Protein Science (1996), 5:2566–2574. Cambridge University Press. Printed in the USA.

Kinetic and crystallographic studies of *Escherichia coli* UDP-*N*-acetylmuramate:L-alanine ligase

JOHN J. EMANUELE, JR., HAIYONG JIN, BRUCE L. JACOBSON, CHIEHYING Y. CHANG, HOWARD M. EINSPAHR, AND JOSEPH J. VILLAFRANCA

Division of Macromolecular Structure, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000

(RECEIVED July 10, 1996; ACCEPTED September 10, 1996)

Abstract

Uridine diphosphate-*N*-acetylmuramate:L-alanine ligase (EC 6.3.2.8, UNAM:L-Ala ligase or *Mur*C gene product) catalyzes the ATP-dependent ligation of the first amino acid to the sugar moiety of the peptidoglycan precursor. This is an essential step in cell wall biosynthesis for both gram-positive and gram-negative bacteria. Optimal assay conditions for initial velocity studies have been established. Steady-state assays were carried out to determine the effect of various parameters on enzyme activity. Factors studied included: cation specificity, ionic strength, buffer composition and pH. At 37 °C and pH 8.0, k_{cat} was equal to 980 ± 40 min^{-1}, while K_m values for ATP, UNAM, and L-alanine were, 130 ± 10, 44 ± 3, and 48 ± 6 μM, respectively. Of the metals tested only Mn, Mg, and Co were able to support activity. Sodium chloride, potassium chloride, ammonium chloride, and ammonium sulfate had no effect on activity up to 75 mM levels. The enzyme, in appropriate buffer, was stable enough to be assayed over the pH range of 5.6 to 10.1. pH profiles of V_{max}/K_m for the three substrates and of V_{max} were obtained. Crystallization experiments with the enzyme produced two crystal forms. One of these has been characterized by X-ray diffraction as monoclinic, space group C2, with cell dimensions a = 189.6, b = 92.1, c = 75.2 Å, β = 105°, and two 54 kDa molecules per asymmetric unit. It was discovered that the enzyme will hydrolyze ATP in the absence of L-alanine. This L-alanine independent activity is dependent upon the concentrations of both ATP and UNAM; k_{cat} for this activity is less than 4% of the biosynthetic activity measured in the presence of saturating levels of L-alanine. Numerous L-alanine analogs tested were shown to stimulate ATP hydrolysis. A number of these L-alanine analogs produced novel products as accessed by HPLC and mass spectral analysis. All of the L-alanine analogs tested as inhibitors were competitive versus L-alanine.

Keywords: alternate substrate; ATPase; bacterial cell wall biosynthesis; crystallization; endpoint assay; *Mur*C; pH profiles

Among the many known antibacterial strategies, interfering with bacterial cell wall formation has been proven to be one of the most effective. In both gram-positive and gram-negative bacteria, the peptidoglycan layer is responsible for bacterial cell wall strength. One component of the peptidoglycan layer, UDP-*N*-acetylmuramate-pentapeptide, is synthesized intracellularly by the *Mur* enzyme family (van Heijenoort, 1995). Uridine diphosphate-*N*-acetylmuramate:L-alanine ligase (EC 6.3.2.8, UNAM:L-Ala ligase; the *Mur*C gene product) catalyzes the formation of the amide bond between UNAM and L-alanine (Fig. 1). An understanding of the mechanism of this enzyme may lead to the development of a novel set of antibiotics.

There has been a growing interest in obtaining structural and mechanistic information about UNAM:L-Ala ligase. An enzyme with this activity was partially purified from *Bacillus subtilis* and *Bacillus cereus* (Hishinuma et al., 1971). A similar enzyme was purified to near homogeneity from *Staphylococcus aureaus* (Mizuno et al., 1973). Recently, UNAM:L-Ala ligase from *E. coli* was over-expressed and purified to homogeneity (Liger et al., 1995; Falk et al., 1996; Gubler et al., 1996). A detailed study of this enzyme's secondary and quaternary structures showed that the enzyme exists in equilibrium between monomeric and dimeric forms, and that the enzyme's specific activity is independent of its oligomerization state (Jin et al., 1996). It has also been shown that during turnover a single [^{18}O]oxygen is transferred from UDP-*N*-acetyl[^{18}O]muramate to the orthophosphate. This observation is

Reprint requests to: Haiyong Jin, Department of Macromolecular Enzymology, H13-01, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543-4000; e-mail: jin@bms.com.

