Properties of α -Amino- ϵ -caprolactam Racemase from *Achromobacter obae*

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α -Amino- ϵ -caprolactam racemase, which occurs in the cytoplasmic fraction of *Achromobacter obae*, has been purified to homogeneity. It has a monomeric structure with a molecular weight of approximately 50,000. The absorption spectrum of the enzyme exhibits maxima at 280 and 412 nm at pH 7.3, and is independent of pH from 6.0 to 8.0. One mole of pyridoxal 5'-phosphate is bound per mol of the enzyme. Incubation of the enzyme with hydroxylamine resulted in the formation of the apoenzyme. D- and L- α -Amino- ϵ -caprolactams are the only substrates. The maximum activity is found at pH 8.8 for both the isomers. Michaelis constants are as follows: 8 mM for D- α -amino- ϵ -caprolactam, 6 mM for L- α -amino- ϵ -caprolactam and 2.1×10^{-7} M for pyridoxal 5'-phosphate. The enzyme is inhibited significantly by CuSO_4 , HgCl_2 , thiol reagents such as N-ethylmaleimide and p-chloromercuribenzoate, and carbonyl reagents (e.g., phenylhydrazine and hydroxylamine). α -Amino- ϵ -caprolactam racemase catalyzes the α -proton exchange of the substrate with deuterium during racemization in deuterium oxide.

α -Amino- ϵ -caprolactam is a chiral heterocyclic compound synthesized chemically, and is used as the starting material for the enzymatic production of L-lysine in industry. The process is composed of two new enzymatic reactions: the stereo-selective hydrolysis of L- α -amino- ϵ -caprolactam to L-lysine by L- α -amino- ϵ -caprolactamase (EC class 3.5.2) of a yeast, and the racemization of α -amino- ϵ -caprolactam by bacterial α -amino- ϵ -caprolactam racemase (EC class 5.1.1).^{1,2)} Thus, L-lysine is produced with a yield of nearly 100%.³⁾ We have purified both the enzymes to homogeneity and reported briefly their fundamental properties.^{4,5)}

Various racemases have been demonstrated in bacteria and actinomycetes, but only a few enzymes have been purified to homogeneity for enzymological studies: pyridoxal 5'-phosphate dependent amino acid racemases,^{6~8)} imino acid racemases with no cofactor requirement,^{9,10)} an ATP dependent phenylalanine racemase¹¹⁾ and a Mg^{++} dependent non-amino

acid racemase, mandelate racemase.¹²⁾ α -Amino- ϵ -caprolactam racemase is unique among racemases in that it acts exclusively on a synthetic non-carboxylic compound i.e., an intramolecular cyclic amide of L-lysine.⁵⁾ We have shown that this enzyme requires pyridoxal 5'-phosphate as a coenzyme.⁵⁾ Thus, the enzyme is the only pyridoxal 5'-phosphate enzyme acting on an α -carboxyamide derivative of an amino acid. We here describe the localization of the enzyme in bacterial cells and the physicochemical and enzymological properties of the enzyme purified from *Achromobacter obae*.

MATERIALS AND METHODS

Materials. D-, L- and DL- α -Amino- ϵ -caprolactams were supplied by Toray Industries Inc., Japan; pyridoxal 5'-phosphate was from Kyowa Hakko Kogyo, Tokyo, Japan; sodium lauryl sulfate especially prepared for protein research, D-cycloserine, and L- and DL-penicillamine were from Nakarai Chemicals, Kyoto, Japan; L-tryptophanamide, L-leucinamide, glycyl-L-leucine, L-

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leucyl-glycine, L-valyl-glycine, L-leucyl-L-alanine, L-valyl-L-alanine, egg white lysozyme, DNase II, RNase A and the standard proteins for molecular weight determinations were from Sigma; and 2-methyl-3-benzothiazolone-hydrazone·HCl was from Aldrich Chemicals. Other chemicals were analytical grade reagents.

