CRYSTALLIZATION AND SOME PROPERTIES OF 3,4-DIHYDROXYPHENYLACETATE-2,3-OXYGENASE

H. Kita, M. Kamimoto and S. Senoh

The Institute of Food Chemistry, Osaka, Japan

T. Adachi and Y. Takeda

Laboratory of Biochemistry, Dental School, Osaka University, Osaka, Japan

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Several enzymes, which catalyze the oxidative cleavage of the benzene rings of catechol derivatives, have been reported, but the purification of these enzymes has been quite difficult, owing to their instability. Finally in 1963 the crystallization of metapyrocatechase (1) and pyrocatechase (2) was reported by Hayaishi's group of Kyoto University.

With regard to the metabolism of p-hydroxyphenylacetic acid, it has been suggested from the experiments in vivo that p-hydroxyphenylacetic acid is degraded either via 3,4-dihydroxyphenylacetic acid to β-carboxymethyl-cis, cis-muconic acid (3) or via homogentisic acid (4). We have recently elucidated the metabolic pathway of p-hydroxyphenylacetic acid with the partially purified enzyme preparations as shown in Fig. I (5).

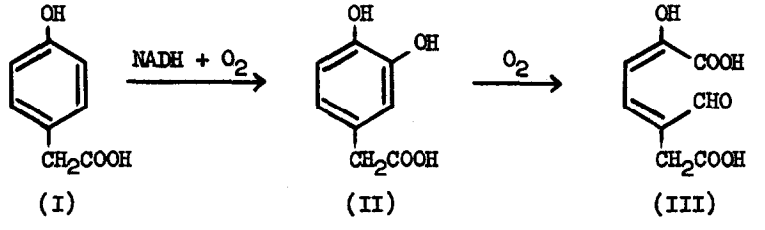

Fig. I. Oxidative Pathway of p-Hydroxyphenylacetic Acid.

During the course of these investigations we have succeeded in the isolation and crystallization of 3,4-dihydroxyphenylacetate-2,3-oxygenase (6), which catalyzes the formation of α-hydroxy-δ-carboxymethylmuconic semialdehyde (III) by the oxidative ring fission of 3,4-dihydroxyphenylacetic acid (II) in its 2,3 position. The present paper describes the purification and crystallization of 3,4-dihydroxyphenylacetate-2,3-oxygenase from cells of Pseudomonas ovalis and some of its properties.

The bacteria was grown in a medium containing 0.25% $(NH_4)_2HPO_4$, 0.15% KH_2PO_4, 0.5% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% yeast extract and 0.15% p-hydroxyphenylacetic acid with vigorous aeration at 30°. The p-hydroxyphenylacetate-adapted cells were stored as an acetone powder at -20°. Acetone powder (20 grams) was suspended in 400 ml of M/80 tris buffer (pH 7.5), mixed with 200 μg of deoxyribonuclease, and extracted overnight at 5°. The supernatant fluid (crude extract) was separated from the residue by centrifugation at 10,000 x g for 10 min.

To the crude extract, 67.5 grams of ammonium sulfate were added and the resulting precipitate was removed by centrifugation. Ammonium sulfate (51.5 grams) was then added to the supernatant. The resulting precipitate was collected by centrifugation and dissolved in 55 ml of M/80 tris buffer (pH 7.5) containing 3% ammonium sulfate.

Cold acetone (33.7 ml) was added to the ammonium sulfate-fractionated enzyme solution at -10°, and the precipitate formed was removed by centrifugation at -10° followed by the addition of 21.3 ml of cold acetone to the supernatant. The resulting precipitate was collected by centrifugation and suspended in 29 ml of M/80 tris buffer (pH 7.5). The insoluble material was removed by centrifugation.

The clear supernatant solution of the acetone-fractionated enzyme was diluted with 3 volumes of distilled water and applied to an alumina C_γ column (2.4 x 18 cm) consisting of 350 mg of alumina C_γ gel and 24 g of crystalline cellulose (Avicel, Asahikasei Ltd.). The column was first washed with M/80 tris buffer (pH 7.5) to remove the bulk of the inactive

protein and then with the same buffer containing 2% ammonium sulfate until the effluent was free from protein. The enzyme was then eluted with M/80 tris buffer (pH 7.5) containing 5% ammonium sulfate. The purification of the enzyme is summarized in Table I. The enzyme preparation thus purified showed a single shlieren boundary in the ultracentrifuge.

