First publ. in: Archives of Microbiology 161 (1994), 1, pp. 25-32

Hydroquinone degradation via reductive dehydroxylation of gentisyl-CoA by a strictly anaerobic fermenting bacterium

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Received: 10 May 1993/Accepted: 8 July 1993

Abstract. Anaerobic degradation of hydroquinone was studied with the fermenting bacterium strain HQGö1. The rate of hydroquinone degradation by dense cell suspensions was dramatically accelerated by addition of $NaHCO_3$. During fermentation of hydroquinone in the presence of $^{14}C-Na_2CO_3$ benzoate was formed as a labelled product, indicating an initial *ortho*-carboxylation of hydroquinone to gentisate. Gentisate was activated to the corresponding CoA-ester in a CoA ligase reaction at a specific activity of 0.15 $\mu mol \times min^{-1} \times mg\ protein^{-1}$. Gentisyl-CoA was reduced to benzoyl-CoA with reduced methyl viologen as electron donor by simultaneous reductive elimination of both the *ortho* and *meta* hydroxyl group. The specific activity of this novel gentisyl-CoA reductase was 17 $nmol \times min^{-1} \times mg\ protein^{-1}$. Further degradation to acetate was catalyzed by enzymes which occur also in other bacteria degrading aromatic compounds via benzoyl-CoA.

Key words: Anaerobic degradation – Aromatic compounds – Gentisate – Carboxylation – Reductive elimination – Benzoyl-CoA pathway

Many phenolic compounds of simple or complex structure are synthesized in the secondary metabolism of higher plants. Because of their surface active properties and membrane-destabilizing effect the majority of non-polymeric phenols are found as glycoside derivatives. This is also true for hydroquinone, the most widely distributed representative of simple phenols which is transformed in a reaction with uridine diphosphate glucose to the glycoside arbutin (Conn 1964). In contrast to other hydroxy aromatics, hydroquinone and its carboxyl derivative gentisic acid occur also as free compounds in many plant tissues (Pridham 1965).

Anaerobic degradation of aromatics proceeds generally through reductive destabilization of the aromatic π-electron structure, but only few compounds are known to be subject to direct ring reduction. Most of them have to be transformed first into one of three central intermediates. Many novel enzyme activities catalyzing carboxylations, reductive eliminations, oxidations, and CoA activations have been found to be involved in such modifications of a broad variety of mononuclear aromatics (Evans and Fuchs 1988; Schink et al. 1992). For phenolic compounds, two different strategies are known: (*i*) Resorcinol and phloroglucinol degradation proceeds through direct reduction and subsequent hydrolytic cleavage of the aromatic ring (Kluge et al. 1990; Brune and Schink 1992). (*ii*) Carboxylation, CoA-activation, and reductive dehydroxylation are the key reactions in anaerobic phenol degradation via benzoyl-CoA (Lack et al. 1991; Gallert et al. 1991).

Since the hydroxyl groups of hydroquinone are in *para* position to each other, direct hydrolytic cleavage of the aromatic ring seems to be unlikely. Also reductive elimination of one of the hydroxyl groups to form phenol as an intermediate is improbable since dehydroxylation has been demonstrated so far only for coenzyme A-linked aromatic compounds. Carboxylation of hydroquinone to gentisate as the first reaction in anaerobic degradation has been proposed earlier for the fermenting bacterium strain HQGö1 (Szewzyk and Schink 1989). This bacterium degrades hydroquinone to benzoate and acetate in pure culture, and oxidizes hydroquinone, gentisate, and benzoate to acetate and CO_2 in the presence of a methanogenic partner bacterium. A sulfate-reducing bacterium able to grow with hydroquinone and gentisate has been described as well (Schnell et al. 1989). So far, nothing is known about the enzymes catalyzing the anaerobic degradation of hydroquinone.

In the present communication we report on new results obtained with strain HQGö1 which allow us to propose a pathway for anaerobic hydroquinone degradation.

Materials and methods

Media and growth conditions

A bicarbonate-buffered, sulfide-reduced mineral medium essentially as described earlier (Widdel and Pfennig 1981; Schink and Pfennig

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1982) was used which contained a 7 vitamin solution (Widdel and Pfennig 1981), trace element solution SL 10 (Widdel et al. 1983) and selenite-tungstate solution (Tschech and Pfennig 1984). Hydroquinone and gentisate were stored as stock solutions in sterile infusion bottles under nitrogen gas, and were added to cultures with syringes. Strain HQGö1 was grown in 500 ml screw cap bottles or in 1-l infusion bottles with 1–3 mM aromatic substrate under a N_2/CO_2 atmosphere at 28 °C. Growth was recorded by measuring OD at 578 nm wavelength.

