Biochemical Evidence for the Formation of a Covalent Acyl-Phosphate Linkage between UDP-*N*-Acetylmuramate and ATP in the *Escherichia coli* UDP-*N*-Acetylmuramate:L-Alanine Ligase-Catalyzed Reaction†

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ABSTRACT: In the peptidoglycan biosynthesis pathway in *Escherichia coli*, UDP-*N*-acetylmuramate:L-alanine ligase (MurC) catalyzes the formation of UDP-*N*-acetylmuramyl-*L*-alanine. A peptide bond is formed in this reaction and an ATP molecule is hydrolyzed concomitantly to produce ADP and orthophosphate. A biochemical approach was devised to elucidate the role of ATP in this reaction. A fusion construct pMAL::*murC* was prepared and the maltose binding protein−UDP-*N*-acetylmuramyl:L-alanine ligase fusion protein was overproduced in *E. coli*/pMal::*murC* upon isopropyl β-thiogalactoside induction. The fusion protein was purified to ≥90% homogeneity by a single-step affinity chromatography. Subsequently, the ligase was released from the maltose binding protein by proteolytic cleavage and was purified to ≥95% homogeneity by an ion-exchange chromatographic step. The kinetic parameters of the regenerated ligase are comparable to those of the purified native enzyme. This ligase was used to investigate the role that ATP plays in the formation of UDP-*N*-acetylmuramyl-L-alanine. UDP-*N*-acetyl[^{18}O]muramate (with ^{18}O located at the carboxylate function only) was prepared by a combination of chemical and enzymatic processes and was used as the substrate of the ligase to probe the reaction mechanism. All reaction products were purified and subjected to liquid chromatographic−mass spectrometric analysis. A single [^{18}O]oxygen was transferred from UDP-*N*-acetyl[^{18}O]muramate to the orthophosphate produced in the reaction. No [^{18}O]oxygen was detected in the adenosine nucleotides recovered from the reaction. These results strongly suggest that this ligase-catalyzed peptide formation proceeds through an activated acyl-phosphate linkage during the reaction process. ATP therefore assists in the process of the peptide bond formation by donating its γ-phosphoryl group to activate the carboxyl group of UDP-*N*-acetylmuramic acid.

Peptidoglycan is an essential component of bacterial cell walls, critical to the maintenance of cell integrity. The peptidoglycan precursor, UDP-*N*-acetylmuramyl pentapeptide (Park, 1987), is constructed in the bacterial cytoplasm and subsequently exported through the cytoplasmic membrane. It is in the periplasmic space that the precursors are finally inserted into the existing murein network (Holtje & Schwarz, 1985; Koch, 1990), resulting in continued network expansion. The pentapeptide moiety of UDP-*N*-acetylmuramyl pentapeptide is synthesized by appending to the lactyl moiety of UDP-*N*-acetylmuramate (UDPNacMur)[1] a group of D- and L-amino acids in alternating steps. In *Escherichia coli*, this involves the sequential addition of L-alanine, D-glutamate, *meso*-DAP, and finally D-alanyl-D-alanine. Each of these reactions is catalyzed by a unique cellular enzyme and is driven by the concomitant hydrolysis of ATP to ADP and orthophosphate (Ito et al., 1966). The role (or roles) that ATP plays in these reactions, however, has not been elucidated to date.

The attachment of L-alanine to UDP-*N*-acetylmuramate has been demonstrated, in several bacterial species, to be catalyzed by UDP-*N*-acetylmuramate:L-alanine ligase (Ito et al., 1966; Hishinuma et al., 1971; Mizuno et al., 1973). Complete purification of this enzyme was first reported from a Gram-positive bacterium, *Staphylococcus aureus* (Mizuno et al., 1973). The gene that encodes this L-alanine adding enzyme in *E. coli* has also been identified, sequenced, and designated *murC* (Ikeda et al., 1990a,b). Due to the low level of MurC activity in *E. coli* cell lysates (Mengin-Lecreulx et al., 1982), only limited biochemical studies have been undertaken (Ishiguro, 1982; Liger et al., 1991). It was not until recently that the purification of the *E. coli* ligase from a ligase-overproducing *E. coli* strain was reported (Liger et al., 1995). In this report we describe alternative methods to overproduce and obtain purified *E. coli* L-alanine-adding enzyme, which allowed us to provide biochemical evidence indicating that the peptide bond formation in the MurC-catalyzed reaction proceeds via an acyl-phosphate covalent

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; ATP, adenosine 5′-triphosphate; β-Me, β-mercaptoethanol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-thiogalactoside; LC-MS analysis, liquid chromatographic−mass spectrometric analysis; MBP, maltose binding protein; MBP−MurC, maltose binding protein−UDP-*N*-acetylmuramate:L-alanine ligase fusion protein; MurC, UDP-*N*-acetylmuramate:L-alanine ligase; NADH, β-nicotinamide adenine dinucleotide, reduced form; ORF, open reading frame; PCR, polymerase chain reaction; PEP, phosphoenolpyruvate; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TE buffer, Tris−EDTA buffer; UDP, uridine 5′-diphosphate; UDPNacG, UDP-*N*-acetylglucosamine; UDPNacG-EP, UDP-*N*-acetylglucosamine enolpyruvate; UDPNacMur, UDP-*N*-acetylmuramate; UDPNacMur-L-Ala, UDP-*N*-acetylmuramyl-L-alanine.

