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Cleavage of formate from ω,4-dihydroxyacetophenone

An unusual oxygen-requiring reaction in the bacterial catabolism of 4-hydroxyacetophenone

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An enzyme, from a soil bacterium grown on 4-hydroxyacetophenone, cleaved the side chain of ω,4-dihydroxyacetophenone between the keto group and the carbon atom bearing a hydroxy group to give 4-hydroxybenzoate and formate. The reaction was O_2-dependent. Partially purified enzyme required no added cofactors for activity.

Besides by the more familiar lyase and hydrolase reactions, cleavage of carbon–carbon bonds in ketonic compounds may proceed by insertion of an oxygen atom by a mono-oxygenase. This yields an ester or lactone, which can then be hydrolysed. Examples of this type of reaction include the bacterial conversion of cyclohexanone into ε-hexanolactone (Norris & Trudgill, 1971) and of tridecanone into undecyl acetate (Forney & Markovetz, 1969) and the fungal conversion of progesterone into testosterone acetate (Rahim & Sih, 1966). This is also the route for the bacterial catabolism of acetophenone with the formation of phenyl acetate followed by its hydrolysis to phenol and acetate (Cripps, 1975). However, related compounds may be attacked in fungi by a hydrolase, producing, for example, phloroglucinol from 2',4',6'-trihydroxyacetophenone (Minamikawa *et al.*, 1970).

We report here the occurrence of a novel reaction for the metabolism of such a ketonic compound in which ω,4-dihydroxyacetophenone is split in an O_2-requiring reaction to 4-hydroxybenzoate and formate as a step in the bacterial catabolism of 4-hydroxyacetophenone.

Materials and methods

Maintenance and growth of organism

The organism, a strain of *Alcaligenes*, was isolated from soil by elective culture on 4-hydroxyacetophenone. The conditions for maintenance and growth were as described by Keat & Hopper (1978), but with 0.5g of 4-hydroxyacetophenone/l as the carbon source in liquid medium.

Preparation of extract

Crude cell extract was prepared by passing a suspension of each g wet weight of cells in 1ml of ice-cold 42mM-KH_2PO_4/NaOH buffer, pH7.0, twice through a pre-cooled French pressure cell with a pressure difference at the orifice of 138MPa. Unbroken cells and large particulate matter were removed by centrifuging at 10000*g* for 20min at 2°C.

DEAE-cellulose chromatography of extract

Crude cell extract (5ml; 24mg of protein/ml) was loaded on a column (4cm × 2cm diam.) of DEAE-cellulose (DE-53; Whatman Chemical Separation, Maidstone, Kent, U.K.) equilibrated with 42mM-KH_2PO_4/NaOH buffer, pH7.0. The column was washed with 25ml of buffer, then with 20ml of buffer containing 0.2M-KCl followed by a linear gradient of 0.2–0.5M-KCl in 80ml of buffer. Fractions of volume 3.5ml were collected. Enzyme activity was monitored spectrophotometrically at 30°C by following the decrease in A_{320} in a 1cm-path-length cuvette containing 250nmol of ω,4-dihydroxyacetophenone in 1ml of 42mM-KH_2PO_4/NaOH buffer, pH7.0. Activity was found in fractions 25–33. Recovery was about 70%.

Determination of acid production by pH-stat

Partially purified enzyme was dialysed against 5mM-KH_2PO_4/NaOH buffer, pH7.0, concentrated by ultrafiltration and 0.3ml (6.7mg of protein/ml) was added to 10ml of distilled water in a stirred reaction vessel. The pH was adjusted to 7.0. Substrate was added, and acid production was monitored by the controlled addition of 5mM-NaOH with an Autotitration Controller (Pye–Unicam, Cambridge, U.K.). The amount of alkali added was noted at 1min intervals.

O_2 stoichiometry

O_2 consumption was measured with a Clark-type oxygen electrode in a stirred vessel at 30°C (Oxygen Monitor model 53; Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). The reaction mixture contained, in 3ml of 42mM-KH_2PO_4/NaOH buffer, pH7.0, 0.34mg of partially purified enzyme and various amounts of substrate.

Chromatography

Chromatography on a Celite column (30cm × 1cm diam.) by the procedure of Swim & Utter (1957) was used to purify the acid products. Products were eluted with 80ml of chloroform followed by 100ml of 2.5% (v/v) butan-1-ol in chloroform and then 100ml of 5.0% (v/v) butan-1-ol in chloroform. Fractions of volume 10ml were collected, and 0.5ml of each was titrated with 5mM-NaOH with 0.04% Phenol Red as indicator.

