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Enzyme reactions involved in anaerobic cyclohexanol metabolism by a denitrifying *Pseudomonas* species

W. Dangel, A. Tschech, and G. Fuchs

Angewandte Mikrobiologie, Universität Ulm, Oberer Eselsberg, D-7900 Ulm, Federal Republic of Germany

Abstract. The enzymes involved in the anaerobic degradation of cyclohexanol were searched for in a denitrifying *Pseudomonas* species which metabolizes this alicyclic compound to CO_2 anaerobically. All postulated enzyme activities were demonstrated in vitro with sufficient specific activities. Cyclohexanol dehydrogenase catalyzes the oxidation of the substrate to cyclohexanone. Cyclohexanone dehydrogenase oxidizes cyclohexanone to 2-cyclohexenone. 2-Cyclohexenone hydratase and 3-hydroxycyclohexanone dehydrogenase convert 2-cyclohexenone via 3-hydroxycyclohexanone into 1,3-cyclohexanedione. Finally, the dione is cleaved by 1,3-cyclohexanedione hydrolase into 5-oxocaproic acid. Some kinetic and regulatory properties of these enzymes were studied.

Key words: Alicyclic compounds – Denitrification – Cyclohexanol dehydrogenase – Cyclohexanone dehydrogenase – 2-Cyclohexenone hydratase – 3-Hydroxycyclohexanone dehydrogenase – 1,3-Cyclohexanedione hydrolase – Phenol – Aromatization

The aerobic microbial metabolism of cyclohexanone and other alicyclic compounds requires molecular oxygen for a monooxygenase catalyzing oxygen insertion into the alicyclic ring; this generates lactones as suitable ring fission substrates (for a review see Trudgill 1984).

Anaerobic degradation of the stable alicyclic structure must use a different mechanism. This problem is of more general interest since aromatic compounds under anoxic conditions were proposed to be reduced to alicyclic compounds which are further metabolized (for reviews see Berry et al. 1987; Evans and Fuchs 1988). Two suggestions how cyclohexanone anaerobically is metabolized were put forward, but so far experimental evidence is lacking: direct ring fission of cyclohexanone yielding caproate (Bakker 1977); or anaerobic hydroxylation of cyclohexanone to give 1,2-cyclohexanediol and subsequent oxidation to 2-hydroxycyclohexanone that is cleaved and oxidized to adipate (Williams and Evans 1975; Balba and Evans 1980).

Recently several strains of facultatively anaerobic denitrifying bacteria were isolated which under anoxic conditions degrade cyclohexanol. Based on identified intermediates and the kinetics of their appearance a novel pathway for anaerobic cyclohexanol degradation was proposed (Dangel et al. 1988; Fig. 2). This paper provides further evidence for the suggested pathway. All required enzymes are demonstrated with specific activities sufficient to explain the growth rate.

Offprint requests to: W. Dangel

Materials and methods

All chemicals were of the highest available purity. Biochemicals were from Boehringer, Mannheim (FRG). SP 1000 + 1% H_3PO_4 on Chromosorb W-AW and 5% Carbowax 20 M on Chromosorb W-AW were from Macherey-Nagel, Düren (FRG). A μ-Bondapak C 18-column (Waters) (3.9 mm × 15 cm) was used for HPLC. Kieselgel 60 was obtained from Merck, Darmstadt (FRG). Alicyclic compounds were from Fluka AG, Buchs (Switzerland). Dihydrophloroglucinol was a gift from Andreas Brune, Tübingen (FRG). 2-Cyclohexenol, 2-hydroxycyclohexanone and 5-oxocaproic acid were from Aldrich, Steinheim (FRG).

Bacterial strains, medium, growth conditions and cultivation

The *Pseudomonas* strain was anaerobically enriched and isolated with cyclohexanol (1 mM) as sole electron donor and carbon source and with nitrate (5 mM) as electron acceptor from anaerobic sewage sludge from the municipal sewage plant in Konstanz, FRG. The strain, the growth conditions and the routine medium composition were described previously (Dangel et al. 1988; Tschech and Fuchs 1987). Stock cultures (75 ml) were grown anaerobically in a phosphate buffered (20 mM) mineral salts medium in 125 ml infusion bottles at 28° C, stored at room temperature and subcultured at weekly intervals. Anaerobic cultivation up to 1 l culture fluid was carried out in 1.25 l infusion bottles sealed with Latex rubber septa. Cultivation up to 10 l was carried out in 10 l glass bottles with rubber septa. Cultures were grown aerobically in 300 ml or 1,000 ml Erlenmeyer flasks on a rotary shaker at 150 rpm and 28° C. Under aerobic conditions nitrate was omitted and NH_4Cl was added as nitrogen source. Cells were harvested by centrifugation and immediately transferred into liquid nitrogen.

