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Initial steps in the fermentation of 3-hydroxybenzoate by *Sporotomaculum hydroxybenzoicum*

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Abstract The anaerobic bacterium *Sporotomaculum hydroxybenzoicum* ferments 3-hydroxybenzoate to acetate, butyrate, and CO_2. 3-Hydroxybenzoate was activated to 3-hydroxybenzoyl-CoA in a CoA-transferase reaction with acetyl-CoA or butyryl-CoA as CoA donors. 3-Hydroxybenzoyl-CoA was reductively dehydroxylated, forming benzoyl-CoA. This reaction was measured in cell-free extracts with cob(I)alamin as low-potential electron donor. No evidence was obtained that cob(I)alamin is the physiological electron donor; however, inhibitor studies indicated involvement of a strong nucleophile in the reaction. Benzoate was degraded by dense cell suspensions without a lag phase until an in situ $\Delta G'$ value <–25 kJ mol^{-1} was reached. Benzoyl-CoA reductase was not detected. Enzyme activities for all reaction steps from glutaryl-CoA to butyryl-CoA, and ATP formation via acetate kinase were detected in cell-free extracts. Glutaconyl-CoA decarboxylase is likely to act as a primary sodium ion pump.

Key words Anaerobic degradation · Aromatic compounds · 3-Hydroxybenzoate · Benzoyl-CoA pathway · Reductive dehydroxylation · Fermenting bacteria · *Sporotomaculum hydroxybenzoicum*

Introduction

Monohydroxybenzoates (2-, 3-, and 4-hydroxybenzoate) are frequently occurring intermediates in the aerobic and anaerobic biodegradation of organic matter (Heider and Fuchs 1997; Schink et al. 2000). Under anoxic conditions, 2- and 4-hydroxybenzoate are degraded by an activation step to the respective CoA-ester in a ligase reaction and by subsequent reductive dehydroxylation leading to benzoyl-CoA as a central intermediate, as studied mainly with nitrate-reducing and anoxygenic phototrophic bacteria (Heider and Fuchs 1997; Harwood et al. 1999). Little is known so far about anaerobic degradation of 3-hydroxybenzoate. Although for this isomer, CoA-thioesterification (Heising et al. 1991; Biegert et al. 1993) and reductive dehydroxylation (Tschech and Schink 1986; Brackmann and Fuchs 1993) were assumed to be the first steps in anaerobic degradation, detailed studies on this pathway are still necessary.

Monohydroxybenzoates can also be degraded by fermenting bacteria. Tschech and Schink (1986) reported on the degradation of all three hydroxybenzoates to acetate, methane, and CO_2 by syntrophic cocultures. Alternatively, 4-hydroxybenzoate can be decarboxylated to phenol as a co-metabolic step during fermentation of amino acids (Zhang et al. 1994). Recently, a strictly anaerobic bacterium, *Sporotomaculum hydroxybenzoicum*, was described which thrives on the fermentation of 3-hydroxybenzoate to acetate, butyrate, and CO_2 in pure culture (Brauman et al. 1998). This reaction is slightly exergonic under standard conditions ($\Delta G°'$=–38 kJ per mol 3-hydroxybenzoate) and does not depend on removal of a fermentation product to become energetically feasible. During degradation of 3-hydroxybenzoate, benzoate accumulates transiently in the medium, indicating that 3-hydroxybenzoate is degraded via the benzoyl-CoA pathway.

In the present communication, we describe key reactions in anaerobic 3-hydroxybenzoate degradation by *S. hydroxybenzoicum* that allow us to propose a degradation pathway for this substrate.

Materials and methods

Cultivation of *Sporotomaculum hydroxybenzoicum*

Sporotomaculum hydroxybenzoicum strain BT (DSM 5475) was obtained from our own culture collection and was grown in 10 l containers under an N_2/CO_2 atmosphere (80/20, v/v) in bicarbonate-buffered (pH 7.6), sulfide- or cysteine-reduced freshwater min-

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eral medium. Growth conditions and medium composition have been described previously in detail (Brauman et al. 1998). The medium contained 10 mM 3-hydroxybenzoate and was inoculated with 10 vol% of pre-culture; crystals of sodium dithionite were added to a final concentration of about 100 µM).

Preparation of cell suspensions and cell-free extracts

Cells were harvested anoxically during exponential growth under an N_2/H_2 atmosphere (95/5, v/v) using an anoxic chamber (Coy Laboratory, Ann Arbor, Mich., USA), washed, and resuspended in degassed 50 mM potassium phosphate or Tris/HCl buffer (pH 7.2–7.5). For measurements of reductive dehydroxylation of 3-hydroxybenzoyl-CoA and assays of benzoyl-CoA reductase, the buffer was pre-reduced with H_2 and 0.005% (w/v) palladium on charcoal as catalyst. This buffer was filtered twice inside the anoxic chamber through filter paper before use in order to remove the catalyst. All experiments with cell suspension were performed anoxically at 30 °C in 5-ml rubber-sealed Hungate vials. The cell density was adjusted to OD_{578}=10. Samples were taken with gastight syringes (Macherey-Nagel, Düren, Germany) and diluted immediately in ice-cold 100 mM H_3PO_4.

