UDP-*N*-acetylmuramyl-L-alanine Functions as an Activator in the Regulation of the *Escherichia coli* Glutamate Racemase Activity†

Hsu-Tso Ho,*,‡ Paul J. Falk,‡ Kerry M. Ervin,‡ Bala S. Krishnan,§ Linda F. Discotto,‡ Thomas J. Dougherty,‡ and Michael J. Pucci‡

Departments of Microbiology and Analytical Chemistry, Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, Connecticut 06492

Received June 27, 1994; Revised Manuscript Received October 24, 1994⊗

ABSTRACT: D-Glutamate is an essential component of the bacterial peptidoglycan. In *Escherichia coli*, the biosynthesis of D-glutamate is catalyzed by a glutamate racemase (encoded by the *dga* gene) and is regulated by UDP-*N*-acetylmuramyl-L-alanine [Doublet et al. (1994) *Biochemistry 33*, 5285], a bacterial peptidoglycan subunit precursor. Investigation was conducted to elucidate the interaction between the enzyme and its regulator. Whole and N-terminal truncated enzymes, encoded by individual constructs containing either a full-length or an N-terminal truncated *dga* gene, were evaluated. In the absence of the regulator, the purified whole enzyme showed a low-level basal racemase activity for which a K_m value of 18.9 mM and a V_{max} of 0.4 μmol/(min·mg) were determined, using D-glutamate as the substrate. Using the same substrate, in the presence of 6.5 μM UDP-*N*-acetylmuramyl-L-alanine, a K_m value of 4.2 mM and a V_{max} of 34 μmol/(min·mg) were measured. Similar kinetic parameters for the activated enzyme were obtained using L-glutamate as the substrate. The N-terminal truncated *E. coli* enzyme, with a 21 amino acid region removed, is similar in size to the *Pediococcus pentosaceus* glutamate racemase. Effects of the regulator on the full-length and the N-terminal truncated enzyme in the dialyzed cell lysate were compared. A host cell line, *E. coli* WM335Δ*recA*, containing a nonfunctional chromosomal *dga* gene was used to minimize the background interference. With 6.5 μM regulator added, the N-terminal truncated enzyme displayed a loss of more than 80% of the activity compared to the full-length enzyme. This loss of activation effect was repeated in the presence of 0.65 and 65 μM regulator. Evaluation of the *Pediococcus* enzyme revealed no detectable effect exerted by UDP-*N*-acetylmuramyl-L-alanine. The activated *E. coli* racemase is at least as efficient as the *Pediococcus* racemase. These results indicate that the regulator is unique to the *E. coli* enzyme and the longer N-terminal region of the enzyme is necessary for its optimal activation. This regulation suggests that a more stringent control of the cytoplasmic level of D-glutamate may be required in Gram-negative bacteria. ^{1}H NMR studies showed that the UDP-*N*-acetylmuramyl-L-alanine-activated *E. coli* racemase catalyzed the exchange of the solvent ^{2}H with the C-2 ^{1}H of the substrate. Site-directed mutagenesis carried out at either of the two conserved cysteine (C96T and C208T) residues resulted in inactivation of the enzyme. These two cysteine residues may be involved in the extraction and readdition of the C-2 proton in the racemization process. The UDP-*N*-acetylmuramyl-L-alanine-activated *E. coli* glutamate racemase appears to follow a deprotonation/reprotonation mechanism as reported for *Lactobacilli* glutamate racemase. However, the latter was reported not to be regulated by this regulator and to have significantly higher efficiency.

Peptidoglycan is an important bacterial cell wall component which maintains the structural integrity of the cells against an elevated internal osmotic pressure. D-Glutamic acid is one of the essential components that is unique to bacterial peptidoglycan. In the cytoplasmic biosynthesis of the peptidoglycan precursor subunit (Scheme 1), D-glutamic acid is attached to UDP-*N*-acetylmuramyl-L-alanine (UDP-NacMur-L-Ala)[1] through a peptide linkage to the alanine moiety to form UDP-*N*-acetylmuramyl-L-alanyl-D-glutamic acid (Park, 1987). Subsequently, one of a number of amino acids such as diaminopimelic acid or lysine is then added to the γ-carboxyl group of D-glutamic acid depending on the bacterial species, followed by the addition of D-Ala-D-Ala to form UDP-*N*-acetylmuramyl pentapeptide. This is transferred to a lipid carrier and transported through the cytoplasmic membrane to be incorporated into the preexisting peptidoglycan sacculus (Matsuhashi, 1994). The enzyme(s) responsible for the cytoplasmic biosynthesis of D-glutamic

† This work was supported by the Bristol-Myers Squibb Pharmaceutical Research Institute.

* Correspondence should be addressed to this author [telephone (203) 284-6862; Fax (203) 284-6771].

‡ Department of Microbiology.

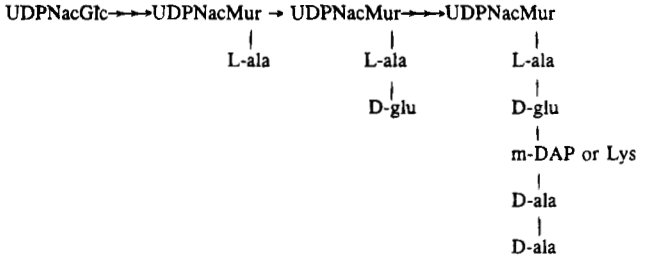
§ Department of Analytical Chemistry.

⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1995.

[1] Abbreviations: UDPNacMur, UDP-*N*-acetylmuramic acid; UDP-NacMur-L-Ala, UDP-*N*-acetylmuramyl-L-alanine; UDPNacMur-L-Ala-D-Glu, UDP-*N*-acetylmuramylalanyl-D-glutamic acid; NADP, nicotinamide adenine dinucleotide 3′-phosphate; D-Ala-D-Ala, D-alanyl-D-alanine; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; PMSF, phenylmethanesulfonyl fluoride; FPLC, fast protein liquid chromatography; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; CD, circular dichroism; wb NMR, wide bore nuclear magnetic resonance; LC−MS, liquid chromatography−mass spectrometry; TMS, tetramethylsilane.

