The Glutamate Racemase Activity from *Escherichia coli* Is Regulated by Peptidoglycan Precursor UDP-*N*-acetylmuramoyl-L-alanine†

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ABSTRACT: The *murI* gene product of *Escherichia coli* was recently identified as the glutamate racemase activity which catalyzes the formation of D-glutamic acid, one of the essential components of bacterial cell-wall peptidoglycan [Doublet et al. (1993) *J. Bacteriol. 175*, 2970–2979]. We here describe the purification to homogeneity and the kinetic properties of this enzyme. *In vitro*, the glutamate racemase activity shows an absolute requirement for UDP-*N*-acetylmuramoyl-L-alanine (UDP-MurNAc-L-Ala), the substrate of the D-glutamic acid-adding enzyme which catalyzes the subsequent step in the pathway for peptidoglycan synthesis. The affinity of the enzyme for this activator is particularly high (K_D = 4 μM) and specific, since no other peptidoglycan precursor from UDP-GlcNAc to UDP-MurNAc-pentapeptide is an effector. Minor chemical modifications of the UDP-MurNAc-L-Ala molecule, such as the reduction of the uracyl moiety, suppress its activating effect. This specific *in vitro* requirement most likely represents the physiological mechanism which regulates the activity of the glutamate racemase *in vivo*. It adjusts the formation of D-glutamic acid to the requirements of peptidoglycan synthesis and avoids an excessive racemization of the intracellular pool of L-glutamic acid.

The rigid, shape-determining material in bacterial cell walls is a giant polymer of periodic structure, named peptidoglycan or murein, consisting of two amino sugars and at least four amino acids (Park, 1987; Rogers et al., 1980). Its biosynthesis is a complex process involving many different cytoplasmic and membrane steps.

D-Glutamic acid appears as one of the components specific of all bacterial cell-wall peptidoglycan structures. It is incorporated into nucleotide peptidoglycan precursors by its addition to UDP-*N*-acetylmuramoyl-L-alanine (UDP-MurNAc-L-Ala) (Figure 1), a reaction catalyzed by the D-glutamic acid-adding enzyme (the *murD* gene product) (Mengin-Lecreulx et al., 1989; Pratviel-Sosa et al., 1991). The enzymatic mechanism by which D-glutamic acid is produced in bacteria has been investigated for a limited number of species. Two different routes have been identified, a transamination process catalyzed by a D-alanine aminotransferase (EC 2.6.1.21) using D-alanine and α-ketoglutarate as substrates, as observed in *Bacillus* species (Martinez-Carrion & Jenkins, 1965; Tanizawa et al., 1989), or a direct conversion of L-glutamic acid to D-glutamic acid catalyzed by a glutamate racemase (EC 5.1.1.3), as demonstrated in *Lactobacillus* and *Pediococcus* species (Nakajima et al., 1986, 1988). We recently investigated in detail this problem in *Escherichia coli* (Doublet et al., 1992, 1993). First, the essential gene *murI* required for the synthesis of D-glutamic acid was identified as a previously sequenced open reading frame of unknown function lying in the 90-min region of the chromosome, which theoretically encoded a protein of 289 amino acids with a molecular weight of 31 500 Da (Doublet et al., 1992; Brosius et al., 1981). More recently, the *murI* gene product was overproduced to a high level in cells carrying appropriate plasmids and was clearly identified as a glutamate racemase (Doublet et al., 1993).

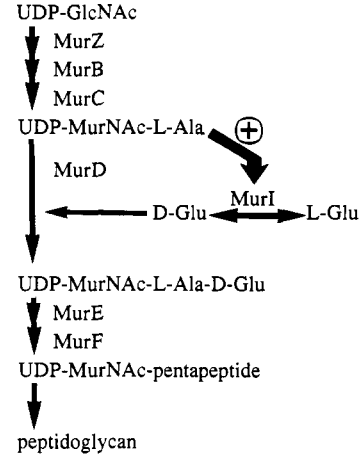

FIGURE 1: Biosynthesis and cellular utilization of D-glutamic acid in *E. coli*. The sign (+) indicates the activating effect of UDP-MurNAc-L-Ala on glutamate racemase. For more details on the *mur* (murein) genes and enzymes involved in this reaction sequence, see Rogers et al., (1980) and Park (1987).

