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Anaerobic degradation of 4-hydroxybenzoate: reductive dehydroxylation of 4-hydroxybenzoyl-CoA and ATP formation during 4-hydroxybenzoate decarboxylation by the phenol-metabolizing bacteria of a stable, strictly anaerobic consortium

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Abstract 4-Hydroxybenzoate was activated with coenzyme A by cells of a strictly anaerobic, phenol-degrading mixed culture to 4-hydroxybenzoyl-CoA, which was reductively dehydroxylated to benzoyl-CoA with reduced benzylviologen as an electron donor. The specific activity of the 4-hydroxybenzoyl-CoA ligase in cell-free extracts of the culture was 100–200 nmol $min^{-1} mg^{-1}$, that of 4-hydroxybenzoyl-CoA reductase 14.5 nmol $min^{-1} mg^{-1}$. An increased growth yield of the phenol-degrading mixed culture of 1.8 g/mol with 4-hydroxybenzoate in comparison to phenol as the substrate was found previously and indicated energy generation by decarboxylation of 4-hydroxybenzoate. Addition of 4-hydroxybenzoate to cell suspensions of the mixed culture resulted in a rapid increase of the cellular ATP level. The proton ionophore carbonylcyanide *m*-chlorophenylhydrazone and the H^+-ATPase inhibitor dicyclohexylcarbodiimide prevented an increase of cellular ATP levels during 4-hydroxybenzoate decarboxylation, whereas the sodium ionophore monensin and the putative Na^+-ATPase inhibitor ouabain revealed no effect. This was taken as good evidence for the generation of a proton gradient across the membrane by decarboxylation of 4-hydroxybenzoate and ATP formation by H^+-ATPase.

Introduction

Although more than 1000 phenolic compounds are components of plant and crop biomass (Harborne 1988) from the point of view of environmental protection the chemically produced phenols cause problems. Pollution of soil, surface and ground water with such compounds occurs, e.g. during production of paper, wood preservatives etc. and during processing of mineral oil and coal (Fedorak and Hrudey 1988).

Anaerobic degradation of phenolic compounds may proceed either via a direct reduction (Balba and Evans 1980; Gorny et al. 1992; Brune and Schink 1992) or via carboxylation (Knoll and Winter 1987; Tschech and Fuchs 1987; Sharak Genthner et al. 1989; Zhang et al. 1990; Schnell and Schink 1991; Bisaillon et al. 1991; Gorny and Schink 1994).

After carboxylation of phenol and derivatives, activation to the respective thioester and reductive elimination of the hydroxyl group(s) (Glöckler et al. 1989; Gorny and Schink 1994) were the next steps, and benzoyl-CoA was generated as a central intermediate. The π-electron system was destabilized by thioester formation, facilitating ring reduction and cleavage. As an alternative to this pathway, Bisaillon et al. (1993) reported a direct dehydroxylation of carboxylated phenols without formation of CoA thioester.

Since the syntrophic phenol-degrading culture grew with a better growth yield on 4-hydroxybenzoate than on phenol (Gallert and Winter 1993) and 4-hydroxybenzoate was primarily and almost stoichiometrically decarboxylated to phenol, decarboxylation of 4-hydroxybenzoate might be an example of the conservation of small portions of energy during decarboxylation of aromatic substances. The decarboxylation of 4-hydroxybenzoate was only sufficient for a free-energy change ($\Delta G^{0\prime}$) of -36.76 kJ/mol (according to Thauer et al. 1977), whereas a $\Delta G^{0\prime}$ of -70 to -100 kJ/mol would be required for the formation of 1 mol ATP by substrate-level phosphorylation (Schink 1988). Thus, the increased growth yield with 4-hydroxybenzoate in comparison to phenol as a substrate might indicate the generation of an ion gradient across the cytoplasmic membrane for conservation of small portions of energy, as known for instance for *Klebsiella pneumoniae* during decarboxylation of oxaloacetic acid (Dimroth 1980). In

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this paper we describe enzyme activities in crude extracts of the syntrophic consortium, that are essential for phenol and 4-hydroxybenzoate degradation, and report increased intracellular ATP levels during decarboxylation of 4-hydroxybenzoate. Furthermore, evidence for the participation of a proton gradient for ATP synthesis is presented.

