Arch Microbiol (1990) 155:52–55

Archives of
Microbiology

Enzymatic evidence for involvement of the methylmalonyl-CoA pathway in propionate oxidation by *Syntrophobacter wolinii*

Frans P. Houwen*, Jeannette Plokker, Alfons J. M. Stams, and Alexander J. B. Zehnder

Department of Microbiology, Agricultural University of Wageningen, Hesselink van Suchtelenweg 4, 6703 CT Wageningen, The Netherlands

Received June 19, 1990/Accepted August 20, 1990

Abstract. Enzyme measurements were carried out with crude cell-free extracts of the propionate oxidizing coculture of *Syntrophobacter wolinii* and *Desulfovibrio* G11. Using cell-free extracts of a pure culture of *Desulfovibrio* G11 as a blank, most of the enzymes involved in the methylmalonyl-CoA pathway for propionate oxidation, including a propionyl-CoA: oxaloacetate transcarboxylase, were demonstrated in *S. wolinii*.

Key words: *Syntrophobacter wolinii* – Propionate oxidation – Syntrophic coculture – Propionyl-CoA: oxaloacetate transcarboxylase – Methylmalonyl-CoA pathway – *Desulfovibrio*

Propionate is an important intermediate in conversion of complex organic material to methane and carbon dioxide (Kaspar and Wuhrmann 1978; Gujer and Zehnder 1983). Propionate is oxidized to acetate and carbon dioxide, and the electrons formed in this oxidation are disposed of by reduction of protons to hydrogen (Zehnder 1978; Bryant 1979) or, as proposed recently, by reduction of bicarbonate to formate (Boone et al. 1989). Because of unfavourable energetics, propionate oxidation is only possible if the reduced products hydrogen and formate are removed by methanogens. This implies that under methanogenic conditions propionate oxidation can only be carried out by obligately syntrophic consortia of bacteria. Up to now only a few syntrophic propionate oxidizing cocultures have been described (Boone and Bryant 1980; Koch et al. 1983; Mah et al. 1990; Houwen et al. 1990).

Studies with ^{14}C- and ^{13}C-labelled propionate have indicated that syntrophic propionate oxidation to acetate proceeds via the methylmalonyl-CoA pathway, a route in which propionyl-CoA, methylmalonyl-CoA, succinyl-CoA, succinate, fumarate, malate, oxaloacetate, pyruvate and acetyl-CoA are intermediates (Buswell et al. 1951; Koch et al. 1983; Schink 1985; Robbins 1988; Houwen et al. 1987, 1990). This pathway is common in propionate-forming anaerobes and is involved in the anaerobic oxidation of propionate by *Desulfobulbus propionicus* (Stams et al. 1984; Kremer and Hansen 1988). Up to now, direct enzymatic evidence for the operation of the methylmalonyl-CoA pathway in syntrophic propionate oxidizing bacteria is lacking. In this paper we present the results of enzyme activity measurements in a coculture of *Syntrophobacter wolinii* and *Desulfovibrio* G11 described by Boone and Bryant (1980). A pure culture of the *Desulfovibrio* species was used as a blank to calculate the specific enzyme activities in the propionate oxidizer.

Materials and methods

Organisms and cultivation

The defined sulfidogenic biculture of *Syntrophobacter wolinii* and *Desulfovibrio* G11 (Boone and Bryant 1980) was obtained from the German Collection of Microorganisms (Braunschweig, FRG) (DSM 2805) and was cultivated in a medium containing (in g/l unless otherwise stated): sodium propionate 1.9; Na_2SO_4, 2.9; $NaHPO_4 \cdot 2H_2O$, 0.53; KH_2PO_4, 0.41; NH_4Cl, 0.3; NaCl, 0.3; $CaCl_2 \cdot 2H_2O$, 0.11; $MgCl_2 \cdot 6H_2O$, 0.1; $NaHCO_3$, 4; $Na_2S \cdot 9H_2O$, 0.24; yeast extract, 0.2; 1 ml of a tenfold concentrated trace element solution described by Pfennig and Lippert (1966); 1 ml of the vitamin solution described by Stams et al. (1983); 1 ml of a mixture of Na_2MoO_4, Na_2WoO_4 and Na_2SeO_3 (each 0.1 mM) in 10 mM NaOH. Sodium lactate (1 mM) was added to stimulate the sulfate reducing bacterium