Abbreviations: UNAM, uridine diphosphate-*N*-acetylmuramate; UNAMA, uridine diphosphate-*N*-acetylmuramate-L-alanine; ADP, adenosine diphosphate; ATP, adenosine 5′-triphosphate; DTT, dithiothreitol; BTP, 1,3 bis[Tris(Hydroxymethyl)-methlamino] propane; MES, 2[N-Morpholino]ethan-sulfonic acid; Tris, Tris(Hydroxymethyl)aminomethane; H-Ala-OMe, 2-amino methyl propionate; H-Ala-OBzl, 2-amino benzyl propionate; H-Ala-NH_2, 2-amino propionamide; H-Ala-N-MeOH, 2-amino N-hydroxymethyl propionamide; H-Ala-OtBu, 2-amino t-butyl propionate; H-Ala-OEt, 2-amino ethyl propionate.

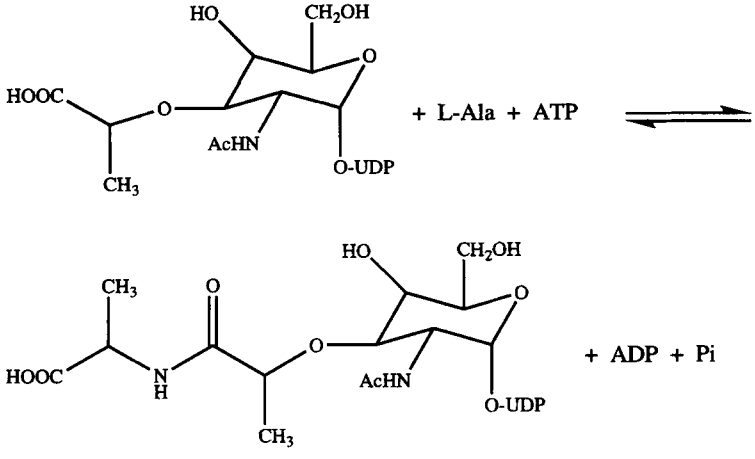

Fig. 1. The reaction catalyzed by UDP-*N*-acetylmuramate: L-alanine ligase.

consistent with catalysis proceeding through an activated acyl-phosphoryl intermediate (Falk et al., 1996). Using an endpoint assay to measure total enzymatic activity over a 30-min time period, the effect of pH, temperature, $MgCl_2$, and phosphate concentration on activity was studied (Liger et al., 1995). A similar study measured activity over a time period over which 50% of the substrate was consumed (Gubler et al., 1996). However, no study has been published outlining the conditions and assay methods necessary for the collection of data under initial velocity conditions.

Kinetic data obtained under initial velocity conditions, in conjunction with transient-state kinetic data, are necessary to establish the kinetic and ultimately the chemical mechanism of an enzyme (Cleland, 1986; Johnson, 1992). In order to perform such studies it is necessary to establish conditions under which the enzyme is both stable and active. It is also necessary to have in place an assay method sensitive enough to measure the amount of product made at times before more than 10% of the substrate is consumed at sub-saturating levels of substrates. The assay method described in this study separates labeled [^{14}C]-UNAMA from unreacted [^{14}C]L-alanine, and is able to determine the rate of UNAMA formation when less than 10% of the substrates were consumed. With this method, it is possible to quantitate the amount of ^{14}C-UNAMA formed at less than 15 s, at substrate concentrations equal to approximately 25% of their K_m values. Under initial velocity conditions, the effect on activity of ionic strength, various cations, and pH was determined.