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linkage between the carboxyl group of UDP-*N*-acetylmuramate and the γ-phosphoryl group of ATP.

EXPERIMENTAL PROCEDURES

Materials. Plasmid pUC::*murC* containing the ORF of *murC* gene was generously provided to us by Dr. T. Dougherty, Bristol-Myers Squibb Co. The pMALc2 fusion vector and factor Xa were purchased from New England Biolabs, Inc. Oligonucleotide primers for PCR were synthesized at Bristol-Myers Squibb Co. dNTPs were purchased from U.S. Biochemical Corp. Vent DNA polymerase and reaction buffer for PCR were purchased from New England Biolabs. Ligases and restriction enzymes were from Boehringer Mannheim. Molecular cloning protocols were based on those described by Sambrook et al. (1989). Protein assay reagents were from Bio-Rad. SDS−PAGE molecular mass standards were from Integrated Separation Systems. Polyacrylamide gels (12%) were from Novex Co. [^{18}O]H_2O (98%) was obtained from Isotec Inc. UDP-*N*-acetylglucosamine (UDPGNac), phosphoenolpyruvate (PEP), NADPH, ATP, pyruvate kinase, and lactate dehydrogenase were from Sigma Chemical Co.

Construction of pMALc2::murC. *MurC* DNA was PCR-amplified by using pUC18::*murC* plasmid DNA as template and the following synthetic oligonucleotide primers: 5′ATCTAG**TCTAGA**ATGAATACACAACAATTGGCA (N-terminus) and 5′ACGTACA**CTGCAG**TTATCAGTCATGTTGTTCTTCC (C-terminus). The N-terminal primer encoded an *Xba*I restriction site (boldface type) immediately before the *murC* start codon (underlined). The C-terminal primer included a *Pst*I restriction site (boldface type) just 3′ of the termination site of the *murC* gene. The PCR-amplified DNA product was subjected to agarose gel electrophoresis, excised, minced, and transferred into a CoStar SpinX filter containing TE buffer, pH 8.0. Following an overnight incubation at 4 °C, the DNA was separated by centrifugal filtration at 14 000 rpm for 4 min. The purified PCR product was concentrated, digested with *Xba*I and *Pst*I restriction enzymes, and ligated to pMALc2 which had been digested with the same enzymes. The resulting expression plasmid, pMALc2::*murC*, was confirmed by restriction digestion and DNA sequence analysis of the vector/insert junctions.

Overproduction and Purification of MBP−MurC Fusion Protein. The MBP−MurC fusion protein overproducing *E. coli* strain was prepared by transforming the *E. coli*/JM109 cells with the pMalc2::*MurC* fusion plasmid. The transformed cells were plated onto a LB agar plate containing 50 μg/mL ampicillin and incubated at 30 °C overnight. Colonies were selected to evaluate the expression level of the fusion protein. Overproduction of MBP−MurC was confirmed by SDS−PAGE analysis of cells grown up in 5-mL cultures at 37 °C, with and without IPTG induction.

MBP−MurC fusion protein was overproduced in the IPTG-induced *E. coli* JM109/pMalc2::*murC* cells. Two flasks of 500 mL of freshly prepared SOC medium, containing 50 μg/mL ampicillin, were inoculated each with a 5-mL overnight culture of *E. coli* JM109/pMALc2::*murC* (grown in TB medium with 50 μg/mL ampicillin added). The cultures were grown at 37 °C with constant aeration. When A_{600nm} of each culture reached 0.6, IPTG was added at a final concentration of 1 mM. The cultures were incubated for an additional 2 h to maximize protein expression. All the following steps were carried out at 4 °C. The cells were harvested by centrifugation at 4000*g* for 20 min. Cell pellets were combined and resuspended in 30 mL of lysis buffer (20 mM Tris-HCl, pH 8.0, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1 mM PMSF, 1 mM benzamidine, and 10 mg/mL AEBSF). Following sonication and centrifugation at 15000*g* for 30 min, the supernatant was diluted 3−4-fold with the lysis buffer. The fusion protein in the crude cell lysate was absorbed onto 12−14 mL of amylose resin by gently mixing the resin−lysate suspension for 1 h. Subsequently the resin was washed with 100 mL of column wash buffer (10 mM Tris-HCl, pH 7.4, 200 mM NaCl, 10 mM β-ME, 1 mM EDTA, 1 mM DTT, and protease inhibitors as above). MBP−MurC fusion protein was then eluted from the resin with 10 mM maltose in the same buffer.