To detect this activity in crude extracts of *E. coli*, we initially used a convenient coupled assay in which the D-glutamic acid produced from the L-isomer by the glutamate racemase was quantitatively converted to UDP-MurNAc-L-Ala-D-Glu by the MurD activity in the presence of UDP-MurNAc-L-Ala, ATP, and Mg^{2+} (Doublet et al., 1993). However, when a direct assay was carried out without UDP-MurNAc-L-Ala, no D-glutamic acid was detectable, a result suggesting that the latter nucleotide precursor could be an effector of the racemase activity.

We here describe the purification to homogeneity and the kinetic properties of the glutamate racemase from *E. coli*. Evidence is provided that the activity of this enzyme is regulated *in vivo* by the peptidoglycan precursor UDP-MurNAc-L-Ala, the other substrate of the D-glutamic acid-

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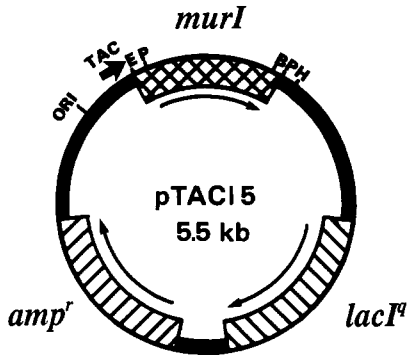

*Eco*RI
↓ MurI →
AGGAAACAGCGATGAATTCATGAGACAGTCT

FIGURE 2: Construction of plasmid pTACI5. This plasmid was constructed by inserting the 0.9-kb *Eco*RI-*Dra*I fragment from pTACI2 plasmid (Doublet et al., 1993) into the *Eco*RI and *Sma*I sites of the pTTQ18 cloning vector. Restriction sites are shown for enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and P, *Pst*I. The junction between vector and *E. coli* DNA sequences at the *Eco*RI site is shown at the bottom. The ribosome-binding site (underlined) and the *murI* coding sequence are indicated.

adding enzyme which catalyzes the subsequent step in the pathway for peptidoglycan synthesis.

MATERIALS AND METHODS

Strains, Plasmids, and Growth Conditions. The *E. coli* strain JM83 [*ara*δ(*lac-proAB*) *rpsL thi* Φ80d*lacZ*δM15] (Yanisch-Perron et al., 1985) was used as the host for plasmids and for the large-scale purification of the overproduced *murI* gene product. When screening plasmid inserts for the presence of a functional *murI* gene, the D-glutamic acid-requiring strain WM335 of *E. coli* B was used (Hoffman et al., 1972; Lugtenberg, et al., 1973). Cloning vector pTTQ18 was purchased from Pharmacia, and plasmid pTACI2 has been previously described (Doublet et al., 1993). LB medium (Miller, 1972) was used for growing cells and was supplemented, when required, with D-glutamic acid (100 μg/mL), thymine (100 μg/mL), and the antibiotics ampicillin (100 μg/mL) or streptomycin (50 μg/mL). Growth was monitored at 600 nm.

General DNA Techniques and E. coli Cell Transformation. Small-and large-scale plasmid isolations were carried out by the alkaline lysis method, and plasmids were further purified by using cesium chloride–ethidium bromide gradients (Sambrook et al., 1989). Standard procedures for endonuclease digestions, ligation, and agarose electrophoresis were used (Sambrook et al., 1989). Usually, *E. coli* cells were made competent for transformation with plasmid DNA by the method of Dagert and Ehrlich (1979).