Uridine diphosphate-*N*-acetylmuramate:L-alanine ligase will hydrolyze ATP if L-alanine is absent as long as UNAM is present. The ATPase activity measured in the absence of L-alanine was only 4% of the activity measured in the presence of saturating levels of L-alanine. The substrate specificity of this enzyme was examined under initial velocity conditions. Values of k_{cat} and k_{cat}/K_m for a number L-alanine analogs were measured using the previously reported coupled enzyme assay (Jin et al., 1996). Novel products were recovered and authenticated from enzyme reactions containing some of these compounds. Three of these compounds were tested as inhibitors; all three were competitive inhibitors versus L-alanine.

Structural studies of the UNAM:L-alanine ligase are also important to understanding of the reaction mechanism. The enzyme has no significant sequence homology to proteins of known three-dimensional structure, and is likely to represent a new class of protein fold. Therefore, UNAM:L-Ala ligase is an excellent candidate for X-ray crystallographic analysis and we have undertaken experiments to crystallize the enzyme to support structural studies.

Results

Assay conditions

Values of k_{cat} and K_m determined in either Tris-HCl or BTP at overlapping pH values were within the limits of experimental error, identical to each other. At pH values of 5.6, 8.0, and 10.1, half-life time values were 13 min, 180 h, and 25 h, respectively (Fig. 2). Since the time required to run either the continuous or the endpoint assay is on the order of 60 s, the enzyme is stable enough to be studied under initial velocity conditions over this pH range.

Metal requirements

The chloride salts of 10 different divalent cations were tested to access their effect on enzyme activity in the presence of sub-

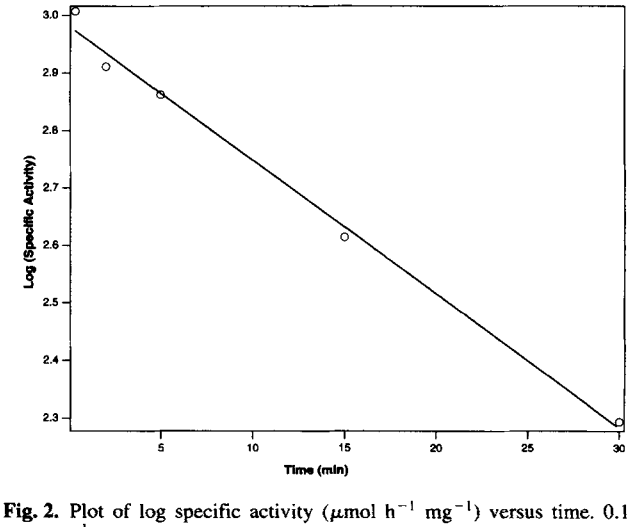

Fig. 2. Plot of log specific activity (μmol h^{-1} mg^{-1}) versus time. 0.17 mg mL^{-1} of enzyme was held at 37 °C in 50 mM AMT pH 5.6, buffer containing 25 mM $(NH_4)_2SO_4$, 2.5 mM β-mercaptoethanol, 1 mM DTT. Aliquots were withdrawn at specified times and assayed for activity using the continuous assay at pH 8.0 and 37 °C. The line is a fit of the data to Equation 3.

saturating amounts of all three substrates. In the absence of added metal there was no detectable activity. Only magnesium, manganese, and cobalt were able to support activity. No detectable activity was observed for the assay mixtures that contained either 1 mM or 40 mM concentrations of the following metal chlorides: $ZnCl_2$, $NiCl_2$, $CdCl_2$, $HgCl_2$, $BaCl_2$, $CaCl_2$, or $FeCl_2$. The largest increase in activity was measured in the presence of 1 mM $MnCl_2$, the specific activity in this assay was 710 ± 30 U/mg compared to a value of 300 ± 10 U/mg measured with 1 mM $MgCl_2$, and 200 ± 5 U/mg measured with 1 mM $CoCl_2$. When the concentration of divalent cation was increased to 40 mM there was no detectable activity with $CoCl_2$. The specific activity measured with the magnesium salt dropped to 270 ± 10 U/mg while the activity measured with $MgCl_2$ rose to 360 ± 15 U/mg. One unit of activity corresponds to 1 μmol UNAMA formed per hour.

