ENZYMATIC AROMATIZATION OF 4-KETOCYCLOHEXANECARBOXYLIC ACID TO p-HYDROXYBENZOIC ACID*

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Corynebacterium cyclohexanicum grows well on cyclohexane carboxylic acid as the sole carbon source, and accumulates a large amount of 4-ketocyclohexanecarboxylic acid in the culture medium at an early growth phase. When this keto acid was aerobically incubated with a crude extract of the organism, an acidic product was produced. Under anaerobic conditions, the same product was obtained using $K_3Fe(CN)_6$ as electron acceptor. The product was identified as p-hydroxybenzoic acid by means of gas-liquid chromatography, infrared spectroscopy, and mass spectroscopy. Two pathways are proposed for the aromatization of 4-ketocyclohexanecarboxylic acid.

Introduction

The metabolism of aromatic compounds has been extensively studied and their degradation pathways have been well established (1,2). Recently, by use of mutants, labile initial intermediates involved in degradation of benzoic acid have successfully been isolated and identified (3).

Cyclohexane derivatives, reduced counterparts of aromatics, however, have not been studied much and very little is known about their metabolic fate. This laboratory is engaged in a study of the metabolism of cyclohexanecarboxylic acid by a Corynebacterium. Earlier experimental results with resting cells have suggested that an aromatization step should be involved in the metabolism (4). Now this postulate has been substantiated by results obtained with cell-free extracts. The essential data are reported in this communication.

Experimental

Microorganism and its cell-free extracts: Corynebacterium cyclohexanicum, previously isolated and identified, was cultured in a medium containing 0.5% cyclohexanecarboxylic acid, 0.01% yeast extract, and inorganic salts as previously described (4). The incubation was carried out for 2 days at 30°C on a rotary shaker. The cells were then centrifuged, washed with cold 0.85% NaCl solution, and suspended in 10 mM potassium phosphate buffer (pH 7.0). Cell-free extracts were prepared from the suspension by treating with ultrasound as before (5).

Reaction conditions: The 2.5 ml reaction mixture containing 4-ketocyclohexanecarboxylic acid, 5 μmoles; phosphate buffer (pH 7.0), 100 μmoles; and cell-free extract, 4.5 mg protein was incubated in a Warburg vessel at 30°C. The reaction

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was followed by observing the oxygen uptake on a Warburg Manometer. The amount of oxygen required for the oxidation of the keto acid was determined separately by a polarographic oxygen sensor (Yellow Spring Instrument Co., Yellow Spring, Ohio).

Isolation of the product: When oxygen uptake leveled off, four runs of the reaction mixture were combined and acidified with 6N HCl. The acidified mixture was saturated with $(NH_4)_2SO_4$ and extracted with diethyl ether overnight. The ether was evaporated under vacuum to obtain the product.

Analytical procedure: Samples were methylated for gas-liquid chromatography by reaction with diazomethane for 1 min (6). Two chromatographic columns were used; one, 6 ft by 1/8 in O.D. stainless steel tubing packed with 2.5% SE-30 coated on Chromosorb G (100-120 mesh)(Applied Science Laboratories, State College, Penn.), and the other, 6 ft by 1/8 in packed with 7% ethyleneglycol adipate polymer on Chromosorb W (100-120 mesh)(Applied Science Laboratories). The SE-30 column used for most experiments was operated at 55°C for 2 min then heated at the rate of 4°C/min to the final temperature of 200°C. The polyester column was used only for determination of equivalent chain length isothermally at 180°C. Equivalent chain length (7) was calculated relative to the methyl esters of normal fatty acids (Applied Science Laboratories) as previously described (8).

Mass spectra were produced by a Perkin-Elmer Model 270 GC-MS system operated at 70 eV and infrared spectra were run with solid film samples in a Perkin-Elmer Model 221 infrared spectrophotometer.

Chemicals: Cyclohexanecarboxylic acid (Aldrich Chemical Co., Milwaukee, Wisconsin) was purified by fractional distillation and the middle fraction, boiling at 121.5°C 13 mm Hg, was used. 4-Ketocyclohexanecarboxylic acid was prepared from p-hydroxybenzoic acid (9). Methyl esters of p-hydroxybenzoic acid and p-methoxybenzoic acid were prepared by reacting with methanol in the presence of H_2SO_4. p-Hydroxybenzoic acid, p-methoxybenzoic acid, and Diazald were purchased from Aldrich.

Results

Tokuyama and Kaneda found that when Corynebacterium cyclohexanicum is grown on cyclohexanecarboxylic acid a large amount of 4-ketocyclohexanecarboxylic acid accumulates in the culture medium only at an early growth phase (unpublished observation). Thus the metabolism of the keto acid was investigated.