Cell-free extracts were prepared by disruption at 140 MPa in a cooled French pressure cell under anoxic conditions. The extract was centrifuged twice at 20,000×*g* for 15 min to remove cell debris. Membranes and the cytoplasmic fraction were separated by ultracentrifugation (1 h at 100,000×*g*, 4 °C).

Enzyme assays

Enzymes were assayed under anoxic conditions at 20–22 °C unless specified otherwise. All enzyme activities are expressed as nmol substrate transformed per mg protein, and assays were carried out at least in triplicate. Protein was quantified as described by Bradford (1976), with bovine serum albumin as standard.

Acyl-CoA synthetase was tested as described earlier (Schnell and Schink 1991). In addition, a photometric assay was performed to check for AMP or ADP formation by a coupled enzyme assay (Geissler et al. 1988).

3-Hydroxybenzoate CoA-transferase was measured discontinuously by HPLC analysis, or by a continuous photometric assay (modified after Buckel 1986). The reaction mixture for the discontinuous assay contained acetyl-CoA or butyryl-CoA (0.5–1.0 mM) as CoA donor and 3-hydroxybenzoate (0.5–1.0 mM) as acceptor in degassed 50 mM potassium phosphate buffer (pH 7.0). The photometric assay contained 100 mM sodium acetate, 1.0 mM 5,5′-dithiobis(2-nitrobenzoate), and 0.1 mM 3-hydroxybenzoyl-CoA in 50 mM potassium phosphate buffer (pH 7.0). CoASH was liberated from formed acetyl-CoA via endogenous phosphotransacetylase, and the absorbance of the resulting thiol was followed at 412 nm (ε=14 mM^{-1} cm^{-1}). In cases of low phosphotransacetylase activity, e.g., in fractionation experiments, 1.0 mM oxaloacetate and 20 µg citrate synthase were added in order to release CoASH.

Benzoate CoA-transferase was measured with the same assays except that 3-hydroxybenzoate or 3-hydroxybenzoyl-CoA was replaced by benzoate or benzoyl-CoA, respectively.

Tests for dehydroxylation of 3-hydroxybenzoyl-CoA were performed in an anoxic chamber with the reaction vessels at 30 °C. The reaction mixture contained 0.1–1.0 mM chemically synthesized 3-hydroxybenzoyl-CoA and 800 µM cob(I)alamin in 50 mM Tris/HCl or potassium phosphate buffer (pH 7.2), pre-reduced with H_2/Pd-catalyst. The reaction was followed discontinuously by HPLC analysis. In addition to cob(I)alamin, NAD(P)H, $FADH_2$, $FMNH_2$, titanium(III) citrate, methyl viologen, benzyl viologen, dithionite, and dithioerythritol were tested as potential electron donors. The viologens and flavins were pre-reduced by dithionite or titanium(III) citrate.

Benzoyl-CoA reductase (EC 1.3.99.15) was assayed for under an N_2 atmosphere in 5-ml rubber-sealed Hungate vials with H_2/Pd-reduced 50 mM potassium phosphate or Tris/HCl buffer (pH 7.2). The benzoyl-CoA concentration was 0.1 mM. In contrast to the previously described test for benzoyl-CoA reductase in cell-free extracts of *Thauera aromatica* (Koch et al. 1993), we used ^{12}C-benzoyl-CoA instead of ^{14}C-labeled benzoyl-CoA. The same potential electron donors tested for dehydroxylation of 3-hydroxybenzoyl-CoA were applied. MgATP (5 mM), phosphoenolpyruvate (10 mM) plus pyruvate kinase (1 U), or acetyl-CoA (1 mM) were added in some assays. Samples (100 µl) were taken with gastight syringes and immediately transferred to 5 µl 5 M KOH, heated (80 °C, 20 min), neutralized by 5 µl 5 M H_3PO_4, centrifuged (15,800×*g*, 10 min), and analyzed by HPLC at 233 nm.

Glutaryl-CoA dehydrogenase (EC 1.99.7) was assayed after Stams et al. (1984). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.2), 1 mM $K_3Fe(CN)_6$ ($\varepsilon_{420\ nm}$= 0.9 mM^{-1} cm^{-1}), 0.1 mM phenazine-methosulfate, and 0.5 mM glutaryl-CoA.

Glutaconyl-CoA decarboxylase (EC 4.1.1.70) was assayed with a test system modified after Buckel (1986). The assay mixture contained 50 mM potassium phosphate buffer (pH 7.2), 2.5 mM $MgCl_2$, 2.5 mM dithioerythritol, 0.2 mM CoASH, 0.1 mM acetyl phosphate, 1 mM NAD^+, and auxilliary enzymes from *Acidaminococcus fermentans*. The reaction was followed at 334 nm upon addition of 1 mM K_2-glutaconate. Assays for sodium dependency of glutaconyl-CoA decarboxylase were carried out in potassium phosphate buffer containing initially less than 50 ppm Na^+ and amended with various amounts of NaCl from stock solutions.