Preparation of cell-free extracts

Cells were harvested by centrifugation, washed once with 50 mM phosphate buffer with 2 mM $MgCl_2$, pH 7.1, and stored as wet cell paste anaerobically at −20° C. Cells were thawed and brought into a French Press compartment in an anaerobic glove box with a gas

* *Present address*. Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, S-750 07 Uppsala, Sweden

Offprint requests to: A. J. M. Stams

Table 1. Specific activities ($\mu mol \times min^{-1} \times mg^{-1}$ protein) of enzymes detected in the *Syntrophobacter-Desulfovibrio* coculture (column 1) and in a pure culture of the *Desulfovibrio* species (column 2). The third column represents the calculated values for *S. wolinii* (see text)

Enzymes	Coculture	Sulfate reducer	*S. wolinii*
Propionate kinase	0.120	– [a]	0.123
Propionyl-CoA carboxylase	<0.02	–	<0.02
Propionyl-CoA: oxaloacetate transcarboxylase	0.519	–	0.535
Succinate thiokinase	0.315	–	0.325
Propionyl-CoA: succinate CoA transferase	<0.005	–	<0.005
Succinate dehydrogenase ($Fe(CN)_6^{3-}$)	0.069	–	0.071
Fumarate reductase (BV^+) [d]	0.013		
Fumarase:			
fumarate disappearance	1.04	–	1.07
fumarate formation	0.144	0.049	0.147
Malate dehydrogenase:			
NADH-dependent	2.33	0.036	2.40
NADPH-dependent	0.191	ND [b]	NC [c]
Malic enzyme	0.30	0.13	0.31
Pyruvate carboxylase	<0.001	–	<0.001
Pyruvate dehydrogenase (BV^{2+}) [e]	0.019	0.260	0.012
Phosphotransacetylase	2.03	0.107	2.09
Acetate kinase	0.116	0.331	0.109
Hydrogenase (MV^{2+}) [f]	1.21	1.83	1.19
Formate dehydrogenase (BV^{2+}) [e]	2.11	1.71	2.12
NADH dehydrogenase (MTT) [g]	0.020	0.008	0.02
Formate hydrogen lyase	0.0043	0.0093	0.0041

[a] Not detected; [b] not determined; [c] not calculated; [d] BV^+, reduced benzyl viologen; [e] BV^{2+}, oxidized benzyl viologen; [f] MV^{2+}, oxidized methyl viologen; [g] MTT, 3-(4'5'-dimethyl-thiazol-2-yl)-2,4-diphenyltetrazolium bromide

Enzymes not detected: Isocitrate lyase, pyruvate formate lyase, phosphoenolpyruvate carboxylase, propionate thiokinase, acetate thiokinase, succinate kinase

phase of N_2/H_2 (96:4). The cells were broken at 1360 bar and the cell debris was removed by centrifugation at 4000 rpm for 20 min. The supernatants were stored oxygen-free at 0°C in glass tubes sealed with butyl rubber stoppers.

Enzyme assays

Enzyme assays were carried out anaerobically at 37°C in a LKB 4053 kinetics spectrophotometer ultrospec K (Pharmacia Nederland b.v., Woerden), Cuvettes (1 ml) were closed with rubber stoppers and made anaerobic by flushing with N_2. Anaerobic buffer (unless otherwise state, Tris-HCl at the same molarity and pH as the buffer mentioned in the cited references) and all other solutions were brought into the cuvette by syringe.