4-Hydroxybenzoic acid was identified by t.l.c. on precoated silica-gel GHLF plates (Analtech, Newark, NJ, U.S.A.) with solvents A [benzene/dioxan/acetic acid (90:25:4, by vol.) (Pastuska, 1961)] and B [ethyl formate/light petroleum (b.p. 60–80°C)/propionic acid (30:70:15.4, by vol.) (Ronkainen, 1963)]. Compounds were located by viewing under u.v. light for materials that quenched the fluorescent indicator in the plates and by spraying with diazotized *p*-nitroaniline (Smith, 1960). Formic acid was identified by chromatography on paper with solvents C [ethanol/aq. NH_3 (sp.gr. 0.88)/water (20:1:4, by vol.) (Quayle *et al.*, 1961)] and D [propan-1-ol/aq. NH_3 (sp.gr. 0.88) (3:2, v/v) (Isherwood & Hanes, 1953)]. Acids were located by spraying with 0.04% Bromocresol Purple.

Absorption spectra

Samples were mulled in Nujol and their i.r.-absorption spectra recorded with a Perkin–Elmer Infracord spectrophotometer.

All u.v.-absorption measurements were made with a Pye–Unicam SP8-150 spectrophotometer.

Assays

A quantitative measure of 4-hydroxybenzoate was made by comparison of the intensity of the absorption peak at 245nm in its u.v.-absorption spectrum at pH7.0 with that of authentic material of known concentration. Formate was assayed quantitatively in reaction mixtures by the method of Barker & Somers (1966).

Chemicals

ω,4-Dihydroxyacetophenone was prepared by the procedure of Robertson & Robinson (1928). 4-Hydroxyphenylglyoxal was prepared by the SeO_2 oxidation of 4-hydroxyacetophenone by following the procedure described by Vogel (1956) for phenylglyoxal preparation from acetophenone. 4-Hydroxybenzoylformate was prepared by the cold-permanganate oxidation of 4-hydroxyacetophenone (Businelli, 1950). 4-Hydroxyacetophenone was from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). 4-Hydroxybenzaldehyde, 4-hydroxybenzoic acid and methyl 4-hydroxybenzoate were from BDH Chemicals.

Results

Activity in extracts

Addition of 3μmol of ω,4-dihydroxyacetophenone to a reaction mixture containing 0.5ml of crude cell extract (32mg of protein/ml) and 1.0ml of 42mM-KH_2PO_4/NaOH buffer, pH7.0, in a Warburg respirometer resulted in rapid O_2 uptake (16μl of O_2/min). After oxidation ceased the mixture was deproteinized by acidification and centrifugation, and extracted with diethyl ether. After removal of the ether, examination of the products by t.l.c. in solvents A and B showed 4-hydroxybenzoic acid as the product. This correlated with the spectrophotometric demonstration of activity when 118μg of crude-extract protein was added to a 1cm-path-length cuvette containing 0.1mM-ω,4-dihydroxyacetophenone in 3.0ml of 42mM-KH_2PO_4/NaOH buffer, pH7.0, and the spectrum from 200 to 400nm was recorded repeatedly. This showed a gradual conversion, over about 11min, of the substrate's spectrum into that of 4-hydroxybenzoate at the same concentration. No added cofactors were required, and, under similar conditions, there was no activity with 4-hydroxyacetophenone, 4-hydroxyphenylglyoxal, 4-hydroxybenzoylformate, methyl 4-hydroxybenzoate or 4-hydroxybenzaldehyde. No activity was seen when extract from succinate-grown bacteria was used.

The enzyme attacking ω,4-dihydroxyacetophenone was partially purified by chromatography on DEAE-cellulose, to which it bound quite tightly at pH7.0, requiring a high concentration of KCl for its elution. Even after this treatment no added cofactors were required for the conversion of substrate into 4-hydroxybenzoate, and there was no indication of any other intermediate in the process (Fig. 1). Boiled enzyme gave no activity.

A cuvette containing ω,4-dihydroxyacetophenone in buffer (as in Fig. 1) was made anaerobic by bubbling with N_2 for 5min and sealed with a rubber septum. Injection of 37μg of enzyme gave no apparent activity, with the substrate spectrum remaining unchanged. After 10min the cuvette was opened and shaken to introduce O_2. This resulted immediately in a gradual change of the

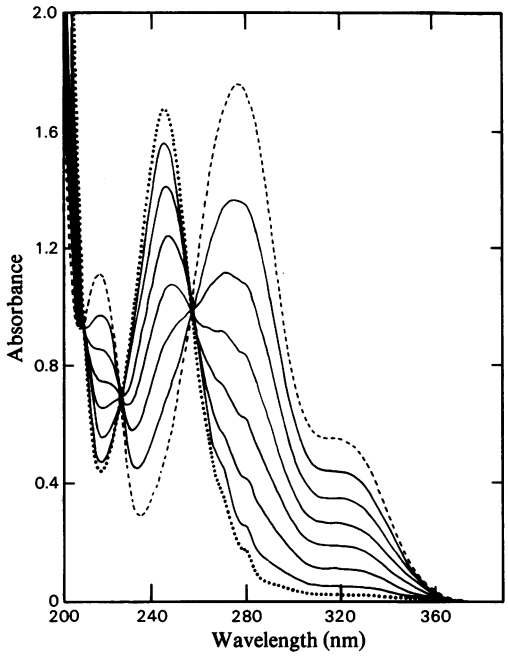


Fig. 1. *Spectrophotometric demonstration of the enzymic conversion of ω,4-dihydroxyacetophenone into 4-hydroxybenzoate*

