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Overexpression, Purification, and Characterization of UDP-*N*-Acetylmuramyl:L-Alanine Ligase from *Escherichia coli*

MARCEL GUBLER,* YVONNE APPOLDT, AND WOLFGANG KECK

Pharmaceutical Research, Department of Infectious Diseases, F. Hoffmann-La Roche AG, CH-4002 Basel, Switzerland

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UDP-*N*-acetylmuramyl:L-alanine ligase from *Escherichia coli* was overexpressed more than 600-fold and purified to near homogeneity. The purified enzyme was found to ligate L-alanine, L-serine, and glycine, as well as the nonnatural amino acid β-chloro-L-alanine, to UDP-*N*-acetylmuramic acid. On the basis of (i) the specificity constants of the enzyme determined for L-alanine, L-serine, and glycine and (ii) the levels of these amino acids in the intracellular pool, it was calculated that the rates of incorporation of L-serine and glycine into peptidoglycan precursor metabolites could maximally amount to 0.1 and 0.5%, respectively, of the rate of L-alanine incorporation.

The pathway for the biosynthesis of peptidoglycan precursor metabolites involves (i) reactions for the synthesis of UDP-activated amino sugars and (ii) reactions for the synthesis of the peptide moiety of UDP-*N*-acetylmuramyl-pentapeptide (2). Four nonribosomal amino acid ligases catalyze the sequential addition of L-alanine, D-glutamate, *meso*-diaminopimelic acid (mDAP) or L-lysine (in gram-positive cocci), and D-alanyl-D-alanine dipeptide to UDP-*N*-acetylmuramic acid (UDP-MurNAc). The amino acid-adding enzymes have been characterized in cell extracts or in partially purified form from a wide variety of bacterial species (3, 7, 10, 15–18, 20). Their ubiquitous occurrence indicates that all bacteria synthesizing peptidoglycan cell walls possess this set of four amino acid-adding enzymes.

This work reports the overexpression, purification, and substrate specificity of UDP-MurNAc:L-alanine ligase from *Escherichia coli*. Similar work on the same enzyme was published (14) while this paper was being written.

UDP-MurNAc:L-alanine ligase catalyzes the addition of the first amino acid to the lactyl group of UDP-MurNAc as follows: UDP-MurNAc + L-alanine + ATP→UDP-MurNAc-L-alanine + ADP + P_i. The excellent stability of the enzyme preparation and its high specific activity, in conjunction with the use of a coupled spectrophotometric assay, allowed the determination of the kinetic constants K_m and V_{max} of the L-alanine-adding enzyme in the presence of different amino acid substrates. Investigation of the kinetic properties of the enzyme elucidated why biosynthesis of peptidoglycan precursor metabolites in *E. coli* is highly specific in spite of the fact that UDP-MurNAc:L-alanine ligase accepts L-serine and glycine, besides L-alanine, as substrates.

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Overexpression and purification of UDP-MurNAc:L-alanine ligase. The *murC* gene from *E. coli*, which codes for UDP-MurNAc:L-alanine ligase, was amplified by PCR and cloned in the expression vector pTrc99A (1). Phage lambda clone 110 from the Kohara library of *E. coli* W3110 (11) served as the template for the PCR amplification; the oligonucleotides murC1 (5′-CGCATGCCATGGATACACAACAATTGGCA-3′) and murC2 (5′-GCTCTAGAGTCGACAGGATACGC GTC-3′) were used as primers. The underlined nucleotides in the oligonucleotides murC1 and murC2 represent cleavage sites for *Nco*I and *Sal*I, respectively. The PCR-amplified 1.6-kb DNA fragment was ligated to pTrc99A after digestion of both vector and insert DNA with *Nco*I and *Sal*I. Introduction by PCR of the *Nco*I cloning site at the 5′ end of the *murC* coding region resulted in the substitution of aspartic acid for asparagine at position 2 of the MurC polypeptide when expressed from the final plasmid construct pRGM106 (Fig. 1).

For the purification of UDP-MurNAc:L-alanine ligase, *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.) harboring pRGM106 was grown in Luria-Bertani broth in the presence of ampicillin (100 μg/ml) at 37°C. Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM when the cell density (A_{600}) of the culture reached 0.8. After an additional 3 h of cultivation, the cells were harvested. The cell extract prepared by sonication and ultracentrifugation (100,000 × *g*) exhibited UDP-MurNAc:L-alanine ligase activity of 0.76 μmol min^{-1} mg^{-1} as determined by an assay based on thin-layer chromatography (TLC) (see below). This represents more than a 600-fold increase in activity over that measured with extracts from *E. coli* W3110 wild-type cells.

