COENZYME CONTENT OF PURIFIED ALANINE RACEMASE FROM PSEUDOMONAS

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Alanine racemase, induced in Pseudomonas putida by growth on DL-alanine, was purified about 1000-fold to homogeneity. The purified enzyme contains approximately one molar equivalent of pyridoxal phosphate by absorbance, fluorescence, and microbiological assay. No evidence for the presence of a flavin coenzyme was found.

Alanine racemase was the first amino acid racemase to be studied enzymatically (Wood and Gunsalus, 1951). Racemases for other common amino acids have subsequently been detected in various bacteria (for references before 1964, see Adams and Norton, 1964; also Markovetz et al, 1966; Soda et al, 1967). In certain of these enzymes, including the alanine racemase first studied (Wood and Gunsalus, 1951), pyridoxal phosphate has been implicated as a coenzyme by inhibition or resolution studies. No direct evidence, however, has been reported for pyridoxal phosphate as a component of racemases for primary amino acids; such observations would require high absolute purity of the racemase studied. The question of coenzyme content is made more cogent by the finding that active preparations of racemases for secondary amino acids, hydroxyproline-2-epimerase (Adams and Norton, 1964) and proline racemase (Cardinale, 1965), lack pyridoxal phosphate, and also by the conclusion that partly-purified glutamic racemase of Lactobacillus fermenti (Tanaka et al, 1961) and alanine racemase of Bacillus subtilis (Diven et al, 1964) require a flavin coenzyme for activity.

The present communication describes the purification to apparent homogeneity of an inducible alanine racemase from Pseudomonas putida. From both absorption spectrophotometry, fluorescence measurements, and microbiological assays, the purified enzyme contains approximately one mole of pyridoxal phosphate per mole of enzyme. In contrast, no stoichiometrically-significant quantity of flavin could be detected spectrally or fluorometrically in the purified enzyme.

Growth of Cells, Enzyme Assay and Purification - A strain of P. putida (ATCC 15070), earlier used for studies of hydroxyproline

metabolism, was grown in mineral media of composition previously described (Yoneya and Adams, 1961), containing 0.05% yeast extract (Difco) and 0.2% DL-alanine as the only organic additions. Cells were grown in 2-liter flasks (1 liter of medium) with shaking, or in carboys with forced aeration, at 30° for 18 - 20 hours; cells (2 to 4 g per liter) were washed and then extracted by sonic lysis in 5 volumes of potassium phosphate, 0.005 M, pH 7.4.

Alanine racemase was assayed in a 2-step procedure. Components of Incubation I (25°, 10 min.) were Tris, 0.1 M, pH 7.8; L-alanine, 0.1 M; and enzyme, in a final volume of 1 ml. Incubation I was stopped by heating at 100° for 2 min; D-alanine formed was measured in Incubation II, which contained a small sample of Incubation I; Tris, 0.1 M, pH 7.8; DPNH, 0.2 mM; crystalline kidney D-amino acid oxidase (Calbiochem), 0.05 mg; in a final volume of 1 ml. The reaction was begun by adding crystalline lactate dehydrogenase (Sigma), 0.01 ml; pyruvate formed from D-alanine was estimated by the fall in DPNH absorbance. One unit of enzyme is the quantity forming 1 μmole of D-alanine per minute in Incubation I.

Crude cell extracts derived from cultures in alanine media contained about 15 times more enzyme per mg of protein than after growth in similar media containing glucose as carbon source. Growth on L-alanine, D-alanine, or DL-alanine, ranging from 0.2 to 1.0%, led to no reliable differences in cell yield or enzyme content.