*Preparation of UDP-N-Acetylmuramate:*L*-Alanine Ligase*. The affinity chromatography-purified MBP−MurC fusion protein was concentrated in a Centriprep 10 concentrator to a concentration of 7.5 mg/mL and then treated with factor Xa (w/w, 0.2% of fusion protein) at 4 °C. The regeneration of the ligase from the fusion protein was monitored by SDS−PAGE (12% polyacrylamide gel) analysis. After incubation at 4 °C for 5 days the fusion protein was completely cleaved to form MBP and free ligase. The enzyme was purified from the mixture by FPLC using a MonoQ HR 5/5 anion-exchange column. Elution of the enzyme from the column was achieved with a three-step Tris-HCl buffer/NaCl gradient: 0−5 mL (2.5 mM NaCl), 5−20 mL (2.5−500 mM NaCl), and 20−30 mL (500−1000 mM NaCl) at a flow rate of 1 mL/min. Fractions containing the ligase were pooled, concentrated, and stored at −20 °C with 20% glycerol and 1 mM β-ME added.

*Assay of UDP-N-Acetylmuramic Acid:-*L*-Alanine Ligase Activity*. An endpoint assay was developed and the amount of UDP-*N*-acetylmuramyl-L-alanine (UDPNacMur-L-Ala) formed in a reaction mixture was analyzed using a strong anion-exchange HPLC. The reactions were carried out at ambient temperature. In a 100-μL enzymic reaction mixture, the following components were included: 100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 2.5 mM β-ME, 1 mM UDPNacMur, 1 mM L-alanine, 2 mM ATP, and a predetermined amount of ligase. The reactions were stopped with 10 μL of 2 N HCl during the time of linear product formation. After removal of enzyme by centrifugal filtration with a Millipore Ultrafree MC 10 000 NMWL concentrator, the protein-free filtrate was analyzed by HPLC. HPLC analysis was carried out on a Waters HPLC equipped with a Phenomenex Optisil 10-μm SAX column (250 × 4.6 mm), two Model 590 pumps, and an on-line Waters 490E programmable multiwavelength detector. The column was eluted with a 150 mM potassium phosphate buffer, pH 3.5, at 1.5 mL/min. Elution of uridine- or adenine-containing entities was monitored by the absorbance at 262 nm. Under these conditions the retention times of UDPNacMur-L-Ala, UDPNacMur, and ADP were 4.7, 5.3, and 10.5 min, respectively. ATP was retained on the column until the column was washed with 1 M potassium phosphate, pH 3.5. The areas under the peaks of both the substrates and products were measured to calculate the enzyme activity.

Determination of the K_m and V_{max} values of UDPNacMur and L-alanine was carried out by varying the concentration of a single substrate while keeping all other ingredients at the same concentrations as mentioned above. Evaluation of the ligase inhibitors was carried out with the concentrations of UDPNacMur and L-alanine in the reaction mixture reduced

to 100 and 50 μM, respectively. Potential inhibitors of the ligase were added individually to the reaction mixtures at 10 mM final concentration.

Preparation of [^{18}O]Phosphoenolpyruvate. Exchange of the [^{18}O]oxygen from [^{18}O]H_2O into [^{16}O]PEP was carried out according to the procedure described by O'Neal et al. (1983) with minor modification. Eighty milligrams of PEP was dissolved in 0.75 mL of [^{18}O]H_2O in a 1.5-mL Eppendorf vial to which 95 μL of 12 N HCl in [^{18}O]H_2O was then added. The vial was sealed and heated in a boiling water bath for 5.5 min. After fast cooling of the solution in an ethanol−ice bath, the solvent was removed under vacuum in a SpeedVac vacuum concentrator. The residue was neutralized with 0.9 mL of 1 M Tris to a final pH of 8, diluted to 50 mL with H_2O, and loaded onto a 0.7- × 9-cm Dowex AG1-X4 column. The column was washed in the following order: 8 mL of H_2O, 40 mL of 30 mM HCl, and 24 mL of 50 mM HCl. [^{18}O]PEP was then eluted with 38 mL of 100 mM HCl. The fractions containing [^{18}O]PEP were pooled and dried overnight in a SpeedVac vacuum concentrator. The residue was redissolved in 1 mL of Millipore Milli Q system purified water and the pH was adjusted to 8 by adding KOH. The amount of [^{18}O]PEP obtained and its percent ^{18}O enrichment were determined by a coupled pyruvate kinase/lactate dehydrogenase assay and by LC-MS analysis, respectively.