UDP-MurNAc:L-alanine ligase was purified from an extract containing 335 mg of soluble protein in buffer A (50 mM Tris-HCl, pH 7.5; 20 mM KCl; 1 mM dithiothreitol [DTT]; 0.1 mM EDTA). The 4-step purification procedure included (i) precipitation of nucleic acids in the cell extract by protamine sulfate (final concentration, 0.2%), (ii) precipitation of UDP-MurNAc:L-alanine ligase by $(NH_4)_2SO_4$ (final concentration, 40%), (iii) dye-ligand chromatography on Matrex Gel Green A, and (iv) dye-ligand chromatography on Matrex Gel Red A (both kinds of Matrex Gel from Amicon, Beverly, Mass.). The enzyme was eluted with gradients of 20 mM to 2 M KCl in buffer A. Fractions containing enzymatic activity were pooled, dialyzed against buffer A, and concentrated in a stirred cell by using a YM30 membrane (30,000-M_r cutoff) from Amicon. The

* Corresponding author. Mailing address: F. Hoffmann-La Roche AG, Room 70/103, CH-4002 Basel, Switzerland. Phone: 41-61-6873515. Fax: 41-61-6882729. Electronic mail address: marcel.gubler@roche.com.

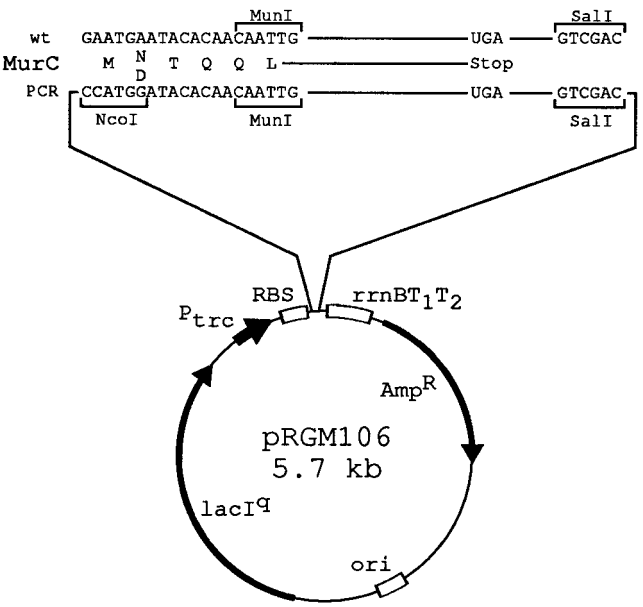

FIG. 1. Map of the UDP-MurNAc:L-alanine ligase expression plasmid pRGM106. The wild-type (wt) and the PCR-amplified (PCR) nucleotide sequences of the 5′ end of the *murC* gene are aligned to show the mutations generated by the cloning procedure. As a consequence, the MurC protein, when expressed from pRGM106, has a substitution of aspartic acid for asparagine at position 2 of its amino acid sequence. P_{trc}, *trc* promoter; RBS, ribosome binding site; $rrnBT_1T_2$, transcriptional terminators of the *rrnB* operon from *E. coli*.

final yield of purified enzyme was 28.6 mg, and the specific activity was 3.7 μmol min^{-1} mg^{-1}. In subsequent experiments, after determination of optimal reaction conditions, a higher specific activity of 7.67 μmol min^{-1} mg^{-1} was measured for UDP-MurNAc:L-alanine ligase. This represents a 4.9-fold purification of the enzyme with a yield of 42%. In comparison with the specific activity of the cell extract from the nonoverproducing strain W3110, an almost 3,000-fold purification was achieved. Figure 2 documents the efficiency of the individual purification steps and shows that the final enzyme preparation is essentially free of contaminating *E. coli* proteins.

