(2)

STRUCTURE AND FUNCTION OF GLUTAMATE RACEMASE

MAKOTO ASHIUCHI

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CONTENTS

ABBREVIATION

ATP	adenosine 5'-triphosphate
Cys	cysteine
DMSO	dimethyl sulfoxide
DTNB	5, 5'-dithiobis-2-nitrobenzoate
E.	*Ecsherichia*
ESR	electron spin resonance
FPLC	fast protein liquid chromatography
Gln	glutamine
Gly	glycine
His	histidine
HPLC	high performance liquid chromatography
Ile	isoleucine
IPTG	isopropyl-1-thio-β-D-galactopyranoside
L.	*Lactobacillus*
2-ME	2-mercaptoethanol
NAD^+	nicotinamide adenine dinucleotide
NADH	dihydronicotinamide adenine dinucleotide
NTCB	2-nitrothiocyanobenzoate

ORF	open reading frame
P.	*Pediococcus*
PCR	polymerase chain reaction
Phe	phenylalanine
PLP	pyridoxal 5'-phosphate
PMSF	phenylmethanesulfonyl fluoride
S.	*Streptococcus*
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
Tris-HCl	tris(hydroxymethyl) aminomethane hydrochloride
Val	valine

INTRODUCTION

Racemization is a unique reaction; both substrate enantiomers are racemized substantially at the same rate. Racemases and epimerases have been found widely in organisms including bacteria, yeasts and mammals. However, amino acid racemases occur solely in bacteria, in which they play an important role in the formation of D-amino acids (Adams, 1976). Bacterial amino acid racemases can be classified into two groups; pyridoxal 5'-phosphate (PLP) dependent and independent racemases. The first group includes most of amino acid racemases such as alanine racemase (EC 5.1.1.1) (Adams, 1976), arginine racemase (EC 5.1.1.9) (Yorifuji *et al.*, 1971; Yorifuji and Soda, 1971), and low substrate specificity amino acid racemase (EC 5.1.1.10) (Soda *et al.*, 1969). The reaction mechanisms of their enzymes in this group are relatively well elucidated; the enzymes require PLP as a coenzyme (Soda *et al.*, 1986), and the racemase reaction is facilitated by formation of internal and external Schiff base intermediates. On the other hand, the second group includes proline racemase (EC 5.1.1.4) (Cardinale and Abeles, 1968), 4-hydroxyproline epimerase (EC 5.1.1.8) (Ramaswamy, 1984), diaminopimelate epimerase (EC 5.1.1.7) (Wiseman and Nichols, 1984), glutamate racemase (EC 5.1.1.3) (Narrod and Wood, 1952; Glaser, 1960; Diven, 1969), and aspartate racemase (EC 5.1.1.13) (Lamont *et al.*, 1972). Their reaction

mechanisms and active site structures have not been characterized (Yamauchi *et al.*, 1992; Choi *et al.*, 1992; Gallo *et al.*, 1993; Tanner *et al.*, 1993). However, the enzymes of the second group have a common charactristic; they do not contain carbonyl moiety or metal, and have sulfhydryl groups as catalytic bases. The active sites of proline racemase (Rudnick and Abeles, 1975), 4-hydroxyproline epimerase (Ramaswamy, 1984), and diaminopimerate epimerase (Higgins *et al.*, 1989), have been proved by thiol reagents and specific inhibitors.

Glutamate racemase (Narrod and Wood, 1952; Glaser, 1960; Diven, 1969) catalyzes racemization of glutamate, and is found in lactic acid bacteria. The racemase was purified to homogeneity from *Pediococcus pentosaceus* (Nakajima *et al.*, 1986), and shown to be independent of any cofactors including PLP and metal ions (Nakajima *et al.*, 1988). The gene encoding the enzyme of *P. pentosaceus* was cloned (Choi *et al.*, 1991) and sequenced (Choi *et al.*, 1994). The gene has a 795-nucleotides open reading frame, and encodes 265 amino acid residues which form a monomeric protein (*Mr*= 29,143) (Choi *et al.*, 1991). The enzyme was demonstrated to contain two essential cysteinyl residues by means of chemical modification (Choi *et al.*, 1992) and site-directed mutagenesis (Yoshimura *et al*, 1993). The amino acid sequence of the enzyme showed significant similarity with those of globin family proteins, in particular in the regions

corresponding to the heme binding pocket. In this paper, I describe that the structure of glutamate racemase of *P. pentosaceus* is similar to those of globin family proteins, and the enzyme binds stoichiometrically an equimolar amount of hemin to form an inactive complex (Choi *et al.*, 1994), and discuss a molecular evolutional relationship among the enzyme and globin family proteins.

Recently, the gene complementing the D-glutamate auxotrophy of the *Escherichia coli* WM335 was identified and isolated from the chromosomal DNA of *E. coli* K-12, and designated as *murI* (Doublet *et al.*, 1992) and later as *dga* (Dougherty *et al.*, 1993). Nucleotide sequence of the gene has revealed that the gene corresponds to a previously sequenced open reading frame, ORF1 (Brosius *et al.*, 1981). The *murI* gene product was predicted to consist of 289 amino acid residues with a molecular weight of 31,504 (Brosius *et al.*, 1981), and contained a large number of hydrophobic amino acid residues. However, no function of the gene product has been characterized (Doublet *et al.*, 1992). I found that the amino acid sequence of the enzyme of *P. pentosaceus* shows significant homology with that of the *murI* gene product. Gallo and Knowles (Gallo and Knowles, 1993) also found that the amino acid sequence of glutamate racemase of *Lactobacillus fermenti* showed 30% homology with that of the *murI* gene product. I show that the *murI* gene is expressed by introducing *tac* promoter and the ribosome binding

site upstream the initiation codon of the gene, and demonstrate that the gene product is glutamate racemase (Yoshimura *et al.*, 1993; Doublet *et al.*, 1993). Additionally, since the *murI* gene product, glutamate racemase of *E. coli* contains a large number of hydrophobic amino acid residues, the enzyme seems to form inclusion bodies *in vivo*. I have also shown that the co-production of molecular chaperon, GroESL decreased in the amount of inclusion bodies formed and increased the enzyme activity (Ashiuchi *et al.*, 1995).

In this study, I show that the highly conserved regions formed in glutamate racemase genes exsist on the chromosomes of various kinds of bacteria, and then discuss the physiological function of bacterial glutamate racemases. On the other hand, D-glutamate is an important component of peptidoglycans of bacterial cell walls as well as D-alanine produced by alanine racemase, but their D-amino acids are believed to be not physiologically essential for mammals. Accordingly, glutamate racemase participating in the biosynthesis can be a new target for antibiotic development, and a specific inhibitor of the racemase possibly serves as an efficient antibiotics for bacterial strains such as methicillin resistant *Staphylococcus aureus* (MRSA). Previously, I found that serine *O*-sulfate (John and Fasella, 1969; Ueno *et al.*, 1982; Like *et al.*, 1982), an analog of glutamate acts as a suicide substrate of the *P. pentosaceus* racemase (Ashiuchi *et al.*, 1993).

This suicide substrate reaction will serve as a model for target-inactivation reactions which are indispensable for the design of new antibiotics. I also discuss here a mechanism of the suicide substrate reaction of glutamate racemase of *P. pentosaceus* with seine *O*-sulfate.

CHAPTER 1

Bacterial Glutamate Racemase has Highly Sequence Similarity with Myoglobins and Forms An Equimolar Inactive Complex with Hemin

Glutamate rasemase (EC 5.1.1.3) catalyzes racemization of both enantiomers of glutamate, and occurs in lactobacilli. A product, D-glutamate is an integral component of bacterial peptidoglycans (Narrod and Wood, 1952; Glaser, 1960; Diven, 1969). The racemase was purified from *P. pentosaceus* and shown to be independent of coenzymes including PLP, which is usually required as the coenzyme for various amino acid racemases (Nakajima *et al.*, 1986). The gene encoding the enzyme of *P. pentosaceus* was cloned (Choi *et al.*, 1991) and sequenced (Choi *et al.*, 1994). The enzyme was demonstrated to have two cysteinyl residues as catalytic bases by chemical modification of sulfhydryl groups (Choi *et al.*, 1992) and site-directed mutagenesis (Yoshimura *et al.*, 1993).

Proline racemase, 4-hydroxyproline epimerase, and diaminopimelate epimerase also are PLP-independent enzymes, and operate racemization or epimerization of the substrate by action of an essential cysteinyl residue at the active site. Higgins *et al.* (1989) have indicated that their enzymes show sequence homology with each other around the cysteinyl residue at the active site, and proposed that they have derived

from a common evolutionary origin. Recently, Yohda *et al.* (1991) cloned and sequenced the aspartate racemase gene of *Streptococcus thermophilus*, and then purified and characterized the enzyme overproduced in the transformant cells. Aspartate racemase of *S. thermophilus*, which has a composite active site formed at the interface of two identical subunits, is also independent of cofactors and contains an essential cysteinyl residue per a subunit (Okada *et al.*, 1991; Yamauchi *et al.*, 1992). On the other hand, the primary structure of glutamate racemase shows no similarity with those of the above three PLP-independent racemases, but has highly homology with that of this aspartate racemase. Very interestingly, the amino acid sequence of glutamate racemase also shows highly sequence similarity with that of bovine myoglobin. I found that the enzyme binds tightly an equimolar amount of hemin which results in loss of the enzyme activity and an ESR spectrum of the complex is similar to that of bovine erythrocyte hemoglobin. Aspartate racemase has little sequence homology with myoglobin and does not bind hemin. I show the possibility that the tertiary structure of glutamate racemase of *P. pentosaceus* is similar to those of globin family proteins. In this thesis, the molecular evolutional relationship among the enzyme and globin family proteins is also discussed.

EXPERIMENTAL PROCEDURES

Materials L-Glutamate oxidase was a gift from Dr. H. Kusakabe of Yamasa Shoyu. L-Glutamate dehydrogenase was purchased from Boehringer Mannheim. An Excellulose^{TM} GF-5 column was from Pierce. An elements standard kit was obtained from Shimadzu. All other chemicals were of analytical grade.

Inhibition by Hemin Glutamate racemase and aspartate racemase activities (0.5 unit each) were assayed by incubation in a mixture (0.5 ml) composed of 50 mM Tris-HCl (pH 7.5), various concentrations (0.01-0.1 mM) of hemin and 25 mM substrate (D-glutamate or D-aspartate) at 37°C for 10 min. L-Glutamate or L-aspartate formed during the assay, was determined with L-glutamate oxidase (Kusakabe *et al.*, 1980). Inhibition of glutamate rasemase activity by different concentrations of hemin was examined as follows. The reaction mixture (1.0 ml) contained 100 mM Tris-HCl (pH 8.0), 5.0 mM NAD^+, 5.0 units of L-glutamate dehydrogenase, 58 μg of glutamate racemase, different concentrations of hemin, and various concentrations of D-glutamate. The rate of L-glutamate formation was followed at 25°C by measurement of NADH absorption at 340 nm in same manner as described previously (Ashiuchi *et al.*,1993). The data were plotted as the reciprocal of initial rate of L-glutamate formation against different concentrations of

hemin, and the *Ki* value was estimated from a Dixon plot of the data (Dixon and Webb, 1979).

Stoichiometry of Hemin Binding with Glutamate Racemase Various amounts of glutamate racemase (58, 116, 174, 232 and 290 nmol) were incubated in a solution (1.0 ml) containing 3.2 mM hemin and 50 mM Tris-HCl (pH 7.5) at 25°C for 1 h. This reaction solution was isocratically chromatographed on an Excellulose^{TM} GF-5 column equilibrated with 50 mM Tris-HCl (pH 7.5). Protein concentrations were determined with a Bio-Rad protein assay kit. Enzyme concentration as also determined with 5,5'-dithiobis-2-nitrobenzate (DTNB): the enzyme contains three cysteinyl residues per mol. Enzyme bound hemin was determined by measurement of atomic absorption of iron with a Shimadzu AA-670G atomic absorption spectrophotometer equipped with a graphite furnace atomizer. An average of seven independent determinations were taken.

UV-visible and ESR spectra of glutamate racemase-hemin complex UV-visible spectra of oxidized (dotted line; 0.20 mM) and reduced (solid line; 0.18 mM) forms of the glutamate racemase-hemin complex in 50 mM Tris-HCl (pH 7.5) were taken. The oxidized form was prepared, and then reduced with sodium

dithionite according to a published method (Chen and Asada, 1989). ESR spectra of the glutamate racemase-hemin complex (B, 0.25 mM) and bovine erythrocyte hemoglobin (C, 250 mM) in 50 mM Tris-HCl (pH 7.5) were measured with a JEOL JES-REzx ESR spectrometer: microwave frequency, 9.28 MHz; microwave power, 5 mW; modulation amplitude, 5.0 G; sample temperature, 77 K.

RESULTS AND DISCUSSION

Sequence similarity with other amino acid racemases The glutamate racemase gene of *P. pentosaceus* consists of a 795-nucleotides open reading frame, and encodes 265 amino acid residues which form a monomeric protein (*Mr*=29,143) (Figure 1). The deduced amino acid sequence of the active site of the glutamate racemase of *P. pentosaceus* was compared with those of PLP-independent amino acid racemases and epimerases, namely proline racemase (Cardinale and Abeles, 1968; Rudnick and Abeles, 1975), 4-hydroxyproline epimerase (Ramaswamy, 1984), diaminopimelate epimerase (Wiseman and Nichols, 1984; Higgins *et al.*, 1989), and aspartate racemase (Lamont *et al.*, 1972; Okada *et al.*, 1991; Yohda *et al.*, 1991; Yamauchi *et al.*, 1992) (Figure 2). Only aspartate racemase showed considerable sequence homology with glutamate racemase. Linear alignment of their sequences by introducing gaps to maximize identity revealed an overall homology of 14% (Figure 3). However, sequence homology in the internal region (69-192 of the glutamate racemase sequence) was much higher; 31 of 124 residues being common. If the mutationally allowed substitutions for similar residues were taken into consideration, the similarity score increased to 68% in this region. In particular, the sequences around the two cysteinyl residues (^{74}Cys and ^{184}Cys of glutamate racemase) were highly

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   1  aatgctggtg gaaatggtgc cacgattgtt cattttaaat aatccttata aaatgtaagc
  61  taattatttc atttgctacg caaagactgg tatagttaac gtaacaaatt gtgaaagagg
 121  gtaattagaa tggttgaagc tattacagat gcaaattttg aagaaaaaac aaatactggc
 181  gtaacactaa ctgatttttg ggctacatgg tgtggacctt gtcggatgca atcacctgta
 241  gttgaacaac ttgctgatga aatgggcgat aaagtttcat tttctaagat ggatgttgat
 301  caaaaccctg aaacagcaag aaactttgga attatgagta tcccaacact cttggtaaaa
 361  aaagacggag cagtcgttga ttcaatcgtg ggataccatt ctaaagaaca attagctaag
 421  attttagacc aatacattta attaaattga ggttaagtag aaaccaattt atcaataatt
 481  ggtttctttt tttatagagt taggtgggta acatgctccc ttttttttact gaaatttagt
 541  attatggatg tatgtataat tttatttagt gggggaatta aa  ATG GAT AAT CGT CCA ATT
                                                    Met Asp Asn Arg Pro Ile

 601  GGT TTT ATG GAT TCA GGC GTC GGT GGT CTG ACA GTT GTC AAA ACA GCT CAA AAA CTG TTA
      Gly Phe Met Asp Ser Gly Val Gly Gly Leu Thr Val Val Lys Thr Ala Gln Lys Leu Leu

 661  CCT AAT GAA GAA ATT ATC TTT ATT GGA GAT GAA GCA AGA ATG CCG TAT GGT CCT CGT CCC
      Pro Asn Glu Glu Ile Ile Phe Ile Gly Asp Glu Ala Arg Met Pro Tyr Gly Pro Arg Pro

 721  ACA GCA GAA GTC GTT GAA TTT TCA CGA CAG ATG GCG TCA TTT TTA ATG ACT AAA AAT ATT
      Thr Ala Glu Val Val Glu Phe Ser Arg Gln Met Ala Ser Phe Leu Met Thr Lys Asn Ile

 781  AAG GCG CTA GTG ATT GCA TGT AAT ACT GCG ACC AAC GCG GCG TTA GCG GTT TTA CAA GCT
      Lys Ala Leu Val Ile Ala Cys Asn Thr Ala Thr Asn Ala Ala Leu Ala Val Leu Gln Ala

 841  GAA TTA CCC ATC CCA GTA ATT GGG GTG ATT TTA CCT GGC GCA ATT GCA GCT AAT AGG CAA
      Glu Leu Pro Ile Pro Val Ile Gly Val Ile Leu Pro Gly Ala Ile Ala Ala Asn Arg Gln

 901  ACT AAA AAT CAA AAA ATT GGA GTT ATT GCT ACA CTA GGC ACA ATT AAA TCT GAG GCT TAC
      Thr Lys Asn Gln Lys Ile Gly Val Ile Ala Thr Leu Gly Thr Ile Lys Ser Glu Ala Tyr