Materials and methods

Organisms and growth conditions

The defined mixed phenol-degrading culture contained the phenol-metabolizing non-motile rod, 1.0–1.2 × 8–15 μm in size, the benzoate-metabolizing motile rod, 0.6–0.8 × 1.5–5 μm in size, a few *Desulfovibrio* sp. cells and *Methanosprillum hungatei*. The phenol-degrading rods formed benzoate from phenol via 4-hydroxybenzoate, 4-hydroxybenzoyl-CoA and benzoate (Gallert et al. 1991; Gallert and Winter 1992). With benzoate as the substrate the phenol-metabolizing rod was lost upon a few transfers. Media and growth conditions for the phenol- and benzoate-degrading cultures were the same as reported previously (Knoll and Winter 1989; Auburger and Winter 1992).

Preparation of cell suspensions and cell-free extracts

Cells were harvested from exponentially growing cultures under anaerobic conditions in a WKF 50K centrifuge (Gesellschaft für elektrophysikalischen Gerätebau, Brandau, Germany). After centrifugation for 30 min at 8000 rpm and 20°C, cell pellets were washed in 50 mM potassium phosphate buffer pH 7.5, containing 2 mM cysteine. Cells were resuspended in the same buffer for experiments requiring dense suspensions.

Cell-free extracts were prepared anaerobically from cell suspensions, mixed 1:1 with glass beads (0.2 mm diameter, Braun Melsungen, Germany), by ultrasonication for 30 min in iced water. The cell suspensions was prepared in screw-capped glass vials under a N_2 atmosphere. Cells were broken in a Bandelin Sonorex RK100 ultrasonicator (Bandelin Electronics, Berlin, Germany) and debris as well as glass beads removed by centrifugation for 10 min at 5000 rpm and 4°C in a Sorvall RC2B centrifuge (rotor SS34, Sorvall, Heidelberg, Germany). The clear supernatant fraction was taken as cell-free extract.

Chemicals

4-Hydroxybenzoyl-CoA was synthesized from CoASH and the corresponding anhydride according to Merkel et al. (1989).

The ATP-monitoring kit was obtained from Colora (Lorch, Germany), enzymes from Boehringer (Mannheim, Germany), inhibitors and other chemicals from Sigma (München, Germany).

Enzyme assays

4-Hydroxybenzoyl-CoA ligase activity was measured in a photometric assay according to Geissler et al. (1988). The potassium-reduced assay mixture (no potassium salts added, but a little cross-contamination by impurities of other chemicals and by crude extract possible) contained 100 mM TRIS/HCl buffer pH 7.8, 2 mM cysteine, 5 mM $MgCl_2$, 1 mM ATP, 0.4 mM CoASH, 0.4 mM NADH + H^+, 1 mM phosphoenolpyruvate, 1 U myokinase, 1 U pyruvate kinase, 1.5 U lactate dehydrogenase, 0.5 mM 4-hydroxybenzoate and 5–50 μg cell-free extract. The oxidation of NADH + H^+ was measured photometrically at 37°C at a wavelength of 365 nm. Omission of potassium was essential to repress decarboxylation of 4-hydrobenzoate by the decarboxylating activity of phenolcarboxylase, which was a potassium-requiring enzyme of the mixed culture (Gallert and Winter 1992).

4-Hydroxybenzoyl-CoA-reductase activity was assayed by quantification of substrate and product by high-performance liquid chromatography. The reduction of 4-hydroxybenzoyl-CoA to benzoyl-CoA with reduced benzylviologen as an electron donor was measured in 50 mM potassium phosphate buffer pH 7.5, containing 2 mM cysteine, 25 mM $MgCl_2$, 0.5 mM benzylviologen, 0.25 mM 4-hydroxybenzoyl-CoA and 50 μg cell-free extract. To test other electron mediators, benzylviologen was replaced by 0.5 mM reduced methylviologen or NADH + H^+.