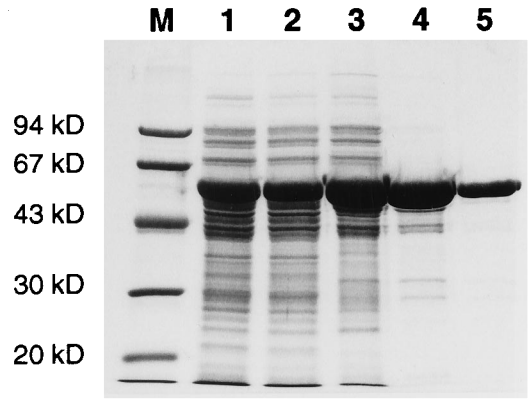

FIG. 2. Denaturing sodium dodecyl sulfate-polyacrylamide gel analysis of UDP-MurNAc:L-alanine ligase at different stages of purification from *E. coli* XL1-Blue(pRGM106). After electrophoresis through a 12% acrylamide gel, the proteins were stained with Coomassie brilliant blue. Lanes: M, molecular mass markers; 1, crude cell extract (100,000 × *g* supernatant); 2, supernatant after protamine sulfate precipitation; 3, proteins precipitating at 40% $(NH_4)_2SO_4$; 4, pool of fractions after dye-ligand Matrex Gel Green A affinity chromatography; 5, pool of fractions after dye-ligand Matrex Gel Red A affinity chromatography.

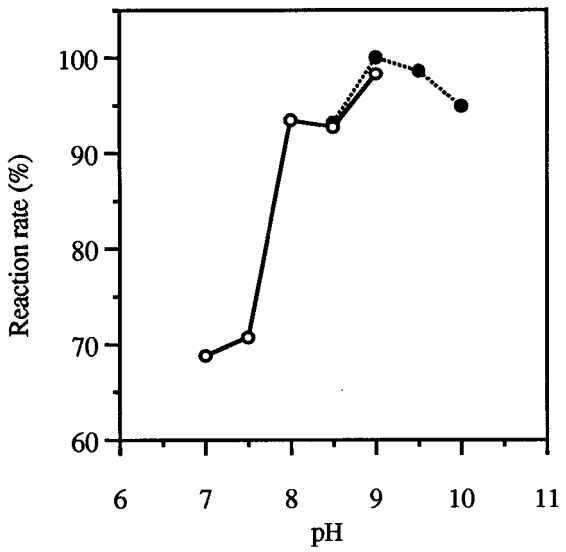

FIG. 3. Dependence of UDP-MurNAc:L-alanine ligase activity on pH. Reaction rates were determined with L-alanine as the amino acid substrate. Open circles represent reaction rates determined in Tris-HCl buffer (pH 7.0 to 9.0), and filled circles represent reaction rates determined in Bicine-NaOH buffer (pH 8.5 to 10.0).

Determination of optimal reaction conditions for UDP-MurNAc:L-alanine ligase. Enzyme activity was measured by TLC analysis of ^{14}C-labelled reaction products. The standard reaction was measured (at 37°C) in 20-μl reaction mixtures containing 50 mM Tris-HCl (pH 8.5), 20 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 1 mM DTT, 5 mM ATP, 1 mM UDP-MurNAc (prepared as described in references 5 and 9), 1 mM L-[^{14}C]alanine (9.26 mCi/mmol), and enzyme. The quantity of enzyme was adjusted so that less than 50% of the substrates were consumed in 5 min at 37°C. Reactions were terminated by the addition of 5 μl of glacial acetic acid, and 1-μl aliquots were spotted onto cellulose TLC plates (Merck, Darmstadt, Germany). After development in ethanol–1 M ammonium acetate (pH 3.8) (5:2), radioactive spots were visualized on a Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.) and quantified by using ImageQuant software from Molecular Dynamics.

Dependence of the enzyme activity on pH was determined from pH 7.0 to pH 9.0 in Tris-HCl buffer and from pH 8.5 to pH 10.0 in Bicine-NaOH buffer. The enzyme was found to be most active at alkaline pH between 8.0 and 10.0 with an optimum at pH 9.0, as shown in Fig. 3.

Either Mg^{2+} or Mn^{2+} was found to be essential for UDP-MurNAc:L-alanine ligase activity. For $MgCl_2$ the activity was maximal at 20 mM, while for $MnCl_2$ it was maximal at 5 mM, though reaching only 50% of the activity measured in the presence of 20 mM $MgCl_2$. $MgCl_2$ and $MnCl_2$ at concentrations above 20 and 5 mM, respectively, reduced the activity of the enzyme.

$(NH_4)_2SO_4$ at concentrations between 5 and 25 mM stimulated the activity of UDP-MurNAc:L-alanine ligase by 15%. NaCl and KCl below 100 mM had no effect on enzyme activity, while higher concentrations inhibited the enzyme.

