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Differential expression of enzyme activities initiating anoxic metabolism of various aromatic compounds via benzoyl-CoA

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Abstract. The regulation of the expression of enzyme activities catalyzing initial reactions in the anoxic metabolism of various aromatic compounds was studied at the whole cell level in the denitrifying *Pseudomonas* strain K 172. The specific enzyme activities were determined after growth on six different aromatic substrates (phenol, 4-hydroxybenzoate, benzoate, *p*-cresol, phenylacetate, 4-hydroxyphenylacetate) all being proposed to be metabolized anaerobically via benzoyl-CoA. As a control cells were grown on acetate, or aerobically on benzoate. The expression of the following enzyme activities was determined:

"Phenol carboxylase", as studied by the isotope exchange between $^{14}CO_2$ and the carboxyl group of 4-hydroxybenzoate; 4-hydroxybenzoyl-CoA reductase (dehydroxylating); *p*-cresol methylhydroxylase; 4-hydroxybenzyl alcohol dehydrogenase; 4-hydroxybenzaldehyde dehydrogenase; coenzymeA ligases for the aromatic acids benzoate, 4-hydroxybenzoate, phenylacetate, and 4-hydroxyphenylacetate; phenylglyoxylate: acceptor oxidoreductase and 4-hydroxyphenylglyoxylate: acceptor oxidoreductase; aromatic alcohol and aldehyde dehydrogenases.

The formation of most active enzymes is strictly regulated; they were only induced when required, the basic activities being almost zero. The observed whole cell regulation pattern supports the postulate that the enzyme activities play a role in anoxic aromatic metabolism and that the compounds are degraded via the following intermediates: Phenol → 4-hydroxybenzoate → 4-hydroxybenzoyl-CoA → benzoyl-CoA; 4-hydroxybenzoate → 4-hydroxybenzoyl-CoA → benzoyl-CoA; benzoate → benzoyl-CoA; *p*-cresol → 4-hydroxybenzaldehyde → 4-hydroxybenzoate → 4-hydroxybenzoyl-CoA → benzoyl-CoA; phenylacetate → phenylacetyl-CoA → phenylglyoxylate → benzoyl-CoA plus CO_2; 4-hydroxyphenylacetate → 4-hydroxyphenylacetyl-CoA → 4-hydroxyphenylglyoxylate → 4-hydroxybenzoyl-CoA plus CO_2 → benzoyl-CoA.

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Key words: Aromatic compounds – Anaerobic aromatic metabolism – *Pseudomonas* K 172 – Phenol – 4-Hydroxybenzoate – *p*-Cresol – Phenylacetate – Benzoate – Coenzyme A thioester

It is well established that the initial reactions in aerobic metabolism of stabile aromatic structures generally are hydroxylations catalyzed by monooxygenases, and that ring cleavage requires oxygen for dioxygenase reactions. Only a few reactions by which hydroxyl groups are introduced from water are known (see below). In contrast, the anoxic attack of aromatic compounds is little understood (recent reviews by Berry et al. 1987; Evans and Fuchs 1988; Tschech 1989).

A variety of novel oxygen-independent reactions have recently been found changing the substitution of the aromatic nucleus, e.g. by formation of coenzyme A thioesters of aromatic acids (Ziegler et al. 1987, 1989; Geissler et al. 1988; Nozawa and Maruyama 1988; Merkel et al. 1989), carboxylations (Tschech and Fuchs 1987, 1989; Rudolphi et al. 1991; Knoll and Winter 1989), reductive dehydroxylations (Glöckler et al. 1989) or other reductive or hydrolytic substitutions (Schink 1988; Tschech 1989), alpha-oxidation of $-CH_2-COOH$ groups (Seyfried 1989; Sembiring and Winter 1989; Seyfried et al. 1991), or isomerization by transhydroxylation (Krumholz and Bryant 1988; Brune and Schink 1989).

Central intermediates of anaerobic metabolism recognized so far are benzoyl-CoA (e.g. Tschech and Fuchs 1989), possibly also 3-CH_3-benzoyl-CoA (Rudolphi et al. 1991), resorcinol (Tschech and Schink 1985; Kluge et al. 1990), and phloroglucinol (Krumholz et al. 1987; Haddock and Ferry 1989). Their further metabolism probably proceeds via ring reduction reactions although this has been shown only for resorcinol (Kluge et al. 1990) and phloroglucinol (Krumholz et al. 1987; Haddock and Ferry 1989), compounds which *per se* are easily reduced since they already have the oxidation status of a β-oxo-

compound. In vitro benzoyl-CoA reduction to an unknown product has been observed (Jürgen Koch, Werner Dangel, Georg Fuchs, unpublished results) but the exact nature of the reaction and its product(s) remains to be elucidated.