Experiments with dense cell suspensions

Cells were harvested in the late logarithmic phase of growth ($OD_{578} = 0.25-0.27$) under anoxic conditions in a Sorvall RC-5B centrifuge (Du Pont de Nemours, Bad Homburg, Germany) at $9000 \times g$ for 30 min at 4 °C. Manipulations were carried out in an anoxic chamber (Coy, Ann Arbor, Mich., USA) under N_2/H_2 (95:5). Gas-tight centrifuge bottles were incubated in the chamber for at least 24 h before use. After centrifugation pellets were washed in potassium phosphate or 50 mM MOPS/KOH buffer, pH 7.0, supplemented with 2 mM DTE and 5 mM $MgCl_2$, and suspended in the same buffer after a further centrifugation step. The cell density was adjusted to $OD_{578\,nm} = 10$. Experiments with cell suspensions were performed anoxically in 5 ml rubber-sealed Hungate tubes under N_2 or N_2/CO_2 (90:10). Samples were taken with syringes and diluted immediately in 0.1 M H_3PO_4.

Incorporation of $^{14}CO_2$ was tested with cell suspensions, too. The assay mixture contained 100 mM potassium phosphate buffer, pH 7.0, 2.75 mM hydroquinone or gentisate, 2 mM DTE, 20 mM $NaHCO_3$, 10 mM $MgCl_2$, and 0.5 mM $MnCl_2$. Experiments were started by injecting $Na_2{}^{14}CO_3$ (150 kBq × ml^{-1}, specific radioactivity 7.5 kBq × μmol^{-1}). Samples were taken with syringes and handled as described (Tschech and Fuchs 1989).

Preparation of cell extracts

Cell extracts were prepared anoxically by French pressure cell treatment at 138 MPa of fresh cells harvested as decribed above, or from frozen cells which were stored in liquid nitrogen. Alternatively, crude extract was obtained by lysozyme treatment (15 mg lysozyme per g dry cell matter plus 10 mM EDTA) for 30 min at 25 °C. Cell debris was removed by centrifugation at $15000 \times g$ for 30 min at 4 °C. Membranes were separated from cytoplasm by centrifugation at $420000 \times g$ for 30 min in an Optima TL ultracentrifuge (Beckman, Munich, Germany) and resuspended in buffer. All steps were carried out in reduced buffers (2 mM DTE) appropriate for the respective enzyme assay.

Enzyme assays

Enzyme assays were carried out under anoxic conditions at 25 °C either in tests discontinuously analyzed by high pressure liquid chromatography (HPLC), or in continuous photometric assays. The crude extract used for the assays was freshly prepared or stored in liquid nitrogen no longer than 3 days.

Acyl-CoA synthetase was assayed as described earlier (Schnell and Schink 1991). The assay mixture contained 50 mM potassium phosphate buffer, pH 7.0, with 0.5 mM CoASH, 0.5–1 mM substrate, 10 mM $MgCl_2$ and 1–2 mM ATP. In addition, a photometric assay was performed measuring the AMP formed in the CoA ligase reaction by a coupled enzyme assay (Geissler et al. 1988).

Carboxylation of hydroquinone was tested following the exchange of ^{14}C of [^{14}C]-carbonate into the carboxyl group of gentisate or other aromatic acids in a discontinuous assay (Tschech and Fuchs 1989).

Enzymes catalyzing a *reductive dehydroxylation* of aromatic CoA-esters were assayed discontinuously by quantification of both substrate and product by HPLC. Tests were performed in 100 mM potassium phosphate or 50 mM HEPES buffer, pH 7.0, with 2 mM DTE, 5 mM $MgCl_2$, and 0.1–1 mM of electron donor. In some cases, reduced viologens were prepared with 10 mM formate as electron donor using the formate dehydrogenase present in the cell extract. Reduction of 2-hydroxybenzoyl-CoA and 3-hydroxybenzoyl-CoA was tested by adding 0.5 mM substrate to a test buffer with a suitable electron donor. Gentisyl-CoA reduction was assayed in a combined assay including the CoA ligase reaction to generate gentisyl-CoA, or by adding purified, enzymatically synthetisized gentisyl-CoA to the assay mixture. In addition, gentisyl-CoA reductase was measured photometrically following the oxidation of reduced benzyl viologen (0.15 mM) at 578 nm. The assay mixture contained 0.3 mM gentisyl-CoA, and was started by adding either cell extract or substrate. The reaction products were analyzed by HPLC.

Benzoyl-CoA : acceptor CoA transferase was tested with benzoyl-CoA (0.5–2.5 mM) as CoA-donor and gentisate or acetate (each 0.5–10 mM) as acceptor in 50 mM potassium phosphate buffer, pH 7.0, with 2 mM DTE. Changes in the concentrations of CoA esters were quantified by HPLC.

