Anaerobic degradation of malonate via malonyl-CoA by *Sporomusa malonica*, *Klebsiella oxytoca*, and *Rhodobacter capsulatus*

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Abstract

Anaerobic decarboxylation of malonate to acetate was studied with *Sporomusa malonica*, *Klebsiella oxytoca*, and *Rhodobacter capsulatus*. Whereas *S. malonica* could grow with malonate as sole substrate ($Y = 2.0 \text{ g} \cdot \text{mol}^{-1}$), malonate decarboxylation by *K. oxytoca* was coupled with anaerobic growth only in the presence of a cosubstrate, e.g. sucrose or yeast extract ($Y_s = 1.1\text{--}1.8 \text{ g} \cdot \text{mol}^{-1}$ malonate⁻¹). *R. capsulatus* used malonate anaerobically only in the light, and growth yields with acetate and malonate were identical. Malonate decarboxylation in cell-free extracts of all three bacteria was stimulated by catalytic amounts of malonyl-CoA, acetyl-CoA, or Coenzyme A plus ATP, indicating that actually malonyl-CoA was the substrate of decarboxylation. Less than 5% of malonyl-CoA decarboxylase activity was found associated with the cytoplasmic membrane. Avidin (except for *K. oxytoca*) and hydroxylamine inhibited the enzyme completely, EDTA inhibited partially. In *S. malonica* and *K. oxytoca*, malonyl-CoA decarboxylase was active only after growth with malonate; malonyl-CoA: acetate CoA transferase was found as well. These results indicate that malonate fermentation by these bacteria proceeds via malonyl-CoA mediated by a CoA transferase and that subsequent decarboxylation to acetyl-CoA is catalyzed, at least with *S. malonica* and *R. capsulatus*, by a biotin enzyme.

Abbreviations: CoASH – Coenzyme A, EDTA – ethylenediamine tetraacetate

Introduction

Decarboxylation of dicarboxylic acids to the corresponding monovalent acids releases a small amount of free energy ($\Delta G_0' = -20\text{--}30 \text{ kJ} \cdot \text{mol}^{-1}$; Thauer et al. 1977) that can be exploited by certain fermenting bacteria to cover their entire energy demand for growth. In the case of succinate fermentation to propionate by *Propionigenium modestum* (Schink & Pfennig 1982), a membrane-bound methylmalonyl-CoA decarboxylase enzyme acts as primary sodium pump and conserves the decarboxylation energy as a sodium ion gradient which drives ATP synthesis by a sodium-pumping ATPase (Hilpert et al. 1984). The decarboxylating enzyme in anaerobes fermenting glu-

tarate to butyrate and isobutyrate (Matthies & Schink 1992a) is stimulated by sodium ions, and acts obviously as a sodium-translocating primary sodium pump as well (Matthies & Schink 1992b). Contrary, in oxalate decarboxylation to formate (Allison et al. 1985; Smith et al. 1985; Dehning & Schink 1989a) the oxalyl-CoA decarboxylase enzyme is not membrane-bound (Baetz & Allison 1989), and membrane energization is accomplished through an electrogenic oxalate/formate antiporter (Anantharam et al. 1989). A similar concept appears to underly energy conservation in malolactic fermentation (Kolb et al. 1992; Poolman 1990).

Growth of fermenting bacteria on the basis of malonate decarboxylation to acetate was reported recently (Breznak et al. 1988; Dehning & Schink 1989b; Dehn-

ing et al. 1989; Janssen & Harfoot 1990; Janssen 1991). Biochemical studies on *Citrobacter diversus* revealed that malonyl-CoA is formed through a ligase reaction, prior to decarboxylation to acetyl-CoA. Conservation of the decarboxylation energy appeared to be mediated through a membrane-bound process (Janssen & Harfoot 1992).

In the present communication, evidence is provided that malonate decarboxylation to acetate in *Sporomusa malonica*, *Klebsiella oxytoca*, and *Rhodobacter capsulatus* involves activation to malonyl-CoA through a CoA transferase, and that the malonyl-CoA decarboxylases are, at least in two cases, biotin enzymes. The biochemistry of malonate decarboxylation and the energy conservation mechanism of all these strains are basically different, therefore, from the malonate decarboxylase system described recently for *Malonomonas rubra* where the free acid is decarboxylated after enzyme activation through acetylation (Hilbi et al. 1992).