Thiokinase activity with propionate (E.C. 6.2.1.17), acetate (E.C. 6.2.1.1) and succinate (E.C. 6.2.1.5) were measured (at pH 8.5) as described by Oberlies et al. (1980). Kinase activities with these substrates (E.C. 2.7.2.1 for acetate kinase) were measured in the same way but with the omission of HSCoA. Propionyl-CoA: oxaloacetate transcarboxylase (E.C. 2.1.3.1), fumarase (E.C. 4.2.1.2), malate dehydrogenase (NADH-dependent, E.C. 1.1.1.37 and NADPH-dependent, E. C. 1.1.1.82) in the direction of malate formation, malic enzyme (E.C. 1.1.1.39) and succinate dehydrogenase (E.C. 1.3.99.1) with ferricyanide in a 100 mM Tris-HCl buffer, pH 7.4) were measured according to Stams et al. (1984). Fumarate reductase (E.C. 1.3.1.6) was assayed with reduced benzylviologen or NADH following the procedure of Boonstra et al. (1975). Hydrogenase (E.C. 1.12.1.2) was assayed as described for CO dehydrogenase by Daniels et al. (1977). Pyruvate dehydrogenase (E.C. 1.2.4.1) and formate dehydrogenase (E.C. 1.2.1.2) were measured according to Odom and Peck (1981). Pyruvate carboxylase (E.C. 6.4.4.1) was assayed according to Scrutton et al. (1969), phosphoenolpyruvate carboxylase (E.C. 4.1.1.31) after Maeba and Sanwal (1969); isocitrate lyase (E.C. 4.1.3.1) after Dixon and Kornberg (1959); phosphotransacetylase (E.C. 2.3.1.8) after Oberlies et al. (1980) and NADH dehydrogenase (E.C. 1.6.99.3) was measured in a HEPES/KOH buffer, pH 7.5, with 0.05% Triton X-100 after Bergsma et al. (1982). Propionyl-CoA:succinate CoA transferase was measured after Hilpert et al. (1984) in a 10 mM Na-arsenate buffer, pH 7.0. Propionyl-CoA carboxylase (2.1.3.1) was assayed, following NADH-oxidation at 340 nm, in a 100 mM Tris-HCl buffer, pH 8.0, containing: $KHCO_3$, 50 mM, KCl 100 mM, $MgCl_2$ 4 mM, glutathione 2 mM, ATP 2 mM, phosphoenolpyruvate 1 mM, NADH 0.6 mM, lactate dehydrogenase 40 U, pyruvate kinase 10 U and propionyl-CoA 0.5 mM. The overall reaction from succinate to propionate was followed in a coupled assay as described by Stams et al. (1984). Both a 60 mM Tris-HCl buffer, pH 7.4, and a 87.5 mM phosphate buffer, pH 7.5, were used. Formate hydrogen lyase activity (E.C. 1.2.1.2) was measured in vials (25 ml) closed with butyl rubber stoppers. For this purpose 1 ml of cell-free extract was added to 2 ml 100 mM phosphate buffer, pH 7.0, and hydrogen formation was measured after addition of formate to give a concentration of 40 mM. Pyruvate formate lyase (E.C. 2.3.1.54) was measured in a buffer as described by Jungermann and Schön (1974), with 2 mM HSCoA and 30 mM pyruvate. 0.5 ml cell-free extract was added to 1 ml assay buffer in vials (10 ml) closed with butyl rubber stoppers.

Analytical methods

Protein in cell-free extracts was determined according to Bradford (1976). Hydrogen was measured gaschromatographically as described before (Houwen et al. 1988).

Materials.

All chemicals used were of analytical grade. Biochemicals and enzymes were purchased from Boehringer, Mannheim, FRG and Sigma Chemical, St. Louis, USA. Gases were obtained from Hoek

Loos, Schiedam, The Netherlands. Palladium catalyst was a gift of BASF, Arnhem, The Netherlands.

Results and discussion

The results of enzyme measurements in cell-free extracts of the *Syntrophobacter-Desulfovibrio* coculture are summarized in the first column of Table 1. The second column represents enzyme activities as measured in cell-free extracts of a pure culture of *Desulfovibrio* G11. In the coculture the relative number of propionate oxidizers was very constant (Houwen et al. 1990). This made it possible to determine the contribution of *S. wolinii* to the total protein in the coculture. Based on mean protein content per cell in the pure culture of the sulfate reducer (8.2×10^{-11} mg), the total protein content of the concentrated coculture (62.4 mg/ml from 4.8×10^{10} cells/ml) and the relative number of the sulfate reducer in the coculture (55%), it was calculated that about 97% of the protein in the coculture was from the propionate oxidizing bacterium. Column 3 (Table 1) gives the calculated specific enzyme activities of the syntrophic propionate degrader.

