Distribution of Glutamate Racemase in Lactic Acid Bacteria and Further Characterization of the Enzyme from *Pediococcus pentosaceus*

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We investigated the distribution of glutamate racemase (EC 5.1.1.3) in bacteria, and found that the enzyme occurs exclusively in lactic acid bacteria. *Pediococcus pentosaceus* IFO 3182 produces the enzyme most abundantly. The enzyme purified from extracts of the *Escherichia coli* clone cells carrying the plasmid pICR221, which contains the enzyme gene of *P. pentosaceus*, does not require cofactors for its catalytic activity [N. Nakajima, K. Tanizawa, H. Tanaka and K. Soda, Agric. Biol. Chem., 50, 2823 (1986)]. This cofactor dispensability was re-confirmed with the enzyme purified from cell extracts of *P. pentosaceus*. On the basis of kinetic parameters obtained by measurements of the initial velocities, the glutamate racemase reaction was categorized into a typical racemization with a calculated equilibrium constant of 1.05. d- and L-Glutamate were the specific substrates for the enzyme, although L-homocysteinesulfinate, a sulfur analog of L-glutamate, slightly served as a substrate. L-3-Aminoadipate behaved as a competitive inhibitor in the racemization of glutamate. The enzyme was inactivated significantly by treatment with various thiol-blocking reagents.

Since the discovery of the enzymatic racemization of alanine by alanine racemase (EC 5.1.1.1), a variety of amino acid racemases and epimerases have been demonstrated, mainly in bacteria, and studied in some detail. Most amino acid racemases contain pyridoxal 5'-phosphate as an essential cofactor, while some of them require no cofactors. Although intensive studies have been done on the reaction mechanisms of various pyridoxal 5'-phosphate-dependent racemases, those of the cofactor-independent enzymes are not well understood.

Glutamate racemase (EC 5.1.1.3) catalyzes the interconversion of L- and D-glutamate, presumably providing the latter enantiomer for the construction of the peptidoglycan layer of bacterial cell walls. Despite its important role in D-glutamate biosynthesis, the enzyme has been reported to occur only in a very few bacterial species such as *Lactobacillus fermenti*. The cofactor requirement for the enzyme activity has also been ambiguous. Recently, we cloned the glutamate racemase gene from a lactic acid bacterium, *Pediococcus pentosaceus*, into *Escherichia coli*, and established an efficient purification procedure for the enzyme on the basis of the high expression of the gene in *E. coli*. We obtained no evidence for the presence of pyridoxal 5'-phosphate or FAD in the purified enzyme.

Although proteins, in general, should be identical in structures and properties irrespective of whether the genes coding them are expressed on plasmids or on the parent chromosomes, it seemed important to us to re-examine the cofactor requirement of glutamate racemase from the original *P. pentosaceus* in...
view of the marked discrepancy between the first proposal that FAD is a cofactor\(^\text{10}\) of the enzyme and its negation thereafter.\(^\text{11}\) In the study reported here, we purified the enzyme from extracts of \(P.\) \(pentosaceus\) cells and found that the enzyme activity is independent of cofactors as was the enzyme from the clone cells.\(^\text{12}\) Distribution of glutamate racemase in bacteria and further characterization of the \(P.\) \(pentosaceus\) enzyme are also described.

**MATERIALS AND METHODS**

*Materials.* \(L\)-Homocysteine-sulfinic acid was synthesized from \(L\)-homocystine (Sigma) by an application of the method for \(L\)-cysteinesulfinate synthesis of Emiliozzi and Pichat.\(^\text{13}\) Deuterated water (99.85 atom \% \(^2\)\(H\)) was obtained from CEA, Saclay, France. \(L\)-Glutamate oxidase from *Streptomyces* sp. X119-6 was kindly provided by Dr. H. Kusakabe, Yamasa Shoyu Co., Choshi, Japan.\(^\text{14}\) \(D\)-Glutamate oxidase was purified from cell extracts of *Aspergillus usus* IFO 3371 as reported previously.\(^\text{15}\) All other chemicals were of analytical grade.

