Cloning and Expression in *Escherichia coli* of the Glutamate Racemase Gene from *Pediococcus pentosaceus*

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The glutamate racemase (EC 5.1.1.3) gene of a lactic acid bacterium, *Pediococcus pentosaceus*, was cloned into *Escherichia coli* C600 with a vector plasmid, pBR322. The requirement of L-glutamate for the growth of *E. coli* in the minimum medium containing D-glutamate and the formation of a red pigment in a coupled enzyme reaction mixture were used to select clones expressing glutamate racemase activity. Glutamate racemase overproduced as 0.3~2.0% of the total soluble proteins in a clone carrying the plasmid pICR221, 10.3 kb of DNA, was purified from cell extracts about 130-fold to homogeneity. The purified enzyme has a molecular weight of about 40,000 and is a single polypeptide chain. Glutamate is the sole substrate for the enzyme. Unlike many other amino acid racemases, glutamate racemase is devoid of cofactors: there is no evidence for pyridoxal 5'-phosphate or FAD in the ultraviolet spectrum of the purified enzyme, and the enzyme is not inactivated by carbonyl reagents such as hydroxylamine and sodium borohydride.

The d-forms of glutamic acid and alanine are important amino acid components in the peptidoglycan layer of bacterial cell walls. D-Alanine is formed from L-alanine by alanine racemase (EC 5.1.1.1), which is ubiquitously found in bacteria. In contrast, D-glutamic acid is mainly produced from z-ketoglutarate and D-alanine by D-amino acid aminotransferase (EC 2.6.1.21), which is also widespread. However, in *Lactobacilli*, glutamate racemase (EC 5.1.1.3) seems to function in the direct biosynthesis of D-glutamate from L-glutamate, since these bacteria apparently have no D-amino acid aminotransferase activity.

Amino acid racemases generally require pyridoxal 5'-phosphate as a cofactor (see ref. 10 for a review), although some enzymes such as diaminopimelate epimerase and phenylalanine racemase are independent of added cofactors. Glutamate racemase purified from *Lactobacillus fermenti* was originally reported to require FAD as a cofactor, whereas FAD was later found to be an inhibitor of the enzyme purified to homogeneity from the same bacterium. Thus, enzymological properties including the cofactor requirement of glutamate racemase remain to be settled.

Our preliminary experiments indicated that the purified glutamate racemase of a lactic acid bacterium, *Pediococcus pentosaceus*, requires neither FAD nor pyridoxal 5'-phosphate for the enzyme activity. However, since the *P. pentosaceus* enzyme had to be purified nine thousand fold to homogeneity, little protein was available for detailed studies on its enzymological properties. In this paper we report cloning of the glutamate racemase gene of *P. pentosaceus* into *Escherichia coli*. The high expression of the gene in *E. coli* has made the enzyme purification more easy. We have confirmed that the enzyme is independent of added cofactors.

**EXPERIMENTAL PROCEDURES**

*Strains and media. P. pentosaceus* IFO 3182 was used as a donor strain of the gene and *E. coli* C600 r<sub>x</sub><sup>−</sup> m<sub>y</sub><sup>−</sup> thi thr
leu, the host strain for plasmid constructions. Transformed E. coli cells were grown in L-broth (1% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose; pH 7.2) supplemented with 2% agar and appropriate antibiotics. Antibiotic concentrations used for the selection of transformants were 50 µg/ml of ampicillin and 15 µg/ml of tetracycline. P. pentosacaeus was grown anaerobically at 37°C in a medium (pH 7.2) containing 2.0% polypeptone, 1.0% glucose, 0.5% meat extract, 0.5% yeast extract, 0.2% KH₂PO₄, and 0.2% K₂HPO₄.

