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Electron transport phosphorylation driven by glyoxylate respiration with hydrogen as electron donor in membrane vesicles of a glyoxylate-fermenting bacterium

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Abstract The syntrophically glycolate-fermenting bacterium in the methanogenic binary coculture FIGlyM was isolated in pure culture (strain FIGlyR) with glyoxylate as sole substrate. This strain disproportionated 12 glyoxylate to 7 glycolate, 10 CO₂, and 3 hydrogen. Glyoxylate was oxidized via the malyl-CoA pathway. All enzymes of this pathway, i.e. malyl-CoA lyase/malate: CoA ligase, malic enzyme, and pyruvate synthase, were demonstrated in cell-free extracts. Glycolate dehydrogenase, hydrogenase, and ATPase, as well as menaquinones as potential electron carriers, were present in the membranes. Everted membrane vesicles catalyzed hydrogen-dependent glyoxylate reduction to glycolate [86–207 nmol min⁻¹ (mg protein)⁻¹] coupled to ATP synthesis from ADP and P_i [38–82 nmol min⁻¹ (mg protein)⁻¹]. ATP synthesis was abolished entirely by protonophores or ATPase inhibitors (up to 98 and 94% inhibition, respectively) indicating the involvement of proton-motive force in an electron transport phosphorylation driven by a new glyoxylate respiration with hydrogen as electron donor. Measured reaction rates in vesicle preparations revealed a stoichiometry of ATP formation of 0.2–0.5 ATP per glyoxylate reduced.

Key words Electron transport phosphorylation · Respiration · Membrane vesicles · Anaerobic degradation · Glyoxylate · Glycolate · Hydrogen · Syntrophy

Abbreviations BES 2-Bromoethanesulfonate · CCCP Carbonylcyanide *m*-chlorophenylhydrazine · DCCD *N,N'*-Dicyclohexylcarbodiimide · DCPIP 2,6-Dichlorophenolindophenol · DTE Dithioerythritol · TCS 3,5,4',5'-Tetrachlorosalicylanilide · SF 6847 3,5-Di-*tert*-butyl-4-hydroxybenzylidenemalonitrile

Introduction

A defined syntrophic coculture, strain FIGlyM consisting of a gram-positive, spore-forming rod and *Methanospirillum hungatei* strain SK, ferments glycolate stoichiometrically to methane and CO₂ (Friedrich et al. 1991). Oxidation of glycolate to 2 CO₂ + 3 H₂, as carried out by the fermenting bacterium in the coculture, is an endergonic reaction under standard conditions ($\Delta G^{\circ'} = +36$ kJ/mol glycolate). Therefore, the P_{H₂} has to be kept low by a hydrogen-scavenging partner (e.g., a methanogenic bacterium) to support growth. Only glycolate is utilized by the fermenting bacterium, and thus growth is possible only in coculture with a hydrogen-consuming partner.

All enzymes necessary to metabolize glycolate have been detected in cell-free extracts of glycolate-grown cells of the glycolate-fermenting bacterium (Friedrich et al. 1991; Friedrich and Schink 1993). The degradation pathway includes oxidation of glycolate to glyoxylate, condensation of glyoxylate with acetyl-CoA to malyl-CoA, ATP formation via substrate level phosphorylation using the energy-rich thioester bond of malyl-CoA, and oxidative decarboxylation of malate to pyruvate and of pyruvate to acetyl-CoA, thus regenerating the glyoxylate acceptor for a new reaction cycle. Electrons from oxidation reactions are released as molecular hydrogen. Direct proton reduction ($E^{\circ'} = -414$ mV) with electrons from glycolate oxidation ($E^{\circ'} = -92$ mV) is in thermodynamic equilibrium with a P_{H₂} of 1 μ Pa, which cannot be maintained by the syntrophic partner. Therefore, a reversed electron transport is required to drive this reaction. Glycolate dehydrogenase, hydrogenase, and ATPase have been shown to be involved in proton gradient-dependent hydrogen formation from glycolate in membrane vesicles of the glycolate-fermenting bacterium, thus providing direct proof for such a reversed electron transport (Friedrich and Schink 1993). The electron carriers involved in electron transport could not be proven directly, but membranes of the glycolate-fermenting bacterium contain menaquinones as potential electron carriers (Friedrich and Schink 1993).

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This investigation reports on the isolation of the glycolate-fermenting bacterium in pure culture with glyoxylate as sole growth substrate and the metabolism of glyoxylate. Glyoxylate respiration with hydrogen as electron donor is described as a new type of anaerobic respiration.