Enzymatic Synthesis of UDP-N-Acetylglucosamine[^{18}O]-Enolpyruvate and UDP-N-Acetyl[^{18}O]muramate. UDP-NacG-[^{18}O]EP was prepared by incubating [^{18}O]PEP with UDP-*N*-acetylglucosamine in the presence of UDP-N-acetylglucosamine enolpyruvate transferase (MurZ) (Falk, 1994; Dhalla et al., 1995) at 37 °C. The reaction mixture (7 mL) contained 100 mM Tris-HCl, pH 7.5, 10 mM [^{18}O]PEP, 20 mM UDPNacG, 5 mM DTT, and 1 mg of MurZ. The progress of the reaction was monitored by HPLC analysis of 10-μL aliquots of the reaction mixture over time. Elution was carried out with a 75 mM potassium phosphate buffer, pH 3.5, at 1.5 mL/min and was monitored on-line at 262 nm. The retention times for UDPNacG, UDPNacG-EP, UDPNacMur, and UDP-*N*-acetylmuramyl-L-alanine (UDP-NacMur-L-Ala) were 8, 26, 20, and 18 min, respectively.

When the conversion of PEP to UDPNacG-EP was completed, NADPH (20 mM), KCl (20 mM), and MurB (0.4 mg/mL) (Benson et al., 1993; Falk, 1994) were added directly to the UDPNacG-[^{18}O]EP reaction solution to prepare UDPNac[^{18}O]Mur. The reaction mixture was incubated further at 37 °C and the formation of UDPNacMur was confirmed by HPLC analysis. UDPNac[^{18}O]Mur was purified by reverse-phase HPLC using a Bio-Rad HiPore RP 318 column (250 × 21.5 mm), eluting with 50 mM ammonium formate buffer (pH 5.0) at 5 mL/min. Eluted fractions containing UDPNac[^{18}O]Mur still contained some unreacted UDPNacG. These fractions were pooled and further purified using the same reverse-phase column, this time eluting with 50 mM ammonium formate at pH 3.5. Fractions containing UDPNac[^{18}O]Mur with >95% purity were pooled and dried on a SpeedVac vacuum concentrator. A small sample was subjected to LC-MS analysis to determine the percent [^{18}O]-oxygen content.

UDP-N-Acetylmuramate:L-Alanine Ligase-Catalyzed Reaction, Using UDPNac[^{18}O]Mur, L-Alanine, and ATP as the Substrates. The ligase-catalyzed reaction was carried out by incubating a reaction mixture which contained 50 mM Tris-HCl (pH 8.75), 4.5 mM ATP, 8 mM L-alanine, 2.5 mM

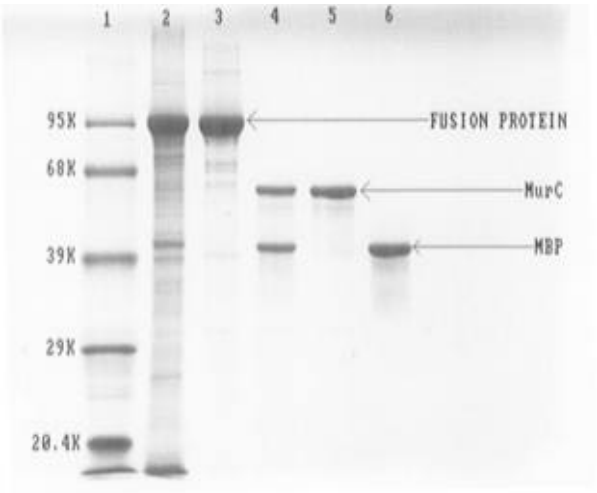


FIGURE 1: SDS−PAGE analysis of the purification of the MBP−MurC fusion protein and UDP-*N*-Acetylmuramate:L-alanine ligase from *E. coli*/pMAL::*murC*. Lane 1, molecular weight standards; lane 2, crude cell lysate; lane 3, MBP−MurC fusion protein after amylose resin affinity chromatography purification; lane 4, proteolytic cleavage of fusion protein with factor Xa; lane 5, purified UDP-*N*-acetylmuramate:L-alanine ligase; lane 6, maltose binding protein (MBP).

β-ME, 20 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 4 mM UDPNac-[^{18}O]Mur, and 0.2 mg of ligase at 37 °C overnight (Ho et al., 1995). Excess L-alanine was used to drive the reaction in the forward direction. At the completion of the reaction (verified by HPLC analysis), the reaction mixture was loaded onto a DEAE-cellulose column (2.5 × 60 cm) which was preequilibrated with 10 mM triethylammonium bicarbonate (TEAB) buffer, pH 7.8. The column was first washed with several column volumes of 50 mM TEAB and then eluted stepwise with increasing concentrations of TEAB (340 mL, 100 mM; 1 L, 125 mM; 660 mL, 175 mM; and 600 mL, 300 mM). Uridine and adenine nucleotide-containing fractions were identified by absorbance at 260 nm. Fractions containing orthophosphate were identified with malachite green reagent (Lanzetta et al., 1979). Fractions containing individual components were pooled accordingly. After removal of excess elution buffer, the identity of each product was verified by HPLC as described above.

