CRYSTALLINE AMINO ACID RACEMASE WITH LOW SUBSTRATE SPECIFICITY

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Summary The preparation of crystalline amino acid racemase with low substrate specificity from Pseudomonas striata is described. The enzyme is homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis. The molecular weight is 110,000. The enzyme exhibits absorption maxima at 280 and 420 mμ, and requires pyridoxal 5'-phosphate for the maximal activity. Leucine, α-aminobutyric acid, alanine, norvaline, norleucine, arginine, methionine, lysine, and ethionine are racemized by this enzyme in the order of increasing the reactivity.

A few amino acid racemases have been purified to homogeneity; alanine racemase from Pseudomonas sp.(1) and from Pseudomonas putida (2), proline racemase from Clostridium sticklandii (3), and arginine racemase (4).

Recently, evidence was obtained for the occurrence of a new amino acid racemase catalyzing the conversion of either D or L isomers of leucine and α-aminobutyric acid to the racemates in the cell-free extract of Pseudomonas striata (5). In the present communication, we report the purification, crystallization of this enzyme, and some of its properties. The substrate specificity is shown to be extremely low.

Purification and Crystallization

Ps. striata AKU 0813 was grown in the medium containing 1.0% peptone, 1.0% meat extract and 0.5% NaCl. The pH was adjusted to about 7.2 with potassium hydroxide. The cultures were grown at 30° for 20 hours under aeration. The harvested cells were washed twice with 0.85% sodium chloride solution. The yield of cells was approximately 7.5 g wet weight per liter of the medium. All subsequent operations were carried out at 0-5°.

Step I. The washed cells (about 1050 g wet weight) were suspended in 1 liter of 0.01 M potassium phosphate buffer, pH 7.0, containing 2 x 10^{-5} M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol, and subjected, in 250 ml portions, to sonication in a 19-kc Kaijo Denki oscillator for 20 minutes. The intact cells and cell debris were removed by centrifugation.

Step II. The supernatant solution (1395 ml) was brought to 30% saturation with ammonium sulfate, and centrifuged at 17,000 g for 30 minutes. Ammonium sulfate was added to the supernatant to 55% saturation. The precipitate collected by centrifugation, was dissolved in 300 ml of 0.01 M potassium phosphate buffer, pH 7.0, containing 10^{-5} M pyridoxal 5'-phosphate. The enzyme solution was dialyzed against 100 volumes of the same buffer. The insoluble materials formed during the dialysis was discarded.

Step III. The enzyme solution (400 ml) was placed on two DEAE-cellulose columns (4 x 40 cm) equilibrated with the dialysis buffer. After the columns were washed thoroughly with 0.01 M potassium phosphate buffer, pH 7.0, containing 0.05 M sodium chloride and 10^{-5} M pyridoxal 5'-phosphate, the enzyme was eluted with the buffer supplemented with 0.1 M sodium chloride and 10^{-5} M pyridoxal 5'-phosphate. The active fractions (450 ml) were pooled, concentrated by addition of ammonium sulfate (65% saturation), and dialyzed against 100 volumes of 0.01 M potassium phosphate buffer, pH 7.0, containing 10^{-5} M pyridoxal 5'-phosphate.

Step IV. The dialyzed enzyme solution was applied to a DEAE-cellulose column (2 x 56 cm) equilibrated as stated above. After the chromatography described in Step III, was repeated, the active fractions (440 ml) were combined and concentrated by addition of ammonium sulfate (65% saturation). The precipitate was dissolved in a minimum volume of 5 mM potassium phosphate buffer, pH 7.0, containing 10^{-5} M pyridoxal 5'-phosphate.

Step V. The enzyme solution was applied to a Sephadex G-200 column (2 x 53 cm) buffered with 5 mM potassium phosphate buffer, pH 7.0, containing 10^{-5} M pyridoxal 5'-phosphate, and eluted with the same buffer (Fig. 1).