Materials and methods

Bacterial strains and cultivation

Sporomusa malonica (DSM 5090) was isolated and described recently (Dehning et al. 1989). *Klebsiella oxytoca* (ATCC 8724) was obtained from the American Type Culture Collection (Rockville, MD, USA). *Rhodobacter capsulatus* (DSM 1710) was provided by Prof. Dr. N. Pfennig, Universität Konstanz, Germany.

S. malonica was grown in 130 ml serum bottles at 30° C in anoxic freshwater mineral medium buffered with 30 mM sodium bicarbonate, reduced with 1 mM cysteine/HCl, and supplemented with 0.05% (w/v) yeast extract (Dehning et al. 1989). *K. oxytoca* was cultivated anaerobically in the same medium in 100 ml screw-cap bottles at 37° C. For aerobic growth in shaken Erlenmeyer flasks, this medium was used without cysteine and bicarbonate, but with 1 mM Na₂SO₄ and 50 mM Na-K-phosphate buffer, pH 6.9. *R. capsulatus* was grown anaerobically in flat 250 ml bottles with medium for nonsulfur phototrophs in light of approx. 1000 lux at 23–28° C (Pfennig & Trüper 1981). Substrates were added from sterile neutralized stock solutions before inoculation.

Growth was determined by measuring turbidity at 580 nm (*S. malonica*, *K. oxytoca*) or 650 nm (*R. capsulatus*) in 23 ml screw-cap tubes in a Spectronic 20 spectrophotometer (Milton Roy Co., Rochester, NY,

USA). Cell dry matter formation was calculated via turbidity using a specific conversion factor obtained by direct determination of dry cell matter grown in 1000 ml cultures.

Preparation of cell suspensions and cell fractionation

Cultures were harvested at the late exponential growth phase, washed, resuspended in the assay buffer, and kept in serum bottles under N₂-atmosphere on ice. Cells of *S. malonica* were anoxically centrifuged in the culture vessels. Cell-free extracts were prepared in an N₂-flushed French press cell (Aminco, Silver Springs, OH, USA) at 130 MPa, followed by anoxic centrifugation at 5000 × *g* for 20 min. Cytoplasmic fraction and membrane fraction were separated by ultracentrifugation at 200,000 × *g* for 1 h. The membrane pellet was resuspended with a syringe, washed once with the same buffer, and both fractions were kept in N₂-flushed serum vials. Alternatively, the outer cell membrane was removed by cold EDTA treatment (Dimroth & Thomer 1986).

Measurement of enzyme activities

CO₂ formation from malonate was assayed with fresh cell suspensions and extract fractions at 25° C in 0.5 ml assay mixture (see legends) under nitrogen (1.2 bar pressure) in 25 ml serum bottles sealed with butyl rubber stoppers. All additions were made from anoxic stock solutions with microliter syringes. 2–6 parallel reactions were started by addition of 20–50 mM Na₂- or K₂-malonate. Over a period of 30–130 min (minimal interval 2.5 min), 300 µl gas samples were taken after vigorous shaking.

CO₂ was quantified with a Carlo Erba Vega 6000 gas chromatograph (Milano, Italy). The temperature of the HWD 430 thermal conductivity detector block was 170° C and of the filament 280° C. Columns (2 m × 2 mm) were packed with 60/80 Carbosieve S II (Supelco, Sulzbach, Germany) and heated at 180° C. Carrier gas was helium at a flow rate of 45 ml min⁻¹. The detection limit was 1 nmol CO₂ per sample.

Decarboxylation activities were determined from the initial linear slope defined by 3–5 points. For calibration, 10 or 20 µl of 100 mM NaHCO₃ was added to 0.5 ml of the respective assay buffer. All figures show typical results of 3–4 independent assays.

Photometric enzyme assays were performed under anoxic conditions in rubber-sealed, N₂-gassed 1 cm

cuvettes at 23° C using a Hitachi 100-40 photometer (Tokyo, Japan). Assay mixtures contained 10 to 200 μ g cell extract protein. Malonyl-CoA:acetate CoA transferase (EC 2.8.3.3.) was determined according to Hilpert et al. (1984) and malonyl-CoA synthetase (EC 6.2.1.7) was assayed by the method described by Kim & Bang (1985).