Glutaryl-CoA dehydrogenase (EC 1.3.99.7) was assayed after Stams et al. (1984). The assay mixture contained 50 mM potassium phosphate buffer, pH 7.2, 2 mM DTE, 1 mM $K_3Fe(CN)_6$ ($\varepsilon_{420} = 0.9$ mM$^{-1} \times$ cm^{-1}), 0.1 mM phenazine-methosulfate, and 0.5 mM glutaryl-CoA.

Glutaconyl-CoA decarboxylase (EC 4.1.1.70) was assayed after Buckel (1986). The assay mixture contained 100 mM Tris-HCl, pH 7.5, 2 mM DTE, 0.2 mM CoA, 0.1 mM acetyl phosphate, and 1 mM NAD. Auxiliary enzymes from *Acidaminococcus fermentans* were provided by Prof. Dr. W. Buckel, Marburg, Germany (Buckel 1986). The reaction was followed at 365 nm upon addition of 1 mM glutaconate.

Crotonase (EC 4.2.1.17) was measured following the decrease of crotonyl-CoA at 263 nm (Moskowitz and Merrick 1969).

3-Hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) was measured following NADH oxidation at 365 nm. The assay mixture contained 100 mM potassium phosphate buffer pH 7.0, 0.3 mM acetoacetyl-CoA, and 0.3 mM NADH (Bergmeyer 1983).

β-Ketothiolase (EC 2.3.1.16) was measured by a standard method (Lynen and Ochoa 1953; Stern 1956).

Phosphotransacetylase (EC 2.3.1.8) and *acetate kinase* (EC 2.7.2.1) were assayed after Bergmeyer (1983). *Benzoate kinase* was measured the same way, with 100 mM benzoate as substrate.

Carbon monoxide dehydrogenase (EC 1.2.99.2) was measured recording benzyl viologen reduction upon addition of carbon monoxide at 578 nm (Dickert and Thauer 1978). *Hydrogenase* (EC 1.18.99.1) and *formate dehydrogenase* (EC 1.2.1.2) were assayed in the same way with hydrogen or 10 mM formate as electron donor.

Protein was quantified as described by Bradford (1976), with bovine serum albumin as standard.

Analytical methods

Aromatic compounds, CoASH, CoA esters, ATP, ADP, and AMP were analyzed and quantified by HPLC (Brune und Schink 1990). Separation was performed with an Ultrasphere-ODS column (4.6 × 150 mm, Beckman Instruments, München, Germany) and gradients or mixtures of 100 mM ammonium phosphate buffer (pH 2.6) and methanol as the mobile phase. Peaks were analyzed by a computer program (System Gold, Beckman Instruments) and quantified by comparison with internal and external standards. Aromatic CoA esters were identified through their retention times and UV spectra at various pH values (2.5, 7 and 11) after separation by HPLC (Webster et al. 1974).

^{14}C-Benzoate was analyzed by HPLC, too. In addition to the UV detector, a RAMONA-5 radioactivity HPLC monitor analyzer (Raytest, Straubenhardt, Germany) was used. Detection of radioactivity was performed with a glass scintillator flow cell (net volume 400 μl) and 1-^{14}C-benzoate as standard.

Acetate and hydrogen were analyzed by gas chromatography (Platen and Schink 1989).

Chemicals

2-Hydroxybenzoyl-CoA, 3-hydroxybenzoyl-CoA, and gentisyl-CoA were synthesized from CoASH and the corresponding anhydrides (Simon and Shemin 1953; Merkel et al. 1989). In addition, gentisyl-CoA was prepared enzymatically using crude cell extract of strain HQGöl in a CoA ligase reaction by incubation for 30 min. The reaction was stopped by centrifugation at $3000 \times g$ in a centriprep concentrator (Amicon, Witten, Germany) for 60 min. The filtrate contained 0.3–0.45 mM gentisyl-CoA which was purified by HPLC.

All other chemicals were obtained from Alfa products, Karlsruhe, Germany: Boehringer, Mannheim, Germany; Fluka, Neu-Ulm, Germany; Merck, Darmstadt, Germany; Serva, Heidelberg, Germany; and Sigma, Deisenhofen, Germany.

Results

Dependence of hydroquinone fermentation on CO_2

Strain HQGöl converted hydroquinone to acetate and benzoate without significant production of hydrogen. During this fermentation a phenolic compound is converted to an aromatic acid, which requires carboxylation and reduction of the substrate molecule. The influence of sodium bicarbonate (50 mM) on the rate of hydroquinone and gentisate degradation was tested in experiments with dense cell suspensions. The degradation rate of the carboxylated derivative gentisate was exactly the same in the presence or absence of bicarbonate. In contrast, the hydroquinone concentration in bicarbonate-free assays did not decrease at all for more than 2 h, and thereafter did so only slowly. In the presence of bicarbonate, degradation followed linear kinetics (Fig. 1a, b).

