nm) operated at 25°C. Gas samples (100 or 50 µl) were withdrawn from headspaces of incubation vessels with gas-tight syringes (Dynatech Precision Sampling Corporation) and immediately analysed. Chromatograms were recorded and analyzed with a D2500 Chromato-Integrator (Merck).

Determination of organic acids

Glycolate, glyoxylate, and formate were quantified with a System Gold high-pressure liquid chromatograph (Beckman), as described previously (Friedrich et al., 1991), equipped with a ORH-801 organic acids column (300 ×6.5 mm; Interaction Chemicals Inc.) with 10 mM H_2SO_4 as eluent at a flow rate of 0.5 ml/min at 25°C. Compounds were detected using either a Beckman 166 detector at 206 nm or a refractive index detector (Erma).

Determination of enzyme activities

All spectrophotometric enzyme assays were performed at 30°C in cuvettes using anoxic or reduced buffers under an atmosphere of N_2 (or H_2 as indicated). Additions to the enzyme assays were made from anoxic stock solutions with

Table 1. Mid-redox potentials, wavelengths, absorption coefficients, and concentrations of electron acceptors/donors used in spectrophotometric assays.

Electron carrier	$E^{\circ\prime}$	Wavelength	$\Delta\varepsilon$	Concentration in assay
	mV	nm	$mM^{-1} \cdot cm^{-1}$	mM
Methyl viologen	−440	578	9.7	5
Benzyl viologen	−360	578	8.65	5
$NADP^+$/NADPH	−324	365	3.5	1/0.3
NAD^+/NADH	−320	365	3.4	1/0.3
Menadione	− 1[a]	260/280[b]	7.8	0.2
Methylene blue	+ 11	570	13.1	0.1
Duroquinone	+ 35[a]	260/280[b]	8.3	0.2
1.4-Naphthoquinone	+ 64[a]	260/280[b]	11.9	0.2
2,3-Dimethoxy-5-methyl-1,4-benzoquinone	+162[a]	280/290[b]	9.6	0.2
Cl_2Ind	+220	578	7.6	0.2
Ferricyanide	+360	420	0.9	1

[a] Schnorf, 1966.
[b] Dual-wavelength measurement (Lemma et al., 1990).

Table 2. Glycolate and hydrogen oxidation activities of the membrane fraction of strain FlGlyM with various electron acceptors. n.d. = not determined.

Electron acceptor	Specific activity of	
	glycolate dehydrogenase	hydrogenase
	U/mg	
Methyl viologen	n.d.	5.9
Benzyl viologen	0.6	38.5
Menadione	4.6	42.4
Methylene blue	4.3−5.6	41.3
1,4-Naphthoquinone	1.05	62.3
Duroquinone	0.62	22.4
2,3-Dimethoxy-5-methyl-1,4-benzoquinone	0.30	13.5
Cl_2Ind + PMS	1.95	n.d.
Cl_2Ind	1.67	n.d.
Ferricyanide + PMS	4.3	n.d.
Ferricyanide	0.6	n.d.

gas-tight syringes (Macherey-Nagel). General assay conditions were as described previously (Friedrich et al., 1991).

Enzyme activities were measured spectrophotometrically as reduction or oxidation of electron carriers at the wavelength indicated in Table 1. Dual-wavelength measurements were performed with a Kontron double-beam spectrophotometer. One unit of enzyme activity is the amount catalyzing the conversion of 1 µmol substrate/min.

Glycolate:acceptor oxidoreductase activity was measured with various electron acceptors. All assay mixtures contained 50 mM Tris/HCl pH 8.5, 10 mM sodium glycolate and electron acceptors (Table 1). The reaction was started either with 2 mg protein/l or 0.4 mg membrane protein/l. A small amount (≈10 µM) of sodium dithionite was added to slightly reduce the assay mixture containing methylene blue and benzyl viologen prior to the start of the reaction.

Hydrogenase:acceptor oxidoreductase activity was anayzed following the reduction of benzyl viologen, methyl viologen (Diekert and Thauer, 1978), methylene blue (Table 1), or water-soluble quinones with hydrogen (for quinones and concentrations, see Table 1). The reaction was started by addition of protein. Dithionite was added as described above.