Benzoyl-CoA has been proposed to be central intermediate not only in anaerobic metabolism of benzoate but also e.g. of phenol (Tschech and Fuchs 1989), 4-hydroxybenzoate (Glöckler et al. 1989), *p*-cresol (Hopper 1978; Bossert et al. 1989; Rudolphi et al. 1991), phenylacetate, and 4-hydroxyphenylacetate (Seyfried et al. 1991). The benzoate carboxyl group thereby can be formed in three quite different ways: (*1*) by carboxylation of phenolic compounds, (*2*) by anaerobic hydroxylation of aromatic CH_3-groups para to phenolic OH-groups, or, (*3*) by anaerobic alpha-oxidation of $-CH_2-COOH$ side chains of phenylacetyl(-coenzyme A) (Fig. 1). Enzymes involved in anaerobic metabolism of these six aromatic compounds have recently been uncovered but have not yet been purified. Their biological role should be verified by studies of mutants. As an alternative investigation of the differential regulation of their activity on the transcriptional level may corroborate conclusions drawn from other type of experiments.

In this investigation the expression of a dozen of enzyme activies of anaerobic metabolism has been studied in cells grown on the six aromatic substrates mentioned above. These enzyme activities have been proposed to catalyze the initial reactions ultimately leading to benzoyl-CoA. Cells were included for comparison which were grown anaerobically on acetate, or aerobically on benzoate. The bacterial strain studied, *Pseudomonas* sp. K 172, is able to grow on a variety of different mononuclear aromatic compound with nitrate as the electron acceptor (Tschech and Fuchs 1987). It is able also to grow aerobically on most though not all of these aromatic compounds. The regulatory properties of the putative enzymes of anaerobic aromatic metabolism adds further substantial evidence for their biological role and thus for the proposed intermediates.

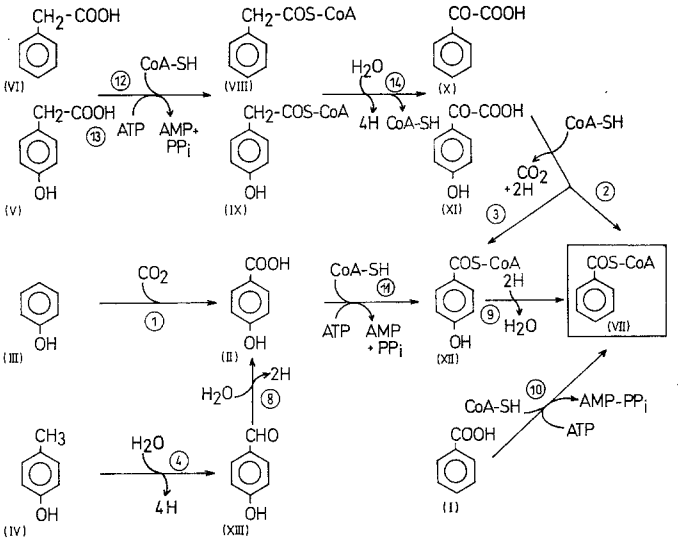

Fig. 1. Proposed initial reactions involved in *Pseudomonas* K 172 in anaerobic metabolism of benzoate (*I*), 4-hydroxybenzoate (*II*), phenol (*III*), *p*-cresol (*IV*), 4-hydroxyphenylacetate (*V*), and phenylacetate (*VI*). These and other aromatic compounds are sugested to be metabolized via benzoyl-CoA (*VII*). Phenylacetyl-CoA (*VIII*), 4-hydroxyphenylacetyl-CoA (*IX*), phenylglyoxylate (*X*), 4-hydroxyphenylglyoxylate (*XI*), 4-hydroxybenzoyl-CoA (*XII*) and 4-hydroxybenzaldehyde (*XIII*) are intermediates. The anoxic alpha-oxidation of the carboxymethyl group in phenylacetyl-CoA or 4-hydroxyphenylacetyl-CoA (reaction 14) has not been demonstrated in vitro. However, whole cells were shown to produce substantial amounts of phenylglyoxylate and 4-hydroxyphenylglyoxylate, respectively, when grown on phenylacetate and 4-hydroxyphenylacetate, respectively. For reactions 1–13, see Table 1 and the text. Enzymes: (*1*) "Phenol carboxylase" system, (*2*) and (*3*) (4-hydroxy)-phenylglyoxylate:acceptor oxidoreductase (decarboxylating), (*4*) *p*-cresol methylhydroxylase, (*8*) 4-hydroxybenzaldhyde dehydrogenase, (*9*) 4-hydroxybenzoyl-coenzyme A reductase (dehydroxylating), (*10*) benzoyl-coenzyme A synthetase (AMP-forming), (*11*) 4-hydroxybenzoyl-coenzyme A synthetase (AMP-forming), (*12*) and (*13*) (4-hydroxy)phenylacetyl-coenzyme A synthetase (AMP-forming), (*14*) hypothetical (4-hydroxy)phenylacetyl-coenzyme A dehydrogenase system (hydroxylating)

