Kinetic Mechanism of the *Escherichia coli* UDPMurNAc-Tripeptide D-Alanyl-D-alanine-Adding Enzyme: Use of a Glutathione *S*-Transferase Fusion

Matt S. Anderson,* Suzanne S. Eveland, H. Russell Onishi, and David L. Pompliano*

Department of Enzymology, Merck Research Laboratories, P.O. Box 2000, Rahway, New Jersey 07065

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ABSTRACT: The D-alanyl-D-alanine-adding enzyme encoded by the *murF* gene catalyzes the ATP-dependent formation of UDP-*N*-acetylmuramyl-L-γ-D-Glu-*meso*-diaminopimelyl-D-Ala-D-Ala (UDP-MurNAc-tripeptide). *MurF* has been cloned from *Escherichia coli* and expressed as a glutathione *S*-transferase (GST) fusion using the *tac* promoter-based pGEX-KT vector. From induced, broken cell preparations, highly active fusion was recovered and purified in one step by affinity chromatography. The purified fusion protein was strongly inhibited by substrate UDPMurNAc-tripeptide, a response unaltered by changes in assay pH or by cleavage from the fusion partner. However, this effect was suppressed by the addition of 0.5 M NaCl. Initial velocity and dead-end inhibitor studies with the fusion enzyme were most consistent with a sequential ordered kinetic mechanism for the forward reaction in which ATP binds to free enzyme, followed by tripeptide and D-Ala-D-Ala in sequence prior to product release. Reported homologies between the MurF protein and the three preceding steps of cytoplasmic murein biosynthesis, MurC, -D, and -E, [Ikeda *et al.* (1990) *J. Gen. Appl. Microbiol. 36*, 179−187], raise the prospect that all of these enzymes will be found to proceed via this mechanism.

The bacterial cell wall or peptidoglycan consists of a single polymeric molecule that surrounds and contains the plasma membrane and cytoplasm of the cell. This structure is responsible for maintaining cell shape and integrity against an osmotic pressure of at least 4 atm. The study of its biosynthesis has its roots in Park and Johnson's descriptions of the effects of penicillin on bacterial cell walls in the 1940s (Park & Johnson, 1949). Later, Strominger and co-workers elucidated many other aspects of wall synthesis including the involvement of a lipid carrier in the pathway (Anderson *et al.*, 1965; Dietrich *et al.*, 1967).

Historically, this biosynthetic pathway has been divided on functional grounds into three parts or stages (Figure 1). During Stage I, the building blocks of the peptidoglycan polymer are constructed in the cytoplasm. Stage II sees the attachment and functionalization of these units on the membrane for final placement during Stage III where they reach growth sites and are incorporated into the cell wall. The enzymes that catalyze these steps have been identified in extracts from a variety of bacteria and many have been purified to homogeneity (Bugg & Walsh, 1992). The sequences and locations of the structural genes (*murABC-DEFG, mraY, alr,* and *ddlA*) that encode these enzymes are known in *Escherichia coli*. In fact, many of them are clustered in the 2 min region of the *E. coli* genome (Errington, 1993). Temperature sensitive alleles for each of the genes listed above have demonstrated the essentiality of the enzymatic functions they encode (Lugtenberg & Schijndel-van Dam, 1972; Lugtenberg *et al.*, 1972). Cognate *mur* gene sequences from a wide variety of bacterial genera are known. Thus, permeative inhibitors of pathway enzymes should be broad spectrum antibacterial agents. Indeed, the antibiotic fosfomycin acts by inhibiting MurA, pharmacologically validating this enzyme (and the pathway) as a therapeutic target.

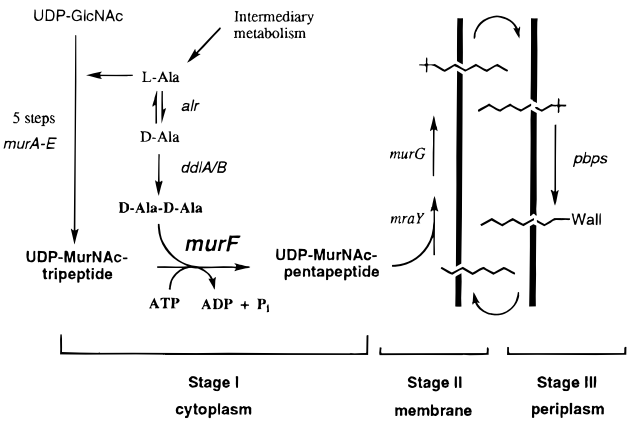

FIGURE 1: Cell wall peptidoglycan biosynthesis in bacteria. The *murF*-encoded UDP-MurNAc-tripeptide, D-Ala-D-Ala ligase (in boldface), catalyzes the formation of completed Stage I intermediates from two interrelated biosynthetic pathways. The reaction utilizes the three substrates, ATP, UDP-MurNAc-tripeptide, and D-Ala-D-Ala, and releases three products, ADP, inorganic phosphate, and UDP-MurNAc-pentapeptide. In the figure, ⁓ represents bactoprenol monophosphate and +⁓ represents lipid intermediate II. Evidence for this pathway has been extensively reviewed (Bugg & Brandish, 1994; Bugg & Walsh, 1992).