Materials and methods

Organisms and cultivation

Strain FIGlyM (DSM 6945) was from our own culture collection. *Methanospirillum hungatei* strain SK was obtained from Prof. F. Widdel, Bremen, Germany. Bacteria were grown in a bicarbonate-buffered freshwater medium as described earlier (Friedrich et al. 1991), reduced with either 1 mM Na₂S or 2 mM cysteine. For isolation, the agar shake dilution method was applied (Widdel and Pfennig 1984). Purity of cultures was checked microscopically after growth in mineral medium with glyoxylate (5 mM), or in semi-solid complex medium (AC-medium; Difco, Ann Arbor, Mich., USA) at normal and at fivefold diluted concentration.

Growth experiments were carried out in 120-ml serum flasks filled with 50 ml mineral medium under an atmosphere of N₂/CO₂ (80:20, v/v) at a temperature of 30°C; samples were taken with syringes.

Preparation of cell-free extracts and membrane vesicles

Strain FIGlyR was grown under an atmosphere of N₂/CO₂ (80:20, v/v) in 1.25-l infusion bottles filled with 1 l mineral medium reduced with 2 mM cysteine as described before (Friedrich and Schink 1993). Glyoxylate (final concentration 25 mM) was fed as sole growth substrate in portions of 5 mM during growth. Cells were harvested in the late exponential growth phase, and cell-free extracts and spheroplasts were prepared as described previously (Friedrich and Schink 1993). Everted membrane vesicles were generated by passing spheroplasts four times through a French pressure cell at 43 MPa pressure. After removal of cell debris, subcellular fractions were obtained by ultracentrifugation (Optima TL-ultracentrifuge equipped with a TLA-100.4-rotor; Beckman, München, Germany) at 417,000 × g for 30 min.

Enzyme assays

Spectrophotometric enzyme assays were performed under N₂ atmosphere at 30°C in rubber-stoppered cuvettes as described before (Friedrich et al. 1991; Friedrich and Schink 1993).

Glycolate dehydrogenase (EC 1.1.99.14) activity was determined according to Friedrich and Schink (1993), *malyl-CoA lyase* (EC 4.1.3.24)/*malate:CoA ligase* (EC 6.2.1.9) according to Friedrich et al. (1991), *malic enzyme* (EC 1.1.1.40) according to Stams et al. (1984), *pyruvate synthase* (EC 1.2.7.1) according to Odom and Peck (1981), and *hydrogenase* (EC 1.12.99.-) according to Diekert and Thauer (1978). *ATPase* (EC 3.6.1.34) was analyzed according to Vogel and Steinhart (1976).

Glyoxylate reductase (EC 1.1.1.26) was analyzed with NAD(P)H ($\epsilon_{365\text{nm}} = 3.5$) mM⁻¹ cm⁻¹) as electron donor. The assay mixture contained 50 mM potassium phosphate buffer (pH 6.5), 2.5 mM dithioerythritol (DTE), 5 mM MgCl₂ and 0.3 mM NAD(P)H. The reaction was started either with protein or with 10 mM glyoxylate.

NAD(P)H:acceptor oxidoreductase (EC 1.6.99.1) was measured following the reduction of benzyl viologen ($\epsilon_{578\text{nm}} = 8.65$ mM⁻¹ cm⁻¹) or methylene blue ($\epsilon_{570\text{nm}} = 13.1$ mM⁻¹ cm⁻¹) with NAD(P)H. The assay mixture contained 50 mM potassium phosphate buffer (pH 6.5), 2.5 mM DTE, 0.3 mM NAD(P)H, and 5 mM benzyl viologen or 0.1 mM methylene blue. NADPH oxidation with protons as electron acceptors was determined with the same assay mixture, but artificial electron acceptors were omitted. The reactions were started by addition of protein.

Assay for glyoxylate reduction with hydrogen

The assay was performed under an atmosphere of hydrogen (0.15 MPa) in 5-ml glass vials sealed with butyl rubber septa and containing 50 mM potassium phosphate buffer (pH 6.5), 2.5 mM DTE, 5 mM Na₂-ADP, 5 mM MgCl₂, and 10 mM glyoxylate. Alternatively, NADPH (0.5 mM) was checked as electron donor for glyoxylate reduction. The reaction was started with membrane protein (0.2–0.8 mg). Samples (100 µl) were withdrawn with gas-tight syringes (Unimetrics; Macherey-Nagel, Düren, Germany), placed on ice immediately, and analyzed subsequently by HPLC.