Materials and methods

Materials

Chemicals were reagent grade. Biochemicals were from Boehringer (Mannheim, FRG). [^{14}C]Na_2CO_3 (specific radioactivity 1.85 GBq × mmol^{-1}) was purchased from Amersham Buchler (Braunschweig, FRG). Gases were from Linde (Höllriegelskreuth, FRG) or Nüssle (Ulm-Söflingen, FRG). The *Pseudomonas* strain K 172 was that described by Tschech and Fuchs (1987).

Bacterial growth

Pseudomonas K 172 was grown anaerobically on mineral salt medium at 28°C, with different organic substrates as carbon sources and with nitrate (20 mM) as the electron acceptor (Tschech and Fuchs 1987). The inoculum was pregrown at least once under the conditions of the experiment. The substrates tested were: Phenol (2 mM) plus CO_2; 4-OH-benzoate (5 mM); benzoate (5 mM); phenylacetate (5 mM); 4-OH-phenylacetate (5 mM); *p*-cresol (2 mM); acetate (20 mM). Phenol and *p*-cresol were repeatedly fed. One batch of cells was grown aerobically with benzoate (5 mM) as carbon source. Cells were harvested by centrifugation and stored frozen in liquid nitrogen.

Cell extracts and ammonium sulfate precipitation

All steps were done anaerobically. Cells (3 g wet weight) were washed once in 50 mM imidazole/HCl buffer (pH 7.0) containing 2 mM dithioerythritol, suspended in 3 ml of the same buffer to which DNAse I was added, and passed through a French press at 137 MPa. Extract was centrifuged at 4°C for 1 h at 100,000 *g*. The supernatant was immediately used or stored frozen at −20°C for maximally one week under N_2 head space. Ammonium sulfate precipitations (60% saturation) were done at 4°C using saturated ammonium sulfate solution pH 7; for assay of coenzyme A ligases extracts may better first be precipitated at 30% saturation. The precipitate obtained at 60% saturation was dissolved in 50 mM potassium phosphate buffer pH 7.

Enzyme assays

The enzyme activities were determined at 30°C normally under strictly anaerobic conditions under a N_2 head space, except for

coenzyme A ligases and pyridine nucleotide-dependent dehydrogenases. Protein was determined by the biuret method (Goa 1953).

Coenzyme A ligases

The ATP-, coenzyme A-, and aromatic substrate-dependent formation of acyl-CoA and AMP (plus presumably pyrophosphate) was followed spectrophotometrically at 365 nm by coupling the AMP formation via myokinase, pyruvate kinase, and lactate dehydrogenase reaction to the oxidation of 2 mol NADH per mol aromatic acid activated:

Aromatic acid + Mg^{2+}-ATP → aromatic acyl-CoA + AMP + PP_i
AMP + ATP → 2 ADP
2 ADP + 2 phosphoenolpyruvate → 2 ATP + 2 pyruvate
2 pyruvate + 2 NADH + 2 H^+ → 2 lactate + 2 NAD^+.

The ammonium sulfate precipitated protein fraction was used since cell extracts contained soluble inhibitory compound(s) the nature of which is unknown which precipitate at 30% ammonium sulfate saturation. The aerobic assay mixture (1 ml) contained: 100 mM Tris/HCl buffer pH 7.8; 2 mM DTE; 30 mM KCl; 20 mM $MgCl_2$; 1 mM ATP; 1 mM phosphoenolpyruvate; 0.4 mM NADH; 5 mM aromatic substrate; 17 nkat myokinase; 17 nkat pyruvate kinase; 25 nkat lactate dehydrogenase; 10 µl extract (0.2–0.4 mg protein).