Construction of pTACI5. The pTACI2 plasmid carrying the *E. coli murI* gene cloned downstream of a *tac* promoter in the pBTac1 vector has been previously described (Doublet et al., 1993). The 0.9-kb *Eco*RI-*Dra*I fragment from pTACI2 was inserted into the *Eco*RI and *Sma*I sites of the plasmid vector pTTQ18 to generate plasmid pTACI5 (Figure 2).

Preparation of Crude Enzyme. Cells of JM83 harboring the plasmid pTACI5 were grown exponentially (1-L culture) at 37 °C in LB-ampicillin medium. When the optical density of the culture reached 0.1, isopropyl β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM, and growth was continued for 6 h (final OD = 0.8). Cells were harvested in the cold and washed with 40 mL of cold 0.02 M potassium phosphate buffer, pH 7, containing 0.1% β-mercaptoethanol and 1 mM DL-glutamic acid (standard buffer). The wet cell pellet was suspended in 10 mL of the same buffer and sonicated in the cold until disruption of all cells. The resulting suspension was centrifuged at 4 °C for 30 min at 200000*g* in a Beckman TL100 centrifuge. The supernatant was dialyzed overnight at 4 °C against 100 volumes of the standard buffer, and the resulting solution (10 mL; 8 mg of protein/mL), designated crude enzyme, was stored at −20 °C. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of proteins was performed as previously described (Laemmli & Favre, 1973), using 13% polyacrylamide gels. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Assays for Glutamate Racemase Activity. (i) Direct Assay. Particularly during enzyme purification, an end-point assay for glutamate racemase was used. The interconversion of glutamic acid isomers was followed in a reaction mixture containing 100 mM Tris-HCl buffer, pH 7.8, either L-[^{14}C]), glutamic acid or D-[^{14}C]glutamic acid (2000 Bq, varying from 0.1 to 40 mM), glycerol (4%, v/v β-mercaptoethanol (5 mM), and UDP-MurNAc-L-Ala (40 μM) in a total volume of 100 μL. After 30 min at 37 °C, samples were heated at 95–100 °C for 10 min. Denatured protein was removed by centrifugation in a microcentrifuge for 10 min at 14 000 rpm, and aliquots (50 μL) from the supernatant were treated with Marfey's chiral reagent (Marfey, 1984). The ratio of glutamic acid isomers was determined after the separation of the resulting diastereoisomers by a previously described HPLC procedure (Doublet et al., 1993).

(ii) D → L *Coupled Assay.* In this assay, the racemase reaction was followed at 30 °C with D-glutamic acid as the substrate, using a continuous method based on the aminotransferase assay of Rej (1982) which was recently adapted for the glutamate racemase from *Lactobacillus* species (Gallo & Knowles, 1993). The racemase activity was coupled to L-glutamate dehydrogenase/NAD^+ and diaphorase/*p*-iodonitrotetrazolium violet (INT). Rates were determined by following the absorbance at 500 nm in a reaction mixture containing 50 mM Tris-HCl buffer, pH 7.8, NAD^+ (10 mM), ADP (3.75 mM), INT (2 mM), UDP-MurNAc-L-Ala (40 μM), D-glutamic acid (varying from 0.1 to 40 mM), β-mercaptoethanol (5 mM), glycerol (4%, v/v), L-glutamate dehydrogenase (60 units), diaphorase (5 units), and glutamate racemase in a total volume of 1 mL. The molar extinction coefficient of the reduced tetrazolium anion was determined by measuring the absorbance change at 500 nm induced by the addition of known amounts of L-glutamic acid in an assay mixture where D-glutamic acid and glutamate racemase were omitted.