0006-2960/95/0434-2464$09.00/0

Scheme 1



acid in both Gram-positive and Gram-negative bacteria has (have) been a focal point of intense research interest.

Significant progress has been made in understanding the Gram-positive bacterial D-glutamic acid biosynthesis pathway. In Gram-positive bacteria, D-glutamic acid is produced by either of two reactions, transamination (Martinez-Carrion et al., 1965; Yonaha et al., 1975, Tanizawa et al., 1989) or racemization (Nakajima et al., 1986, 1988). The biosynthesis of D-glutamic acid by a cofactor-independent glutamate racemase in two Gram-positive organisms, *Lactobacillus fermenti* (Gallo & Knowles, 1993; Tanner et al., 1993) and *Pediococcus pentosaceus* (Nakajima et al., 1986, 1988; Ashiuchi et al., 1993), has been elucidated in great detail. However, the enzyme that catalyzes the biosynthesis of D-glutamate in the Gram-negative bacterium, *Escherichia coli*, has eluded investigators until recently (Dougherty et al., 1993; Yoshimura et al., 1993; Doublet et al., 1993, 1994). The gene required for D-glutamate biosynthesis in *E. coli*, designated *dga* or *murI*, was identified and shown to have significant sequence homology with the gene encoding the *Pediococcus* glutamate racemase (Pucci et al., 1994). Moreover, the enzyme was shown to catalyze the racemization of glutamate (Yoshimura et al., 1993; Doublet et al., 1993) and was reported by Doublet et al. to have an absolute requirement for UDP-*N*-acetylmuramyl-L-alanine. UDP-*N*-acetylmuramyl-L-alanine appears to be required by *E. coli* glutamate racemase for D-glutamic acid formation in addition to its role as the recipient molecule for D-glutamate. In this way UDP-*N*-acetylmuramyl-L-alanine may exert an important regulatory effect early in the peptidoglycan biosynthesis pathway in *E. coli*.

In this report we attempt to elucidate the interaction between UDP-*N*-acetylmuramyl-L-alanine and the *E. coli* enzyme using both the native enzyme and an N-terminal truncated enzyme. Kinetic evaluation of the purified native enzyme, in the presence and absence of UDP-*N*-acetylmuramyl-L-alanine, was also carried out to investigate how UDP-*N*-acetylmuramyl-L-alanine affects the efficiency of *E. coli* glutamate racemase. Solvent isotope exchange studies and site-directed mutagenesis were conducted to probe if UDP-*N*-acetylmuramyl-L-alanine-activated *E. coli* glutamate racemase still follows a similar two-base proton extraction/readdition mechanism as reported for the *Lactobacillus* glutamate racemase.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pRDD020, which contains the *E. coli* glutamate racemase gene (*dga*) cloned into a pUC18 plasmid, and *E. coli* JM109/pICR222 that overproduced the *P. pentosaceus* glutamate racemase were described previously (Pucci et al., 1993). *E. coli* WM335, a D-glutamate auxotroph described by Lugtenberg et al. (1973), was obtained from W. Messer. WM335Δ*rec*A was constructed by P1 phage transduction using a phage lysate containing Δ*rec*A linked to Tn10. After selection on tetracycline, colonies were screened for UV light sensitivity. Plasmid pICR222 was obtained from K. Soda. Expression vector pKK223-3 was from Pharmacia LKB Biotechnology Inc., and pBluescript expression vector was from Stratagene. Ligases and restriction enzymes were from Boehringer Mannheim. Protein assay reagent was from Bio-Rad. SDS−PAGE molecular weight standards were from Integrated Separation Systems. Polyacrylamide gel (12%) was from Novex Co. *Proteus* L-glutamate dehydrogenase was from Sigma Chemical Co. L-[^{14}C]alanine was from Amersham Co.

Preparation of Native and Truncated E. coli Glutamate Racemase. Overexpression and Purification of E. coli Glutamate Racemase. The *E. coli dga* gene from pRDD020 was PCR amplified and subcloned into the pKK223-3 expression vector as described by Yoshimura et al. (1993). *E. coli* JM109 cells were transformed with the pKK223-3::*dga* construct in order to overproduce *E. coli* glutamate racemase. A 5-mL overnight culture of *E. coli* JM109/pKK223-3::*dga* in LB medium with 50 μg/mL ampicillin was inoculated into 500 mL of fresh LB medium containing ampicillin and grown at 37 °C for 4.5 h, at which point IPTG was added to a final concentration of 1 mM. After incubation for an additional 3 h at 37 °C to maximize protein expression, the cells were harvested by centrifugation at 4000*g* for 20 min. Cell pellets were combined and resuspended in 15 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 10% glycerol, 0.1% 2-mercaptoethanol, 0.1 mM PMSF, 1 mM benzamidine, 10 mg/mL AEBSF, and 1 mM EDTA (standard buffer). The cell suspension was sonicated and centrifuged at 15000*g* for 30 min. The supernatant containing the soluble proteins was removed and diluted to 50 mL with the standard buffer. Glutamate racemase was initially resolved from most of the other proteins by FPLC using a Q-Sepharose HiLoad 26/10 column (Pharmacia). The column was loaded with the cell extract and washed with the standard buffer for 20 min, and then the enzyme was eluted with a 0−0.5 M NaCl linear gradient in the standard buffer at a flow rate of 8 mL/min. Fractions containing glutamate racemase activity (determined by the spectrophotometric assay described below) were combined and concentrated in Centriprep-10 filters down to 4 mL. The concentrated enzyme solution was then loaded on a HiLoad 16/60 Superdex 200 gel filtration column and eluted with the standard buffer containing 0.5 M NaCl. Fractions containing enzyme activity were pooled and concentrated again to 3.1 mL. A 1-mL aliquot of the concentrated enzyme solution was diluted to 10 mL with the standard buffer and resolved on a MonoQ HR 5/5 column using a three-step standard buffer/NaCl gradient: 0−5 mL (25 mM NaCl), 5−20 mL (25−500 mM NaCl), and 20−30 mL (500 mM NaCl). Fractions containing the racemase activity were concentrated using a Centricon-10 filter and stored at 4 °C in standard buffer containing 20% glycerol.