DNA base composition

The G+C content of the DNA was determined by direct quantitation of the nucleosides with HPLC after enzymatic degradation of the DNA (Mesbah et al. 1989), using phage λ DNA as standard. DNA was isolated from a 1-l culture of glyoxylate-grown cells of strain FIGlyR using the NaOH method for cell disruption (Mesbah et al. 1989).

Chemical analyses

Methane and hydrogen were quantified by gas chromatography (Platen and Schink 1987; Friedrich and Schink 1993); samples containing less than 0.2 nmol hydrogen were analyzed using a RGD2 reduction gas detector (Techmaton, Düsseldorf, Germany) (Friedrich and Schink 1993).

Glycolate, glyoxylate, and formate were measured by HPLC as described previously (Friedrich et al. 1991), using an ORH-801 organic acids column (300 × 6.5 mm; Interaction Chemicals, Mountain View, Calif., USA) with 5 mM H₂SO₄ as eluent at a flow rate of 0.6 ml/min at 50°C. Compounds were detected by a refractive index detector (Erma, Tokyo, Japan).

ATP, ADP, and AMP were determined by HPLC employing ion-pair chromatography (Litters and Schmelzeisen-Redeker 1989). Separation was performed on an Ultrasphere-ODS column (150 × 4 mm, Beckman, München, Germany) with 80 mM KH₂PO₄ (adjusted to pH 6.0 with 1 M KOH), and 5 mM tetrabutylammonium hydrogen sulfate/methanol (77:23, v/v) as eluent at a flow rate of 1.25 ml/min. Adenosine derivatives were detected at 260 nm wavelength and analyzed as described earlier (Friedrich et al. 1991).

Protein determination

Protein was determined by the microprotein assay (Bradford 1976) with bovine serum albumin as standard.

Chemicals

All chemicals were of analytical or reagent grade quality and were obtained from Biomol, Ilvesheim, Germany; Boehringer, Mannheim, Germany; Fluka, Neu-Ulm, Germany; Eastman Kodak, Rochester, N.Y., USA; Merck, Darmstadt, Germany; Serva, Heidelberg, Germany; Sigma, Deisenhofen, Germany; or Wako-Chemie, Neuss, Germany. Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Isolation

The syntrophically glycolate-oxidizing bacterium in the binary methanogenic coculture FIGlyM was enriched with glyoxylate as sole growth substrate. Transferred sub-

Fig. 1 Phase contrast photomicrograph of strain FIGlyR (bar 10 μm)

cultures resumed growth much faster in cysteine-reduced (2 mM) mineral medium than in sulfide-reduced (1 mM) mineral medium. In cysteine-reduced medium the coculture grew with glyoxylate as substrate if glyoxylate was added in small amounts (1 mM) to a coculture growing with glycolate. Transfers of glyoxylate-adapted cocultures resumed growth immediately, and glyoxylate could be added up to 5 mM in one step. Attempts to isolate the fermenting bacterium from the coculture by agar shake dilution series with glyoxylate as substrate failed. Colony formation was observed only in the first tube of a dilution series, but all colonies contained *M. hungatei*. Therefore, cultures were grown in the presence of 2-bromoethanesulfonate (2 mM; BES) to inhibit growth of *M. hungatei*. After several transfers in the presence of BES, *M. hungatei* was diluted out, and the fermenting bacterium grew in pure culture with glyoxylate.

Again, attempts to isolate the fermenting bacterium from the axenically grown culture by the agar dilution method failed. No colony formation was observed, even after 3 months of incubation. Purity of this culture was checked after growth with glyoxylate in AC medium, or after growth with glyoxylate in mineral medium without BES in the presence of formate as a possible growth substrate for *M. hungatei*. In all cases, *M. hungatei* was not observed microscopically, and no CH_4 was formed.

Fig. 2 Time course of glyoxylate fermentation by strain FIGlyR. OD_{500} (open circles), glyoxylate (filled diamonds), glycolate (filled circles), hydrogen (open triangles)

After reassociation of the glyoxylate-grown pure culture with *M. hungatei*, glycolate was degraded completely to CH_4 , proving that the fermenting bacterium isolated from coculture FIGlyM was identical with the glycolate-oxidizing bacterium. The strain was named FIGlyR (Fig. 1).

