2,3-Dihydroxybenzoate pathway in *Pseudomonas putida*

^1H n.m.r. study on the ring-cleavage site

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1. Ring cleavage of 2,3-dihydroxybenzoate by cell-free extracts of *Pseudomonas putida* leads to 2-hydroxy-6-oxo-(2Z,4E)-hexa-2,4-dienoic acid and CO_2 . 2. The ^1H n.m.r. spectrum of the ring-fission product obtained in a $^2\text{H}_2\text{O}$ solution suggests that the extra-diol cleavage occurs between C-3 and C-4.

Ribbons & Watkinson (1968) and Galli *et al.* (1975) found that 2,3-dihydroxybenzoate was oxidized with CO_2 evolution by cell-free extracts of *Pseudomonas* strains to an α -hydroxymuconic semi-aldehyde whose chemical and physical properties are the same as those described by Kojima *et al.* (1961) for the extra-diol ring-fission product of catechol.

Ribbons & Senior (1970) assumed that 2,3-dihydroxybenzoate underwent a cleavage between C-3 and C-4 by analogy with the site of cleavage of 2,3-dihydroxy-4-methylbenzoate.

The present report is concerned with the determination of the ring-cleavage site of 2,3-dihydroxybenzoate by ^1H n.m.r. analysis of the ring-fission product obtained in presence of $^2\text{H}_2\text{O}$ with a *Pseudomonas putida* cell-free extract.

Materials and methods

Organism

A micro-organism, previously isolated and provisionally assigned to the genus *Achromobacter* (Galli *et al.*, 1975), was used. This strain has been again submitted to identification according to Bergey's Manual (1974) and now definitely ascribed to *Pseudomonas putida*.

For maintenance and cell-free-extract preparations, the organism was cultured on Raymond & Davis (1960) mineral-salts medium solidified with 1.8% (w/v) Difco agar (Difco Laboratories, Detroit, MI, U.S.A.) and supplemented with 0.1% (w/v) of 2,3-dihydroxybenzoate as the only carbon and energy source.

Cell-free extracts

Freshly harvested cells, suspended in 0.05 M-potassium phosphate buffer, pH 7, were crushed at 4–5°C in an Aminco French Pressure Cell (American Instrument Co. Inc., Washington, DC, U.S.A.) at 1.42×10^4 kPa. The crude extract was incubated with 10 μg of deoxyribonuclease (bovine pancreas) (Boehringer–Mannheim G.m.b.H.) for 15 min at 0°C. After clarification by centrifuging, the supernatant solution, taken as source of soluble enzymes, was diluted to give a total protein content of 20 mg/ml, determined by the method of Layne (1957), with bovine serum albumin as a standard (BDH Chemicals, Poole, Dorset, U.K.).

Manometric determinations

O_2 uptake by cell-free extracts was determined by conventional Warburg manometry at 30°C. The reaction mixture contained, in a final volume of 3 ml, 2.0 ml of 0.05 M-potassium phosphate buffer, pH 7, 3 μmol of substrate and 1 ml of cell-free extract. The CO_2 evolution was determined by the method of Umbreit *et al.* (1961).

Spectral analyses

U.v. spectra were recorded in a Beckman model 24/25 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, U.S.A.).

^1H n.m.r. spectra were recorded on an XL-100 Varian spectrometer for approx. 0.2 M-solutions in $[\text{H}]$ chloroform/ $[\text{H}]$ dimethyl sulphoxide (1:1, v/v). All chemical shifts are quoted as p.p.m. downfield from internal tetramethylsilane.

Chemicals

2,3-Dihydroxybenzoic acid was obtained from Aldrich-Europe, Beerse, Belgium, and purified by sublimation (95°C at 400 Pa); catechol was from BDH Chemicals; $^2\text{H}_2\text{O}$ (99.75 atom-% of $^2\text{H}_2\text{O}$) was from Merck, A.-G., Darmstadt, Germany. All other chemicals were of analytical grade or the purest available.

Results

Cell-free-extracts experiments

Cell-free extracts of *Ps. putida* grown on 2,3-dihydroxybenzoate oxidized 2,3-dihydroxybenzoate with an O_2 uptake of 1 mol/mol of substrate, the evolution of 1 mol of CO_2 and the formation of a yellow compound whose u.v. spectrum showed λ_{max} 375 nm at pH 11 shifting to 320 nm at pH 2. No activity was detected when the same enzyme extract was incubated with catechol. Moreover the 2,3-dihydroxybenzoate oxygenase activity was completely inhibited by the presence of 0.001 mM-catechol. In addition, when the cell-free extracts were incubated anaerobically with 2,3-dihydroxybenzoate no CO_2 evolution was detected.