Acetate CoA-transferase (EC 2.8.3.8) was measured with the same photometric assay as used for 3-hydroxybenzoate CoA-transferase, except that 3-hydroxybenzoyl-CoA was replaced by butyryl-CoA.

Butyryl-CoA dehydrogenase (EC 1.3.99.2), phosphotransacetylase (EC 2.3.1.8.), and acetate kinase (EC 2.7.2.1) were assayed as described by Bergmeyer (1983), and hydrogenase (EC 1.18.99.1) was measured according to Diekert and Thauer (1978).

Effect of potential inhibitors on 3-hydroxybenzoate degradation

The influence of alkylating agents on the degradation of 3-hydroxybenzoate was tested in dense cell suspensions. Propyl iodide was added from 1–10 mM ethanolic stock solutions to cell suspensions in Hungate vials wrapped in aluminum foil. For treatment of propyl iodide-amended cell suspensions with light, a cold halogen lamp was used. Methyl iodide, iodoacetamide, iodoacetate, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), or *N*-tosyl phenylalanine chloromethyl ketone (TPCK) was added to cell suspensions from 1–50 mM methanolic stock solutions. The effect of N_2O on 3-hydroxybenzoate degradation was investigated by changing the gas phase in the test vials from N_2 to N_2O. Incubation time was 5 min for alkylating agents and 30 min for N_2O. The concentration of 3-hydroxybenzoate or benzoate as control was 1 mM in all assays.

Chemical analyses

Aromatic compounds, CoASH, and CoA-esters were analyzed by C18 reversed-phase HPLC as described previously (Brune and Schink 1990). Aromatic CoA-esters were identified by their retention times, UV absorption spectra (Webster et al. 1974), and identification of the aromatic acid after alkaline hydrolysis (KOH at pH 12, 80 °C, 20 min). Acetate and butyrate were measured according to Galushko et al. (1999). Hydrogen concentrations were determined by gas chromatography (Wallrabenstein and Schink 1994). Corrinoids were determined by HPLC analysis in cell-free extracts containing 144–172 mg protein according to Kengen et al. (1988). Eluting compounds were scanned on-line with a Beckman 168 diode array detector (Beckman Instruments, Munich) within the range of 200–600 nm wavelength. Cyanocobalamin was used as standard. Sodium in assays for glutaconyl-CoA decarboxylase was measured with an atomic absorption spectrophotometer (3030B, Perkin Elmer, Norwalk, Conn., USA) at 589 nm.

Chemicals

3-Hydroxybenzoyl-CoA was synthesized after Wieland (see Decker 1959), modified according to Merkel et al. (1989), in 20 mM bicarbonate buffer (pH 7.2) under an N_2/CO_2 atmosphere (80/20, v/v) without final alkali treatment. Acetyl-CoA and butyryl-CoA were synthesized from CoASH and the corresponding anhydrides (Simon and Shemin 1953). All CoA-esters were purified by HPLC. CoA-ester-containing fractions were evaporated to dryness and dissolved in 10 mM potassium phosphate buffer (pH 6.0).

Cob(I)alamin and cob(II)alamin were prepared by electrochemical reduction of aquocob(III)alamin as described by Kreft and Schink (1994). Titanium(III) citrate stock solutions were prepared in an anoxic chamber after Zehnder and Wuhrmann (1976) and contained 90 mM Ti^{3+} chelated by 100 mM citrate. KOH, KH_2PO_4, and K_2HPO_4 for testing sodium dependence of glutaconyl-CoA decarboxylase were from Merck (Darmstadt, Germany) and contained a maximum of 20 ppm and 50 ppm sodium. All other chemicals and gases were of the highest purity available and from standard commercial sources.

Calculations

The change in Gibbs free energy ($\Delta G'$) for benzoate degradation under non-standard conditions was calculated using the measured end-point concentrations of substrate and products according to the following equation:

$$\Delta G = \Delta G^\circ + RT\ln\frac{[CH_3COO^-]\,[CH_3(CH_2)_2COO^-]\,[H_2]\,[HCO_3^-]}{[C_6H_5COO^-]}$$

where R is the gas constant, T the temperature, $[H_2]$ the H_2 partial pressure in atmospheres, and the other formulae in brackets the molar concentrations of the respective compounds. The change in standard Gibbs free energy ($\Delta G^{\circ\prime}$) for benzoate degradation ($C_6H_5COO^- + 4H_2O \rightarrow CH_3COO^- + CH_3(CH_2)_2COO^- + H_2 + CO_2 + H^+$) is +25.8 kJ mol^{-1} (after Thauer et al. 1977; Kaiser and Hanselmann 1983). pH values did not shift significantly (<0.1 units) and the H^+ activity was therefore considered to be constant.

