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PURIFICATION AND PROPERTIES OF γ-OXALOMESACONATE HYDRATASE FROM PSEUDOMONAS OCHRACEAE GROWN WITH PHTHALATE

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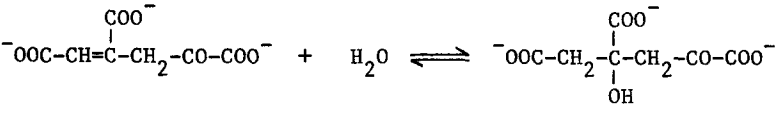
SUMMARY: Pseudomonas ochraceae produces inducibly a hydro-lyase which catalyzes the reversible conversion of γ-oxalomesaconate into (-)-γ-oxalocitramalate. The enzyme has been purified to homogeneity from the bacteria grown with phthalate. The enzyme was a dimeric protein (pI≒4.9) with a M_r of 68,000 and showed a high specificity for γ-oxalomesaconate (Km=14 μM) and (-)-γ-oxalocitramalate (Km=6.4 μM). Equilibrium constant for the hydration of γ-oxalomesaconate at pH 8.0 and 24°C was 2.5. Various thiols activated the enzyme.

The microorganisms metabolize phthalate through protocatechuate (1-3). In nonfluorescent pseudomonads protocatechuate is initially converted to 4-carboxy-2-hydroxymuconate 6-semialdehyde (4-6). Two different pathways have been proposed for the subsequent metabolism of the semialdehyde. Hydrolytic fission of the semialdehyde followed by aldol cleavage yields formate and pyruvate (7). Alternatively, the semialdehyde undergoes, successively, dehydrogenation, hydrolysis, hydration and aldol cleavage to give pyruvate and oxalacetate (3,8,9). This communication describes the purification and properties of a hydro-lyase involved in the latter pathway. The enzyme is inducibly produced in Pseudomonas ochraceae and mediates the reversible conversion of γ-oxalomesaconate ((E)-3-carboxy-5-oxo-2-hexenedioic acid) into (-)-γ-oxalocitramalate (4-carboxy-4-hydroxy-2-oxoadipic acid):



The enzyme appears to be highly specific for γ-oxalomesaconate and γ-oxalomesaconate hydratase is proposed as the trivial name for this enzyme.

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MATERIALS AND METHODS

Materials: γ-Oxalomesaconate, (±)-γ-oxalocitramalate, γ-oxalocrotonate and 2-pyrone-4,6-dicarboxylate were prepared as described previously (9,10). Vinylpyruvate was prepared by the method of Collinsworth et al. (11).
Bacteria: P. ochraceae was grown with phthalate as a sole carbon source and harvested as reported previously (12).
Enzyme assay: γ-Oxalomesaconate exhibits an absorption maximum at 265 nm (ε=6.0 $mM^{-1}cm^{-1}$) in Tris-acetate, pH 8.0, while (±)-γ-oxalocitramalate is virtually transparent at this wavelength. Based on this finding, the enzyme assay was performed by following A_{265} with a Hitachi 200-10 spectrophotometer. Unless otherwise noted, the enzyme activity was determined on hydration of γ-oxalomesaconate with a mixture (1.0 ml) containing 0.1 M Tris-acetate, pH 8.0, 0.1 mM γ-oxalomesaconate and enzyme (0.069-0.685 μg). The reaction was carried out at 24°C in a quartz cuvette (light path, 1 cm). Under these conditions, the initial rate of reaction was directly proportional to the amount of the enzyme used. One unit of enzyme was defined as the amount of enzyme that transforms 1 μmol of substrate per min. Protein was determined by the method of Bennett (13) with bovine serum albumin as a standard.
Purification procedures: All manipulations were carried out at 0-4°C unless otherwise specified. The bacterial cells (about 100 g) were suspended with 300 ml of 0.02 M K-phosphate, pH 7.0 (buffer A) and treated in a Tomy UR-150P ultrasonic disintegrator for 10 min. The sonic extract was centrifuged at 25,000 x g for 15 min. The supernatant solution was held at 55°C for 3 min and then rapidly cooled to 4°C. The solution was clarified by centrifugation. Then, solid $(NH_4)_2SO_4$ was added to the solution to 50 % saturation. The precipitate formed was collected by centrifugation at 20,000 x g for 30 min, suspended in 60 ml of buffer A and dialyzed against 2 liters of buffer A. After centrifugation to remove insoluble materials, the solution was placed onto a DEAE-cellulose column (3 x 23 cm) equilibrated with buffer A. The column was washed with 800 ml of buffer A, and the enzyme was eluted with a linear gradient obtained with 1 liter of buffer A containing 0-0.35 M KCl; 20-ml fractions were collected. The active fractions (Fr. Nos. 21 and 22) were combined and dithiothreitol was added to a final concentration of 1 mM. The solution was treated with $(NH_4)_2SO_4$, and the precipitate obtained between 50 and 65 % saturation was dissolved in 3.2 ml of 0.02 M K-phosphate, pH 7.0, containing 1 mM dithiothreitol and 10 % (v/v) ethylene glycol (buffer B). The solution was applied to a Sephadex G-200 column (1.5 x 45 cm) equilibrated with buffer B. The column was washed with the same buffer; 2.6-ml fractions were collected. The active fractions (Fr. Nos. 14-21) were combined and applied to the second DEAE-cellulose column (1.4 x 25 cm) equilibrated with buffer B. The enzyme was eluted with a linear gradient obtained with 200 ml of buffer B containing 0-0.2 M KCl; 2.6-ml fractions were collected. The active fractions (Fr. Nos. 40-42) were pooled and dialyzed against buffer B. The hydratase thus prepared could be stored at -20°C for, at least, 1 month without appreciable loss in activity.