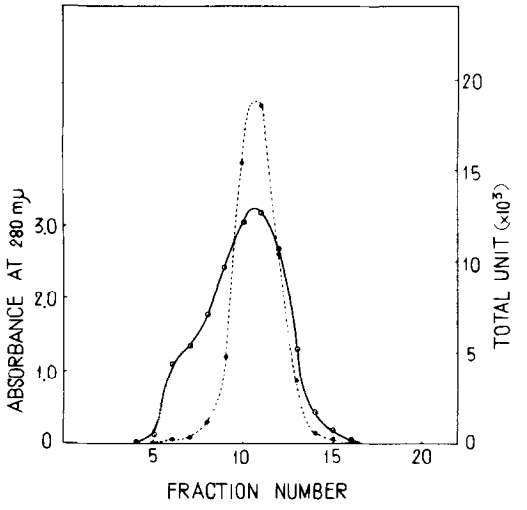

Figure 1. Elution diagram of a typical Sephadex G-200 column. The enzyme was assayed in the reaction system containing L-methionine as a substrate according to the procedure described previously (5). (○), Absorbance at 280 mμ; (●), Total activity.

The active fractions (50 ml) were pooled and concentrated by addition of ammonium sulfate (65% saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate buffer, pH 7.0, containing 10^{-5} M pyridoxal 5'-phosphate.

Step VI. Ammonium sulfate was added gradually to the enzyme solution until a faint turbidity was obtained. The pH of solution was kept constant at 7.0 with 14% ammonium hydroxide solution. On standing overnight at about 3°, crystal formation occurred. The crystals took the form of thin rectangular leaflets with a light yellow color. A summary of the purification procedure is presented in Table I.

Properties

The crystalline enzyme is homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis (Fig. 2). The sedimentation coefficient of the enzyme, calculated for water at 20° and zero protein concentration, is 4.45 S. The molecular weight was determined by the

TABLE I. PURIFICATION OF AMINO ACID RACEMASE WITH LOW SUBSTRATE SPECIFICITY

Step	Fraction	Total Protein (mg)	Total Units	Specific Activity	Yield (%)
I.	Crude extract	80,800	236,390	2.926	100
II.	Ammonium sulfate fractionation	21,200	115,500	5.456	48.8
III.	First DEAE-cellulose chromatography	1,090	88,770	81.4	37.5
IV.	Second DEAE-cellulose chromatography	285	56,430	198.0	23.9
V.	Sephadex G-200 chromatography	89	48,290	543.4	20.4
VI.	Crystallization	36.8	26,070	708.4	11.0

The enzyme was assayed in the reaction system containing L-methionine as a substrate according to the procedure described previously (5). One unit of the enzyme is defined as the amount of enzyme that catalyzes the formation of 1 μmole of the antipode per minutes. Specific activity is expressed as units per mg of protein. Protein was determined by the method of Lowry et al. (6), or estimated from the absorbance at 280 mμ.

sedimentation equilibrium method (8). Assuming a partial specific volume of 0.74, a molecular weight of 110,000 ± 400 was obtained. The enzyme exhibits absorption maxima at 280 and 420 mμ. No appreciable spectral shifts occurred when pH was varied. Incubation of the enzyme with 0.01 M hydroxylamine solution (pH 7.0), followed by dialysis against 0.01 M potassium phosphate buffer, pH 7.0, resulted in the formation of apoenzyme. The apoenzyme exhibits no peak at 420 mμ, and can be reactivated by addition of pyridoxal 5'-phosphate. The substrate specificity of the enzyme was found to be extremely low in comparison with other amino acid racemases studies so far, e. g., proline racemase (3) and arginine racemase (4).