Isolation of the 2,3-dihydroxybenzoate ring-fission product obtained in the presence of $^2\text{H}_2\text{O}$

To isolate a sufficient amount of 2,3-dihydroxybenzoate ring-fission product, a large-scale incubation was carried out as follows. Two Erlenmeyer flasks, each containing 24 ml of cell-free extract of *Ps. putida* grown on 2,3-dihydroxybenzoate, 41 ml of 0.05 M-potassium phosphate buffer, pH 7, and 2.5 ml of 0.01 M-2,3-dihydroxybenzoate, were incubated at 28°C by shaking. A further eleven 2.5 ml portions of 2,3-dihydroxybenzoate solution were successively added at 15 min intervals. To follow the transformation, 1 ml samples of the incubation mixture were removed and spectrophotometrically assayed for the presence of the ring-fission product absorbing at 375 nm at pH 11. At the end of incubation, the reaction mixture was deproteinized by adding an equal volume of a 10% (w/v) metaphosphoric acid solution; the precipitate was discarded by centrifugation and the supernatant was saturated with NaCl and extracted several times with peroxide-free diethyl ether. The ethereal extracts were dried by cooling at -20°C and then separated from ice by filtration at -20°C. The resulting solution was evaporated to dryness at room temperature under vacuum. The crude yellow product was then dissolved in methanol, and the resulting solution was filtered and evaporated to dryness at room temperature under reduced pressure leaving a yellow powder.

The same experiment was subsequently carried

out using $^2\text{H}_2\text{O}$ in place of water for preparing the potassium phosphate buffer and the 2,3-dihydroxybenzoate solution; water was only used to prepare the metaphosphoric acid solution, so that at the end of incubation the reaction mixture was based on water/ $^2\text{H}_2\text{O}$ (1 : 1, v/v).

^1H n.m.r. analyses of α -hydroxymuconic semialdehydes produced either in water or in $^2\text{H}_2\text{O}$ solution

To test possible exchange with $^2\text{H}_2\text{O}$ of the protons of 2,3-dihydroxybenzoate, we first recorded the ^1H n.m.r. spectrum after adding this compound to the buffer solution in $^2\text{H}_2\text{O}$, without cell-free extract, and then working up and isolating as usual. On the basis of the spectral information, it was clear that the protons of the aromatic nucleus were not exchanged with $^2\text{H}_2\text{O}$ under our experimental conditions.

Table 1 shows the results for the ^1H n.m.r. spectrum of α -hydroxymuconic semialdehyde (I) (Fig. 1), obtained from 2,3-dihydroxybenzoate in water. Irradiation of the aldehydic proton $\text{H}_{(1)}$ at 9.35 p.p.m. caused a collapse of the double of doublets of the olefinic proton $\text{H}_{(2)}$ at 6.05 p.p.m. into a doublet, due to coupling by the olefinic proton $\text{H}_{(3)}$ ($J_{2,3} = 15 \text{ Hz}$).

Table 2 reports the ^1H n.m.r. spectrum of the ring-fission product obtained in $^2\text{H}_2\text{O}$ and Table 3

Table 1. ^1H n.m.r. data of α -hydroxymuconic semialdehyde (I)

The numbering of protons refers to Fig. 1.

Type	δ (p.p.m.)	Multiplicity	J (Hz)
$\text{H}_{(1)}$	9.35	d	$J_{1,2}$ 8.0
$\text{H}_{(2)}$	6.05	dd	$J_{1,2}$ 8.0; $J_{2,3}$ 15.0
$\text{H}_{(3)}$	7.52	dd	$J_{3,4}$ 12.0; $J_{2,3}$ 15.0
$\text{H}_{(4)}$	6.21	d	$J_{3,4}$ 12.0

Fig. 1. 2-Hydroxy-6-oxo-(2Z,4E)hexa-2,4-dienoic acid, the ring-fission product of 2,3-dihydroxybenzoate

Table 2. ^1H n.m.r. data of the ring-fission product obtained in the presence of $^2\text{H}_2\text{O}$

The numbering of protons refers to Fig. 1. At the end of the incubation, the reaction mixture was based on water/ $^2\text{H}_2\text{O}$ (1:1, v/v).

Integration	Type	δ (p.p.m.)	Multiplicity	J (Hz)
0.5 H	H ₍₁₎	9.35	d	$J_{1,2}$ 8.0
0.5 H	H ₍₁₎	9.35	s	—
0.5 H	H ₍₂₎	6.05	dd	$J_{1,2}$ 8.0; $J_{2,3}$ 15.0
0.25 H	H ₍₃₎	7.52	dd	$J_{2,3}$ 15.0; $J_{3,4}$ 12.0
0.25 H	H ₍₃₎	7.52	d	$J_{2,3}$ 15.0
0.25 H	H ₍₃₎	7.52	d	$J_{3,4}$ 12.0
0.25 H	H ₍₃₎	7.52	s	—
0.5 H	H ₍₄₎	6.21	d	$J_{3,4}$ 12.0

Table 3. ^1H n.m.r. spectrum recorded after adding compound (I) to the buffer solution in $^2\text{H}_2\text{O}$

The numbering of protons refers to Fig. 1.