Reverse reaction. By using enzymatically synthesized UDP-MurNAc-L-[^{14}C]alanine as the substrate (prepared as described in reference 19), it was found that UDP-MurNAc:L-

TABLE 1. Kinetic constants of UDP-MurNAc:L-alanine ligase for UDP-MurNAc, ATP, and different amino acids used as substrates

Substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
UDP-MurNAc[a]	0.058	—[b]	—	—
ATP[a]	0.092	—	—	—
L-Alanine	0.087	7.67	6.85	78.7
L-Serine	1.99	12.76	11.40	5.7
Glycine	7.89	13.08	11.68	1.5
β-Chloro-L-alanine	0.173	12.42	11.10	64.1

[a] Determined with L-alanine as the amino acid substrate.
[b] —, value correlates with that given for L-alanine.

alanine ligase also catalyzes the reverse reaction, i.e., the hydrolysis of UDP-MurNAc-L-alanine. The reaction was assayed (at 37°C) in 10-μl reaction mixtures containing 50 mM Tris-HCl (pH 8.5), 20 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 1 mM DTT, 2 mM ADP, 10 mM Na_2HPO_4, 0.7 mM UDP-MurNAc-L-[^{14}C]alanine, and 1.36 μg of purified UDP-MurNAc:L-alanine ligase. Reaction products were analyzed by TLC and quantified as was done for the forward reaction. The rate of the reverse reaction was 0.048 μmol min^{-1} mg^{-1} at a substrate concentration of 0.7 mM, which represents less than 1% of the rate of the forward reaction at identical substrate concentrations.

Inhibition of UDP-MurNAc:L-alanine ligase. The TLC-based method was also used to assay for inhibitors of UDP-MurNAc:L-alanine ligase. All inhibitors were tested at a concentration of 1 mM. Feedback inhibition was investigated by assaying UDP-MurNAc:L-alanine ligase activity in the presence of three different UDP-MurNAc peptides which are precursor metabolites further downstream in the pathway of peptidoglycan biosynthesis. Only UDP-MurNAc-L-alanine, the immediate reaction product of the L-alanine-adding enzyme, inhibited the ligation of L-alanine to UDP-MurNAc at a high level (29.8% inhibition). The metabolites UDP-MurNAc-tripeptide and UDP-MurNAc-pentapeptide (prepared from *Bacillus cereus* T as described in reference 12) only marginally inhibited the ligation reaction (7 and 2% inhibition, respectively). L-Serine and glycine at a concentration of 1 mM inhibited UDP-MurNAc:L-alanine ligase by 4.1 and 5.2%, respectively, while the stereoisomer D-alanine did not inhibit the ligation of L-alanine at all. Among the five nonnatural alanine analogs tested [aminomethylphosphonic acid, (*R*−)-aminoethylphosphonic acid, (*S*+)-aminoethylphosphonic acid, alanyl-L-1-aminoethylphosphonic acid, and β-chloro-L-alanine], only β-chloro-L-alanine inhibited ligation of L-alanine to UDP-MurNAc at a significant level (32.8% inhibition).

Substrate specificity and kinetic properties of UDP-MurNAc:L-alanine ligase. Initial experiments carried out with the TLC-based enzyme assay and ^{14}C-labelled L-alanine, L-serine, and glycine as substrates confirmed the finding of Liger et al. (13) that the enzyme ligates L-alanine, L-serine, and glycine to UDP-MurNAc with a high degree of efficiency. Subsequently, we used a coupled spectrophotometric assay which allowed the determination of ligase activities with nonradiolabelled amino acids or amino acid analogs as substrates. Consumption of ATP by UDP-MurNAc:L-alanine ligase was measured by coupling ADP formation to NADH consumption via the pyruvate kinase and lactate dehydrogenase reactions and monitoring the decrease in A_{340} (extinction coefficient of NADH, 6.22 mM^{-1} cm^{-1}). One-milliliter reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 1 mM DTT, 2 mM ATP, 2 mM phosphoenolpyruvate, 0.4 mM NADH, 0.6 mM UDP-MurNAc, 1 mM individual amino acid substrates, and 0.9 U of the coupling enzymes pyruvate kinase and lactate dehydrogenase. Assay mixtures were equilibrated at 37°C for 5 min before the reactions were started by the addition of 2.7 μg of purified UDP-MurNAc:L-alanine ligase.