Fixation of $^{14}CO_2$ in fermentation products

Hydroquinone and gentisate (each 2.75 mM) were fermented by dense cell suspensions to form up to 0.9 mM benzoate with either substrate. During fermentation in the presence of [^{14}C]-Na_2CO_3 formation of benzoate and increase of ^{14}C-label in the fermentation products were monitored by HPLC. Unfortunately, the preparation procedure stripped acid-volatile products from the reaction mixture, and therefore acetate as a further labelled product could not be quantified. With hydroquinone (2.75 mM) as substrate, the ^{14}C-label in benzoate increased parallel to benzoate formation (Fig. 2a). Since the specific radioactivity was 7.5 kBq per µmol of total available CO_2 from HCO_3^-, 6.75 kBq was expected in the 0.9 mM benzoate formed. We measured 6.95 kBq. Incorporation of ^{14}C-label into benzoate was much lower with gentisate as substrate but a low time-linear ^{14}C incorporation was observed as well (Fig. 2b).

In assays with crude extracts of hydroquinone-grown cells, a carboxylating enzyme could not be detected, neither by HPLC measurement of reaction products nor by measuring ^{14}C incorporation into acid-stable products

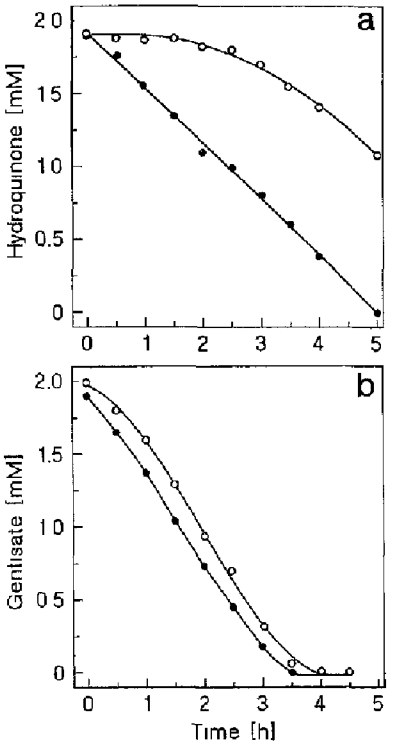

Fig. 1. Degradation of **a** hydroquinone and **b** gentisate by dense cell suspensions in the absence of CO_2 (○) and with the addition of 50 mM $NaHCO_3$ (●)

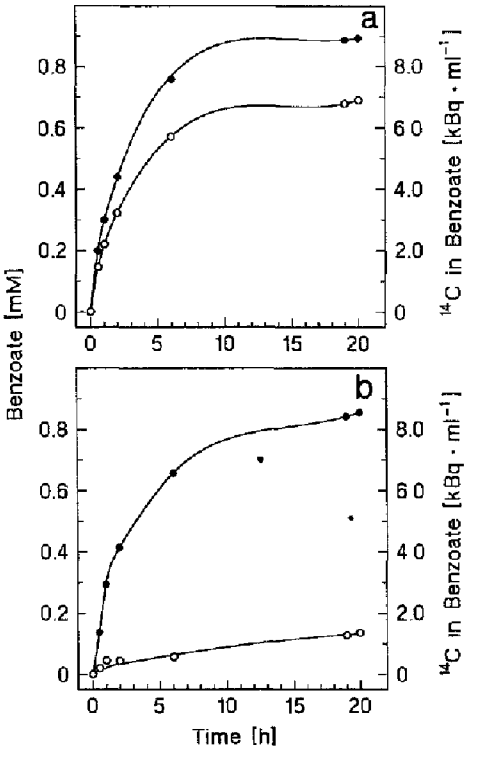

Fig. 2. Formation of ^{14}C-benzoate during fermentation of **a** hydroquinone and **b** gentisate by cell suspensions of strain HQGöl. Symbols: (●) benzoate, (○) ^{14}C label in benzoate

from hydroquinone and [^{14}C]-Na_2CO_3. Cofactors such as biotin, thiamin pyrophosphate, pyridoxal phosphate, pyridoxamine hydrochloride, pyridoxal hydrochloride, or vitamin B_6 (50–100 µM each) had no effect. No measurable exchange between the carboxyl group of gentisate and $^{14}CO_2$ was catalyzed by cell-free extract either. The protein concentration in these experiments was 0.1–2.5 mg × ml^{-1}.