Analytical methods

Protein was quantified by a modified microbiuret method (Zamenhoff 1957) using bovine serum albumin as standard. Acetate and malonate were analysed by gas chromatography as described earlier (Dehning et al. 1989; Dehning & Schink 1989b). Sodium ion concentrations were determined with an Orion Na^+ -electrode (model Ross 84-11, Colora, Lorch, Germany). The standard addition method was applied according to the manufacturer's protocol. The detection limit was 10 μ M NaCl.

Chemicals

Tetrameric avidin and biotin were obtained from Fluka (Neu-Ulm, Germany). Biochemicals were purchased from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany) or Boehringer (Mannheim, Germany). All chemicals were of analytical grade and obtained from Fluka or Merck (Darmstadt, Germany).

Results

Growth with malonate

The homoacetogenic strict anaerobe *Sporomusa malonica* grows by fermentation of malonate as sole carbon and energy source in freshwater medium as shown previously (Dehning et al. 1989). Malonate is degraded stoichiometrically to acetate and CO_2 ; the growth yield is 2.0 g dry weight $\cdot$ mol malonate $^{-1}$.

Klebsiella oxytoca (ATCC 8724) grew aerobically in mineral medium with malonate as sole organic substrate (results not shown). For anaerobic growth, a second substrate was required. With yeast extract at low concentrations (0.05–0.1% (w/v) or sucrose (0.5–1.0 mM), but not with glucose, cell yields of *K. oxytoca* increased linearly with increasing malonate concentrations in the medium (Fig. 1). During growth, the pH of the medium remained constant, and malonate up to 20 mM concentration was stoichiometrical-

Fig. 1. Effect of malonate on cell yields of *K. oxytoca* after growth with sucrose or yeast extract in anoxic mineral medium. Δ 1 mM and $\blacktriangle$ 0.5 mM sucrose, $\circ$ 0.1% and $\bullet$ 0.05% (w/v) yeast extract, $\square$ without additional substrate.

Fig. 2. CO_2 formation from malonate by cell suspensions, cell extracts, cytoplasmic or membrane fractions of *S. malonica*. All assay mixtures contained in 0.5 ml: 50 mM potassium phosphate buffer, pH 7.0, 3 mM MgCl_2 , 2 mM dithioerythritol, 30 mM Na_2 -malonate, 0.5 mM malonyl-CoA, 3 mM ATP, and 3–10 mg protein. $\bullet$ cell suspension, $\circ$ cell-free extract, $\blacksquare$ cytoplasmic and $\square$ membrane fraction, $\blacktriangle$ without malonate, + 20 mM acetate.

Table 1. Characteristics of malonate decarboxylation by the strains tested. Assays of 0.5 ml in 25 ml serum bottles contained 50 mM K-phosphate buffer, pH 7.0, 3 mM MgCl₂, and 1–10 mg protein. CO₂ formation was measured by gas chromatography after addition of 20–50 mM Na₂-malonate. Cell extracts were prepared by French press disruption, cell fractions by ultracentrifugation. The variation between 3–4 independent assays was about 10%.

		<i>S. malonica</i>		<i>K. oxytoca</i>		<i>R. capsulatus</i>	
Specific activity [nmol CO ₂ min ⁻¹ mg protein ⁻¹]	suspension	500		550		80	
	extract	70	130 ^{a)}	1500	1700 ^{b)}	18	20 ^{c)}
	cytoplasm	60	110 ^{a)}	1600	2000 ^{b)}	14	17 ^{c)}
	membranes	2	2 ^{a)}	20	20 ^{b)}	<1	1 ^{c)}
50% inhibition by hydroxylamine [mM] ^{d)}		90		80		100	
Maximal stimulation by malonyl-CoA [mM]		0.5 ^{e)}		0.05 ^{f)}		0.5 ^{g)}	
% inhibition by addition of 20 mM EDTA		80		20		10	
50% inhibition by avidin [μg·mg cytoplasmic protein ⁻¹]		27		no		36	
Stimulation by 5 mM NaCl ^{h)}	suspension	1.3-fold		no		no	
	extract	no		no		no	