ATPase was measured recording the oxidation of NADH with a coupled enzyme assay according to Vogel and Steinhart (1976). The assay mixture (950 µl) contained 100 mM Tris/HCl pH 7.8, 0.3 mM Na_2NADH, 3 mM phospho*enol*-pyruvate (tricyclohexylammonium salt), 5 U pyruvate kinase, 20 U lactate dehydrogenase, and 7 mM Na_2ATP. The reaction was started by addition of 30−300 µg protein.

Malic enzyme activity was determined following $NADP^+$ reduction upon addition of L-malate (modified after Stams et al., 1984). The assay mixture contained 100 mM Tris/HCl pH 7.8, 1 mM Na_2NADP^+, 10 mM $MgCl_2$, and 10−100 µg protein. The reaction was started by addition of 10 mM sodium L-malate.

Extraction and determination of menaquinones

Menaquinones were extracted from lyophilized cells using the method of Collins et al. (1977). After repeated extraction with petrolether/methanol mixture (1:1), the extract was dried under reduced pressure, and resuspended in petrolether/methanol. Quinones were identified by thin-layer chromatography (silica gel 60 F_{254}; Merck). Menaquinones were identified by Dr R. M. Kroppenstedt (Braunschweig), using HPLC analysis (Kroppenstedt, 1985). Menaquinones were quantified with vitamin K_1 as standard (after Tamaoka et al., 1983) by HPLC analysis using a Beckman HPLC system equipped with a LiChrospher 100 RP-18-endcapped column (250×4 mm; Merck) and methanol/isopropanol mixture (65:35) as eluent (1 ml · min^{-1}) (modified after Kroppenstedt, 1985).

Assay for H_2 formation with everted vesicle preparations

The assay was performed under an atmosphere of N_2 in 16-ml Hungate tubes sealed with a butyl rubber septum, and containing 125 mM Tris/HCl pH 8.0, prereduced with 2.5 mM dithioerythritol, 10 mM $MgCl_2$, 4 mM ATP, and 10 mM glycolate. The reaction was started with membrane protein. H_2 formation was followed by gas chromatography.

RESULTS

The glycolate-fermenting bacterium could be grown in pure culture (strain FlGlyR) with glyoxylate as substrate which was disproportionated to glycolate, CO_2 and small amounts of hydrogen (data not shown).

All enzyme studies were performed with crude extracts or subcellular fractions from the glycolate-fermenting bacterium either from differentially lysed cells of the coculture (strain FlGlyM) or from the pure culture of the glycolate-fermenting bacterium (strain FlGlyR).

Enzymes involved in glycolate oxidation

In crude extracts with a protein concent 10 times higher than in previous studies (Friedrich et al., 1991) and under strictly anoxic conditions, a glycolate dehydrogenase activity

Table 3. Distribution of membrane-associated enzyme activities in subcellular fractions of the fermenting bacterium in the coculture, strain FlGlyM. DH = dehydrogenase, MB = methylene blue, MV = methyl viologen.

Enzyme activity	Source	Activity	Protein	Specific activity	Proportion in source
		U	mg	U/mg	%
Glycolate/MB DH	extract	14.9	15.53	0.96	100
	cytoplasm	0.9	12.4	0.073	6
	membrane	14.5	3.1	4.03	97
Hydrogenase MB	extract	303	15.53	19.5	100
	cytoplasm	578	12.4	46.6	190
	membrane	87	3.1	28.2	29
Hydrogenase MV	extract	71.5	15.53	4.6	100
	cytoplasm	100	12.4	8.1	140
	membrane	16.8	3.1	5.4	23
ATPase	extract	7.2	24.4	0.3	100
	cytoplasm	3.4	21	0.16	48
	membrane	3.6	3.4	1.05	50
Malic enzyme ($NADP^+$)	extract	144.2	16.7	8.7	100
	cytoplasm	141.6	15.9	8.9	98
	membrane	0.5	1.2	0.42	0.4

was found. In membrane fractions glycolate was oxidized to glyoxylate and methylene blue, benzyl viologen, dichloroindophenol (Cl_2Ind), various quinones, or $K_3[Fe(CN)_6]$ were reduced (Table 2). The specific activity with the latter was significantly enhanced in the presence of catalytic amounts of 0.1 mM phenazine methosulfate (PMS). Neither NAD^+, $NADP^+$, acetylpyridine–adenine dinucleotide ($E^{\circ\prime} = -248$ mV) nor FAD and FMN were reduced.