Table I

Purification of 3,4-Dihydroxyphenylacetate-2,3-oxygenase

Enzyme Fraction	Volume ml	Activity* units	Protein mg	Specific activity**	Recovery
Crude extract	375	8250	6487	1.27	100
Ammonium sulfate fraction 33-55% satn.	56	7616	2195	3.49	92
Acetone fraction 38-50%	28.7	7378	362	20.4	89.4
Cγ column chromatography	24.3	3423	56.1	61.0	41.5

*The assay was performed spectrophotometrically by measuring the increase in optical density at 380 mμ at 20°. This is the absorption maximum of the reaction product.

One unit of enzyme is arbitrarily defined as the amount of enzyme that produces an increase in absorbancy at 380 mμ of 1.0 in 10 seconds at 20°.

**Specific activity is defined as the number of units per mg protein.

Crystallization of the enzyme was performed as follows. To the dilute enzyme solution (4 mg protein/ml) obtained by Cγ column chromatography, finely powdered ammonium sulfate was added to 45% saturation. The turbidity was removed by centrifugation and the supernatant solution was stored at 5°. After several days, small colorless, rhombic plates had formed (Fig. II). The specific activity of the crystalline enzyme remained the same after crystallization.

The sedimentation constant (S_{20},w) of the enzyme was 7.14 cm/sec and its molecular weight was calculated to be approximately 100,000 assuming a partial specific volume of 0.75. The absorption spectrum of

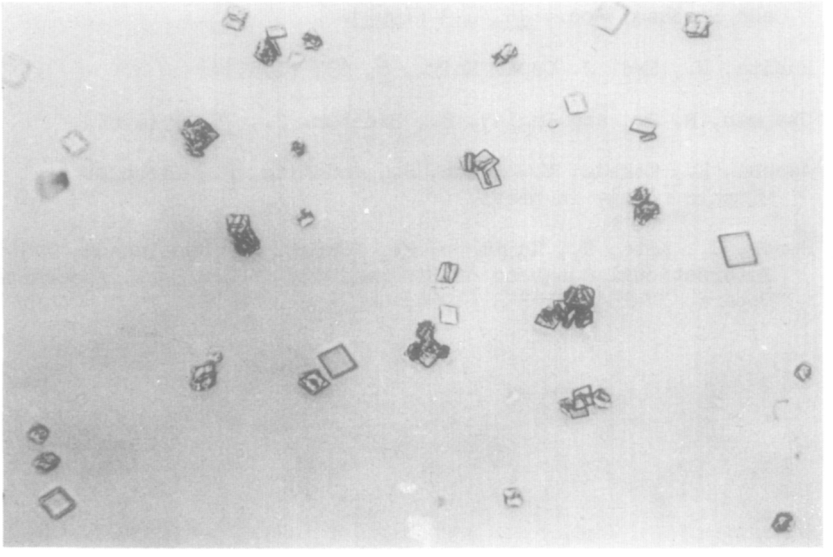

Fig. II. The Crystalline 3,4-Dihydroxyphenylacetate-2,3-oxygenase

the enzyme showed a maximum at 280 mμ and a shoulder at 292 mμ, but no evidence was found for the presence of a chromophore.

Preliminary results from colorimetric determinations with o-phenanthroline suggest that the enzyme contains approximately 5 atoms of iron per molecule of enzyme protein. Moreover, the possibility of the participation of the iron in the enzyme reaction is suggested from the inhibition experiments with chelate reagents such as 8-hydroxyquinoline and o-phenanthroline. This is further confirmed in that the enzyme inactivated by the treatment with hydrogen peroxide is restored to its original activity by ferrous ion.

The details of the properties of the crystalline 3,4-dihydroxyphenylacetate-2,3-oxygenase will be published elsewhere.

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