Oxygen requirement: When 4-ketocyclohexanecarboxylic acid was incubated with a cell-free extract of the organism, 1.04 moles of oxygen was consumed per mole of acid.

Identification of the product: The product, which is acidic, was extracted with diethyl ether from the reaction mixture at pH 2. Evaporation of the ether extract

Table I. Identification of the product as p-hydroxybenzoic acid

	Equivalent chain length		Mass spectrometric peaks (m/e)					
	Column used							
	SE-30	EGA*	Parent	Base	Others			
Methylated product								
major component	11.50	22.6	152 (37%)**	121 (100%)	93 (24%)	65 (25%)	39 (19%)	
minor component	10.37	15.5	166 (39%)	135 (100%)	136 (10%)	108 (11%)	92 (17%)	77 (20%)
Methyl p-hydroxy-benzoate	11.43	22.5	152 (36%)	121 (100%)	93 (25%)	65 (24%)	39 (20%	
Methyl p-methoxy-benzoate	10.37	15.5	166 (35%)	135 (100%)	136 (10%)	108 (11%)	92 (15%)	77 (19%)

* Ethyleneglycol adipate polymer

** Intensity relative to the base peak

gave a white crystalline compound. Upon methylation by a short exposure with diazomethane the infrared spectrum indicated the presence of an aromatic ester function (a strong absorption for ketonic bond at 1670 cm^{-1} and for the conjugated double bond at 1570 and 1600 cm^{-1}). Gas-liquid chromatography of the methylated product on SE-30 gave two peaks, neither of them corresponding to the methyl ester of 4-ketocyclohexanecarboxylic acid. The mass spectrum analysis indicated that the two compounds were methyl p-hydroxybenzoate (the major peak component), and methyl p-methoxybenzoate (the second peak component, 5% of the major one). Identification of the compounds was confirmed from mass spectral data and gas-liquid chromatographic comparison of retention temperature and equivalent chain length to those of authentic samples (Table I). That the product was composed of a single component, namely, p-hydroxybenzoic acid, was established by the fact that a sample of p-hydroxybenzoic acid also gave two peaks when methylated with diazomethane in a ratio identical to that obtained with the product.

Anaerobic experiments: If the incubation was carried out anaerobically under helium, no p-hydroxybenzoic acid was detected. The addition of $K_3Fe(CN)_6$, 25 μ moles, to the incubation mixture produced about the same yield of p-hydroxybenzoic acid from an anaerobic incubation as from the aerobic incubation.

Discussion

Mitoma, Posner and Leonard (10) have shown that cyclohexanecarboxyl CoA is aromatized to benzoate by guinea pig liver mitochondria in the absence of any co-factors. A further study by Babior and Bloch (11) showed that 1-cyclohexenecarboxyl-CoA was a direct intermediate, and that the required oxygen merely served as a terminal electron acceptor and could be replaced by phenazine methosulfate or potassium ferricyanide. The present communication provides an additional example of the aromatization of cyclohexanecarboxylic acid in which it is converted to 4-keto-cyclohexanecarboxylic acid before aromatization takes place. Apparently no CoA ester is involved. In the present system, also, oxygen can be replaced by another electron acceptor.

The following two pathways are postulated for the aromatization of 4-ketocyclohexanecarboxylic acid to p-hydroxybenzoic acid.

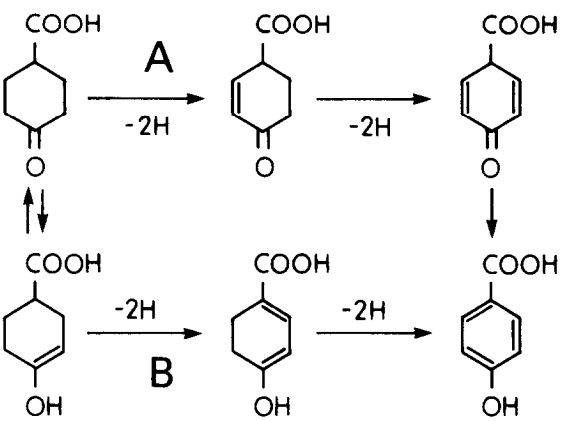

Fig. 1. Probable pathways for the aromatization of 4-ketocyclohexanecarboxylic acid to p-hydroxybenzoic acid

Many desaturating enzymes introduce a double bond which is conjugated to a carboxyl carbon (12). The scheme shown is based on this. Pathway A includes the direct desaturation of the keto acid to form a double bond conjugated to the carbonyl carbon. A further introduction of a conjugated double bond forms a dienone before it is isomerized to p-hydroxybenzoic acid. Pathway B includes the desaturation of enol-form of the keto acid to form a dienol before it is oxidized to p-hydroxybenzoic acid. A further study to differentiate the two pathways is in progress.

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