Specific activities of glycolate dehydrogenase in crude extracts with methylene blue as electron acceptor were 0.3–0.96 U · mg protein^{-1} and were in the range calculated for the physiological activity (0.24 U · mg protein $^{-1}$) in an exponentially growing coculture ($Y_s = 3.7$ g · mol glycolate^{-1}, $\mu = 0.027$ h^{-1}). Thiol group-oxidizing agents such as $CuCl_2$ (4 mM; Cypionka and Dilling, 1986) or exposure to air oxygen (for less than 10 min) destroyed glycolate-oxidizing activity entirely.

If cells were lysed in the presence of 10 mM $MgCl_2$, almost all the glycolate dehydrogenase activity (97%; Table 3) was found in the membrane fraction.

In addition to glycolate dehydrogenase, significant amounts of hydrogenase and ATPase activity were found to be membrane-bound (Table 3). Interestingly, the activity of hydrogenase was considerably higher in the cytoplasmic fraction than in the crude extract. Malic enzyme, as a cytoplasmic marker, was found almost exclusively in the cytoplasmic fraction, indicating successful separation of membranes from the cytoplasm.

Glyoxylate-grown cells of the fermenting bacterium, strain FlGlyR, also catalyzed the oxidation of small amounts of glycolate in the absence of a H_2-scavenging partner until a p_{H_2} of 100 Pa was reached. We therefore investigated the enzyme activities involved in glycolate oxidation to H_2. Glycolate dehydrogenase, hydrogenase, and ATPase activities were membrane-bound enzyme activities comparable to those measured in strain FlGlyM (data not shown), proving that these enzyme activities detected in the coculture did not originate from *M. hungatei*.

Menaquinones in membranes of the glycolate-fermenting bacterium and quinone reactivity of enzymes involved in electron transport

Dry cells of the glycolate-fermenting bacterium grown in coculture (strain FlGlyM) as well as from the pure culture (strain FlGlyR) were extracted with a mixture of petrolether and methanol. Extracts were subjected to HPLC analysis revealing menaquinones (MK) 7–10 in both strains, MK 9 being the major fraction (FlGlyM: 1.9 nmol MK · mg dry cells^{-1}, 46% MK 9; FlGlyR: 3.06 nmol MK · mg dry cells^{-1}, 58% MK 9).

To check whether menaquinones mediated electron transport between glycolate oxidation and proton reduction, the reaction of glycolate dehydrogenase and hydrogenase in the membrane fraction with water-soluble quinones was investigated (Table 2). Glycolate oxidation with menadione as acceptor yielded the highest specific activity of all quinones tested, comparable to methylene blue or PMS/ferricyanide reduction (Table 2). Benzoquinones such as duroquinone or 2,3-dimethoxy-5-methyl-1,4-benzoquinone were reduced at much lower activities than the naphtoquinones. With hydrogen as electron donor, the nonsubstituted 1,4-naphtoquinone was reduced more rapidly than the substituted menadione (Table 2).

H_2 formation from glycolate with everted membrane vesicles

Membrane vesicle preparations catalyzed H_2 evolution from glycolate in the presence of ATP (Fig. 2). No H_2 formation was observed if either ATP or glycolate or membrane protein was omitted. Glyoxylate, as a product of glycolate oxidation,was identified by HPLC analysis, and was formed in stoichiometric amounts with hydrogen (data not shown). Under optimized conditions (10 mM $MgCl_2$, 125 mM Tris/HCl pH 8.0, 2.5 mM dithioerythritol, 10 mM glycolate and 6 mM ATP) highest specific activities of H_2 formation (307 nmol · min^{-1} · mg membrane protein^{-1}) were measured

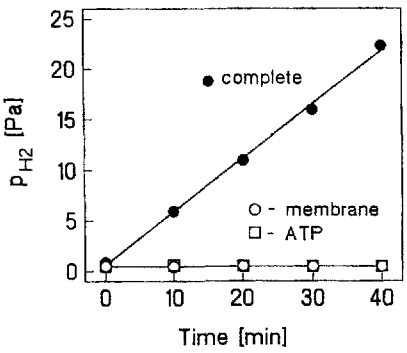

Fig. 2. Time course of ATP-driven H_2 formation from glycolate by membrane fractions of the glycolate-fermenting bacterium. The assay was performed at 25°C in 16-ml Hungate tubes containing 0.5 ml of the following assay mixture under an atmosphere of N_2: 125 mM Tris/HCl pH 8.0, 2.5 mM dithioerythritol, 10 mM $MgCl_2$, 10 mM sodium glycolate, 4 mM ATP, 30 µg protein (membrane fraction). Reactions were started by addition of protein. In control experiments without glycolate no hydrogen was produced either.