Results

Degradation of 3-hydroxybenzoate and benzoate by dense cell suspensions

Dense cell suspensions converted 3-hydroxybenzoate to benzoate, acetate, butyrate, CO_2, and small amounts of H_2 at a rate of 43 nmol min^{-1} (mg protein)$^{-1}$. Compared to conversion of 3-hydroxybenzoate by growing cultures (Brauman et al. 1998), higher amounts of benzoate were formed (up to 20% of the 3-hydroxybenzoate added). Benzoate formation started immediately after addition of 3-hydroxybenzoate to the cell suspensions and proceeded at a rate of 9 nmol min^{-1} (mg protein)$^{-1}$. After 3-hydroxybenzoate was consumed, the benzoate concentration decreased slowly. Changing the gas phase from N_2 to H_2 reduced the 3-hydroxybenzoate degradation rate [18 nmol min^{-1} (mg protein)$^{-1}$] and the relative amount of benzoate formed increased up to 60% of the 3-hydroxybenzoate added (acetate and butyrate were not monitored).

Cell suspensions of *S. hydroxybenzoicum* grown with 3-hydroxybenzoate degraded benzoate under N_2 atmosphere to acetate, butyrate, CO_2, and hydrogen at a rate of 15 nmol min^{-1} (mg protein)$^{-1}$. Degradation started without a lag phase but stopped when a $\Delta G'$ value <- 25 kJ mol^{-1} was reached (Table 1). Increasing the hydrogen partial pressure in the assays up to 100% H_2 led to substantially lower conversion rates and less benzoate consumption; flushing the gas phase with N_2 for 20 min after benzoate degradation ceased resulted in further benzoate consumption until again a $\Delta G'$ value of less than – 25 kJ per mol was reached.

Table 1 Benzoate degradation by dense cell suspensions of *Sporotomaculum hydroxybenzoicum*. Final concentrations were assayed after 6 h incubation

Initial benzoate concentration (mM)	Final concentrations				Residual $\Delta G'$ (kJ mol^{-1})[b]
	Benzoate (mM)	Acetate (mM)	Butyrate (mM)	H_2 (atm)	
1.03	$<0.2\cdot10^{-3}$[a]	0.9	0.8	$8\cdot10^{-7}$	–25
2.00	1.05	0.93	0.89	$9.8\cdot10^{-7}$	–44.5
4.98	3.85	1.2	1.15	$1.1\cdot10^{-6}$	–45.5

[a] A benzoate concentration of 0.1 μM was used for calculation
[b] A final bicarbonate concentration of 1 mM was assumed for all assays

Activation of 3-hydroxybenzoate and benzoate in cell-free extracts

Both 3-hydroxybenzoate and benzoate were activated to the corresponding CoA-esters in CoA-transferase reactions in cell-free extracts (Fig. 1, Table 2). CoA donors were acetyl-CoA, butyryl-CoA, crotonyl-CoA, and glutaryl-CoA. The activities were highest with acetyl-CoA

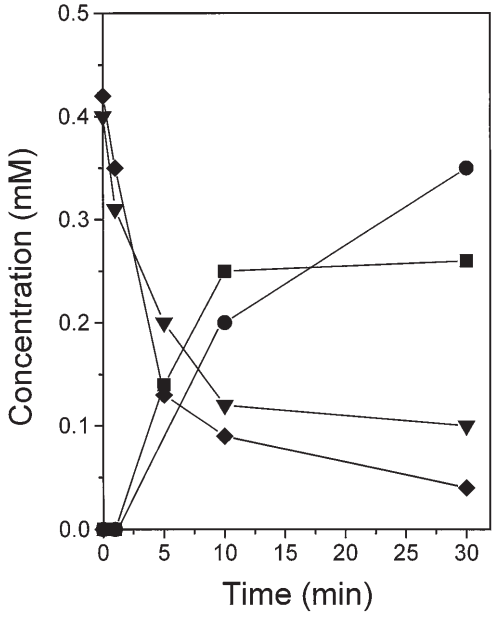

Fig. 1 Activation of 3-hydroxybenzoate (▼) to 3-hydroxybenzoyl-CoA (■) with acetyl-CoA (◆) as CoA donor by cell-free extract of *Sporotomaculum hydroxybenzoicum*. Formation of acetate is indicated (●)

Table 2 Enzyme activities detected in *S. hydroxybenzoicum*

Enzyme	Specific activity [nmol min^{-1} (mg protein)$^{-1}$]
3-Hydroxybenzoate CoA-transferase	224–247[a]
Benzoate CoA-transferase	234–243[a]
3-Hydroxybenzoyl-CoA reductase	4
Glutaconyl-CoA decarboxylase	15–18
Glutaryl-CoA dehydrogenase	31–37
Butyryl-CoA dehydrogenase	156–167
Acetate CoA-transferase	75–98
Phosphotransacetylase	6450–6600
Acetate kinase	170–250
Hydrogenase	20
In vivo substrate turnover rate	46