4-OH-Benzoyl-CoA reductase (dehydroxylating)

The 4-OH-benzoyl-CoA-dependent oxidation of reduced benzyl viologen was followed spectrophotometrically at 578 nM:

4-Hydroxybenzoyl-CoA + 2 benzyl $viologen_{reduced}$ → benzoyl-CoA + H_2O + 2 benzyl $viologen_{oxidized}$.

The anaerobic assay mixture (1 ml) contained: 50 mM potassium phosphate buffer pH 7; 0.2 mM 4-OH-benzoyl-CoA; sodium dithionite to reduce the viologen dye until a A_{578} of approximately 2 was reached; 10 µl extract (0.2–0.4 mg protein). Since the extracts sometimes contained thioesterase activity, the substrate could be reformed by endogenous coenzyme A ligase when 2 mM ATP, 2.5 mM $MgCl_2$, and 0.2 mM coenzyme A was included. This was important when the stoichiometry between 4-OH-benzoyl-CoA added to benzyl viologen reduced was to be determined.

(4-OH)-Phenylglyoxylate: acceptor oxidoreductase

Assay a. The (4-OH)-phenylglyoxylate- and coenzyme A-dependent reduction of benzyl viologen was followed spectrophotometrically at 578 nm:

Phenylglyoxylate + coenzyme A + 2 benzyl $viologen_{oxidized}$ → benzoyl-CoA + CO_2 + 2 benzyl $viologen_{reduced}$.

Correspondingly 4-OH-phenylglyoxylate was used. The anaerobic assay mixture (1 ml) contained: 200 mM potassium phosphate buffer pH 7.8; 0.8 mM coenzyme A; 1 mM methyl viologen; 0.1 mM phenylglyoxylate or 4-OH-phenylglyoxylate; 10 µl extract (0.2–0.4 mg protein).

Assay b. The phenylglyoxylate- or 4-OH-phenylglyoxylate-dependent fixation of ^{14}C from $^{14}CO_2$ into acid-stabile compounds (phenylglyoxylate or 4-OH-phenylglyoxylate), a type of isotope exchange reaction catalyzed by 2-oxoacid:acceptor oxidoreductases (2-oxoacid synthases), was followed:

Phenylglyoxylate + $^{14}CO_2$ → [carboxy-^{14}C]phenylglyoxylate + $^{12}CO_2$.

Correspondingly 4-OH-phenylglyoxylate was used. The anaerobic assay mixture (1 ml) contained: 50 mM imidazole/HCl buffer pH 7; 2 mM phenylglyoxylate or 4-OH-phenylglyoxylate; 5 mM $MgCl_2$; 0.1 mM coenzyme A; 20 µl extract (0.4–0.8 mg protein); 25 µmol [^{14}C]$NaHCO_3$ added per assay (14 kBq; specific radioactivity 560 Bq × µmol^{-1} total 'CO_2'; when the symbol 'CO_2' is used throughout this paper, no distinction is made between gaseous CO_2 or dissolved CO_2, HCO_3^- or $CO_3^=$). The determination of acid-stabile ^{14}C was as described for "phenol carboxylase".

"Phenol carboxylase" studied by $^{14}CO_2$:4-OH-benzoate isotope exchange reaction

The 4-hydroxybenzoate-, Mn^{2+}-, and K^+-dependent ^{14}C incorporation from $^{14}CO_2$ into acid-stabile 4-OH-benzoate was determined:

4-Hydroxybenzoate + $^{14}CO_2$ → [carboxy-^{14}C]4-hydroxybenzoate + $^{12}CO_2$.

The anaerobic standard assay mixture (2 ml) contained: 50 mM imidazol/HCl buffer pH 7.0, 0.5 mM $MnCl_2$, 2 mM 4-OH-benzoate, 20 mM KCl, 50 µmol 'CO_2' (100 µl 0.5 M $NaHCO_3$ added per assay), 12.5 kBq ^{14}'CO_2' (final specific radioactivity 250 Bq × µmol^{-1} 'CO_2'), 40 µl extract (0.8–1.6 mg protein); head space was 95% N_2 plus 5% H_2 (Formiergas). From the amount of ^{14}C fixed and the initial specific radioactivity of ^{14}'CO_2' the molar amount of CO_2 exchanged was calculated. The materials used were preincubated over night in an anaerobic chamber. The assay was performed in stoppered glass vials containing 2 ml assay mixture, leaving 3.8 ml gas space. The complete assay but without ^{14}'CO_2' was prepared at 0°C in an anaerobic chamber. The ^{14}'CO_2' stock solution was prepared by adding 25 µl of $Na_2{}^{14}CO_3$ solution (375 kBq; 1.85 GBq × mmol^{-1}) to 3 ml anaerobic 1 mM NaOH solution. After preincubation of the vials at 30°C for 10 min in a shaking water bath outside the anaerobic chamber, 100 µl each of the ^{14}'CO_2' stock solution was added to the individual 2 ml assay mixtures to start the isotope exchange reaction. Samples (200 µl) were taken after 7 min and 14 min incubation with ^{14}'CO_2', and pipetted into Eppendorf caps containing 20 µl 3 M perchloric acid. The precipitate was centrifuged down, and from the clear supernatant 150 µl were transferred into scintillation vials containing 150 µl 1 M $KHCO_3$ solution. 60 µl 10 M formic acid was added, and the solution was gassed with CO_2 gas for 10 min under the hood in order to remove $^{14}CO_2$. Then, 4 ml scintillation cocktail was added.