Acyl-CoA ligase activity

Several aromatic acids were activated to the corresponding CoA-esters by extracts of hydroquinone-grown cells of strain HQGö1. Gentisate was converted to gentisyl-CoA in a reaction which was linear with time only during the first 3–4 min. Besides gentisate, also benzoate was activated at high specific activities (Table 1). In contrast, salicylate and 3-hydroxybenzoate were converted only with comparably low activity. AMP rather than ADP was formed from ATP. After ATP or CoASH in the assay mixture was used up the concentration of the product decreased at a rate of 0.07 µmol × min^{-1} × mg protein^{-1}.

The acyl-CoA ligase was the only enzyme forming gentisyl-CoA. No evidence of a CoA transferase using either acetyl-CoA or benzoyl-CoA as CoA-donor was found.

Reduction of gentisyl-CoA

Reduction of gentisyl-CoA, salicylyl-CoA, or 3-hydroxybenzoyl-CoA by cell extract of strain HQGö1 was studied. Only gentisyl-CoA was found to be subject to reductive dehydroxylation. A time course of a discontinuous assay is shown in Fig. 3. Decrease of gentisyl-CoA started immediately upon addition of the electron donor, with a corresponding nearby stoichiometric increase in benzoyl-CoA concentration. No other aromatic CoA-ester could be detected during the reaction indicating that both hydroxyl groups were removed in one single step. Besides benzoyl viologen and methyl viologen no other electron donor tested proved suitable (Table 2). The highest specific activity was 17 nmol × min^{-1} × mg protein^{-1} measured with reduced methyl viologen as electron donor.

The gentisyl-CoA reductase activity was sensitive to oxygen. After treatment with a weak air stream for two minutes only 10–15% of the initial activity remained. Activity could not be restored by addition of a reductant.

Table 1. Specific activities of acyl-CoA synthetase with several aromatic substrates detected in crude cell extracts of strain HQGö1 grown with hydroquinone

Substrate	Specific activity [nmol × min^{-1} × mg protein^{-1}]
Benzoate	174–177
2-Hydroxybenzoate	9.4–12.3
3-Hydroxybenzoate	16–19.6
4-Hydroxybenzoate	<0.1
Gentisate	150–166

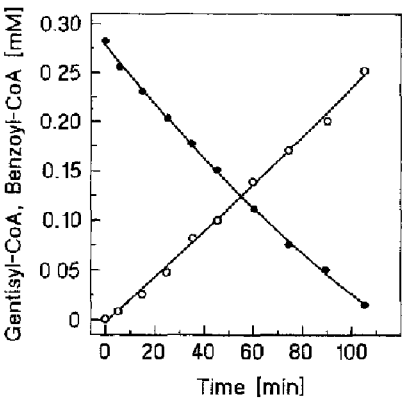

Fig. 3. Reductive dehydroxylation of gentisyl-CoA (●) to benzoyl-CoA (○) with reduced methyl viologen as electron donor (0.15 mg protein × ml^{-1})

Table 2. Specific activity of gentisyl-CoA reductase with various electron donors in cell-free extracts of strain HQGö1 grown with hydroquinone

Electron donor	Specific activity [nmol × min^{-1} × mg protein^{-1}]
Methyl viologen	9.6–17.2
Benzyl viologen	6.6–9.9
Ti(III) citrate	1–2
NADH/H$^+$ or NADPH/H$^+$	<0.1
Ferredoxin[a] *(Clostridium pasteurianum)*	<0.1

[a] Reduced by addition of dithionite

The localization of gentisyl-CoA reductase was determined with fractionated crude cell extracts prepared by lysozyme/EDTA treatment. 3-Hydroxybutyryl-CoA reductase and hydrogenase were used as marker enzymes for the soluble and membrane fractions, respectively. 3-Hydroxybutyryl-CoA dehydrogenase activity was recovered almost entirely in the soluble fraction whereas 24% of total hydrogenase activity was found to be membrane-bound. Twelve percent of the gentisyl-CoA reductase activity detected in crude extracts was measured in the membrane fraction while some 30% of the activity was lost during the fractionation process.