[a]Measured with both discontinuous and continuous assays

and butyryl-CoA, with values between 220 and 250 nmol min^{-1} (mg protein)$^{-1}$; with the two other CoA donors, activity was about 10–15% of these values. No activity was detected after exclusion of one assay component or with either 3-hydroxybutyryl-CoA or succinyl-CoA as potential CoA donors. 3-Hydroxybenzoate was converted to 3-hydroxybenzoyl-CoA also with benzoyl-CoA as CoA donor and vice versa. Activation of 2- and 4-hydroxybenzoate with acetyl-CoA as CoA donor could also be measured; however, activities were significantly lower [12–27 nmol min^{-1} (mg $protein^{-1}$)] than those with 3-hydroxybenzoate or benzoate. After ultracentrifugation of the cell-free extract, up to 45% of 3-hydroxybenzoate and benzoate CoA-transferase activity were found in the membrane fraction. Washing the membrane fraction with 0.5 M NaCl and 0.5 mM EDTA in the buffer released both activities into the supernatant (Table 3). The activity of 3-hydroxybenzoate CoA-transferase was not affected by alkylating reagents such as iodoacetamide (1 mM), iodoacetate (1 mM), 5,5′-dithiobis(2-nitrobenzoate) (1 mM), and propyl iodide (500 μM).

The CoA-transferase activities were the only reactions forming 3-hydroxybenzoyl-CoA or benzoyl-CoA. No indication of an acyl-CoA ligase activity reacting with either 3-hydroxybenzoate or benzoate was found.

Reductive dehydroxylation of 3-hydroxybenzoyl-CoA to benzoyl-CoA

Reduction of 3-hydroxybenzoyl-CoA was studied with cell-free extracts of *S. hydroxybenzoicum* in the presence of various reducing agents. Only cob(I)alamin proved to be a suitable electron donor. Initial activity with cob(I)alamin was 4 nmol min^{-1} (mg protein)$^{-1}$ (Table 2), which is about 10% of the in vivo rate of 3-hydroxybenzoate degradation [46 nmol min^{-1} (mg $protein^{-1}$)]. No activity was found with dithionite, titanium(III) citrate, reduced methyl viologen, reduced benzyl viologen, NAD(P)H, $FADH_2$, $FMNH_2$, or dithiothreitol. Also, in combination with cob(I)alamin at low concentration or after addition of ATP (5 mM), none of the reductants mentioned could serve as electron donors. No activity was measured with heat-denatured cell-free extract (80 °C, 15 min) plus cob(I)alamin. A time course of a discontinuous assay for benzoyl-CoA formation with cob(I)alamin as electron donor is depicted in Fig. 2. The assay was started with 3-hydroxybenzoyl-CoA after pre-incubation of the reaction mixture with cob(I)almin for 5 min. Decrease of 3-hydroxybenzoyl-CoA started immediately after its addition to the assay, with a corresponding increase in benzoyl-CoA and small amounts of free 3-hydroxybenzoate. Free benzoate appeared later in the course of the reaction. Conversion of 3-hydroxybenzoyl-CoA to benzoyl-CoA was not complete after incubation for up to 240 min, even though cob(I)alamin was not limiting. The reaction was not hampered if cob(II)alamin (0.1 mM) was added to the assay mixture from the beginning. The 3-hydroxybenzoyl-CoA reductase activity was rather unstable with time, with about a 10% decrease in activity per hour under

Table 3 Distribution of 3-hydroxybenzoate CoA-transferase, benzoate CoA-transferase, 3-hydroxybenzoyl-CoA reductase, and glutaconyl-CoA decarboxylase in *S. hydroxybenzoicum* after fractionation of cell-free extract by ultracentrifugation (1 h at 100,000×*g*, 4 °C). Malate-dehydrogenase was used as a marker enzyme. After ultracentrifugation, 99% of malate-dehydrogenase activity was recovered in the supernatant and <1% in the pellet

Fraction	Total activity (%)			
	3-Hydroxybenzoate CoA-transferase	Benzoate CoA-transferase	3-Hydroxybenzoyl-CoA reductase	Glutaconyl-CoA decarboxylase
Crude extract	100[a]	100[b]	100[c]	100[d]
Supernatant	34–45	39–42	79–89	64–81
Pellet	52–62	59–65	0	17–32
Combined supernatant plus pellet	98–102	97–99	81–84	95
Pellet washed with 250 mM NaCl and 0.5 mM EDTA in the buffer	<1	<1	–	–
Pellet washed with 1% Triton X-100 in the buffer	–	–	–	<2
Washing buffer	49–63	55–61	–	13–35

[a]100%=239 nmol min^{-1} (mg protein)$^{-1}$
[b]100%=227 nmol min^{-1} (mg protein)–1
[c]100%=4 nmol min^{-1} (mg protein)$^{-1}$
[d]100%=18 nmol min^{-1} (mg protein)$^{-1}$