The enzyme in sonic extracts was recovered entirely in the supernatant fluid after 90-min. centrifugation at 100,000 g. The latter supernatant (usually in batches of 150 - 200 ml) was treated with 0.2 volumes of freshly-dissolved 1% protamine sulfate and the resultant precipitate was discarded. The protamine supernatant was adjusted to pH 8.9 with dilute ammonia and brought to 0.2 saturation with solid ammonium sulfate. A small precipitate was discarded and the supernatant solution was treated with more ammonium sulfate to reach 0.45 saturation. The precipitate, dissolved in a minimum volume of potassium phosphate, 0.05 M, pH 7.4., was passed through a column (3 x 80 cm) of Sephadex G-100; the column was washed with potassium phosphate, 0.005 M, pH 7.4. Fractions high in enzyme were pooled, adjusted to pH 6.2 with dilute acetic acid, and treated with calcium phosphate gel (Sigma), 14 mg (dry weight) per mg of protein. The centrifuged gel was discarded and the supernatant fluid was passed through a column (0.9 x 24 cm) of DEAE-cellulose. The column was eluted with linearly-increasing NaCl in 0.005 M potassium phosphate, pH 7.4; the enzyme emerged at about 0.15 M NaCl. Pooled enzyme-containing fractions were dialyzed overnight against 0.005 M potassium phosphate, pH 7.4, and passed through a column of Ecteola (0.9 x 22 cm)

TABLE I PURIFICATION OF ALANINE RACEMASE

Fraction	Volume	Total Protein[a]	Total Units	Specific Activity
	ml	mg		units/mg
1. Spinco Supernatant	170	1700	5400	3.2
2. Protamine Supernatant	200	730	4900	6.6
3. Ammonium Sulfate	4.5	82	4100	50
4. Sephadex Filtrate	28	16	2800	175
5. Calcium Phosphate	30	8.4	2700	320
6. DEAE Eluate, dialyzed	18	2.0	1300[b]	650
7. Ecteola Eluate	14	0.1	368[c]	3680

[a]Protein was determined turbidimetrically (Stadtman et al, 1951) in Fractions 1 - 3, and by absorbance at 280 and 260 mμ in Fractions 4 and 5. Fractions 6 and 7 required the method of Waddell (1956) before concentration.

[b]Total units were 1900 before overnight storage and dialysis.

[c]An additional 700 units with a specific activity of 1500 were obtained.

The column was eluted with linearly-increasing NaCl in 0.005 M potassium phosphate, pH 7.4. The enzyme emerged at about 0.07 M NaCl. Table I summarizes the purification steps for a specific preparation.

Purity and Properties of Enzyme - The enzyme appeared stable until Fraction 6 (Table I) and required neither thiols nor EDTA. Fractions 6 and 7 were unstable, particularly Fraction 7, which lost significant activity on storage at -15° for several days, or on concentration by ultrafiltration or lyophilization. Additon of DL-alanine, thiols, EDTA or pyridoxal phosphate during storage had no stabilizing effect.

Fraction 7 repeatedly gave a single sharp protein-staining band on polyacrylamide gel electrophoresis (Fig 1). Although no activity was eluted from this band (presumably due to the instability of the