^{a)} + 0.5 mM malonyl-CoA + 3 mM ATP; ^{b)} + 0.1 mM malonyl-CoA; ^{c)} + 0.5 mM malonyl-CoA;

^{d)} cell suspensions with 2–5 mg protein; ^{e)} 1.2 mg protein + 3 mM ATP; ^{f)} 0.8 mg protein;

^{g)} 1.0 mg protein; ^{h)} 50 mM Tris/HCl, pH 7.5, 20 mM K₂-malonate; background: 0.5 mM Na⁺ (sodium electrode).

ly decarboxylated to acetate. The malonate-dependent molar growth yield increase obtained was 1.5–1.8 g dry weight·mol⁻¹ in the presence of yeast extract, and 1.1–1.6 g·mol malonate⁻¹ with sucrose as co-substrate (Fig. 1).

Rhodobacter capsulatus did not grow with malonate or acetate anaerobically in the dark. Anaerobically in the light, however, malonate (1 mM) was assimilated and supported growth. Growth yields (48 g dry weight·mol⁻¹) and rates ($t_d = 0.038$ – 0.046 h⁻¹) were identical with either malonate or acetate as substrate.

Activities of malonate decarboxylation

Malonate-decarboxylating activities of the three bacterial strains were assayed with cell suspensions, cell-free extracts, and with cytoplasmic and membrane fractions. Typical kinetics of malonate-dependent CO₂ formation by the various cell fractions of *S. malonica* are shown in Fig. 2, and mean values of the specific activities for all three strains are listed in Table 1.

With *S. malonica*, a decarboxylating activity of 500 ± 60 nmol·min⁻¹·mg protein⁻¹ was determined in cell suspensions, and about 25% of this activity was found in crude cell extract in the presence of malonyl-CoA and ATP at low concentration. The calculated rate of malonate degradation by exponentially growing cells of *S. malonica* is $2.1 \mu\text{mol} \cdot \text{min} \cdot \text{mg protein}^{-1}$, based on a molar growth yield of $2.0 \text{ g} \cdot \text{mol}^{-1}$ and growth rate of 0.124 h^{-1} . The specific decarboxylating activity of *K. oxytoca* cell extract was about 1.7 – $2.0 \cdot \text{mol CO}_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ (as determined with malonyl-CoA); in cell suspensions, only 550 ± 50 nmol CO₂·min⁻¹·mg protein⁻¹ was measured. *R. capsulatus* showed low decarboxylation rates of 80 ± 10 nmol CO₂ (min·mg protein)⁻¹ in cell suspensions and 25% of this activity in cell extracts with malonyl-CoA as cosubstrate. Obviously the measurement conditions in the cell-free system are not optimal; this is also indicated by major fluctuations in measurable activity with different cell batches. The decarboxylating activity was always found nearly exclusively in the cytoplasmic fraction; only with *S. malonica*, a

Fig. 3. Influence of avidin on CO₂ formation from malonate by cell extracts and cytoplasmic fractions of A *S. malonica* and B *R. capsulatus*. Assay mixtures contained in 0.5 ml: 50 mM potassium phosphate buffer, pH 7.0, 3 mM MgCl₂, 25 mM Na₂-malonate, 1 mg protein, and 0.5 mM malonyl-CoA (A: + 3 mM ATP). □, ■ cell-free extract, ○, ● cytoplasmic fractions, ■, ● controls with biotin-saturated avidin.

small part (3–5%) of the total activity turned out to be membrane-bound.

In the absence of malonate or with acetate as substrate, no CO₂ formation was observed. Assays with *K. oxytoca* had to be kept strictly anoxic, and assay bottles with *R. capsulatus* cells were wrapped in aluminum foil for light protection.

Cell suspensions of *S. malonica* grown with fructose showed only 1–3% of the specific malonate-decarboxylating activity exhibited by cells grown with malonate. Sucrose-grown cell suspensions and cell extracts of *K. oxytoca* had 3–5% of the activity found in cells cultivated with sucrose in the presence of 20 mM malonate.