Preparation of cell extract

Cell extracts were prepared from 1–2 g cells (wet weight) which were suspended under anaerobic conditions in 2–

4 ml 100 mM Tris HCl buffer pH 7.8 containing 0.2 mg DNAase I. Cells were desintegrated by passage through a cooled French pressure cell with a pressure difference of 138 MPa. Unbroken cells and debris were removed by anaerobic centrifugation at 100,000 × *g* for 1 h.

Enzyme assays

All enzyme assays were done anaerobically at 30° C in 1.5 ml glass or quarz glass cuvettes (d = 1 cm) with rubber stoppers containing 1 ml of anaerobic reaction mixture and cell extract (0.1 – 0.5 mg of protein). Gas phase was nitrogen. Routinely the enzyme activities were followed photometrically as follows: Cyclohexanol dehydrogenase: Tris HCl pH 7.8 (100 mM), NAD^+ (0.8 mM) plus alicyclic alcohols (1 mM) (forward reaction) and NADH (0.8 mM) plus alicyclic ketones (1 mM) (back reaction), respectively; absorbance change at 365 nm was measured. Cyclohexanone dehydrogenase: Tris HCl pH 7.8 (100 mM), 2,6-dichlorophenol indophenol (100 µM), cyclohexanone (1 mM) or other cyclic ketones (1 mM); absorbance change at 578 nm was followed. 2-Cyclohexenone hydratase and 3-hydroxycyclohexanone dehydrogenase: Tris HCl pH 7.8 (100 mM), methylene blue (50 µM), 2-cyclohexenone (1 mM); absorbance change at 578 nm was determined. 1,3-Cyclohexanedione hydrolase: Tris HCl pH 7.8 (100 mM), 1,3-cyclohexanedione (50 – 100 µM), absorbance change at 276 nm was followed. In addition to the photometric assays, substrates and products of the enzymatic reactions were analyzed by various chromatographic methods (see below). Cyclohexanone oxygenase was measured as described by Donoghue and Trudgill (1975) by following the oxygen and cyclohexanone dependent NADPH oxidation at 365 nm and 30° C. Malate synthase and isocitrate lyase were measured as described by Dixon and Kornberg (1959).

Chromatographic methods

Preparation of samples. Typically enzyme reactions (1 ml assay mixture) were terminated by injection of 50 – 100 µl H_2SO_4 (50% v/v) and protein was removed by centrifugation. For thin-layer chromatography and gas-liquid chromatography the reaction products additionally were extracted with chloroform. Solutions of acidic reaction products were first methylated according to the procedure of Laanbroek et al. (1977) and then extracted with chloroform. The chloroform layer was evaporated to small volume for thin-layer chromatography (TLC).

Identification and determination of reaction products: Alicyclic compounds, the methylester of 5-oxocaproic acid and phenol were assayed by TLC, gas-liquid chromatography (GLC) and high-performance liquid chromatography (HPLC). GLC and HPLC were performed essentially as described in a previous paper (Dangel et al. 1988). A 1.8 m × 3 mm glass column was packed with either SP 1,000 + 1% H_3PO_4 (oven temperature 120° C, N_2 carrier gas 25 ml · min^{-1}) (I) or Carbowax 20 M (oven temperature 110° C, N_2 carrier gas 35 ml · min^{-1}) (II). The retention times in min were (system in parenthesis): Cyclohexanol 2.8 (I), 4.4 (II); cyclohexanone 2.0 (I), 3.1 (II); 2-cyclohexenone 3.5 (I), 5.5 (II); 1,2-cyclohexanediol (trans) 66.0 (II); 2-hydroxycyclohexanone dimer 6.9 (I), 11.4 (II); ε-caprolactone 21.5 (I), 37.3 (II); 5-oxocaproic acid methylester 9.5 (I), 17.0 (II). Reversed phase HPLC with UV detection (254 nm) was performed on a µ-Bondapak C 18-column (Waters) with isopropanol-ammoniumacetate buffer (100 mM pH 4.3) as solvent at a flow rate of 1 ml · min^{-1}. The retention times in min were: 1,3-Cyclohexanedione 3.2; 2-cyclohexenone 4.6; phenol 7.3. Thin-layer chromatography of cyclohexanone, 2-cyclohexenone and the methylester of 5-oxocaproic acid was carried out on 0.2 mm thick layers of Kieselgel 60 developed with ethylacetate/toluene (5/2, v/v) (I) or with chloroform (100%) (II). The R_F-values were (solvent in parenthesis): Cyclohexanone 0.52 (I), 0.35 (II); 2-cyclohexenone 0.43 (I), 0.26 (II); 5-oxocaproic acid methylester 0.46 (I), 0.21 (II); ε-caprolactone was not detectable. Compounds were detected by spraying with 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2 M HCl and observing the yellow derivatives thus formed. In GLC and HPLC analysis, peak areas were integrated with a Hitachi integrator.