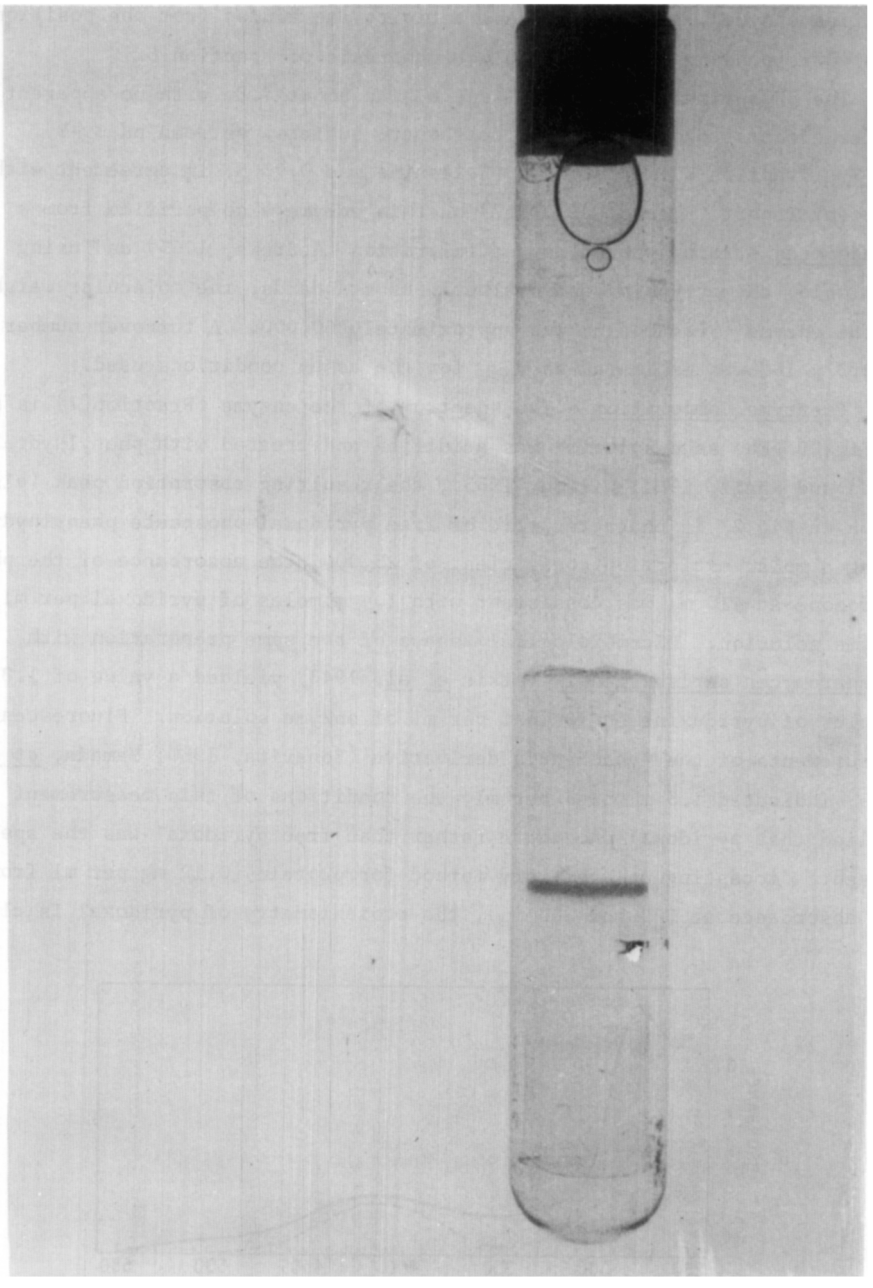

Fig. 1. Polyacrylamide gel electrophoresis of Fraction 7.

A sample of Fraction 7, 20-fold concentrated and containing about 12 micrograms of protein, was electrophoresed under the conditions of Davis (1964) using 7% polyacrylamide and pH 8.9 (Tris) and pH 9.5 (Tris) in the stacking gel and running gel respectively. The gel was stained with Coomassie blue.

purified enzyme), alanine racemase activity was eluted from the position of a corresponding band on gel electrophoresis of Fraction 6.

The pH optimum (Fractions 1 and 6) was about 7.8, with no apparent buffer effects (phosphate, Tris, carbonate buffers, between pH 6 -9). The K_m (Fraction 6) measured for L-alanine was 0.03 M, in agreement with a recent report (Free et al, 1967) on this enzyme also purified from a Pseudomonas strain. Based on gel filtration (Andrews, 1965) and using myoglobin, chymotrypsin, and ovalbumin as standards, the molecular weight of the enzyme (Fraction 6) was approximately 60,000. A turnover number of 2.3×10^4 was estimated as V_{max} for the assay conditions used.