When the effect of various metals was tested with all substrates present at saturating levels, the results were somewhat different. Under these conditions, in the presence of 1 mM metal, activities with Mg^{2+}, Mn^{2+}, and Co^{2+} were; 580 ± 30, 650 ± 30, and 530 ± 40 μmol h^{-1} mg^{-1}, respectively. In the presence of 40 mM metal the values measured with Mg^{2+}, Mn^{2+} and Co^{2+} were; 850 ± 60, 430 ± 40, and 160 ± 30 μmol h^{-1} mg^{-1}, respectively.

At 1.4 mM UNAM, 1.5 mM L-alanine, and in the presence of either 1 or 20 mM ATP, $MgCl_2$ over the concentrations range of 1–100 mM had no significant effect on activity (Fig. 3). The effect of $MnCl_2$ concentration on activity was more dramatic. Peak activity at 1 mM ATP was observed at a $MnCl_2$ concentration of about 1.0 mM (data not shown).

Effect of ionic strength

Sodium chloride, potassium chloride, ammonium chloride, and ammonium sulfate were tested to determine their effect on enzyme activity (Fig. 4). The effect on activity of all salts were tested over the concentration range of 10 to 400 mM. All four salts had little effect on activity up to 75 mM. Above 120 mM, the ammonium salt appeared to reduce activity by about 20%, while the sodium salt at this concentration appeared to increase activity by about 20%.

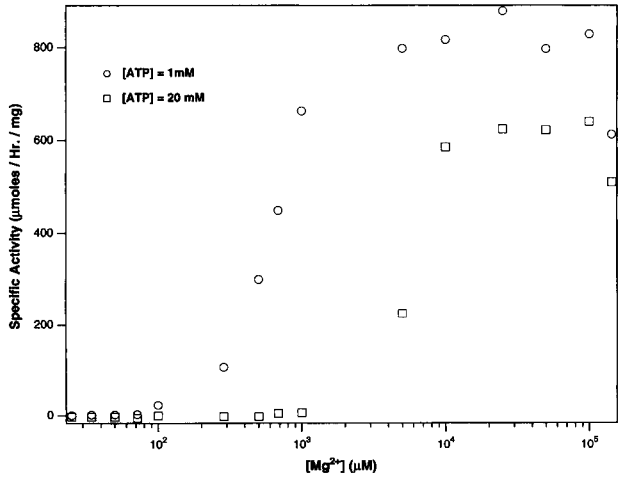

Fig. 3. Plot of specific activity (μmol h^{-1} mg^{-1}) versus log(μM) $MgCl_2$ concentration. Assays were performed at 37 °C, in 100 mM Tris HCl pH 8.0 buffer supplemented with 25 mM $(NH_4)_2SO_4$, 2.5 mM β-mercaptoethanol, 1 mM DTT. Each reaction mixture contained 1.5 mM UNAM, 1.4 mM L-alanine, and either 1 mM ATP or 20 mM ATP.