Morphological and cytological properties of strain FIGlyR

Cells of strain FIGlyR were gram-positive, slightly curved rods, $2.0\text{--}3.5 \times 0.45 \mu\text{m}$ in size (Fig. 1). Endospore formation was observed after syntrophic growth with glycolate without delay; glyoxylate-grown cultures formed only a few spores, even after several months of incubation. Poly-(β)-hydroxybutyrate or other inclusion bodies were not detected. The G + C content of the DNA was $46.7 \pm 0.15 \text{ mol}\%$.

Physiology

Strain FIGlyR oxidized glyoxylate to CO_2 and H_2 and reduced part of the glyoxylate to glycolate. Hydrogen accu-

Table 1 Stoichiometry of fermentation and growth yields of strain FIGlyR. Growth experiments were carried out in 120-ml serum flasks filled with 50–60 ml mineral medium under a N_2/CO_2 (80:20, v/v) atmosphere

Glyoxylate concentration (mM)	Glyoxylate degraded (μmol)	Optical density reached ΔE ₅₀₀	Cell dry mass formed (mg)	Substrate assimilated ^a (μmol)	Products formed (μmol)		Electron recovery (%)	Molar growth yield (g/mol)
					Glycolate	H ₂		
FIGlyR								
3	162	0.08	0.93 ^b	38	70	29	98	5.7
7	382	0.151	1.75 ^b	72	154	38	84	4.6
Per Glyox1 ^d								
10	512	0.072	0.92 ^c	38	140	547	103	1.8

^a Glyoxylate assimilated was calculated according to the formula: $17 \text{ C}_2\text{H}_2\text{O}_3 \rightarrow 4 <\text{C}_4\text{H}_7\text{O}_3> + 3 \text{ H}_2\text{O} + 18 \text{ CO}_2$

^{b, c} Cell dry matter values were calculated via cell density using an experimentally determined conversion factor (^b 0.1 $\text{OD}_{500} = 21 \text{ mg dry matter}$; ^c 0.1 $\text{OD}_{500} = 25 \text{ mg dry matter/l}$)

^d Data taken from Friedrich and Schink (1991)

mulated up to a P_{H_2} of 1,000 Pa (Fig. 2). The doubling time of strain FIGlyR was 33 h ($\mu = 0.021 \text{ h}^{-1}$; Fig. 2), which was shorter than that for growth of the coculture with glycolate ($t_d = 51 \text{ h}$; Friedrich et al. 1991). If initial glyoxylate concentrations higher than 5 mM were applied for growth, the lag phase was considerably longer or the culture did not resume growth at all. Therefore, the fermentation balance was analyzed only with low substrate concentrations (Table 1). With constant gas volume of the culture vessel, an increase in glyoxylate supply resulted in a shift of the fermentation stoichiometry towards more glycolate formation, whereas the amount of H_2 formed was the same in all cases (Table 1). Cysteine acted only as reducing agent and sulfur source and was not dissimilated as the fermentation balance demonstrates (Table 1). Strain FIGlyR did not grow with glycolate in pure culture, or with other intermediates of the malyl-CoA pathway (Friedrich et al. 1991), i.e., malate or pyruvate, or with fumarate, succinate, or H_2/CO_2 in cysteine-reduced medium. No growth occurred with glyoxylate in the presence of H_2/CO_2 (80:20, v/v) in the gas phase.

Enzymes involved in glyoxylate degradation

Enzymes involved in glycolate degradation were detected in crude extracts of the pure culture FIGlyR at activities sufficient to account for dissimilatory turnover of glyoxylate by an exponentially growing culture [$136 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$]. All enzyme activities of the malyl-CoA pathway of glyoxylate degradation were detected; malyl-CoA lyase/malate:CoA ligase [$0.95 \text{ U (mg protein)}^{-1}$, measured as reaction chain], malic enzyme [$10.2 \text{ U (mg protein)}^{-1}$, Table 2], and pyruvate synthase [$0.17 \text{ U (mg protein)}^{-1}$] were present at activities comparable to those found previously in crude extracts of the coculture FIGlyM grown on glycolate (Friedrich et al. 1991).

A membrane-bound glycolate dehydrogenase activity reacting with methylene blue, benzyl viologen, DCPIP, ferricyanide, and water-soluble quinones, which was found previously in cells grown syntrophically with glycolate (Friedrich and Schink 1993), was also found in glyoxylate-grown cells of the pure culture (Table 2); hydrogenase and ATPase were also present at significant activities in the membrane fraction (Table 2). Malic enzyme as a cytoplasmic marker was almost entirely recovered in the cytoplasmic fraction, indicating successful separation of both cell fractions.

