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Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA in a denitrifying, phenol-degrading *Pseudomonas* species

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The initial reactions in anaerobic degradation of phenol to CO_2 have been studied in vitro with a denitrifying *Pseudomonas* strain grown with phenol and nitrate in the absence of molecular oxygen. Phenol has been proposed to be carboxylated to 4-hydroxybenzoate [(1987) Arch. Microbiol. 148, 213–217]. 4-Hydroxybenzoate was activated to 4-hydroxybenzoyl-CoA by a coenzyme A ligase. Cell extracts also catalyzed the reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA with reduced benzyl viologen as electron donor. This enzyme, benzoyl-CoA:(acceptor) 4-oxidoreductase (hydroxylating) (EC 1.3.99.-), has not been reported before. The data suggest that phenol and 4-hydroxybenzoate are anaerobically metabolized by this strain via benzoyl-CoA.

Anaerobic degradation; Aromatic compound; Phenol; Hydroxybenzoyl-CoA, 4-; Benzoyl-CoA:(acceptor) 4-oxidoreductase (hydroxylating); (*Pseudomonas*)

1. INTRODUCTION

Degradation of phenol or benzoate by aerobic microorganisms has been studied in some detail; aerobic metabolism requires molecular oxygen for oxygenase-catalyzed attack on the stable aromatic ring structure (review [2]). However, these compounds are also completely mineralized to CO_2 in the total absence of molecular oxygen by different anaerobes (reviews [3–5]). The enzymes involved under anoxic conditions in the proposed reductive attack on the aromatic ring structure are unknown.

We are currently studying anaerobic phenol degradation with pure cultures of a denitrifying *Pseudomonas* species which is able to grow anaerobically with phenol as sole source of cell carbon and electrons. Phenol is oxidized to CO_2 and concurrently nitrate is reduced to N_2 via the intermediate nitrite. Initial studies suggested that phenol is carboxylated to 4-hydroxybenzoate [1]. 4-Hydroxybenzoate or its coenzyme A thioester is also formed anaerobically by oxidation of *p*-cresol [6], of 4-hydroxyphenyl acetate [7], and from tyrosine-derived compounds [8], among others.

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2. MATERIALS AND METHODS

Sephacryl S200 was obtained from Pharmacia (Freiburg); XAD 4, 50–100 μm, and benzoyl-CoA from Serva (Heidelberg); reversed-phase C_{18}-Lichrosorb column (25 × 0.4 cm) from Merck (Darmstadt); 10% SP 1000, 1% H_3PO_4 on Chromosorb WAW from Machery and Nagel (Düren, FRG); and coenzymes from Boehringer (Mannheim).

2.1. *Growth of bacteria*

Pseudomonas strain K 172 was grown at 30°C in the absence of molecular oxygen in mineral salts medium, with 1 mM phenol and 4 mM nitrate as sole sources of energy and cell carbon. The substrates were repeatedly fed to the batch culture [1,9]. The generation time was 20 h corresponding to a specific growth rate of 5.8×10^{-4} min^{-1}. The growth yield was 42 g cell material formed per mol phenol metabolized. Growth determination, cell harvesting and storage were as described [1,9].

2.2. *Preparation of cell extracts*

Cell extracts were prepared anaerobically in 50 mM potassium phosphate buffer (pH 7), 2 mM $MgCl_2$, 1 mM DTE by French press treatment and 100000 × *g* centrifugation (supernatant) [9].

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2.3. *4-Hydroxybenzoyl-CoA synthetase assay*

Synthetase activity was determined as in [9]. Formation of AMP was determined according to [10].