 961  CCA AAG GCT TTA GCT GAA ATT AAT ACC AAA TTA CGT GCT TAT CCG GTA GCA TGC CAA GAA
      Pro Lys Ala Leu Ala Glu Ile Asn Thr Lys Leu Arg Ala Tyr Pro Val Ala Cys Gln Glu

1021  TTT GTA GAA ATT GCT GAA AAA AAT GAA CTT CAT ACA ACG GCA GCT CAA AAA GTT ATG AAT
      Phe Val Glu Ile Ala Glu Lys Asn Glu Leu His Thr Thr Ala Ala Gln Lys Val Met Asn

1081  GAA AAA CTA GCT GAG TTT AGG CAA GAT CAA ATT GAT ACT TTG ATT TTA GGC TGC ACT CAT
      Glu Lys Leu Ala Glu Phe Arg Gln Asp Gln Ile Asp Thr Leu Ile Leu Gly Cys Thr His

1141  TTT CCG CTT TTA GAA GAA GGT ATC CAA GCA GCC GTT GGG CCT GAT GTT ACT TTA GTA GAT
      Phe Pro Leu Leu Glu Glu Gly Ile Gln Ala Ala Val Gly Pro Asp Val Thr Leu Val Asp

1201  CCA GGA GTG GAG ACC GTT CAC CAA TTA ATT GAA ATT TTA ACA AAA CAA GCG CTT CAA CAT
      Pro Gly Val Glu Thr Val His Gln Leu Ile Glu Ile Leu Thr Lys Gln Ala Leu Gln His

1261  GCA GAA GGT CCT AAA GCG CAG GAT CAA TAT TAT TCA ACG GGT AAT ATT AAG AAT TTT GAA
      Ala Glu Gly Pro Lys Ala Gln Asp Gln Tyr Tyr Ser Thr Gly Asn Ile Lys Asn Phe Glu

1321  GAA ATA GCG CGG ACA TTC TTA AAT CAA GAT CTA AGA GTT GAA GAA GTT AAA ATT GAC TAG
      Glu Ile Ala Arg Thr Phe Leu Asn Gln Asp Leu Arg Val Glu Glu Val Lys Ile Asp Stop

1381  gagaaattag aatgaataat gaaattttga ttgccacgaa aaatgatgga aaattaaaag
1441  aatttaaaca aatttttgaa caaaagggaa ttgttgtaaa atcactgaaa gatattaatg
1501  atgacgttga aatagttgaa aatggcttgt cgtttgaaga aaatgcccgt ttaaaagcag
1561  acggatacgc taaatcaatt ggtattccag tccttgccga tgattcaggt ttggaaatcg
1621  atgctttgaa tggacgccca ggaatttttt cggcgcgtta cgccggtgat cacaatgatg
1681  cagctaataa tgccaaagtt cttagcgaat tgggtggcat tccagatgaa aaacgtacag
1741  caacgtttca ttcaaccgta gttgtgcgca aaccagatgg aagtgagtta gtggcaaatg
1801  gaaatc
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Figure 1. Nucleotide sequence of the glutamate racemase gene of *P. pentosaceus* and the deduced amino acid sequence of the enzyme. Essential cysteinyl residues in catalysis are shown by *boldface* type. The possible ribosome binding site is *underlined*.

-L-V-I-A-C-N-T-A-T-

-L-I-L-G-C-T-H-F-P-

-I-V-L-T-C-N-T-A-

-S-T-C-G-T-G-S-

-S-P-C-G-T-

-G-V-A-Q-C-G-N-G-A-

Figure 2. Comparisons of amino acid sequences around essential cysteinyl residue of PLP-independent amino acid racemases.

Glutamate Racemase (^{73}Cys)

Glutamate Racemase (^{184}Cys)

Aspartate Racemase (^{84}Cys)

Proline Racemase

4-Hydroxyproline Epimerase

Diaminopimelate Epimerase

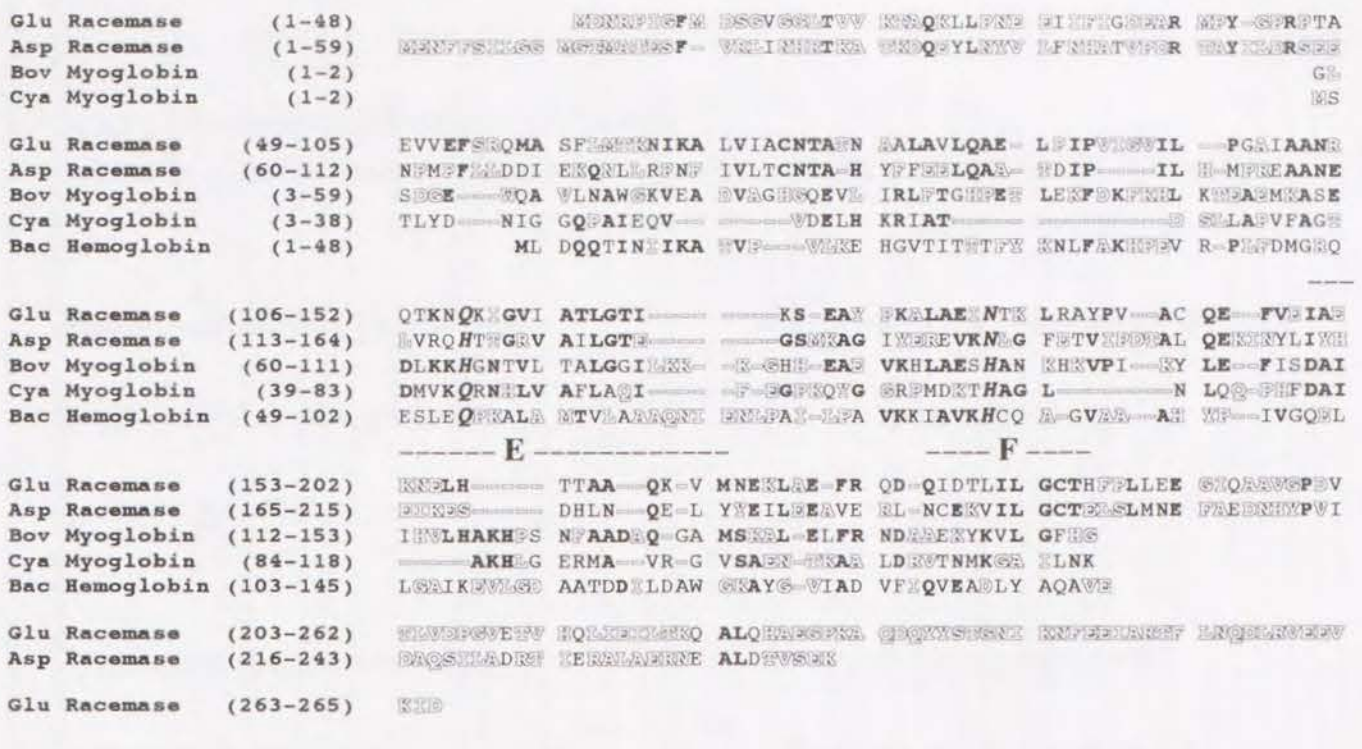


Figure 3. Linear alignment of the amino sequences of glutamate racemase (Glu Racemase), aspartate racemase (Asp Racemase), bovine myoglobin (Bov Myoglobin), bacterial hemoglobin from *Vitreoscilla* (Bac Hemoglobin), and cyanobacterial myoglobin from *Nostoc commune* (Cya Myoglobin). The sequences were aligned by introducing gaps (*hyphen*) to maximize identities according to the methods of Dayhoff *et al.*.

homologous. Both enzymes contain an essential cysteinyl residue as reported previously, suggesting that either ^{74}Cys or ^{184}Cys, or both play an essential role in catalysis.

Sequence similarity with other proteins Glutamate racemase shows the highest sequence homology with bovine myoglobin among various proteins registered in the National Biomedical Research Foundation and the Swissprot protein sequence data-banks. The homologous region between glutamate racemase and myoglobin occurs mainly in the region between ^{46}Phe and ^{150}Gly of bovine myoglobin, which corresponds to the region from ^{92}Val to ^{183}Gly of glutamate racemase (Figure 3). Twenty-seven of the 92 residues of glutamate racemase are common to the corresponding residues of the myoglobin. The homology score is 52% in this region, if the similar residues of permissible mutational substitution are taken into account. The amino acid sequences of myoglobins from various sources except for abalone, *Sulculus diversicolor aquatilis* (Suzuki and Takagi, 1992), are highly conserved. The abalone myoglobin shows highly homology with human indoleamine 2,3-dioxygenase, but not with other myoglobins (Suzuki and Takagi, 1992). I found no significant sequence homology between the abalone myoglobin and glutamate racemase. Homology scores between glutamate racemase and the other myoglobins were similar: 21-27% identity in the range of the 92 amino acid

residues. Cyanobacterial myoglobin from *Nostoc commune* (Potts *et al.*, 1992) showed the lowest sequence homology (21%) with glutamate racemase. Significant sequence similarities were also found between glutamate racemase and other globin family proteins such as hemoglobins in this same region (Figure 3). However, bacterial hemoglobin from *Vitreoscilla* (Wakabayashi *et al.*, 1986) shows the lowest homology with glutamate racemase among various hemoglobins examined.

Proteins homologous with bovine myoglobin in primary structure were also searched by means of the same data-banks. The homology is dependent on the kind of proteins and their sources: myoglobins from other sources, 38-85%; α and β-chains of mammalian hemoglobins, 21-31%; *Vitreoscilla* hemoglobin (Wakabayashi *et al.*, 1986), 24%; *Nostoc* myoglobin (Potts *et al.*, 1992), 16%; glutamate racemase, 26% (in the range between ^{46}Phe and ^{150}Gly of bovine myoglobin). Bovine myoglobin shows higher homology with glutamate racemase than prokaryotic myoglobin and hemoglobin. Aspartate racemase (Yohda *et al.*, 1991) was also homologous with bovine myoglobin in the region from ^{102}Ile to ^{196}Gly corresponding to that from ^{46}Phe to ^{150}Gly of bovine myoglobin: 14 residues were common between the two proteins (Figure 3). However, this sequence similarity was much lower than that found between glutamate racemase and bovin myoglobin.

Effects of hemin on glutamate racemase activity The homologous range (residue numbers, 46-150) of bovine myoglobin contains the regions corresponding to E and F helices, which constitute its heme binding pocket (Imai, 1988) (Figure 3). E7 of the E helix of bovine myoglobin, ^{64}His ,which is essential in binding molecular oxygen (Imai, 1988) was replaced by Gln in the bacterial myoglobin (Potts *et al.*, 1992) and the bacterial hemoglobin (Wakabayashi *et al.*, 1986). A homologous Gln occurs as ^{110}Gln in glutamate racemase. Moreover, ^{68}Val of E11, which is highly conserved among globin family proteins, is also conserved as ^{114}Val. Accordingly, I examined the interaction of glutamate racemase and aspartate racemase with hemin. When the enzymes were assayed in the presence of various concentrations of hemin, only glutamate racemase was inhibited by hemin (Figure 4). The inhibition was concentration dependent. A plot of the reciprocal of glutamate racemase activity against hemin concentrations showed that hemin produces a mixed-type inhibition (Figure 5). The *Ki* value for hemin was estimated to be about 3.7 μM from the data. When glutamate racemase was incubated with hemin at various concentrations, a stoichiometric complex was formed and isolated by gel filtration (Figure 6). However, no appreciable amount of hemin was bound with asparate racemase under the same conditions. The complex of glutamate racemase with hemin was reduced with dithionite. UV-

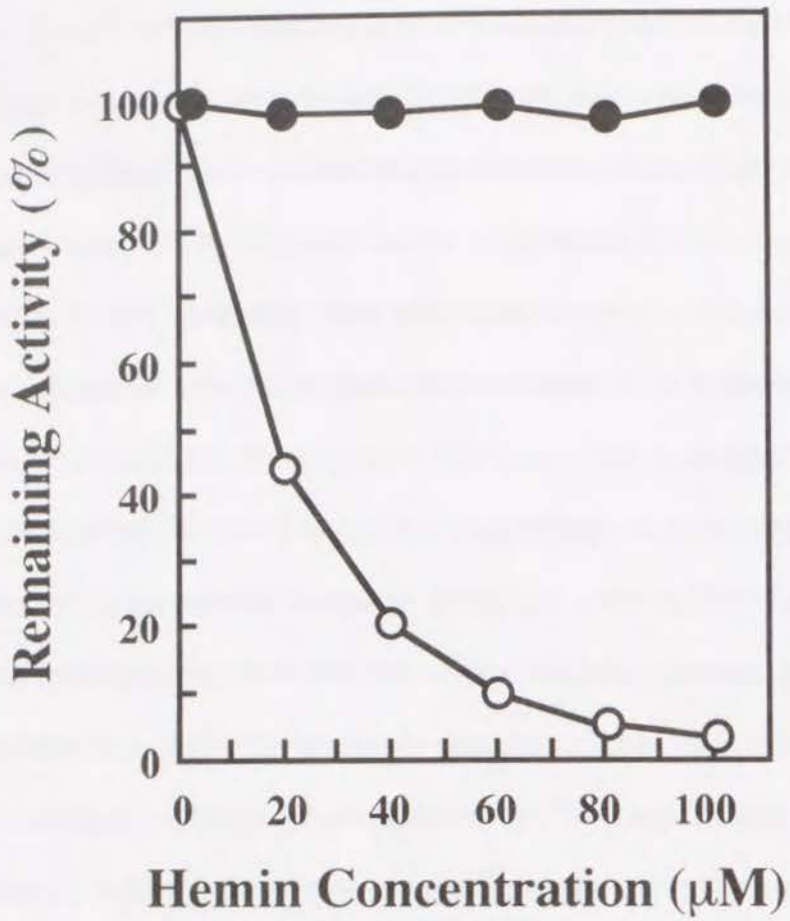


Figure 4. The effect of hemin concentration on glutamate racemase and aspartate racemase activities. Glutamate racemase (○) and aspartate racemase (●) activities were determined by L-glutamate oxidase coupling assay.

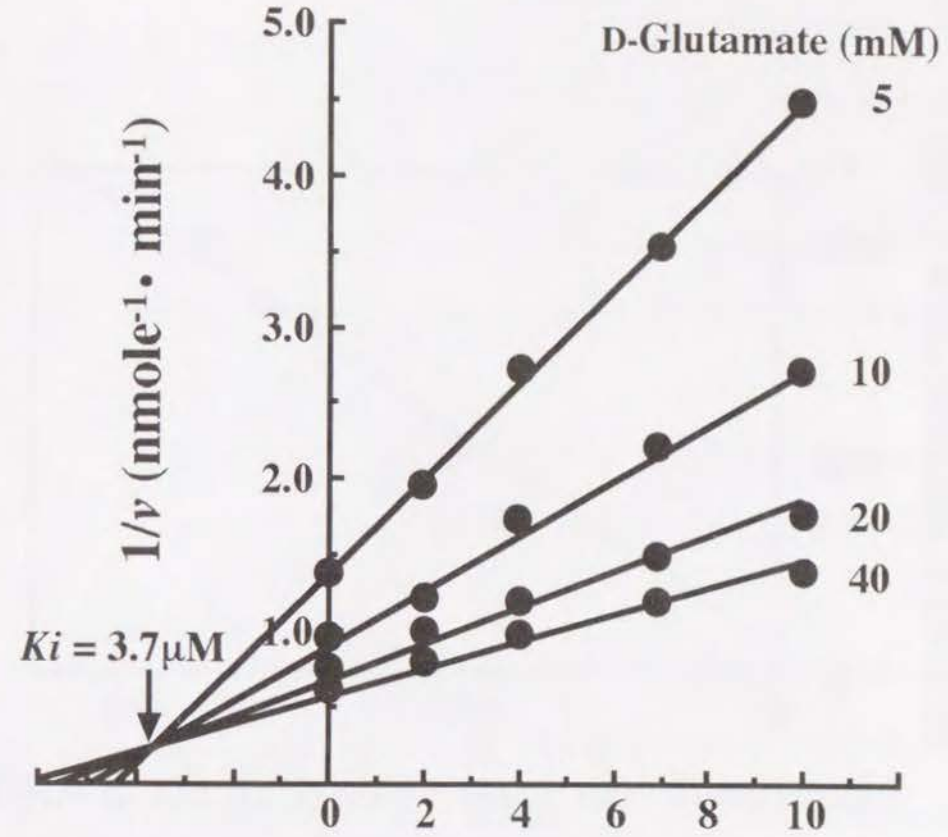


Figure 5. Inhibition of glutamate racemase activity by different hemin concentrations. Glutamate racemase activity in indicated conditions was determined by L-glutamate dehydrogenase coupling assay. Velocity (v) refers to initial glutamate racemase activity (nmole of L-glutamate formed per min). Data were plotted $1/v$ against hemin concentration, and the *Ki* was estimated as described by Dixon and Webb.