Coenzyme Composition - The spectrum of the enzyme (Fraction 7) is shown in Fig 2. The same solution was acidified and treated with phenylhydrazine (Wada and Snell, 1961; Wilson, 1963); the resulting absorption peak (also shown in Fig 2) is characteristic of free pyridoxal phosphate phenylhydrazone Using a molar extinction coefficient of 25,000, the absorbance of the phenylhydrazone at 410 mμ was consistent with 1.4 mμmoles of pyridoxal per ml of enzyme solution. Microbiological assays of the same preparation with Saccharomyces carlsbergensis (Atkin et al, 1943) yielded a value of 1.8 mμmoles of pyridoxine equivalent per ml of enzyme solution. Fluorescence measurements of the cyanohydrin derivative (Bonavita, 1960; Yamada, et al, 1966) indicated 1.5 mμmoles per ml; the conditions of this measurement implied that pyridoxal phosphate rather than free pyridoxal was the species present. Accepting an arbitrary method for protein (0.12 mg per ml from the absorbance at 280 and 260 mμ), the stoichiometry of pyridoxal is closest

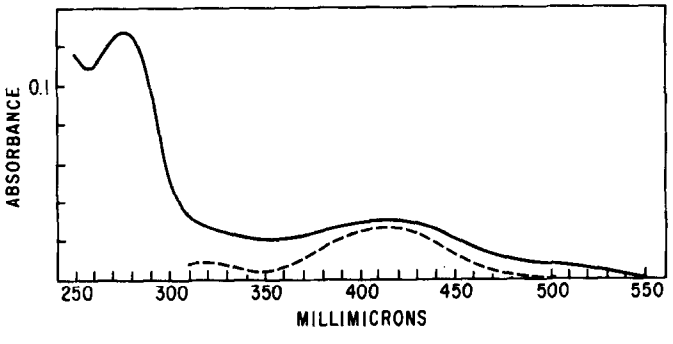

Fig. 2. Spectra of enzyme and phenylhydrazine-treated enzyme.

Fraction 7 was concentrated 17-fold (final protein concentration, 0.12 mg per ml). Spectra were obtained on a Cary Model 11 with full scale corresponding to 0.1 absorbance unit. Solid line: spectrum in 0.005 M potassium phosphate, pH 7.4. Dashed line: spectrum after treatment with acid phenylhydrazine (Wilson, 1963) relative to a reagent blank with dilute phosphate buffer in place of enzyme.

to 1 mole per mole of enzyme.

Fluorometric examination of two separate preparations of purified enzyme by the method of Burch (1957) gave only a few percent of the fluorescence yield expected for a molar equivalent of either FMN or FAD. It could be argued that alanine racemase may bind a flavin coenzyme too tightly for release by trichloracetic acid; however, no indication of flavin absorbance was seen in spectra of the purified enzyme, either directly or in difference spectra after treatment with sodium hydrosulfite.

Discussion - The findings above support the conclusion that alanine racemase from Pseudomonas contains at least one molar equivalent of pyridoxal phosphate, but no flavin. Earlier kinetic and resolution experiments have suggested pyridoxal phosphate as a coenzyme for racemases in general and for alanine racemase from Pseudomonas in particular (Adams and Norton, 1964; Free, et al, 1968). The observations presented here may be the first direct evidence for this coenzyme as a component of an amino acid racemase. Our failure to detect a flavin coenzyme does not support the interpretation of data for other racemases of unknown purity (Tanaka et al, 1961; Diven et al, 1964).

Induction of alanine racemase by growth on alanine as a carbon source suggests that L-alanine may be metabolized via conversion to D-alanine, comparable to the obligatory role of hydroxyproline epimerase in hydroxyproline metabolism by the same cell strain (Gryder and Adams, 1969). Our strain resembles P. aeruginosa (Marshall and Sokatch, 1968) in that washed particles rapidly oxidize D-alanine but not L-alanine, while whole cells or homogenates oxidize both antipodes. Furthermore, we have found that at 10^{-4} M, aminooxyacetate (reported by Free et al (1967) to be a potent inhibitor of alanine racemase in another Pseudomonas strain) inhibits the oxidation of L-alanine, but not D-alanine, both in whole cells and homogenates. These findings are consistent with racemization as an obligatory step in L-alanine metabolism, but do not exclude a possible independent oxidation of L-alanine by a soluble, aminooxyacetate-inhibitable system, e.g. one requiring a transaminase.

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