The fractions (Fr. Nos. 31-36) in the first DEAE-cellulose column chromatography contained 4-hydroxy-4-methyl-2-oxoglutarate aldolase [EC 4.1.3.17], which catalyzes the aldol cleavage of (±)-γ-oxalocitramalate into pyruvate and oxalacetate in the presence of $MgCl_2$ (9,14). These fractions were pooled, concentrated by ammonium sulfate fractionation and were used without further purification.
Analytical methods: The molecular weight of the hydratase was determined by gel filtration with a column (1.6 x 55 cm) of Sephadex G-200 as described previously (9), except that 0.02 M K-phosphate, pH 7.0, containing 1 mM dithiothreitol was used in place of 0.02 M K-phosphate, pH 8.0. Polyacrylamide disc gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing were conducted as described by Takács and Kerese (15), Weber and Osborn (16) and Vesterberg (17), respectively. Optical rotation was measured at 589 nm with a Jasco polarimeter DIP-SL (sensitivity, ±0.002°).

RESULTS AND DISCUSSION

Purification of the hydratase: P. ochraceae uses various organic acids as growth substrates. The crude extracts prepared from the bacteria grown with the aromatic carboxylates such as phthalate, terephthalate, m-hydroxybenzoate and p-hydroxybenzoate showed the high activity (0.039-0.064 unit/mg) of γ-oxalomesaconate hydratase, while virtually no activity (less than 0.002 unit/mg) could be detected in the extract of succinate-grown bacteria. These results suggest that the bacteria produces the hydratase inducibly. The hydratase was purified from the bacteria grown with phthalate (Table 1). The crude extract contained the 2-pyrone-4,6-dicarboxylate hydrolase which catalyzed the interconversion of 2-pyrone-4,6-dicarboxylate and γ-oxalomesaconate (10). The heat treatment of the crude extract removed the majority of the hydrolase without significant loss of the hydratase and resulted in a marked increase in activity of the hydratase. The purified hydratase showed a single protein band coinciding the enzyme activity when subjected to electrophoresis on 7.5 % polyacrylamide disc gel (pH 7.5) (Fig. 1). The electrophoretic mobility of the enzyme in pH 8.9 gel was higher than that in pH 7.5 gel, but the enzyme was considerably inactivated.