*Enzyme assay.* The glutamate racemase activity was routinely measured spectrophotometrically in the \(d\)-to \(L\)-glutamate direction as described previously.\(^\text{12}\) The reaction rate of racemization was also measured directly by the change in optical rotation at 365 nm with a Perkin-Elmer 241 polarimeter. The reaction mixture contained 100 \(\mu\)mol of Tris–HCl buffer (\(pH\) 8.0), 100 \(\mu\)mol of \(d\)- or \(L\)-glutamate, and enzyme in a final volume of 1.0 ml. Racemization of other substrate amino acids was measured polarimetrically as well. For calculation of kinetic parameters, \(d\)- or \(L\)-glutamate produced from the counterpart was measured enzymatically with \(L\)- or \(D\)-glutamate oxidase,\(^\text{14,15}\) respectively, and peroxidase; hydrogen peroxide produced by the oxidase reaction was measured with 4-aminoantipyrine in the presence of phenol and peroxidase (horseradish, Sigma).\(^\text{16}\) The reaction mixture (1 ml) was composed of 100 \(\mu\)mol of potassium phosphate buffer (\(pH\) 7.4), various amounts of \(d\)- or \(L\)-glutamate, and glutamate racemase. The reaction was done at 37°C for 10 min, and stopped by heating in a boiling water bath for 3 min. A solution (2 ml) containing 26 \(\mu\)mol of phenol, 1.6 \(\mu\)mol of 4-aminoantipyrine, 20 units of peroxidase, and 1 unit of \(d\)- or \(L\)-glutamate oxidase was added and the mixture was further incubated at 37°C for 20 min. The red quinoneimine dye formed was measured by absorbance at 500 nm with a Carl Zeiss PMQII spectrophotometer. The protein assay and definition of the enzyme unit were as described previously.\(^\text{12}\)

*Culture conditions.* Bacterial strains including lactic acid bacteria were cultivated anaerobically (or aerobically) at 37°C for about 15 hr in a medium (\(pH\) 7.2) containing 2.0%, polypeptone, 1.0% glucose, 0.5% yeast extract, and 0.5% meat extract.

**RESULTS AND DISCUSSION**

*Distribution of glutamate racemase in bacteria.* We have investigated the distribution of glutamate racemase in various kinds of bacteria including both aerobes and anaerobes. As shown in Table I, the enzyme activity was found only in several lactic acid bacteria, i.e., *Pediococcus*, *Lactobacillus*, and *Leuconostoc* species. No activity was found at all in *Bacillus*, *Escherichia*, or *Clostridium* species. Among lactic acid bacteria showing the glutamate racemase activity, *P. pentosaceus*, the

**Table I. Distribution of Glutamate Racemase in Lactic Acid Bacteria**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Specific activity* ((\times 10^{-3} \text{ units/mg}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pediococcus acidilactici IFO 3076</td>
<td>2.7</td>
</tr>
<tr>
<td>Pediococcus acidilactici IFO 12218</td>
<td>1.8</td>
</tr>
<tr>
<td>Pediococcus pentosaceus IFO 3182</td>
<td>5.9</td>
</tr>
<tr>
<td>Leuconostoc lactis IFO 12455</td>
<td>2.4</td>
</tr>
<tr>
<td>Lactobacillus casei subsp. casei</td>
<td>1.1</td>
</tr>
<tr>
<td>Lactobacillus casei subsp. rhamnosus</td>
<td>1.0</td>
</tr>
<tr>
<td>Lactobacillus plantarum IFO 3070</td>
<td>2.3</td>
</tr>
<tr>
<td>Lactobacillus fermentum IFO 3071</td>
<td>2.3</td>
</tr>
<tr>
<td>Lactobacillus sp. IFO 3955</td>
<td>3.0</td>
</tr>
</tbody>
</table>

same species that we used as a donor of the glutamate racemase gene for cloning,\textsuperscript{12} produced the enzyme most abundantly. When 1.0\% (w/v) D- or L-glutamate was added to the cultivation medium for these lactic acid bacteria, the specific activity of glutamate racemase was unaffected (data not shown). This shows that the enzyme is formed constitutively in the bacteria. Although D-glutamate can also be synthesized by transamination between D-alanine and \(\alpha\)-ketoglutarate, as has been demonstrated with many bacterial species, no lactic acid bacteria is known to have a D-amino acid aminotransferase (EC 2.6.1.21).\textsuperscript{17} Thus, the exclusive occurrence of glutamate racemase in lactic acid bacteria suggests that the enzyme plays a principal role in the formation of D-glutamate, an essential component of the peptidoglycan layer of cell walls, in these bacteria.