In *E. coli*, the MurF protein catalyzes the formation of the last metabolite of Stage I synthesis, UDP-MurNAc-pentapeptide,[1] directly from the tripeptide by ligation of a D-alanyl-D-alanine moiety in an ATP-utilizing reaction (Bugg & Walsh, 1992) (Figure 1). Recombinant enzyme has been purified to apparent homogeneity from *E. coli* and partially characterized (Duncan *et al.*, 1991). This enzyme—UDP-MurNAc-tripeptide: D-alanine-D-alanine ligase—sits at a crucial junction in peptidoglycan synthesis, mobilizing

* Corresponding authors: Matt S. Anderson, (908) 594-3801, e-mail: matt_anderson@merck.com, fax (908) 594-6645; David L. Pompliano, (908) 594-3023, e-mail: david_pompliano@merck.com, fax (908) 594-1188.

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precursors for Stage II synthesis from two separate substrate supply pathways.

We report the purification in one step of a glutathione *S*-transferase::D-Ala-D-Ala-adding enzyme (GST::MurF) fusion protein in highly active form. Preliminary kinetic studies with the purified enzyme revealed the presence of severe substrate inhibition by UDPMurNAc-tripeptide independent of the GST fusion partner, suggesting that the mechanistic analysis of this three substrate enzyme was going to be rather complex. However, variation of assay conditions led to the discovery that high concentrations of salt were able to supress the observed substate inhibition. Under these conditions, steady-state kinetic measurements were used to distinguish between various mechanistic possibilities and to determine the relative order of substrate binding. Such an understanding of the MurF kinetic mechanism is an important first step to help guide the design of enzyme inhibitors and provides a framework in which to evaluate such inhibitory compounds.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were obtained from Promega. *Pfu* polymerase was obtained from Stratagene. S-Linked glutathione−agarose, glutathione, D-Ala-D-Ala, Type 3 rabbit muscle pyruvate kinase, β-NADH, ATP, and phospho(enol)pyruvate were obtained from Sigma. Hog muscle lactate dehydrogenase, AMP-PCP and AMP-PNP were obtained from Boehringer Mannheim. UDP-MurNAc-tripeptide (*meso*-A_2pm-containing) was isolated from *Bacillus megaterium* (Nakatani *et al.*; 1968). UDP-MurNAc-tripeptide (L-lysine-containing) was isolated from *Staphylococcus aureus* in the same manner. Remaining minor impurities were removed using the reversed phase HPLC system of Flouret *et al.* (1981). D-Ala-D-Lac was the generous gift of Dr. Lihu Yang (Merck Research Laboratories).

Bacterial Strains. All strains used were of *E. coli*. Strain BL21 was purchased from Novagen. XL-1 Blue (*recA*$^-$) was obtained from Stratagene. Plasmid pGEX-KT has been described (Hakes and Dixon, 1992).

Assay of D-Ala-D-Ala Ligase Activity (MurF Activity). Production of ADP was monitored spectrophotometrically using a coupled assay system of pyruvate kinase (converting PEP to pyruvate with regeneration of ATP) and lactate dehydrogenase (converting pyruvate to lactate) with a corresponding loss of NADH absorbance at 340 nm. Initially, assays were performed in 1 mL volume using a Cary 14, but were later adapted to a microtiterplate format, using a Molecular Devices SPECTRAmax 250 microplate reader. Results obtained in each setting were identical. Typically, assay mixtures contained Tris, pH 8.6 (100 mM), KCl (40 mM), $MgCl_2$ (10 mM), NaCl (500 mM), β−NADH (125 μM), lactate dehydrogenase (10 μg), pyruvate kinase (10 μg, 4.1 units), PEP (2.5 mM), BSA (1 mg/ml), varied concentrations of ATP, D-Ala-D-Ala, and UDP-MurNAc-tripeptide, and sufficient enzyme (typically 2−4 nM) to produce a change within 0.1−1.5 μM in NADH concentration per minute at 30 °C in a final volume of 200 μL (light path length of 0.55 cm). An extinction coefficient of 6220 M^{-1} cm^{-1} for NADH absorbance at 340 nm was used to calculate specific activities. Determination of kinetic parameters for the unstable substrate D-Ala-D-Lac was performed with freshly dissolved material. Velocities were linear over 30 min.