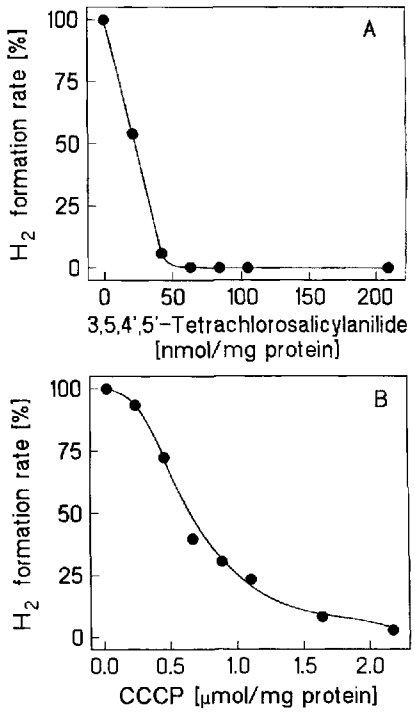

Fig. 3. Effect of the protonophores 3,5,4′,5′-tetrachlorosalicylanilide (A) and CCCP (B) on H_2 formation activity from glycolate in everted vesicles of the glycolate-fermenting bacterium. Assay conditions were as in Fig. 2, but the assay volume was 1 ml. Inhibitors dissolved in ethanol (final concentration 0.5%) were added 20 min before start of the reaction with ATP. In (A), 100% activity = 32 nmol H_2 · mg membrane protein^{-1}; in (B), 100% activity = 173 nmol H_2 · min^{-1} · mg protein^{-1}.

which were in the same range as the glycolate oxidation rate of a growing culture. The H_2 formation rate was linear with increasing protein concentration (data not shown) and was observed only in the membrane fraction (31–307 nmol H_2 · min^{-1} · mg membrane protein^{-1}) and the crude extract (25 nmol H_2 · min^{-1} · mg membrane protein^{-1}), but not in the cytoplasmic fraction.

ATP-dependent H_2 formation from glycolate was possible also with everted membrane vesicles of glyoxylate-grown cells of the pure culture (strain FlGlyR). H_2 formation rates were in a similar range as with vesicles from cells grown syntrophically with glycolate (44–119 nmol H_2 · min^{-1} · mg membrane protein^{-1}).

Inhibition of H_2 formation by protonophores and ATPase inhibitors

Protonophores such as 3,5,4′,5′-tetrachlorosalicylanilide, CCCP, and SF 6847 are known to dissipate the Δp across cytoplasmic membranes. H_2 formation from glycolate was abolished by small amounts of these uncouplers (Fig. 3) at concentrations typically uncoupling proton gradient-driven processes (Heytler, 1979). As shown by inhibitor titration (Fig. 3A), 3,5,4′,5′-tetrachlorosalicylanilide inhibited H_2 formation completely at a concentration of 84 nmol · mg protein^{-1} with 50% inhibition at 17 nmol · mg protein^{-1}, whereas CCCP had to be applied up to 2.2 µmol · mg protein^{-1} for full inhibition (Fig. 3B). The CCCP titration curve was hyperbolic with 50% inhibition at 0.66 µmol CCCP · mg membrane protein^{-1}. SF 6847 was the most effective inhibitor tested: full inhibition was achieved with 43 nmol SF6847 · mg membrane protein^{-1} (Table 4). These results indicate that a membrane potential was involved in H_2 formation from glycolate.