The results as shown in Table 1 are in agreement with the involvement of the methylmalonyl-CoA pathway for propionate oxidation by *S. wolinii*. The high activity of propionyl-CoA:oxaloacetate transcarboxylase and the absence of isocitrate lyase activity excludes a possible route via α-OH-glutarate (Wegener et al. 1968); in such a pathway the isocitrate lyase but not the transcarboxylase plays a key role (Rabin et al. 1965). The occurrence of the methylmalonyl-CoA pathway with a transcarboxylase is supported by label patterns found during propionate conversion in the presence of both [3-^{13}C]-propionate and $H^{13}CO_3^-$ (Houwen et al. 1990).

The transcarboxylase activity measured in *S. wolinii* is comparable to the activity in extracts of *D. propionicus* (Stams et al. 1984). However, attempts to measure the overall conversion from succinate to propionate in a similar way (with 5 times more protein) as reported for *D. propionicus*, were not successful. The reason for this is not known the more so as whole cells did interconvert succinate and propionate (Houwen et al. 1990).

In this study enzyme activities present in *S. wolinii* were calculated by subtracting specific activities of enzymes of different extracts. It remains unknown whether artifacts are introduced in this manner. Because *Desulfovibrio* G11 was grown on hydrogen and sulfate in the presence of propionate, the method is reliable if an enzyme is absent in the pure culture as is the case with e.g. the crucial enzymes transcarboxylase and succinate thiokinase. However, if an enzyme is present in the pure culture, results should be interpreted with caution. The low activities of a pyruvate dehydrogenase calculated for *S. wolinii* may be an example of this. The high protein content of *S. wolinii* (97%) in the total protein of the cell-free extract was certainly favourable for the demonstration of most of the enzymes of the methylmalonyl-CoA pathway in *S. wolinii*.

Acknowledgements. The authors thank M. S. M. Jetten for valuable advise and W. Roelofsen for technical assistance.

References

Bergsma J, Dongen MBM van, Konings WN (1982) Purification and characterization of NADH dehydrogenase from *Bacillus subtilis*. Eur J Biochem 128:151–157

Boone DR, Bryant MP (1980) Propionate-degrading bacterium, *Syntrophobacter wolinii* sp. nov. gen. nov., from methanogenic ecosystems. Appl Environ Microbiol 40:626–632

Boone DR, Johnson RL, Liu Y (1989) Diffusion of the interspecies electron carriers H_2 and formate in methanogenic ecosystems, and applications in the measurement of K_M for H_2 and formate uptake. Appl Environ Microbiol 55:1735–1741

Boonstra J, Huttunen MT, Konings WN (1975) Anaerobic transport in *Escherichia coli* membrane vesicles. J Biol Chem 250:6792–6798

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bryant MP (1979) Microbial methane production – theoretical aspects. J Anim Sci 48:193–201

Buswell AM, Fina L, Müller H, Yahiro A (1951) Use of C^{14} in mechanism studies of methane fermentation. II. Propionic acid. J Am Chem Soc 73:1809–1811

Daniels L. Fuchs G, Thauer RK. Zeikus JG (1977) Carbon monoxide oxidation by methanogenic bacteria. J Bacteriol 132:118–126

Dixon GH, Kornberg HL (1959) Assay methods for key enzymes of the glyoxylate cycle. Biochem J 72:3p

Gujer W, Zehnder AJB (1983) Conversion processes in anaerobic digestion. Water Sci Tech 15:127–167

Hilpert W, Schink B, Dimroth P (1984) Life by a new decarboxylation-dependent energy conservation mechanism with Na^+ as coupling ion. EMBO J 3:1665–1670

Houwen FP, Dijkema C, Schoenmakers CHH, Stams AJM, Zehnder AJB (1987) ^{13}C-NMR study of propionate degradation by a methanogenic coculture. FEMS Microbiol Lett 41:269–274