Growth medium and cultural conditions. *A. obae* FERM-P 776, supplied by Toray Industries Inc., Japan, was grown aerobically at 28°C for 19 hr in a medium containing L-aspartate and DL- α -amino- ϵ -caprolactam as described previously.⁵⁾ The cells harvested were washed twice with 0.85% NaCl and subsequently with 10 mM potassium phosphate buffer (pH 7.2) containing 20 μ M pyridoxal 5'-phosphate, 0.25 M sucrose and 0.01% 2-mercaptoethanol. The washed cells were stored at -20°C until use.

Separation of membrane from cytoplasmic fractions. The membrane and the cytoplasmic fractions were prepared as described previously.¹³⁾ The cells were washed with 10 mM potassium phosphate buffer (pH 7.2) containing 20 μ M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol. One gram (wet weight) of the freshly grown cells were suspended in 2.0 ml of 50 mM Tris-HCl buffer (pH 8.0). Ethylenediaminetetraacetate (disodium salt) (7.5 μ Mol) and egg white lysozyme (1 mg) were added to the cell suspension. After incubation at 25°C for 40 min, the suspension was frozen and thawed twice to lyse the cells. Magnesium chloride (15 μ Mol), DNase II and RNase A (0.2 mg each) were added to the lysed cells, followed by incubation at 25°C for 2 hr. The solution was centrifuged at 14,000 $\times$ g at 0°C for 40 min. The membrane pellet was suspended in 1.5 ml of 50 mM Tris-HCl buffer (pH 8.0) and washed by centrifugation. Both the supernatant fractions were combined.

Enzyme preparation. α -Amino- ϵ -caprolactam racemase was purified from a cell-free extract of *A. obae* FERM-P 776 as described previously.⁵⁾ The specific activity of the homogeneous preparation was approximately 378 units per mg of protein.

Enzyme and protein assays. α -Amino- ϵ -caprolactam racemase was assayed by the polarimetric rotation change of D- or L- α -amino- ϵ -caprolactam or by an enzymatic method with L- α -amino- ϵ -caprolactamase (EC class 3.5.2) and L-lysine- α -oxidase (EC class 1.4.3) as described previously.⁵⁾

One unit of enzyme is defined as the amount of enzyme that catalyzes the racemization of 1 μ Mol of D- α -amino- ϵ -caprolactam per min. The specific activity was expressed as units per mg of protein. Protein was determined by the method of Lowry *et al.*,¹⁴⁾ with bovine serum albumin as a standard. Alanine racemase activity was assayed according to Rosso *et al.*,⁶⁾ and the unit is defined as described above for α -amino- ϵ -caprolactam racemase. The reaction mixture (2.0 ml; pH 8.0) contained

100 μ Mol of L-alanine, 200 μ Mol of Tris-HCl buffer (pH 8.0), 20 nmol of pyridoxal 5'-phosphate and an aliquot of cytoplasmic or membrane fraction. After incubation at 37°C for 1 hr, the reaction was stopped by heating at 100°C for 5 min. D-Alanine formed was determined with a reaction mixture (2.0 ml) containing 2.5 μ Mol of NADH, 200 μ Mol of Tris-HCl buffer (pH 8.0), 0.12 unit of crystalline D-amino acid oxidase, 5 units of lactate dehydrogenase, and an aliquot of the above reaction mixture.

Polyacrylamide gel electrophoresis. Disc gel electrophoresis was performed in 7.5% polyacrylamide gel.¹⁵⁾ After electrophoresis, protein was stained with Coomassie Brilliant Blue R-250. To measure the enzyme activity in the gel, the electrophoresis buffer was supplemented with 0.25 M sucrose, 20 μ M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol. The gel was electrophoresed for 30 min before use.

Isoelectric focusing. Isoelectric focusing of the enzyme was carried out in polyacrylamide gel by the method of Righetti and Drysdale¹⁶⁾ with a carrier ampholite with a pH range of 3.5~10.0 at 4°C.

Spectrophotometry. Absorption spectra were taken with a Union SM-401 recording spectrophotometer.