\textbf{Purification and cofactor dispensability of the enzyme from P. pentosaceus}

To re-confirm our previous important conclusion\textsuperscript{12} that glutamate racemase is independent of cofactors such as FAD once proposed for the enzyme from \textit{L. fermenti}\textsuperscript{10} and pyridoxal 5\' -phosphate, an essential cofactor of many other amino acid racemases,\textsuperscript{2} we purified the enzyme from cell extracts of \textit{P. pentosaceus}. Products from the same gene that is carried either on the chromosome or on a plasmid, and expressed in different host bacterial species might have different structures and properties, although such a possibility is rather low. The overall purification steps of glutamate racemase from \textit{P. pentosaceus} were essentially identical with those reported for the purification from \textit{E. coli} clone cells.\textsuperscript{12} However, the purification required a 9000-fold enhancement of the specific activity in marked contrast with that from \textit{E. coli} clone cell extracts (about 130-fold),\textsuperscript{12} because \textit{P. pentosaceus} cell extracts had a specific glutamate racemase activity of only \(5.9 \times 10^{-3}\) units/mg protein (see Table I). The enzyme purified from \textit{P. pentosaceus} showed a specific activity of 22 units/mg, which is comparable to that (26 units/mg) of the enzyme from \textit{E. coli}/pICR22,\textsuperscript{12} and was homogeneous upon 7.5\% polycrylamide gel electrophoresis (data not shown). The yield of the enzyme was 0.75 mg (16.2 units) from 800 g (wet weight) of \textit{P. pentosaceus} cells with a 7.5\% overall recovery of total activity.

The absorption and fluorescence spectra of the purified enzyme were those characteristic of a simple protein, as was shown previously.\textsuperscript{12} Furthermore, the addition of FAD, FMN, NAD\(^{+}\), NADP\(^{+}\), or pyridoxal 5\' -phosphate (1 mM) into the assay mixture resulted in neither activation nor inhibition of the enzyme, corresponding with our previous data.\textsuperscript{12} Thus, the cofactor dispensability of glutamate racemase was clearly demonstrated again with the preparation obtained from cell extracts of the original bacterium, \textit{P. pentosaceus}.

\textbf{Further characterization of the glutamate racemase reaction}

Among amino acids occurring in proteins, the enzyme acted specifically on glutamic acid;\textsuperscript{12} others including aspartic acid, asparagine, and even glutamine were all inert when the reaction was assayed polarimetrically. Similarly, various glutamate analogs (L-homocysteinate, L-\(\alpha\)-aminoacidipate, L-glutamate-\(\gamma\)-methylenelester, \(\gamma\)-methyl-L-glutamate, N-acetyl-L-glutamate, L-\(\alpha\)-hydroxylglutарате, and L-cysteinesulfinate) did not serve as a substrate for glutamate racemase. Thus, the enzyme has a very high structural specificity for substrates, while it shows no stereospecificity for the configuration at the \(\alpha\)-carbon of glutamate (see below). The high substrate specificity is comparable to that of a pyridoxal 5\' -phosphate-dependent enzyme, alanine racemase (EC 5.1.1.1),\textsuperscript{18} and contrasts with that of another pyridoxal 5\' -phosphate-dependent one, the amino acid racemase with broad substrate specificity (EC 5.1.1.10).\textsuperscript{19} Among cofactor-independent enzymes thus far studied, diaminopimelate epimerase (EC 5.1.1.7) has been reported to have a very narrow substrate specificity.\textsuperscript{20} To further map the
active site structure of glutamate racemase, we synthesized l-homocysteinesulfinic acid, a sulfinate analog of L-glutamate, and tested its reactivity for the enzymatic racemization by the polarimetric method. The enzyme was found to racemize L-homocysteinesulfinate at a rate of about 10% of that of L-glutamate. The bovine liver glutamate dehydrogenase is also known to act on L-homocysteinesulfinate at a rate of 65% relative to the oxidation of L-glutamate.21 Taking account of this result and that both the sulfonate analog of L-glutamate (L-homocysteinate) and the sulfinate analog of L-aspartate (L-cysteinesulfinate) do not serve as a substrate at all, the active site structure of glutamate racemase may be depicted in such a manner that the binding site for the distal carboxyl group of glutamate can also bind the sulfinate group (−SO_2H) but not the sulfonate group (−SO_3H) and it is located at a relatively inflexible distance of a three-carbon chain from the binding site for the α-carbon atom being racemized. In accordance with this idea, no inhibition of the racemization of glutamate was found with the non-substrate amino acids described above (at 50 mM) except for L-α-amino adipate (homoglutamate), which was shown to be a weak competitive inhibitor with a large inhibition constant (K_i = 78 mM).