Cloning of MurF from E. coli Strain MB 2884. The MurF reading frame was cloned by PCR from *E. coli* strain MB2884 based upon the reported DNA sequence in Accession Number X55034. The host vector for *murF* was pGEX-KT, a *tac* promoter-based vector designed to express cloned inserts as a fusion with glutathione *S*-transferase of *Schistosoma japonicum* (Hakes & Dixon, 1992; Smith & Johnson, 1988). This vector incorporates a linker and thrombin cleavage site between these two domains to allow cleavage and removal of the fusion partner after purification of the expressed protein using glutathione-agarose. PCR primers to the N-terminal and C-terminal regions of the *murF* gene were designed to allow unidirectional ligation of the resulting PCR fragment into these vectors. The 5′ primer for cloning into pGEX-KT incorporated a G/C clamp, a *Bam*HI site immediately 5′ of the initiating ATG codon and 18 additional bases of coding sequence 5′-GGGCCCATCGTA GGATC-CATGATT AGCGTAACC CTTAGC-3′. The 3′ primer incorporated a G/C clamp, an *Eco*RI site, tandem stop codons and 16 additional bases of anticoding sequence 5′-CCCGGGATC GTGAATTCT TACTAACATGTC CCAT-TCTCC-3′. PCR reactions contained 200 ng MB 2884 genomic DNA template, 1 μM of each primer, 200 μM dNTP's, and 2.5 units of *Pfu* DNA polymerase in 20 mM Tris-HCl (pH 8.75), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 100 μg/mL bovine serum albumin. PCR conditions were 35 cycles of denaturation at 97 °C for 30 s, annealing at 65 °C for 30 s, and elongation at 72 °C for 2 min. This was followed by a 10 min polishing at 72 °C. The major PCR product (1395 bp) was purified using Wizard PCR Preps DNA Purification System (Promega), digested with *Bam*HI and *Eco*RI, and ligated into similarly digested pGEX-KT vector. Transformation of the ligation reaction into electroporation-competent Epicurian Coli XL-1 Blue cells (Stratagene) yielded ampicillin resistant colonies. The pGEXmurF plasmid, identified as a transformant containing a 1395 bp insert, was isolated by an alkaline lysis procedure (Wizard Minipreps DNA Purification System; Promega) and tranformed into competent BL21 cells (Novagen) with ampicillin selection just prior to use.

Expression and Purification of Recombinant GST::MurF Protein. BL21 cells containing the pGEXmurF plasmid were grown at 30 °C in LB medium containing ampicillin (100 μg/mL) until the culture reached $OD_{600} = 0.5$. Expression of the fusion protein was induced with IPTG (1 mM) for 2 h. The cells were harvested by centrifugation (5000*g* for 15 min at 4 °C), resuspended in 1/40 volume 50 mM HEPES (pH 7.5)/10 mM $MgCl_2$, and lysed by a single passage through a French pressure cell at 18 000 psi. Cellular debris was removed by centrifugation (20 000*g* for 15 min at 4 °C). The supernatant was mixed with S-linked glutathione agarose (pre-equilibrated with PBS buffer) at 4 °C for 2 h and then washed with 50 mM HEPES, pH 7.5, containing 10 mM

[1] Abbreviations: *meso*-A_2pm, *meso*-2,6-diaminopimelic acid; AMP-PCP, adenylyl 5′-(β,γ-methylenediphosphonate); AMP-PNP, adenylyl imidodiphosphonate; D-Ala-D-Ala, D-alanyl-D-alanine; GS-MurF, glycylseryl-MurF protein; HPLC, high performance liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; lipid intermediate II, bactoprenylpyrophosphoryl-MurNAc-pentapeptide-GlcNAc; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PK-LDH, pyruvate kinase−lactate dehydrogenase; SDS, sodium dodecyl sulfate; MurNAc, *N*-acetylmuramic acid; UDP-MurNAc-tripeptide (*meso*-A_2pm-containing), UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm; UDP-MurNAc-tripeptide (L-lysine-containing), UDP-MurNAc-L-Ala-γ-D-Glu-L-lys; UDP-MurNAc-pentapeptide, UDP-MurNAc-L-Ala-γ-D-Glu-*meso*-A_2pm-D-Ala-D-Ala.

$MgCl_2$. The GST::MurF fusion protein was recovered from the column with a buffer of 50 mM Tris HCl, pH 7.5, 10 mM glutathione, and 10% glycerol. The enzyme was stored in aliquots at −80 °C.