ATP formation coupled to glyoxylate reduction with hydrogen in membrane vesicles

Membrane vesicle preparations of strain FIGlyR catalyzed H_2 -dependent glyoxylate reduction to glycolate (Fig. 3A). ATP was formed from ADP and P_i if glyoxylate was reduced with H_2 to glycolate in membrane vesicles (Fig. 3B). No ATP formation occurred if glyoxylate or hydrogen was omitted, indicating that ATP was not formed from ADP by adenylate kinase. During complete glyoxy-

Table 2 Distribution of membrane-associated enzyme activities in subcellular fractions of strain FIGlyR. Electron donors or acceptors for redox reactions are given in parentheses (*DH* dehydrogenase, *MB* methylene blue, *MV* methyl viologen)

Enzyme activity	U	Protein (mg)	Specific activity (U/mg)	Proportion in source (%)
Glycolate DH (MB)				
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	11.4	36.6	0.31	100
Cytoplasm	6.8	21	0.32	56
Membrane	4.9	12	0.41	43
Malic enzyme (NADP⁺)				
Extract	373	36.6	10.2	100
Cytoplasm	229	21	10.9	61
Membrane	4.5	12	0.4	1.2

Fig. 3 Time course of glyoxylate reduction with **A** H_2 and **B** ATP formation in everted membrane vesicles of strain FIGlyR. A complete assay mixture (filled symbols) contained: 9 mM glyoxylate, 5 mM ADP, 0.15 MPa H_2 in the gas phase and 0.43 mg membrane protein. As a control, H_2 was omitted (open symbols). Glyoxylate (open and filled diamonds), glycolate (open and filled triangles), ADP (open and filled squares), and ATP (open and filled circles)

late reduction to glycolate, ATP accumulated to concentrations of 1.5 mM and was later hydrolyzed to ADP and P_i . A stoichiometry of 0.25 to 0.5 ATP per glyoxylate was deduced from rates of glyoxylate reduction with hydrogen [86–267 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$] versus ATP formation [38–82 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$].

At low concentrations of the protonophores tetrachlorosalicylanilide (TCS), carbonylcyanide *m*-chlorophenyl-

Table 3 Effect of protonophores and the ATPase inhibitor DCCD on H_2 -dependent glyoxylate reduction and on respiration-dependent ATP formation. Inhibitors dissolved in ethanol (final concentration 0.5%) were added 20 min prior to the start of the reaction with glyoxylate (CCCP carbonylcyanide *m*-chlorophenyl-hydrazone, DCCD *N,N'*-dicyclohexylcarbodiimide, TCS 3,5,4',5'-tetrachlorosalicylanilide)

Treatment	Inhibitor concentration [nmol (mg protein) ⁻¹]	Glyoxylate reduction [nmol min ⁻¹ (mg protein) ⁻¹]	Remnant activity (%)	ATP formation [nmol min ⁻¹ (mg protein) ⁻¹]	Remnant activity (%)
None	0	259	100	63	100
TCS	59	118	46	1.3	2
TCS	118	94	36	1.3	2
DCCD	59	177	68	6.7	11
DCCD	118	186	72	3.9	6
None	0	126	100	55	100
TCS	0.74	116	92	53	96
TCS	1.5	111	88	21	38
TCS	3	105	83	1.7	3
TCS	7.4	90	71	0.8	1.5
CCCP	7.4	105	83	57	104
CCCP	15	124	98	47	85
CCCP	74	121	96	17	31

Fig. 4 Effect of the protonophore 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalonitrile (SF 6847) on respiration-dependent ATP formation activity (filled circles) and on H_2 -dependent glyoxylate reduction activity (open triangles). Assay conditions were as in Fig. 3. SF 6847 dissolved in ethanol was added 20 min before the start of the reaction with glyoxylate and ADP. For ATP formation, 100% activity = 49 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$; for glyoxylate reduction, 100% activity = 107 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$