2.4. *4-Hydroxybenzoyl-CoA reductase assay*

Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA was assayed using four different methods. The anaerobic assay at 37°C was performed in an anaerobic glove box (95% N_2, 5% H_2) using the following assay mixture (1 ml): 50 mM potassium phosphate buffer (pH 7), 2.5 mM $MgCl_2$, 0.5 mM benzyl viologen, dithionite solution to give a $\Delta A_{555nm} = 2$, 10 μl extract (0.4 mg protein); the reaction was initiated by addition of 0.2 mM 4-hydroxybenzoyl-CoA. The consumption of 4-hydroxybenzoyl-CoA and production of benzoyl-CoA were followed in 4 different ways: (i) Substrate-dependent oxidation of reduced benzyl viologen was photometrically monitored at 555 nm ($\epsilon_{555nm} = 12\ mM^{-1} \cdot cm^{-1}$). (ii) Substrate consumption was determined spectrophotometrically in air-oxidized samples. 4-Hydroxybenzoyl-CoA exhibits an absorbance maximum at 330 nm at pH > 12, which is not shown by benzoate, 4-hydroxybenzoate, or benzoyl-CoA [11]. The absorbance decrease at 330 nm was partially due to hydrolysis of the coenzyme A thioester catalyzed by endogenous thioesterase. (iii) Substrate consumption and benzoyl-CoA formation were evaluated by HPLC. (iv) Formation of benzoyl-CoA was followed indirectly by determining benzoate after acid hydrolysis (4 N H_2SO_4) of the thioesters in the assay. Benzoate was identified and quantitated by GLC.

The assay was stopped either by adding H_2SO_4 or NaOH, or by air oxidation.

2.5. *Partial purification of 4-hydroxybenzoyl-CoA synthetase*

Extract from 2.3 g cells (100000 × *g* supernatant) was fractionated at 60% $(NH_4)_2SO_4$ saturation, the dissolved protein was passed over a Sephacryl S200 column (82 × 1.6 cm), the synthetase-containing fractions were combined, and the enzyme was precipitated again with $(NH_4)_2SO_4$ (60% saturation) [10].

2.6. *Preparation of 4-hydroxybenzoyl-CoA*

4-Hydroxybenzoyl-CoA was enzymatically synthesized using the partially purified synthetase and purified essentially according to [10].

2.7. *Analytical methods*

Protein was determined by the method of Bradford [12]. Coenzyme A thioesters were separated and quantitated by HPLC on a reversed-phase C_{18}-LiChrosorb column (25 × 0.4 cm) using 27% aqueous methanol as solvent (flow rate 0.7 $ml \cdot min^{-1}$). Photometric detection was at 254 nm. For spectra see [11]. Benzoate was identified and quantitated by GLC on 10% SP 1000, 1% H_3PO_4 on chromosorb WAW (1.5 m × 3 mm). Conditions: N_2 carrier gas, 30 $ml \cdot min^{-1}$; flame ionization detection; oven, 180°C; injector, 250°C; detector, 260°C.

3. RESULTS

3.1. *Activation of 4-hydroxybenzoate to 4-hydroxybenzoyl-CoA*

Cell extracts of phenol-grown cells catalyzed the activation of 4-hydroxybenzoate according to: 4-hydroxybenzoate + ATP + CoA ⟶ 4-hydroxybenzoyl-CoA + AMP + PP_i. The specific activity at pH 7 was determined as 5 nmol 4-hydroxybenzoyl-CoA formed $\cdot min^{-1} \cdot mg^{-1}$ protein; this activity was underestimated due to inhibitory compounds in the extract. The reaction was strictly dependent on the three substrates and Mg^{2+}. In a coupled enzymatic assay, AMP + ATP were converted to 2 ADP via myokinase, and ADP was determined using pyruvate kinase and lactate dehydrogenase; 2 mol NADH were oxidized per mol 4-hydroxybenzoate and CoA, respectively, indicating that the products were AMP and PP_i rather than ADP and P_i.

The 4-hydroxybenzoyl-CoA ligase was partially purified to a specific activity of 0.7 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein and was used for the enzymatic preparation of 4-hydroxybenzoyl-CoA. The oxygen-insensitive, soluble enzyme had an M_r value equal to 70000 as determined by gel filtration on Sephacryl S200. The apparent K_m value for 4-hydroxybenzoate was 30 μM.