p-Cresol methylhydroxylase

The *p*-cresol-dependent reduction of 2,6-dichlorophenolindophenol plus phenazine methosulfate was determined spectrophotometrically at 578 nm (Hopper 1976):

p-Cresol + H_2O + 2 $dichlorophenolindophenol_{oxidized}$ → 4-hydroxybenzaldehyde + 2 $dichlorophenolindophenol_{reduced}$.

The anaerobic assay mixture (1 ml) contained: 50 mM potassium phosphate pH 8; 0.15 mM dichlorophenolindophenol; 0.3 mM phenazine methosulfate; 0.5 mM *p*-cresol; 10 µl extract (0.1–0.2 mg protein).

Benzyl alcohol dehydrogenase

The benzyl alcohol-dependent NAD^+ reduction or the benzaldehyde-dependent NADH oxidation were followed spectrophotometrically at 365 nm:

benzyl alcohol + NAD^+ → benzaldehyde + NADH + H^+.

The aerobic assay mixture (1 ml) contained: 50 mM potassium phosphate buffer pH 8; 0.2 mM NAD^+ or NADH; 1 mM benzyl alcohol or benzaldehyde; 10 µl extract (0.1–0.2 mg protein). Correspondingly 4-hydroxybenzyl alcohol was tested.

Benzaldehyde dehydrogenase

The benzaldehyde-dependent or 4-hydroxybenzaldehyde-dependent reduction of $NADP^+$ was followed spectrophotometrically at 365 nm:

Benzaldehyde + $NADP^+$ + H_2O → benzoate$^-$ + 2 H^+ + NADPH.

The aerobic assay mixture (1 ml) contained: 50 mM potassium phosphate buffer pH 7; 0.2 mM $NADP^+$; 1 mM benzaldehyde or 4-OH-benzaldehyde; 10 µl extract (0.1–0.2 mg protein). Correspondingly 4-hydroxybenzaldehyde was tested.

4-Hydroxybenzaldehyde dehydrogenase

The benzaldehyde- or 4-hydroxybenzaldehyde-dependent reduction of dichlorophenolindophenol in the presence of NAD^+ was measured at pH 9 as described by Chalmers and Fewson (1989).

Synthesis of 4-OH-benzoyl-CoA. 4-OH-benzoyl-CoA was synthesized enzymatically and purified as described before (Glöckler et al. 1989).

Determinations. ^{14}C was determined in a liquid scintillation counter using external standardization. Bacterial growth was followed by determining the apparent absorption increase at 578 nm, corrected for the absorption of the medium; samples with $A_{578\,nm} > 0.5$ were diluted with 100 mM phosphate buffer pH 7. Protein was determined by the Biuret method (Goa 1953).

Results and discussion

Growth of Pseudomonas K 172 with different substrates, aerobically or anaerobically

The denitrifying *Pseudomonas* sp. strain K 172 was grown anaerobically at 28° C with nitrate as the electron acceptor on the same mineral salt medium which differed only in the organic substrate. Nitrate was reduced to N_2 via nitrite which transiently and stoichiometrically accumulated in the growth medium resulting in a biphasic growth curve. The aromatic substrates phenol, 4-hydroxybenzoate, benzoate, *p*-cresol, phenylacetate, and 4-hydroxyphenylacetate, as well as acetate were completely oxidized to CO_2 under the anoxic conditions tested. It should be noted, however, that the strain is able to oxidize e.g. benzoate both under oxic or anoxic conditions whereas phenol was metabolized only anaerobically. For comparison in a separate experiment cells were therefore grown also aerobically on benzoate as sole carbon source. The generation time depended on the substrate and was 18 h with phenol (Tschech and Fuchs 1987), corresponding to a specific growth rate of 6.4×10^{-4} min^{-1}.