¹H-NMR studies. The enzyme reaction in ²H₂O was conducted as follows with a reaction mixture (2.0 ml; pD 8.2) containing 200 μ Mol of the substrate and 40 nmol of pyridoxal 5'-phosphate in 99.75% ²H₂O (Merck). The enzyme (6.5 units) was added to the mixture below 0°C. An aliquot (0.5 ml) of the reaction mixture was transferred to an NMR tube and immediately frozen. The rest of the reaction mixture was used to measure the racemization of the substrate with a recording polarimeter as described previously.⁵⁾ The reaction was started by thawing the reaction mixture rapidly at 37°C. At appropriate time intervals ¹H-NMR spectra and peak integrals were taken with a JEOL JNM MH 100 spectrometer.

RESULTS

Intracellular localization of α -amino- ϵ -caprolactam racemase

A. obae FERM-P 776 cells cultivated at 28°C for 19 hr were treated with lysozyme, and the cell lysate was separated into cytoplasmic and membrane fractions by ultracentrifugation. The cytoplasmic fraction contained about 75% of the total α -amino- ϵ -caprolactam racemase activity. The activity detected in the membrane fraction (25%) is probably due to incomplete cell lysis during lysozyme treat-

ment. We determined alanine racemase activity of both the fractions. This activity was found mostly in the membrane fraction: cytoplasmic fraction, 0.04; and membrane fraction, 0.14 unit. These indicate that α -amino- ϵ -caprolactam racemase is localized in the cytoplasm, whereas alanine racemase is in the bacterial cell membrane.

Purity and molecular properties

The purified enzyme was shown to be homogeneous upon ultracentrifugation and sodium lauryl sulfate (SDS)-polyacrylamide gel electrophoresis.⁵⁾ The sedimentation coefficient ($S_{20,w}$) was 4.28 S at 20°C with 10 mM potassium phosphate buffer (pH 7.2) containing 20 μ M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol at a protein concentration of 1.2 mg. It, however, showed a faint protein band in addition to the main band on the gel electrophoresed under the conditions of Davis¹⁵⁾ (Fig. 1A). The isoelectric points of the major and minor bands were estimated to be 5.9 and 5.5, respectively. To determine the activity of these proteins, 50 μ g of the purified enzyme was electrophoresed in 7.5% polyacrylamide gel (0.4 $\times$ 70 mm). After electrophoresis, the gel was immediately sliced in 2 mm sections, and was dipped in 2.0 ml of the standard reaction mixture. After incubation at 37°C for 18 hr, the optical rotation change of the reaction mixture was measured. Another gel was used for protein staining by Coomassie Brilliant Blue R-250. Both the protein bands were found to show the enzyme activity (Fig. 1B). However, the two proteins were not separated by SDS-gel electrophoresis,^{17,18)} gel filtration, ion exchange and other chromatographies. Therefore, the faint band is an artifact developed during disc gel electrophoresis or an inherent subform of the enzyme.

The molecular weight of the enzyme was approximately 51,000⁵⁾ by the Sephadex G-200 gel filtration method.¹⁹⁾ The Stokes radius of the enzyme was calculated to be 31×10^{-8} cm. The molecular weight of the polypeptide chain was determined to be about 49,000 by the method of Weber and Osborn.¹⁷⁾ These results

FIG. 1. Disc Gel Electrophoresis of Purified α -Amino- ϵ -caprolactam Racemase.

A, disc gel electrophoresis of 50 μ g of enzyme (in 7.5% polyacrylamide gel).¹⁵⁾ B, relative activity of the enzyme in the unstained gel slices (2 mm) and relative absorbance of the stained gel at 660 nm measured with a Shimadzu dual wavelength TLC Scanner (Model CS 900).

suggest that the enzyme is composed of a single polypeptide chain.