$(cHxN)_2C$, an inhibitor of prokaryotic proton- and Na^+-translocating F_1F_0-ATPases (Linnett and Beechey, 1979; Heise et al., 1992; Kluge et al., 1992) was found to inhibit ATP-driven H_2 formation from glycolate. Titration of H_2 formation activity resulted in a hyperbolic inhibition curve with 50% inhibition at 55 nmol $(cHxN)_2C$ · mg membrane protein^{-1} (Fig. 4). Also ATPase activity in membranes was inhibited by $(cHxN)_2C$ at similar concentrations (Fig. 4). Diethylstilbestrol, another F_1F_0-ATPase inhibitor (McEnery and Pedersen, 1986), effectively inhibited H_2 formation as well with 70% inhibition at 90 nmol diethylstilbestrol · mg membrane protein^{-1}. Sodium vanadate, an inhibitor of E_1E_2-ATPases (Pedersen and Carafoli, 1987), did not cause any inhibition (Table 4).

Glycolate dehydrogenase and hydrogenase activities were not affected by inhibitors employed in H_2 formation assays (data not shown).

To check for possible involvement of Na^+ in reversed electron transport from glycolate to protons, we tested the effect of amiloride which has been shown to inhibit Na^+/H^+ antiport activity competitively both in eukaryotic and prokaryotic organisms (Krulwich, 1983; Müller et al., 1987; Udagawa et al., 1986). H_2 formation was not affected by amiloride up to 250 µM (4.2 µmol amiloride · mg membrane protein^{-1}; Table 4). Amiloride (250 µM) did not prevent the inhibition of H_2 formation by the protonophore 3,5,4′,5′-tetrachlorosalicylanilide (Table 4). Monensin (843 nmol · mg protein^{-1}), a Na^+/H^+ antiporter, and *N,N′*-dibenzyl-*N,N′*-diphenyl-1,2-phenylenedioxydiacetamide (ETH 157; 898 nmol · protein^{-1}), a Na^+ ionophore, caused inhibition (36% and 25%, respectively) at high concentrations, but failed to abolish H_2 formation entirely (Table 4).

Effect of quinone analogs on ATP-driven H_2 formation

Menaquinones in the membranes of the glycolate-fermenting bacterium could act as electron carriers mediating between glycolate dehydrogenase and hydrogenase. Unfortunately, extraction and reconstitution experiments (Ernster et

Table 4. Effect of various inhibitors on H_2 formation from glycolate by everted vesicles of the glycolate-fermenting bacterium. Assay conditions were the same as in Fig. 3. Activities in control assays without treatment varied depending on the respective preparation. TCS = 3,5,4′,5′-tetrachlorosalicylanilide.

Treatment	Inhibitor	H_2 formation	Remnant activity
	nmol · mg protein^{-1}	nmol · min^{-1} · mg protein^{-1}	%
None	0	33	100
Sodium vanadate	1050	32	97
Diethylstilbestrol	87	9.3	28
Diethylstilbestrol	433	1.3	4
Diethylstilbestrol	866	0.2	0.6
None	0	17	100
SF 6847	9	14	82
SF6847	43	0	0
SF6847	87	0	0
None	0	56	100
Amiloride	4220	50	89
Amiloride + TCS	4220 34	0.09	0.2
Monensin	843	36	64
None	0	86	100
ETH 157	449	67	78
ETH157	898	65	75
None	0	33	100
Rotenone	209	29	88

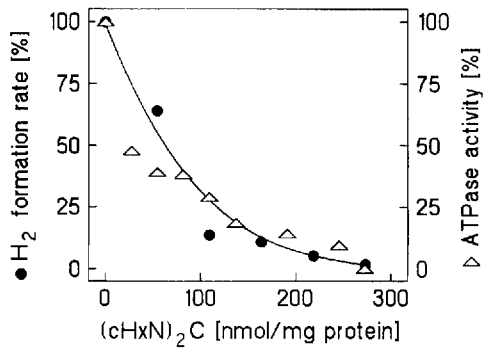

Fig. 4. $(cHxN)_2C$ inhibition of ATP-driven H_2 formation from glycolate (●) and of ATPase (△) in everted vesicles of the glycolate-fermenting bacterium. Assay conditions were the same as in Fig. 3. $(cHxN)_2C$ dissolved in ethanol was added 20 min before start of assay. For H_2 formation, 100% = 91 nmol H_2 · min^{-1} · mg membrane protein^{-1}; for ATPase, 100% = 230 nmol ATP · min^{-1} · mg membrane protein^{-1}.