Native PAGE and cyclohexanol dehydrogenase activity staining

Native polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli (1970) in 8% gels. Extracts (100,000 × *g* supernatant) of cyclohexanol grown cells containing cyclohexanol dehydrogenase activity (0.8 µmol · min^{-1} · mg^{-1} protein) and a standard protein calibration kit (Pharmacia, Freiburg, FRG) containing tyreoglobulin (690,000), ferritin (440,000), katalase (232,000), lactate dehydrogenase (140,000), bovine serum albumin (67,000) were applied. For activity staining the gel was transferred into 40 ml of 30 mM Na-pyrophosphate buffer pH 8.8 containing NAD^+ (40 mg), phenazine methosulphate (0.4 mg) and nitro blue tetrazolium chloride (10 mg). The staining reaction was started with cyclohexanol (1 mM), 1,2-cyclohexanediol (trans) (1 mM) or 1,3-cyclohexanediol (cis, trans) (1 mM), respectively. When bands appeared, the staining mixture was replaced by water. Standard proteins were visualized by Coomassie blue staining. Protein was determined using the Microbiuret method (Goa 1953) with bovine serum albumin as standard.

Results

It was proposed that in the *Pseudomonas* species strain K 601 anaerobic cyclohexanol (I) metabolism proceeded via the intermediates cyclohexanone (II), 2-cyclohexenone (III), 3-hydroxycyclohexanone (IV), 1,3-cyclohexanedione (V) and 5-oxocaproic acid (VI) (see Discussion, Fig. 2). The enzymes catalyzing these hypothetical reactions have been searched for in extracts from cells anaerobically grown with cyclohexanol plus nitrate.

Enzymes of anaerobic cyclohexanol metabolism

All enzymes required for the postulated pathway were detected (Table 2). As will be shown (see Discussion) their specific activities could account for the specific growth rate with this alicyclic substrate. All enzymes were detected in the 100,000 × *g* supernatant of cell extract, and enzyme activities were not affected by oxygen. Routinely, activities were determined at 30° C (growth temperature, 28° C) in 100 mM Tris HCl buffer pH 7.8. The enzyme activities were stable for several days when extracts were stored frozen at −20° C.

Table 1. Substrate specificity of cyclohexanol dehydrogenase activity in cell free extracts of *Pseudomonas* sp. K 601. The percentage refers to a specific activity of 0.8 μmol cyclohexanol oxidized · min^{-1} · mg^{-1} protein = 100% (forward reaction) and 1.9 μmol cyclohexanone reduced · min^{-1} · mg^{-1} protein = 100% (back reaction), respectively

Substrate (1 mM)	Activity (%)
With NAD (forward reaction)	
Cyclohexanol	100
2-Cyclohexenol	142
1,2-Cyclohexanediol (trans)	15
1,3-Cyclohexanediol (cis, trans)	14
1,4-Cyclohexanediol	36
1,2-Cycloheptanediol (trans)	25
1-Hexanol	7
1-Heptanol	32
With NADH (back reaction)	
Cyclohexanone	100
2-Cyclohexenone	1
Cyclopentanone	4
Cycloheptanone	10
2-Hydroxycyclohexanone (dimer)	50
1,3-Cyclohexanedione (in MES 100 mM pH 6.0)	3
1,2-Cyclohexanedione	3
1,4-Cyclohexanedione	240

Not metabolized were the following compounds. *Forward reaction*: 2-hydroxycyclohexanone (dimer); 1,3,5-trihydroxycyclohexane (cis, cis); menthol; EtOH, 1-propanol, 1-butanol, 1-pentanol. *Back reaction*: ε-caprolactone; 2-cyclopentenone; 1,3-cyclohexanedione (in Tris pH 7.8); 1,3-cyclopentanedione; 5-oxocaproate

Cyclohexanol dehydrogenase

Cells contained a NAD^+ dependent alcohol dehydrogenase which was specific for alicyclic alcohols and oxidized cyclohexanol to cyclohexanone. Enzyme activity with $NADP^+$ as cosubstrate was less than 5% of that with NAD^+. A stoichiometry of 1 mol NADH oxidized per mol cyclohexanone reduced was observed in the reverse reaction (data not shown). Under saturating substrate concentrations the cyclohexanol dehydrogenase had a specific activity (pH 7.8) of 0.8 μmol cyclohexanol oxidized · min^{-1} · mg^{-1} protein, with NADH of 1.9 μmol cyclohexanone reduced · min^{-1} · mg^{-1} protein.