**DNA preparation.** P. pentosacaeus grown in 2 liters was harvested in the late log phase (about 20 hr), and after lysing the cells with N-acetylmuramidase SG and lysosyme (Seikagaku Kogyo, Tokyo) the chromosomal DNA was isolated essentially as described by Saito and Miura. Plasmid DNA from E. coli was prepared by a modification of the method of Oka. After removal of RNA by gel filtration on a Bio Gel A-5m (Bio-Rad Laboratories) column (1.0 × 40 cm), covalently closed circular plasmids were purified by banding in a cesium chloride-ethidium bromide equilibrium gradient. Ethenidum bromide was removed by n-butanol extraction, then dialysis against 10 mM Tris–HCl buffer (pH 7.5) containing 0.2 mM EDTA. Minipreparations of recombinant plasmids were obtained by the alkaline-SDS lysis procedure.

**Digestion of DNA with restriction endonuclease.** A restriction enzyme, HindIII, was obtained from Takara Shuzo, Kyoto, and used to digest DNA under the conditions recommended by the supplier; the fragments were analyzed on 0.7% agarose gels in a Tris-borate–EDTA buffer system containing 0.5 µg ethidium bromide per ml. For digestion of plasmid DNA, the enzyme was used at 2 units per µg of DNA, and incubations were done at 37°C for 16 hr.

**Ligation and transformation.** Total genomic DNA (18 µg) from P. pentosacaeus was partially digested (at 37°C for 30 min) with HindIII (10 units), and the resultant fragments were ligated with T₄ DNA ligase (10 units, Takara Shuzo, Kyoto) into the HindIII site of pBR322 (6 µg), which had been treated with calf intestinal alkaline phosphatase (25 units, Boehringer Mannheim GmbH). Ligation was done at 13°C for 16 hr in 66 mM Tris–HCl buffer (pH 7.6) containing 6.6 mM MgCl₂, 10 mM dithiothreitol, and 0.5 mM ATP. E. coli was transformed as described by Mandel and Higa.

**Selection of clones that express glutamate racemase activity.** Among the transformants of E. coli C600 that do not grow in a medium containing D-glutamate as a sole nitrogen source, but grows well in a medium containing L-glutamate. Therefore, on the assumption that clones producing glutamate racemase would assimilate D-glutamate, screening was done by planting colonies on 2% agar plates of the minimum medium containing 20 g of D-glutamate, 1 g of glycerol, 1 mg of thiamine, 10 mg of L-leucine, 20 mg of L-threonine, 50 mg of MgSO₄·7H₂O, 5 mg of MnCl₂·4H₂O, 0.25 mg of FeSO₄·7H₂O, 0.5 mg of CaCl₂, 0.5 mg of yeast extract, 20 mmol of potassium phosphate buffer (pH 7.2), and 25 mg of ampicillin per liter.

**Procedure A.** A micromethod for the direct assay of glutamate racemase in transformed cells was established. After transferring cells of each colony into wells of a tissue culture plate (Corning) with toothpicks, they were lysed at 37°C for 20 min in a 1-ml solution containing 10 mg of egg white lysozyme, 10 mM EDTA, and 10 mM potassium phosphate buffer (pH 7.2). The glutamate racemase assay mixture (2 ml) composed of 50 µmol of D-glutamate, 100 µmol of potassium phosphate buffer (pH 7.2), 21 µmol of phenol, 1.2 µmol of 4-aminooantipyrine, 100 units of horseradish peroxidase, and 20 units of L-glutamate oxidase of Streptomyces sp. X119-6, which was kindly provided by Dr. H. Kusakabe, Yamasa Shoyu Co., was added to the wells, and incubation was continued at 37°C. The glutamate racemase activity could be detected by the formation of a deep red color due to the coupled reaction of 4-aminooantipyrine, phenol, and peroxidase with hydrogen peroxide formed from L-glutamate, a racemization product of D-glutamate, by the action of L-glutamate oxidase.