Preparation of the Truncated E. coli Glutamate Racemase. When amino acid sequences of the glutamate racemases from *E. coli* and *P. pentosaceus* were compared, a 21 amino acid peptide extension was identified at the N-terminus of the *E. coli* racemase. An N-terminal sequence (21 amino acids) truncated enzyme was prepared by PCR amplification of the *dga* gene from pRDD020 using a pair of oligonucleotide

primers: 5′**GGAAAG**CAGCTATGGAACCACGTCCCACCGTG (N terminus) and 5′GAATTCCTGACCGCGCAACATTCAAC (C terminus). A designed ribosomal binding site (boldface type) and a new start (underlined) with a methionine residue replacing the native serine residue were introduced in the N-terminal primer. The full-length *dga* gene as a control was prepared using a second N-terminal primer, 5′TAGCCAACCTGTTCGACAAAG (starts at 24 bp upstream from the first ATG start codon to include the ribosome binding site), which was paired with the above C-terminal primer to amplify the complete gene. The complete *dga* and truncated genes were individually ligated with pUC19 vector at the *Hin*cII restriction site. Clones in the proper orientation with the *lac* promoter were expressed in *E. coli* WM335Δ*recA* cells. Crude cell lysate obtained after sonication was dialyzed against 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM D-glutamate, 5 mM DTT, 100 μg/L benzamidine, and 1 mM AEBSF at 4 °C. Glutamate racemase activity in the dialyzed cell extracts was measured in both the presence and absence of the activator.

Sequencing of Gene Inserts Prepared by PCR Amplification. DNA was sequenced by the dideoxyribonucleic acid chain termination method of Sanger et al. (1977). The Sequenase 2.0 kit was used according to the manufacturer's protocol (United States Biochemical Corp.). Starting primers were the universal and reverse primers of pUC19, continuing with internal sequence-specific primers. DNA and protein sequence information was analyzed with the GENEPRO computer program, version 6.10 (Riverside Enterprises), with access to the GeneBank sequence database (release 82) and the PIR sequence database (release 40).

Enzymatic Synthesis of UDP-N-acetylmuramyl-L-alanine. Since UDP-*N*-acetylmuramyl-L-alanine is not readily available, a system was devised to yield quantities for this study. UDPNacMur-L-Ala was synthesized enzymatically from UDP-*N*-acetylmuramic acid (UDPNacMur) (Benson et al., 1993) and L-alanine, using partially purified L-alanine adding enzyme (Liger et al., 1991). The reaction mixture contained 25 mM Tris-HCl, pH 7.5, 1 mM ATP, 5 mM $MgSO_4$, 40 mM NH_4SO_4, 20 mM KCl, 2.5 mM β-mercaptoethanol, 0.26 mM UDPNacMur, 3 mM L-alanine, and L-alanine adding enzyme. The reaction mixture was kept at 37 °C for 2 h. UDPNacMur-L-Ala was isolated by DEAE-cellulose chromatography. Reaction products were eluted with a 0.1−0.3 M linear triethylammonium bicarbonate buffer gradient at pH 7.8. Fractions containing material with an absorbance at 260 nm were further analyzed by HPLC as described later. A 100-μL aliquot of an identical reaction mixture spiked with [^{14}C]-L-Ala also was used to assist in the identification of UDPNacMur-[^{14}C]-L-Ala by HPLC analysis. A sample of purified UDPNacMur-L-Ala was subjected to NMR and LC−MS analysis to further confirm the structural identity of the product.

The enzymatic conversion of UDPNacMur to UDPNacMur-L-Ala was quantitative. ^{1}H NMR spectra demonstrated that the proton chemical shifts of protons from the UDPNacMur moiety were consistent with those identified by Benson et al. An additional ^{1}H signal (doublet) corresponding to the α-methyl group of L-alanine was present in the 1.30−1.35 ppm region.

Assays for Glutamate Racemase Activity. (*A*) D *to* L. A continuous assay for the formation of L-glutamate from D-glutamate was established by coupling glutamate racemase with L-glutamate dehydrogenase in the assay mixture (Nakajima et al., 1987) and monitoring the reduction of NADP to NADPH. Change of absorbance at 340 nm was monitored on a Beckman DU 7400 spectrophotometer. Reaction mixtures usually contained 25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 2.5 mM NADP, 6.5 μM UDPNacMur-L-Ala, 30 units of L-glutamate dehydrogenase, 5 mM D-glutamate, and a predetermined amount of glutamate racemase. For kinetic evaluation of D- to L-glutamate racemization, up to 10 mM D-glutamate was used in the assay. To evaluate the effect of UDPNacMur-L-Ala on the racemase activity, 0−65 μM effector was used.