A molar growth yield of 42 g cells (dry matter) formed per mol phenol metabolized has been reported (Tschech and Fuchs 1987). Based on the assumption that 1 g cell dry matter X contains approximately 600 mg protein, the specific rate of substrate S (phenol) metabolism ($-dS/dt$) is 25 nmol phenol metabolized min^{-1} mg^{-1} total cell protein. This value was calculated from the equation $-dS/dt = (\mu/Y) \cdot X$ which correlates substrate consumption per time unit with the specific growth rate µ, the molar growth yield Y, and the amount of cells X growing on that substrate. Enzymes involved in the anaerobic aromatic pathways should exhibit this or a higher specific activity. Cells were harvested before they reached stationary growth phase and were stored frozen in liquid nitrogen.

Specific activities of enzymes of anaerobic aromatic metabolism in cells grown with different substrates, aerobically or anaerobically

Cell extracts were analyzed for enzymes catalyzing initial reactions of anaerobic aromatic metabolism which would finally lead to benzoyl-CoA, the proposed common intermediate of the substrates tested. The pathways supported by our previous investigations are presented in Fig. 1. The results of the enzyme investigations are shown in Table 1. Most enzymes required according to this proposal were present at specific activities as high as 25 nmol · min^{-1} · mg^{-1} cell protein or higher and therefore can account for growth on these substrates via the proposed reactions. In addition all enzyme activities appear to be regulated at the transcription level probably by induction, and the regulation pattern fits well to the proposed role of the individual enzymes. Several remarkable features were observed:

"Phenol carboxylase" activity, which was measured as $^{14}CO_2$:4-hydroxybenzoate isotope exchange reaction, was only present when cells were grown on phenol. The enzyme is thought to carboxylate phenol to 4-hydroxybenzoate, and the isotope exchange reaction represents a freely reversible partial reaction of this biological Kolbe-Schmitt carboxylation (A. Lack, unpublished). Virtually no activity could be measured in cells grown on other aromatic substrates (for *o*-cresol carboxylation see below). This is a strong further biological argument for the specific role of "phenol carboxylase" in phenol metabolism. Phenol appears to induce the "phenol carboxylase" system only. The enzymes required for activation of 4-hydroxybenzoate (4-hydroxybenzoyl-coenzyme A synthetase) and reductive conversion to benzoyl-CoA (4-hydroxybenzoyl-CoA reductase) appear to be induced sequentially, probably by the carboxylation product (4-hydroxybenzoate) and by its coenzyme A thioester, respectively. This follows from the fact that cells grown with phenol contained "phenol carboxylase", 4-hydroxybenzoate coenzyme A ligase, and 4-hydroxybenzoyl-CoA reductase; cells grown on 4-hydroxybenzoate contained the CoA ligase and the reductase; and cells grown on compounds leading to 4-hydroxybenzoyl-CoA (4-hydroxyphenylacetate) (see below) only contained the reductase. Carboxylation may not be restricted to phenol, but similarly *o*-cresol (Rudolphi et al. 1991) may be carboxylated and *o*-cresol may act as inducer for the same or a similar enzyme system. Possibly other phenolic compounds such as *m*-cresol (Roberts et al. 1990) or even aniline (B. Schink, Tübingen, personal communication) are anaerobically degraded via initial carboxylation.

Table 1. Specific activities of enzymes of anoxic aromatic metabolism in extracts of *Pseudomonas* K 172 after growth on different substrates. The aromatic compounds used as growth substrates all have been proposed to be metabolized anaerobically via benzoyl CoA as the central intermediate. A scheme which presents the enzymes (represented by the numbers of this table) and their carbon substrates and products is given in Fig. 1. (–) = no significant activity was seen with cell extract; in general this means the activity was $\leq 2\%$ of the maximal value reported. For assay conditions see Materials and methods. n.d. = not determined. DCPIP = Dichlorophenolindophenol, PMS = Phenazine methosulfate