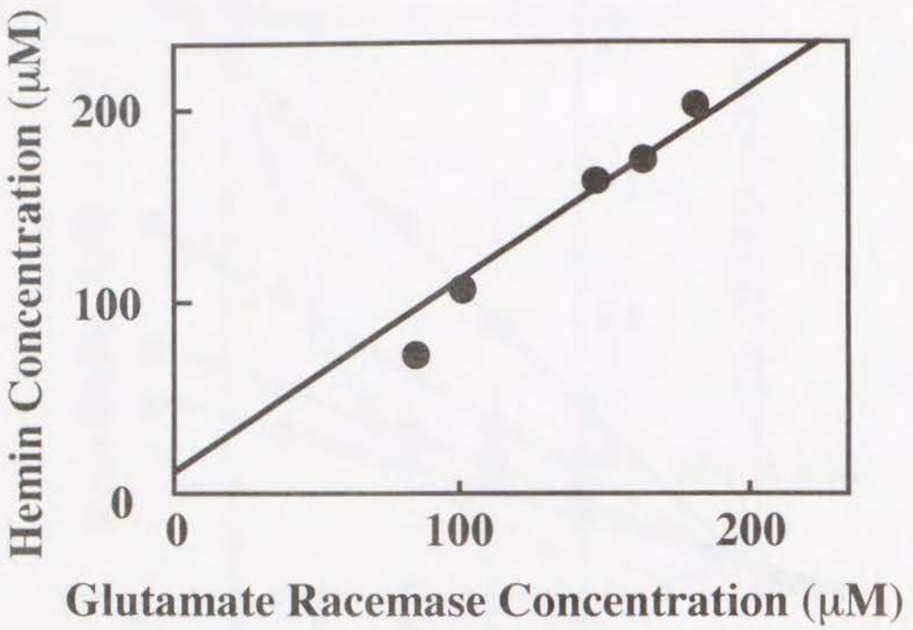


Figure 6. Stoichiometry of hemin binding with glutamate racemase. Enzyme concentration and enzyme bound hemin were determined by DTNB titration and measurement of atomic absorption of iron with a Shimazdu AA-670G atomic absorption spectrophotometer. An average of seven independent determinations were taken.

visible spectra of both oxidized and reduced forms of the complex were characteristic of hemoproteins (Figure 7). The ESR spectrum of the oxidized form resembled that of hemoglobin under the same conditions (Figure 8). Thus, glutamate racemase resembles hemoglobins in having a hemin binding pocket, in which two nitrogen atoms of some amino acid residues are probably ligated to iron in the coordination complex with hemin.

Hemin inhibits glutamate racemase either by binding near the active site, or at some other site where the binding causes a conformational change of the active site.

Proline racemase, 4-hydroxyproline epimerase and diaminopimelate epimerase contain an essential cysteinyl residue, and show sequence similarity with each other in the moiety around the cysteinyl residues. Higgins *et al.* (1989) proposed that their enzymes have evolved from a common ancestral protein. Glutamate racemase as well as aspartate racemase also contains an essential cysteinyl residue, but shows no sequence homology to their three enzymes. However, highly sequence similarity in the regions of two cysteinyl residues occurs between glutamate racemase and aspartate racemase. It is suggested that glutamate racemase and aspartate racemase have derived from a common evolutionary origin which is different from the common ancestor for proline racemase, 4-hydroxyproline epimerase and diaminopimelate epimerase.

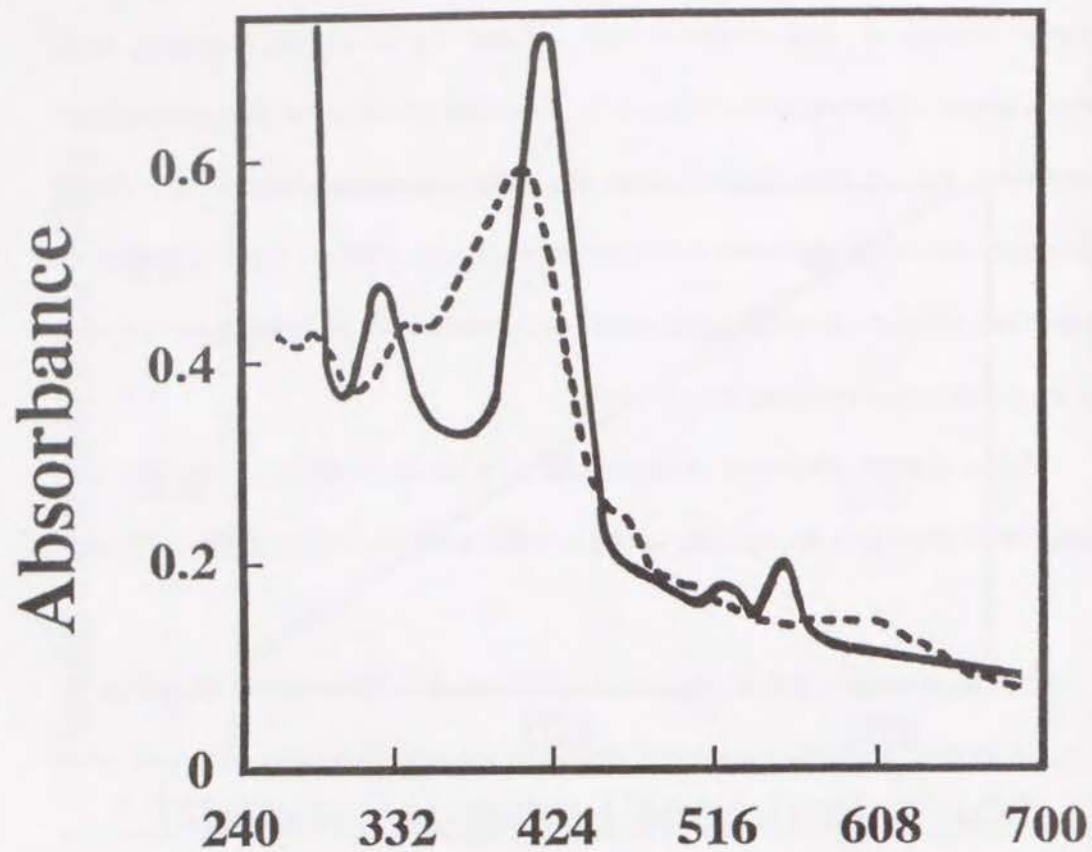


Figure 7. UV-visible spectra oxidized (*dotted line*) and reduced (*solid line*) forms of the glutamate racemase-hemin complex. The reduced form was prepared with sodium dithionite according the method of Chen and Asada.

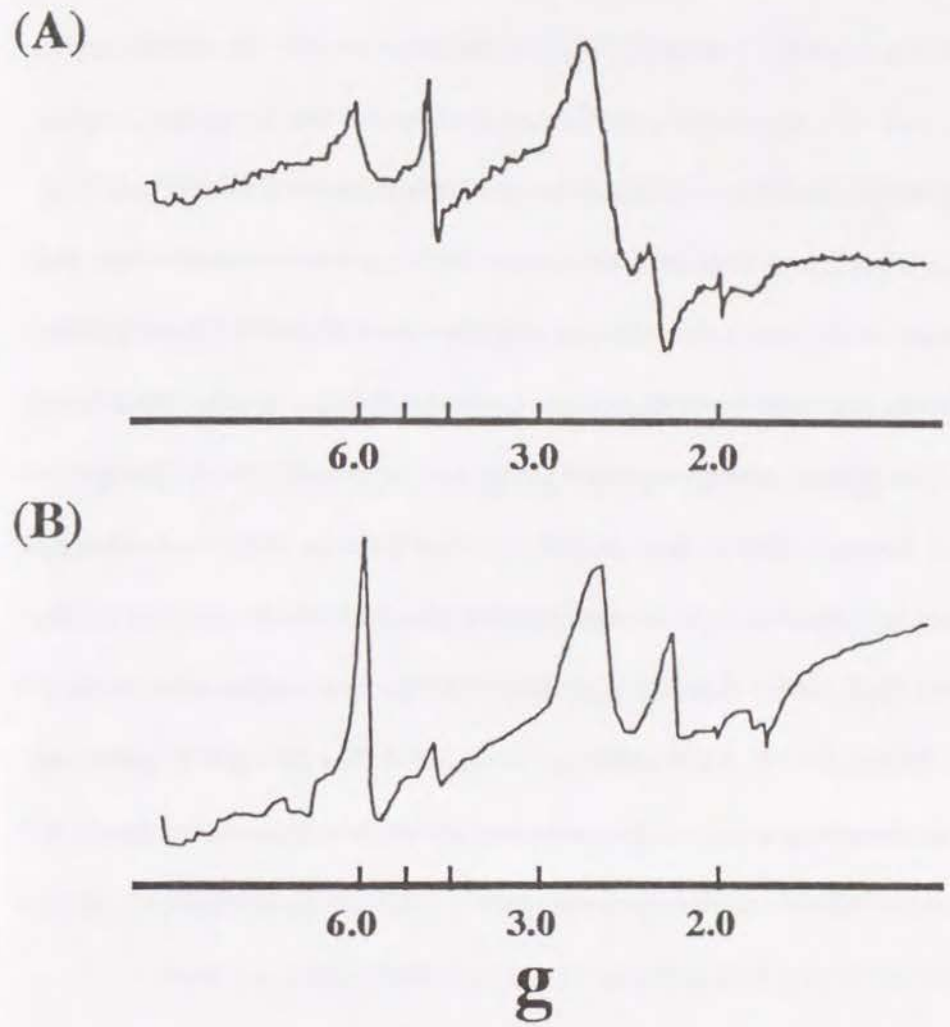


Figure 8. ESR spectra of the glutamate racemase-hemin complex (A) and bovine erythrocyte hemoglobin (B). The spectra were measured with a JEOL JES-REzx ESR spectrometer: microwave frequency, 9.28 MHz; microwave power, 5 mW; modulation amplitude, 5.0 G; sample temperature, 77K.

The highly sequence homology of glutamate racemase with the globin family proteins, in particular myoglobins, and formation of its inactive equimolar complex with hemin, suggest that the enzyme may be derived from an evolutionary origin of globin family proteins. Aspartate racemase also may have evolved from the common ancestral protein, but its structure may have been altered more extensively than glutamate racemase by divergence. Lactic acid bacteria may have been producing glutamate racemase and aspartate racemase, namely globin family-like proteins that diverged from an ancestral globin protein after the ability to synthesize hemin was lost. Alternatively, lactic acid bacteria inherently never produced hemin (Teuber and Geis, 1981; Kandler and Weiss, 1986), and acquired from other organisms the gene for the globin family proteins, which then diverged to glutamate racemase and aspartate racemase. Whatever may be the case, glutamate racemase is proved to be a microbial enzyme that is structurally similar to globin family proteins and to stoichiometrically bind hemin to form a catalytically inactive complex.

SUMMARY

The glutamate racemase gene of *P. pentosaceus* consists of a 795-nucleotides open reading frame, and encodes 265 amino acid residues which form a monomeric protein (*Mr*=29,143). The amino acid sequence of the enzyme shows highly similarity with that of aspartate racemase from *S. thermophilus*, in particular in the regions around the two cysteinyl residues. Neither racemase requires cofactors, but they contain essential cysteinyl residues (Yohda *et al.*, 1991). I have found also significant sequence homology between the glutamate racemase and mammalian myoglobins, in particular in the regions corresponding to E and F helices, which constitute a heme binding pocket of myoglobins. Glutamate racemase is bound with an equimolar amount of hemin to be inactivated. Aspartate racemase shows a low sequence homology with myoglobins, but is not bound with hemin.

CHAPTER 2

Expression and Distribution of Bacterial Glutamate Racemase Genes

Bacterial cell walls contain several kinds of D-amino acids as components of peptidoglycans. D-Glutamate is incorporated into peptidoglycan through its addition to UDP-*N*-acetylmuramyl-L-alanine, a peptidoglycan precursor, and this reaction is catalyzed by UDP-*N*-acetylmuramyl-L-alanine-D-glutamate ligase (EC 6.3.2.9) (Mengin *et al.*, 1989). D-Glutamate can be produced by both enzyme reactions of D-amino acid aminotransferase (EC 2.6.1.21) (Thorne *et al.*, 1955; Thorne and Molnar, 1955; Meadow and Work, 1958; Kuramitsu and Snoke, 1962; Tanizawa *et al.*, 1989) and glutamate racemase (EC 5.1.1.3) (Ayenger and Roberts, 1952; Narrod and Wood, 1952; Glaser, 1960; Diven, 1969; Nakajima *et al.*, 1988; Gallo and Knowles, 1993). However, D-amino acid aminotransferase occurs only in bacilli, and glutamate racemase is found only in lactobacilli, and the physiological functions of their enzymes have not been fully elucidated. It is unknown if their roles are biosynthetic, biodegradative or both. The biosynthesis of D-glutamate in other bacteria such as *E. coli* has not been shown. Recently, the gene responsible for the D-glutamate biosynthesis in *E. coli* was identified and

designated as *murI* (Doublet *et al.*, 1992) and later as *dga* (Dougherty *et al.*, 1993). The gene complements the D-glutamate auxotrophy of *E. coli* strain, WM335. The *murI* gene has corresponded to a sequenced open reading frame, ORF1 (Brosius *et al.*, 1981). The *murI* gene product was predicted to consist of 289 amino acid residues with a molecular weight of 31,504 (Brosius *et al.*, 1981), and contained a large number of hydrophobic residues. However, attempts were made to identify the enzyme activity of the gene product without success (Doublet *et al.*, 1992).

Recently, the gene encoding glutamate racemase of *P. pentosaceus* was cloned (Choi *et al.*, 1991) and sequenced (Choi *et al.*, 1994). I found that the amino acid sequence of the enzyme of *P. pentosaceus* shows significant homology with that of the *murI* gene product. I made the *murI* gene overexpressed, and demonstrated that the *murI* gene product is glutamate racemase (Yoshimura *et al.*, 1993; Doublet *et al.*, 1993). I here provide the evidence that the *murI* gene is expressed to produce glutamate racemase, which is involved in the synthesis of D-glutamate. Additionally, I show that the glutamate racemase genes are distributed among various kinds of bacterial strains, and discuss here the physiological functions of the enzyme.

EXPERIMENTAL PROCEDURES

Strains The following bacteria were used; *Acinetobacter calcoaceticus* IFO12552, *Bacillus subtilis* IFO14192, *Clostridium butylicum* IFO13949, *Escherichia coli* IFO3301, *Pseudomonas putida* IFO14164, *Pediococcus pentosaceus* IFO12318, *Staphylococcus aureus* IFO3060.

Materials Custom-made oligonucleotide primers for PCR were from Greiner Japan. Taq DNA polymerase, PCR buffer and $MgCl_2$ solution were purchased from Gibco BRL. dNTP Mixture and a SUPREC^{TM}-01 were obtained from Takara Shuzo. A Taq dye deoxy^{TM} terminator cycle sequencing kit was from Applied Biosystems. All restriction enzymes and DNA ligation kit were purchased from Takara Shuzo. Plasmid pKK223-3 was obtained from Pharmacia. All reagents for DNA synthesis were from Applied Biosystems. A Bio-Rad Protein Assay reagent and a SDS-PAGE standard were from Bio-Rad; standard proteins for gel filtration from Oriental Yeast. A 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's Reagent) was from Pierce. L-Glutamate oxidase was a gift from Dr. H. Kusakabe of Yamasa Shoyu. The Kohara ordered phage bank, E11C11 (Kohara *et al.*, 1987) was a kind gift from Dr. Y. Kohara of National Institute of Genetics, Japan. All other chemicals were of analytical grade.

Construction of plasmids pGR2 and pGR3 E11C11 Phage DNA was digested with *Eco*RI and *Hin*dIII. The plasmid pGR2 was obtained by insertion of the resultant 1.9-kb fragment containing *murI* gene into pKK223-3. Plasmid pGR3 contained the open reading frame of *murI* gene (ORF1) with a designed ribosome binding sequence. This was constructed by PCR with primers, GRP-1 (N-terminus) and GPR-2 (C-terminus), which were synthesized on an Applied Biosystems 381A DNA synthesizer. Sequences of the primers were as follows.

GRP-1; 5'-AGCCAGAATTC**AGGA**CAAAGACCATGAGAC-3'

GRP-2; 5'-CAACATT CCTGCAGATCAGCCTAAA-3'

GRP-1 contained an *Eco*RI restriction site (*underlined*) and a designed ribosome binding sequence (*boldface type*). The PCR mixture (100 µl) consisted of 8 µmole of Tris-HCl buffer (pH 8.3), 2 µmole of $(NH_4)_2SO_4$, 0.5 µmole of $MgCl_2$, 20 pmol of each dNTP, 2.5 units of Taq DNA polymerase, 1 µg of E11C11 Phage DNA (as a template), and each 100 pmol of GRP-1 and -2. The reaction mixture was heated at 94°C for 1 min (for denaturation), then cooled rapidly to 52°C with 1 min-hold (for annealing), and incubated at 72°C for 4 min (for extension). The programmed temperature shift was repeated 40 times. The plasmid pGR3 was constructed by replacement of the 38-bp EcoRI-PstI fragment of PGR2 by the corresponding fragment excised from the PCR product.