(iii) L → D *Coupled Assay.* In this assay, the racemase reaction was coupled to that of the D-glutamic acid-adding enzyme (Pratviel-Sosa et al., 1991) which leads to the formation of UDP-MurNAc-L-Ala-D-Glu. Typical incubations were performed at 37 °C for 30 min in 100 mM Tris-HCl buffer, pH 8.6, containing L-[^{14}C]glutamic acid (2000 Bq, varying from 0.1 to 10 mM), UDP-MurNAc-L-Ala (1 mM), ATP (6 mM), $MgCl_2$ (12 mM), purified D-glutamic acid-adding enzyme (0.1 μg), β-mercaptoethanol (5 mM), glycerol (4%, v/v), and glutamate racemase in a total volume of 40 μL. The reaction was stopped by addition of 10 μL of glacial acetic acid, and radioactive products were separated

by high-voltage paper electrophoresis and quantitated as previously described (Doublet et al., 1993; Mengin-Lecreulx et al., 1982). One unit of enzyme activity was defined as the amount which catalyzed the formation of 1 nmol of product (L- or D-glutamic acid) in 1 min.

Purification of Glutamate Racemase from Strain JM83 (pTACI5). The crude extract (80 mg of protein, 62 500 units of glutamate racemase activity) secured from strain JM83 (pTACI5) as described above was loaded onto a column (12.5- × 2.5-cm) of DEAE-Trisacryl-M (IBF, Villeneuve-la-Garenne, France) equilibrated with buffer A (20 mM potassium phosphate buffer, pH 7.4, containing 0.1% β-mercaptoethanol, 1 mM DL-glutamic acid, and 10% (v/v) glycerol). The elution was run at a flow rate of 1 mL/min, first with 50 mL of buffer A and then with a linear gradient (500 mL) of NaCl (0–500 mM) in buffer A. Fractions (10 mL) were collected and assayed for glutamate racemase activity. The most active fractions, which were eluted at 150 mM NaCl, were pooled (15 000 units, 10 mg of protein), and the buffer was exchanged with buffer B (2 mM potassium phosphate buffer, pH 7.4, containing the same additives as in buffer A) by repeated concentration and dilution on PM10 membranes (Amicon, Beverly, MA). Then, this protein solution was concentrated to 2 mL and loaded onto a column (10- × 2.6-cm) of hydroxylapatite-Ultrogel (IBF) equilibrated with buffer B. The elution was run at a flow rate of 1 mL/min, first with 60 mL of buffer B and then with a linear gradient (400 mL) of potassium phosphate (2–250 mM). Fractions (10 mL) were collected and assayed for glutamate racemase activity. The most active fractions, which were eluted at 90 mM potassium phosphate, were pooled (9500 units 2.5 mg of protein), concentrated as above to 1 mL, and dialyzed against buffer C (buffer A with 100 mM potassium phosphate). This protein solution was loaded onto a column (59- × 1-cm) of Ultrogel-AcA44 equilibrated with buffer C, and the elution was run at a flow rate of 0.1 mL/min. Fractions (1 mL) containing pure glutamate racemase (4000 units 1.6 mg of protein) were pooled and stored at –20 °C. The molecular mass of the purified racemase was determined by FPLC on a calibrated column of Superose 12 HR 10/30 (Pharmacia), as described previously (Michaud et al., 1987).

RESULTS AND DISCUSSION

Overproduction and Purification of E. coli Glutamate Racemase. To obtain a high overproduction of glutamate racemase, plasmid pTACI5 carrying the *murI* gene expressed under the control of a *tac* promoter was constructed (Figure 2). As compared to the previous pTACI2 construct (Doublet et al., 1993), it offered several advantages: a higher copy number, the presence of the $lacI^q$ gene on the vector to more efficiently repress the expression of the *murI* gene in the absence of inducer, and the deletion of the whole sequence downstream from *murI* in the chromosomal insert. The 200-bp distal region contained the strong promoters of the *rrnB* operon whose cloning into multicopy vectors was not tolerated by cells and often resulted in plasmid rearrangements (Doublet et al., 1993; Erdei et al., 1983). Upon induction with 1 mM IPTG, a high level of the 31 500-Da MurI protein was produced in cells carrying the pTACI5 plasmid (Figure 3). This induction was accompanied by a decrease in the bacterial growth rate, but this was most probably a nonspecific effect of the high rate of synthesis of an unnecessary protein, which finally accounts for 10–15% of the total soluble cell proteins. This overproduction allowed the purification of the glutamate racemase in three simple chromatographic steps to apparent