Stability, effect of pH and temperature

We have reported the conditions for stabilization of α -amino- ϵ -caprolactam racemase in the cell-free extract of *A. obae*, and have shown that the enzyme retained approximately 90% of its initial activity in the presence of 0.25 M sucrose after storage at -20°C for 4 months.²⁰⁾ The purified enzyme can be stored at -20°C for at least 6 months in the presence of 0.25 M sucrose in 10 mM potassium phosphate buffer (pH 7.3, containing 20 μ M pyridoxal 5'-phosphate and 0.01% 2-mercaptoethanol) with little loss of activity, when the protein concentration is more than 0.3%, whereas it is inactivated significantly by freezing in a dilute solution (e.g., less than 0.1%). Thus, a 1% enzyme solution was routinely stored at -20°C in presence of 0.25 M sucrose until use.

The maximum enzyme activity was observed at pH 8.8 for both D- and L- α -amino- ϵ -caprolactams. The activity in glycine-KCl-KOH buffer²¹⁾ was higher than that in Tris-HCl, tricine-KOH and sodium pyrophosphate buffers. The enzyme was stable in the pH range of 6~9 when assayed after incubation

at 25°C for 20 min. The initial velocity during the first 5 min incubation was measured at different temperatures with D- α -amino- ϵ -caprolactam as a substrate at pH 8.0 (potassium phosphate buffer). The maximum activity was found at 45~65°C. The enzyme was heated in 10 mM potassium phosphate buffer (pH 7.2) at different temperatures for 20 min, and then the remaining activities were measured. The enzyme retained the following activities: 60°C, 100%; 65°C, 95%; 70°C, 85%; 75°C, 60% and 80°C, 25%.

Absorption spectrum of the enzyme and reduction with sodium borohydride

The holoenzyme exhibited absorption maxima at 280 (ϵ , 58,300) and 412 nm (ϵ , 11,700) at pH 7.3 (Fig. 2A). No appreciable spectral shifts were caused by varying the pH between 6 and 8. Reduction of the enzyme with sodium borohydride by the dialysis method of Matsuo and Greenberg²²) affected both the absorption spectrum (Fig. 2B) and the activity. The reduced enzyme was catalytically inactive and the addition of pyridoxal 5'-phosphate did not reverse the inactivation. This suggests that the borohydride reduces the aldimine linkage formed between the 4-formyl group of pyridoxal 5'-phosphate and the ϵ -amino group of a lysine residue of the enzyme to yield an aldamine linkage as in other pyridoxal 5'-phosphate enzymes.^{8,22})

Resolution and reconstitution of the enzyme and pyridoxal 5'-phosphate content

Pyridoxal 5'-phosphate was required for maximum activity of the enzyme. Approximately 30% of the coenzyme was removed from the enzyme by dialysis against 10 mM potassium phosphate buffer (pH 7.3) for 18 hr. Full resolution of the enzyme was performed as follows. The enzyme was incubated with 10 mM hydroxylamine (pH 7.3) at 4°C for 12 hr, followed by dialysis against 1000 volumes of 10 mM potassium phosphate buffer (pH 7.3) at 4°C for 18 hr. The enzyme thus treated had no detectable activity in the absence of added pyridoxal 5'-phosphate, and no

FIG. 2. Absorption Spectra of α -Amino- ϵ -caprolactam Racemase.

Curve A, the holoenzyme (0.03 mg/ml) in 10 mM potassium phosphate buffer (pH 7.3); Curve B, the enzyme reduced with sodium borohydride and dialyzed; Curve C, the apoenzyme in 10 mM potassium phosphate buffer (pH 7.3). Other conditions are given in the text.

longer exhibited the absorption maximum peak at 412 nm (Fig. 2C). Activity was fully restored by addition of 20 μ M pyridoxal 5'-phosphate.

Pyridoxal 5'-phosphate bound to the enzyme was determined by fluorometric method of Bonavita.²³) An average pyridoxal 5'-phosphate content of 1 mol/50,000 g of the protein was obtained; one mol of pyridoxal 5'-phosphate is bound per mol of the enzyme.