No	Enzyme activity	Specific enzyme activity [nmol · min^{-1} · mg^{-1} protein] in extracts of *Pseudomonas* K 172 grow on							
		Benzoate	Benzoate (+ O_2)	4-OH-Benzoate	Phenol	*p*-Cresol	4-OH-Phenyl-acetate	Phenyl-acetate	Acetate
1	"Phenol carboxylase" ($^{14}CO_2$: 4-OH benzoate exchange)	–	–	–	126	–	–	–	–
2	Phenylglyoxylate: Acceptor Oxidoreductase								
	– Phenylglyoxylate oxidation (Benzyl viologen)	–	–	–	–	–	4.6	40.9	–
	– $^{14}CO_2$ exchange	–	–	–	–	–	3.7	13.5	
3	4-OH-Phenylglyoxylate: Acceptor Oxidoreductase								
	– 4-OH-Phenylglyoxylate oxidation (Benzyl viologen)	–	–	–	–	–	3.3	29	–
	– $^{14}CO_2$ exchange	–	–	–	–	–	9.3	22	–
4	*p*-Cresol Methylhydroxylase (DCPIP + PMS)	–	–	–	–	42	–	–	–
5	Benzyl alcohol dehydrogenase (NAD^+)	48	–	–	17	52	–	145	13
6	4-Hydroxybenzyl alcohol dehydrogenase (NAD^+)	124	–	–	35	111	–	304	51
7	Benzaldehyde dehydrogenase ($NADP^+$, pH 7)	7	112	–	11	48	6	85	12
	(NAD^+ + DCPIP, pH 9)	60	–	21	–	n.d.	130	70	95
8	4-Hydroxybenzaldehyde dehydrogenase (NAD^+ + DCPIP, pH 9)	61	–	77	–	54	74	–	71
9	4-Hydroxybenzoyl-CoA reductase (dehydroxylating) (reduced Benzyl viologen)	–	–	23	20	16	13	–	–
10	Benzoyl-CoA synthetase (AMP forming)	39	–	6	60	40	23	39	–
11	4-Hydroxybenzoyl-CoA synthetase (AMP forming)	7	–	15	26	43	7	3	1
12	Phenylacetyl-CoA synthetase (AMP forming)	2	–	2	44	9	35	49	–
13	4-Hydroxyphenylacetyl-CoA synthetase (AMP forming)	1	–	–	2	–	11	–	–

4-Hydroxybenzoyl-CoA reductase (dehydroxylating) was present only in cells grown on phenol, 4-hydroxybenzoate, *p*-cresol, or 4-hydroxyphenylacetate. This regulatory pattern is exactly in line with the proposed order of reactions in the metabolism of these apparently so different compounds, as can be seen from Fig. 1. All four compounds were proposed to be oxidized via 4-hydroxybenzoyl-CoA necessitating its reductive conversion to benzoyl-CoA. The enzyme was active with reduced benzyl viologen, the physiological electron donor remains to be shown. The data suggest sequential induction by 4-hydroxybenzoyl-coenzyme A since 4-hydroxy-

benzoate appears not to be formed as free intermediate in 4-hydroxyphenylacetate metabolism (4-hydroxyphenylglyoxylate: acceptor oxidoreductase forms 4-hydroxybenzoyl-coenzyme A rather than 4-hydroxybenzoate).

p-Cresol methylhydroxylase was present only in *p*-cresol grown cells. The enzyme reacted with phenazine methosulfate plus dichlorophenol indophenol as electron acceptor pair. *p*-Cresol methylhydroxylase is known since long as the enzyme initiating aerobic *p*-cresol metabolism (Hopper 1978). It is one of the few examples where the introduction of hydroxyl groups into aromatic molecules are brought about by dehydrogenase-type of enzymes acting with water as oxygen source (see also 4-chlorobenzoate metabolism via 4-hydroxybenzoate, Müller et al. 1984; note that these two reactions probably follow different mechanisms). Another type of anaerobic "hydroxylations" are common in the metabolism of heterocyclic compounds such as purines, pyridines, or furanoic acid (Trudgill 1984; Koenig and Andreesen 1989), but a dehydrogenase reaction is also required for anaerobic alpha-oxidation (hydroxylation) of the side chain of phenylacetyl(-coenzyme A) to phenylglyoxylate (Seyfried et al. 1991).