Enzymes of anaerobic benzoate degradation, β-oxidation, and ATP formation

Cell extracts of hydroquinone-grown cells catalyzed all reactions necessary for acetate formation from glutaryl-CoA (Table 3). Glutaryl-CoA dehydrogenase activity and glutaconyl-CoA decarboxylase activity were present as well as enzymes for β-oxidation of fatty acids. In contrast to the high activities detected for these enzymes, phosphotransacetylase and acetate kinase could be detected only at very low activities. No enzyme forming

Table 3. Enzyme activities in extracts of hydroquinone-grown cells of strain HQGö1

Enzyme	Specific activitiy [nmol × min^{-1} × mg protein^{-1}]
Glutaryl-CoA dehydrogenase	142–165
Glutaconyl-CoA decarboxylase	55–59
Crotonase	12660–13300
3-Hydroxybutyryl-CoA dehydrogenase	5450–5510
β-Ketothiolase	5110–5190
Phosphotransacetylase	45–60
Acetate kinase	12–20
Formate dehydrogenase (BV)	1450–1500
Hydrogenase (BV)	375–466
CO dehydrogenase (BV)	0

benzoate from benzoyl-CoA was detectable. No benzoyl-CoA: acetate CoA transferase, benzoyl-CoA-gentisate: CoA transferase, or benzoate kinase activity was found.

Discussion

The phenolic non-carboxylated compound hydroquinone was fermented to acetate and to the non-hydroxylated aromatic acid benzoate by the fermenting bacterium strain HQGö1. Two modification reactions had to take place at the aromatic ring, therefore: (*i*) carboxylation, and (*ii*) reductive dehydroxylation.

The first step in anaerobic hydroquinone degradation appears to be a carboxylation in *ortho* position to one hydroxyl group. The only possible product of this reaction is gentisate. Experiments with resting cells indicated that hydroquinone degradation was significantly accelerated by addition of bicarbonate. Degradation of the carboxyl derivative gentisate was not influenced by the bicarbonate addition. Further evidence of a carboxylation reaction was obtained by following the incorporation of radioactively labelled CO_2. ^{14}C-Labelled benzoate was produced in nearby stoichiometric amounts only during hydroquinone fermentation. Incorporation of radioactive label into benzoate was much slower during gentisate degradation indicating an isotope exchange by the carboxylating enzyme (Tschech and Fuchs 1989). Therefore, fixation of CO_2 occurred as a consequence of hydroquinone carboxylation to gentisate.

Since the carboxylation of hydroquinone is an endergonic process (Table 4, (3)) it was not surprising that the cell yield Y_s [g mol^{-1}] of strain HQGö1 was 3.6 g mol^{-1} higher with gentisate than with hydroquinone (Szewzyk and Schink 1989). If Y_{ATP} [g × mol ATP^{-1}] is assumed to be 10 g × mol ATP^{-1} (Stouthamer 1979) and the free energy for irreversible ATP synthesis to be 70 kJ × mol ATP^{-1} (Thauer et al. 1977), strain HQGö1 would gain 25 kJ per mol more from gentisate than from hydroquinone. This is exactly the energy amount required for hydroquinone carboxylation (Table 4, (3)).

Carboxylation of aromatic compounds has been reported for anaerobic metabolism of e.g. phenol (Lack et al. 1991), aniline (Schnell and Schink 1991), *o*-cresol (Bisaillon et al. 1991; Rudolphi et al. 1991) or *m*-cresol (Roberts et al. 1990). All these compounds are carboxylated in *para*-position to a hydroxyl or amino substituent. Hydroquinone carboxylation is the first described *ortho*-carboxylation of a phenolic compound. Unfortunately, no assay has yet been established to detect hydroquinone carboxylase in cell extracts. However, proof of the respective enzyme in the other carboxylation reactions in cell-free extracts is still missing as well.

Formation of benzoate from gentisate requires an elimination of the *ortho* and the *meta* hydroxyl group. Reductive eliminations of hydroxyl or amino groups has been observed so far only after activation of the aromatic acid to the respective CoA-ester which results in the destabilization of the mesomeric π-electron system (Schink et al. 1992). In cell extracts of strain HQGö1, an acyl-CoA synthetase activity for gentisate and for benzoate was detected at high activity. The reactions were linear with time only for a few minutes, due to the presence of an esterase activity (70 nmol × min^{-1} × mg protein^{-1}) in the crude cell extract. Since the maximum concentration of gentisyl-CoA was 0.4–0.45 mM, the ester could be prepared in quantitative amounts for further experiments.