Analytical methods

Phenol was analysed with a Packard model 437 gas chromatograph (United Technologies, Frankfurt) using an OV 351 column (Teflon, i.d. 1.5 mm, 2 m length) at 170°C oven temperature for separation with nitrogen as a carrier gas. The flow rate was 30 ml min^{-1}. The detector and injector temperature was 210°C.

Coenzyme A, 4-hydroxybenzoate, benzoate and benzoyl-CoA were separated by reversed-phase high-performance liquid chromatography with an ODS II column (Kontron, Eching) by elution with acetonitrile (A) and 0.2 M ammonium acetate pH 5.0 (B). At a solvent flow rate of 1 ml min^{-1} a linear gradient was applied with a reduction of the proportion of solvent B from 100% to 75% within 25 min and from 75% to 25% within the following 5 min. The amount of solvent B was increased again from 25% to 100% within 5 min and the separation procedure continued for another 5 min at a flow of 100% solvent B. Absorbance of the eluent was measured at 254 nm. The synthesis of 4-hydroxybenzoyl-CoA was proven by comparison of UV spectra of the HPLC peak at pH 6.0 and 12.0 (Webster et al. 1974). 4-Hydroxybenzoyl-CoA was separated from other compounds with an ODS II column at a flow rate of 1 ml min^{-1} and detected by UV spectroscopy at 254 nm. The eluent contained water (A) and methanol (B). An eluent program of 100% solvent A for 3 min, 100%–50% A within 10 min and again 100% A for 10 min was used for separation. The products of 4-hydroxybenzoyl-CoA hydrolysis with 0.1 M NaOH, 4-hydroxybenzoate and CoASH (Schachter and Taggaert 1953), were measured by HPLC using the acetonitrile/ammonium acetate system described above. The benzoyl-CoA was identified by comparison of its retention time with that of authentic benzoyl-CoA and by HPLC analysis of benzoate and CoASH after alkaline hydrolysis.

To monitor ATP formation during decarboxylation of 4-hydroxybenzoate, cell suspensions in 50 mM potassium phosphate buffer pH 7.5 + 2 mM cysteine were supplemented with 0.5 mM 4-hydroxybenzoate after incubation for 15 min at 37°C without 4-hydroxybenzoate. Samples were taken at different times for ATP analysis and prepared as follows: 100 μl cell suspension was mixed with 100 μl 0.5 M NaOH and boiled in an Eppendorf thermomixer (Hamburg, Germany) for 5 min. After thermal denaturation the pH was adjusted to 7.0 with 40 μl 1 M HCl. A 50-μl sample was mixed with 400 μl water and 50 μl ATP-monitoring reagent (the luciferin/luciferase system from *Photinus pyralis*) and the luminescence was measured in a luminometer (Bio Orbit 1250, Colora) at 562 nm. The transformation of oxyluciferin to luiferin produced a linear signal in a concentration range of 5–75 nM ATP. For testing the effects of uncouplers and inhibitors on cellular ATP levels, cell suspensions were incubated for 3 min with 4-hydroxybenzoate in the presence of ethanol and inhibitory substances were added 3 min later from ethanolic stock solutions. The quenching of CCCP (carbonylcyanide *m*-chlorophenylhydrazone) and DCCD (dicyclohexylcarbodiimide) was corrected using samples with ATP from stock solutions at authentic concentration.

Protein was quantified colorimetrically according to Bradford (1976) with bovine serum albumin as a standard.

Results

4-Hydroxybenzoyl-CoA ligase activity in cell-free extracts of the phenol-degrading mixed culture

4-Hydroxybenzoyl-CoA ligase activity in cell-free extracts of the phenol-degrading mixed culture was only detectable in an assay system without explicitely added potassium ions. Omission of potassium ions was essential since, in the presence of K^+, 4-hydroxybezoate was the preferred substrate of a 4-hydroxybenzoate-decarboxylating activity (reverse reaction of the phenol carboxylase), which was active in cell-free extracts (Gallert and Winter 1992). 2-Hydroxybenzoate and 3-hydroxybenzoate were not activated by 4-hydroxybenzoyl-CoA ligase. Cell-free extracts of the benzoate-degrading mixed culture could not form 4-hydroxybenzoyl-CoA from 4-hydroxybenzoate. The specific activity of the AMP-forming 4-hydroxybenzoyl-CoA ligase of the phenol-degrading mixed culture was 100–200 nmol min^{-1} mg^{-1}.

Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA by cell-free extracts

Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA by cell-free extracts of the phenol-degrading mixed culture proceeded almost stoichiometrically in the initial test phase (Fig. 1). The non-stoichiometric increase of benzoyl-CoA after 40 min and finally its cleavage to benzoate and CoASH was a result of thioesterase activities of the crude cell-free extract. After 120 min of incubation little residual benzoyl-CoA was left and, together with the benzoate/CoASH, accounted for the amount of 4-hydroxybenzoyl-CoA at time zero. In control assays without cell extract of the phenol-degrading mixed culture or with a crude cell-free extract of the benzoate-degrading mixed culture no dehydroxylation of 4-hydroxybenzoyl-CoA could be measured. In the test system benzylviologen but not methylviologen or NADH + H^+ acted as the electron mediator for dehydroxylation of 4-hydroxybenzoyl-CoA. The physiological electron donor is unknown. The specific activity of the 4-hydroxybenzoyl-CoA reductase was 14.5 nmol min^{-1} mg^{-1}.

ATP formation during 4-hydroxybenzoate decarboxylation

Cell suspensions of the phenol-degrading mixed culture in buffer decarboxylated 4-hydroxybenzoate to phenol

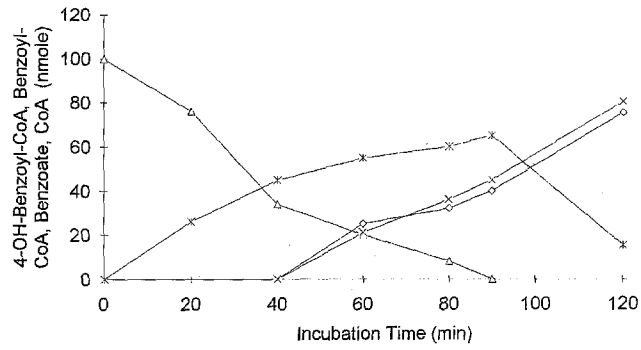

Fig. 1 Time course of dehydroxylation of 4-hydroxybenzoyl-CoA (△) and formation of benzoyl-CoA (*), benzoate (×) and CoASH (◇) by cell-free extracts of the phenol-degrading mixed culture

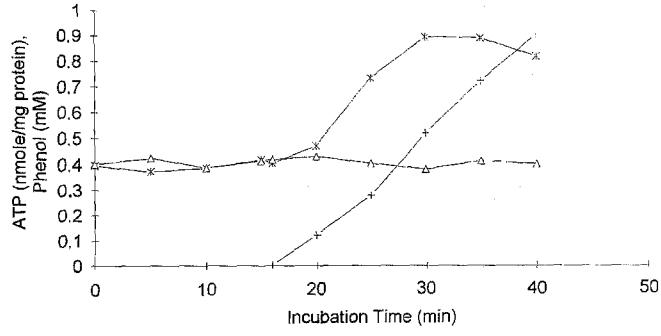

Fig. 2 ATP formation during 4-hydroxybenzoate decarboxylation by cell suspensions of the phenol-degrading mixed culture. Concentrated cell suspensions of the phenol-degrading mixed culture were preincubated at 37°C without substrate. After 15 min 1 mM 4-hydroxybenzoate was added. (△) ATP level without substrate, (*) ATP level in the presence of 4-hydroxybenzoate and (+) phenol formed from 4-hydroxybenzoate

at a rate of 200 nmol min^{-1} mg protein^{-1} at 37°C. Under a H_2/CO_2 gas atmosphere benzoate, an intermediate of anaerobic phenol degradation, accumulated stoichiometrically and no further degradation to acetate, CO_2 and methane occurred. The ability of cell suspensions to degrade 4-hydroxybenzoate and phenol was dependent on rod-shaped bacteria, which accounted for about 10% of the total bacteria of the consortium and which were inhibited by hydrogen (Knoll and Winter 1989).