hydrazone (CCCP), and 3,5-di-*tert*-butyl-4-hydroxybenzylidene malonitrile (SF 6847) ATP formation was uncoupled from glyoxylate reduction entirely (Table 3; Fig. 4). As shown by inhibitor titration, SF 6847 inhibited ATP formation almost completely at a concentration of 7.4 $\text{nmol (mg protein)}^{-1}$ with a remnant ATP formation rate of only 3 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ (6%) with 50% inhibition at 3 $\text{nmol (mg protein)}^{-1}$ (Fig. 4). CCCP inhibited ATP formation at comparably higher concentrations (31% remnant activity at 74 $\text{nmol (mg protein)}^{-1}$). Neither protonophore affected the rate of glyoxylate reduction with hydrogen at all. TCS also uncoupled ATP formation from glyoxylate respiration very effectively at low concentrations up to 7.4 $\text{nmol (mg protein)}^{-1}$, whereas glyoxylate respiration was affected only up to 30% (Table 3). At higher concentrations of up to 118 $\text{nmol (mg protein)}^{-1}$, the effect of TCS on glyoxylate reduction was more pronounced with an inhibition of up to 66% (Table 3).

The ATPase inhibitor dicyclohexylcarbodiimide (DCCD; Linnett and Beechey 1979; Hassinen and Vuokila 1993) uncoupled ATP formation driven by H_2 -dependent glyoxylate reduction almost entirely (94%) at a DCCD concentration of 118 $\text{nmol (mg protein)}^{-1}$, whereas glyoxylate reduction to glycolate was affected only to a minor extent with a remnant activity of 72% (Table 3); glycolate dehydrogenase and hydrogenase activity were insensitive to the applied inhibitors, and the ATPase activity in strain FIGlyR was inhibited by DCCD, as proven previously (Friedrich and Schink 1993). These results indicate that the proton-motive force is involved in ATP formation.

H_2 -dependent $NADP^+$ reduction

During glyoxylate oxidation, electrons are transferred to a ferredoxin-like carrier in the pyruvate synthase ($E^{\circ'} = -470 \text{ mV}$) and to $NADP^+$ in the malic enzyme reaction ($E^{\circ'} = -331 \text{ mV}$). The pure culture releases part of them, preferentially the former ones, as H_2 ($E^{\circ'} = -414 \text{ mV}$), and the remainder is used up in glyoxylate reduction to glycolate.

Membranes and cytoplasmic fractions oxidized NADPH with benzyl viologen and methylene blue, but not with protons as electron acceptors (Table 4). Instead, high activities of H_2 -dependent $NADP^+$ reduction were found in both fractions (Table 4), indicating that the metabolism of NADPH is partly associated with the cytoplasmic membrane. However, no ADP phosphorylation driven by H_2 -dependent $NADP^+$ reduction was detected. A small, apparently glyoxylate-dependent, NADPH-oxidizing activity was found exclusively in the membrane fraction as determined by photometric assay of NADPH oxidation (Table 4). However, this reaction could not be verified in a discontinuous HPLC assay monitoring glyoxylate conversion to glycolate.

Table 4 Distribution of substrate-converting activities in NADP(H) metabolism in sub-cellular fractions of strain FIGlyR (BV benzyl viologen, MB methylene blue)

Activity	Electron acceptor/donor	Source	Activity (U)	Protein (mg)	Specific activity (U/mg)	Proportion in source (%)
Glyoxylate reduction	NADPH	Extract	0.52	22.7	0.023	100
		Cytoplasm	0	15.3	0	0
		Membrane	0.62	3.1	0.2	119
NADPH oxidation	BV	Extract	29.5	22.7	1.3	100
		Cytoplasm	21.1	15.3	1.4	72
		Membrane	9.4	3.1	3.06	32
	MB	Extract	31.2	36.6	0.85	100
		Cytoplasm	5.6	21.5	0.26	18
		Membrane	27.1	10.8	2.51	87
	H ⁺	Extract	<0.02	22.7	<0.001	–
		Cytoplasm	<0.01	15.3	<0.001	–
		Membrane	<0.003	3.1	<0.001	–
H ₂ oxidation	NADP ⁺	Extract	213.5	22.7	9.4	100
		Cytoplasm	98	15.3	6.4	46
		Membrane	54	3.1	17.5	25

Discussion

Strain FIGlyR was isolated in pure culture with glyoxylate as sole carbon and energy source from the syntrophically glycolate-degrading coculture FIGlyM. Although strain FIGlyR could not be transferred into pure culture from a single cell clone, the syntrophic methanogenic partner bacterium was diluted out of the culture by repeated transfers in the presence of the inhibitor 2-bromoethanesulfonate. Lack of growth in agar cultures may be due to accumulation of inhibitory H₂ concentrations (see below). A similar problem was reported also for the syntrophically butyrate-degrading bacterium *Syntrophus buswellii*, which grows in pure culture with crotonate only in liquid medium, but not in agar (Dörner 1992). Growth and isolation of strain FIGlyR with glyoxylate in pure culture prove again that the concept of obligate syntrophy has to be revised. Recently, other strains formerly regarded as obligate syntrophs, such as *Syntrophomonas wolfei*, *Syntrophospora bryantii*, *Syntrophobacter wolinii*, and *Syntrophus buswellii*, have been shown also to grow with partly oxidized substrates in pure culture (Beatty and McInerney 1987; Zhao et al. 1990; Dörner 1992; Wallrabenstein and Schink 1994; Wallrabenstein et al. 1994).

