Bacterial Degradation of \( p \)-Methoxybenzoic Acid

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Received October 29, 1971

Summary. A cell-free system from a \textit{Pseudomonas} sp., strain PM3, catalysed the oxidative demethylation, hydroxylation and subsequent ring cleavage of \( p \)-methoxybenzoate. Demethylation, to yield \( p \)-hydroxybenzoate, involved absorption of 1.0 mole of oxygen/mole of \( p \)-methoxybenzoate, and required reduced pyridine nucleotide (either NADH or NADPH) as cofactor. \( p \)-Hydroxybenzoate was hydroxylated to yield protocatechuate with the absorption of 1 mole of oxygen/mole of substrate, and required NADPH as cofactor. Protocatechuate was oxidized, with absorption of 1 mole of oxygen/mole of substrate, to 3-oxoadipate. The methyl group of \( p \)-methoxybenzoate was removed as formaldehyde, and oxidized to formate and carbon dioxide by formaldehyde dehydrogenase, which required GSH and NAD\(^+\), and formate dehydrogenase, which required NAD\(^+\).

Enzymic \( O \)-demethylation is an important process in the detoxication of many drugs in the animal body (Axelrod, 1955; Brodie \textit{et al.}, 1955), and in the biological decomposition of plant residues essential for soil fertility. Ether cleavage activities of bacterial systems have been investigated by Cartwright and Smith (1967) and by Cartwright and Buswell (1967, 1969) as part of a study of microbial demethylation of model compounds thought to be related to the complex structure of lignin.

Henderson (1957, 1961) and Buswell (1966) respectively, showed that demethylated products arose from the attack on the isomeric methoxybenzoates by fungal cells and by cell-free extracts of bacteria. We have extended the latter work and now describe some properties of cell-free extracts of a soil pseudomonad (strain PM3), isolated in this laboratory, which contain a 4-methoxybenzoate-\( O \)-demethylase. Observations are presented on the degradative pathway of \( p \)-methoxybenzoate, via \( p \)-hydroxybenzoate and protocatechuate, to products involved in the general energy-yielding mechanisms of the cell.

Materials and Methods

\textit{Organism}. A fluorescent pseudomonad (strain PM3) was isolated by enrichment from the soil in the presence of 0.15\% w/v sodium-4-methoxybenzoate as carbon source, and was grown as described previously by Cartwright and Smith (1967).

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Preparation of Cell Extracts. The washed cell paste (1 vol.) was suspended in 0.05 M tris/HCl, pH 7.8 (1.5 vol.), containing 0.0067 M dithiothreitol, cooled in an ice-salt bath, and disrupted by sonication (Bronwill Biosonik III, 20×15 sec at 60% max. power, large tip). The irradiated suspension was centrifuged at 20000 g for 20 min. (Sorvall RC2-B, SS34 rotor) to yield a residue fraction R1 and a supernatant S1, which was further centrifuged at 78000 g for 30 min in a Beckman L2-65B, preparative ultracentrifuge (Type 65 angle rotor) to yield a residue fraction, R2. The supernatant fraction (S2), containing about 35 mg protein per ml, was centrifuged at 270000 g for 3 h (type 65 rotor) to yield supernatant fraction S3 and a residue fraction R3.

Estimations. Protein was estimated by the method of Lowry et al. (1951).
3-Oxoadipate was estimated by catalytic decarboxylation with 4-aminoantipyrine (Sistrom and Stanier, 1953).
Formaldehyde, trapped as semicarbazone, was estimated using chromotropic acid by the method of MacFadyen (1945).
Formate was demonstrated by reduction to formaldehyde with magnesium foil to which was added 0.5 ml conc. HCl dropwise with shaking. The reaction mixture was incubated at 100°C/30 min with 0.18% chromotropate and the spectrum determined between 350–650 nm.

Analytical Methods. The O2 uptake and CO2 production were followed by conventional manometric techniques. Spectrophotometric measurements were made in 1 cm silica cuvettes in a Hitachi-Perkin Elmer 139 Spectrophotometer with a Sargent Welch recorder, and Beckman DK-2 recording spectrophotometer. Infra red spectra of isolated intermediates were obtained in KBr pellets with a Beckman IR-10 spectrophotometer. Melting points (uncorrected) were determined with a Fisher-Johns melting point apparatus.

Chemicals. Most chemicals used as substrates were from commercial sources purified by recrystallisation. NAD + (grade III), NADP + and their reduced forms (NADPH, chemically reduced), GSHP (99% pure), ATP (99–100% pure) and dithiothreitol were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Isovanillie acid (3-hydroxy-4-methoxybenzoic acid) was prepared as described by Cartwright and Smith (1967). Recrystallization from water gave a product, m.p. 245–247°C.

Chromatography. Thin layer chromatography was conducted on Brinkman polygram sheets (MN Silica Gel S-HR/UV254, Brinkmann Instruments, Inc., Westbury, N.Y.) using ethanol-aq. ammonia (Sp. gr. 0.88)-water (20:1:4, by vol.). Phenolic compounds were located by u.v. visualization and by spraying with a 2% w/v solution of 2,6-dichloroquinone-4-chloroimide in ethanol. Oxo acids, run as their 2,4-dinitrophenylhydrazones, were detected either under u.v. light, or under alkaline conditions in visible light.

Buffers. K2HPO4-KH2PO4 and tris/HCl buffers were prepared at the required pH.

Results and Discussion

O-Demethylation of p-Methoxybenzoate

Cartwright et al. (1970) showed that organisms grown on p-methoxybenzoate were induced to metabolize p-methoxybenzoate, p-hydroxybenzoate and protocatechuate. Cartwright and Buswell (1969) described cell-free preparations from a Pseudomonas sp. grown on p-methoxybenzoate which oxidized these substrates but it was difficult to attach