With gentisyl-CoA as substrate, a novel enzyme activity was detected in cell-free extracts of strain HQGö1. With reduced viologen derivatives as electron donor, two hydroxyl groups were removed from gentisyl-CoA without intermediate formation of either salicylyl-CoA or 3-hydroxybenzoyl-CoA. With these monohy-

Table 4. Approximate Gibbs free energy changes of conversion reactions in anaerobic hydroquinone degradation

(1) $3\,C_6H_4(OH)_2 + CO_2 + 6\,H_2O \rightarrow 6\,CH_3COO^- + C_6H_5COO^- + 7\,H^+$
$\Delta G^{0'} = -102$ kJ (mol hydroquinone)$^{-1}$

(2) $3\,C_6H_3(OH)_2COO^- + 3\,H^+ + 6\,H_2O \rightarrow 6\,CH_3COO^- + C_6H_5COO^- + 7\,H^+ + 2\,CO_2$
$\Delta G^{0'} = -125$ kJ (mol gentisate)$^{-1}$

(3) $C_6H_4(OH)_2 + CO_2 \rightarrow C_6H_3(OH)_2COO^- + H^+$
$\Delta G^{0'} = +24$ kJ (mol hydroquinone)$^{-1}$

(4) $C_6H_3(OH)_2COO^- + 2\,NADH + H^+ \rightarrow C_6H_5COO^- + 2\,H_2O + 2\,NAD^+$
$\Delta G^{0'} = -109$ kJ (mol gentisate)$^{-1}$

Gibbs free energies were calculated after Thauer et al. 1977. The $\Delta G f^0$ value for gentisate (−578 kJ per mol) was calculated by comparison with values of the carboxylation of phenol to monohydroxybenzoates

droxy-derivatives as substrates, no dehydroxylation activity was observed. These results indicate that both hydroxyl groups are removed simultaneously in one step.

Gentisyl-CoA reductase was irreversibly inactivated by treatment of cell extract with air. Oxygen sensitivity was described also for other eliminating reductase, e.g. 4-hydroxybenzoyl-CoA reductase (Glöckler et al. 1989) and 2-aminobenzoyl-CoA reductase (Lochmeyer et al. 1992).

Reductive elimination has been observed repeatedly in anaerobic degradation of aromatic compounds. This kind of reaction was shown so far with aromatic CoA-esters substituted in *para*- and *ortho*-position (Glöckler et al. 1989; Rudolphi et al. 1991; Schnell and Schink 1991; Lochmeyer et al. 1992). Gentisyl-CoA reductase is the first enzyme described which removes hydroxyl groups in *ortho* and *meta* position. Studies on 3-hydroxybenzoate degradation allow the conclusion that this single