Activation of malonate

Malonate-decarboxylating activities in cell extracts of all bacteria were stimulated by addition of catalytic amounts of malonyl-CoA (Table 2). The cofactor-dependent increase of activity depended on the protein concentration of the respective cell extracts. The effects of various cofactors and of EDTA, either alone or in combination, on decarboxylation activities in cell extracts are shown in Table 2. In all cases, CO₂ production from malonate was stimulated significantly (up to 20-fold) by catalytic amounts of malonyl-CoA, acetyl-CoA, or CoASH plus ATP. All decarboxylation activities were inhibited to varying extent by 15–

20 mM EDTA. Activities were completely inhibited by addition of 200 mM hydroxylamine to the assay mixtures.

Preincubation of cell extracts of *S. malonica* or *K. oxytoca* with CoASH plus ATP and 3 mM acetate 15 min prior to malonate addition resulted in the respective stimulations without lag phases (data not shown).

Enzyme assays with cell extracts of *S. malonica* and *K. oxytoca* revealed specific activities of a malonyl-CoA:acetate CoA transferase of 800 ± 70 and 40 ± 5 nmol·min⁻¹·mg protein⁻¹. Malonyl-CoA synthetase activity could not be detected.

Characterization of decarboxylating enzymes

Malonate-decarboxylating activity of cell-free extracts of *S. malonica* showed a temperature optimum of 25–30° C. The optimal pH was 7.3–7.6. Similar activities were measured in 50–100 mM phosphate or in Tris buffer. Addition of reductants such as dithioerythritol did not stimulate decarboxylation rates.

To examine whether the decarboxylases are biotin enzymes, avidin was added to cell extracts and cytoplasmic fractions of the three strains 15 min prior to malonate addition. Avidin pre-incubated with an excess of biotin served as control.

Decarboxylation activities of cell extracts and cytoplasmic fractions of *S. malonica* were 50% inhibited by

Table 2. Influence of cofactors and inhibitors on malonate decarboxylation activities in cell extracts of A: *S. malonica*, B: *K. oxytoca* and C: *R. capsulatus*. Assays of 0.5 ml contained 50 mM K-phosphate buffer, pH 7.0, 3 mM MgCl₂, and 30–50 mM Na₂-malonate. Data presented are representative results of 2–3 independent assays.

Cofactor	Concentration [mM]	Activity [nmol CO ₂ min ⁻¹]	% Activity of control
A: <i>S. malonica</i> : 1.2 mg protein			
Control (no further addition)	–	5	100
ATP	3.0	6	120
CoASH	0.5	14	280
CoASH; ATP	0.1; 3.0	39	780
	0.2; 3.0	72	1440
	0.5; 3.0	91	1820
Mal-CoA	0.1	11	220
	0.5	28	560
Ac-CoA	0.5	30	600
Mal-CoA; ATP	0.1; 3.0	45	900
	0.5; 3.0	98	1960
Ac-CoA; ATP	0.5; 3.0	104	2080
EDTA	15.0		15–24
B: <i>K. oxytoca</i> : 0.8 mg protein			
Cofactor, inhibitor			
Control (no further addition)	–	330	100
ATP	3.0	660	200
CoASH	0.2	350	106
CoASH; ATP	0.1; 1.0	1270	385
	0.2; 3.0	1340	406
	0.5; 3.0	1380	418
Mal-CoA	0.01	1350	409
	0.05	1390	421
Ac-CoA	0.01	1290	391
Mal-CoA; ATP	0.01; 3.0	1410	427
	0.05; 3.0	1420	430
EDTA	8	320	97
	20	270	82

85 ± 10 and 27 ± 3 µg avidin·mg protein⁻¹, respectively (Fig. 3A). Activities of cell extracts and cytoplasmatic fractions of *R. capsulatus* were inhibited to 50% by 45 pm 5 and 36 ± 3 µg avidin per mg protein, respectively (Fig. 3B). In the respective control assays, CO₂-formation was not influenced. Avidin up to a ratio

Table 2. continued

Cofactor	Concentration [mM]	Activity [nmol CO ₂ min ⁻¹]	% Activity of control
C: <i>R. capsulatus</i> : 1.1 mg protein			
Control	–	9	100
ATP	3.0	10	110
CoASH	0.2	17	190
	0.5	20	220
CoASH; ATP	0.2; 3.0	18	200
	0.5; 3.0	21	230
Mal-CoA	0.2	18	200
	0.5	20	220
Ac-CoA	0.5	21	230
Mal-CoA; ATP	0.2; 3.0	18	200
EDTA	15		87–91

Mal-CoA: malonyl-CoA; Ac-CoA: acetyl-CoA; nd: not determined.

of 3 mg per mg protein did not affect decarboxylation rates of cell extracts of *K. oxytoca*.