In addition to cyclohexanol and cyclohexanone also alicyclic diols were oxidized and vice versa alicyclic diketones were reduced, although only the 1,3-cyclohexanediol (cis, trans) and 1,3-cyclohexanedione served as growth substrates (Table 1). 1,2-Cyclohexanediol (trans), 1,3-cyclohexanediol (cis, trans) and 1,4-cyclohexanediol were oxidized. In the back reaction a stoichiometry of 2 mol NADH oxidized per mol 2-hydroxycyclohexanone dimer or per mol 1,4-cyclohexanedione was observed; thus both keto groups were reduced. 1,2-Cyclohexanedione and 1,3-cyclohexanedione (pH 6) were reduced only very slowly; in addition 1,3-cyclohexanedione was rapidly hydrolysed (see below), and therefore a stoichiometry could not be established. The enzyme activity was not specific for the C_6 ring; cyclohexanol, 1,2-cycloheptanediol (trans) were oxidized and cyclohexanone, cyclopentanone, cycloheptanone were reduced, respectively.

Although it has not been proven several experimental results suggest that these reactions were catalyzed by one enzyme with relatively low specificity towards the alicyclic alcohols and ketones. First, native PAGE and activity staining revealed one main activity band and a weak second band when assayed with cyclohexanol, 1,2-cyclohexanediol (trans) and 1,3-cyclohexanediol (cis, trans). The minor band comigrated with lactate dehydrogenase standard (M_r 140,000); the main activity banded at somewhat higher molecular weight. Second, under saturating cyclohexanol concentration the addition of all three cyclohexanediols did not increase the rate of NAD^+ reduction. The cyclohexanol dehydrogenase activity exhibited a pronounced pH optimum at pH 10 in glycine-NaOH buffer (approximately 2.5 μmol cyclohexanol oxidized · min^{-1} · mg^{-1} protein) and was rapidly inactivated above pH 10.5. The pH dependence of the back reaction was inverse with an optimum below pH 7. The apparent K_m values of the physiological substrates were 50 μM cyclohexanol and 40 μM NAD^+.

Cyclohexanone dehydrogenase

Cell extracts catalyzed a cyclohexanone dependent reduction of 2,6-dichlorophenol indophenol (DCPIP); NAD^+, $NADP^+$, or methylene blue were not reduced. Substrate consumption and product formation were also analyzed by chromatographic methods. The reaction product chromatographed with authentic 2-cyclohexenone in three independent chromatographic systems, thin layer chromatography, gas liquid chromatography and HPLC. The specific activity of cyclohexanone dehydrogenase in Tris-HCl buffer pH 7.8 was 0.3 μmol cyclohexanone oxidized · min^{-1} mg^{-1} protein. The specific activity at the pH optimum of pH 9.6 was 0.7 μmol · min^{-1} · mg^{-1} protein. Under non-saturating DCPIP concentration (50 μM) half maximal enzyme activity was obtained with 0.16 mM cyclohexanone. The reaction product 2-cyclohexenone (1 mM) inhibited the oxidation of cyclohexanone (1 mM) almost 4-fold. In addition, 2-cyclohexenone was oxidized with DCPIP to phenol at a rate approximately 1/3 of that of cyclohexanone oxidation. Consequently, addition of 2-cyclohexenone to the cyclohexanone dehydrogenase assay diminished the DCPIP reduction rate, whereas vice versa, addition of cyclohexanone to the 2-cyclohexenone dehydrogenase assay increased the DCPIP reduction rate. The final DCPIP reduction rate in both experiments was identical and was the same as in an assay which contained both compounds from the beginning. Since phenol cannot be metabolized by this strain it is believed that 2-cyclohexenone oxidation is a side reaction of cyclohexanone dehydrogenase. This is supported by the identical alkaline pH optimum of both reactions, cyclohexanone and 2-cyclohexenone oxidation. With 1,2-cyclohexanedione (1 mM) a 4.3 times lower and with 1,4-cyclohexanedione (1 mM) a 2.4 times higher DCPIP reduction rate as compared with cyclohexanone (1 mM) was observed. 1,3-Cyclohexanedione, 2-hydroxycyclohexanone dimer, ε-caprolactone, cyclopentanone, 2-cyclopentenone, cycloheptanone or succinate were not oxidized nor were they inhibitory.

2-Cyclohexenone hydratase and 3-hydroxycyclohexanone dehydrogenase

Extracts catalyzed a 2-cyclohexenone (1 mM) dependent methylene blue reduction; NAD^+ and $NADP^+$ were not

Table 2. Specific activities (μmol alicyclic substrate consumed · min $^{-1}$ · mg^{-1} · protein) of enzymes in 100,000 × *g* supernatant involved in the degradation of cyclohexanol in the *Pseudomonas* species strain K 601

Enzyme	Specific activity in extracts of cells		
	Anaerobically grown with cyclohexanol	Anaerobically grown with succinate	Aerobically grown with cyclohexanol
Cyclohexanol dehydrogenase	0.80	0.004	0.31
Cyclohexanone dehydrogenase	0.30	0.003	0.10
2-Cyclohexenone hydratase and 3-hydroxycyclohexanone dehydrogenase	0.27	n.d.	0.18
1,3-Cyclohexanedione hydrolase	0.20	n.d.	0.068
Cyclohexanone oxygenase[a]	0.01	n.d.	0.100