(*B*) L *to* D. For L- to D-glutamate conversion a stepwise assay was designed. The reaction mixture (1 mL), in the first step, contains 25 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 6.5 μM UDPNacMur-L-Ala, and varying amounts of L-glutamate. A predetermined amount of glutamate racemase was added to the solution to start the reaction. Aliquots of 320 μL were taken out at 30, 60, and 120 min and mixed with 12.5 μL of 2 N HCl to quench the reaction. The solutions were then filtered through Millipore Ultrafree-MC (10 000 MW cutoff) filter units to remove the glutamate racemase. Addition of 7.5 μL of 2 N NaOH to the filtrate brought the pH of the solution up to 8.2. In the second step, UDP-*N*-acetylmuramyl-L-alanyl-D-glutamate synthase (the D-glutamate adding enzyme) (Michaud et al., 1987) was used to attach the D-glutamate (produced in the racemase reaction) to UDPNacMur-L-Ala. Reagents needed for the D-glutamate adding enzyme reaction were added to the filtrate to yield final concentrations of 300 μM UDPNacMur-L-Ala, 1 mM ATP, and excess D-glutamate adding enzyme. Tris-HCl, 25 mM, pH 8.2, was added to bring the final volume to 400 μL. The reaction mixture was incubated at 37 °C overnight such that all of the D-glutamate produced in solution was converted to UDPNacMur-L-Ala-D-Glu. After ultrafiltration to remove the protein, an aliquot of 100 μL was analyzed by HPLC to determine the amount of UDPNacMur-L-Ala-D-Glu produced. An HPLC-generated standard curve was produced, by using varying amounts of UDPNacGlc, to quantitate the amount of UDPNacMur-L-Ala-D-Glu produced.

HPLC analysis of uridine sugar nucleotides was carried out with a Waters HPLC equipped with an Optisil 10-μm SAX column (250 × 4.6 mm), two model 590 pumps, and a 490E programmable multiwavelength detector. Reaction products were eluted isocratically with 75 mM potassium phosphate, pH 3.5, at a flow rate of 1.5 mL/min and monitored on-line at 262 nm. In addition, an on-line Radiomatic Flo-One Beta radioactive flow detector was employed when radioactively labeled materials were used. UDPNacMur-L-Ala has a retention time of 21 min, and UDPNacMur-L-Ala-D-Glu was eluted at 29 min.

Circular Dichroism Assay To Determine Glutamate Racemase Substrate Specificity. L-Amino acids of choice were scanned on a JASCO-700 CD spectrometer to identify the λ_{max} values and to determine the specific extinction coefficient. Reaction mixtures contained 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 50 mM amino acid (unless limited by solubility), and 6.5 μM UDPNacMur-L-Ala. Ten micrograms of glutamate racemase was added to start the reaction. Activity as expressed by a change in ellipticity was monitored at λ_{max} over a 30-min period at ambient

temperature. The corresponding slope was measured to determine the reaction rate.

Measurement of Enzyme-Catalyzed Isotope Exchange by ^1H NMR. Reaction mixtures containing 50 mM potassium phosphate buffer, pH 7.5, 2 mM β-mercaptoethanol, 15 μM UDP-*N*-acetylmuramyl-L-alanine, and 10 mM D- or L-glutamate were dried under vacuum. The residue was taken up in 0.5 mL of D_2O, and the drying process was repeated three times. The final residue was dissolved in 0.8 mL of D_2O. ^1H NMR spectra were acquired with a Bruker WM360 wb NMR spectrometer operating at a proton frequency of 360.13 MHz at 25 °C. Twenty microliters of enzyme solution was added to start the reaction. The proton spectra were acquired every 30 min thereafter over a period of several hours. The ^1H chemical shifts were expressed with respect to an external standard (TMS).

Site-Directed Mutagenesis of the Conserved Cysteine Residues and Preparation of Mutant Extracts. The *dga* gene was cloned into the pBluescript expression vector, and site-directed mutagenesis was carried out as described by Sowek et al. (1991). Uracil-incorporated single-stranded DNA (ss DNA) was isolated and used as the template for mutagenesis. HPLC-purified mutant oligonucleotide primers were custom synthesized by Genosys Biotechnologies, Inc. The sequences of the primers for mutation 1 (C96T) and mutation 2 (C208T) are as follows: primer, mutation 1 = 5′ACTGGCAGTGTTGGTAGCGACCACAG; primer, mutation 2 = 5′AGGGAAATGGGTGGTACCCAATACAACG. Changes made in the nucleotide sequences are underlined. Each oligonucleotide was phosphorylated at the 5′ end and then hybridized with the uracil-incorporated ss DNA. Primer extension was performed as described by Kunkel (1987). The resulting double-strand wild-type/mutant heteroduplex plasmids were transformed into *E. coli* JM109. Ampicillin-resistant colonies were lifted onto Immobilon nitrocellulose transfer membranes. Bacterial DNA was liberated from the colonies *in situ* and bound to the nitrocellulose membranes. 5′-^{32}P-labeled mutant oligonucleotides were hybridized with the bacterial DNA overnight. The colonies corresponding to strong signals were picked, and DNA sequencing was performed to confirm the mutation. Clones with the desired changes were inoculated into LB broth to prepare cell lysate for the racemase activity assay as described earlier. Cell extract from *E. coli* JM109/pBluescript::*dga* was used as a control.

RESULTS

Purification of the Cloned E. coli Glutamate Racemase. In order to produce sufficient quantities of purified enzyme for our study, the *dga* gene was cloned into the expression vector pKK223-3. Subsequently, DNA sequencing was carried out to verify that the *dga* gene insert in the pKK223-3::*dga* construct retained the identical DNA sequence as that reported previously (Doublet et al., 1993). After IPTG induction, the major protein present in the *E. coli* JM109/pKK223-3::*dga* lysate was identified by SDS−PAGE to be a protein of 31 000 Da (Figure 1). By comparison of the specific activity in the crude extract of the overproducing strain to that of the purified enzyme, it can be estimated that ≈20% of the protein in the crude extract was glutamate racemase. After two ion-exchange chromatographic steps and one size exclusion chromatographic step, glutamate racemase with >95% homogeneity (by SDS−PAGE, Figure 1) was obtained with an overall yield of 10%. Some loss of specific activity was observed after the MonoQ column chromatography. The enzyme required DTT for activity as assay of enzyme activity in a reaction mixture lacking DTT resulted in an immediate decrease in activity. The enzyme did not appear to contain pyridoxal phosphate, as scanning of a 1 mg/mL racemase solution revealed no absorbance in regions above 350 nm. Addition of pyridoxal phosphate showed no effect on the racemase activity.