Enzyme and Protein Assay Glutamate racemase was assayed with D-glutamate as a substrate by determination of α-ketoglutarate formed from L-glutamate, the product of racemase reaction with L-glutamate oxidase. The reaction mixture (200 μl) containing 20 μmole of Tris-HCl buffer (pH 8.0), 2 μmole of D-glutamate, and enzyme was incubated at 30°C for 1 h. The reaction was terminated by addition of 10 μl of 12 M HCl. After the reaction mixture was neutralized by addition of 20 μl of 6 M NaOH, a 160 μl aliquot was withdrawn from the mixture and incubated at 37°C for 1 h with 20 μmole of Tris-HCl buffer (pH 8.0), and 0.5 units of L-glutamate oxidase in a final volume of 200 μl. The reaction mixture was mixed with 200 μl of 0.066% dinitrophenylhydrazine in 2 M HCl, then stood at room temperature for 10 min and mixed with 1.0 ml of 2 M NaOH. α-Ketoglutarate formed was determined by measurement of an increase in the absorbance at 550 nm. One unit of enzyme was defined as the amount that catalyzes the formation of 1 nmole of product per min.

Glutamate racemase was alternatively assayed with HPLC by determination of L-glutamate formed from D-glutamate after the product was derivatized to a diastereomer with Marfey's reagent (Merfey, 1984). The reaction mixture (1.0 ml) containing 20 μmole of Tris-HCl buffer (pH 8.0), 10 μmole of D-glutamate, and enzyme was incubated at 30°C for 2 h, and the reaction was terminated by addition

of 50 μl of 12 M HCl. After the reaction mixture was neutralized with 100 μl of 6 N NaOH, a 100 μl aliquot was withdrawn, and mixed with 200 μl of 1% Marfey's reagent in acetone, and 40 μl of 1 M $NaHCO_3$ (Nagata *et al.*, 1992). The reaction mixture was incubated at 37°C for 1 h in dark, and the reaction was stopped by addition of 20 μl of 1 M HCl. The reaction mixture was dried up with a Speed Vac Concentrator (Savant), and the residue was dissolved in 1.4 ml of methanol. FDAA-DL-glutamate thus obtained was applied to a TSK ODS-120T column (0.46 X 25 cm, Tosoh) equipped on a Waters 600 HPLC system. The column was developed at a flow rate of 0.6 ml/min with a linear gradient of acetonitrile concentrations (10 to 40%/h) in 50 mM triethylamine-phosphate buffer (pH 2.8) with monitoring the absorbance at 340 nm.

Protein concentration was determined with a Bio-Rad protein assay kit with bovin serum albumin as a standard.

Expression and purification of glutamate racemase *E. coli* JM109 cells were transformed with pGR2 or pGR3. Each transformant was cultured overnight in 5 ml of the Luria-Bertani's broth supplemented with ampicillin (50 mg/ml). The culture was then inoculated into the fresh medium (500 ml) containing 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested

after incubation at 37°C for 10 h. The cells (1.4 g wet weight) were suspended in 5 ml of the standard buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM DL-glutamate, 10% glycerol, 0.1% 2-mercaptoethanol (2-ME), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by sonication. After centrifugation, the supernatant solution was dialyzed against the same buffer and used as a cell extract. The precipitate was resuspended in the standard buffer supplemented with 6 M urea and incubated at 4°C for 16 h. After centrifugation, the supernatant solution was dialyzed against the standard buffer. The resultant solution was designated as a precipitate fraction. The cell extract for purification (3680 mg of protein, 17,000 units of glutamate racemase) was prepared from 34 g (wet weight) of *E. coli* JM109/pGR3 cells and applied to a DEAE-cellulose column (4.8 X 25 cm) equilibrated with the standard buffer. The column was washed with the standard buffer and subsequently with the same buffer containing 0.2 M NaCl. The enzyme was eluted with 1200 ml of a linear gradient of NaCl concentrations (0.2-0.5 M) in the standard buffer. The active fractions (1100 mg of protein, 12800 units) were combined and concentrated, and subjected to gel filtration with a HiLoad 26/60 column (Pharmacia) equipped on a FPLC system (Pharmacia). The column was equilibrated with the standard buffer containing 0.5 M NaCl and developed with the same buffer at a flow rate of 3.5 ml/min. The active fractions (24.8 mg, 467 units) were combined and used as a partially purified enzyme.

Molecular weight determination Molecular weight of glutamate racemase was determined by gel filtration with a TSK-G3000sw column (0.75 X 30 cm, Tosoh) equilibrated with 50 mM Tris-H_2SO_4 buffer (pH 7.5) containing 0.2 M Na_2SO_4, 1 mM DL-glutamate, 10% glycerol, 0.1% 2-ME at a flow rate of 0.1 ml/min. A calibration curve was made with the following proteins: alcohol dehydrogenase (*Mr*=150,000), bovine serum albumin (66,200), carbonic anhydlase (29,000), and cytochrome C (12,400).

Conditions of PCR and Direct Sequencing Bacterial lysate was prepared by lysozyme and sodium dodecyl sulfate (SDS) treatments, and the chromosomal DNA was purified by phenol-chloroform extraction and polyethylene glycol (PEG) precipitation. *In vitro* amplification of the regions homologous to the glutamate racemase genes was carried out by means of PCR with each primer pair of GRPP1-GRPP2 (a) and GRPP3-GRPP4 (b). Sequences of their primers were as follows (nucleotides were shown with *IUB* code).

GRPP1; 5'-GAYTCVGGCKTCGGTGGKYTGWCRGT-3'

GRPP2; 5'-RSTSGCAGTRTTRCAWGCRAYCAC-3'

GRPP3; 5'-TCIGGIKTIGGIGGIYTRWCIGT-3'

GRPP4; 5'-RSTIGCIGTRTTRCAIGCIAYIA-3'

The reaction mixture for PCR (100 μl) consisted of 8 μmole of Tris-HCl buffer (pH 8.3), 2 μmole of $(NH_4)_2SO_4$, 0.2 μmole of $MgCl_2$, 20 pmole of each dNTP, 2.5 units of Taq DNA polymerase, 1μg of bacterial chromosomal DNA (as a template DNA), 100 pmole of those of each primer pair and 5% (v/v) DMSO. The reaction mixture was heated at 96°C for 1min, then cooled rapidly to 60°C (a) or 50°C (b) for 15 sec, and incubated at 72°C for 4 min (for extension). The programmed temperature shift was repeated 25 times. The PCR products were separated from the rest of nucleotides (primers and dNTPs) with a SUPREC™-01. Direct sequencing of the purified PCR products was carried out with an Applied Biosystems Taq dye deoxy™ terminator cycle sequencing kit. The sequencing mixture (20 μl) contained 9.5 μl of cycle sequencing premixture, 100 ng of the purified PCR products (as a template) and 3.2 pmole of the same primer. The reaction for sequencing was performed essentially according to the above program except the extension temperature (60°C). The reaction products were purified by phenol-chloroform extraction and ethanol precipitation at about 25°C. The precipitated products were dissolved in 80% deionized formamide containing 10 mM EDTA (4 μl) and heated at 96°C for 2 min. Nucleotide sequence of the reaction products was determined with an Applied Biosystems 373A DNA sequencer.

RESULTS AND DISCUSSION

Sequence Homology of murI gene-encoding protein of E.coli with glutamate racemase of P. pentosaceus Linear alignment of the amino acid sequence of the *murI* gene-encoding protein of *E. coli* and those of the glutamate racemases of *P. pentosaceus* and *L. fermenti* (Gallo and Knowles, 1993) showed highly sequence homology with each other (Figure 9). The overall sequence homologies between ORF1 and the glutamate rasemases are about 30% and are lower than that between two glutamate racemases (44.5%, 118 out of 265 matchable residues). However, the homologous local sequence of three segments (31-39, 93-100, and 206-214 of the ORF1 sequence) is highly conserved in three enzymes. Both glutamate racemase reactions are considered to proceed through a two-base mechanism (Choi *et al.*, 1992; Tanner *et al.*, 1993). Conversion of ^{73}Cys and ^{184}Cys of glutamate racemase to alanyl residues by site-directed mutagenesis results in a loss of the activity. Therefore, their cysteinyl residues are thought to serve as a catalytic base in both glutamate racemase; the bases probably remove the α-hydrogen from the substrate to result in racemization. Their cysteinyl residues are found in the above homologous segments, and the corresponding cysteinyl residues are conserved in the *murI* gene-encoding protein as ^{95}Cys and ^{209}Cys. These strongly suggest that the *murI* gene-encoding protein is identical

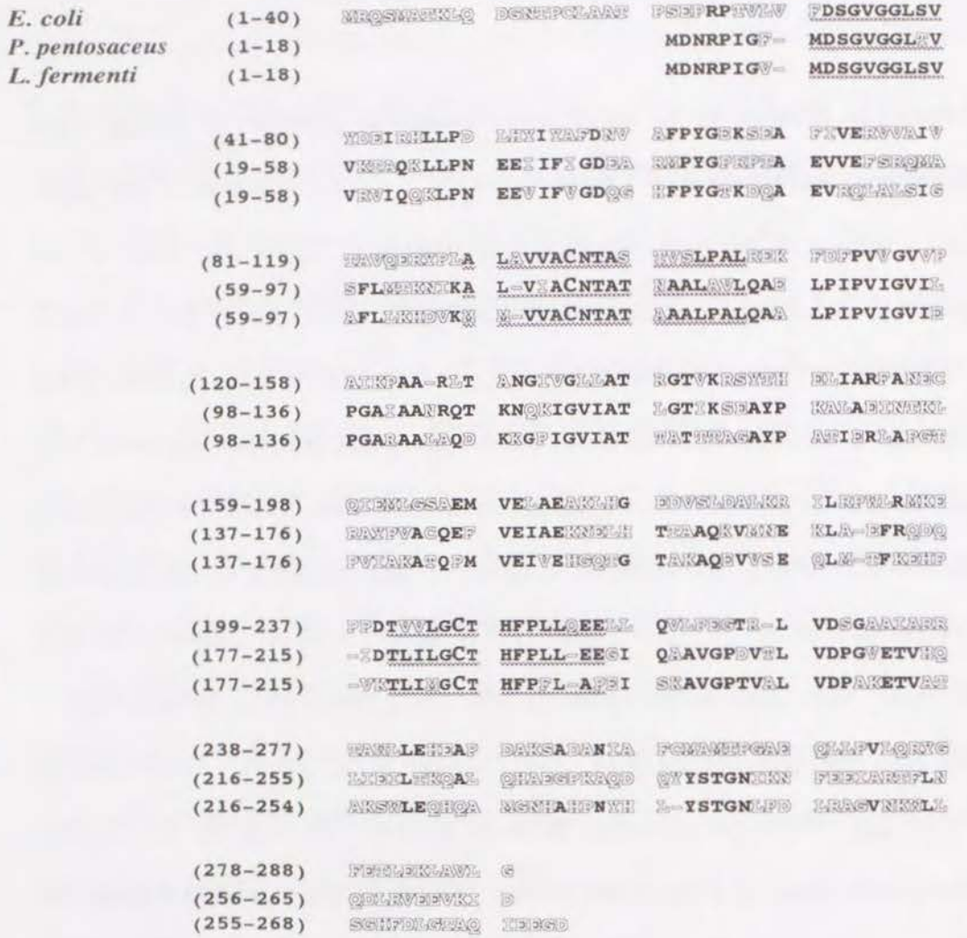


Figure 9. Linear alignment of the amino acid sequence of predicted *murI* gene product of *E. coli* (upper), glutamate racemase of *P. pentosaceus* (middle), and *L. fermenti* (lower). Three sequences were aligned by introducing gaps (*hyphens*) to maximize identities. Common residues in two or three enzymes are shown by *boldface* type. The regions showing high sequence homology in each other are *underlined*.

with glutamate racemase.

Expression and identification of murI gene I constructed the plasmid pGR2 and transformed *E. coli* JM109 with it to obtain the *murI* gene-encoding protein (Figure 10). However, no glutamate racemase activity was detected in the cell extract of *E. coli* JM109/pGR2 cells. SDS-PAGE of the cell extract showed the absence of the protein with a molecular weight of about 31,000; the molecular weight of *murI* gene-encoding protein was calculated as 31,504 based on the sequence. I supposed that failure of the *murI* gene to be expressed was due to its unsuitable structure, in particular lack of the definite ribosome binding sequence upstream the initiation codon of ORF1. Thus, I constructed the plasmid pGR3. This was designed so that the initiation codon is located at 19-bp downstream of the *Eco*RI-site of pKK223-3 with an artificial ribosome-binding region (AGGA and CC next to ATG, Figure 10).

SDS-PAGE of the cell extract of *E. coli* JM109/pGR3 showed the occurrence of protein with a molecular weight of about 31,000 (Figure 11, lane A). When the glutamate racemase of *P. pentosaceus* was overproduced under the control of *tac* promoter in the *E. coli* clone cells, it was found in inclusion bodies (Choi *et al.*, 1991). I found the occurrence of protein with molecular weight of about 31,000 in the precipitate fraction of *E. coli* JM109/pGR3 (Figure 11, lane B). The 31,000

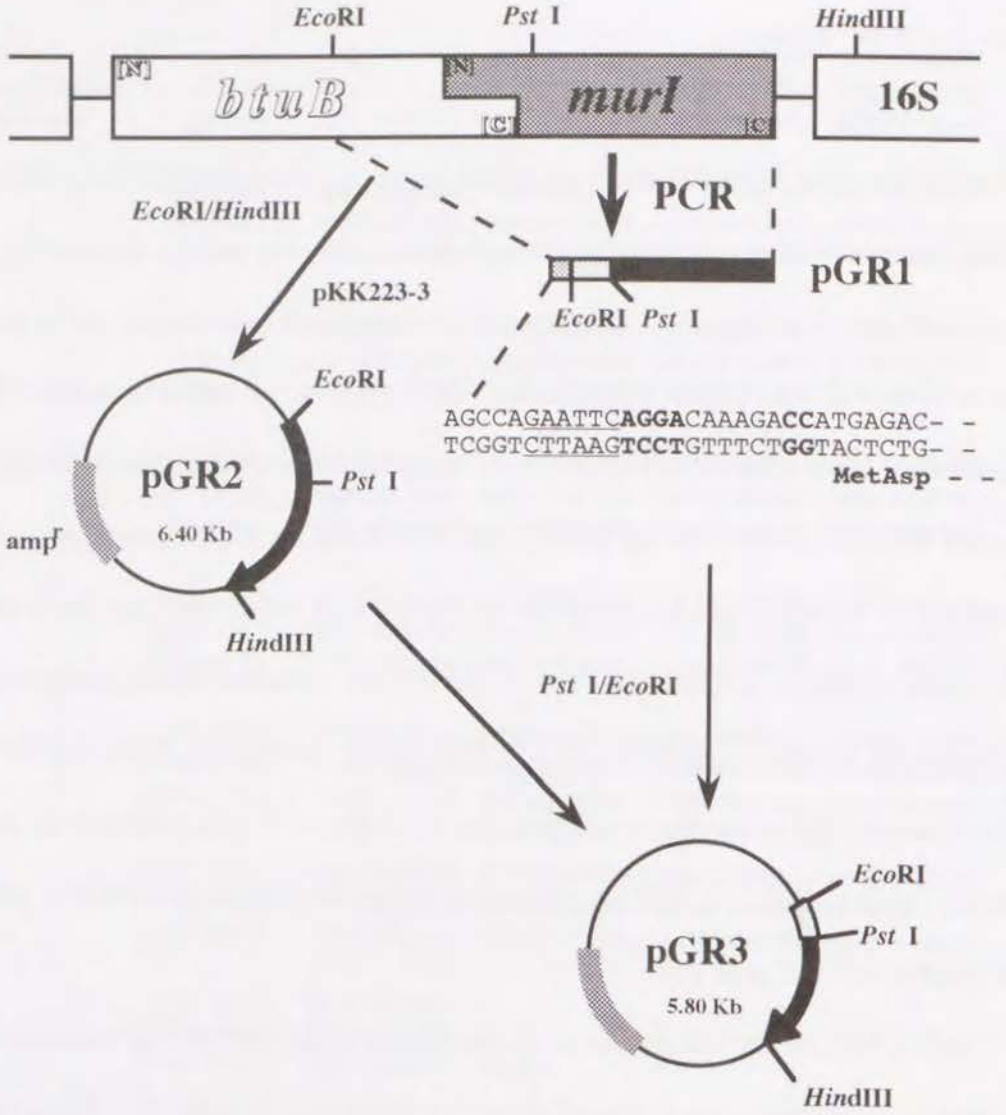


Figure 10. Constraction of plasmids pGR2 and pGR3. The length of DNA fragments shown is arbitary.