Kinetic Analysis of MurF Protein. Steady state kinetic patterns were determined from experiments in which the concentration of one substrate was varied at several fixed concentrations of another substrate, while the third was held constant. These sets of 36 initial velocity pairs were fit as a group by nonlinear least-squares regression analysis using the Marquardt algorithm (Marquardt, 1963). The rate equations used were eq 1 for an intersecting pattern or eq 2 for a parallel pattern. These equations have been derived assuming rapid equilibrium binding of the substrates. Experiments using the nonhydrolyzable ATP analog AMP-PCP were similarly fit to eq 3 in order to model competitive inhibition and eq 4 to model mixed noncompetitive inhibition.

$$v = \frac{VAB}{K_{ia}K_{ib} + K_bA + K_aB + AB} \quad (1)$$

$$v = \frac{VAB}{K_bA + K_aB + AB} \quad (2)$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \quad (3)$$

$$v = \frac{VA}{A(1 + I/K_{ii}) + K(1 + I/K_{is}) + A} \quad (4)$$

RESULTS

Cloning and Purification of GST::D-Ala-D-Ala-Adding Enzyme. A study of bacterial peptidoglycan biosynthesis requires a ready and reproducible source of each enzyme for kinetic studies as well as for the creation of commercially unavailable substrates. For these purposes, the adding enzyme was cloned, expressed, and purified as a fusion with glutathione *S*-transferase (GST), a protein partner that makes possible an affinity purification using glutathione agarose. The plasmid construct, pGEXmurF, was prepared as described under Experimental Procedures. Routine DNA sequencing to verify the identity of the *murF* clone revealed two DNA sequence conflicts—both CG to GC—at codons 61 and 178 with the published sequence (accession number X55034) that resulted in translation changes (Ala61 to Gly, and Arg178 to Ala). These alterations were found in other *E. coli* strains as well (data not shown). The amended nucleotide sequence has been submitted to GenBank under the Accession Number U67891. Molecular weights were calculated on the basis of these changes.

To express the GST::MurF fusion protein, *E. coli* strain BL21, freshly transformed with pGEXmurF, was grown at 30 °C to late log phase and induced for 2 h with IPTG. Analysis of such induced whole cells by SDS−polyacrylamide gel electrophoresis revealed the presence of an approximately 71 kDa protein, in good agreement with the calculated molecular weight of 74.224 kDa for the GST::MurF fusion (data not shown). After French press lysis of the cells, the majority of this protein was recovered in the soluble fraction (lane 2, Figure 2). The fusion was then recovered in a single pass through glutathione agarose in good yield (lanes 3−5, Figure 2). This protein was greater than 97% pure as judged by gel densitometery. The isolated protein was stored in 20 μL aliquots at −80 °C which were thawed once and discarded. Kinetic parameters determined for several preparations of the GST::MurF fusion protein agreed within the error of measurements. Activity was stable under these conditions for at least 6 months.

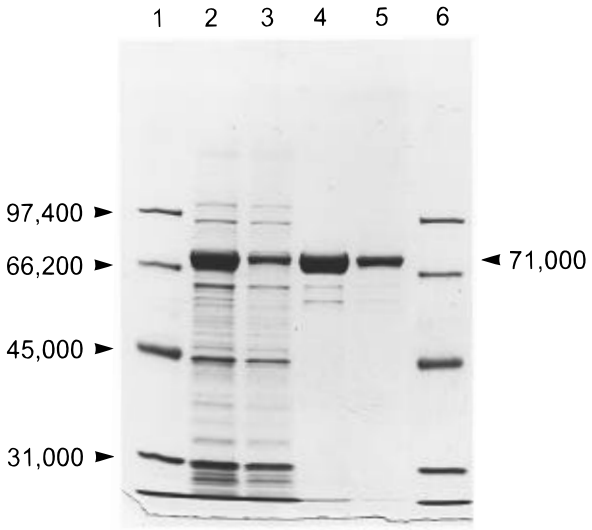


FIGURE 2: Coomassie-stained 10% SDS−polyacrylamide gel (Novex Corp.) detailing GST::MurF purification via affinity chromatography. Lanes 1 and 6: Low molecular weight protein standards (Bio-Rad): phosphorylase B (97 400); bovine serum albumin (66 200), ovalbumin (45 000), carbonic anhydrase (31 000). Lane 2: Soluble protein fraction resulting from French pressure cell disruption of induced BL21/pGEXmurF cells as described under Experimental Procedures. Lane 3: Protein fraction not retained by glutathione agarose (GST−agarose). Lanes 4 and 5: Protein eluted from GST−agarose column in two fractions. The major band at approximately 71 kDa corresponds to the desired GST::MurF fusion protein.

The GST::MurF fusion protein contains a polyglycine spacer between the GST and MurF domains. A thrombin cleavage site has been engineered within this spacer in such a manner that a Gly-Ser extension remains at the N-terminus of the MurF protein after cleavage. Reapplication of the thrombin reaction over glutathione−agarose removes uncut protein and released GST. For comparative purposes, a small amount of such cleaved MurF protein was generated as described under Experimental Procedures. This material was not routinely created due to somewhat erratic yields after cutting and purification. In our hands, the cleaved fusion also had a propensity to copurify with variable amounts of cut GST. Cut samples averaged 80% GS-MurF with the remainder GST and some uncut fusion.