Addition of 4-hydroxybenzoate to the phenol-degrading mixed culture resulted in a subsequent increase of cellular ATP levels, accompanied by phenol formation (Fig. 2). If cell suspensions were incubated in the absence of 4-hydroxybenzoate no changes of the basic ATP level of 0.31 nmol ATP/mg protein occurred. Furthermore, no changes of the ATP level of the benzoate-degrading mixed culture occurred upon incubation with 4-hydroxybenzoate or phenol. The increase of the cellular ATP level during 4-hydroxybenzoate decarboxylation by cell suspensions of the

Table 1 Effect of ionophors and ATPase inhibitors on cellular ATP levels during 4-hydroxybenzoate decarboxylation. Concentrated cell suspensions of the phenol-degrading mixed culture were supplemented with inhibitors/uncouplers 3 min after addition of 1 mM 4-hydroxybenzoate and incubated for another 18 min. Cells were broken and ATP levels as well as 4-hydroxybenzoate-decarboxylating activities were determined (*DCCD* dicyclohexylcarbodiimide, *CCCP* carbonylcyanide *m*-chlorophenylhydrazone)

Inhibitors (150 μM)	Cellular ATP levels (nmol/mg)	4-Hydroxybenzoate-decarboxylating activity (mU/mg)
Without 4-hydroxybenzoate	0.310	0
4-Hydroxybenzoate	0.868	207
4-Hydroxybenzoate + ethanol	0.833	208
4-Hydroxybenzoate + DCCD	0.150	425
4-Hydroxybenzoate + CCCP	0.560	169
4-Hydroxybenzoate + ouabain	0.911	190
4-Hydroxybenzoate + monensin	0.807	186

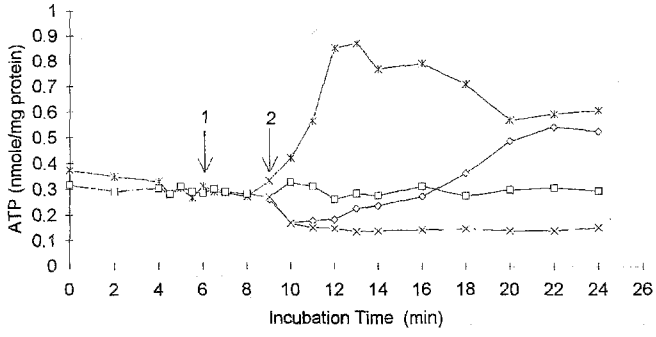

Fig. 3 ATP levels in cells of the phenol-degrading mixed culture in the presence of dicyclohexylcarbodiimide (DCCD) and carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Concentrated cell suspensions of the phenol-degrading mixed culture were incubated at 37°C in the presence of H_2/CO_2 gas atmosphere. After 6 min 1 mM 4-hydroxybenzoate was supplemented (*1* ↓) and after 9 min the ionophors/inhibitors were added (*2* ↓). ATP level without substrate (□), in the presence of 1 mM 4-hydroxybenzoate (*) and in the presence of 1 mM 4-hydroxybenzoate plus DCCD (×) or CCCP (◇)

phenol-degrading mixed culture indicated that the energy from decarboxylation of 4-hydroxybenzoate could be transformed into ATP.

The participation of sodium ions in energy conservation during 4-hydroxybenzoate decarboxylation was excluded, since monensin, an inhibitor of ATP formation by sodium gradients across the cytoplasmic membrane, as well as the putative Na^+-ATPase inhibitor ouabain had no effect on cellular ATP-levels and the 4-hydroxybenzoate-decarboxylating activity respectively (Table 1). In contrast, the proton ionophore CCCP prevented ATP formation (followed by an increase of the ATP concentration) by decarboxylation of 4-hydroxybenzoate to phenol, indicating the importance of proton gradients for ATP synthesis (Fig. 3, Table 1). Addition of DCCD, an inhibitor of the membrane-bound H^+-ATPase complex, reduced cellular ATP levels (Fig. 3) and accelerated 4-hydroxybenzoate decarboxylation (Table 1).