At a fixed concentration of 1 mM, L-alanine, D-alanine, L-serine, glycine, and five different nonnatural alanine analogs were tested as substrates. The measured reaction rates (in micromoles per minute per milligram) were 6.62 for L-alanine, 0 for D-alanine, 4.86 for L-serine, and 1.75 for glycine. Of the nonnatural alanine analogs tested as inhibitors (see above), only β-chloro-L-alanine was used as a substrate. In fact, at a concentration of 1 mM it gave rise to ligase activity of 10.54 μmol min^{-1} mg^{-1}, which exceeded that measured with any other amino acid substrate. In order to investigate the phenomenon of promiscuous substrate specificity of UDP-MurNAc:L-alanine ligase in more detail, we determined the kinetic parameters K_m and V_{max} of the ligation reaction with respect to the different natural amino acids and β-chloro-L-alanine.

At first, however, the K_m values were determined for the indispensable substrates UDP-MurNAc and ATP in the presence of L-alanine. The K_m values for UDP-MurNAc and ATP were found to be 0.058 and 0.092 mM, respectively (Table 1). These K_m values are lower by factors of 2 and 4, respectively, than those obtained by Liger et al. (14). The discrepancy may be due to the fact that these authors used an end point assay while we used a continuous spectrophotometric assay that is more suitable for determining the rate of the UDP-MurNAc:L-alanine ligase reaction. Linearity of double-reciprocal plots of the kinetic data for both substrates indicated that UDP-MurNAc:L-alanine ligase obeys Michaelis-Menten kinetics (plots not shown). With UDP-MurNAc and ATP being kept at fixed, saturating concentrations, reaction rates were then determined for L-alanine, β-chloro-L-alanine, L-serine, and glycine over a concentration range from approximately $K_m/2$ to 10 times the K_m. The K_m values calculated from these data are 0.087 mM for L-alanine, 0.173 mM for β-chloro-L-alanine, 1.99 mM for L-serine, and 7.89 mM for glycine. The maximal reaction rates (V_{max} values) (in micromoles per minute per milligram) for the four different amino acid substrates are 7.67 for L-alanine, 12.42 for β-chloro-L-alanine, 12.76 for L-serine, and 13.08 for glycine. The kinetic parameters, including turnover numbers (catalytic constant [k_{cat}]) and specificity constants (k_{cat}/K_m), are given in Table 1. The K_m for L-alanine was found to be 23- and 91-fold lower than those for L-serine and glycine, respectively. On the other hand, UDP-MurNAc:L-alanine ligase reached maximal reaction rates with L-serine, glycine, or β-chloro-L-alanine as the substrate. These rates are almost double that reached with L-alanine. Graphic representation of reaction rates plotted against amino acid substrate concentrations and double-reciprocal Lineweaver-Burk plots of the same data are shown in Fig. 4.

While we have shown that L-serine and glycine (as well as β-chloro-L-alanine) are ligated to UDP-MurNAc at maximal reaction rates (V_{max}) that exceed the maximal reaction rate reached with the natural substrate L-alanine, investigations about the composition of *E. coli* peptidoglycan did not reveal the presence of, e.g., L-serine or glycine at position 1 of the peptide moiety of muramic acid residues (6). Taking into account (i) the different specificity constants (k_{cat}/K_m) of UDP-MurNAc:L-alanine ligase for L-alanine, L-serine, and glycine and (ii) the levels of these amino acids in the intracellular pool, it becomes clear why L-alanine rather than L-serine or glycine is incorporated into *E. coli* cell walls. Intracellular amino acid contents have been determined (21) in terms of micromoles of