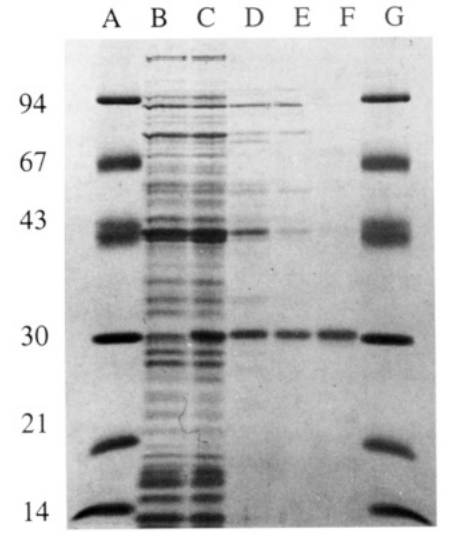

FIGURE 3: Overproduction and purification of *E. coli* glutamate racemase. Crude protein extracts were prepared from exponential-phase cells of JM83 (pTACI5) grown in LB medium for 6 h in the absence or presence of 1 mM IPTG. As described in Materials and Methods, soluble fractions obtained after high-speed centrifugation of the corresponding extracts were analyzed by SDS–PAGE. Molecular weight standards (thousands) indicated on the left are as follows: phosphorylase *b* (94), bovine serum albumin (67), ovalbumin (43), carbonic anhydrase (30), and soybean trypsin inhibitor (21). Lanes: A and G, molecular weight standards; B and C, crude soluble fractions from JM83 (pTACI5) cells grown without or with IPTG, respectively; and D–F, purification of the glutamate racemase after chromatography on DEAE-Trisacryl (lane D), hydroxylapatite-Ultrogel (lane E), and Ultrogel-AcA44 (lane F), respectively.

electrophoretic homogeneity (Figure 3). Starting from 80 mg of total soluble proteins from the MurI overproducing strain, 1.6 mg of pure glutamate racemase was obtained with an overall yield of 7% and a purification factor of 4. When compared with the specific activity of the crude extract from a nonoverproducing strain (Doublet et al., 1993), a purification factor of 920 was estimated. On the basis of its elution volume from a size-exclusion volumn (as compared with standards of known size), the molecular mass of the native racemase was estimated to be approximately 30 000 Da. The active enzyme was therefore believed to be a monomer.

Kinetic Properties of the Purified Glutamate Racemase. The overproduced glutamate racemase appeared to be very labile at every stage of its purification from crude extracts. However, the addition of 10% glycerol, 1 mM DL-glutamic acid, and 1 mM of a reducing thiol to standard buffers efficiently stabilized the enzyme which could be stored in these conditions for a few months at –20 °C without an important loss of activity. This instability was also obvious after 2 h of preincubation at 37 °C, where samples that lacked these additives completely lost the initial activity, whereas samples containing them retained more than 80% of it. Furthermore, as previously observed for the glutamate racemase from other bacterial species (Nakajima et al., 1988; Gallo & Knowles, 1993), the enzyme was completely inactivated in less than 5 min by the thiol-alkylating agent 5,5′-dithiobis(2-nitrobenzoic acid) at 2 mM. These results were consistent with the presence of essential cysteinyl residue(s) in or near the active site of the protein, which may play a catalytic role (proton-abstracting base) in the racemization reaction. The reaction catalyzed by the glutamate racemase from *Lactobacillus fermenti* was recently investigated in detail by Knowles and co-workers (Gallo & Knowles, 1993; Gallo et al., 1993; Tanner et al., 1993). A two-base