**Enzyme and protein assays.** Enzyme assays were done at 37°C. The glutamate racemase activity was measured in the D- to L-glutamate direction by monitoring the production of NADH at 340 nm on a Union Giken SM401 spectrophotometer as the L-glutamate produced was converted to α-ketoglutarate and ammonia by glutamate dehydrogenase (Boehringer Mannheim GmbH). A standard assay mixture contained 50 µmol of D-glutamate, 100 µmol of Tris–HCl buffer (pH 8.0), 5 µmol of NAD⁺, 30 units of glutamate dehydrogenase, and glutamate racemase in 1 ml. Protein was estimated by the method of Lowry et al., with crystalline bovine serum albumin as a standard. For most column fractions, the protein elution patterns were observed through the absorption at 280 nm. A unit of enzyme is defined as the amount of enzyme which catalyzes the formation of 1 µmol of product per min. The specific activity is expressed as units per mg of protein.

**Purification of glutamate racemase.** All operations were done at 0–5°C and the buffer (50 mM Tris–HCl, pH 7.4) used in the chromatographic procedures contained 0.1% 2-mercaptoethanol, 10% glycerol, and 1 mM DL-glutamate, since these additives stabilized the enzyme during purification. After each step, the crude enzyme was concentrated on an Amicon 300 ultrafiltration unit (Amicon Co., Lexington, Mass.) and PM-10 membrane.
Cloning of Glutamate Racemase

All dialyses were done with seamless cellulose bags at 4°C for at least 4 hr.

Step 1: Preparation of crude extract. E. coli C600 carrying the plasmid DNA containing the glutamate racemase gene was grown at 37°C for 3 hr in 20 liters of L-broth supplemented with 25 mg/liter of ampicillin. The cultivation was done in a 30-liter fermentor jar with a 5% inoculum. E. coli cells harvested by centrifugation were washed with 0.85% NaCl. The washed cells (100 g, wet weight) were suspended in 200 ml of 50 mM buffer and disrupted by sonication at 0°C for 30 min with a 50 kHz oscillator (Seiko Denshi Kogyo, Tokyo). The intact cells and debris were removed by centrifugation.

Step 2: Butyl-Toyopearl column chromatography. Ammonium sulfate was added to the enzyme solution to give 30% saturation, and the enzyme was put on a Butyl-Toyopearl (Toyo Soda Manufacturing Co., Tokyo) column (5 x 30 cm) equilibrated with the buffer containing 30% saturated ammonium sulfate. After the column was washed with the same buffer, the enzyme was eluted at a flow rate of 100 ml/hr with a 2-liter linear gradient of ammonium sulfate (30 to 10% saturation). The active fractions were combined and concentrated by ultrafiltration.

Step 3: Cellulofine GCL-2000 column chromatography. The enzyme solution (30 ml) was placed in three 10-ml portions on a Cellulofine GCL-2000 column (2.5 x 120 cm) equilibrated with 50 mM buffer, and the enzyme was eluted at a flow rate of 20 ml/hr. The active fractions were pooled and concentrated by ultrafiltration.

Step 4: TSK-DEAE 5PW high performance liquid chromatography. The enzyme was purified by high performance liquid chromatography on a Toyoda Soda pre-packed 2-inch column of TSK-DEAE 5PW with a Toyoda Soda HLC-837 liquid chromatograph. The program of chromatography and conditions for eluting the enzyme were; 100% A buffer at 0 ~ 12 min, 0 ~ 20% B buffer for 48 min (linear gradient), 100% B buffer at 75 ~ 80 min, and 100% A buffer at 90 min; A buffer, 50 mM Tris-HCl (pH 7.4), and B buffer, A buffer supplemented with 1.0 M KCl; and at room temperature (about 25°C) with a constant flow rate of 30 ml/min. The enzyme solution (30 ml) was put on automatically in 5-ml portions, and the protein elution was monitored at 280 nm. The enzyme eluting around 0.13 M KCl was concentrated by ultrafiltration.