3.2. *Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA*

Cell extracts of phenol-grown cells catalyzed the reductive dehydroxylation of 4-hydroxybenzoyl-CoA with reduced benzyl viologen (BV) (fig.1) according to: 4-hydroxybenzoyl-CoA + $2BV^+_{red}$ + $2H^+$ ⟶ benzoyl-CoA + H_2O + $2BV^{2+}_{ox}$.

4-Hydroxybenzoate was not reduced. NADH, NADPH, $FADH_2$, $FMNH_2$ were ineffective. DTE (2 mM) was inhibitory. The specific activity at pH 7 amounted to 30 nmol benzoyl-CoA formed $\cdot min^{-1} \cdot mg^{-1}$ protein. The enzyme, benzoyl-CoA:(acceptor) 4-oxidoreductase (hydroxylating), was oxygen-sensitive; half of the activity was irreversibly lost in air within a few minutes. The enzyme was recovered in the 100000 × *g* supernatant. The enzyme activity was followed by four independent methods (see section 2). The reaction rate was linearly dependent on protein concentration within the range 0–1 mg protein $\cdot ml^{-1}$ assay, and time-dependent in the range 0–1 min. The half-maximal rate of benzoyl-CoA formation was at 10 μM 4-hydroxybenzoyl-CoA in an enzyme assay where 0.17 mM reduced benzyl viologen was present. The consumption of 4-hydroxybenzoyl-CoA and formation of benzoyl-CoA in a typical assay are shown in fig.2. Due to hydrolysis of the thioester

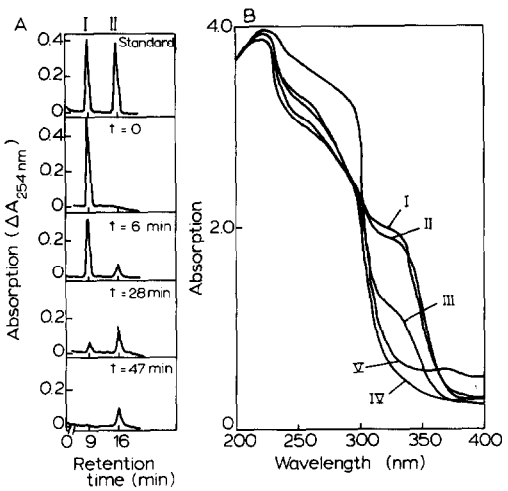

Fig.1. (A,B) Reductive dehydroxylation of 4-hydroxybenzoyl-CoA to benzoyl-CoA with reduced benzyl viologen as electron donor. The reaction was catalyzed by cell extracts of a denitrifying *Pseudomonas* sp. which was grown anaerobically with phenol and nitrate. (A) HPLC analysis of the enzyme assay at different times. Uppermost panel shows standards: 4-hydroxybenzoyl-CoA(I) and benzoyl-CoA(II). (B) Spectroscopic analysis of air-oxidized samples after different reaction times. I, $t = 0$; II, $t = 2.5$ min; III, $t = 14$ min; IV, $t = 37$ min; V, $t = 104$ min.

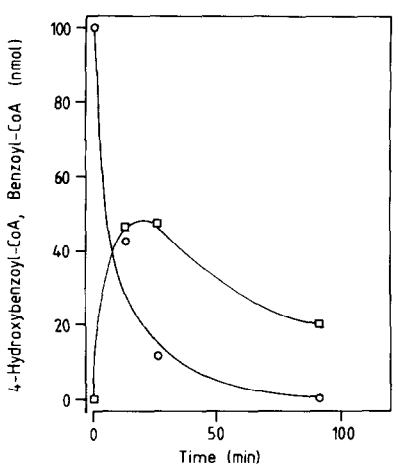

Fig.2. Reduced benzyl viologen-dependent 4-hydroxybenzoyl-CoA consumption and benzoyl-CoA formation catalyzed by extracts of a denitrifying *Pseudomonas* strain. (□) Benzoyl-CoA; (○) 4-hydroxybenzoyl-CoA. Note that the extract also contained a thioesterase activity that cleaved both CoA thioesters; this accounts for some 4-hydroxybenzoyl-CoA consumption and for most of that of benzoyl-CoA. Substrate and product were determined by HPLC using standards as reference.