Activity of the purified fusion protein was monitored using the PK-LDH coupled spectrophotometric assay described by Duncan *et al.* (1990). The purified fusion was devoid of contaminating ATPase activities that might interfere with this assay. No assay signal was detected when any one of the three substrates was removed from the assay. UDPMurNAc-tripeptide (*meso*-A_2pm-containing) from a single 100 L preparation of *Bacillus megaterium*, homogeneous by HPLC (Fluoret *et al.*, 1981) and verified by mass spectrometry, was used for all analyses.

Substrate Inhibition by UDPMurNAc-Tripeptide and Its Suppression by NaCl. Initial experiments to determine approximate values of K_m for the three substrates of the adding enzyme revealed strong substrate inhibition by UDPMurNAc-tripeptide (Figure 3). This effect was not

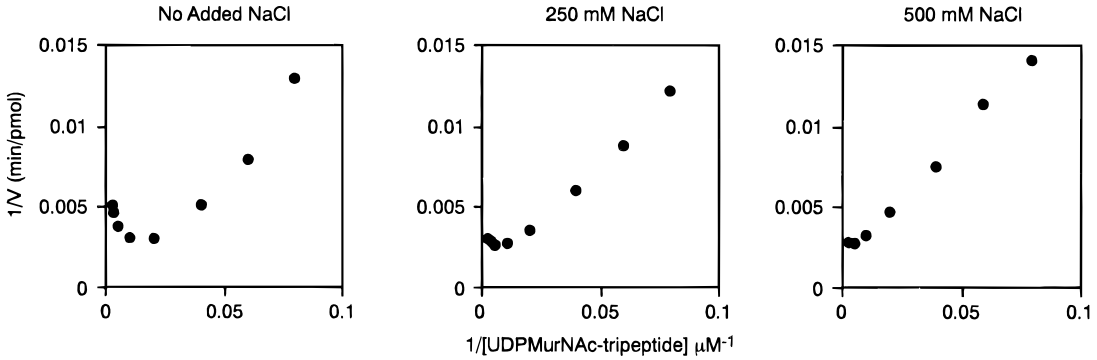


FIGURE 3: Double reciprocal plot of initial velocity versus UDPMurNAc-tripeptide at saturating D-Ala-D-Ala and ATP. Severe substrate inhibition by UDPMurNAc-tripeptide was observed, but was suppressible by addition of salt. Subsequent kinetic analysis was performed in the presence of 0.5 M NaCl.

Table 1: Initial Velocity Patterns for the Forward Reaction

varied substrates	fixed substrate	pattern
tripeptide,[a] D-Ala-D-Ala	ATP, 5 mM	intersecting
ATP, tripeptide	D-Ala-D-Ala, 2 mM	intersecting
ATP, D-Ala-D-Ala	tripeptide, 0.56 mM	parallel
ATP, D-Ala-D-Ala	tripeptide, 0.037 mM	intersecting

[a] UDP-MurNAc-tripeptide (*meso*-A_2pm-containing).

observed with either of the other cosubstrates ATP and D-Ala-D-Ala. Inhibition was unrelieved by changes in assay pH. The related UDPMurNAc-(L-lysine-containing)-tripeptide was an equally effective substrate for the adding enzyme. The insensitivity of the adding enzyme to variants of UDPMurNAc-tripeptide at this position is well-known (Mengin-Lecreulx *et al.*, 1994). Yet, it revealed equally potent substrate inhibition. A sample of the cleaved MurF protein behaved identically. Fortunately for the kinetic analysis, this behavior could be heavily supressed by the inclusion of sodium chloride in the assay mixture. Supression was dose dependent and had little effect on overall reaction velocity (Figure 3). Concentrations of 0.5 M were necessary to sufficiently reduce this problem. All further work was carried out in the presence of 0.5 M NaCl.

The reasons for this inhibition are not clear. One obvious possibility was that the UDPMurNAc-tripeptide might compete with ATP at a single binding site by virtue of the structural similarity between these substrates. As a consequence, the substrate inhibition pattern seen at low salt (Figure 3) and saturating ATP should change significantly when performed at lower concentrations of ATP. Yet, reassay under several sub-K_m concentrations of ATP did not alter the shape of this curve (data not shown). Thus, competition between these two metabolites for a single binding site is not the root cause of the substrate inhibition and suggests that the ATP and UDPMurNAc-tripeptide binding sites are independent.