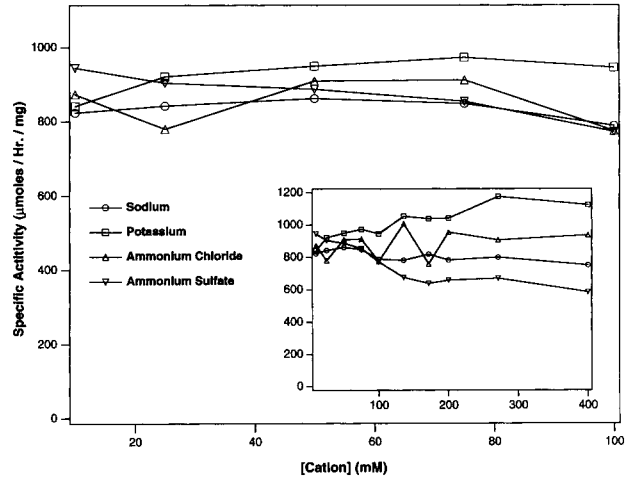

Fig. 4. Plot of specific activity versus salt concentration. Activities were measured in 37 °C, in 100 mM Tris HCl pH 8.0 buffer supplemented with 25 mM $(NH_4)_2SO_4$, 2.5 mM β-mercaptoethanol, 1 mM DTT. Each reaction mixture contained 1.5 mM UNAM, 1.4 mM L-alanine, and 2 mM ATP and one of the following salts; NaCl (open circle), KCl (open square), NH_4Cl_2 (up triangle), or $(NH_4)_2SO_4$, (down triangle).

The effect of pH on V_{max} and V_{max}/K_m values

The effect of pH on the fundamental kinetic constants V_{max} and V_{max}/K_m was studied over the pH range 6.0 to 10.1. Log V_{max} versus pH data fit best to equation 4; activity decreased with a unit slope of one when a group with a pK_a value of 6.1 ± 0.1 was protonated (Fig. 5A).

Plots of log $(V_{max}/K_m)_{ATP}$ values versus pH fit best to equation 5. Protonation of a group with a pK_a value of 6.4 ± 0.2 decreased log $(V_{max}/K_m)_{ATP}$ values with a unit slope of one as did deprotonation of a group with a pK_a value of 9.1 ± 0.2 (Fig. 5B) A similar profile was observed with UNAM; log $(V_{max}/K_m)_{UNAM}$ values decreased with a unit slope of one when a group with a pK_a value of 7.6 ± 0.1 was protonated and when a group with a pK_a value of 8.2 ± 0.1 was deprotonated (Fig. 5C). Qualitatively, the log $(V_{max}/K_m)_{L\text{-}Ala}$ versus pH profile was more like the log V_{max} versus pH profile. The best fit of these data was to Equation 4; values decreased with a unit slope of one when a group with a pK_a value of 7.9 ± 0.4 was protonated (Fig. 5D).

ATPase activity

ATPase activity was measured using the continuous assay. When ATP and enzyme were mixed at pH 8.0 and 37 °C, the rate of ATP hydrolysis was identical to that measured in the absence of enzyme. When enzyme was mixed with ATP and UNAM, the rate of ADP formation was significantly higher than background. The measured L-alanine independent ATPase activity showed Michaelis-Menten kinetics (Fig. 6); the k_{cat} value for ADP formation was 32 ± 1 min^{-1} and the K_m value for ATP was 22 ± 2 μM, while the K_m value for UNAM was 220 ± 33 μM. In the presence of saturating amounts of L-alanine, the k_{cat} was 980 ± 30 min^{-1}, the K_m values for ATP and UNAM and L-alanine were 130 ± 8.0 μM and 44 ± 3 μM, and 48 ± 6, respectively.