Substrate specificity

The enzyme catalyzed exclusively the racemization of α -amino- ϵ -caprolactam as reported previously.⁵) The α -amino-carboxylic acids (50 mM) tested, *i.e.*, L-lysine, L-ornithine, L-arginine, L-histidine, L-tryptophan, L-proline, L-hydroxyproline, L-serine, L-cysteine, and L-methionine were inert. The following amino acid derivatives and amino sugars related to α -amino- ϵ -caprolactam (50 mM) also were not substrates even when an excess amount of enzyme (one unit) was used and the activity was assayed by polarimetry and measurement of α -H exchange (see below): glycine, ϵ -caprolactam, L-tryptophanamide, L-leucinamide, L-homoserine lactone, D-cycloserine, glycyl-L-leucine, L-leucyl-glycine, L-valyl-glycine, L-valyl-L-alanine, L-leucyl-L-alanine, D-glucosamine, D-mannosamine and D-galactos-

amine. The relative activities for D- and L- α -amino- ϵ -caprolactams were found to be 100 and 90, respectively. The apparent K_m values were 8 mM for D- α -amino- ϵ -caprolactam and 6 mM for L- α -amino- ϵ -caprolactam. The K_m value for pyridoxal 5'-phosphate was determined to be 2.1×10^{-7} M when the enzyme was assayed with D- α -amino- ϵ -caprolactam as a substrate after incubation of the apoenzyme with various concentrations of pyridoxal 5'-phosphate in 10 mM potassium phosphate buffer (pH 7.3) at 25°C for 30 min.

Effect of inhibitors

Various compounds were studied for their inhibitory effects on enzyme activity. After substrate analogues and amino acids (25 mM) were incubated at 37°C for 5 min with the standard reaction mixture, its activity was determined. The enzyme was inhibited 40% by ϵ -caprolactam, 30% by L-methionine, 18% by L-histidine, 15% by L-ornithine and L-lysine, and 10% by glycine. The other amino acids (25 mM) such as alanine, leucine, glutamate and proline inhibited the enzyme little. Plots of reciprocals of initial velocities versus reciprocals of L- α -amino- ϵ -caprolactam concentrations at several fixed concentrations of ϵ -caprolactam gave a family of straight lines. This shows that the enzyme is competitively inhibited by ϵ -caprolactam. The apparent K_i value was calculated to be 24.2 mM. Among the metal ions (1 mM) tested, CuSO_4 and HgCl_2 strongly inhibited the enzyme with 100% and 95% inhibition, respectively. Other metal ions (e.g., BaSO_4 , MgSO_4 , NiSO_4) are much weaker inhibitors (inhibition, 5~40%). When the enzyme was incubated with 1 mM HgCl_2 in 10 mM potassium phosphate buffer (pH 7.3) at 25°C for 20 min, 90% of the enzyme activity was lost. CuSO_4 did not inactivate the enzyme under the same conditions. The enzyme lost most of its activity when incubated with 2 mM *N*-ethylmaleimide and 5 μM *p*-chloromercuribenzoate for 20 min at 25°C (inhibition, 90 and 78%, respectively), but was little affected by 2 mM iodoacetate and iodoacetamide. The enzyme was inhibited

FIG. 3. ^1H -NMR Spectra of D- α -Amino- ϵ -caprolactam before and after Incubation with the Enzyme in $^2\text{H}_2\text{O}$.

Enzyme (2 units) was incubated with D- α -amino- ϵ -caprolactam (50 μmol) in a total volume of 0.5 ml (pD 8.2) at 37°C and ^1H -NMR spectra were taken at the indicated times. The pD was adjusted with 0.3 N NaOD, and measured directly with a pH meter at 20°C.

FIG. 4. Enzymatic α -Proton Exchange and Racemization of D- α -Amino- ϵ -caprolactam in $^2\text{H}_2\text{O}$.

The rates of racemization and α -proton exchange were followed with the same reaction mixture as described in the text.

70~100% after a 20-min incubation (25°C) at concentrations of 2 mM by hydroxylamine, phenylhydrazine, D-cycloserine and 2-methyl-3-benzothiazolonehydrazine hydrochloride, which are typical inhibitors of pyridoxal 5'-phosphate enzymes. The activity was fully restored by the addition of pyridoxal 5'-phosphate.