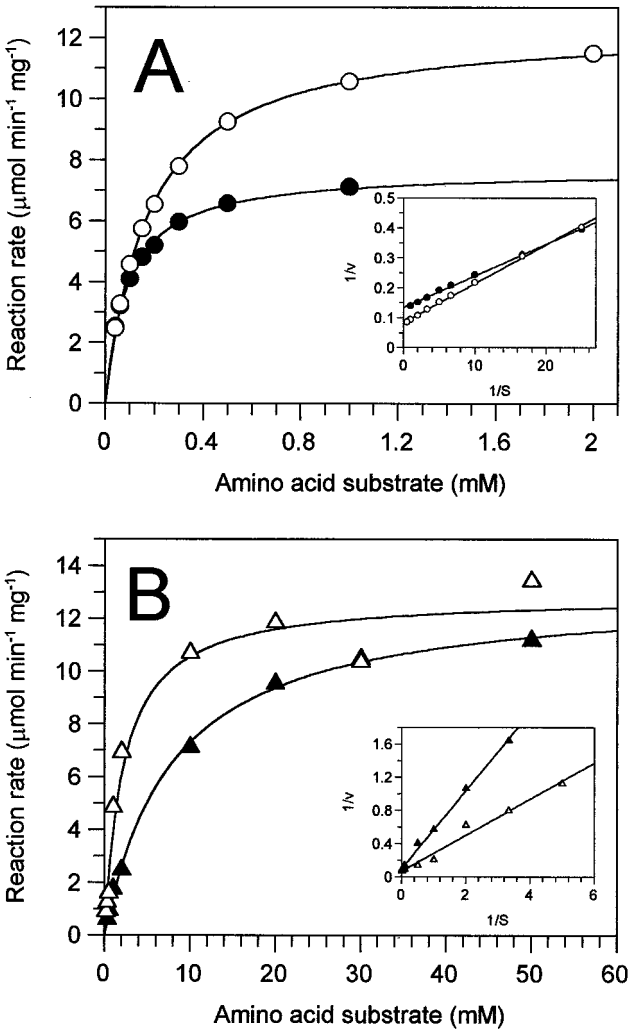

FIG. 4. Plots of UDP-MurNAc:L-alanine ligase reaction rates versus concentrations of the amino acid substrates L-alanine (filled circles) and β-chloro-L-alanine (open circles) (A) and L-serine (open triangles) and glycine (filled triangles) (B). Insets represent double-reciprocal plots of the same data; weights are the fourth powers of the average raw data. The plots were generated by using the nonlinear curve-fitting program GraFit from Erithacus Software Ltd. (Staines, United Kingdom). The kinetic constants K_m and V_{max} were estimated by the least-squares method to fit the Michaelis-Menten equation $V = V_{max} \cdot [S]/K_m + [S]$ by using the same program (S is substrate).

amino acid per gram (dry weight) of *E. coli* cells grown in minimal medium. Assuming a dry weight of 289 mg/liter of culture containing 3.8×10^{11} bacteria and a cell water content of 1.5×10^{-12} ml (15), the amino acid contents translate into intracellular concentrations of 3.4 mM for L-alanine, below 0.05 mM for L-serine, and 0.86 mM for glycine. Because the specificity of an enzyme, i.e., the discrimination between two competing substrates, is determined by the ratios of the specificity constants of the enzyme (k_{cat}/K_m) for the two substrates, the ratio of the reaction rates at which the enzyme consumes two competing substrates can be expressed as follows (4):

$$\frac{V_A}{V_B} = \frac{(k_{cat}/K_m)_A[A]}{(k_{cat}/K_m)_B[B]} \quad (1)$$

where V_A is the reaction rate at a given concentration [A] of substrate A and $(k_{cat}/K_m)_A$ is the specificity constant of the enzyme for substrate A. Substitution in equation 1 of the specificity constants for L-alanine and L-serine and the intracellular pool L-alanine and L-serine levels results in the following:

$$\frac{V_{\text{L-alanine}}}{V_{\text{L-serine}}} = \frac{78.7\ \text{s}^{-1}\ \text{mM}^{-1} \times 3.4\ \text{mM}}{5.7\ \text{s}^{-1}\ \text{mM}^{-1} \times 0.05\ \text{mM}} = \frac{934}{1}$$

This means that the rate at which L-serine is ligated to UDP-MurNAc accounts for about 0.1% of the reaction rate at which L-alanine is ligated to UDP-MurNAc in the *E. coli* cytoplasm. Similarly, calculation of $V_{\text{L-alanine}}/V_{\text{glycine}}$ at physiological substrate concentrations results in a ratio of 207:1, i.e., the reaction rate of glycine incorporation is less than 0.5% that of L-alanine incorporation. Therefore, differential substrate specificity in combination with biased intracellular amino acid concentration ensures highly specific biosynthesis of the peptidoglycan precursor metabolites.

An additional barrier to prevent incorporation of the wrong amino acids into the final peptidoglycan cell wall may be provided by the enzyme adding D-glutamate to UDP-MurNAc-L-alanine. It may be possible that D-glutamate ligase does not accept UDP-MurNAc-L-serine or UDP-MurNAc-glycine as a substrate, thus preventing further elongation of false UDP-MurNAc-amino acid combinations. Similarly, the D-glutamate-adding enzyme may not utilize UDP-MurNAc-β-chloro-L-alanine as a substrate. β-Chloro-L-alanine is known to be an efficient inhibitor of peptidoglycan biosynthesis in *E. coli* (8). When ligated to UDP-MurNAc, β-chloro-L-alanine may block further elongation of the peptide moiety of this peptidoglycan precursor metabolite. Studies of the substrate specificity of UDP-MurNAc-L-alanine:D-glutamate ligase will be needed to elucidate this hypothesis.

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