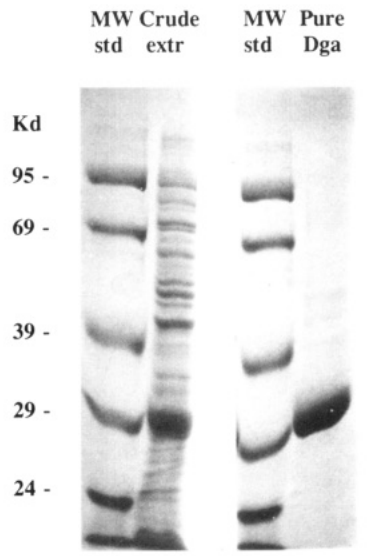


FIGURE 1: SDS−PAGE of crude and purified *E. coli* glutamate racemase. Protein samples were run on a 12% polyacrylamide gel. Molecular mass standards used are as labeled. Lanes labeled MW std are molecular mass standards. The lane labeled Crude extr is the crude cell extract obtained after sonication of the cell suspension. The lane labeled Pure Dga is *E. coli* glutamate racemase recovered after the MonoQ HR 5/5 column chromatography.

Effect of UDPNacMur-L-Ala on the Purified E. coli Glutamate Racemase Activity. Purified *E. coli* glutamate racemase was assayed spectrophotometrically in the presence of 0−65 μM UDPNacMur-L-Ala. When UDPNacMur-L-Ala was omitted, racemase activity was detectable at a low level [400 nmol/(min·mg)]. The addition of UDPNacMur-L-Ala to the reaction mixture resulted in an immediate rate increase in a dose-dependent fashion (Figure 2). No consumption of UDPNacMur-L-Ala could be detected by HPLC analysis throughout the enzyme reaction process. UDPNacMur-L-Ala therefore functioned as a positive effector of *E. coli* glutamate racemase. The kinetic constants for the activated *E. coli* glutamate racemase with D- and L-glutamate as substrates were determined in the presence of 6.5 μM UDPNacMur-L-Ala. The K_m values for D- and L-glutamate were 4.2 mM and 2.1 mM, respectively. The V_{max} measured for the D to L direction was 34 μmol/(min·mg) and for the L to D direction was 23 μmol/(min·mg). In the presence of UDPNacMur-L-Ala both D- and L-glutamate are therefore equally efficient substrates for *E. coli* glutamate racemase. In contrast, without UDPNacMur-L-Ala the K_m of D-glutamate rose to 19.2 mM and V_{max} dropped almost 80-fold to 0.4 μmol/(min·mg). The conversion of L-glutamate to D-glutamate in the absence of the activator also occurred at a similar low level as suggested by both HPLC and NMR studies.

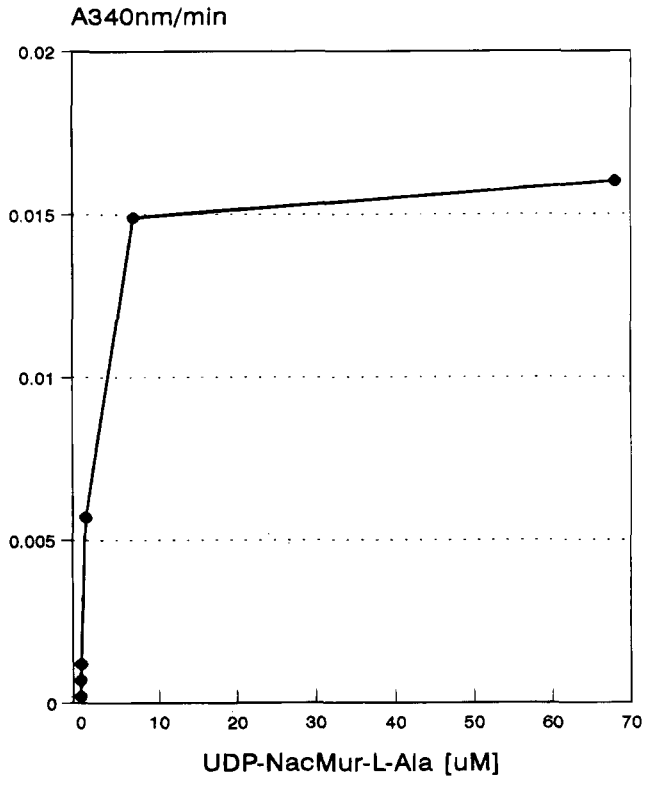


FIGURE 2: Concentration-dependent stimulation of *E. coli* glutamate racemase by UDP-*N*-acetylmuramyl-L-alanine. Formation of L-glutamate from D-glutamate was assayed by coupling the reaction with NADP/L-glutamate dehydrogenase and following the increase in absorbance at 340 nm.

For comparison, the effect of UDPNaCMur-L-Ala on the *Pediococcus* glutamate racemase activity was also evaluated. The *Pediococcus* glutamate racemase displayed identical activity in both the absence and presence of UDPNacMur-L-Ala (data not shown), despite its 28% amino acid sequence similarity to the *E. coli* enzyme.

Effect of UDPNacMur-L-Ala on the N-Terminal Truncated E. coli Glutamate Racemase. Alignment of the amino acid sequences of *E. coli* with *P. pentosaceus* and *L. fermenti* glutamate racemases (Pucci et al., 1994) shows that the *E. coli* enzyme has an additional 21 amino acids at the N-terminus. An N-terminal truncated enzyme was constructed to evaluate the role this region may play with regard to the UDPNacMur-L-Ala-mediated enzyme activation. Removal of the coding region for the N-terminal 21 amino acids of the *E. coli* enzyme was followed by the introduction of a ribosome binding site and a methionine start codon (see Figure 3) to result in a truncated gene product. A full-length gene construct was also prepared as a control. Transformation of *E. coli* WM335Δ*rec*A (a D-glutamate auxotrophic strain with a nonfunctioning chromosomal *dga* gene and deficient in DNA recombination) cells with a construct containing either the full-length or the truncated *dga* gene resulted in the growth of the host cells in minimal medium without D-glutamate supplement. These results indicate the expression of a functioning gene product (full length or truncated) in the host cells. *E. coli* WM335Δ*rec*A also provides a minimal background for the racemase activity assay. Assay of the truncated enzyme, in the presence of the activator (6.5 μM), revealed a reduction of the UDP-NacMur-L-Ala-mediated enzyme activation by more than 80% (Table 1). The same level of reduction effect on the truncated enzyme was observed with UDPNacMur-L-Ala concentrations varying from 0.65 to 65 μM (Figure 4). The truncated enzyme did, however, retain a low level of basal activity. This result suggests that the N-terminal sequence is essential for the interaction between the UDPNacMur-L-Ala activator molecule and *E. coli* glutamate racemase.