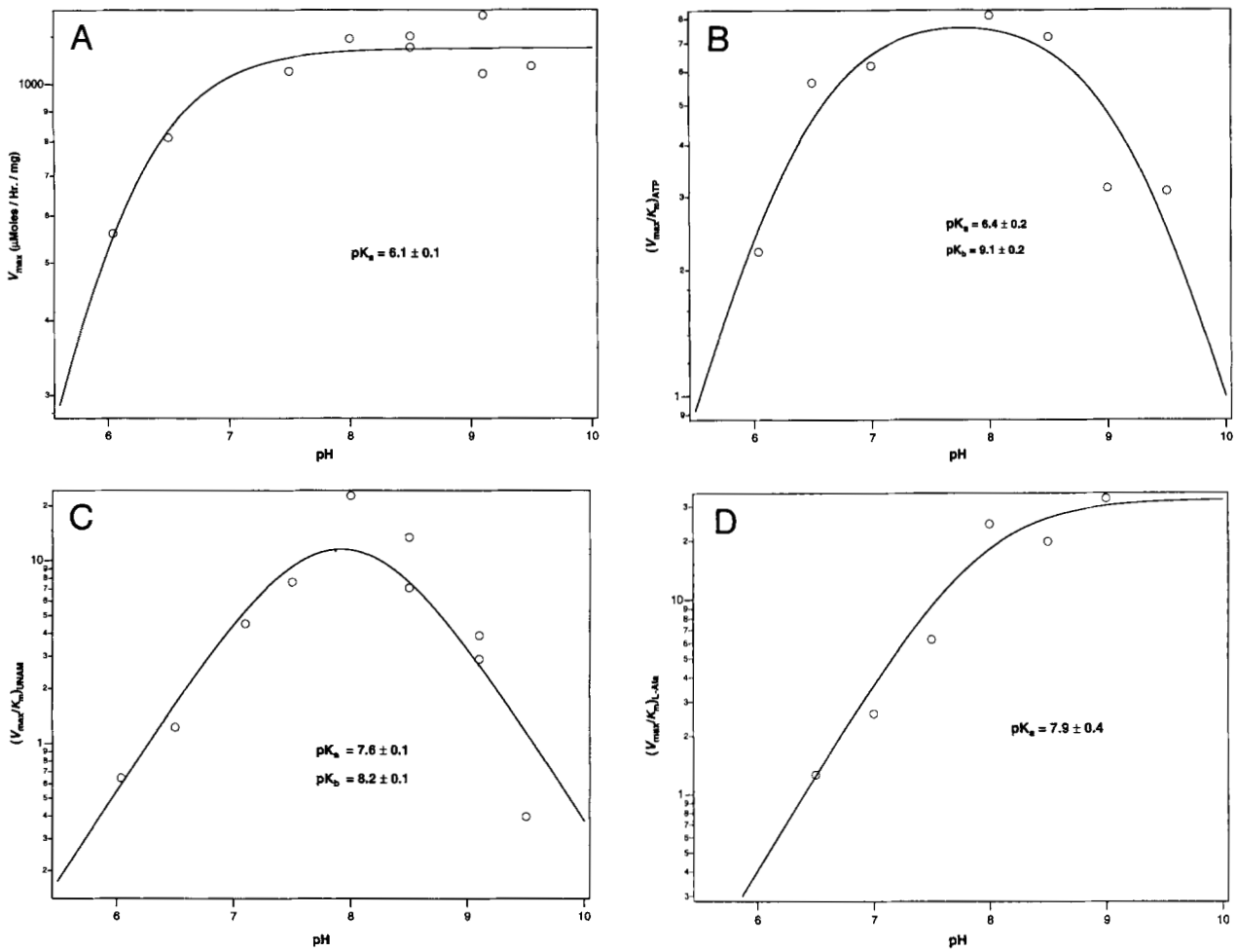

Fig. 5. (**A**) Plot of k_{cat} (min^{-1}) versus pH. The line is a fit of the data to Equation 4. (**B**) $(k_{cat}/K_m)_{ATP}$ plotted versus pH. The line is a fit of the data to Equation 5. (**C**) $(k_{cat}/K_m)_{UNAM}$ plotted versus pH. The line is a fit of the data to Equation 5. (**D**) $(k_{cat}/K_m)_{L\text{-}Ala}$ plotted versus pH. The line is a fit of the data to Equation 4.