Type	δ (p.p.m.)	Multiplicity	J (Hz)
H ₍₁₎	9.35	s	—
H ₍₃₎	7.52	s	—

the ^1H n.m.r. spectrum recorded after adding α -hydroxymuconic semialdehyde (I) to the buffer solution in $^2\text{H}_2\text{O}$, without cell-free extract, and then working up and isolating the product as described; in the latter conditions, protons H₍₂₎ and H₍₄₎ are exchanged with $^2\text{H}_2\text{O}$, whereas the aldehydic proton H₍₁₎ and the proton H₍₃₎ are not.

All ^1H n.m.r. spectra were recorded for [^2H]-chloroform/[^2H]dimethyl sulphoxide solutions of the products obtained as described above, and showed that the chemical purity of these compounds was $\geq 95\%$.

Discussion

The precise site of 2,3-dihydroxybenzoate ring cleavage was unknown because α -hydroxymuconic semialdehyde obtained with *Ps. putida* cell-free extracts could originate by fission between either C-1 and C-2 or C-3 and C-4. Ribbons & Senior (1970) deduced that the ring cleavage probably occurred between C-3 and C-4 by analogy with the site of cleavage of 2,3-dihydroxy-4-methylbenzoate.

The site of ring fission of 2,3-dihydroxybenzoate could be determined by investigating the origin of the aldehydic proton H₍₁₎ of the α -hydroxymuconic semialdehyde product (I). Accordingly, we carried out the reaction with the cell-free extract in the presence of $^2\text{H}_2\text{O}$.

Two possibilities could be expected. In Scheme 1(a) the aldehydic proton is retained, and must therefore be a protium. According to Scheme 1(b),

the aldehydic proton derives from the medium, and must therefore be a deuteron.

Our spectral data are consistent with the Scheme 1(a), which involves an extra-diol cleavage between C-3 and C-4, decarboxylation of the resulting β -oxo acid (II) and isomerization of the double bonds.

This ring-fission process shows a diverse regioselectivity in comparison with the cleavage of 3-methylcatechol (Catelani *et al.*, 1968). The presence on the aromatic nucleus of either $-\text{CO}_2\text{H}$ or $-\text{CH}_3$, which possess opposite inductive effects, causes different sites of ring fission. In the case of 2,3-dihydroxy-4-methylbenzoate (Ribbons & Senior, 1970), both the substituents would direct the cleavage between C-3 and C-4.

From our results the direct decarboxylation of 2,3-dihydroxybenzoate has to be excluded, since catechol is not a substrate for the oxygenase induced by 2,3-dihydroxybenzoate and moreover inhibits this oxygenase activity. On the other hand it is not possible to establish if the decarboxylation of the β -oxo acid (II) is an enzyme-catalysed reaction or a chemical process, because β -oxo acids often decarboxylate under very mild conditions.

Stereochemically the α -hydroxymuconic semialdehyde arising from catechol ring fission was initially assigned a (2*E*,4*Z*)-configuration (Dagley *et al.*, 1960), subsequently altered to α -hydroxy-(2*Z*,4*Z*)-muconic acid semialdehyde (Morgan, 1962). The ring-fission product of 3-methylcatechol in contrast has been assigned a (2*Z*,4*E*)-configuration (Catelani *et al.*, 1968), on the basis of its ^1H n.m.r. spectrum. Our spectral data suggest, for the 2,3-dihydroxybenzoate ring-fission product, the structure of 2-hydroxy-6-oxo-(2*Z*,4*E*)-hexa-2,4-dienoic acid (I), on the basis of the large coupling constant between the H₍₂₎ and H₍₃₎ protons ($J_{2,3} = 15$ Hz) (Catelani *et al.*, 1968; Adam & Balci, 1979). The *trans* configuration of the 4,5-double bond probably arises either during the enzymic process or from the acidic treatment during the extraction of the *cis* precursor, through keto-enol

Scheme 1. 2,3-Dihydroxybenzoate extra-diol cleavage between C-3 and C-4 (a) or between C-1 and C-2 (b)

equilibrations. This hypothesis is supported by the easy exchange with $^2\text{H}_2\text{O}$ of protons $\text{H}_{(2)}$ and $\text{H}_{(4)}$ (Table 3). In this way, the isomerization of the two double bonds affords the thermodynamically more stable stereoisomer (I).

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