Table 1: Loss of Activation Effect of the N-Terminal Truncated *E. coli* Glutamate Racemase[a] by UDPNacMur-L-Ala

glutamate racemase	glutamate racemase activity [nmol/(min·mg)]	
	without UDPNacMur-L-Ala	with UDPNacMur-L-Ala
native enzyme	10	175
truncated enzyme	11	29

[a] Assayed with crude cell extracts after dialysis.

Solvent ^2H−Glutamate α-^1H Exchange and Properties of the Cysteine-to-Threonine Mutants of Glutamate Racemase. Despite the apparent amino acid sequence similarity between *E. coli* glutamate racemase and the known Gram-positive bacterial glutamate racemases, only the *E. coli* enzyme is regulated by UDP-*N*-acetylmuramyl-L-alanine. One would wonder if the UDP-*N*-acetylmuramyl-L-alanine-activated *E. coli* glutamate racemase follows a different racemization mechanism. ^1H NMR analysis of a reaction mixture prepared in D_2O showed an *E. coli* glutamate racemase dependent exchange of the solvent deuterium with the C-2 proton of the substrate glutamate over time. The C-2 proton signal at 3.9 ppm largely disappeared after a 6-h incubation (Figure 5) and so did the *J*-coupling of C-3 proton signal (due to the adjacent C-2 proton) at 2.25 ppm. No exchange with the solvent ^2H was detectable at either C-3 or C-4. Both changes demonstrate that only the C-2 ^1H was exchanged with solvent ^2H and strongly support the conclusion that racemization proceeds through a deprotonation/reprotonation process as reported for both *Lactobacillus* (Gallo et al., 1993) and *Pediococcus* (Nakajima et al., 1988) glutamate racemases, the two cofactor-independent glutamate racemases. As expected, overnight incubation of the *E. coli* glutamate racemase in the absence of UDPNacMur-L-Ala showed minimal solvent isotope exchange.

It has been shown that the two conserved cysteine residues of the *L. fermenti* glutamate racemase serve as the enzymic bases in the racemization reaction (Gallo et al., 1993). Alignment of the amino acid sequences of the glutamate racemases from *E. coli*, *P. pentosaceus*, and *L. fermenti* revealed the presence of the two conserved cysteine residues in the *E. coli* enzyme (Pucci et al., 1994). To probe their function in the *E. coli* enzyme, site-directed mutagenesis was carried out to produce altered enzymes with either C96T and C208T. The cell extracts containing either of the two altered *E. coli* enzymes exhibited no detectable racemase activity above the background racemase activity of *E. coli* JM109, even in the presence of UDPNacMur-L-Ala, whereas the control cell extract from *E. coli* JM109/pBluescript::*dga* (native enzyme) retained normal enzyme activity. Therefore, these cysteine residues are essential for the *E. coli* racemase activity and are probably involved in the two-base racemization process similar to those reported in the case of the *Lactobacillus* glutamate racemase.

Substrate Specificity. The purified *E. coli* glutamate racemase was incubated with several L-amino acids individually, and the change in ellipticity at 204 nm was measured

```
                                  ↓
1     MRQSMATKLQDGNTPCLAATPSEPRPTVLVFDSGVGGLSVYDEIRHLLPDLHY.....
                              **      ******* *       ***
                            * **      *********       ***
2                          MDNRPIGFM-DSGVGGLTVVKTAQKLLPNEEI.....
                           ******* * *** *** **    *   *****
                           ******* * ***********   *   ******
3                          MDNRPIGVM-DSGLGGLSVVRVIQQKLPNEEV.....
```

FIGURE 3: Amino acid sequences of the N-terminal regions of the glutamate racemases of *E. coli* (1), *P. pentosaceus* (2), and *L. fermenti* (3). Exact matches among the sequences are indicated by two asterisks and conservative substitutions by a single asterisk. The common N-terminal box (DSGXGGLXV) is shown in italics for all three sequences. The 21 amino acid N-terminal extension of *E. coli* is shown, as well as the location (arrow) of the serine residue that was changed to a methionine in the truncated *E. coli* racemase construct.

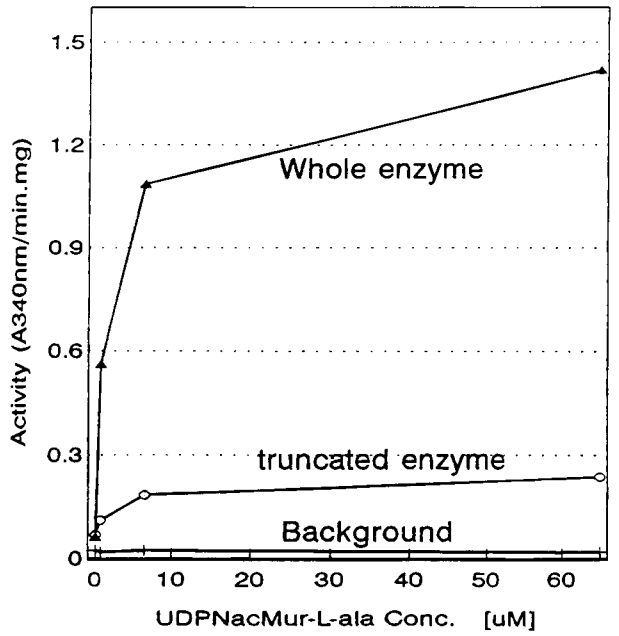


FIGURE 4: Effect of increasing concentrations of UDPNacMur-L-Ala on the full-length and the N-terminal truncated *E. coli* glutamate racemase activity. Enzyme activity was assayed as described in Figure 2.