Discussion

The present study describes further examples of bacteria which can use the free energy of a decarboxylation reaction as their sole energy source for anaerobic growth. Obviously, malonate fermentation is more widespread in nature than expected so far, although this substrate is only of minor importance in natural environments (Bentley 1952). Whereas *Sporomusa malonica* could grow with malonate as sole source of carbon and energy, *Klebsiella oxytoca* depended on a further substrate for growth. Obviously, these facultatively aerobic bacteria cannot base their whole assimilatory metabolism on malonate or acetate, probably because the tricarboxylic acid cycle is switched off under these conditions. We have shown in earlier publications that assimilation of acetate under strictly anoxic conditions requires a functional pathway of acetate oxidation to obtain electrons for reductive acetyl-CoA carboxylation (Dehning & Schink 1989b; Dehning et al. 1989; Brune & Schink 1990). *Rhodobacter capsulatus* uses the citric acid cycle in acetate assimilation, and *S. malonica* can employ its carbon monoxide dehydrogenase system (Wood pathway) in the oxidative direction.

In all three cases studied here the pathway of malonate conversion to acetate included the involvement of malonyl-CoA which was formed from malonate and acetyl-CoA through a CoA transferase reaction. Malonyl-CoA is also formed in malonate fermentation by *Citrobacter diversus* (Janssen & Harfoot 1992), however, in this bacterium the acyl-CoA derivatives were formed through kinase reactions. Energetically, both pathways are equivalent. Contrary to these bacteria, the malonate-fermenting anaerobe *Malonomonas rubra* decarboxylates the free malonic acid in the cell through a new type of enzyme which is activated through acetylation (Hilbi et al. 1992).

As expected, there was no difference in cell yields with the phototrophic *R. capsulatus* after growth with malonate or acetate, since phototrophic growth at sufficient light supply is limited only by the amount of electrons available. The two fermenting bacteria studied exhibited cell yields due to malonate fermentation which were both in the range of 1.1–2.0 g per mol substrate. The small release of free energy (about -20 kJ/mol) by decarboxylation has to be translated into ATP synthesis through a membrane-bound coupling mechanism; it is equivalent to the smallest amount of energy that can be exploited by living organisms, i.e., the amount of energy required to carry a monovalent ion across the energized cytoplasmic membrane against the electric potential (Schink 1988). One could calculate a Y_{ATP} in the range of 3.3–6.0 g from these data which would be at the lower rank of published values (Stouthamer 1979), but is in the same range as yields determined with other bacteria living exclusively on decarboxylation reactions (Schink & Pfennig 1982; Dehning & Schink 1989a, b; Dehning et al. 1989; Matthies & Schink 1992a).

The mechanism by which the decarboxylation reaction couples to ATP synthesis in the bacteria studied here has not been resolved yet. Malonyl-CoA decarboxylase activity was recovered mainly in the cytoplasmic fraction suggesting that this enzyme does not act as a primary ion pump as does methylmalonyl-CoA decarboxylase in *Propionigenium modestum* (Hilpert et al. 1984). Although this finding may be due to artifactual resolution of the respective enzyme from the membrane through the preparation procedure we do not have any positive proof that the decarboxylation reaction itself is directly involved in membrane energetization. Studies with ionophores and transport inhibitors on protoplasts and membrane vesicles prepared from *K. oxytoca* did not yield conclusive results in our hands, and *S. malonica* cells did not allow such

preparations at all. The question remains open as well whether sodium ions or protons are involved in this type of energetical coupling, and whether a primary decarboxylating ion pump or an electrogenic substrate/product transport system creates a charge imbalance across the membrane, as suggested for oxalate fermentation by *Oxalobacter formigenes* (Anantharam et al. 1989).

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