A phenylglyoxylate:acceptor oxidoreductase was present only when cell were grown on phenylacetate or 4-hydroxyphenylacetate. Probably one and the same enzyme also acts on 4-hydroxyphenylglyoxylate which explains best the result that activities towards both substrates were also present in cells grown on 4-hydroxyphenylacetate. Most likely (4-hydroxy)phenylglyoxylate rather than (4-hydroxy)phenylacetate acts as inducer since cells grown on phenylglyoxylate also contain this enzyme (not shown). The lower oxidoreductase activity in 4-hydroxybenzoate-grown cells may be due to later harvest of this batch of cells. Alternatively, the oxidative decarboxylation of the 2-oxoacid may be the rate-limiting step. This assumption could explain the excretion of (4-hydroxy)-phenylglyoxylate by growing cells (Seyfried et al. 1991). The enzyme reacted with benzyl viologen but not with $NAD(P)^+$; its physiological electron acceptor may be ferredoxin as it is in the case of other 2-oxoacid:acceptor oxidoreductases (2-oxoacid synthases) (for reviews see Buchanan 1979; Kerscher and Oesterhelt 1982), but this has not been shown. In addition the enzyme(s) catalyzed the isotope exchange between $^{14}CO_2$ and the carboxyl of phenylglyoxylate or 4-hydroxyphenylglyoxylate. This property is characteristic for "reversible" 2-oxoacid synthases (Blaschkowski et al. 1982) and excludes an "irreversible" pyridine nucleotide-dependent 2-oxoacid dehydrogenase.

Cell extracts displayed aromatic acid:coenzyme A ligase activities (aromatic acyl-coenzyme A synthetases) (AMP forming) towards the four aromatic carboxylic acids tested, which are probably due to at least three different enzymes. The activities could only be measured reliably after ammonium sulfate precipitation of extract protein. An unknown constituent in the extract which can be removed by ammonium sulfate precipitation appears to be a powerful inhibitor of these enzymes (for similar reports see Evans and Fuchs 1988). The pattern of activities towards the different aromatic acids suggests that at least one enzyme each is responsible for benzoate, 4-hydroxybenzoate, and (4-hydroxy)phenylacetate activation, respectively. The data do not exclude the possibility that there is another 4-hydroxyphenylacetate activating enzyme. Since these enzymes are not absolutely specific with respect to its aromatic substrate (Ziegler et al. 1989; Geissler et al. 1988) further study of their whole cell regulation requires purification of each individual enzyme, as has been done for the enzymes activating benzoate or 4-hydroxybenzoate in *Rhodopseudomonas palustris* (Geissler et al. 1988; Merkel et al. 1989). Recently it has been shown that aerobic metabolism of 2-aminobenzoate (Buder and Fuchs 1989; Buder et al. 1989; Langkau et al. 1990) and possibly of phenylacetate (Martinez-Blanco et al. 1990) also may proceed via the coenzyme A thioesters in certain bacteria.

Extracts from most cells contained NAD^+-specific benzyl alcohol dehydrogenase and $NADP^+$-dependent benzaldehyde dehydrogenase activity. The observed stoichiometry was 0.86 mol NADH oxidized per mol benzaldehyde added (note that benzaldehyde is volatile and escapes from solution resulting in the lower stoichiometry). The aldehyde was irreversibly oxidized by the aldehyde dehydrogenase to the aromatic acid rather than to its coenzyme A thioester since the reaction did not require coenzyme A, and NADPH-dependent reduction of benzoyl-CoA to benzaldehyde and coenzyme A could not be measured. The exact role of these activities remains obscure since no clear regulatory pattern can be recognized. The alcohol dehydrogenase acting on benzyl alcohol also appears to act on 4-hydroxybenzyl alcohol as deduced from the fact that the ratio of these two activities was almost constant and their regulatory pattern was similar. Comparison of activity patterns demonstrates that 4-hydroxybenzyl alcohol dehydrogenase activity is not due to *p*-cresol methylhydroxylase. NADP-dependent benzaldehyde dehydrogenase and 4-hydroxybenzaldehyde dehydrogenase probably are two different enzymes since the formation of their activities was differently regulated.

According to Hopper (Hopper 1976) *p*-cresol methylhydroxylation yields 4-hydroxybenzaldehyde which subsequently is oxidized to 4-hydroxybenzoate by an aldehyde dehydrogenase; the intermediary 4-hydroxybenzyl alcohol is oxidized by the methylhydroxylase enzyme itself. Extracts of *p*-cresol grown cells catalyzed the 4-hydroxybenzaldehyde-dependent reduction of dichlorophenolindophenol in the presence of NAD^+ at pH 9; they also contained high NAD(P)H:dichlorophenol indophenol oxidoreductase activity. A stoichiometry of 1.1 mol dichlorophenol indophenol reduced per mol 4-hydroxybenzaldehyde added was measured. This activity was not confined to cells grown on *p*-cresol but was similarly high in cells grown on other substrates. Therefore synthesis of *p*-cresol methylhydroxylase and 4-hydroxybenzaldehyde dehydrogenase activity cannot be coordinated.

Taken together, the data of this first investigation of the regulation of anaerobic aromatic metabolism provide

strong support for the proposed metabolic scheme (see Fig. 1).

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