LC-MS Analysis of Percent ^{18}O Enrichment in Individual Reaction Products. The percent [^{18}O]oxygen atom enrichment in the compounds of interest was characterized by full-scan ion-spray MS analysis, using a Sciex API III triple quadruple mass spectrometer equipped with an ion-spray interface operated in the negative-ion mode. The level of ^{18}O enrichment and number of [^{18}O]oxygen atoms incorporated for each compound was determined by examination of the ^{18}O isotope clusters present in the full-scan mass spectra.

RESULTS

Preparation and Characterization of the Ligase. A fusion plasmid containing the *malE−murC* fused gene was constructed by inserting the *murC* gene into the pMALc2 expression vector at the polylinker site, downstream of and in-frame with the *malE* gene. *E. coli* JM109 cells transformed with the fusion construct overproduced MBP−MurC fusion protein (MW ≈95 kDa) upon IPTG induction (Figure 1). From a 1-L culture 100−150 mg of fusion protein could be recovered after a single amylose resin affinity chromato-

Table 1: Purification of UDP-*N*-Acetylmuramate:L-Alanine Ligase from *E. coli*/pMAL::*murC*

purification steps	total protein (mg/L)	specific activity [nmol/(mg·min)]	yield (%)
crude extract	400	400	100
amylose affinity column	160	1100	110
Mono Q	80	2100	102

Table 2: Comparison of the Kinetic Parameters of the MBP−MurC Fusion Protein, the Regenerated Ligase, and the Native Ligase[a]

enzyme	K_m (μM)		
	UDPNacMur	L-Ala	ATP
MBP−MurC fusion protein	40	24	109
regenerated ligase	55	21	90
native ligase	39	24	140

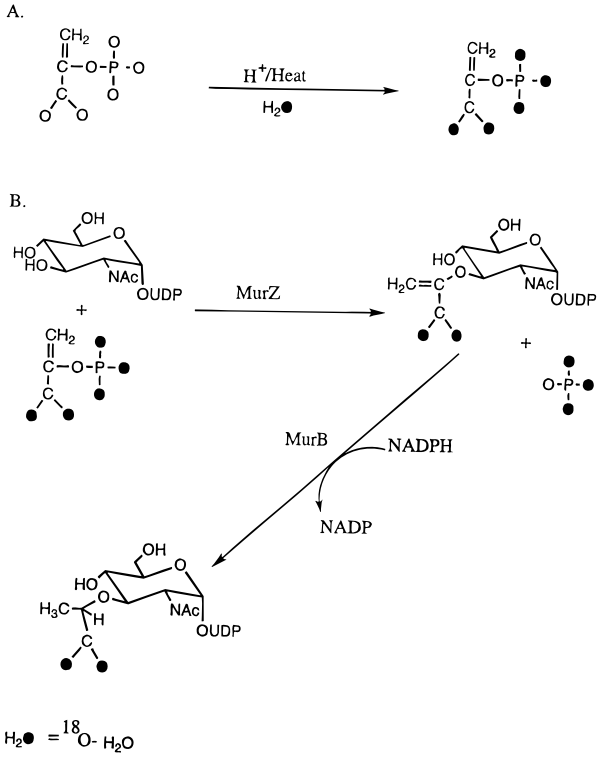
[a] The kinetic parameters of native MurC were determined using ligase partially purified from cell lysate of *E. coli*/pUC::*murC*.

graphic step. The fusion protein obtained this way was ≥90% pure and retained the L-alanine-adding enzyme activity. Proteolytic cleavage of the fusion protein by factor Xa resulted in the complete release of the intact ligase from MBP. Subsequent anion-exchange chromatography effectively resolved the two proteins. The ligase regenerated from the fusion protein was ≥95% homogeneous. SDS−PAGE analysis of the regenerated enzyme showed a single protein band with a molecular mass of 55 kDa (Figure 1). No loss of the specific activity was observed throughout the purification process. An overall yield of ≥80% has been routinely achieved (Table 1).

The kinetic properties of the ligase (regenerated from MBP−MurC fusion protein) were compared with that of the partially purified native ligase. Similar K_m values pertaining to the individual substrates were obtained for both the regenerated and the native enzymes (Table 2). In addition, the K_m and V_{max} values obtained for the MBP−MurC fusion protein, with regard to the L-alanine-adding enzyme activity, were comparable to those of the free ligase (Tables 1 and 2). Therefore the ligase domain in the fusion protein was properly folded and retained its L-alanine-adding enzyme characteristics. Size-exclusion HPLC, using a Bio-Rad Bio-Sil Sec 125-5 column (300 × 7.8 mm), of the fusion protein, the regenerated ligase, and the native ligase indicated that these proteins existed as dimers (data not shown). This result suggested that the presence of the MBP in the fusion protein did not interfere with the physical interaction between the ligase molecules. The observation that the fusion protein has similar L-alanine-adding enzyme efficiency as free ligase is in good agreement with the physical properties observed. The structure−activity relationship of the monomeric and the dimeric enzyme has been investigated and will be reported elsewhere (Jin et al., 1996).