Step 5: MonoQ fast protein liquid chromatography (FPLC). The enzyme (3 ml) dialyzed thoroughly with 50 mM buffer was chromatographed on a MonoQ HR 10/10 anion-exchange column of the Pharmacia FPLC system. The elution was done with a linear gradient of KCl (0 to 0.3 M) in 50 mM buffer at a flow rate of 1 ml/min. The active fractions were pooled and concentrated by ultrafiltration.

Step 6: TSK-phenyl 5PW liquid chromatography. The enzyme solution was saturated with ammonium sulfate (30%) and purified by a Toyoda Soda TSK-Phenyl 5PW column (0.75 x 7.5 cm) in the FPLC system. The enzyme was eluted with a linear gradient of ammonium sulfate (30 to 10%) in 50 mM buffer at a flow rate of 1 ml/min. Ammonium sulfate was removed from the enzyme solution by dialysis against 100 volumes of 50 mM buffer.

RESULTS AND DISCUSSION

Construction of Pediococcus pentosaceus genomic library

Total DNA was isolated from a lactic acid bacterium, P. pentosaceus IFO 3182, and digested with the restriction endonuclease HindIII to yield a population of fragments of average sizes of about 4 to 10 kilobases (kb). The fragments were then ligated into the HindIII site in the cloning vector pBR322, which was dephosphorylated in advance with alkaline phosphatase after the HindIII digestion, and transformed into E. coli C600. A library of about 3,200 transformed (Amp', Tc') clones was obtained.

Isolation of clones that express glutamate racemase activity

The recombinant E. coli library was screened for the expression of P. pentosaceus glutamate racemase gene; of approximately 3,200 Amp'Tc' recombinants examined, 48 clones grew on the agar plates of the minimum medium containing D-glutamate as a sole nitrogen source (Procedure A). The glutamate racemase activity in each clone was then measured directly by activity staining with L-glutamate oxidase (Procedure B). The oxidase produces hydrogen peroxide during oxidation of L-glutamate formed from D-glutamate by the racemase reaction. The sensitive reaction of hydrogen peroxide with 4-aminoantipyrine to form the red quinoneimine dye in the presence of phenol and peroxidase gave a convenient qualitative assay of glutamate racemase in the wells of tissue culture plates with only a small amount of cells. As shown in Fig. 1, three clones formed this red pigment during incubation at 37°C. Since one of them apparently had the highest glutamate racemase activity judging from the rate of red pigment formation, this clone (No. A-5 of the plate)
Fig. 1. Identification of E. coli Clones with Glutamate Racemase Activity.

Transformed E. coli cells were cultured on agar plates of the minimum medium containing D-glutamate and transferred into wells of a tissue culture plate with toothpicks. Cells lysis and enzyme assay were done as described under Experimental Procedures.

was used for further study described below. A plasmid band about 6.0 kb larger than the vector pBR322 was found in this positive clone upon agarose gel electrophoresis of a mini-preparation of the recombinant plasmid. The plasmid DNA was isolated from the clone, and designated pICR221 (10.3 kb, see Fig. 2).

Mapping of restriction endonuclease cleavage sites of pICR221

Digestion of the recombinant plasmid pICR221 with HindIII yielded a 6.0 kb fragment in addition to the 4.3 kb fragment of the vector pBR322. Thus, a 6.0 kb fragment derived from P. pentosaceus genomic DNA was inserted into the HindIII site of pBR322. By evaluating the electrophoretic patterns of pICR221 digested with single and double restriction endonucleases, the internal restriction map of the plasmid pICR221 DNA was obtained (Fig. 2). The inserted 6.0 kb fragment contained four EcoRI sites and two MluI sites, but no sites for SalI, BamHI, or PstI.