[a] Cyclohexanone oxygenase was assayed as described by Donoghue and Trudgill (1975); n.d., not detectable

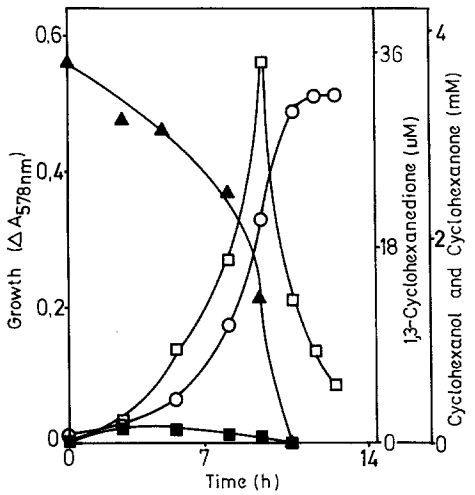

Fig. 1. Aerobic growth of the *Pseudomonas* strain K 601 with cyclohexanol (3.7 mM). NH_4Cl was added as nitrogen source. The culture grew in 100 ml mineral salts medium in a 300 ml Erlenmeyer flask on a rotary shaker at 150 rpm and 28° C. (○) Cell density, (▲) cyclohexanol, (■) cyclohexanone, (□) 1,3-cyclohexanedione

reduced. The reaction product cochromatographed on HPLC with authentic 1,3-cyclohexanedione. Also, a 1,3-cyclohexanediol (1 mM) dependent methylene blue reduction was observed provided that NAD^+ was simultaneously present as electron acceptor for cyclohexanol dehydrogenase. This effect can be explained by NAD^+ serving as electron acceptor for cyclohexanol dehydrogenase which would oxidize 1,3-cyclohexanediol to 3-hydroxycyclohexanone; 1,3-cyclohexanedione was identified as the reaction product also in this experiment. The specific activity at pH 7.8 was 0.27 μmol 2-cyclohexenone converted into 1,3-cyclohexanedione · min^{-1} · mg^{-1} protein. With methylene blue as electron acceptor phenol was not formed from 2-cyclohexenone. These results are consistent with the hypothesis that 2-cyclohexenone hydratase catalyzed the addition of water to the double bound of 2-cyclohexenone. This is followed by oxidation of the resulting hydroxyl group in 3-hydroxycyclohexanone to the keto group in 1,3-cyclohexanedione, catalyzed by 3-hydroxycyclohexanone dehydrogenase. This enzyme could use DCPIP as well as methylene blue as electron acceptor. Since 3-hydroxycyclohexanone is not commercially available both enzymes were measured in one assay starting from 2-cyclohexenone. In this assay methylene blue rather than DCPIP was used as artificial cosubstrate since DCPIP was also reduced in a 2-cyclohexenone-dependent reaction; this reaction, however, was catalyzed by cyclohexanone dehydrogenase and would result in phenol formation.

2-Cyclopentenone (1 mM) completely inhibited the 2-cyclohexenone-dependent methylene blue reduction. Since the hydratase and dehydrogenase reactions could not be followed in separate assays it remains unknown which step was inhibited. Since inhibition was immediately abolished by increasing the 2-cyclohexenone concentration, the hydratase reaction was probably affected by this analogue. During the 2-cyclohexenone-dependent methylene blue reduction catalyzed by the extracts a further reaction product was formed, 5-oxocaproic acid. It was assumed that this was a fission product of 1,3-cyclohexanedione. Therefore, the cleavage of 1,3-cyclohexanedione was investigated.

1,3-Cyclohexanedione hydrolase

Cell extracts catalyzed a hydrolytic cleavage of 1,3-cyclohexanedione. The reaction product after methylation cochromatographed in two systems, thin-layer chromatography and gas liquid chromatography, with the authentic methyl ester of 5-oxocaproic acid. It was assured that the chemical derivatization procedure did not result in cleavage of 1,3-cyclohexanedione. The 1,3-cyclohexanedione hydrolase had an apparent K_m for 1,3-cyclohexanedione of 70 μM; 0.65 μmol 1,3-cyclohexanedione were cleaved · min^{-1} · mg^{-1} protein at the optimal pH of 7.1. The enzyme activity was specific for 1.3-cyclohexanedione. 1,4-Cyclohexanedione, 1,2-cyclohexanedione, 1,3-cyclopentanedione, 1,3-dioxo-5-hydroxycyclohexane (dihydrophloroglucinol), and the 1,3-cyclohexanedione herbicide cycloxidim (a gift from Dr. Pawliczek, BASF) were not cleaved. 1,2-Cyclohexanedione (20 μM) acted as an inhibitor of 1,3-cyclohexanedione hydrolase. Inhibition was reversed by increasing the substrate concentration. Inhibitors of aldolase reactions such as EDTA or borohydride in combination with the substrate had no effect. The reaction was not stimulated by coenzyme A suggesting a hydrolytic rather than thiolytic substrate cleavage.