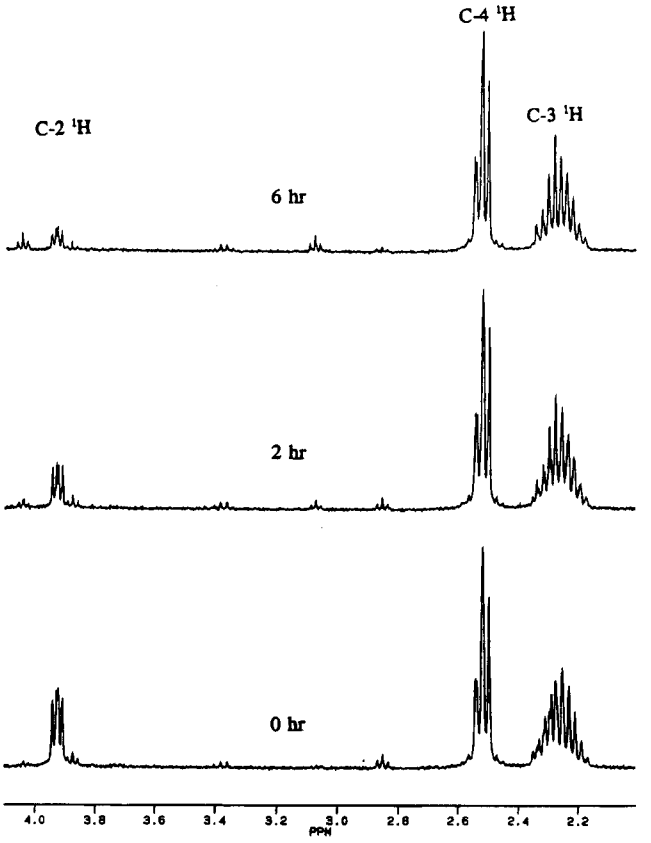


FIGURE 5: ^{1}H NMR spectra of glutamic acid after 0, 2, and 6 h of incubation with *E. coli* glutamate racemase. DTT is essential to maintain the racemase activity. The signals corresponding to the C-2, C-3, and C-4 protons are as labeled.

by CD spectroscopy over time. As shown in Table 2, at a concentration of 50 mM, L-homocysteic acid is a relatively good substrate while L-glutamine is not a substrate. L-α-Aminoadipic acid can also serve as a substrate yet L-aspartic acid cannot. These observations suggest that at least a 5-C backbone and an acidic function at the γ-C are necessary for racemase activity. In contrast, the *Pediococcus* glutamate racemase was reported to accept neither L-homocysteic acid nor L-α-aminoadipic acid as its substrate.

DISCUSSION

E. coli glutamate racemase was found active in the absence of UDPNacMur-L-Ala. A basal level of activity was detectable, and this is increased dramatically when UDP-NacMur-L-Ala was added. UDPNacMur-L-Ala therefore functions as an activator for the racemase. Steady-state kinetic analysis with D-glutamate as the substrate, in the presence and absence of the activator, showed that UDP-NacMur-L-Ala exerts its effect by increasing both the substrate binding affinity and the turnover rate of the enzyme. Comparison of the kinetic parameters obtained for the activated *E. coli* glutamate racemase with those reported for the *Pediococcus* enzyme (Nakajima et al., 1986) indicates that UDPNacMur-L-Ala-activated *E. coli* glutamate racemase is at least as efficient as the *Pediococcus* enzyme, if not more efficient. Our results also showed that, in the presence of UDPNacMur-L-Ala, *E. coli* glutamate racemase catalyzes the interconversion between D- and L-glutamate with equal efficiency in both directions. Studies were also conducted under equilibrium conditions, which confirmed that the reaction reached equilibrium near unity (unpublished data).

Table 2: Substrate Specificity of *E. coli* Glutamic Acid Racemase[a]

compound	concn (mM)	relative reaction rate, $V_{(analog/L\text{-}Glu)} \times 100$
L-glutamic acid	50	100
L-glutamine	50	0
L-aspartic acid	50	0
L-asparagine	17	0
L-α-aminoadipic acid	12.5	14
L-lysine	50	0
L-threonine	50	0
L-homocysteic acid	50	62

[a] Reaction rate of L-glutamate, the natural substrate, is normalized to be 100%. Lower concentrations were used for asparagine and L-α-aminoadipic acid due to their limited solubility.

This enzyme is unique in that its activity is highly stimulated by the presence of a specific peptidoglycan precursor, UDPNacMur-L-Ala, which is also the acceptor molecule in the D-glutamate adding enzyme catalyzed reaction. As one of the precursors in the peptidoglycan biosynthesis pathway and the recipient molecule of D-glutamate in the next step of the peptidoglycan precursors synthesis, UDPNacMur-L-Ala may play an important role in regulating the total synthesis of bacterial peptidoglycan in Gram-negative bacterial cells by controlling glutamate racemase activity.

Despite the significant level of amino acid sequence homology present between the *E. coli* enzyme and the glutamate racemases from *P. pentosaceus* and *L. fermenti*, no activation of the latter enzymes by the activator can be demonstrated [our data and Doublet et al. (1994)]. The *E. coli* enzyme has a 21 amino acid extension at the N terminus as compared to the Gram-positive bacterial racemase. This longer N-terminal region of the enzyme appears to be essential for the optimal stimulation of the enzyme activity by the activator. The N-terminal truncated enzyme, though retaining the basal catalytic activity, is minimally stimulated by the activator (<20% activation). It also appears that the N-terminal 21 amino acid extension is not itself responsible for the inhibition of the racemase activity. Whether this 21 amino acid region binds directly to the activator or assists the binding of the activator to another region of the protein remains to be determined.