Expression of glutamate racemase gene in E. coli

The crude extract prepared from E. coli cells containing the plasmid pICR221, which carries the cloned P. pentosaceus glutamate racemase gene as well as the ampicillin resistance gene from pBR322, had nearly 14 times as much glutamate racemase as P. pentosaceus cells (Table I). The plasmidless E. coli C600 host cells had no glutamate racemase activity. Since the specific activity of the enzyme purified to homogeneity is 26 units per mg protein (see below), the specific activity in the crude extract

Table I. Glutamate Racemase Activity in Crude Extracts of P. pentosaceus and E. coli Carrying Plasmid pICR221

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture conditions</th>
<th>Total act.</th>
<th>Specific act. (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. pentosaceus</td>
<td>anaerobic, 20 hr</td>
<td>0.018</td>
<td>0.006</td>
</tr>
<tr>
<td>IFO 3182</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli C600-pICR221</td>
<td>aerobic, 7 hr</td>
<td>0.90</td>
<td>0.083</td>
</tr>
<tr>
<td>E. coli C600</td>
<td>aerobic, 7 hr</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The bacteria were cultured at 37°C in 50 ml of the medium described in the text. Cells (0.1 to 0.3 g) were disrupted by sonication and cell-free extracts obtained by centrifugation were dialyzed for 12 hr against 50 mM Tris-HCl buffer (pH 7.4). The enzyme activity was measured in the D to L direction at 50 mM D-glutamate.
Cloning of Glutamate Racemase

**Fig. 3.** Changes of Glutamate Racemase Activity during the Growth of *E. coli* carrying pICR221.

The clone was grown aerobically at 37°C in the L-broth (50 ml) for various cultivation times and assayed for glutamate racemase as described in the text: O—O, cell growth measured by the absorbance at 610 nm; and ○—○, specific activity of glutamate racemase.

of the clone indicates that the glutamate racemase encoded by the *P. pentosaceus* gene is overproduced to about 0.3% of the soluble protein when carried on the plasmid pICR221 in *E. coli*. The higher expression of the gene in *E. coli* than in the original bacterium *P. pentosaceus* suggests that the promoter gene of glutamate racemase either functions efficiently in *E. coli* or is strongly controlled in *P. pentosaceus* by its own feedback repression. When examining the effects of cultivation time of the clone on the expression of glutamate racemase (Fig. 3), we found that the enzyme gene was expressed at a level even higher than 0.3% of the soluble protein, the highest specific activity being at the logarithmic phase of the cell growth (3 to 4 hr). The enzyme activity after 3 hr of cultivation (0.52 unit/mg protein) shows that the enzyme corresponds to 2% of the total soluble protein. The enzyme activity decreases rapidly on prolonging the cultivation time. This decrease might be either due to physical unstability of the enzyme or inactivation by proteolytic cleavage.

To establish the optimum culture conditions for the enzyme production, the effects of various carbon and nitrogen sources added to the defined minimum medium on the extent of glutamate racemase gene expression was investigated (Table II). The addition of L- or D-
glutamic acid as a nitrogen source, particularly when glucose was used as a carbon source, induced the enzyme most abundantly, although the specific activity was almost identical with that of L-broth-grown cell extracts. Considering that the higher total enzyme activity is usually advantageous for the purification of enzymes, L-broth was used for the production of enzyme in the following enzyme purification.