To further characterize this enzyme, several L-alanine analogs were evaluated as potential ligase inhibitors to probe the L-alanine binding site specificity. Moderate modification of the side chain of the receptor amino acid substrate was tolerated by the enzyme. L-Cysteine, L-vinylglycine, and β-chloro-L-alanine all demonstrated significant affinity for the enzyme as shown by their ability to effectively inhibit the addition of L-alanine to UDPNacMur (≥90% inhibition). L-Valine, L-leucine, and L-isoleucine, on the other hand, did not inhibit the enzyme activity. Modification of the carboxyl function, such as in *t*-butyl-L-alanine and L-alaninamide, also significantly reduces their binding affinity to the enzyme (≈50% inhibition). Changes made at the -NH_2 group (e.g., *N*-methylalanine and D,L-lactate) resulted in complete loss of affinity (no inhibition observed). The L-isomer of cycloserine inhibited the enzyme activity by more than 30% while the D-isomer exerted no inhibitory effect, indicating the enzyme is selective for acceptor amino acids with L-configuration at C_α. This was further confirmed by the failure of D-alanine to serve as a substrate for the ligase.

Scheme 1: Preparation of (A) [^{18}O]PEP and (B) UDPNac[^{18}O]Mur



The enzyme also showed high selectivity for the ribonucleoside triphosphate, with the reaction only taking place in the presence of ATP, but not with GTP, CTP, or TTP. Optimal activity was observed around pH 8.

Preparation of [^{18}O]PEP and UDPNac[^{18}O]Mur. The exchange of both the carboxylic and the nonbridging phosphoryl [^{16}O]oxygen of [^{16}O]PEP with the solvent [^{18}O]-oxygen was accomplished under acidic conditions at an elevated temperature (Scheme 1A). After purification through an anion-exchange column, the yield of [^{18}O]PEP was 24%. Seventy-two micromoles of [^{18}O]PEP was obtained. LC-MS analysis of a small sample of [^{18}O]PEP revealed the presence of five subpopulations of PEP molecules; these contained 1, 2, 3, 4, and 5 [^{18}O]oxygen atoms/molecule of PEP, respectively. The distribution of the percent [^{18}O]oxygen enrichment of [^{18}O]PEP is summarized in Table 3.

UDPNac[^{18}O]Mur was prepared from [^{18}O]PEP, UDP-*N*-acetylglucosamine, and NADPH by two consecutive enzymatic reactions, without intermediate product isolation (Scheme 1B). The specificity of the enzymatic reactions allowed only the carboxylic function of UDPNac[^{18}O]Mur to be labeled with [^{18}O]oxygen (Scheme 1B). Product conversion in each step was quantitative. The final product UDPNac[^{18}O]Mur was separated from NADP/NADPH and the unreacted UDP-*N*-acetylglucosamine by reverse-phase HPLC. Only fractions containing >90% pure product were pooled and this amounted to 40 μmol of UDPNac[^{18}O]Mur

Table 3: [^{18}O]Oxygen Enrichment in [^{18}O]PEP and UDPNac[^{18}O]Mur Determined by LC-MS Analysis[a]

	% ^{18}O enrichment					
	no ^{18}O	1 ^{18}O atom	2 ^{18}O atoms	3 ^{18}O atoms	4 ^{18}O atoms	5 ^{18}O atoms
[^{18}O]PEP	0.0	1.0	8.1	22.9	38.0	29.9
UDPNac[^{18}O]Mur (**A**)	8.5	38.9	52.6			

[a] The % [^{18}O]oxygen enrichment in UDPNac[^{18}O]Mur (**A**) was used in Table 4 to calculate the projected % [^{18}O]oxygen enrichment of UDPNac[^{18}O]Mur-L-Ala and orthophosphate which were produced in the ligase reaction.