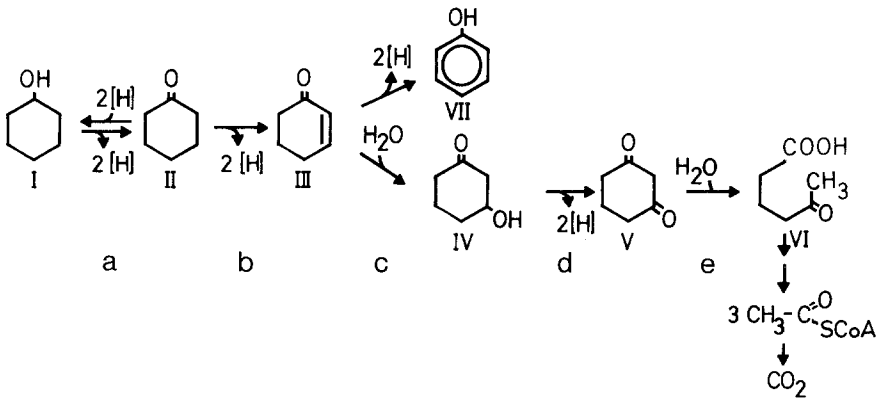

Fig. 2
Proposed pathway of anaerobic degradation of cyclohexanol by the denitrifying *Pseudomonas* spec. strain K 601. Compounds: *I* cyclohexanol, *II* cyclohexanone, *III* 2-cyclohexenone, *IV* 3-hydroxycyclohexanone, *V* 1,3-cyclohexanedione, *VI* 5-oxocaproic acid, *VII* phenol. Enzymes: (*a*) Cyclohexanol dehydrogenase; (*b*) cyclohexanone dehydrogenase; (*c*) 2-cyclohexenone hydratase; (*d*) 3-hydroxycyclohexanone dehydrogenase, (*e*) 1,3-cyclohexanedione hydrolase

Induction of the enzymes

All the assayed enzymes were induced by growth of *Pseudomonas* strain K 601 on cyclohexanol. However, the specific activities were generally three times lower in aerobically grown cells (Table 2). In cells anaerobically grown with succinate plus nitrate the activities were maximally 1% of that of cells anaerobically grown with cyclohexanol plus nitrate.

Aerobic growth with cyclohexanol

In order to test whether the proposed pathway for cyclohexanol metabolism was also used under aerobic growth conditions, the aerobic growth with cyclohexanol was studied (Fig. 1). The specific growth rate μ was approximately $5.9 \times 10^{-3} \cdot \text{min}^{-1}$ (2 h generation time). The culture supernatant was analyzed for substrate and products. Only small amounts of 1,3-cyclohexanedione (40 µM) and of cyclohexanone (130 µM) were transiently formed from cyclohexanol (3.7 mM), but no phenol or 2-cyclohexenone could be detected. The same holds true for growth with cyclohexanone in which case little cyclohexanol instead of cyclohexanone was transiently formed. Crude extracts of aerobically cyclohexanol-grown cells ($100{,}000 \times g$ supernatant) catalyzed an oxygen and cyclohexanone dependent NADPH-oxidation at pH 9.0 and 30° C, suggesting that a cyclohexanone oxygenase as described by Donoghue et al. (1976) is active under aerobic conditions. The specific activity was 0.1 µmol NADPH oxidized $\cdot \text{min}^{-1} \cdot \text{mg}^{-1} \cdot$ protein in crude extracts from aerobically grown cells. In extracts of anaerobically grown cells this activity was only moderate (Table 2).

Key enzymes of the glyoxylate bypass

It is proposed that the cleavage product of anaerobic cyclohexanol degradation, 5-oxocaproic acid, is further metabolized by β-oxidation to 3 molecules of acetyl CoA. Many, though not all, organisms when growing on fatty acids, use the glyoxylate bypass as anaplerotic reaction (Kornberg and Krebs 1957). The key enzymes of this bypass, isocitrate lyase (E.C. 4.1.3.1) and malate synthase (E.C. 4.1.3.2), were observed with sufficient specific activities in anaerobically, cyclohexanol-grown cells. The specific activity of isocitrate lyase was 0.064 µmol isocitrate cleaved to glyoxylate and succinate $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein, and of malate synthase 0.28 µmol malate formed from acetyl CoA and glyoxylate $\cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein.

Discussion

In this contribution all essential enzymes required for anaerobic metabolism of cyclohexanol by a denitrifying bacterium tentatively ascribed to the genus *Pseudomonas* have been demonstrated in vitro. The proposed pathway (Fig. 2) involves oxidation of the alcoholic function, introduction of a double bound in $\alpha-\beta$ position to the carbonyl function, addition of water resulting in a β-hydroxyl group, its oxidation to 1,3-dione, and hydrolytic cleavage of the carbon bond between the two ketone functions.