**Table II. Effects of Carbon and Nitrogen Sources on the Expression of Glutamate Racemase Gene in E. coli-pICR221**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Nitrogen source</th>
<th>Growth ( (A_{610}) )</th>
<th>Specific activity (units/mg)</th>
<th>Total activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(L-Broth)*</td>
<td></td>
<td>0.80</td>
<td>0.520</td>
<td>3.60</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Peptone</td>
<td>1.47</td>
<td>0.029</td>
<td>0.58</td>
</tr>
<tr>
<td>Glycerol</td>
<td>((\text{NH}_4)\text{SO}_4)</td>
<td>0.64</td>
<td>0.064</td>
<td>0.39</td>
</tr>
<tr>
<td>Glycerol</td>
<td>L-Glutamate</td>
<td>0.76</td>
<td>0.336</td>
<td>2.02</td>
</tr>
<tr>
<td>Glycerol</td>
<td>d-Glutamate</td>
<td>0.61</td>
<td>0.384</td>
<td>2.31</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>((\text{NH}_4)\text{SO}_4)</td>
<td>0.50</td>
<td>0.080</td>
<td>0.24</td>
</tr>
<tr>
<td>d-Glutamate</td>
<td>((\text{NH}_4)\text{SO}_4)</td>
<td>0.43</td>
<td>0.096</td>
<td>0.58</td>
</tr>
<tr>
<td>Glucose</td>
<td>Peptone</td>
<td>1.22</td>
<td>0.074</td>
<td>1.54</td>
</tr>
<tr>
<td>Glucose</td>
<td>L-Glutamate</td>
<td>0.68</td>
<td>0.512</td>
<td>3.07</td>
</tr>
<tr>
<td>Glucose</td>
<td>d-Glutamate</td>
<td>0.72</td>
<td>0.176</td>
<td>1.44</td>
</tr>
</tbody>
</table>

* The *E. coli* clone was cultured in 50 ml of L-broth.

**Purification of glutamate racemase from E. coli-pICR221**

Starting with about 100 g wet cells of *E. coli* C600-pICR221, the *P. pentosaceus* glutamate racemase was purified about 130-fold with an overall yield of 4.3% (Table III). The specific activity of the purified enzyme (about 26 units/mg protein) is much higher than the values reported so far.\textsuperscript{14,15} As shown in Fig. 4, the purified enzyme was homogeneous upon
Table III. Purification of Glutamate Racemase from E. coli C600-pICR221

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cell-free extract</td>
<td>9,000</td>
<td>1,800</td>
<td>0.2</td>
<td>1</td>
</tr>
<tr>
<td>2. Butyl-Toyopearl</td>
<td>442</td>
<td>620</td>
<td>1.4</td>
<td>7</td>
</tr>
<tr>
<td>3. Cellulofine</td>
<td>91</td>
<td>420</td>
<td>4.6</td>
<td>23</td>
</tr>
<tr>
<td>GCL-2000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. TSK-DEAE 5PW</td>
<td>21</td>
<td>260</td>
<td>12.0</td>
<td>60</td>
</tr>
<tr>
<td>5. FPLC-MonoQ</td>
<td>7</td>
<td>112</td>
<td>16.0</td>
<td>80</td>
</tr>
<tr>
<td>6. TSK-Pheny1 5PW</td>
<td>3</td>
<td>78</td>
<td>26.0</td>
<td>130</td>
</tr>
</tbody>
</table>

Fig. 4. Polyacrylamide Gel Electrophoresis of the Purified Glutamate Racemase from E. coli-pICR221.

Electrophoretic separation was done on 7.5% polyacrylamide gels by the procedure of Davis26) with 10 µg of the purified enzyme. The direction of migration is from cathode (top) to anode. The gels were stained for protein with Coomassie Brilliant Blue G-250 (A), and for activity with a 2-ml solution containing 0.1 M Tris-HCl buffer (pH 8.0), 0.1 M D-glutamate, 5 mM NAD⁺, 0.064 mM phenazine methosulfate, 0.24 mM nitroblue tetrazolium, and 30 units of bovine liver L-glutamate dehydrogenase (Sigma) (B).