Table 4: Percent [^{18}O]Oxygen Enrichment of the Products Formed in the UDP-*N*-Acetylmuramate:L-Alanine Ligase-Catalyzed Reaction[a]

	% ^{18}O enrichment detected	% ^{18}O enrichment projected
UDPNac[^{18}O]Mur-L-ala (**B**)	73.2	72.0
[^{18}O]orthophosphate (**C**)	31.3	32.7

[a] Values are for 1 ^{18}O atom per molecule. The projected % ^{18}O enrichment is calculated on the basis of the assumption that the two oxygen atoms of the carboxyl group have equal probability of being involved in the activation process. UDPNac[^{18}O]Mur released one of the two carboxyl oxygen atoms during the reaction. Using the % ^{18}O distribution determined in UDPNac[^{18}O]Mur (**A**) as reported in Table 3, the projected % ^{18}O enrichment for (**B**) is calculated: % ^{18}O projected for **B** = {(% ^{18}O of 1-[^{18}O])0.5} + {(% ^{18}O of 2-[^{18}O])1.0} in **A** = {(38.9)(0.5)} + {(52.6)(1.0)} = 72.0%. Projected % ^{18}O enrichment in [^{18}O]orthophosphate was calculated using the same principle above and taking into consideration excess phosphate generated in AMP formation. % ^{18}O projected for **C** = (projected % ^{18}O in **B**){(μmol of UDPNac[^{18}O]Mur-L-Ala)/[(μmol of ADP) + 2(μmol of AMP)]} = (72.0){(30)/[(14) + (26)(2)]} = 32.7%.

recovered. The percent [^{18}O]oxygen atom enrichment of UDPNac[^{18}O]Mur determined by LC-MS is summarized in Table 3. Three subpopulations of UDPNacMur with 0, 1, and 2 [^{18}O]oxygen atoms were observed.

UDP-N-Acetylmuramate:L-Alanine Ligase-Catalyzed Transfer of [^{18}O]Oxygen from UDPNac[^{18}O]Mur to [^{18}O]Orthophosphate. The ligase-catalyzed formation of UDPNac[^{18}O]-Mur-L-ala from UDPNac[^{18}O]Mur and L-alanine was quantitative. The individual components recovered from the reaction mixture included UDPNacMur-L-Ala, AMP, ADP, ATP, and orthophosphate. These components were isolated, quantified, and subjected to LC-MS analysis to determine their percent [^{18}O]oxygen atom enrichment. Thirty micromoles of UDPNac[^{18}O]Mur-L-Ala was recovered along with 14 μmol of ADP and 26 μmol of AMP. [^{18}O]Oxygen was found to be present only in UDPNac[^{18}O]Mur-L-Ala and [^{18}O]orthophosphate, and there was no more than one [^{18}O]-oxygen atom present per molecule. None of the three adenosine nucleotides (AMP, ADP, and ATP) contained any [^{18}O]oxygen. The projected and the LC-MS-determined percent ^{18}O enrichment in the products are summarized in Table 4. The projected percent ^{18}O enrichment for each of the products was calculated assuming that a single phosphorylated intermediate was formed during the reaction. The percent [^{18}O]oxygen enrichment of UDPNac[^{18}O]Mur-L-Ala, as determined by LC-MS, was 73.2%. This value is essentially identical to the percent ^{18}O enrichment (72.0%) projected for this molecule. The LC-MS-determined percent ^{18}O enrichment of [^{18}O]orthophosphate was 31.3%. Although this value was lower than that observed in UDPNac-[^{18}O]Mur-L-Ala, it agreed with the 32.7% ^{18}O enrichment projected when nonspecific hydrolysis of ATP and ADP was taken into account (Table 4). The presence of AMP in the reaction mixture at the end of the reaction period indicated that nonspecific hydrolysis of ATP and ADP also occurred in the setting of prolonged incubation. Excess [^{16}O]orthophosphate generated from the nonspecific hydrolysis of ATP and/or ADP therefore reduced the concentration of [^{18}O]-orthophosphate in the total orthophosphate population. These results showed that a single [^{18}O]oxygen atom from UDPNac-[^{18}O]Mur was transferred to the orthophosphate produced during the reaction and demonstrated the direct involvement of the γ-phosphoryl group of ATP in the amide bond formation process between UDPNacMur and L-alanine.