al., 1969; Kröger and Dadák, 1969) could not be performed because activity of H_2 formation from glycolate was destroyed after the necessary lyophilization of the membrane fraction. Therefore, the effect of quinone analogs on electron transport from glycolate dehydrogenase to hydrogenase was studied. Antimycin A which is known to inhibit the cytochrome-bc_1 segment of the respiratory chain (Rieske and Zaugg, 1962), inhibited H_2 formation effectively. The titration curve showed 50% inhibition at a concentration of 0.22 µmol antimycin A · mg membrane protein^{-1} (Fig. 5). 2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide, another inhibitor of the cytochrome-bc_1 region (Lightbrown and Jackson, 1956),

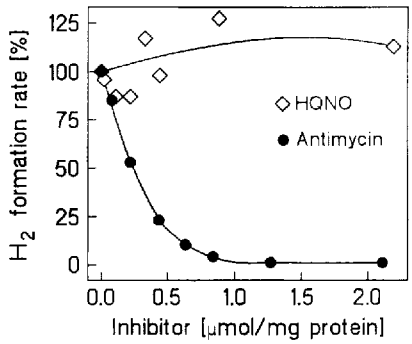

Fig. 5. Effect of antimycin A (●) and 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (◇) on ATP-driven H_2 formation from glycolate by everted membrane vesicles of the glycolate-fermenting bacterium. Assay conditions were the same as in Fig. 3. 2-*n*-Heptyl-4-hydroxyquinoline *N*-oxide (NQNO) and antimycin A (a mixture of A_1 to A_3) dissolved in ethanol were added. For 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide, 100% = 85 nmol H_2 · min^{-1} · mg membrane protein^{-1}; for antimycin A, 100% = 57 nmol H_2 · min^{-1} · mg membrane protein^{-1}.

as well as rotenone (Burgos and Redfearn, 1965), failed to inhibit electron transport (Fig. 5, Table 4).

DISCUSSION

In the present study, a reversed electron transport from glycolate to H_2 was demonstrated to occur in membrane vesicles of a syntrophically proton-reducing bacterium.

The first enzyme in glycolate oxidation, glycolate dehydrogenase, was shown to be membrane-bound, and catalyzed the oxidation of glycolate to glyoxylate with water-soluble quinones and several artificial electron acceptors such as benzyl viologen, methylene blue, Cl_2Ind or ferricyanide. Because of its sensitivity towards O_2, this enzyme was not detected in our first study on the glycolate-fermenting bacterium (Friedrich et al., 1991). Other glycolate dehydrogenases reacting with artificial electron acceptors have been described to occur in various algal species (for an overview, see Suzuki et al., 1991), cyanobacteria (Codd and Stewart, 1973; Tolbert, 1976), *Thiobacillus neapolitanus* (Beudeker et al., 1981), and *Escherichia coli* (Lord, 1972); the enzyme of the latter was shown to be membrane-associated. Aerobic metabolism of glycolate in these organisms is apparently linked to the respiratory chain via unknown electron carriers. Glycolate dehydrogenases reducing glyoxylate to glycolate with NADH or NADPH as electron donor were found in various plants (Zeltich, 1955), a few algal species (Lord and Merrett, 1971; Harrop and Kornberg, 1966), *Thiobacillus neapolitanus* (Saxena and Vishniac, 1970), and in a Gram-negative, glyoxylate-fermenting, strictly anaerobic rod, strain PerGlx1 (Friedrich and Schink, 1991). Reversibility of glycolate dehydrogenase reaction in the rather undefined membrane system of our glycolate-fermenting bacterium has not been demonstrated yet but is likely to occur because of the ability of this bacterium to disproportionate glyoxylate in pure culture (strain FlGlyR) to glycolate, CO_2 and H_2.

Also part of hydrogenase activity (25%) was found to be membrane-bound which could allow an electron transport from membrane-bound glycolate dehydrogenase to the site of proton reduction. Two different hydrogenase activities, one membrane-bound and one cytoplasmic, might be present in the glycolate-fermenting bacterium as indicated by the larger

part of the total hydrogenase activity being found in the cytoplasmic fraction. A similar distribution of membrane-associated enzyme activities was found with the pure culture (strain FlGlyR) of the glycolate-fermenting bacterium confirming that enzyme activities in membrane vesicle preparations originated from the glycolate-fermenting bacterium, not from *M. hungatei*, and that localization of these enzymes is independent of the respective cultivation conditions.