Discussion

With phenol as the substrate, the mixed culture catalysed the carboxylation to 4-hydroxybenzoate, activation to 4-hydroxybenzoyl-CoA and reductive dehydroxylation to benzoyl-CoA (Gallert et al. 1991; Gallert and Winter 1992), which was converted to benzoate by a thioesterase (Fig. 4). 4-Hydroxybenzoate and 4-hydroxybenzoyl-CoA were found ^{14}C-labeled when [U-^{14}C] phenol was supplied (Gallert and Winter 1993). Only cells of the phenol-degrading cultures were able to catalyse this reaction sequence. Activation of 4-hydroxybenzoate to the respective CoA thioester by a 4-hydroxybenzoyl-CoA ligase in cell-free extracts proceeded with a specific activity of 100–200 nmol $min^{-1}\,mg^{-1}$, which was in a similar order to the activities of CoA ligases from other organisms: the 3-hydroxybenzoyl-CoA ligase of a nitrate-reducing bacterium had a specific activity of 190 nmol $min^{-1}\,mg^{-1}$ (Heising et al. 1991), the gentisyl-CoA ligase of strain HQGö1 150–166 nmol $min^{-1}\,mg^{-1}$ (Gorny and Schink 1994) and the 4-hydroxybenzoyl-CoA ligase of strain K172 8 nmol $min^{-1}\,mg^{-1}$ (Biegert et al. 1993).

Activation of 4-hydroxybenzoate to the respective CoA thioester was only measurable if potassium ions were omitted from the test system. In the presence of potassium ions 4-hydroxybenzoate was almost quantitatively decarboxylated by the potassium-requiring 4-hydroxybenzoate decarboxylase activity of crude extracts (Gallert and Winter 1992).

The central intermediate of anaerobic or anoxic degradation of phenols was benzoyl-CoA, which was formed by reductive dehydroxylation or deamination of the CoA thioesters of substituted phenols (Dangel et al. 1991; Brackmann and Fuchs 1993). Cell-free extracts of the syntrophic phenol-degrading culture catalysed reductive dehydroxylation of 4-hydroxybenzoyl-CoA with reduced benylviologen as an electron donor with a specific activity of the reductase of 14.5 nmol min^{-1} mg^{-1}. For comparison, the 4-hydroxybenzoyl-CoA reductase of the nitrate-reducing strain K172 had a

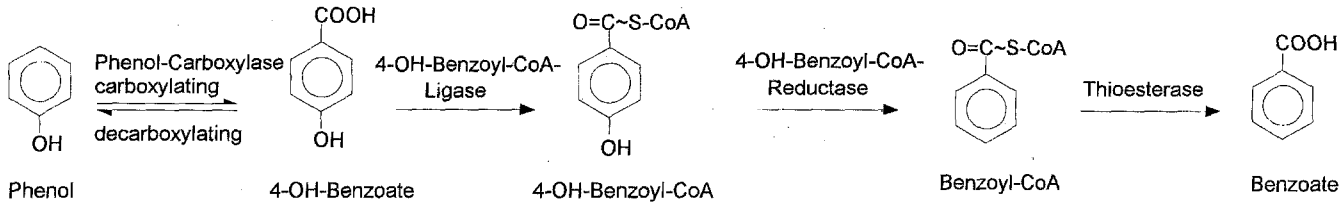

Fig. 4 Reaction sequence of phenol conversion to benzoyl-CoA/benzoate by the phenol-degrading syntrophic culture

specific activity of 30 nmol min^{-1} mg^{-1} (Glöckler et al. 1989), the gentisyl-CoA reductase of strain HQGö1 17 nmol min^{-1} mg^{-1} (Gorny and Schink 1994) and the 4-aminobenzoyl-CoA deaminating enzyme of *Desulfobacterium anilini* 2.1 nmol min^{-1} mg^{-1} (Schnell and Schink 1991).

The growth yield of the phenol-degrading syntrophic culture with 4-hydroxybenzoate as substrate was increased by 1.8 g/mol in comparison to the growth yield with an equimolar amount of phenol (Gallert and Winter 1993). The only difference in the pathway of both substrates was decarboxylation of 4-hydroxybenzoate to phenol. Thus, the increased cell yield must have been due to energy generation by the decarboxylation reaction. Growth at the expense of energy conservation from decarboxylation of succinate (Schink and Pfennig 1982; Denger and Schink 1990), oxaloacetic acid (Smith et al. 1985), malonate (Dehning and Schink 1989; Janssen and Haarfoot 1992) and glutarate (Matthies and Schink 1992) as well as energy conservation during dechlorination of 3-chlorobenzoate (Dolfing 1990) and 2-chlorophenol (Dietrich and Winter 1990) has been reported previously.