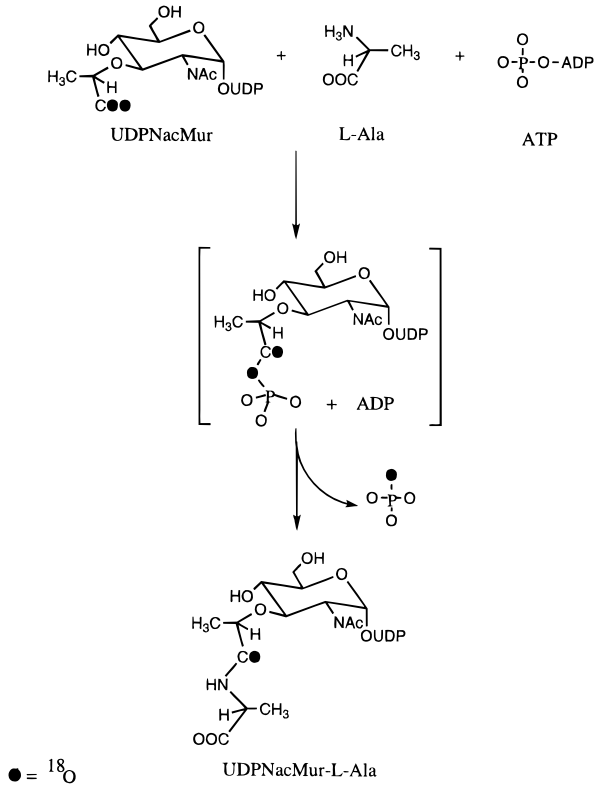
DISCUSSION

E. coli UDP-*N*-acetylmuramate:L-alanine ligase catalyzes the formation of the first peptide linkage in the peptidoglycan biosynthesis pathway. Large quantities of purified enzyme have been obtained (50−80 mg/L of culture). Kinetic analysis of both the fusion protein and the freed ligase showed that these proteins retain comparable ligase activity to that of the native enzyme. Furthermore, the kinetic parameters and other biochemical properties of the ligase we obtained are comparable to those recently reported by Liger et al. (1995). Inhibition studies performed with L-alanine analogs demonstrated the substrate selectivity of MurC which would ensure proper incorporation of the acceptor amino acid in this ribosome-independent peptide synthesis process *in vivo*.

The biosynthesis of bacterial peptidoglycan precursor includes the formation of several peptide bonds independent of ribosomes. The ribosome-independent, enzymatic biosynthesis of peptide bonds generally proceeds through two different mechanisms. In a multienzyme system, the individual acceptor amino acids are sequentially added on to the donor amino acid/peptide through the formation of a series of covalent enzyme−substrate intermediates, of which only the final product can be released into the medium (Lipmann, 1982; Christiansen et al., 1982). On the other hand, the synthesis of some peptides involves a series of unique enzymes which independently catalyze the sequential addition of the acceptor amino acids without the formation covalent enzyme intermediates (Strumeyer & Bloch, 1960; Mullins et al., 1990). In either case, an initial activation of the carboxyl function of the donor substrate at the expense of ATP generally takes place. The cleavage of ATP can take place between either the $\alpha-\beta$ (Lipmann, 1982; Christiansen et al., 1982) or the $\beta-\gamma$ (Strumeyer & Bloch, 1960; Mullins et al., 1990) phosphoryl linkage. The formation of either an acyl-adenylate or acyl-phosphate mixed anhydride intermediate in these reactions has been suggested. Among the enzymes involved in the formation of the *E. coli* peptidoglycan precursor biosynthesis, it has been reported that MurC and MurE (the *m*-DAP-adding enzyme) catalyzed the exchange between the free acceptor amino acid and the corresponding uridine nucleotide product only when both ADP and orthophosphate were present (Michaud et al., 1990; Liger et al., 1995). Although speculation of the presence of an activated acyl-phosphate intermediate was made, no further biochemical elucidation of the mechanism of these reactions has been carried out to date.

We have utilized the enzymes which catalyze the first two committed reactions in the bacterial peptidoglycan biosynthesis pathway to prepare UDPNac[^{18}O]Mur with [^{18}O]-oxygen specifically placed at the carboxyl function of the

Scheme 2: Proposed Route of Transfer of ^{18}O from UDP-*N*-Acetyl[^{18}O]muramate to Orthophosphate in the UDP-*N*-Acetylmuramate:L-Alanine Ligase-Catalyzed Reaction



muramic acid moiety. Using this ^{18}O-labeled substrate, we were able to probe the mechanistic role of ATP in the *E. coli* L-alanine-adding enzyme-catalyzed reaction. Although the enzyme is capable of catalyzing the reverse reaction (Liger et al., 1995), under our reaction conditions the reaction proceeds only in the forward direction. This is supported by the observations that the reaction product, UDPNac[^{18}O]-Mur-L-Ala, fully retained one of the two [^{18}O]oxygen from the ^{18}O-labeled substrate (73.2% ^{18}O enrichment) and that no [^{18}O]oxygen was found to associate with any of the adenosine nucleotide recovered. The transfer of a single [^{18}O]oxygen atom from the ^{18}O-labeled substrate to the inorganic phosphate produced in the reaction strongly indicates that a covalent UDP*N*-acetylmuramyl-phosphate linkage is present in the reaction process. This type of transfer probably takes place through an activated phosphate intermediate (Scheme 2), although the direct isolation of an acyl-phosphate intermediate in a complete reaction mixture has not been successful.

Adequate cytoplasmic biosynthesis of peptidoglycan precursor is vital to bacterial growth. Little has been reported on antibacterial agents targeting enzymes involved in the early steps of peptidoglycan precursor biosynthesis. UDP-*N*-acetylmuramate:L-alanine ligase catalyzes the first of the five peptide-bond formation steps in the pathway. Understanding the reaction mechanism in this step could provide valuable insight into the search and design of novel antibacterial agents. This is now an even more urgent task given the resurgence of antibiotic resistance in pathogenic bacteria against a large number of commonly available antibiotics.

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