Reaction product: The product from γ-oxalomesaconate was identified to be γ-oxalocitramalate by paper chromatography as described previously (9). To determine whether one or both of the enantiomers of γ-oxalocitramalate are enzymatically formed, the enzyme reaction was monitored with a polarimeter

Table 1. Purification of γ-oxalomesaconate hydratase

Fraction	Protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification (-fold)
Crude extract	2,870	0.051	146	1
Heat treatment	895	0.339	303	6.65
DEAE-cellulose (I)	9.93	23.7	235	465
Ammonium sulfate	4.54	43.0	195	843
Sephadex G-200	2.21	75.2	166	1,475
DEAE-cellulose (II)	1.01	108	109	2,118

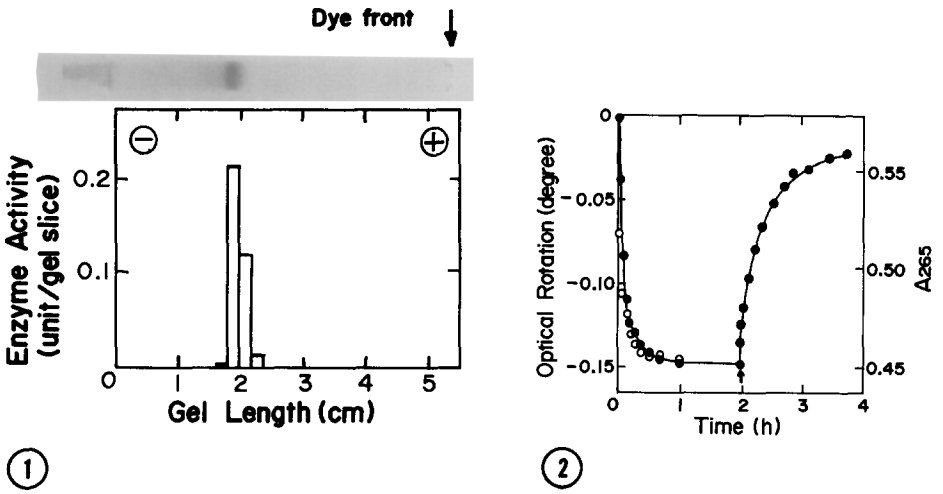

Fig. 1. Disc gel electrophoresis of the purified hydratase. The enzyme (15 μg) was subjected to disc gel electrophoresis at 4°C. Electrophoresis was carried out in duplicate with 7.5 % polyacrylamide gel (pH 7.5). After electrophoresis, one gel was stained for protein with Coomassie brilliant blue. The other was sliced into 2-mm sections. Each piece was extracted at 4°C for 3 h with 0.3 ml of buffer B and was assayed for enzyme activity.

Fig. 2. Change of optical rotation during the enzyme reaction. The mixture (10 ml) containing 0.1 M Tris-acetate, pH 7.6, 29 mM γ-oxalomesaconate and 0.45 mg of the hydratase was incubated at 24°C in a cylindrical cell (light path, 10 cm) and optical rotation (●) at 589 nm was measured at indicated time. At the same time, an aliquot of 10 μl was withdrawn and mixed with 3 ml of 0.2 M Na-acetate, pH 4.0, to stop the reaction, and then A_{265} (○) was measured. At the time marked with an arrow, 0.5 ml of 0.1 M $MgCl_2$ and 0.36 mg (62 units) of 4-hydroxy-4-methyl-2-oxoglutarate aldolase were added.

(Fig. 2). During the reaction, optical rotation at 589 nm turned to the negative side concurrently with the decrease in A_{265}. The reaction was almost complete after 1 h, and both optical rotation and A_{265} remained constant for additional 1 h, indicating that the product was only the (-)-enantiomer. On addition of 4-hydroxy-4-methyl-2-oxoglutarate aldolase and $MgCl_2$ into the reaction mixture, the optical rotation returned to the initial level due to the degradation of (-)-γ-oxalocitramalate to pyruvate and oxalacetate.