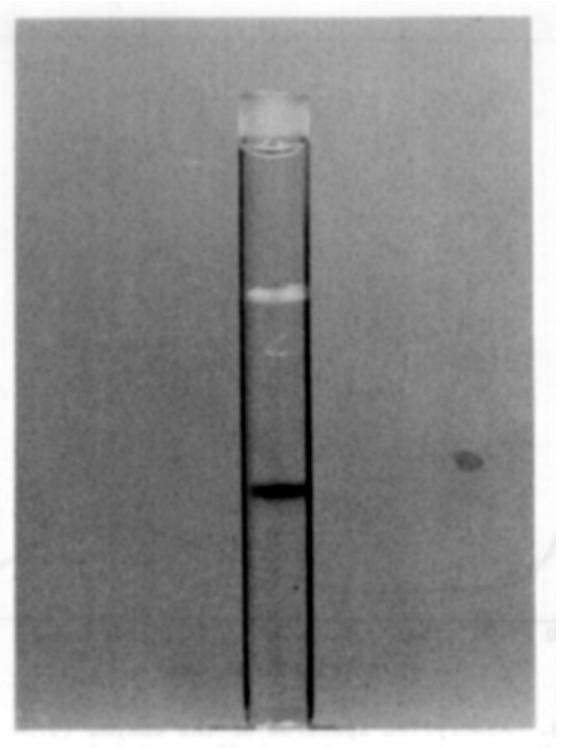

Figure 2. Disc gel electrophoresis of the enzyme. A sample of the crystalline enzyme preparation (16.5 μg) was electrophoresed under the conditions of Davis (7) using 7.5% polyacrylamide gel and Tris-glycine buffer, pH 8.9. After the run, protein was stained with 1.0% Amido-Schwarz in 7% acetic acid.

The relative activity was 1 for L-leucine; 1.8 for L-α-aminobutyric acid; 3.3 for L-alanine; 5.2 for L-norvaline; 5.9 for L-norleucine; 7.5 for D-arginine; 10.6 for L-methionine; 13.2 for D-lysine; and 15.9 for L-ethionine, when the enzyme was assayed at pH 8.3 (in 7/20 M pyrophosphate buffer). The racemizing activities for D-lysine and L-arginine were determined with L-lysine decarboxylase and arginase, respectively. The enzyme has a maximum reactivity in the pH range of 7.5-10.0 for all the substrates. The Km values were calculated to be 3.3×10^{-2} M for L-leucine and L-α-aminobutyric acid, 3.6×10^{-2} M for L-alanine, 3.8×10^{-2} M for L-methionine, and 3.0×10^{-2} M for D-lysine.

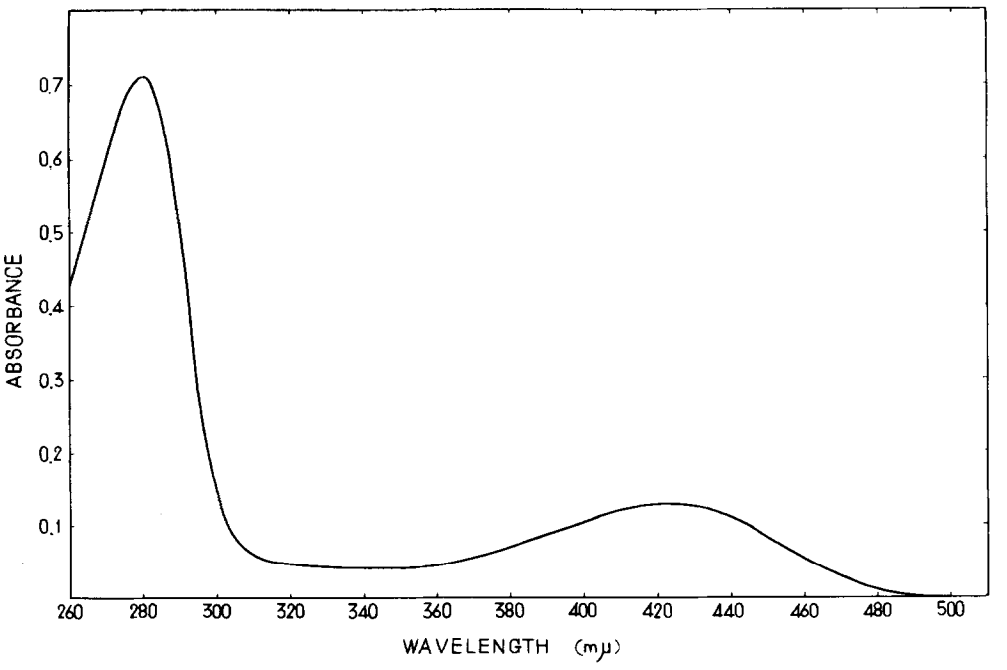

Figure 3. Absorption spectrum of the enzyme (0.84 mg per ml) in 0.01 M potassium phosphate buffer, pH 7.0.

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