mechanism was identified, involving two cysteines as essential catalytic residues at positions 73 and 184 in the 268 amino acid polypeptide (Gallo & Knowles, 1993; Tanner et al., 1993). It was noteworthy that the *E. coli* and *Lactobacillus* gene sequences were 30% identical and that these two cysteine residues and their surrounding regions were particularly well conserved in both species (Gallo & Knowles, 1993). It could thus be assumed that the *E. coli* enzyme also used a two-base mechanism involving cysteines at positions 96 and 208 in the corresponding amino acid sequence. This hypothesis must be confirmed by directed-mutagenesis experiments.

The steady-state kinetic parameters for *E. coli* glutamate racemase were determined in both directions, using identical and optimal assay conditions at 37 °C and pH 7.8. As predicted from the Haldane relationship (Briggs & Haldane, 1925), the chemically symmetric reaction L-glutamate ↔ D-glutamate should give an equilibrium constant of unity, as has been validated with other amino acid racemases (Nakajima et al., 1988; Gallo & Knowles, 1993; Wasserman et al., 1984). The following values were calculated from double-reciprocal plots: for L-glutamate, K_m = 4 mM and V_{max} = 5.9 μmol/min/mg (k_{cat} = 187 min^{-1}); for D-glutamate, K_m = 14 mM and V_{max} = 18.7 μmol/min/mg (k_{cat} = 590 min^{-1}). The K_{eq} of the glutamate racemase reaction calculated from the derived V_{max}/K_m values was 1.1, in close agreement with the theoretical value. These kinetic constants differed substantially from those published for the glutamate racemase from other bacterial species: Gallo and Knowles (1993) reported for the *Lactobacillus* enzyme K_m values for L- and D-glutamic acid of 0.24 and 0.26 mM, respectively, and for the *Pediococcus* enzyme, Nakajima et al. (1988) estimated these constants to 14 and 10 mM, respectively. The k_{cat} values estimated in the present work are 2–5-fold lower than the corresponding values determined with the other glutamate racemase species (Nakajima et al., 1988; Gallo & Knowles, 1993).

Activation by UDP-MurNAc-L-Ala. To detect the glutamate racemase activity in crude extracts and at every stage of its purification, we initially used a coupled (L → D) assay in which the D-glutamic acid produced from the L-isomer was converted to UDP-MurNAc-L-Ala-D-Glu in the presence of UDP-MurNAc-L-Ala, ATP-Mg^{2+}, and purified MurD activity. Surprisingly, when either of the two other assays was used (direct assay or D → L coupled assay, see Materials and Methods), no activity could be detected unless UDP-MurNAc-L-Ala was present, even with the highly active purified enzyme (Figure 4). This peptidoglycan precursor thus appeared to be an essential activator of the glutamate racemase activity. Its presence was required in both L → D and D → L directions, according to similar hyperbolic saturation curves from which a very low K_D values of 4 μM was calculated (data not shown). Consumption of UDP-MurNAc-L-Ala molecules in the course of a racemase assay (by adding purified MurD activity and ATP-Mg^{2+}) immediately abolished the activity of the glutamate racemase (Figure 4), indicating that the activated state of the enzyme was not maintained even for a short time upon removal of the activator from its binding site.