When we monitored the enzymatic racemization of D-glutamate by the polarimetric method, the gross change in optical rotation was the same magnitude as that of L-glutamate. However, this assay method required a high concentration of substrates (>80 mM) and was not sensitive enough to obtain kinetic parameters of the reaction. Thus, we measured the initial velocity for glutamate racemase in both the D to L and the L to D directions by the L- and D-glutamate oxidase methods, respectively. The Km values for D- and L-glutamate were 14 and 10 mM, respectively. The V_max values for the D to L and L to D directions were 40 and 30 μmol·min^{-1}·mg^{-1}, respectively, under identical conditions at pH 7.4. The chemically symmetric reaction L-glutamate → D-glutamate should give an equilibrium constant of unity\(^22\) as has been validated with other amino acid racemases.\(^23,24\) The K_eq of the glutamate racemase reaction was calculated from the derived V/K values and found to be consistent with the theoretical value:

\[
K_{eq} = \frac{V_{max}/Km}{(V_{max}/Km)_{L isomer}} = 1.05
\]

As reported previously, amino acid racemases generally catalyze the exchange of a substrate α-hydrogen with deuterium in deuterium oxide during racemization.\(^25,26\) Figure 1 shows the \(^1\)H-NMR spectral change of D-glutamate observed during incubation with glutamate racemase in \(^2\)H_2O. Substantial amounts of the peak of the α-proton disappeared after 24 hr; the proton was replaced by deuterium, but the β and γ protons were not affected. These results along with the above kinetic data indicate that the reaction is a typical racemization and the enzyme catalyzes deprotonation and reprotonation only at the α-carbon atom of glutamate without its con-
figurational preference. More detailed analysis of the glutamate racemase reaction is currently in progress with a view to confirming an internal proton transfer under single turnover conditions.

**Inactivation by thiol-blocking reagents**

The initial step of amino acid racemase reactions is the proton abstraction from the substrate z-carbon atom, which is mediated by an amino acid residue(s) in the enzyme protein.\(^6\) It has been reported that the cofactor-independent diaminopimelate epimerase contains a single sulfhydryl group which is modified rapidly with a thiol-blocking reagent, iodoacetamide, and is necessary for enzymatic activity.\(^27\) This cysteinyl residue was suggested as a proton-abstraction base in the diaminopimelate epimerase reaction. We also examined the effects of various thiol-blocking reagents on the glutamate racemase activity. As shown in Table II, the enzyme was readily inactivated by brief incubation with these reagents. It is noteworthy that even 2-nitro-5-thiocyanobenzoate, a chemical modification reagent for cysteinyl residues, which introduces a small cyano group on the sulfur atom,\(^28\) leads to the complete inactivation of the enzyme at 0.1 mM, suggesting the presence of a very reactive and essential sulfhydryl group(s) in glutamate racemase. The activity of the inactivated enzyme was restored only partially (about 15%) by incubation with excess thiols such as 10 mM 2-mercaptopethanol and dithiothreitol; the modification by thiol-blocking reagents is largely irreversible. Although the number of the sulfhydryl groups modified by the reagents remains to be studied, the addition of substrate (D- or L-glutamate) significantly protected the enzyme from inactivation; at 0.1 mM 2-nitro-5-thiocyanobenzoate and 50 mM L-glutamate, the remaining activity was about 75% of the untreated enzyme activity. This result suggests that the cysteinyl residue is at or near the active site of the enzyme and it acts as a proton-abstracting base in the glutamate racemase reaction. Thus, it seems to be of particular interest in our future studies to identify the essential cysteinyl residue in the entire primary sequence of glutamate racemase, the identification of which is now under way based on the DNA sequencing of the cloned gene.\(^12\)

**Table II. Effects of Various Thiol-Blocking Reagents on Glutamate Racemase Activity**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Conc. (mM)</th>
<th>Remaining activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>1.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate</td>
<td>1.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>5,5'-Dithiobis(2-nitrobenzoate)</td>
<td>1.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>2-Nitro-5-thiocyanobenzoate</td>
<td>1.0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>0.1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

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**REFERENCES**