Houwen FP, Cheng Guangsheng, Folkers GE, Heuvel WMJG van de, Dijkema C (1988) Pyruvate and fumarate conversion by a methanogenic propionate-oxidizing coculture. In: Lettinga G, Zehnder AJB, Grotenhuis JTC, Hulshoff Pol LW (eds) Granular anaerobic sludge; microbiology and technology. Pudoc, Wageningen, pp 62–70

Houwen FP, Plokker J, Dijkema C, Stams AJM (1990) Syntrophic propionate oxidation. In: Belaich JP, Bruschi M, Garcia JL (eds) Microbiology and biochemistry of strict anaerobes involved in interspecies hydrogn transfer. Plenum Press, New York, pp 281–289

Jungermann K, Schön G (1974) Pyruvate formate lyase in *Rhodosprillium rubrum Ha* adapted to anaerobic dark conditions. Arch Microbiol 99:109–116

Kaspar HF, Wuhrmann K (1978) Kinetic parameters and relative turnovers of some important catabolic reactions in digesting sludge. Appl Environ Microbiol 36:1–7

Koch ME, Dolfing J, Wuhrmann K, Zehnder AJB (1983) Pathways of propionate degradation by enriched methanogenic cultures. Appl Environ Microbiol 45:1411–1414

Kremer DR, Hansen TA (1988) Pathway of propionate degradation in *Desulfobulbus propionicus*. FEMS Microbiol Lett 49:273–277

Maeba P, Sanwal BD (1969) Phosphoenolpyruvate carboxylase from *Salmonella typhimurium*, strain LT2. In: Lowenstein JM (ed) Methods in enzymology, vol 13. Academic Press, New York London, pp 283–288

Mah RA, Xun LY, Boone DR, Arhing B, Smith PH, Wilkie A (1990) Methanogenesis from propionate in sludge and enrichment cultures. In: Belaich JP, Bruschi M, Garcia JL (eds) Microbiology and biochemistry of strict anaerobes involved in interspecies hydrogen transfer. Plenum Press, New York, pp 99–111

Oberlies G, Fuchs G, Thauer RK (1980) Acetate thiokinase and the assimilation of acetate in *Methanobacterium thermoautotrophicum*. Arch Microbiol 128:248–252

Odom JM, Peck HD (1981) Localization of dehydrogenases, reductases, and electron transfer components in the sulfate-reducing bacterium *Desulfovibrio gigas*. J Bacteriol 147:161–169

Pfennig N, Lippert KD (1966) Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch Mikrobiol 55:245–256

Rabin R, Reeves HC, Wegener WS, Megraw RE, Ajl SJ (1965) Glyoxylate in fatty-acid metabolism. Condensations of glyoxylate with fatty acids lead to alternate pathways of fatty-acid metabolism. Science 150:1548–1558

Robbins JE (1988) A proposed pathway for catabolism of propionate in methanogenic cocultures. Appl Environ Microbiol 54:1300–1301

Schink B (1985) Mechanisms and kinetics of succinate and propionate degradation in anoxic freshwater sediments and sewage sludge. J Gen Microbiol 131:643–650

Scrutton MC, Olmsted MR, Utter MF (1969) Pyruvate carboxylase from chicken liver. In: Lowenstein JM (ed) Methods in enzymology, vol 13. Academic Press, New York London, pp 235–249

Stams AJM, Veenhuis M, Weenk GH, Hansen TA (1983) Occurrence of polyglucose as a storage polymer in *Desulfovibrio* species and *Desulfobulbus propionicus*. Arch Microbiol 136:54–59

Stams AJM, Kremer DR, Nicolay K, Weenk GH, Hansen TA (1984) Pathway of propionate formation in *Desulfobulbus propionicus*. Arch Microbiol 139:167–173

Wegener WS, Reeves HC, Rabin R, Ajl SJ (1968) Alternate pathways of metabolism of short-chain fatty acids. Bacteriol Rev 32:1–26

Zehnder AJB (1978) Ecology of methane formation. In: Mitchell R (ed) Water pollution microbiology, vol 2. Wiley, New York, pp 349–376