The UDP-MurNAc-L-Ala effect was highly specific, since none of the six other peptidoglycan nucleotide precursors from UDP-GlcNAc to UDP-MurNAc-pentapeptide was an effector when tested at 1 mM (Figure 4). The effect of some modifications of the UDP-MurNAc-L-Ala structure on the activating effect was also investigated. 1-Phospho-MurNAc-L-Ala and MurNAc-L-Ala derivatives obtained by enzymatic hydrolysis and chemical synthesis, respectively (Michaud et al., 1987), appeared totally inactive at 0.1 mM. The dihydro derivative obtained by reduction of the uracyl moiety (Michaud et al., 1987) was a very weak activator at 0.1 mM (only 6% of the enzyme activity observed with UDP-MurNAc-L-Ala at a 10-fold lower concentration) (Figure 4). These results clearly indicated that all parts of the UDP-MurNAc-L-Ala structure might be implicated in its binding to the enzyme. The different precursors and analogues were also tested as inhibitors of the racemase activity, using the D → L coupled assay and a nonsaturating concentration (0.5 μM) of activator UDP-MurNAc-L-Ala. UDP-MurNAc, UDP-MurNAc-L-Ala-D-Glu, and UDP-MurNAc-pentapeptide were shown to inhibit the enzyme activity by 45%, 75%, and 60%, respectively, when added at 0.2 mM. At the same concentration, UDP, MurNAc-L-Ala, and 1-phospho-MurNAc-L-Ala also inhibited the enzyme by 60%, 25%, and 85%, respectively. These results indicated that these various analogues had some affinity for the activator-binding site of the enzyme. As expected for a competitive process, the inhibitory effect of these compounds was completely abolished when using a 50 μM saturating concentration of UDP-MurNAc-L-Ala.

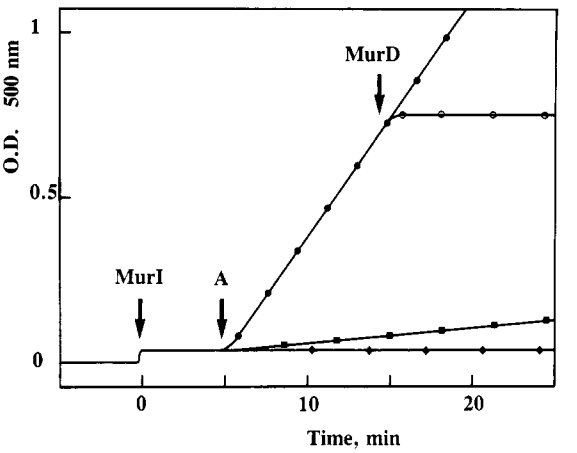

FIGURE 4: Activation of glutamate racemase by UDP-MurNAc-L-Ala. The activity was followed using the continuous D → L coupled assay. The initial addition (t = 0) of glutamate racemase (MurI) to the reaction mixture from which UDP-MurNAc-L-Ala was omitted only provided a slight increase in the OD at 500 nm which corresponds to the consumption of the L-glutamic acid already present in the enzyme dilution buffer. The activator (A) UDP-MurNAc-L-Ala or one of its structural analogues was added 5 min later, at a 0.1 mM concentration: (●) + UDP-MurNAc-L-Ala, (■) + dihydro-UDP-MurNAc-L-Ala, (♦) + either phospho-MurNAc-L-Ala, MurNAc-L-Ala, or any one of the other nucleotide precursors from UDP-GlcNAc to UDP-MurNAc-pentapeptide, and (○) addition (at t = 15 min) of a mixture of pure D-glutamic acid-adding enzyme (MurD) and ATP-Mg^{2+}, in the course of an assay with UDP-MurNAc-L-Ala.

Physiological Role of the Activation by UDP-MurNAc-L-Ala. In vitro, the glutamate racemase from *E. coli* showed an absolute requirement for UDP-MurNAc-L-Ala to catalyze the interconversion of glutamate isomers. As little as an 8 μM concentration of this nucleotide precursor was enough for the complete activation of the enzyme. It was previously shown that the pool level of UDP-MurNAc-L-Ala in exponentially growing cells of *E. coli* was ca. 10 μM (Mengin-Lecreulx et al., 1982; Doublet et al., 1992). The fact that physiological concentrations of this precursor could activate the enzyme *in vitro* supported our proposal that UDP-MurNAc-L-Ala modulates *in vivo* the glutamate racemase activity and adjusts the rate of D-glutamic acid synthesis to the peptidoglycan requirements.