Kinetic parameters: The enzyme reaction was found to be reversible. The equilibrium for the interconversion of γ-oxalomesaconate and (-)-γ-oxalocitramalate was established at 24°C in Tris-acetate, pH 8.0, starting with either 0.09 mM γ-oxalomesaconate or 0.18 mM (±)-γ-oxalocitramalate (Fig. 3). Both forward and reverse reactions occurred monophasically and were complete after 30 min. The amounts of γ-oxalomesaconate and (-)-γ-oxalocitramalate in the

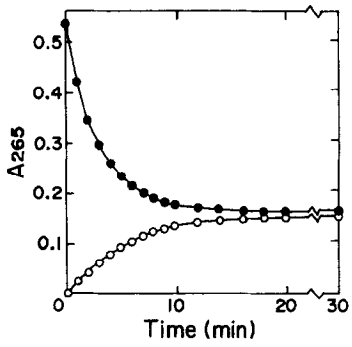

Fig. 3. Approach to equilibrium of the reaction catalyzed by γ-oxalomesaconate hydratase. The mixture (1.0 ml) containing 0.1 M Tris-acetate, pH 8.0, 0.34 μg of the hydratase and 0.09 mM γ-oxalomesaconate (●) or 0.18 mM (±)-γ-oxalocitramalate (○) was incubated at 24°C and A_{265} was monitored.

equilibrium were estimated from A_{265}. The average value for the equilibrium constant, equal to [(-)-γ-oxalocitramalate]/[γ-oxalomesaconate], obtained from several determinations was 2.5. Both forward and reverse reactions showed hyperbolic kinetics. The kinetic parameters of the enzyme reactions were determined by Lineweaver-Burk double reciprocal plots; Km and Vmax values were 14 μM and 120 units/mg, respectively, for the hydration of γ-oxalomesaconate, and were 6.4 μM and 17.4 units/mg, respectively, for the dehydration of (-)-γ-oxalocitramalate. The equilibrium constant was calculated from these values to be 3.2 by Haldane's equation (18). This value is in good agreement with that determined directly.

Substrate specificity: To examine the substrate specificity of the hydratase, various acids (0.06-2.0 mM) were tested for the hydratase-dependent absorption change between 240 and 340 nm. The enzyme was highly specific for γ-oxalomesaconate and (-)-γ-oxalocitramalate, and none of the following acids served as substrate: fumarate, maleate, crotonate, vinylpyruvate, mesaconate, γ-oxalocrotonate, 2-pyrone-4,6-dicarboxylate, citrate, L-malate, (±)-citramalate, cis- and trans-aconitate.

General properties of the hydratase: The hydratase exhibited a simple protein absorption spectrum, showing a maximum at 280 nm ($A_{280}^{1\%}_{1\,cm}$=10.2) with a

shoulder at 290 nm and a minimum at 250 nm. A M_r of 68,000 was obtained by analytical gel filtration on Sephadex G-200, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave a M_r of 37,000, suggesting that the enzyme was composed of two identical subunits. The enzyme had a pI of 4.9 and was most active at pH 8.4 with 50 % activity at pH 7.2 and 9.6 in Tris-acetate. Various thiols (1 mM) activated the enzyme about 2-fold, whereas various thiol reagents (1 mM) inhibited the enzyme at various degrees. The relative activities observed were as follows: dithiothreitol 204; cysteine 181; reduced glutathione 176; 2-mercaptoethanol 166; $HgCl_2$ 0.0; p-chloromercuribenzoate 0.0; N-ethylmaleimide 68.6; iodoacetamide 88.2; and iodoacetate 92.2. Among various metal ions (1 mM) tested, $ZnCl_2$ and $CuSO_4$ inhibited 91-96 % activity, possibly due to the chelation of metal ion with γ-oxalomesaconate. $CoCl_2$ and $NiCl_2$ inhibited the enzyme to lesser extents (46-48 %). Mohr's salt, $MnCl_2$, $FeCl_3$, $CaCl_2$ and $MgCl_2$ were ineffective. No apparent effect was detected with various metal chelating agents (1 mM) such as EDTA, 2,2'-bipyridine, 8-quinolinol and tiron. It has been known that Fe^{2+} and Mn^{2+} activate the several hydro-lyases in conjunction with thiols (11,19,20). In contrast, γ-oxalomesaconate hydratase did not show any Fe^{2+}- or Mn^{2+}-dependent activation in the presence of dithiothreitol under anaerobic conditions.

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