Several arguments are in favour of the suggested pathway:

1. The organism is able to grow with each of the postulated intermediates of the pathway: cyclohexanol, cyclohexanone, 2-cyclohexenone, 1,3-cyclohexanedione, 5-oxocaproic acid; 3-hydroxycyclohexanone was not available for testing. This argument does not hold for hypothetical intermediates of other suggested pathways (Bakker 1977; Williams and Evans 1975).

2. Several intermediates have been found during growth in the culture medium, and the kinetics of their appearance and consumption are fully consistent with the proposed order of reactions.

3. The enzymes are induced by cyclohexanol under anaerobic conditions and, less pronounced, under aerobic conditions.

4. In vitro the specific activities of all enzymes tested were higher than that of the overall rate of cyclohexanol metabolism by anaerobically growing cells. From the equation $-\frac{ds}{dt} = \frac{\mu}{Y} \cdot X$ a specific rate of cyclohexanol consumption can be calculated amounting to 0.06 µmol cyclohexanol $\text{min}^{-1} \cdot \text{mg}^{-1}$ protein (specific growth rate constant $\mu = 1.65 \cdot 10^{-3} \cdot \text{min}^{-1}$) (7 h generation time); molar growth yield $Y = 58$ g dry cell material formed per mol cyclohexanol metabolized (partly to phenol); the protein content of dry cell material was assumed to be 50%. In addition, the substrate concentrations for half maximal enzyme activities were in a physiological range.

5. The proposed enzymic reactions are variations – yet interesting ones – of known biochemical principles and appear to be sound in that respect.

6. The demonstration of key enzymes of the glyoxylate bypass offers an explanation how 5-oxocaproic acid is further metabolized by β-oxidation to 3 molecules of acetyl CoA.

None of the enzymes has been purified and, except for cyclohexanol dehydrogenase (E.C. 1.1.1.–), has been reported before. Several aspects of anaerobic cyclohexanol metabolism remain to be studied: So far the electron acceptor of cyclohexanone dehydrogenase and of 3-hydroxycyclohexanone dehydrogenase as well as the stereochemistry of some reactions cannot be specified. This study was also not aimed at clarifying how cyclohexanol was utilized aerobically. The transient formation of 1,3-cyclohexanedione – though in low concentrations – suggests that cyclohexanol may partly be oxidized by the suggested anaerobic pathway even under aerobic conditions; the enzymes required were shown to be oxygen-insensitive. Although the level of most enzyme activities was 2–3 times lower under aerobic conditions, the specific activities would possibly be sufficient. In addition, we observed an oxygen- and cyclohexanone-dependent NADPH oxidation by crude extract from aerobically grown cells, suggesting that a cyclohexanone oxygenase as described by Donoghue et al. (1976) is active under aerobic conditions.

Cyclohexanol dehydrogenase appears not to be specific for cyclohexane derivatives but also alicyclic C_5-, or C_7-rings as well as 1-hexanol and 1-heptanol are slowly oxidized. Similar catalytic properties as well as the alkaline pH optimum have been reported for cyclohexanol dehydrogenase (measured in the direction of alcohol oxidation) from aerobic bacteria (Donoghue and Trudgill 1975, Trower et al. 1985).

Cyclohexanone dehydrogenase, an oxidoreductase acting on the CH-CH group (E.C. 1.3.–.–), catalyzes a reaction common for many flavoproteins. The enzyme does not couple with pyridine nucleotides; it is probably responsible for the gratuitous production of the dead-end product phenol by oxidizing unspecifically also 2-cyclohexenone. 2-Cyclohexenone is known to be rather toxic (Fischman et al. 1981; Chung et al. 1988). Surprisingly, this enzyme would not react with the corresponding ketones of cyclopentane or cycloheptane. It would be interesting to study the evolutionary relationship of that enzyme and also of 2-cyclohexenone hydratase, a carbon-oxygen lyase (E.C. 4.2.1.–), to the enzymes involved in β-oxidation of fatty acids.

3-Hydroxycyclohexanone dehydrogenase, an oxidoreductase acting on the CH-OH group in β-position to the carbonyl group (E.C. 1.1.–.–) is not a common pyridine nucleotide dependent alcohol dehydrogenase. In vitro, DCPIP ($E_o' = +0.27$ V) and methylene blue ($E_o' = +0.011$ V) served as electron acceptor. A relatively positive electron acceptor, e.g., a quinone or cytochrome, may be required to ensure the complete oxidation of the acidic alcohol function in β-position to the keto function.