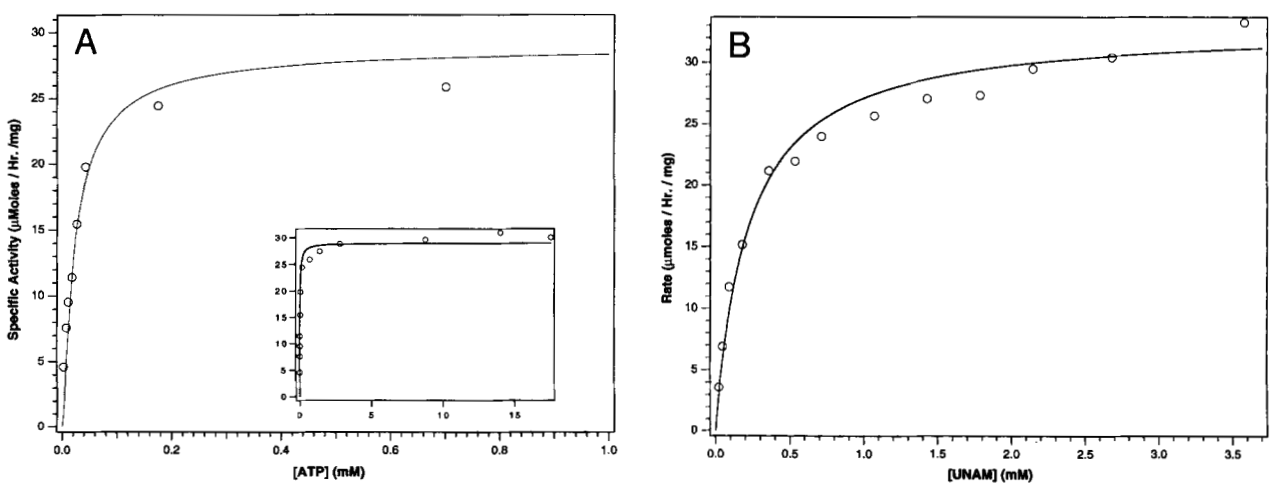

Fig. 6. L-Alanine independent hydrolysis of ATP plotted as a function of substrate concentration. Data were collected at pH 8.0 and 37 °C using the continuous assay. The lines are fits of the data to Equation 1. (**A**) Specific activity plotted as a function of ATP concentration. Insert shows specific activity examined over a wider range of ATP concentration. (**B**) Specific activity plotted as a function of UNAM concentration.

Table 1. *Kinetic constants measured for L-alanine analogs*[a]

Substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\ \mu M^{-1}$)
L-Alanine	980 ± 30	48 ± 6	20 ± 6
L-Serine	1,500 ± 100	1,200 ± 300	1.2 ± 0.3
L-Cysteine	1,200 ± 100	3,800 ± 500	0.30 ± 0.05
Glycine	1,300 ± 100	10,000 ± 1,000	0.14 ± 0.02
L-Threonine	1,400 ± 500	25,000 ± 13,000	0.006 ± 0.004
L-Valine	1,300 ± 100	70,000 ± 14,000	0.017 ± 0.004
L-Isoleucine	310 ± 100	40,000 ± 24,000	0.008 ± 0.006
L-Leucine	—	>42,000	—
H-Ala-OMe	1,700 ± 100	500 ± 100	3.3 ± 0.6
H-Ala-OBz	810 ± 20	360 ± 30	1.6 ± 0.1
H-Ala-OEt	910 ± 20	590 ± 50	2.2 ± 0.3
H-Ala-OtBu	1,300 ± 100	200 ± 10	6.0 ± 0.6
H-Ala-NH_2	1,100 ± 100	7,900 ± 600	0.14 ± 0.01
H-Ala-N-MeOH	700 ± 30	16,000 ± 1,000	0.043 ± 0.003
2-amino-N-butyric acid	1,500 ± 100	4,300 ± 300	0.32 ± 0.02
DL-Propargylglycine	1,200 ± 100	1,100 ± 100	1.1 ± 0.1
L-Vinylglycine	920 ± 50	440 ± 80	2.1 ± 0.4
β-cyano-L-alanine	1,600 ± 100	190 ± 10	8.0 ± 0.5
β-chloro-L-alanine	1,200 ± 100	140 ± 10	9.0 ± 0.6
β-alanine	150 ± 10	46 ± 10	0.055 ± 0.0012
N-(2-aminopropionyl) piperdine	370 ± 40	62,000 ± 15,000	0.0055 ± 0.0016

[a]Activity was measured by following the rate of ADP formation using the coupled enzyme assay, as described in Materials and methods. Only two significant digits are listed.