Enzymatic incorporation of deuterium into substrates

Figure 3 shows the ^1H -NMR spectral change of D- α -amino- ϵ -caprolactam observed

during incubation with the enzyme in $^2\text{H}_2\text{O}$. A peak of the α -proton disappeared substantially after 130 min; the proton was replaced by deuterium, but the β , γ , δ and ϵ protons were not affected. In order to compare the rate of α -proton exchange with the rate of racemization, logarithms of the peak integral values were plotted against times as shown in Fig. 4. The rates of α -proton exchange and racemization were calculated to be $1.7 \times 10^{-2} \text{ min}^{-1}$ and $2.17 \times 10^{-2} \text{ min}^{-1}$, respectively. When the L-isomer was used as a substrate, the rate of α -proton exchange was also lower than that of racemization.

DISCUSSION

Among various racemases thus far studied, the alanine racemase of *Escherichia coli* is the only enzyme whose intracellular localization has been demonstrated.^{24,25} We have shown that *Achromobacter obae* contains at least two distinct kinds of racemases; alanine racemase that is localized in the membrane, and α -amino- ϵ -caprolactam racemase that is in the cytoplasm.

As judged by the substrate specificity, α -amino- ϵ -caprolactam racemase belongs to a group of racemases (EC class 5.1.1) acting on amino acids and derivatives.⁶⁻¹⁰ The amino acid racemases so far studied act exclusively on amino acids.²⁶ α -Amino- ϵ -caprolactam racemase is the first example of a racemase which does not act on amino acids, but on an amino acid derivative.

Pyridoxal 5'-phosphate enzymes catalyzing racemization, transamination, decarboxylation, elimination, replacement and some other reactions of amino acids have been demonstrated,²⁷ but α -amino- ϵ -caprolactam racemase is the only racemase which acts on an amino acid derivative having no carboxyl group. L-Tyrosine ethyl ester was not a substrate for the tyrosine phenol lyase (EC 4.1.99.2) of *Clostridium tetanomorphum*.²⁸ Cyclic derivatives of amino acid amides inhibit alanine (EC 2.6.1.2) and aspartate (EC 2.6.1.1) aminotransferases^{29,30} but are inert as sub-

strates for them. Tryptophanase (EC 4.1.99.1) from *E. coli* does not catalyze α,β -elimination of L-serine methyl ester, but is inhibited by this compound.³¹ No pyridoxal 5'-phosphate enzymes acting on amines have been demonstrated except the ω -amino acid : pyruvate aminotransferase of *Pseudomonas sp.*³² This aminotransferase catalyzes transamination of mono- and diamines as well, but the most efficient and inherent substrate is β -alanine. Thus, α -amino- ϵ -caprolactam racemase is unique among pyridoxal 5'-phosphate enzymes in that the inherent substrate is an amine containing no free carboxyl groups.

Labilization of the α -hydrogen during catalysis is a characteristic feature of most reactions catalyzed by pyridoxal 5'-phosphate enzymes such as transamination and elimination.²⁷ α -Amino- ϵ -caprolactam racemase catalyzes α -hydrogen exchange of the substrate with the solvent deuterium during racemization. The α -H is abstracted from the pyridoxal 5'-phosphate-substrate Schiff base by a base at the active site to form an achiral intermediate.²⁷ The proton attached to the base is probably exchanged with a solvent deuterium, which is incorporated into the α -carbon of the product, D- or L- $[\alpha\text{-}^2\text{H}]\alpha$ -amino- ϵ -caprolactam. On some occasions, however, the proton abstracted from the α -carbon of the substrate is probably returned to the achiral intermediate to produce D- or L- $[\alpha\text{-}^1\text{H}]\alpha$ -amino- ϵ -caprolactam, because the rate of α -proton exchange was lower than that of racemization for both the isomers of α -amino- ϵ -caprolactam. The detailed mechanism of the enzyme reaction is currently under investigation.

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