Strain FIGlyR dismutated glyoxylate to glycolate, CO₂, and H₂, approximately according to the following equation:

Glycolate was used for growth only in coculture with a hydrogen scavenger (Friedrich et al. 1991).

Biochemistry

The pathway of glyoxylate degradation by strain FIGlyR was elucidated by analysis of key enzymes (Fig. 5). Glyoxylate was oxidized via the malyl-CoA pathway (i.e., via malyl-CoA lyase and malate:CoA ligase and malic enzyme and pyruvate synthase. All these enzymes were detected at activities comparable to those of cells grown syntrophically with glycolate (Table 2; Friedrich et al. 1991). Membrane-bound glycolate dehydrogenase was also demonstrated at activities in the same range as those of syntrophically grown cells. In the pure culture, this enzyme obviously catalyzed glyoxylate reduction to glycolate. The stoichiometry of glyoxylate fermentation (Eq. 1) revealed that a major part of reducing equivalents from glyoxylate oxidation served as electron donor for glyoxylate reduction to glycolate; only 12–27% were liberated as H₂, depending on the initial glyoxylate concentration (Table 1).

Reversibility of the glycolate dehydrogenase reaction was demonstrated with membrane vesicles: glyoxylate was reduced with hydrogen as electron donor, probably involving electron transport from hydrogenase to glycolate dehydrogenase with menaquinones as electron carriers. The latter were previously found in glycolate- and glyoxylate-grown cells, but direct evidence of their participation in electron transport has not yet been obtained (Friedrich and Schink 1993). NADPH was tested as an alternative electron donor for glyoxylate reduction. Photometric analysis revealed an apparently glyoxylate-dependent NADPH oxidation, but this activity was not confirmed in attempts to measure NADPH-dependent glyoxylate conversion to glycolate by HPLC. Thus, direct re-oxidation of NADPH with glyoxylate as electron acceptor seems unlikely. Nevertheless, some of the electrons from malic enzyme reaction end up in glyoxylate reduction to

Fig. 5 Hypothetical pathway of glyoxylate degradation by strain FIGlyR

glycolate, as indicated by the fermentation balance. So far, the path of these electrons is still unknown, but H_2 might be an intermediate, as suggested by the following findings, NADPH-oxidizing activities with benzyl viologen and methylene blue, and high activities of H_2 -dependent $NADP^+$ reduction were both found to be partly membrane-associated. A possible link of NADPH to the H_2 pool ($E' = -353$ mV at P_{H_2} of 1,000 Pa) would interfere directly with the malic enzyme reaction [$E' = -331$ mV calculated with $CO_2(g)$ after Thauer et al. 1977]. The P_{H_2} did not exceed 1,000 Pa during growth of strain FIGlyR, even with increased glyoxylate concentrations, and strain FIGlyR was unable to grow with glyoxylate (5 mM) in the presence of excess H_2 (80 kPa). Obviously, reduction of the $NADP^+$ pool by excess H_2 prevents malate oxidation and thereby inhibits glyoxylate fermentation. This may also be the reason why the pure culture of strain FIGlyR did not grow in agar cultures (see above).

We have shown previously that membrane vesicles of glyoxylate-grown cells catalyzed ATP-dependent H_2 formation from glycolate [$30\text{--}307$ nmol min^{-1} (mg protein) $^{-1}$] (Friedrich and Schink 1993). Now we demonstrate ATP formation due to glyoxylate reduction with H_2 in membrane vesicles at a comparable rate. The F_1F_0 -ATPase inhibitor DCCD abolished glyoxylate reduction-driven ATP formation almost entirely (94% inhibition, Table 3). In addition, we have shown previously DCCD inhibition of ATPase activity (Friedrich and Schink 1993). Glyoxylate reduction with hydrogen was influenced by DCCD only to a minor extent (32% inhibition; Table 3). It is well known that DCCD also interferes with several other proteins of bioenergetic relevance, such as cytochrome-*c* oxidase, ubiquinol:cytochrome-*c* oxidoreductase, NADH:ubi-