Kinetic Characterization of D-*Ala*-D-*Ala-Adding Enzyme*. Although a discussion for the kinetic analysis of terreactant systems is beyond the scope of this paper, a thorough review of such systems has been published (Viola & Cleland, 1982). Initial velocity kinetic studies were performed varying two of the three substrates and holding the third one constant. The resulting patterns are summarized in Table 1. Combinations of UDPMurNAc-tripeptide with either ATP or D-Ala-D-Ala were intersecting at all levels of the fixed substrate, indicating a lack of product dissociation steps between substrate binding steps. However, the kinetic pattern observed with varied ATP vs D-Ala-D-Ala was a function of the UDP-MurNAc-tripeptide concentration. At subsaturating levels of this substrate, the pattern was distinctly intersecting, but when the nonvaried substrate was held at saturating concentrations, the pattern became essentially parallel. The data are consistent with a mechanistic step that is made functionally irreversible by the presence of high UDPMurNAc-tripeptide and confirms the placement of this metabolite between the ATP and D-Ala-D-Ala binding steps. These patterns are consistent with ordered binding of the three substrates prior to substrate release.

A priori, this ordered sequence could occur in either order, and these data alone do not allow distinction between these two possibilities. The products P_i and UDPMurNAc-pentapeptide displayed no inhibition at millimolar levels, consistent with published studies on purified recombinant enzyme (Duncan *et al.*, 1990). Product inhibition studies with ADP were not feasible with the coupled enzyme system. In order to fix a binding order, we employed the nonhydrolyzable ATP analogs AMP-PCP and AMP-PNP. In tests with the coupling system alone, these compounds did not interfere with the ability of the PK-LDH coupling system to respond to added ADP. However, this test revealed significant ADP contamination in the AMP-PNP. Since the AMP-PCP was not contaminated with ADP, it was used for all further studies.

AMP-PCP proved to be a potent inhibitor of ATP hydrolysis and was competitive with ATP as anticipated (Figure 4, panel A; Table 2). In the presence of $2 \times K_m$ ATP, variation of AMP-PCP gave an intersecting pattern with varied UDPMurNAc-tripeptide (Figure 4, panel B; Table 2, expt 1) consistent with the ability of this inhibitor to interact with an enzyme form to which the tripeptide can bind. Further, the ordered binding model predicts that inhibition by AMP-PCP with D-Ala-D-Ala as the varied substrate should show a change from a mixed noncompetitive form to an increasingly uncompetitive form as the concentration of UDPMurNAc-tripeptide increases from subsaturating to saturating conditions. Accordingly, assays performed with varied AMP-PCP and D-Ala-D-Ala at fixed UDPMurNAc-tripeptide concentrations of $0.5 \times K_m$, $3 \times K_m$, and $7 \times K_m$ yielded velocity data which became increasingly free of a noncompetitive component (Table 2, expt. 2); that is, the inhibition pattern became more parallel. These data provide compelling evidence for initial binding of ATP to the free enzyme.

The ordered binding model of Scheme 1 is analogous to that used by D-Ala-D-Ala ligase of *E. coli* in which an acyl phosphate intermediate is used to activate the carboxylate

Table 2: Kinetic Parameters for Reaction Inhibited with the Nonhydrolyzable ATP Analog AMP-PCP

varied components	fixed components	pattern[a]	K_{ii} (μM)	K_{is} (μM)
expt 1				
AMP-PCP, ATP	tripeptide,[b] 0.312 mM; D-Ala-D-Ala, 2 mM;	C	4.15 ± 0.15	
AMP-PCP, tripeptide[b]	ATP, 0.33 mM; D-Ala-D-Ala, 2 mM	NC	7.4 ± 0.38	33.6 ± 4.6
expt 2				
AMP-PCP, D-Ala-D-Ala	tripeptide, 0.039 mM; ATP, 0.33 mM	NC	17.0 ± 0.88	20.8 ± 2.9
AMP-PCP, D-Ala-D-Ala	tripeptide, 0.312 mM; ATP, 0.33 mM	NC	10.7 ± 0.32	68.3 ± 15.0
AMP-PCP, D-Ala-D-Ala	tripeptide, 0.555 mM; ATP, 0.33 mM	NC	12.6 ± 0.71	146 ± 106

[a] C, competitive; NC, noncompetitive. [b] UDP-MurNAc-tripeptide (*meso*-A_2pm-containing).