We found that H_2 formation from glycolate with everted membrane vesicles of the glycolate-fermenting bacterium depended strictly on the presence of ATP. Inhibitors of F_1F_0-ATPase, such as $(cHxN)_2C$ and diethylstilbestrol, abolished ATP hydrolysis by the membrane-bound ATPase and also H_2 formation from glycolate, indicating involvement of a proton-translocating ATPase, probably of the F_1F_0 type. In addition, we found that protonophores inhibited ATP-driven H_2 formation from glycolate. These data suggest that proton reduction with electrons from glycolate oxidation is driven by reversed electron transport which is powered by the proton motive force generated via ATP hydrolysis by a H^+ translocating ATPase. Direct evidence of involvement of protonmotive force to drive thermodynamically unfavourable reactions in energy metabolism was shown for *Methanosarcina barkeri* (Bott et al., 1986; Bott and Thauer, 1987) and for *Desulfuromonas acetoxidans* (Paulsen et al., 1986); in the latter, menaquinone was demonstrated to be involved in reversed electron transport during succinate oxidation with sulfur or NAD^+ as electron acceptor.

In membrane fractions of our glycolate-fermenting bacterium, menaquinone was found which could serve as physiological electron carrier between glycolate oxidation and proton reduction. This hypothesis is supported indirectly by reactivity of glycolate dehydrogenase and hydrogenase with water-soluble quinones, and inhibition of H_2 formation by antimycin A (although, only at comparably high concentrations; Rieske and Zaugg, 1962), while 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide and rotenone failed to inhibit this electron transport. Because of their mid-redox potential of -74 mV, menaquinones are suitable electron acceptors for glycolate oxidation ($E^{\circ\prime} = -92$ mV). Direct evidence of menaquinone involvement in this electron transport system has not yet been obtained. Cytochromes were not detected in crude extracts by redox difference spectroscopy (Friedrich et al., 1991).

Only recently, reversed electron transport systems have been described which use Na^+ as coupling ion, e.g. in the methanogenic strain Göl metabolizing methanol (Müller et al., 1988) and in anaerobically grown *Klebsiella pneumoniae* (Pfenniger-Li and Dimroth, 1992). The possibility of Na^+ involvement in glycolate oxidation could not be ruled out entirely: Na^+ ionophores such as monensin and ETH 157 affected H_2 formation from glycolate to some extent, but total inhibition was not observed, even at high inhibitor concentrations (Table 4). Nonetheless, high sensitivity of ATP-dependent H_2 formation towards the protonophores CCCP, 3,5,4′,5′-tetrachlorosalicylanilide and SF 6847, and lack of alleviation of protonophor inhibition by amiloride indicate strongly that this reversed electron transport system is driven exclusively by protons.

So far, reversed electron transport driven by $\Delta\mu_{H^+}$ has been known to occur only in assimilatory metabolism of purple phototrophic bacteria (Knaff, 1978), aerobic chemolithotrophs (Aleem, 1977) and mitochondria (Klingenberg and Schollmeyer, 1960), and in dissimilatory metabolism of methanogenic (Bott et al., 1986; Bott and Thauer, 1987), acetogenic (Diekert et al., 1986), sulfur-reducing (Paulsen et al., 1986), and sulfate-reducing bacteria (Pankhania et al., 1988). Involvement of a reversed electron transport in the proton-reducing *Syntrophomonas wolfei* during fermentation of butyrate has been postulated (Thauer and Morris, 1984) but has not yet been proven experimentally. Proton-reducing glycolate-oxidizing bacteria have to deal with a similar problem: the initial reaction oxdizing glycolate to glyoxylate ($E^{\circ\prime} = -92$ mV) is also very endergonic with protons as electron acceptors:

$$\text{Glycolate}^- \rightarrow \text{glyoxylate}^- + H_2, \qquad \Delta G' = +38 \text{ kJ/mol glycolate} \tag{2}$$

(at a p_{H_2} of 10 Pa).

We therefore hypothesize that this reaction requires a reversed electron transport driven by a transmembrane proton potential. It is open at present how many protons have to cross the membrane per pair electrons transported. Since glycolate oxidation through the pathway depicted in Fig. 1 yields only 1 mol ATP/mol substrate by substrate level phosphorylation, a maximum of 2 mol protons/mol substrate (corresponding to 0.67 mol ATP; Schink, 1988) can be invested into reversed electron transport.

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