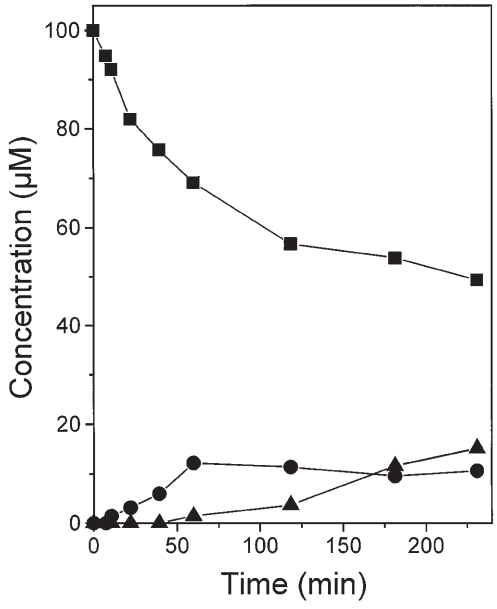

Fig. 2 Reductive dehydroxylation of 3-hydroxybenzoyl-CoA (■) to benzoyl-CoA (●) with cob(I)alamin as electron donor by cell-free extract of *S. hydroxybenzoicum*. Benzoate (▲) was formed as a side product. The difference between the decrease in the 3-hydroxybenzoyl-CoA concentration and the yield of benzoyl-CoA plus benzoate is due to slow hydrolysis of the former to 3-hydroxybenzoate and HSCoA (not shown), and slight absorption of benzoyl-CoA by components in the cell-free extracts

N_2 atmosphere. This loss in activity could not be prevented by addition of enzyme-stabilizing compounds, e.g., 10% glycerol, to the extract. The activity was also sensitive towards oxygen. Treatment of the cell-free extract with a weak air stream for 5 min resulted in complete loss of the activity, and the activity could not be recovered by restoring reducing conditions. Applying a stream of N_2 gas instead of air had no effect.

After ultracentrifugation, 3-hydroxybenzoyl-CoA reductase activity was found entirely in the soluble fraction, with 10–20% activity loss during the fractionation process. This loss could not be alleviated by combining soluble and membrane fractions and was similar to that observed if the cell-free extract was stored on ice without further treatment.

Inhibition of 3-hydroxybenzoate degradation by alkylating agents, and corrinoid determination

The influence of inhibitors of cob(I)alamin-catalyzed reactions on the rate of 3-hydroxybenzoate degradation was determined in dense cell suspensions (Table4). Propyl iodide (see Ljungdahl and Wood 1982, and references therein) strongly inhibited degradation of 3-hydroxybenzoate already at low concentrations and after a short incubation time, but benzoate degradation was not inhibited. However, inhibition of 3-hydroxybenzoate degradation by propyl iodide could not be alleviated by illumination of the cell suspension, and light had no effect on control assays without propyl iodide. Several other alkylating agents inhibited 3-hydroxybenzoate degradation, but benzoate degradation was not impaired or only to a small extent. The extent of the inhibition of 3-hydroxybenzoate degradation increased with the reactivity of these agents towards nucleophiles. Addition of methanol or ethanol alone (max. 0.5%, v/v) had no effect. Changing the gas phase in the vials from N_2 to N_2O (100% gas phase; Schrauzer and Stadlbauer 1975) had no effect on the rate of 3-hydroxybenzoate degradation. Catalysis of reductive dehydroxylation via an enzyme-bound corrinoid in the reduced Co(I) state would probably be inhibited by cob(II)alamin (Kreft and Schink 1994). This was not the case for 3-hydroxybenzoyl-CoA dehydroxylation by *S. hydroxybenzoicum*.

Table 4 Influence of potential inhibitors on 3-hydroxybenzoate and benzoate degradation by dense cell suspensions of *S. hydroxybenzoicum*. *TLCK* N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, *TPCK* *N*-tosyl phenylalanine chloromethyl ketone

Compound	Concentration (μM)	Residual activity (%)	
		3-Hydroxy-benzoate	Benzoate
Methyl iodide	20	0	89
Propyl iodide	50	0	95
Propyl iodide/ illumination	50	0	97
TLCK	200	13	100
TPCK	200	79	100
Iodoacetamide	100	0	89
Iodoacetate	100	0–6	81
N_2O	100% gas phase	100	Not determined

We also determined the presence of corrinoids in this bacterium by HPLC analysis of methanolic extracts treated with KCN. No evidence of corrinoids in *S. hydroxybenzoicum* was obtained. The UV/visible spectrum of cyanocobalamin in 50 mM potassium phosphate buffer (pH 7.2) as a standard could be recorded after injection of 10 pmol of this compound into the HPLC. This amount corresponds to a minimum intracellular corrinoid concentration in the range of 20 nM that could be detected in our assays, as calculated with an estimated intracellular volume of 3.0 μl (mg protein)$^{-1}$ (Kreke and Cypionka 1992).