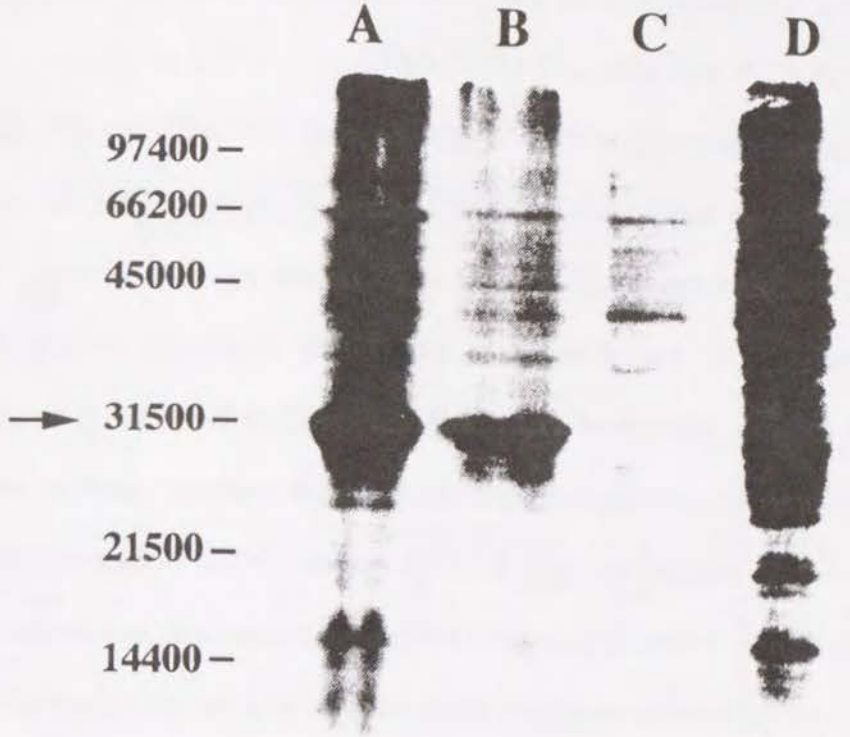


Figure 11. SDS-PAGE of the cell extract and precipitate fraction of *E. coli* JM109/pGR3 and JM109/pKK223-3. Each cell extract (150 μg of protein) and precipitate fraction (100 μg of protein) were run on an SDS-PAGE (12.5 % acrylamide). *lane A*, cell extract of *E. coli* JM109/pGR3; *lane B*, precipitate fraction of *E. coli* JM109/pGR3; *lane C*, cell extract of *E. coli* JM109 /pKK223-3; *lane D*, precipitate fraction of *E. coli* JM109 /pKK223-3.

protein band was found neither in the precipitate fraction nor in the cell extract of *E. coli* JM109/pKK223-3 (Figure 11, lanes C and D).

The glutamate racemase activity of extracts of *E. coli* JM109/pGR3 and JM109/pKK223-3 cells was determined with L-glutamate oxidase. Only the cell extract of JM109/pGR3 showed the activity (specific activity; 0.53 units/mg of protein). The activity was detected in neither the precipitate fraction of JM109/pGR3 nor the extracts of *E. coli* JM109/pKK223-3. The glutamate racemase activity was confirmed by HPLC after the reaction product was derivatized to form a diastereomer with Marfey's reagent. When D-glutamate was incubated with the cell extract of *E. coli* JM109/pGR3, L-glutamate was produced (Figure 12). L-Glutamate was not formed in the reaction with the cell extract of *E. coli* JM109/pKK223-3. The results demonstrated that the *murI* gene encodes glutamate racemase, and its product is glutamate racemase.

Molecular weight of glutamate racemase encoded by murI gene

Glutamate racemase encoded by *murI* gene was purified about 4 fold as described above. The purity of the enzyme was estimated to be about 70% by SDS-PAGE. A molecular weight of the partial purified enzyme was determined to be about 64,000 by means of gel filtration with a TSK-G3000sw column. SDS-PAGE showed that each active fraction eluted from TSK-G3000sw column contained a

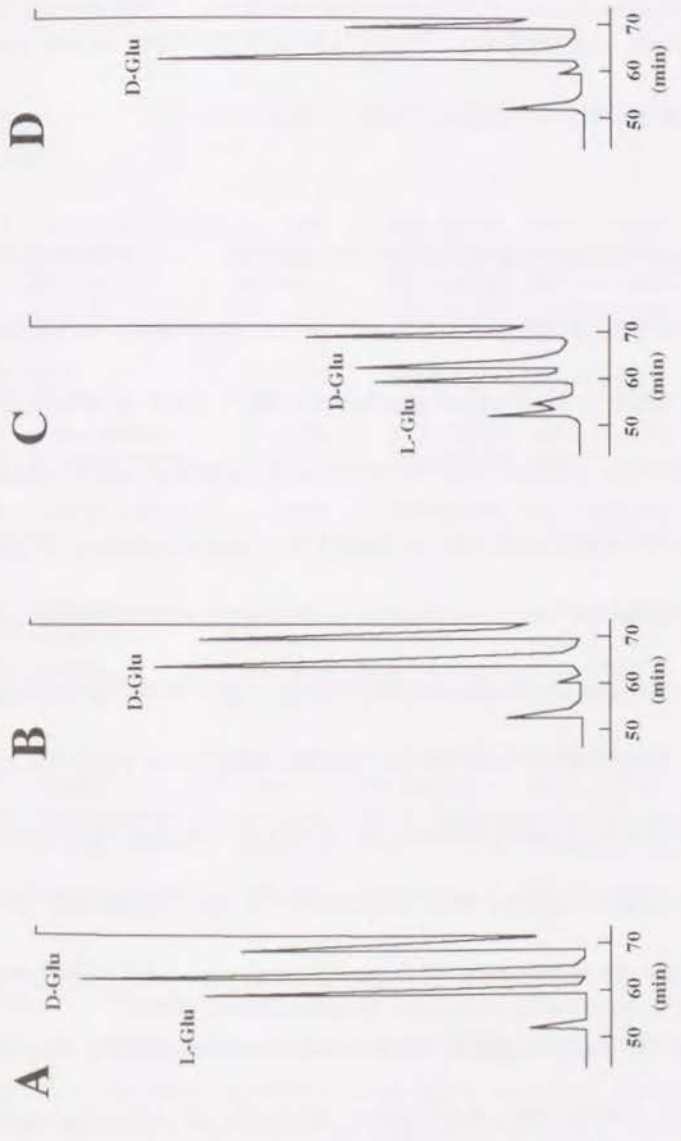


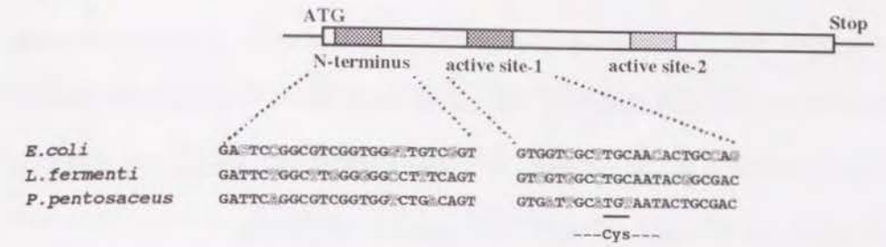
Figure 12. Conversion of D-glutamate to L-glutamate by the cell extract of *E. coli* JM109/pGR3.

The reaction mixture contained 9.2 mg (as protein) of cell extract of *E. coli* JM109 pGR3 or 12 mg (as protein) of cell extract of *E. coli* JM109 pKK223-3. *A*, authentic D- and L-glutamate; *B*, reaction with cell extract of *E. coli* JM109 pGR3, zero time; *C*, reaction with cell extract of *E. coli* JM109 pGR3, 2h; *D*, reaction with cell extract of *E. coli* JM109 pKK223-3, 2h.

protein with a molecular weight of 31,000. Thus, glutamate racemase of *E. coli* consists of two identical subunits encoded by *murI* gene. Glutamate racemase of *P. pentosaceus* is a monomer (Choi *et al.*, 1991), but its primary structure is homologous with that of the *E. coli* enzyme. This is the first example of the same kind of amino acid racemase having the distinct subunit structures.

Distribution of Bacterial Glutamate Racemase Genes Three highly conserved regions found in the glutamate racemase genes previously sequenced were found (Brosius *et al.*, 1981; Gallo and Knowles, 1993; Choi *et al.*, 1994) (Figure 13 A). Oligonucleotide primers for *in vitro* amplification were derived from the nucleotide sequences of indicated two highest homologous regions(Figure 13 A). Under this PCR conditions, double-stranded DNA fragments of about 200 base pairs were robustly amplified from all chromosomal DNAs from the bacterial strains used. Nucleotide sequences of DNA fragments amplified from several bacterial chromosomal DNAs were determined by a direct sequencing method. Linear alignment of the deduced amino acid sequences of the amplified DNA fragments and those of both corresponding regions of four previously described glutamate racemases (Yoshimura *et al.*, 1993; Gallo and Knowles, 1993; Doublet *et al.*, 1993; Pucci *et al.*, 1995) and *murI* gene products of *Mycobacterium leprae* shows considerable sequence homology with one another (Figure 13 B).

(A)



(B)

A. calcoaceticus	(p1-p38)	SG▒GGLSV	YDEIRHLLPD	LHYIYAFDNV	AFPYGEKAEA
B. subtilis	(p1-p38)	SG▒GGLSV	AKEIMRQLP▒	E▒IIYVGDT▒	RCPYGPRPEE
P. putida	(p1-p38)	SG▒GGLSV	YDEIRHLLPD	LHYIYAFD▒V	AFPYGEKAEA
S. aureus	(p1-p38)	SG▒GGLSV	A▒EIMRQLPN	ETIYYLGDIG	RCPYGPRPGE
E. coli	(33-70)	SGVGGLSV	YDEIRHLLPD	LHYIYAFDNV	AFPYGEKSEA
L. fermenti	(11-48)	SG▒GGLSV	VR▒IQ▒▒LPN	EE▒IFVGD▒G	▒FPYG▒KD▒A
P. pentosaceus	(11-48)	SGVGGLTV	VK▒▒Q▒LLPN	EEIIF▒GD▒A	R▒PYGPRP▒A
S. haemolyticus	(10-47)	SGVGGLTV	AKEIMRQLPN	ETIYYLGDIA	RCPYGPRPG▒
M. leprae (MurI)	(13-50)	SGVGGLTV	AR▒I▒DQLPD	E▒I▒YVGDTG	▒▒PYGP▒S▒P

A. calcoaceticus	(p39-p68)	▒IVERVVAIV	TAVQERYPVA	LAV▒ACNTA▒
B. subtilis	(p39-p68)	EVLQYT▒EL▒	N▒LLE▒H▒IK	MLV▒ACNTA▒
P. putida	(p39-p68)	FIVERVVAIV	TAVQERYPVA	LAV▒ACNTA▒
S. aureus	(p39-p67)	▒VKQYTVEIA	▒KLME-FDIK	MLV▒ACNTA▒
E. coli	(70-100)	FIVERVVAIV	TAVQERYPLA	LAVVACNTAS
L. fermenti	(49-77)	EVRQ▒AL▒I▒	▒FLL-▒HDVK	M▒VVACNTAT
P. pentosaceus	(49-77)	EVVEF▒▒Q▒A	▒FLM-▒▒▒IK	▒LVIACNTAT
S. haemolyticus	(47-76)	EVKQFT▒QLA	NKLM▒-FDIK	MLVIACNTAT
M. leprae (MurI)	(50-80)	EIR▒▒ALAIC	▒▒LV▒R-▒VK	▒LVIACNTAS

Figure 13. ***In vitro*** **amplification of the regions homologous to the glutamate racemase genes from the chromosomes of various kinds of bacterial strains**. (A) A proposed structure of bacterial glutamate racemases. Highly conserved regions among the enzymes of *E. coli*, *L. fermenti* and *P. pentosaceus* are *boxed*. (B) Linear alignment of deduced amino acid sequences of the amplified DNA fragments and those of corresponding regions of known glutamate racemases. Common residues in one another were shown by *boldface* type. Amino acid residues deduced from the primer sequences were *underlined*.

These suggest that the nucleotide sequences corresponding to glutamate racemase genes exsist on the chromosomes of various bacterial strains and the gene products participate closely in the biosynthesis of D-glutamate as an essential component of peptidoglycans of bacterial cell walls (Yoshimura *et al.*, 1993).

In this work, I have shown the glutamate racemase activity of the *murI* gene-encoding protein in *E. coli* JM109 cells transformed with the plasmid pGR3 which has a designed ribosome binding region, and also demonstrated that the highly homologous regions of the glutamate racemase genes exsist on various kinds of bacterial chromosomes. These supported that glutamate racemase synthesizes D-glutamate as the direct precursor of D-glutamyl residue of bacterial peptidoglycans. However, the enzyme activity has not been determined in the cells of almost all bacterial strains except lactic acid bacteria. One possible explanation for that is that the production and activation of bacterial glutamate racemases are commonly regulated at a limited level *in vivo*. For example, in structure of the *E. coli* glutamate racemase gene, the open reading frame overlaps by 66 bp the end of the preceding *btuB* gene (Brosius *et al.*, 1981; Heller and Kandner, 1985), and lacks a definite ribosome binding region, which may cause the lower gene expression efficiency. Additionally, rate of glutamate racemization by the enzyme is also very low; the catalytic efficiency of the *P. pentosaceus* enzyme shows about only 0.2%

of that of the alanine racemase of *Bacillus stearothermophilus* (Choi *et al.*, 1991; Toyama *et al.*, 1991), and that of *E. coli* is further low by analogy of the *P. pentosaceus* enzyme. Furthermore, it was reported that the glutamate racemase of *E. coli* has markedly activated by addition of UDP-*N*-acetylmuramyl-L-alanine that is a peptidoglycan precursor (Doublet *et al.*, 1994, Ho *et al.*, 1995). In contrast, the racemase of *P. pentosaceus* was not activated by the activator.

The results suggest that D-glutamate, which is indispensable for almost all of bacteria as a constituent of the peptidoglycans, is directly produced from its L-enantiomer by bacterial glutamate racemases. Since its physiological role is not known in mammals and other organisms, a specific inactivator for the enzyme is possibly used as a new antibiotic showing highly selective toxicity even for refractory infectious-bacteria.

SUMMARY

The *murI* (*dga*) gene of *E. coli* is required for the biosynthesis of D-glutamate, an essential component of bacterial peptidoglycans (Doublet *et al.*, 1992; Dougherty *et al.*, 1993), but its gene product has not been identified. I found that the amino acid sequence of protein deduced from the nucleotide sequence of open reading frame of *murI* gene shows significant homology with that of glutamate racemase of *P. pentosaceus*. The gene was ligated into a plasmid, pKK223-3 with a designed ribosome binding site, and expressed in *E. coli* JM109 cells. Glutamate racemase was produced in the transformant cells, whereas the enzyme was not found in the host cells. In this study, I partially purified the enzyme to characterize it. The enzyme consists of two identical subunits with a molecular weight of about 31,000 in contrast to the *P. pentosaceus* enzyme, a monomer protein. Furthermore, I have also found three highly conserved regions formed in the glutamate racemase genes previously sequenced (Brosius *et al.*, 1981; Gallo and Knowles, 1993; Choi *et al.*, 1994) and shown that their conserved regions exsist on the chromosomes of various kinds of bacteria.

CHAPTER 3

In vivo Effect of GroESL on the Folding of Glutamate Racemase of *Escherichia coli*

Glutamate racemase (EC 5.1.1.3) is considered to play an important role in the biosynthesis of D-glutamate, which is an essential component of peptidoglycans of the bacterial cell walls (Perkins, 1963). The enzyme is thus considered to be a target for antibiotic action (Ashiuchi *et al.*, 1993), and a study of its detailed reaction mechanism is essential to design and develop specific inhibitors. It is applicable to the enzymatic synthesis of various D-amino acids (Nakajima *et al.*, 1988). It is important to obtain a large amount of the enzyme in order to study the reaction mechanism and to the application of the enzyme.

Glutamate racemase from *P. pentosaceus* (Nakajima *et al.*, 1988) and *L. fermenti* (Gallo and Knowles, 1993) requires no coenzymes including PLP. Recently, *murI* gene of *E. coli*, which is required for the biosynthesis of D-glutamate, has been shown to encode glutamate racemase (Yoshimura *et al.*, 1993; Doublet *et al.*, 1993; Dougherty *et al.*, 1993). The predicted *murI* gene product consists of 289 amino acid residues with a molecular weight of 31,504 (Brosius *et al.*, 1981), and contains a large number of hydrophobic amino acid residues

(Doublet *et al.*, 1992). I constructed the expression vector (pGR3) by introducing the ribosome binding site (Yoshimura *et al.*, 1993). However, overproduction of the enzyme in *E. coli* JM109 resulted in the formation of inclusion bodies, and only little activity was found in the soluble fraction of the transformed cells. The inclusion bodies of the glutamate racemase of *P. pentosaceus*, which were over-produced by *E. coli* transformant cells were solubilized effectively with urea (Choi *et al.*, 1991). However, the insoluble enzyme of *E. coli* could not be solubilized with urea *in vitro* into an active form.