Solvent isotope exchange studies reveal a C-2 proton extraction/addition process that is both enzyme and UDP-NacMur-L-Ala dependent. The minimal level of solvent isotope exchange in the absence of UDPNacMur-L-Ala coincides with the small percentage of racemization when UDPNacMur-L-Ala was omitted from the reaction mixtures. Replacement of either of the two conserved cystein residues with a threonine by site-directed mutagenesis resulted in complete loss of enzyme activity. A similar deprotonation/reprotonation process, like the one reported for *Lactobacillus* glutamate racemase, therefore may be required for the enzymatic racemization of glutamate in *E. coli*, with or without UDPNacMur-L-Ala. The activation effect by UDPNacMur-L-Ala was equally demonstrated for both the "D to L" and "L to D" conversion. Therefore, this effector is not controlling the direction of the racemization. The level of UDPNAcMur-L-Ala present in the cytoplasm could be a mechanism to control the racemization process and regulate D-glutamate levels to be consistent with the needs of peptidoglycan synthesis. In the meantime the low-level activity displayed by the enzyme with no activator or low levels of the activator present would help to avoid the overproduction of cellular D-glutamic acid, which may be toxic to *E. coli* cell growth.

These results demonstrate a major difference between this Gram-negative bacterial glutamate racemase and the known Gram-positive bacterial racemases. The significance of this difference in bacterial cell wall synthesis should be investigated further. Since Gram-negative bacteria have a much thinner peptidoglycan layer than do Gram-positive bacteria, more stringent control of the cytoplasmic level of D-glutamate may be required in Gram-negative bacteria.

ACKNOWLEDGMENT

We appreciate Dr. D. Bonner, Dr. R. Kessler, and Dr. R. White for their critical reading of the manuscript. We thank Dr. K. Volk for obtaining the MS spectrum and Ms. M. D'Andrea for obtaining the NMR spectra.

REFERENCES

Ashiuchi, M., Yoshimura, T., Esaki, N., Ueno, H., & Soda, K. (1993) *Biosci. Biotech. Biochem. 57*, 1978−1979.

Benson, T. E., Marquardt, J. L., Marquardt, A. C., Etzkorn, F. A., & Walsh, C. T. (1993) *Biochemistry 32*, 2024−2030.

Doublet, P., van Heijenoort, J., & Mengin-Lecreulx, D. (1992) *J. Bacteriol. 174*, 5772−5779.

Doublet, P., van Heijenoort, J., Bohin, J.-P., & Mengin-Lecreulx, D. (1993) *J. Bacteriol. 175*, 2970−2979.

Doublet, P., van Heijenoort, J., & Mengin-Lecreulx, D. (1994) *Biochemistry 33*, 5285−5209.

Dougherty, T. J., Thanassi, J., & Pucci, M. J. (1993) *J. Bacteriol. 175*, 111−116.

Gallo, K. A., & Knowles, J. R. (1993) *Biochemistry 32*, 3981−3990.

Gallo, K. A., Tanner, M. E., & Knowles, J. R. (1993) *Biochemistry 32*, 3991−3997.

Kunkel, T. A. (1987) in *Current Protocol in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) Greene Publishing Associates and Wiley Interscience, New York.

Liger, D., Blanot, D., & van Heijenoort, J. (1991) *FEMS Microbiol. Lett. 80*, 111−116.

Lugtenberg, E. J. J., Wijsman, H. J. W., & van Zaane, D. (1973) *J. Bacteriol. 114*, 499−506.

Martinez-Carrion, M., & Jenkins, W. T. (1965) *J. Biol. Chem. 240*, 3547−3552.

Matsuhashi, M. (1994) in *Bacterial Cell Wall* (Ghuysen, J. M., & Hakenbeck, R., Eds.) *New Comprehensive Biochemistry*, Vol. 27, pp 55−71, Elsevier, Amsterdam and New York.

Michaud, C., Blanot, D., Fluoret, B., & van Heijenoort, J. (1987) *Eur. J. Biochem. 66*, 631−637.

Nakajima, N., Tanizawa, K., Tanaka, H., & Soda, K. (1986) *Agric. Biol. Chem. 50*, 2823−2830.

Nakajima, N., Nakajima, N., Tanizawa, Katsuyuki, T., Tanaka, H., & Soda, K. (1988) *Agric. Biol. Chem. 52*, 3099−3104.

Park, J. T. (1987) *E. coli and Salmonella typhimurium Cellular and Molecular Biology* (Neidhart, F. C., Ed.) pp 663−671, American Society for Microbiology, Washington, DC.

Pucci, M. J., Novotny, J., Discotto, L. F., & Dougherty, T. J. (1994) *J. Bacteriol. 176*, 528−530.

Sanger, F., Niklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.

Sowek, J. A., Singer, S. B., Ohringer, S., Malley, M. F., Dougherty, T. J., Gougoutas, J. Z., & Bush, K. (1991) *Biochemistry 30*, 3179−3188.

Tanizawa, K., Katsuyuki, T., Tanaka, H., & Soda, K. (1987) *Anal. Lett. 20*, 471−478.

Tanizawa, K., Asano, S., Masu, Y., Kuramitsu, S., Kagamiyama, H., Tanaka, H., & Soda, K. (1989) *J. Biol. Chem. 264*, 2450−2454.

Tanner, M. E., Gallo, K. A., & Knowles, J. R. (1993) *Biochemistry 32*, 3998−4006.

Yonaha, K., Misono, H., Yamamoto, T., & Soda, K. (1975) *J. Biol. Chem. 250*, 6983−6989.

Yoshimura, T., Ashiuchi, M., Esaki, N., Kobatake, C., Choi, S.-Y., & Soda, K. (1993) *J. Biol. Chem. 268*, 24242−24246.

BI941406P