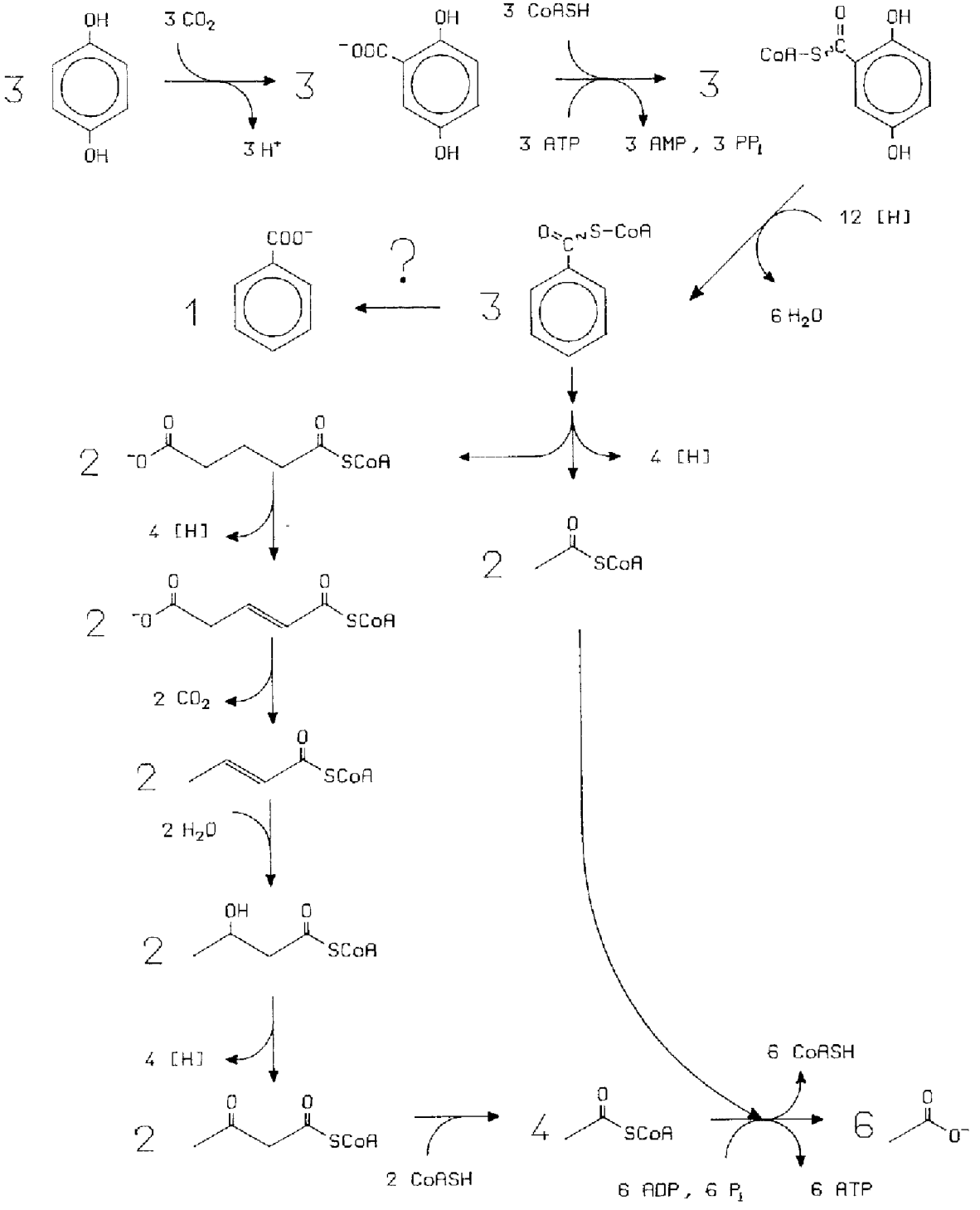

Fig. 4. Pathway of anaerobic hydroquinone degradation by the fermenting strain HQGö1 as proposed from the results in this study

meta group is also eliminated by reduction (Heising et al. 1991; Tschech and Schink 1986) but the reductase enzyme involved could not yet be detected in in vitro assays. Elimination of a hydroxyl group in *ortho*-position has never been described. Lack of reductive elimination of a single *ortho*- or *meta*-hydroxyl group, but simultaneous removal of both substituents in one single reaction indicates that this type of elimination is governed by a basically different reaction mechanism.

In an earlier paper (Tschech and Schink 1986) we speculated that reductive dehydroxylation could contribute to the energy budget of bacteria fermenting hydroxybenzoates. This could also be true for strain HQGö1, based on the free energy changes calculated for the reduction of gentisic acid to benzoic acid (Table 4, (4)). Theoretically, more than 1 mol ATP could be obtained by reducing 1 mol of gentisate to benzoate with NADH as electron source, which is the physiological electron donor regenerated by β-oxidation of fatty acids. Up to twelve percent of the gentisyl-CoA reductase activity detected in crude extracts was found in the membrane fraction. This could indicate that this enzyme activity might be associated with an energy-conserving electron transport phosphorylation. Unfortunately, a possible contribution of this reaction to the total energy budget is difficult to assess because gentisyl-CoA reductase is the only reaction accepting electrons relased during benzoyl-CoA oxidation, and is therefore indirectly involved in ATP formation via phosphotransacetylase and acetate kinase as well.

Enzymes necessary for the oxidation of benzoyl-CoA to acetate were detected in cell-free extracts. Glutaryl-CoA dehydrogenase and glutaconyl-CoA decarboxylase are key enzymes involved in anaerobic benzoate degradation, and are present in phototrophic and denitrifying bacteria growing anaerobically with aromatic compounds (Härtel et al. 1993). In cell extracts of strain HQGö1, both enzymes were measured at activities high enough for the calculated in vivo conversion rate of 54 $nmol \times min^{-1} \times mg\ protein^{-1}$, as well as enzymes of fatty acid β-oxidation, formate dehydrogenase, and hydrogenase. Phosphotransacetylase and acetate kinase were measured at the in vivo rate or below. We checked for other acetate-forming enzymes such as reversible acetyl-CoA ligase (ADP forming), but without success.

So far an enzyme forming benzoate from benzoyl-CoA could not be detected in cell-free extract. Obviously a benzoyl-CoA-acceptor: CoA transferase activity was not involved but there was also no indication of a benzoate kinase activity generating ATP via benzoyl phosphate (Dörner and Schink 1991).

On the basis of the results of the present study we propose a pathway of hydroquinone degradation by strain HQGö1 as depicted in Fig. 4. This pathway includes two novel reactions transforming the substrate to benzoyl-CoA: (*i*) a carboxylase acting in *ortho*-position to a hydroxy ring substituent, and (*ii*) a reductive dehydroxylation removing hydroxy substituents in *ortho* and *meta* position simultaneously in one reaction step.

Strain HQGö1 was deposited in the Deutsche Sammlung von Mikroorganismen (DSM), Braunschweig, Germany under No. 8423

Acknowledgements. The authors are grateful to Prof. Dr. W. Buckel, Marburg, for supplying auxiliary enzymes for the assay of glutaconyl-CoA decarboxylase. Special thanks are due to Dr. P. H. Janssen for reading the manuscript. This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

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