Recently, it was reported that molecular chaperons co-produced prevent heterologous proteins produced by *E. coli* clone cells from formation of inclusion bodies (Goloubiniff *et al.*, 1989). Co-expression of the gene of molecular chaperons, *dnaK* and *groESL* caused an increase in solubility of human procollagenase produced in *E. coli* (Lee and Olins, 1992). Co-production of human growth hormone with DnaK significantly reduced the formation of inclusion bodies and the extent of aggregation of the hormone proteins (Blum *et al.*, 1992). I here show that co-expression of *groESL* gene with the *murI* gene brought out an increase in solubility of glutamate racemase of *E. coli in vivo* into an active form.

EXPERIMENTAL PROCEDURES

Materials The restriction enzymes, and T4 DNA ligation kit were purchased from Takara Shuzo. Vector plasmid pKK223-3 and pACYC184 were from Pharmacia and Nippon Gene, respectively. A Bio-Rad Protein Assay reagent and a SDS-PAGE standard proteins were from Bio-Rad. L-Glutamate oxidase was a gift from Dr. H. Kusakabe of Yamasa Shoyu. All other chemicals were of analytical grade.

Preparation of cell extracts and precipitate fractions *E. coli* JM109 cells were transformed with pGR3 alone, pGR3 and pACYC184, or pGR3 and pKY206 which contains the *groESL* gene (Mizobata *et al.*, 1992). Plasmid pKY206 was derived from pACYC184, and can coexist in the same cell with pGR3 derived from pKK223-3. Cells of transformant were cultured in 5 ml of LB broth containing ampicilin (concentration, 50 mg/ml) or ampicilin plus tetracycline (12.5 mg/ml) at 37°C overnight. A 100 μl-portion of the overnight culture was used for inoculation of 100 ml of LB broth containing appropriate antibiotics, and the culture was carried out at 37°C. When the turbidity of the culture broth at 600 nm reached to approximately 0.2, IPTG was added to the culture broth to a final concentration of 1 mM to induce the production of glutamate racemase, and then the cultivation

was carried out at 37°C. A 1 ml-portion of culture broth was withdrawn every 2 h, and growth curve of transformant was determined by measurement of the turbidity of culture broth at 600 nm. Cells were harvested and suspended in 2.0 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM DL-glutamate, 0.1 mM PMSF, 10% glycerol, 0.5 mM $MgCl_2$ and 1 mM ATP, and disrupted by sonication. The resulting lysate was incubated at 37°C for 10 min in order to release glutamate racemase from the complex with GroESL, and centrifuged at 8,000 rpm for 15 min with a Beckman J-21 centrifuge. The supernatant solution was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM DL-glutamate, 0.1 mM PMSF, 10% glycerol and 0.1% 2-ME at 4°C for 12 h and used as a cell extract. The precipitate was incubated with 0.5 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM DL-glutamate, 0.1 mM PMSF, 10% glycerol and 6 M urea at 4°C for 1 h, and centrifuged at 12,000 rpm for 10 min at 4°C with a Sakuma M-160-IV centrifuge. The supernatant solution was dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 1 mM DL-glutamate, 0.1 mM PMSF, 10% glycerol and 0.1% 2-ME at 4°C for 12 h and used as a precipitate fraction.

Assay of the enzyme Glutamate racemase activity was determined with a coupled system with L-glutamate oxidase as described previously (Yoshimura *et*

al., 1993). L-Glutamate formed by the racemase was converted to α-ketoglutarate by L-glutamate oxidase. α-Ketoglutarate formed was determined with 2,4-dinitrophenylhydrazine.

Purification of glutamate racemase The cell extract containing GroESL (80 μg of protein) was applied to Mono Q™ HR 10/10 column equilibrated with the standard buffer consisting of 50 mM Tris-HCl (pH 7.5), 1 mM DL-glutamate, 0.1% 2-ME, 10% glycerol, 0.1 mM PMSF, and 1mM ATP. The enzyme was eluted with a 100-ml linear gradient of 0-0.5 M NaCl in the standard buffer with a flow rate of 1.0 ml/min, and each 2-ml fraction of eluant was collected.

RESULTS AND DISCUSSION

Effect on the productivity Growth and glutamate racemase activity of transformant cells containing pGR3 alone, pGR3 and pKY206, or pGR3 and pACYC184 were compared with one another (Figure 14). The glutamate racemase activity of the cell extract from the transformant containing both pGR3 and pKY206 was about 2-4 times higher than those of cells containing pGR3 alone, or both pGR3 and pACYC184. The specific activity of the enzyme of the cell extract from the transformant containing both pGR3 and pKY206 was about 28.0 units/mg, and that of cells containing pGR3 alone was about 6.5 units/mg. The value of the cell extract from the cell containing both pGR3 and pACYC184 was similar to that of the transformant containing pGR3 alone. The results suggest that co-production of GroESL elevates the racemase activity in the transformant cells. The specific activity of the precipitate fraction obtained from the transformant harboring only pGR3 was lower than 10% of that of the cell extract, though the amount of glutamate racemase in the precipitate fraction was much higher than that in the cell extract (Figure 15).

Effect on the solubility The cell extracts (150 μg of protein) and the precipitate fractions (100 μg of protein) from both transformants were

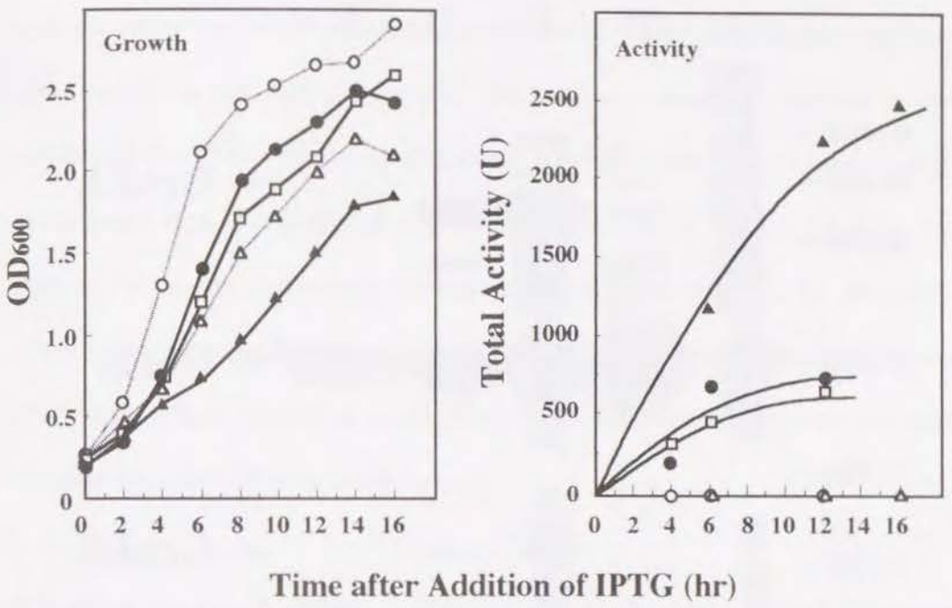


Figure 14. Effect of co-production Gro ESL on the growth and the productivity of glutamate racemase of transformant cells. Growth of the transformant cells containing pKK223-3 (○), pKY206 (△), pGR3 (●), pGR3 and pKY206 (▲), or pGR3 and pACYC184 (□) were determined by measurement of the turbidity of culture broth at 600 nm. Glutamate racemase activity in cell extract was determined with L-glutamate oxidase coupling assay.

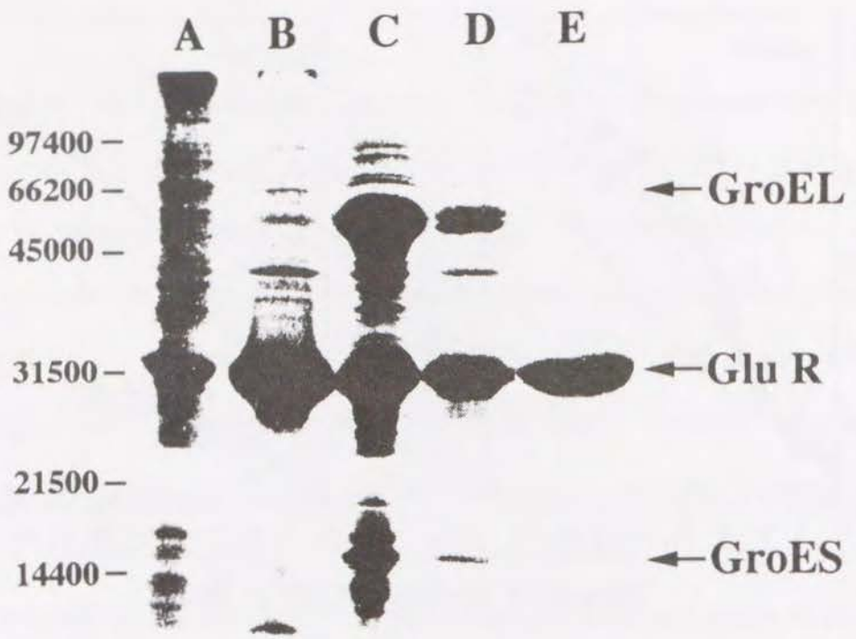


Figure 15. SDS-PAGE of the cell extract and precipitate fraction of *E. coli* JM109/pGR3 and JM109/pGR3+pKY206. *lane A*, cell extract of *E. coli* JM109/pGR3; *lane B*, precipitate fraction of *E. coli* JM109/pGR3; *lane C*, cell extract of *E. coli* JM109/pGR3+pKY206; *lane D*, precipitate fraction of *E. coli* JM109/pGR3+pKY206; *lane E*, purified enzyme (70.0 U/mg).

electrophoresed on an SDS-PAGE (12.5% acrylamide). The protein overproduced with the molecular weight of 31,000 corresponding to that of glutamate racemase was observed in each lane (Figure 15). The amount of glutamate racemase in the cell extract produced with the co-production of GroESL (Figure 15, lane C) was much larger than that produced without the co-production (Figure 15, lane A). Ratio of the amount of glutamate racemase in the cell extract to that in the precipitate fraction was increased by co-expression with the *groESL* gene (Figure 15, lane A-D). These indicate that the co-production of GroESL prevents glutamate racemase from the formation of inclusion bodies.

Effect on the purification Glutamate racemase can be purified effectively from the cell extract containing GroESL (Figure 15, lane C) with a Pharmacia FPLC system equipped with Mono Q^{TM} HR 10/10 column (Figure 16). The peaks in the chromatography of cell extract with GroESL (Figure 16, A) were more clearly separated than those of the cell extract without GroESL (Figure 16, B). The purity of the enzyme purified from the cell extract produced with co-production with GroESL was estimated to be higher than 95% by SDS-PAGE (Figure 15, lane E). About 15 μg of the enzyme was obtained from 100-ml cell culture with about 2.5 fold purification and 50% recovery.

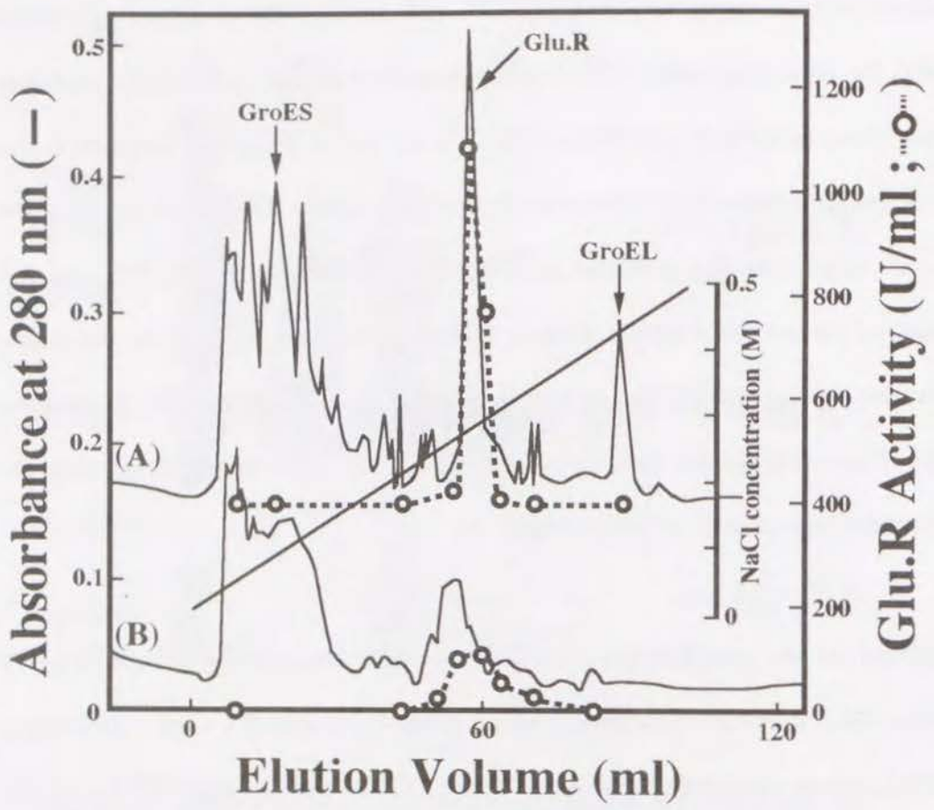


Figure 16. Purification of glutamate racemase of *E. coli* in the cell extract with (A) and without (B) co-production of GroESL by FPLC with Mono Q™ HR 10/10 Column.

The groESL gene was constitutively expressed under the conditions we used. Therefore, a considerable amount of GroESL protein is probably accumulated before the expression of glutamate racemase gene is induced by IPTG under the conditions used for the production of enzyme. When the glutamate racemase gene expression is induced at the beginning of the cultivation, almost all the products are formed in the inclusion bodies, and no activity is practically observed in the cell extract (less than 0.03 units/mg). These suggest that the GroESL level is a limiting factor controlling inclusion body formation of glutamate racemase.

It is suggested that in a *E. coli* cell a newly synthesized and premature protein forms a complex with DnaK/DnaJ and then protein is transferred to GroEL/GroES dependent on GrpE and ATP hydrolysis (Linger *et al.*, 1994; Gregerov *et al.*, 1992). This seems to be important for proper folding and/or assembly of protein *in vivo*. In the present study, however, the overproduction of GroESL protein resulted in a decrease in the amount of inclusion bodies and a significant increase in that of the active enzyme. This fact suggests that a molecular species of this enzyme, which is prone to aggregate *in vivo* is more precisely recognized by GroESL rather than by DnaK/DnaJ. However, it remains to be proved if co-production of DnaK/DnaJ is effective in this system. Recombinant proteins overproduced are often formed in a form of inclusion bodies in the host cells. In addition, it is sometimes difficult to solubilize the inclusion bodies into active forms

in vitro. GroESL facilitates the folding of a variety of proteins (Kawata *et al.*, 1994), thus the procedure shown in this work is applicable to solubilization of other proteins into an active form.

SUMMARY

The over-expression of the *murI* gene, which encodes the glutamate racemase of *E. coli* resulted in the formation of inclusion bodies of the enzyme, and little activity was found in the soluble fraction of the transformant cells. The co-expression of groESL gene with *murI* gene caused an *in vivo* solubilization of glutamate racemase in an active form. I isolated the active enzyme and purified it effectively.

CHAPTER 4

Suicide Substrate Reaction of Glutamate Racemase of *Pediococcus pentosaceus* with Serine *O*-sulfate

Several kinds of D-amino acids occur in peptidoglycans of bacterial cell walls (Perkins, 1963). D-Glutamate is a kind of them and can be synthesized from D-alanine and α-ketoglutarate by D-amino acid aminotransferase (EC 2.6.1.21) (Thorne *et al.*, 1955; Thorne and Molnar, 1955; Meadow and Work, 1958; Kuramitsu and Snoke, 1962; Tanizawa *et al.*, 1989) and also from L-glutamate by glutamate racemase (EC 5.1.1.3) (Ayenger and Roberts, 1952; Narrod and Wood, 1952; Glaser, 1960; Diven, 1969; Nakajima *et al.*, 1988; Gallo and Knowles, 1993). However, both enzymes were found only in a few bacterial species, the former in bacilli and the latter in lactobacilli, and no functions of their enzymes have been clarified. Recently, a previously reported ORF1 (Brosius *et al.*, 1981), the *murI* (*dga*) (Doublets *et al.*, 1992; Dougherty *et al.*, 1993) gene was found to complement the D-glutamate auxotrophy of the *E. coli*, WM335. I have shown that the gene encodes glutamate racemase of *E. coli* (Yoshimura *et al.*, 1993; Doublets *et al.*, 1993) to synthesize D-glutamate *in vivo*, and suggested that glutamate racemases play an important role in the biosynthesis of D-glutamate in

almost all bacteria. In fact, I have found that highly homologous regions to those of known glutamate racemase genes are distributed in the chromosomes of various bacterial strains.