Benzoyl-CoA reductase

Attempts to measure benzoyl-CoA reductase in cell-free extracts of *S. hydroxybenzoicum* were not successful, even though various conditions were employed. The applied system of measuring benzoate after alkaline treatment of the samples was sufficient to detect concentration differences of 1 μM. Monitoring benzoate instead of benzoyl-CoA was necessary because of slight adsorption of the latter to components of the cell-free extract. This resulted in a decrease in the benzoyl-CoA concentration measured due to precipitation and sedimentation during the centrifu-

gation steps in sample preparation for HPLC analysis. Benzoate was not reduced with the electron donors used for testing benzoyl-CoA reduction.

Enzymes involved in anaerobic benzoate degradation subsequent to ring cleavage and in ATP formation

In cell-free extracts of *S. hydroxybenzoicum*, enzyme activities for all reaction steps from glutaryl-CoA to butyryl-CoA could be detected (Table 2). Activities were at least in the range of the in vivo rate of 3-hydroxybenzoate degradation. Glutaconyl-CoA decarboxylase was studied in further detail. It was detected mainly in the membrane fraction after ultracentrifugation of the crude extract and could be released only after addition of detergent (Table3). The activity depended on the sodium ion concentration in the assay. In assays containing 120 μM Na^+, only one-sixth of the activity was measured as compared to assays with 20 mM Na^+. The K_m for Na^+ was calculated to be approximately 1 mM. Furthermore, a possible dependence of glutaconyl-CoA decarboxylase on biotin was investigated. Cell-free extract incubated for 15 min with avidin (0.5 mg ml^{-1}) exhibited only minimal glutaconyl-CoA decarboxylase activity [<0.1 nmol min^{-1} (mg protein) $^{-1}$]; addition of avidin pre-incubated with biotin (5 mM) had only an insignificant effect on the activity [13–16 nmol min^{-1} (mg protein)$^{-1}$].

Enzymes involved in ATP formation from acetyl-CoA or butyryl-CoA via acetate CoA-transferase were detected at sufficient activities in cell-free extracts of *S. hydroxybenzoicum* (Table2); hydrogenase was measured at an activity of 20 nmol min^{-1} (mg protein)$^{-1}$.

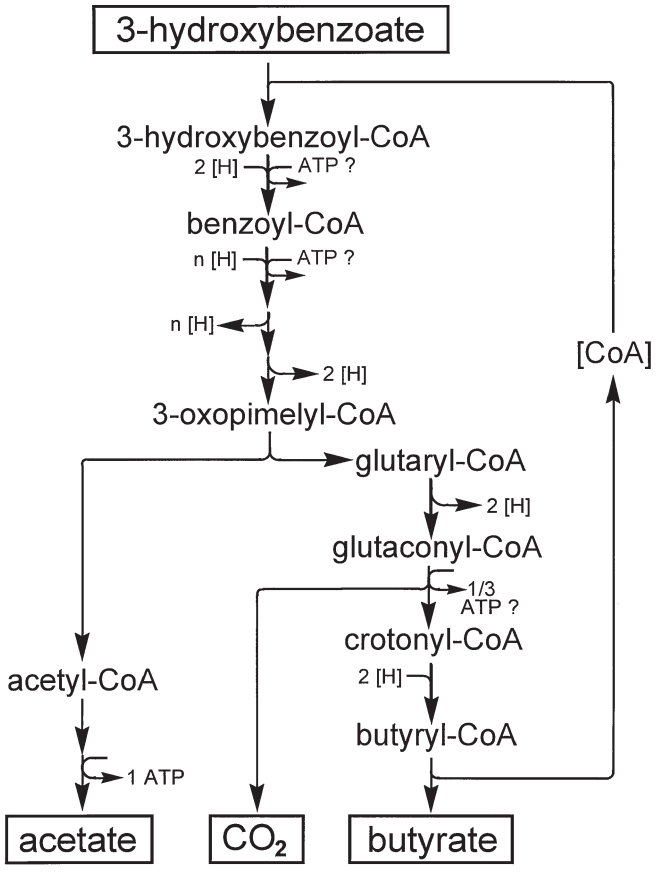

Fig. 3 Proposed pathway of 3-hydroxybenzoate fermentation by *S. hydroxybenzoicum*. For activation of 3-hydroxybenzoate either butyryl-CoA or acetyl-CoA could serve as CoA donor. The precise nature of the reaction product of benzoyl-CoA reduction is not clear

Discussion

3-Hydroxybenzoate is fermented by the anaerobic bacterium *S. hydroxybenzoicum* according to the following equation ($\Delta G^{\circ\prime}$ value calculated after Thauer et al. 1977; Kaiser and Hanselmann 1983):

$C_6H_4(OH)COO^- + 3H_2O \rightarrow CH_3COO^- + CH_3(CH_2)_2COO^- + CO_2 + H^+ \quad \Delta G^{\circ\prime} = -38$ kJ per mol 3-hydroxybenzoate

Benzoate accumulates transiently in the growth medium (Brauman et al. 1998). We suggest that 3-hydroxybenzoate is degraded by *S. hydroxybenzoicum* via benzoyl-CoA as the central intermediate (Fig. 3). Two features render the proposed metabolism of 3-hydroxybenzoate by this bacterium interesting: (1) the modification reactions forming benzoyl-CoA from 3-hydroxybenzoate, and (2) the metabolism of benzoyl-CoA in a fermenting bacterium.