1,3-Cyclohexanedione hydrolase catalyzes a less common hydrolytic reaction resulting in the cleavage of a carbon-carbon bond (E.C. 3.7.–.–). This reaction appears to be irreversible in aqueous solution. The chemical reaction would require heating at 100° C in 2 M NaOH (Meek et al. 1953). The active species of 1,3-cyclohexanedione, which may exist in 4 isomeric (diketo, dienol, ketoenol, ketoenolate) forms (Meek et al. 1953), is not known. There are very few carbon-carbon hydrolases; they mostly catalyze the hydrolysis of 3-oxocarboxylic acids. One interesting parallel is the cleavage of dihydrophloroglucinol which has recently been reported (Krumholz et al. 1987). The enzyme in our strain had a different substrate specificity.

The bacterial anaerobic metabolism of cyclohexane derivatives may be of interest for several reasons. First, the anaerobic degradation of aromatic compounds most likely proceeds via reduction of the aromatic nucleus resulting in a cyclohexane or cyclohexene derivative (for reviews see Berry et al. 1987; Evans and Fuchs 1988). Principles similar to those disclosed by this work may be underlying the final steps of the anoxic aromatic metabolism. Second, several herbicides and pesticides, besides many naturally occuring compounds, are alicyclic compounds. The knowledge of their anaerobic metabolism therefore is of some practical significance. Third, as shown here the anaerobic metabolism of cyclohexanol introduces a double bound, and the hydratase reaction may lead to a stereospecific addition of water (e.g., giving the trans-hydroxy stereoisomer). Some of the enzymic reactions may turn out to be technologically useful.

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References

Bakker G (1977) Anaerobic degradation of aromatic compounds in the presence of nitrate. FEMS Microbiol Lett 1:103–108

Balba MT, Evans WC (1980) The methanogenic biodegradation of catechol by a microbial consortium: evidence for the production of phenol through cis-benzenediol. Biochem Soc Trans 8:452–453

Berry DF, Francis AJ, Bollag JM (1987) Microbial metabolism of homocyclic and heterocyclic aromatic compounds under anaerobic conditions. Microbiol Rev 51:43–59

Chung FL, Roy KR, Hecht StS (1988) A study of reactions of α, β-unsaturated carbonyl compounds with deoxyguanosine. J Org Chem 53:14–17

Dangel W, Tschech A, Fuchs G (1988) Anaerobic metabolism of cyclohexanol by denitrifying bacteria. Arch Microbiol 150: 358–362

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

Donoghue NA, Norris DB, Trudgill PW (1976) The purification and properties of cyclohexanone oxygenase from *Norcardia globerula* Cl 1 and *Acinetobacter* NCIB 9871. Eur J Biochem 63:175–192

Donoghue NA, Trudgill PW (1975) The metabolism of cyclohexanol by *Acinetobacter* NCIB 9871. Eur J Biochem 60:1–7

Evans WC, Fuchs G (1988) Anaerobic degradation of aromatic compounds. Ann Rev Microbiol 42:289–317

Fischman CM, Udey MC, Kurtz M, Wedner HJ (1981) Inhibition of lectin-induced lymphocyte activation by 2-cyclohexene-1-one: decreased intracellular glutathione inhibits an early event in the activation sequence. J Immunol 127:2237–2262

Goa J (1953) A microbiuret method for protein determination: determination of total protein in cerebrospinal fluid. Scand J Clin Lab Invest 5:128

Kornberg HL, Krebs HA (1957) Synthesis of cell constituents from C_2-units by a modified tricarboxylic acid cycle. Nature (Lond) 179:988–991

Krumholz LR, Crawford RI, Hemling ME, Bryant MP (1987) Metabolism of gallate and phloroglucinol in *Eubacterium oxidoreducens* via 3-hydroxy-5-oxohexanoate. J Bacteriol 169: 1886–1890

Laanbroek HJ, Kingma W, Veldkamp H (1977) Isolation of an aspartate-fermenting, free-living *Campylobacter* species. FEMS Microbiol Lett 1:99–102

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature (Lond) 227:680–685

Meek EG, Turnball JH, Wilson W (1953) Alicyclic compounds. III. Ultra-violet absorption acidity, and ring fission of cyclohexane-1:3 diones. J Chem Soc London, Part III:2891–2894

Trower MK, Buckland RM, Higgins R, Griffin M (1985) Isolation and characterization of a cyclohexane-metabolizing *Xanthobacter* sp. Appl Environ Microbiol 49:1282–1289

Trudgill PW (1984) Microbial degradation of the alicyclic ring. In: Gibson DT (ed) Microbial degradation of organic compounds. Dekker, New York, pp 131–175

Tschech A, Fuchs G (1987) Anaerobic degradation of phenol by pure cultures of newly isolated denitrifying pseudomonads. Arch Microbiol 148:213–217

Williams RJ, Evans WC (1975) The metabolism of benzoate by *Moraxella* species through anaerobic nitrate respiration. Biochem J 148:1–10

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