An interesting feature is that this specific requirement had not been previously observed with the purified enzymes from

Lactobacillus and *Pediococcus* species (Gallo & Knowles, 1993; Choi et al., 1991). It was essential to know whether the activities detected in the latter bacterial species corresponded to basal levels that were in fact also sensitive to activation by UDP-MurNAc-L-Ala or if they corresponded to UDP-MurNAc-L-Ala-insensitive activities. A sample of purified glutamate racemase from *L. fermenti*, kindly provided by M. Tanner (Tanner et al., 1993), was assayed in that way and did not exhibit any specific requirement. It was quite surprising that the *Lactobacillus* and *E. coli* enzymes were not regulated in a similar manner, considering that their amino acid sequences were 30% identical. However, it should be mentioned that the *E. coli* racemase is larger than the *Lactobacillus* enzyme (289 amino acids versus 268) and that this difference mainly consists in a 22 amino acid extension of the N-terminal extremity in the *E. coli* racemase. It is tempting to imagine that this N-terminal extension could be involved in the specific binding of UDP-MurNAc-L-Ala to the enzyme and in the regulation here described. Construction of appropriate racemase mutants is now required to bring an answer to this problem.

To the best of our knowledge, the activation of *E. coli* glutamate racemase by UDP-MurNAc-L-Ala provides the first example of a regulated amino acid racemase activity in bacteria. The reason for such a tight regulation of D-glutamic acid synthesis in *E. coli* is unclear. L-Glutamic acid is one of the most abundant amino acids in the cell content of *E. coli*, which is used in many different metabolic pathways and for protein synthesis. It was shown that its pool level was greatly influenced by osmotic conditions, increasing up to 10-fold in growth media of high ionic strength (Measures, 1975). As compared, the D-isomer has a very minor cellular utilization since it accounts for 13% in mass of the peptidoglycan structure, a macromolecule which in turns represents only 1% of the bacterial dry weight (Mengin-Lecreulx & van Heijenoort, 1985). The regulation of the glutamate racemase activity thus avoids an excessive racemization of this large pool of L-glutamic acid and is consistent with the previous observation that the pool of free glutamic acid mainly consists (at more than 95%) of the L-isomer (Mengin-Lecreulx et al., 1982; Doublet et al., 1993). A significant increase in the pool of D-glutamic acid was previously observed when the glutamate racemase was overproduced to very high levels in *E. coli* cells, but even in these conditions, the L-isomer was largely predominant (Doublet et al., 1993). Both the UDP-MurNAc-L-Ala requirement and the low abundance of glutamate racemase in the cell content of *E. coli* (Doublet et al., 1993; Baliko et al., 1988) should be considered as factors limiting the wasteful production of D-glutamic acid in this bacterial species.

An eventual toxicity of D-glutamic acid on cell metabolism could also support the necessity for such a regulatory mechanism. Certain D-amino acids are known to inhibit bacterial growth and to interfere with late steps of peptidoglycan synthesis when added at a high concentration to the culture medium (Caparros et al., 1992). Owing to the poor uptake of D-glutamic acid by *E. coli* cells which is mediated by permeases that are specific for the L-isomer (Deguchi et al., 1989), the latter hypothesis was not further investigated. Venetianer and co-workers (Balikó et al., 1988; Balikó & Venetianer, 1993) also previously attempted to learn the function of the *murI* gene. They observed that inactivating or overexpressing this gene in growing cells resulted in aberrations in nucleoid separation during cell division as well as decreased negative superhelicity of reporter plasmids (Balikó & Venetianer, 1993). How a perturbation of D-glutamic acid biosynthesis could result in such a phenotype is yet unclear, but it could also explain the necessity of a tight regulation of the glutamate racemase activity.

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