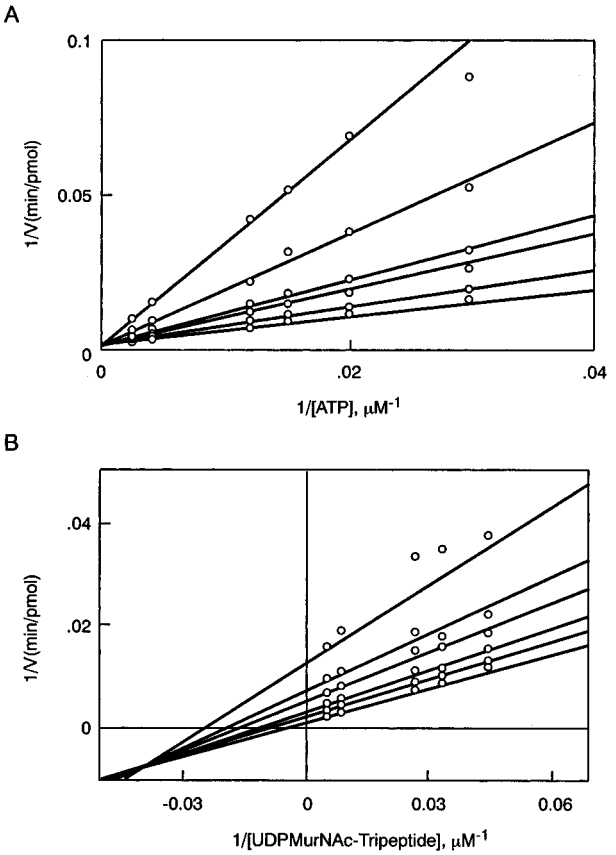
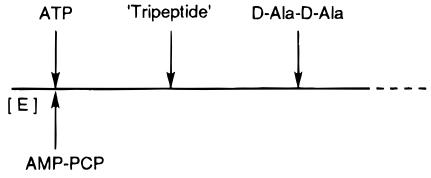


FIGURE 4: Analysis of inhibition by the nonhydrolyzable ATP analog AMP-PCP. Panel A: Double reciprocal plot of initial velocity versus ATP at saturating D-Ala-D-Ala and UDPMurNAc-tripeptide. AMP-PCP was varied over the range 2−40 μM. Panel B: Double reciprocal plot of initial velocity versus UDPMurNAc-tripeptide at saturating D-Ala-D-Ala and 2 × K_m ATP (330 μM). AMP-PCP was varied over the range 0−45 μM.

Scheme 1



of the nascent amide bond (Mullins *et al.*, 1990). An acyl phosphate intermediate has been identified in catalysis of the related MurC protein as well (Falk *et al.*, 1996). If such an intermediate were to form on the MurF protein prior to the binding of D-Ala-D-Ala, it might be possible to detect ADP formation if this product were to diffuse from the enzyme at a measurable rate. MurF activity assays were carried out at saturating ATP and UDPMurNAc-tripeptide in the absence of D-Ala-D-Ala, but less than 0.2% of the corresponding rate (detection limit) observed with saturating D-Ala-D-Ala was measurable. Thus, if this metabolite forms under these conditions, it is tightly held, consistent with a sequential ordered mechanism.

Table 3: Kinetic Constants for D-Ala-D-Ala-Adding Enzyme

substrate	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
ATP	164	1.18×10^5
UDPMurNAc-tripeptide[a]	78	2.49×10^5
D-Ala-D-Ala	208	9.33×10^4
D-Ala-D-Lac	267	7.27×10^4

[a] UDP-MurNAc-tripeptide (*meso*-A_2pm-containing).

Kinetic parameters are summarized in Table 3. V_{max} for the purified fusion enzyme was 15.7 ± 2.5 μmol min^{-1} mg^{-1}. k_{cat} for the GST::murF fusion was determined to be 19.4 s^{-1}, almost 50% higher than that (13 s^{-1}) reported for the purified recombinant protein (Duncan *et al.*, 1990), but similar to a later report (18 s^{-1}) by Bugg and co-workers (Bugg *et al.*, 1991). The values of K_m for these substrates are in excellent agreement with those reported previously for the purified adding enzyme of Duncan *et al.*, (1990), but less so with a partially purified preparation (Michaud *et al.*, 1987). In particular, the value of K_m(UDPMurNAc-tripeptide) for the partially purified material (11 μM) disagrees with both our result and that (70 μM) for the purified material. This discrepancy may be a consequence of substrate inhibition by this species, which may not have been readily apparent due to a scarcity of this substrate. The values of K_m(D-Ala-D-Ala) are roughly equal to the pool levels of this precursor in *E. coli* (200 μM; Mengin-Lecreulx *et al.*, 1982), consistent with evolutionary expectations (Fersht, 1974). D-Ala-D-Lac and D-Ala-D-Ala were similarly efficient substrates as reported earlier (Bugg *et al.*, 1991).