Conversion of 3-hydroxybenzoate to benzoyl-CoA

As the first step in the degradation pathway, 3-hydroxybenzoate was activated to 3-hydroxybenzoyl-CoA in a CoA-transferase reaction with acetyl-CoA or butyryl-CoA as CoA donors. Use of a CoA-transferase rather than a CoA-ligase (AMP-forming) reaction for substrate activation has important energetic implications for this fermenting bacterium, since only one, rather than two, ATP equivalents are consumed. To our knowledge, this is the first report of a CoA-transferase acting on benzoic acid compounds. An activation of benzoate via a CoA-transferase was discussed for a sulfate-reducing bacterium (Kuever et al. 1993), however, without experimental proof. Involvement of CoA-transferases acting on ω-carboxy groups of side chains of aromatic compounds has been reported for the anaerobic degradation of toluene (Leutwein and Heider 1999) and *m*-cresol (Müller et al. 1999).

3-Hydroxybenzoyl-CoA was reductively dehydroxylated to benzoyl-CoA, as measured in cell-free extracts with cob(I)alamin as the low-potential electron donor. The standard redox potential of the couple 3-hydroxybenzoic acid/benzoic acid is –102 mV (calculated after Thauer et al. 1977), and the redox potential for the respective CoA-ester couple should not be substantially different. The mechanistically arduous dehydroxylation of 3-hydroxybenzoyl-CoA might require electrons from a

low-potential donor. However, no evidence was obtained that a corrinoid with Co in the +1 oxidation state is also the physiological electron donor. Furthermore, it is not understood why reductive dehydroxylation of 3-hydroxybenzoyl-CoA was measurable only with cob(I)alamin in the test assay but not with any other low-potential electron donor tested. The strong inhibition by alkylating agents of 3-hydroxybenzoate but not of benzoate degradation or the 3-hydroxybenzoate CoA-transferase reaction might indicate that a potent nucleophile is necessary for reductive dehydroxylation of 3-hydroxybenzoyl-CoA. The nucleophile could attack the benzene ring with subsequent removal of the hydroxy group. This concept is different from reduction of 4-hydroxybenzoyl-CoA to benzoyl-CoA by nitrate-reducing bacteria, which proceeds through two successive one-electron transfers with transient formation of an organic radical (Brackmann and Fuchs 1993; Buckel and Keese 1995; El Kasmi et al. 1995).

Benzoyl-CoA metabolism in *S. hydroxybenzoicum*

Several lines of evidence strongly indicate that benzoyl-CoA is a true intermediate of 3-hydroxybenzoate degradation by *S. hydroxybenzoicum*. Dense suspensions of 3-hydroxybenzoate-grown cells degraded benzoate without a lag phase to acetate, butyrate, CO_2, and H_2. Enzymes necessary for formation of benzoyl-CoA from 3-hydroxybenzoate or benzoate were detected in cell-free extracts, and all enzyme activities downstream of glutaryl-CoA dehydrogenase were found as well (Fig. 3, Table 2).

In *T. aromatica*, benzoyl-CoA is reduced to cyclohexa-1,5-diene-1-carboxyl-CoA (Koch et al. 1993) by benzoyl-CoA reductase (Boll and Fuchs 1995), with ferredoxin as electron donor (Boll and Fuchs 1998). This is an endergonic reaction that is driven by hydrolysis of 2 mol ATP per mol substrate converted (Boll et al. 1997). In *S. hydroxybenzoicum*, benzoyl-CoA reduction has to be carried out in a different manner due to the limited energy budget for this bacterium. One mol ATP per mol substrate converted is gained intermediately by the acetate kinase reaction. Glutaconyl-CoA decarboxylase displayed properties that were shown for the corresponding enzymes in *A. fermentans* and other fermenting bacteria (Buckel 1986; Beatrix et al. 1990; Matthies and Schink 1992; Schöcke and Schink 1999). These enzymes are primary sodium ion pumps. It can be hypothesized that the decarboxylase in *S. hydroxybenzoicum* generates a Na^+ ion motive force as well. This would be equivalent to 1/3 mol ATP per mol substrate converted (Dimroth and Schink 1998). There is no evidence for any additional means of energy conservation in *S. hydroxybenzoicum*. Apparently, *S. hydroxybenzoicum* gains intermediately less than 2 mol ATP equivalents per mol 3-hydroxybenzoate converted. This is insufficient to account for benzoyl-CoA reduction as it was described for *T. aromatica*. Similarly, the fermenting bacterium *Syntrophus gentianae* achieves benzoyl-CoA reduction most likely with less ATP expenditure than *T. aromatica* (Schöcke and Schink 1999). The precise nature of benzoyl-CoA reduction in fermenting bacteria, e.g., product formation, remains to be elucidated.

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