Characterization of glutamate racemase purified from E. coli-pICR221

There was no distinction between glutamate racemase purified from E. coli-pICR221 and the enzyme from P. pentosaceus (unpublished data) in the following enzymological properties. Glutamic acid is the sole substrate for the enzyme with a Km value of 15 mM for the D-enantiomer. The enzyme has maximum reactivity at 40°C and at around pH 8.0. The enzyme irreversibly loses activity on storage at pH 6 to 8 in the absence of 10% glycerol, 1 mM D,L-glutamate and a reducing thiol such as 2-mercaptoethanol. This inactivation might be partly due to air oxidation of a free thiol residue(s) which is necessary for the enzyme activity; the activity is completely lost by brief incubation with various sulfhydryl reagents (e.g., 0.1 mM p-chloromercuribenzoate, 5,5'-dithiobis(2-nitrobenzoate), and 2-nitro-5-thiocyanobenzoate). The molecular weight of the enzyme was estimated to be about 40,000 by the high-performance gel permeation method (Fig. 5A). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate gave a single band that had an estimated molecular weight of 40,000 (Fig. 5B). These results suggest that the enzyme is composed of a single polypeptide chain.

The availability of pure enzyme in quantity resulting from the cloning and high expression in E. coli has motivated us to study the cofac-
Cloning of Glutamate Racemase

Fig. 5. Molecular Weight Measurement of the Purified Glutamate Racemase.

A: The molecular weight of the enzyme was measured by high-performance liquid chromatography with an Asahipak GS-520 column (0.75 x 60 cm) (Asahi Kasei Manufacturing Co., Tokyo). Retention time was plotted against molecular weight in a semilogarithmic scale. The position of glutamate racemase is shown by an open circle. The standard proteins (Oriental Yeast Co., Osaka) used were: a, glutamate dehydrogenase (M.W. = 290,000); b, lactate dehydrogenase (M.W. = 142,000); c, enolase (M.W. = 70,000); d, adenyate kinase (M.W. = 32,000); and e, cytochrome c (M.W. = 12,400).

B: The molecular weight of the subunit was estimated by disc gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate by the method of Laemmli.27) Relative mobility was plotted against subunit molecular weight in a semilogarithmic scale. The standard proteins (Pharmacia) used were: a, phosphorylase b (M.W. = 97,400); b, bovine serum albumin (M.W. = 68,000); c, ovalbumin (M.W. = 43,000); d, carbonic anhydrase (M.W. = 30,000); e, soybean trypsin inhibitor (M.W. = 21,000); and f, a-lactalbumin (M.W. = 14,400).

Fig. 6. Absorption and Fluorescence Spectra of Glutamate Racemase.

The absorption spectrum (A) was taken at 0.8 mg/ml of the enzyme in 50 mM Tris-Cl buffer (pH 7.4) with a Union Giken SM401 recording spectrophotometer. The fluorescence emission spectrum (B) was recorded by exciting at 280 nm with a Hitachi MPF-4 spectrofluorometer.

The electronic spectra of purified glutamate racemase also indicate the absence of pyridoxal 5'-phosphate, which is known as a bound cofactor of several amino acid racemases.10) If pyridoxal 5'-phosphate were present in 1:1 stoichiometry, the pyridoximine form of the enzyme would have been expected to have an absorbance peak near 420 nm with an extinction coefficient of approximately 5000 M⁻¹ cm⁻¹.28) This peak is clearly absent. In addition, the racemase is not inhibited by 10 mM hydroxylamine or by 1 mM phenylhydrazine, both of which are typical inhibitors of pyridoxal 5'-phosphate enzymes, and we have observed no inactivation when 10 mM sodium borohydride was included in the glutamate racemase assay. The specific activity of the enzyme was not affected by dialysis, and the activity was not increased by the addition of 0.1 mM pyridoxal 5'-phosphate to assay solutions. There seems to be no possibility, therefore, that glutamate racemase could be an FAD- or pyridoxal 5'-phosphate-dependent enzyme. Thus, glutamate racemase is analogous to diaminopimelate epimerase11) and proline racemase29) in dispensability of cofactors. However, our preliminary observation that the proton translocation from the substrate to product is mediated by a single-base mechanism suggests a difference from these.
enzymes using a two-base mechanism.\textsuperscript{11,29)}

Analyses of the glutamate racemase reaction as well as the presence of a sulfhydryl group(s) essential for catalytic activity will be reported elsewhere.

REFERENCES