The spectrum of 0.1 mM-ω,4-dihydroxyacetophenone, in 3ml of 42mM-KH_2PO_4/NaOH buffer, pH7.0, in a 1cm-path-length cuvette at 30°C, was recorded against a buffer blank (------). Enzyme (5 μl of partially purified enzyme, 0.7mg of protein/ml) was added to both cuvettes, and the spectra at 15min intervals are shown (——). The spectrum when there was no further change (·····) is identical with that of 4-hydroxybenzoate.

spectrum to that of 4-hydroxybenzoate, indicating a requirement for O_2 in the reaction. When O_2 uptake during the reaction was measured with the oxygen electrode, an average of 0.84 μmol of O_2 was utilized/μmol of substrate.

The amount of acid produced during the reaction was measured with a pH-stat. Addition of 5 μmol of ω,4-dihydroxyacetophenone resulted in the total consumption of 2ml of 5mM-NaOH (over a period of 13min), giving a stoichiometry of 2 molecules of acid produced/molecule of substrate. Thus there is another acid produced besides 4-hydroxybenzoic acid, and, since it will contain only one carbon atom, this is probably formic acid.

Identification of reaction products

To produce sufficient material for identification, a reaction mixture, containing in 10ml of 0.1M-KH_2PO_4/NaOH buffer, pH7.0, 5.2mg of partially purified enzyme and 200 μmol of ω,4-dihydroxyacetophenone, was incubated with shaking in a water bath at 30°C for 4.25h. It was then acidified with HCl to pH3.0, and extracted continuously with diethyl ether for 5h. The ether extract was dried over anhydrous Na_2SO_4 and then evaporated. When the products were separated by column chromatography on Celite, peaks were eluted in the same positions as standard 4-hydroxybenzoic acid (in 2.5% butan-1-ol in chloroform) and formic acid (in 5.0% butan-1-ol in chloroform). From the titres, most of the theoretical yield of 4-hydroxybenzoic acid, but slightly less than 50% of the formic acid, had been extracted by the ether. The fractions corresponding to each acid were pooled, each was extracted with 20ml of 0.1M-NH_3, and the water and excess NH_3 were then removed on a rotary evaporator. The product corresponding to 4-hydroxybenzoate was dissolved in water, acidified with HCl and extracted with diethyl ether. The ether extract was dried over anhydrous Na_2SO_4 and evaporated. The solid product gave the same u.v.- and i.r.-absorption spectra as authentic 4-hydroxybenzoic acid, to which it also corresponded on t.l.c. with solvents A and B. The other product corresponded to authentic ammonium formate when examined by paper chromatography in solvents C and D.

Table 1. *Stoichiometry of product formation from ω,4-dihydroxyacetophenone*

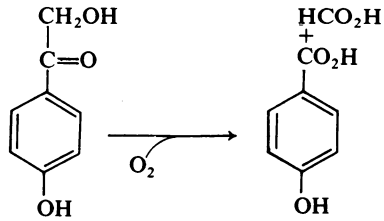
Reaction mixtures containing ω,4-dihydroxyacetophenone in 3.0ml of 0.1M-KH_2PO_4/NaOH buffer, pH7.0, and 0.2ml of partially purified enzyme (0.88mg of protein/ml), together with a control without substrate, were incubated with shaking at 30°C for 2.5h. They were then assayed for 4-hydroxybenzoate and formate.

Substrate consumed (μmol)	Products formed (μmol)	
	4-Hydroxybenzoate	Formate
12	11.0	11.0
24	22.0	23.6

Measurement of product formation showed that 1 molecule of 4-hydroxybenzoate and 1 molecule of formate were produced for each molecule of ω,4-dihydroxyacetophenone that reacted (Table 1).

Discussion

The results are consistent with the reaction shown in Scheme 1, in which both formate and 4-hydroxybenzoate were identified as reaction products and a requirement for O_2 was demonstrated. The stoichiometry approached the unitary relationship required for the reaction shown in Scheme 1, which constitutes an unusual mode of cleavage for this type of compound. Oxidative attack on the bond between carbon atoms bearing oxo and hydroxy groups does occur in the formation of tartaric acid from 4-oxogluconic acid (Kotera *et al.*, 1972), but with the intermediate formation of



Scheme 1. *Proposed enzymic reaction*

an ester in a reaction analogous to the monooxygenase conversions mentioned in the introduction. In all of these cases an external electron donor was required, whereas no similar additions were necessary for the reaction described in the present paper, even after DEAE-cellulose chromatography of the enzyme and with addition of very small volumes of enzyme solution to reaction mixtures. This lack of a requirement for added cofactors for O_2-dependent cleavage between these groups makes the reaction more analogous to the rather unusual type of oxygenase activity exhibited by ribulose-1,5-bisphosphate carboxylase, in which ribulose 1,5-bisphosphate is split into glycerate 3-phosphate and glycollate 2-phosphate (Lorimer, 1981). However, further comparisons await a more detailed study of the purified enzyme.

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