In concentrated cell suspensions of the phenol-degrading mixed culture the intracellular ATP level increased by approximately 0.5 nmol/mg protein after the addition of 4-hydroxybenzoate. If no 4-hydroxybenzoate was added to the cell suspensions the ATP level remained constant under otherwise identical incubation conditions. In *Citrobacter diversus* the ATP level increased by 4.5–6.0 nmol ATP/mg protein during decarboxylation of succinate (Janssen 1992) and in strain DCB-1 by 6.0 nmol ATP/mg protein during dechlorination of 3-chlorobenzoate (Dolfing 1990). The increase of the intracellular ATP pool of "only" 0.5 nmol/mg in the phenol-degrading culture during decarboxylation of 4-hydroxybenzoate seemed to be comparatively little. However, taking into consideration that the phenol degrader comprised only 10% of the total population of the consortium, then the increase of the ATP level during decarboxylation of 4-hydroxybenzoate was in the same order as reported for other decarboxylation reactions.

The free energy from decarboxylation of 4-hydroxybenzoate at a pH of 6.5, a reaction temperature of 37°C and the equilibrium concentrations of substrate and products was estimated as $\Delta G^{\circ\prime} = -36.67$ kJ/mol (according to Thauer et al. 1977; ΔG_f° values for phenol and 4-hydroxybenzoate were calculated from increments according to Landolt–Börnstein 1961). Under physiological conditions the formation of 1 mol ATP required a free energy of between -70 kJ/mol and -100 kJ/mol (Schink 1988), which was generally available during substrate-chain phosphorylation. For smaller amounts of free energy, ion gradients across the cytoplasmic membrane were responsible for ATP generation.

In *Klebsiella pneumoniae* and *Salmonella typhimurium* the energy from decarboxylation of oxalic acid was conserved as ATP through the formation of a sodium gradient and the action of a sodium pump (Wifling and Dimroth 1989). Other examples of primary sodium pumps were the methylmalonyl-CoA decarboxylase of *Veillonella alcalescens* (Hilpert and Dimroth 1982) and of *Propionigenium modestum* (Hilpert et al. 1984), the glutaconyl-CoA decarboxylase of *Acidaminococcus fermentans* (Buckel and Semmler 1985) and of *Peptostreptococcus asaccharolyticus* (Wohlfarth and Buckel 1985).

The involvement of a sodium ion gradient in energy conservation of the phenol-degrading mixed culture during 4-hydroxybenzoate decarboxylation was excluded by application of ion-specific ionophors and of uncouplers. The intracellular ATP level and the rate of phenol formation were not reduced by the sodium ionophor monensin and the Na^+-ATPase inhibitor ouabain respectively.

If concentrated cell suspensions were incubated with the proton ionophor CCCP no change in the ATP level was observed initially, while in the absence of CCCP the ATP level increased. However, after some time the H^+ gradient could apparently be restored and the ATP level began to increase slowly, but the decarboxylation rate of 4-hydroxybenzoate was lower than without inhibitor. This might indicate that protons were involved in the uptake of 4-hydroxybenzoate. The pK of 4-hydroxybenzoate at 19°C and a pH of 7 was 4.48, indicating that most of the 4-hydroxybenzoate was present as the carbanion at this pH. Thus, for uptake of 4-hydroxybenzoate a symport with protons might be an appropriate mechanism.

The H^+-ATPase inhibitor DCCD prevented ATP formation, but increased the decarboxylation rate of 4-hydroxybenzoate, as expected if a proton gradient was involved in ATP formation. Decarboxylation and energy conservation were apparently uncoupled by

DCCD. Thus, ATP generation and 4-hydroxybenzoate decarboxylation must be separate activities of the cytoplasmic membrane.

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