quinone oxidoreductase, and energy-linked transhydrogenases due to its reactivity with carboxyl residues (Hassinen and Vuokila 1993); this might be a rationale for the observed 30% inhibition of H_2 -dependent glyoxylate reduction by DCCD. Nevertheless, ATP formation was already inhibited to a large extent, whereas glyoxylate was still reduced with 70% remnant activity, indicating involvement of proton-translocating ATPase. In addition, protonophores such as CCCP, SF 6847, and TCS also inhibited ATP formation. With the exception of TCS at concentrations higher than 10 nmol (mg protein) $^{-1}$, glyoxylate reduction was not affected by these protonophores. Interference of TCS with targets other than the proton-motive force has only recently been reported for luciferase (Peinemann et al. 1989). We conclude that ATP was formed from ADP and P_i by a proton-translocating ATPase, probably of the F_1F_0 -type; this reaction was driven by a proton-motive force generated by the electron transport from glyoxylate to glycolate with H_2 as electron donor. This electron transport phosphorylation represents the reversal of the reversed electron transport from glycolate to glyoxylate and H_2 that was found recently in syntrophic glycolate oxidation (Friedrich and Schink 1993).

Energetics

Synthesis of ATP from ADP and P_i under quasi-reversible conditions requires a minimum of -44 kJ per mol in a living cell (Thauer et al. 1977), and irreversible ATP synthesis consumes up to -70 kJ per mol (Schink 1991). Under standard conditions, glyoxylate respiration with H_2 as electron donor is sufficiently exergonic ($\Delta G^{\circ} = -62$ kJ per mol glyoxylate) to drive electron-transport-linked

synthesis of one ATP per glyoxylate reduced, and we showed ATP synthesis under comparable conditions with everted membrane vesicles. A stoichiometry of 0.2–0.5 ATP synthesized per glyoxylate reduced was deduced from rates of ATP formation and glyoxylate reduction, indicating that ATP synthesis and glyoxylate respiration were not strictly coupled. This may be due to the membrane vesicle preparation used that might have consisted of everted vesicles, right-side-out vesicles, and open membrane fragments; phosphorylation is expected to be carried out only in everted vesicles (Kröger and Winkler 1981). Glyoxylate fermentation by strain FIGlyR according to Eq. 1 yields –29 kJ per mol glyoxylate. Under physiological conditions (i.e., 1.5 mM glyoxylate, 1.5 mM glycolate, 30 mM HCO₃⁻, and 600 Pa H₂), growth with glyoxylate according to Eq. 1 yields –73 kJ per mol glyoxylate. According to the reaction sequence outlined in Fig. 5, 0.4 mol ATP per mol glyoxylate is synthesized by substrate level phosphorylation, and 0.13–0.3 mol ATP per mol glyoxylate by glyoxylate respiration based on the phosphorylation stoichiometry measured with membrane vesicles. The overall ATP gain of 0.5–0.7 mol ATP per mol glyoxylate metabolized is in good agreement with Y_s of 4.6–5.7 g per mol glyoxylate measured, assuming that 10 g cell dry matter is formed per mol ATP (Stouthamer 1979). Glyoxylate fermentation by strain PerGlyox1 originally isolated with glyoxylate as substrate yielded only 1.8 g per mol glyoxylate, although glyoxylate was degraded via the same pathway (Table 1; Friedrich and Schink 1991). The difference in yield is probably due to the inability of strain PerGlyox1 to conserve energy by glyoxylate respiration. Instead, a small amount of glycolate (1 glycolate per 4 glyoxylate) is formed by an NADH-dependent glyoxylate reductase, and ATP is synthesized only by substrate level phosphorylation (Friedrich and Schink 1991). This strain cannot oxidize glycolate syntrophically, and the glycolate dehydrogenase reaction operates only toward glycolate formation. Glyoxylate respiration in strain FIGlyR contributes to energy conservation, and this strain grows obviously closer to thermodynamic equilibrium than strain PerGlyox1 in which glycolate reduction only increases the reaction entropy. The possible advantage of the latter strategy still needs to be elucidated.

The phylogenetic status of strain FIGlyR will be defined in detail in a forthcoming study based on 16S rRNA sequence analysis. Strain FIGlyR has been deposited with Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany, under no. DSM 8271.

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