DISCUSSION

We desired to work with a form of the D-Ala-D-Ala-adding enzyme that was both highly functional and easily purified with potential for retrieval of a more "native" polypeptide if required. The use of a GST fusion satisfied these requirements. The very close concurrence of the kinetic parameters reported here for the GST::MurF fusion protein with those reported for recombinant protein isolated without a fusion partner (Duncan, 1991) validates this approach for this enzyme. Preliminary studies on cleaved and enriched MurF protein showed little variation from results obtained with the fusion. In principle, such an approach could be used to garner many of the enzymes of peptidoglycan metabolism through a single focused purification strategy.

The kinetic data are consistent with an ordered binding of substrates to MurF in the sequence ATP, UDPMurNAc-tripeptide, D-Ala-D-Ala as summarized in Scheme 1. This is supported by the intersecting patterns observed between all pairs of substrates dictating a lack of product release until all three are bound. However, in the presence of high

concentrations of tripeptide, the ATP versus D-Ala-D-Ala pattern became parallel, suggesting that the tripeptide adds between the ATP and the D-Ala-D-Ala. Inhibition by the ATP analog, AMP-PCP, is competitive with ATP, but noncompetitive with respect to tripeptide. If ATP were binding after the tripeptide, this pattern would have been uncompetitive. Moreover, AMP-PCP is a noncompetitive inhibitor versus D-Ala-D-Ala when subsaturating tripeptide concentration allows a reversible connection between the enzyme form that binds the ATP analog and the enzyme form that binds the varied substrate D-Ala-D-Ala. Since AMP-PCP is a dead-end inhibitor, a slope effect in the double reciprocal plot is only seen if it combines upstream from the varied substrate. That places the binding of the D-Ala-D-Ala after the binding of ATP. Also, when one interrupts the reversible connection between the downstream D-Ala-D-Ala and the ATP analog-binding form of the enzyme (by increasing the tripeptide concentration), the inhibition becomes uncompetitive. Unfortunately, the PK-LDH assay is incompatible with product inhibition studies using ADP which, by analogy with the mechanism of D-alanyl-D-alanine ligase, is likely to show competitive effects against ATP (Mullins *et al.*, 1990).

Previous work with D-Ala-D-Ala ligase has clearly demonstrated the occurrence of an acyl phosphate intermediate in catalysis as a means of activating the carboxylate toward the amine nucleophile (Mullins *et al.*, 1990). By analogy, the *meso*-A_2pm α carboxylate of the tripeptide should be the site of phosphorylation by ATP. The kinetic patterns using ATP and D-Ala-D-Ala as varied substrates were dependent upon relative UDPMurNAc-tripeptide concentrations. By mass action and energetic considerations, high levels of this metabolite must drive such a phosphorylation step irreversibly. The lack of detectable ADP release from the enzyme in the absence of D-Ala-D-Ala is also consistent with the sequential kinetic model. If an acyl phosphate intermediate forms on the enzyme prior to binding of D-Ala-D-Ala, the ADP consequently formed is not released.

The adding enzyme lies at a metabolic junction between D-Ala-D-Ala and UDPMurNAc-tripeptide formation pathways. Regulation of the D-Ala-D-Ala branch could occur through product inhibition by this metabolite, slowing the flow of alanine skeletons into the cell wall (Duncan *et al.*, 1990). Substrate inhibition of the adding enzyme by UDPMurNAc-tripeptide was an unexpected finding. It is not known whether this also occurs in vivo. If so, it may act as a means of slowing metabolic flux through the UDPMurNAc-tripeptide forming branch under conditions of elevated L-alanine concentrations. Alternatively, this inhibition could merely arise from inappropriate binding of the tripeptide to the active site in vitro, but this site appears not to be coincident with the ATP binding site. In this scenario, high salt concentrations suppress nonspecific interactions and improve the fidelity of the enzyme−substrate interaction.

The peptidoglycan biosynthetic pathway has long been described as suitable ground for the development of antibiotics. In this regard, the four amide bond forming enzymes of UDPMurNAc-pentapeptide synthesis, the MurC, -D, -E, and -F proteins, have been shown to bear significant blocks of homology (Ikeda *et al.*, 1990). These likely reflect elements of shared macromolecular structure necessary for substrate recognition and/or catalytic function. Indeed, the use of an acyl phosphate intermediate in catalysis by the MurC protein (UDPMurNAc:L-alanine ligase) has recently been reported (Falk *et al.*, 1996). The elucidation of a kinetic mechanism for these enzymes is an aid in defining and confirming the nature of enzyme−substrate complexes and likely enzyme intermediates occurring during catalysis. Such data should illuminate recurring themes of binding and catalysis used by this and other functionally related enzymes. In conjunction with biophysical data and/or modeling of acceptable substrates and existing leads, this information will provide a guide for the construction of new substructures suitable for development as antibacterial agents. Given sufficient homology between the *mur* genes, such inhibitors could conceivably act at several points in the pathway. Such chemotherapeutic agents may have lessened potential to acquire resistance through mutation(s) in a single target protein.

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