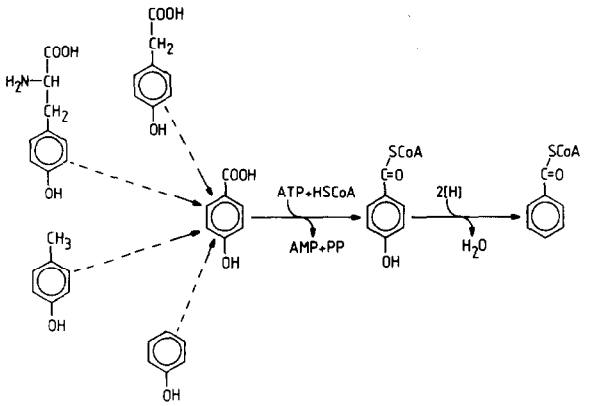

Fig.3. Proposed role of reductive dehydroxylation of 4-hydroxybenzoyl-CoA in anaerobic degradation of phenol and some other aromatic compounds.

bond, more 4-hydroxybenzoyl-CoA was consumed than benzoyl-CoA formed, and benzoyl-CoA disappeared.

4. DISCUSSION

We have described the reductive removal of an aromatic hydroxyl group by a novel enzyme tentatively named 4-hydroxybenzoyl-CoA reductase (dehydroxylating) or benzoyl-CoA:(acceptor) 4-oxidoreductase (hydroxylating). The enzyme acts on an aromatic CH–CH bond (EC 1.3.99.-), a reaction to which no direct parallel in biochemistry is known (see below). The substrate, 4-hydroxybenzoyl-CoA, is formed in a coenzyme A ligase reaction. The specific activities in phenol-grown cells and the apparent K_m values of both enzymes make them likely to play a role in anaerobic phenol and 4-hydroxybenzoate metabolism. The growing culture metabolized phenol at a rate of 27 nmol · $min^{-1} \cdot mg^{-1}$ protein which is comparable to the specific enzyme activities.

Phenol has been proposed to be carboxylated by 'phenol carboxylase', an enzyme catalyzing a biological Kolbe-Schmidt reaction [1,9]. The data suggest that phenol is carboxylated to 4-hydroxybenzoate, followed by activation of the product to 4-hydroxybenzoyl-CoA, and its reductive dehydroxylation to benzoyl-CoA. Benzoyl-CoA, a central metabolite of anaerobic degradation of many aromatic compounds, may then be reduced

to a cyclohexane carboxyl-CoA derivative [13,14]. Reductive dehydroxylation has been shown at the whole cell level with pure cultures for different hydroxylated aromatic compounds [15–17]. Phenol conversion to benzoate in bacterial enrichment cultures [18] may proceed as noted. 4-Hydroxybenzoate or 4-hydroxybenzoyl-CoA formed anaerobically from precursors other than phenol (fig.3) may also be metabolized via benzoyl-CoA. However, evidence was presented for different metabolism of 4-hydroxybenzoate and benzoate in a phototrophic bacterium [19].

So far only 2-furoyl-CoA dehydrogenase [2-furoyl-CoA:(acceptor) 5-oxidoreductase (hydroxylating) (EC 1.3.99.8] has been described [20] which catalyzes a reaction similar to reductive dehydroxylation of 4-hydroxybenzoyl-CoA. Other dehydrogenases (EC 1.5), which formally introduce a hydroxyl group from water, mostly act on the CH-NH group of donors. Among these substrates are xanthine, inosine 5′-phosphate, nicotinic, isonicotinic, 2-hydroxyisonicotinic and picolinic acids, and nicotine (for literature see [21]). Others act on a CH_2 group (EC 1.17), e.g. in *p*-cresol forming 4-hydroxybenzyl alcohol [22] and possibly in (4-hydroxy)phenyl acetate [forming (4-hydroxy)phenyl glyoxylate (Seyfried, B., Ulm, unpublished)]. An enzyme catalyzing the reductive deamination of glycine to acetate is glycine reductase [23].

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