Glutamate racemase is independent of any coenzymes including PLP. No mechanisms of PLP-independent amino acid racemase reactions (Yamauchi *et al.*, 1992; Choi *et al.*, 1992; Gallo *et al.*, 1993; Tanner *et al.*, 1993; Cardinale and Abeles, 1968; Rudinick and Abeles, 1975; Wiseman and Nichols, 1984) are elucidated. While D-glutamate, which is produced by the racemase reaction is an integral component of bacterial peptidoglycans, the amino acid is considered to be not so important for mammals. Accordingly, the enzyme participating in the biosynthesis can be a target for development of new antibiotics, and the study on the inactivation reaction will contribute to it. I have purified the enzyme of *P. pentosaceus* (Nakajima *et al.*, 1986) from *E. coli* JM 109 transformant cells introducing the expression vector (pICR223) (Choi *et al.*, 1991) to elucidate the reaction mechanism of glutamate rasemase and to design mechanism-based inactivators possibly useful as new antibiotics. I have found also that glutamate racemase is inactivated by L-serine *O*-sulfate (Ashiuchi *et al.*, 1993) that is a suicide substrate of several PLP-dependent enzymes (John and Fasella, 1969; Ueno *et al.*, 1982; Like *et al.*, 1982). In this thesis, I describe a proposed mechanism of the suicide substrate reaction of glutamate racemase with serine *O*-sulfate.

EXPERIMENTAL PROCEDURES

Materials Both enantiomers of serine *O*-sulfate were synthesized by the procedure of Tudball (Tudball, 1962). D- and L-[1-^{14}C]Serine *O*-sulfate (3.7 MBq/mmole) were synthesized in the same way from D- and L-[1-^{14}C]serine purchased from Muromachi Kagaku Kogyo (1.85 GBq/mmole), respectively. Ethyleneimine was synthesized according to the procedure of Reeves *et al.* (Reeves *et al.*, 1951). A protein assay kit was from Bio-Rad, and an Excellulose^{TM} GF-5 desalting gel column was from Pierce. L-Glutamate dehydrogenase (GDH) (20 mg/ml) and L-lactate dehydrogenase (LDH) (5 mg/ml) were from Boehringer Mannheim. Glutamate racemase of *P. pentosaceus* was purified from *E. coli* clone cells harboring pICR223 carrying the enzyme gene as reported previously (Choi *et al.*, 1991). Lysyl endopeptidase and an amino acid sequencing kit were from Shimadzu Seisakusho. All other chemicals were of analytical grade.

Enzyme and Protein Assay Glutamate racemase activity was determined with L-glutamate dehydrogenase as reported previously (Choi *et al.*, 1991). The mixture (1.0 ml) contained 100 mM Tris-HCl buffer (pH 8.0), 5 mM NAD^+, 0.05 units of L-glutamate dehydrogenase and 0.05 nmole of glutamate racemase. The

reaction was started by addition of 10 μmole of D-glutamate after preincubation at 37°C and was followed by measurement of an initial rate of increase in absorbance at 340 nm with a Shimadzu MPS-3000 spectrophotometer. Inactivation of glutamate racemase with serine *O*-sulfate was examined as follows. Glutamate racemase (10 nmole) was incubated in 1.0 ml of 700 mM Tris-HCl (pH 8.0) containing 100 μmole of each enantiomer of serine *O*-sulfate at 37°C. A 50-μl aliquot of the reaction mixture was withdrawn, and the racemase activity was determined (Choi *et al.*, 1991).

α,β–Elimination reaction of serine *O*-sulfate was measured by determination of pyruvate formed with L-lactate dehydrogenase (Ashiuchi *et al.*, 1993). The mixture (1.0 ml) contained 700 mM Tris-HCl buffer (pH 8.0), 0.4 mM NADH, 50 units of L-lactate dehydrogenase and 0.5 nmole of glutamate racemase. The reaction was started by addition of 100 μmole of D- or L-serine *O*-sulfate after preincubation at 37°C and was followed by measurement of an initial rate of decrease in absorbance at 340 nm. The apparent pseudo-first order rate constant for the inactivation, k, was obtained from Guggenhiem plot of the progress curve for the α,β-elimination reaction with the equation, $k=0.693/t_{1/2}$, where $t_{1/2}$ denotes the time (in minutes) required for inactivation of one-half amount the enzyme initially present.

Protein concentration was determined with a Bio-Rad protein assay kit with bovin serum albumin as a standard.

Stoichiometry of Reaction Products The racemase (10 nmole) was incubated at 37°C in 1.0 ml of 700 mM Tris-HCl (pH 8.0) containing 100 μmole of D- or L-serine *O*-sulfate. A 50-μl aliquot was withdrawn for the determination of pyruvate and ammonia formed. Pyruvate was determined with 2,4-dinitrophenyl-hydrazine (Friedmann and Haugen, 1943), and ammonia was determined with Nessler's reagent (Okamoto and Morino, 1973).

Incorporation Reaction of ^{14}C-Labeled Serine O-sulfate The incorporation of radioactivity from [1-^{14}C]serine *O*-sulfate into the racemase was examined as follows. The mixture (1.0 ml) containing 700 μmole of Tris-HCl (pH 8.0), 100 μmole of either D- or L-enantiomer of [1-^{14}C]serine *O*-sulfate and 30 nmole of the enzyme was incubated at 37°C, and a 250-μl aliquot was withdrawn to determine radioactivity incorporated into the enzyme. The enzyme was denatured with 6 M urea and then dialyzed against 700 mM Tris-HCl buffer (pH 8.0) at 5°C for 24 h. The ^{14}C-labeled enzyme was isolated by gel filtration with an Excellose^{TM} GF-5 column, and the radioactivity was determined with a Packerd

Tri-Carb 300C liquid scintillation system.

Effect of 2-Nitrothiocyanobenzoate (NTCB) The enzyme (5.0 nmole each) was incubated with 20 nmole of NTCB in 0.1 ml of 700 mM Tris-HCl buffer (pH 8.0) at 37°C for 10 min, and an 10-μl aliquot was withdrawn to determine the enzyme activity in the α,β-elimination of serine *O*-sulfate (Ashiuchi *et al.*, 1993).

DTNB Titration Glutamate racemase (50 nmole) was incubated with 250 μmole of each enantiomer of serine *O*-sulfate in 2.5 ml of 700 mM Tris-HCl buffer (pH 8.0) at 37°C. A 500-μl aliquot of the reaction mixture was withdrawn and dialyzed against 700 mM Tris-HCl buffer (pH 8.0) at 5°C for 24 h. The enzyme in an aliquot (10 nmole) was denatured completely with 6 M guanidine hydrochloride. The cysteinyl residues of the enzyme were determined with DTNB by measurement of nitromercaptobenzoate formed at 412 nm.

Preparation and Identification of Peptide Fragments containing Amino Acid Residue modified with Serine O-sulfate Conversion of cysteinyl residues into S-(β-aminoethyl) cysteinyl residues was carried out by the method of Raftery and Cole (Raftery and Cole, 1966). The native enzyme and the enzyme modified with serine *O*-sulfate (30 nmole each) were denatured with 500

μl of 1 M Tris-HCl (pH8.0) containing 6 M guanidine hydrochloride and 1% 2-ME. After the reaction mixture was stood at room temperature (about 25°C) for 1 h, each 5 μl of ethyleneimine neat solution was added to the reaction mixture three times intermittently with 10 min interval. After 2 h, the reaction mixture was dialyzed against 1% ammonium bicarbonate solution and then against water at 4°C for 24 h each. The resulting solution was lyophilized with an Eyela freeze dryer FD-80. Site-specific digestion of lysyl and S-(β-aminoethyl)cysteinyl residues of the enzymes by lysyl endopeptidase was performed as follows. The enzyme (30 nmole) modified with ethyleneimine was suspended in 200 μl of 10 mM Tris-HCl (pH 9.0), and 0.15 nmole of lysyl endopeptidase was added to the solution. The reaction mixture was incubated at 37°C for 6 h and lyophilized.

Peptide fragments of the enzymes digested with lysyl endopeptidase were isolated by reversed phase chromatography with a Waters 600E HPLC system equipped with a Puresil 5μ C18 120 Å (4.6 X 150 mm) column. The enzyme (about 1.0 mg) digested with lysyl endopeptidase was dissolved in 100 μl of 0.1% TFA, and an aliqout of 20 μl was applied to reversed phase HPLC. A linear gradient was prepared with 0.1% TFA supplemented with acetonitrile by increase in the concentration of a acetonitrile from 0 to 60% for 60 min, and the flow rate was adjusted to 1.0 ml/min. Detection of the peptide fragments was carried out by

increase in absorbance at 215 nm. The eluant was fractionated and amino acid sequence of the target fragment was determined with a Shimadzu PPSQ-10S protein gas-phase protein sequencer.

RESULTS AND DISCUSSION

Inactivation of Glutamate Racemase with Serine O-sulfate

Glutamate racemase of *P. pentosaceus* was inactivated by incubation with serine *O*-sulfate (Table I). A semi-logarithmic plot of the remaining activity against time gave a straight line; the inactivation followed a pseudo-first order kinetic. The enzyme was irreversibly inactivated; no activity was recovered by dialysis of the inactivated enzyme. Additionally, when the glutamate racemase was incubated with serine *O*-sulfate, essentially equimolar amounts of pyruvate and ammonia were formed (Table I). This shows that the glutamate racemase catalyzes the α,β-elimination of both enantiomers of serine *O*-sulfate. They act as the substrates and also as the inactivators; suicide substrates of the enzyme. This suicide substrate will be a model to design mechanism-based inactivators for glutamate racemase.

Kinetics The Michaelis constants, *Km*, of the glutamate racemase for serine *O*-sulfate in the α,β-elimination reaction were calculated by the double reciprocal plot of initial rate of the pyruvate formation against concentration of serine *O*-sulfate. The corresponding constants, *Kinact*, were also obtained by the double reciprocal plot of the apparent pseudo-first order rate constant for the inactivation (*k*) against concentration of the amino acid. The values *Km* and *Kinact* were

Table I. Formation of Pyruvate and Ammonia from Serine *O*-sulfate in Catalysis of Glutamate Racemase.

Time of Reaction (hour)	Remaining Activity[a] (%)	Product Formed (μmole/ml)	
		Pyruvate	Ammonia
D-Serine *O*-sulfate			
0	100	0	0
0.5	60	2.7	2.8
1	40	3.6	3.4
2	20	3.7	3.4
L-Serine *O*-sulfate			
0	100	0	0
0.5	85	1.0	0.9
1	75	1.7	1.6
2	60	2.2	2.0

[a]Glutamate racemase activity was determined with L-glutamate dehydrogenase as described in MATERIALS AND METHODS.

consistent with each other; 200 mM for D-enantiomer and 100 mM for L-enantiomer. This indicates that both α,β-elimination and inactivation occur through a common intermediate; so called a suicide substrate reaction occurs. The obtained data also showed that the value of reactivity (*kcat*/*Km*) for the α,β-elimination of D-enantiomer is about 5 fold higher than that of L-enantiomer. On the other hand, the *Km* value for D-serine *O*-sulfate in the α,β-elimination was about 40 fold higher than that for D-glutamate in the racemization; serine *O*-sulfate as the substrate shows very low affinity for the enzyme. Comparison of the rate of α,β-elimination with the rate of inactivation yields the turnover number of α,β-elimination required for inactivating 1 molecule of the enzyme. Thus 1 molecule of the enzyme was inactivated during approximately 650 turnovers of α,β-elimination in both reactions with D- and L-enantiomer.

Covalent Incorporation of ^{14}C-Labeled Serine O-sulfate The enzyme was incubated with [1-^{14}C]serine *O*-sulfate at 37°C for indicated times. As shown in Table II, the result strongly suggests that inactivated enzyme is modified with an equimolar amount of a derivative of serine *O*-sulfate. Both activities in the racemization of glutamate and the α,β-elimination of serine *O*-sulfate were lost. These indicate that an essential amino acid residue for catalysis of both reactions was modified with a derivative of serine *O*-sulfate.

Table II. Incorporation of Radioactivity from [1-^{14}C]Serine *O*-sulfate into Glutamate Racemase during Inactivation Reaction.

Time of Reaction (hour)	Remaining Activity (min^{-1})		^{14}C Incorporated (moles/mole of enzyme)
	Racemization of D-Glutamate	α,β-Elimination of D- Serine *O*-sulfate	
D-Serine *O*-sulfate			
0	1600 (100%)[a]	30 (100%)	0
0.5	1300 (80%)	24 (80%)	0.20
2	480 (30%)	9.0 (30%)	0.80
24	160 (10%)	6.0 (20%)	1.1
L-Serine *O*-sulfate			
0	1400 (100%)	30 (100%)	0
6	700 (50%)	18 (60%)	0.60
24	280 (20%)	9.0 (30%)	1.3

[a]A parenthesized percentage shows a relative remaining activity of the enzyme.

Effect of NTCB on Enzyme Activity After the enzyme was incubated with enough amounts of NTCB, the cysteinyl residues was modified almost completely. The activity was markedly lost by addition of NTCB. The result suggests that the cysteinyl residues play an important role in α,β-elimination of serine *O*-sulfate.

Modification of Cysteinyl Residues with Serine O-sulfate Rate of modification of cysteinyl residues of the enzyme with a derivative of serine *O*-sulfate was determined by DTNB titration. The amount of cysteinyl residues of the enzyme decreased with decrease in glutamate racemase activity (Fig. 17), and the enzyme was inactivated almost completely when 35% of total amount of cysteinyl residues was lost. Since the enzyme contains three cysteinyl residues, one mole of cysteinyl residue per mole of the enzyme was modified with a derivative of serine *O*-sulfate, probably α-aminoacrylate.

Identification of Amino Acid Residue modified with Serine O-sulfate Peptides containing the amino acid residues modified with a derivative of serine *O*-sulfate were prepared as follows. The modified enzyme was denaturated with guanidine hydrochloride, and then modified further with ethyleneimine. The modified protein was digested with lysyl endopeptidase, and peptide fragments

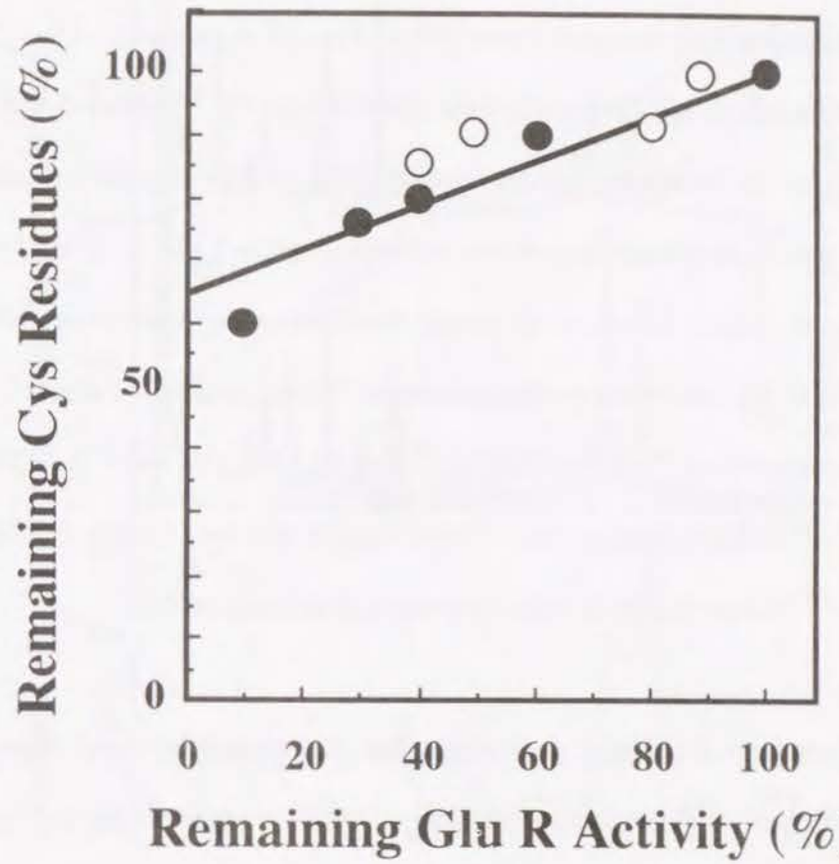


Figure 17. Modification of cysteinyl residues of glutamate racemase with serine *O*-sulfate. Glutamate racemase (50 nmole) was incubated at 37 °C in 2.5 ml of 700 mM Tris-HCl buffer (pH8.0) containing 100 mM D-serine *O*-sulfate (●) and L-serine *O*-sulfate (○). Rate of modification of cysteinyl residues of the enzyme was determined by a DTNB titration.

obtained were isolated by reversed phase HPLC (Fig. 18 A, and B). Two ^{14}C-labeled peptides (about 150 dpm each) were isolated from the ^{14}C-labeled enzyme (about 500 dpm) on the elution volume corresponding to that of Peptide I and II (Fig. 18 C). Amino acid sequences of two peptides (Peptide I and II) formed only by modification with a derivative of serine *O*-sulfate were determined with a Shimadzu PPSQ-10S gas-phase protein sequencer. The sequences of Peptide I and II were determined to be ^{68}ALVIA^{73}XNTA^{77}XNAALAVLQAE and ^{169}LAEFRQ-DQIDTLILG^{184}XTHFP, respectively. These suggest that two cysteinyl residues (^{73}Cys and ^{184}Cys) are modified with a derivative of serine *O*-sulfate.

Role of Cysteinyl Residues in Glutamate Racemization and Suicide Substrate Reaction with Serine O-sulfate To elucidate further the role of cysteinyl residues of the glutamate racemase, I have applied the mutant enzymes with alanyl residues substituted for cysteinyl residues (^{73}Cys, ^{144}Cys and ^{184}Cys) to both racemization and α,β-elimination. Two mutant enzymes (C73A and C184A) catalyzed neither the racemization of glutamate nor the exchange of α-hydrogen of glutamate with deuterium in D_2O, but the activities of another mutant enzyme (C144A) in their reactions were similar to those of the wild-type enzyme (Choi *et al.*, 1992). On the other hand, both the wild-type and two mutant enzymes (C73A and C184A) catalyzed α,β-elimination of serine *O*-sulfate and

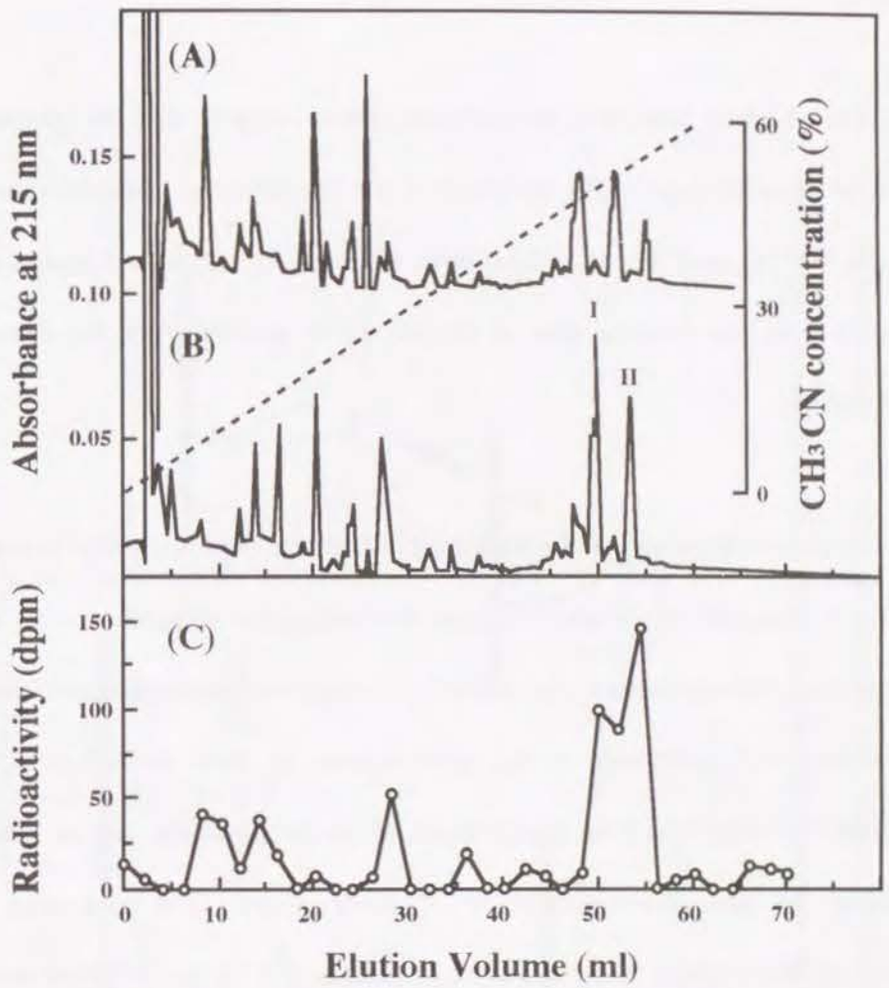


Figure 18. Isolation of peptide fragments containing an amino acid residue modified with serine *O*-sulfate. The native enzyme (A) and the enzymes modified with 100 mM serine *O*-sulfate (B) or [1-^{14}C]serine *O*-sulfate (C) were modified further with ethyleneimine neat solution and digested with lysylendopeptidase. The resulting peptides were separated with reversed phase HPLC. The amino acid sequences of two peptides (Peptide I and II) containing amino acid residue modified with serine *O*-sulfate were determined with a Simadzu PPSQ-10S gas-phase protein sequencer. Two ^{14}C-labeled peptides (about 150 dpm each) were isolated from the enzyme modified with [1-^{14}C]serine *O*-sulfate (about 500 dpm).

were inactivated while catalyzing the reaction. These suggest that the glutamate racemization occurred concertedly by action of the two essential cysteinyl residues (^{73}Cys and ^{184}Cys), and the α,β-elimination of serine *O*-sulfate is accomplished by action of only one catalytic base, a thiolate anion derived from the essential cysteinyl residue.

Since D-glutamate, an essential component of bacterial peptidoglycans is not so important for mammals, glutamate racemase involved in the biosynthesis can be a novel target for antibiotic action. Accordingly, the study on inactivation mechanism of the enzyme will contribute to the development of new antibiotics. Our observations indicated that both enantiomers of serine *O*-sulfate act as suicide substrates of the glutamate racemase of *P. pentosaceus*. The racemase was inactivated by modification of two cysteinyl residues (^{73}Cys and ^{184}Cys) with a derivative of serine *O*-sulfate; both activities in the racemization of glutamate and the α,β-elimination of serine *O*-sulfate were lost. A proposed mechanism for inactivation of the enzyme with serine *O*-sulfate is illustrated in Fig. 19. α-Hydrogen of serine *O*-sulfate is perhaps removed by the thiolate anion. When the following elimination of the sulfate group occurs, an α-aminoacrylate is probably produced. Majority of the product is spontaneously hydrolyzed to pyruvate and ammonia, and α,β-elimination of serine *O*-sulfate is accomplished. However, α-

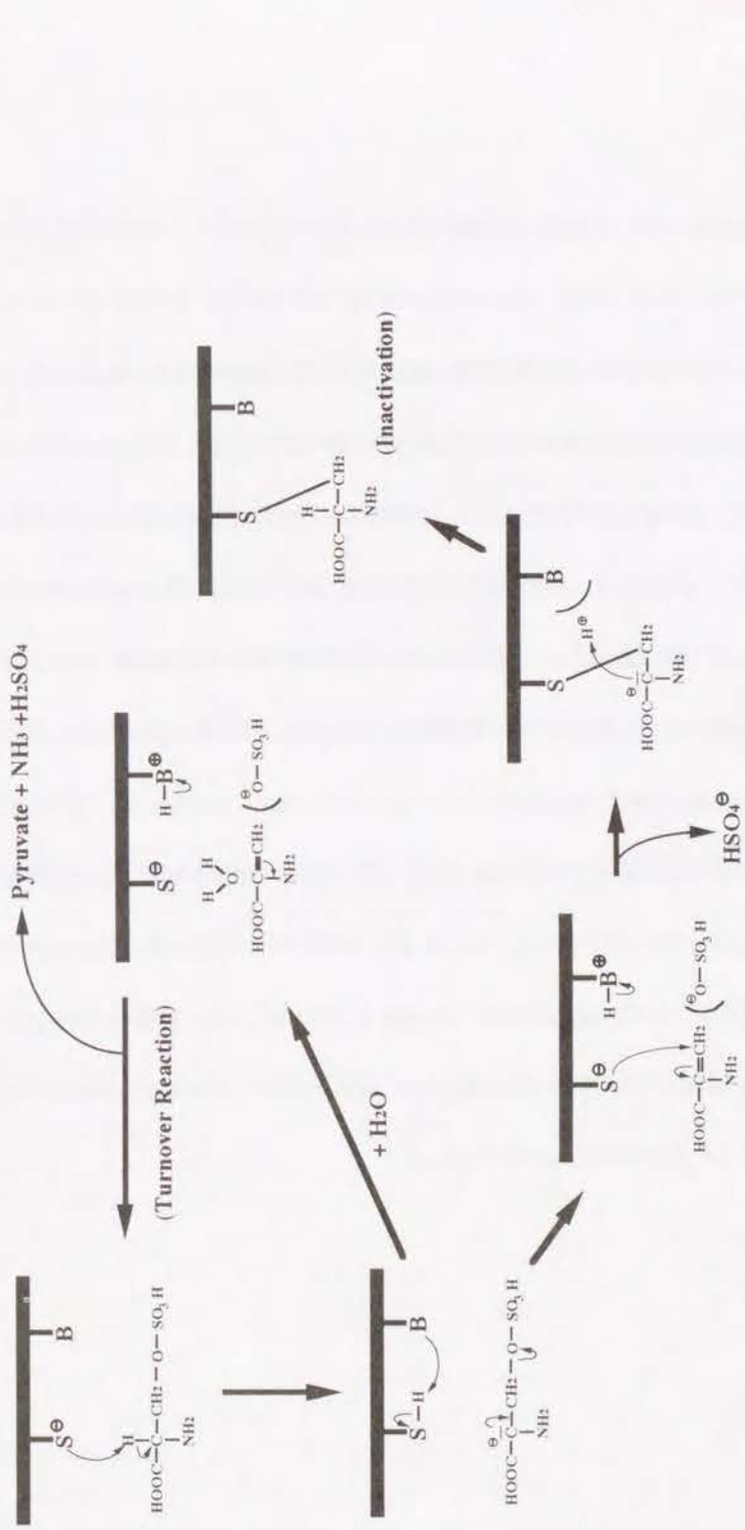


Figure 19. A proposed mechanism for the suicide substrate reaction of glutamate racemase with serine *O*-sulfate. Amino acid residues showing S and B are an essential cysteinyl residue and an amino acid residue as an acceptor in proton relay from a thiol of the cysteinyl residue, respectively.

aminoacrylate is reactive and readily modified by nucleophilic functional groups such as a thiol. On the other hand, dissociation of sulfhydryl group of essential cysteinyl residues is probably an important step for the enzyme to catalyze α,β-elimination of serine *O*-sulfate and to be modified by following Michael reaction. However, sulfhydryl groups will not be spontaneously dissociated under the conditions at pH 8.0. Therefore, the active site of glutamate racemase probably includes an amino acid residue (Fig. 19, residue B) functioning as an acceptor in proton relay from a thiol of the essential cysteinyl residue, which acts as the base in α-deprotonation of serine *O*-sulfate.

This is the first example showing that two essential cysteinyl residues of glutamate racemase play an important role in the suicide substrate reaction with serine *O*-sulfate. This reaction can serve as a model for target-inactivation reactions which are indispensable to design new antibiotics, and will be a key to clarify the mechanism of glutamate racemization.

SUMMARY

Glutamate racemase of *P. pentosaceus* catalyzes α,β-elimination of serine *O*-sulfate to produce equimolar amounts of pyruvate, ammonia, and sulfuric acid. The enzyme was inactivated by this amino acid while catalyzing the α,β-elimination, and was covalently modified with [1-^{14}C]serine *O*-sulfate. The activity for α,β-elimination of serine *O*-sulfate was lost due to modification of thiol groups with NTCB. The amounts of cysteinyl residues of the enzyme decreased by incubation with serine *O*-sulfate, and both of two essential cysteinyl residues (^{73}Cys and ^{184}Cys) were modified with this amino acid. On the other hand, two mutant enzymes (C73A and C184A), which did not catalyze racemization of glutamate and exchange of α-hydrogen of glutamate with deuterium in D_2O, catalyzed α,β-elimination of serine *O*-sulfate and were inactivated while catalyzing the reaction.

CONCLUSIONS

The glutamate racemase gene of *P. pentosaceus* consists of a 795-nucleotides open reading frame, and encodes 265 amino acid residues which form a monomeric protein. The sequence shows significant homology with that of aspartate racemase from *S. thermophilus*: it requires no cofactors and contains an essential cysteinyl residue (Yohda *et al.*, 1991). The two racemases are structurally similar to each other, in particular in the regions around the two cysteinyl residues. I have also found significant sequence homology between glutamate racemase and mammalian myoglobins, in particular in the regions corresponding to a heme binding pocket of myoglobins. Glutamate racemase is bound with an equimolar amount of hemin to be inactivated, and aspartate racemase shows a low sequence homology with myoglobins, but is not bound with hemin. These suggest that glutamate racemase may be derived from an evolutionary origin of globin family proteins. Aspartate racemase also may have evolved from the common ancestral protein, but its structure may have been altered more extensively than glutamate racemase by divergence.

In this work, I have shown that the amino acid sequence deduced from the nucleotide sequence of *murI* (*dga*) gene that is required for the biosynthesis of D-glutamate as an essential component of peptidoglycan in *E. coli* (Doublet *et al.*,

1992; Dougherty *et al.*, 1993) has significant homology with that of glutamate racemase of *P. pentosaceus*. The gene was ligated into a plasmid, pKK223-3 with a designed ribosome binding site, and expressed in *E. coli* JM109 cells. Glutamate racemase was produced in the transformant cells, whereas the enzyme was not found in the host cells. I partially purified the enzyme to characterize it. The enzyme consists of two identical subunits with a molecular weight of about 31,000. I have also found three highly conserved regions in the glutamate racemase genes previously sequenced (Brosius *et al.*, 1981; Gallo and Knowles, 1993; Choi *et al.*, 1994) and shown that their conserved regions exsist on the chromosomes of various kinds of bacterial strains. These indicate that D-glutamate, which is indispensable for almost all bacterial strains as a constituent of peptidoglycans (Perkins, 1963), is directly produced from its L-enantiomer by glutamate racemase.

Glutamate racemase is thus considered to be a target for antibiotic action (Ashiuchi *et al.*, 1993), and a study of its detailed reaction mechanism is essential to design and develop specific inhibitors. In addition, it is applicable to the synthesis of various D-amino acids (Nakajima *et al.*, 1988). Therefore, it is important to obtain a large amount of enzyme in order to study mechanism and application of the enzyme. I have constructed an overproducer by introducing *murI* gene of *E. coli*, but the overexpression of the gene resulted in formation of

inclusion bodies of the enzyme that contains a large number of hydrophobic amino acid residues (Doublet *et al.*, 1992), and little activity was found in the soluble fraction of the transformant cells. I have shown that the co-expression of groESL gene with *murI* gene caused solubilization of the enzyme in an active form. I have isolated the active enzyme and purified it effectively. Recombinant proteins overproduced are often formed in a form of inclusion bodies in the host cells, and it is sometimes difficult to solubilize the inclusion bodies into active forms *in vitro*. GroESL facilitates the folding of a variety of proteins (Kawata *et al.*, 1994). Thus, the procedure shown in this work is probably applicable to solubilization of other proteins into an active form.

Since D-glutamate, an essential component of bacterial peptidoglycans is not so important for mammals and other organisms, glutamate racemase participating in the biosynthesis can be a new target for antibiotic action. Accordingly, the study on inactivation mechanism of glutamate racemase will contribute to the development of new antibiotics showing highly selective toxicity even for refractory infectious-bacteria. In this study, I have shown that both enantiomers of serine *O*-sulfate serve as suicide substrates of glutamate racemase. The enzyme was inactivated by serine *O*-sulfate while catalyzing the α,β-elimination, and was covalently modified with [1-^{14}C]serine *O*-sulfate. The activity for α,β-elimination of serine *O*-sulfate

was lost by modification of thiol groups with NTCB. The amounts of cysteinyl residues of the enzyme decreased by incubation with serine *O*-sulfate, and both of two essential cysteinyl residues (^{73}Cys and ^{184}Cys) were modified with a derivative of serine *O*-sulfate. Additionally, two mutant enzymes (C73A and C184A), which do not catalyze racemization of glutamate and exchange of α-hydrogen of glutamate with deuterium in D_2O, catalyzed α,β-elimination of serine *O*-sulfate and were inactivated while catalyzing the reaction. These suggest that the glutamate racemization occurred concertedly by action of the two essential cysteinyl residues, and α,β-elimination of serine *O*-sulfate is accomplished by action of only one catalytic base, a thiolate anion derived from the essential cysteinyl residue.

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