Kinetic properties in the presence of L-alanine analogs

A number of alanine analogs were tested as substrates for the enzyme. The rate of ADP formation was followed using the continuous assay; k_{cat} and k_{cat}/K_m values for a number of these compounds was determined (Table 1). In agreement with results obtained with the *S. aureus* enzyme (Mizuno et al., 1973) none of the D-enantiomers tested were substrates.

To determine if L-alanine induced ATP hydrolysis was accompanied by biosynthetic activity, samples of reactions run with selected alternate substrates were tested for the presence of novel products. As identified by HPLC and confirmed by mass spectrometric analysis, all alternate substrates tested formed novel products (Table 2).

β-alanine, β-CN-L-alanine, and L-vinylglycine were tested for their ability to inhibit the incorporation of ^{14}C-labeled L-alanine into UNAMA. Inhibition patterns obtained with all three compounds fit best to the equation for competitive inhibition (Equation 3). K_{is} values for β-alanine, β-CN-L-alanine, and L-vinylglycine versus L-alanine were 110 ± 20, 3.3 ± 1.1, and 5.8 ± 1.9 mM, respectively.

Crystallography

Two crystal forms of the UNAM:L-Ala ligase were identified by large-scale screening of crystallization conditions by vapor diffusion. Crystals of the first form were grown from 28–30% (w/v) polyethylene glycol (MW 8000) solutions in 0.18 M Na/K tartrate buffered at pH 7.0 with 0.1 M imidazole. These crystals are very thin plates and have, to this point, remained too small for further analysis. Crystals of the second form (Fig. 7) were obtained from 24–26% (w/v) polyethylene glycol monomethyl ether (MW 5000) solutions in 0.2 M $MgCl_2$, buffered at pH 9.0 with 0.1 M Bicine and containing 3% (v/v) dioxane.

X-ray diffraction data collected from the second crystal form are consistent with a monoclinic system, space group C2, with cell dimensions $a = 189.6$, $b = 92.1$, $c = 75.2$ Å, a beta angle of 105°, and two 54 kDa molecules, possibly forming a dimer, per asymmetric unit. These crystals scatter to about 3.5 Å resolution. Native crystals can be flash-cooled directly from crystallization solutions containing 10% (v/v) glycerol. Work continues to improve and capitalize on these crystal forms for structure studies.

Table 2. *Evidence for the formation of products after reaction of UDP-N-acetylmuramate:L-alanine ligase, UNAM, and ATP*

Substrate	Retention time[a] (min)	Molecular weight[b] (g mol^{-1})
UNAM (Substrate)	2.5	79
L-alanine	7.0	750
β-alanine	5.5	750
2-amino-N-butyric acid	4.7	764
DL-Propargylglycine	4.3	774
β-cyano-alanine	4.7	775
L-vinylglycine	6.2	762
β-chloro-alanine	12.3	784

[a]Retention times were determined by HPLC, absorbence was monitored at 262 nm. HPLC conditions were the same as those used to authenticate UNAM.

[b]Molecular weights were determined by negative ion mass spectrometry.

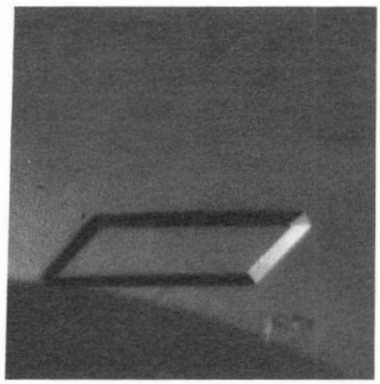

Fig. 7. A typical second form of UNAM:L-Ala ligase crystal (space group C2 with cell dimensions $a = 189.6$, $b = 92.1$, $c = 75.2$ Å, a beta angle of 105°).

Discussion

To obtain kinetic data under initial velocity conditions requires an assay method sensitive enough to follow the rate of reaction when relatively small amounts substrate are consumed. The method described in this study meets this criterion. Using this method, a small amount of labeled [^{14}C]-UNAMA can be separated from unreacted [^{14}C]L-alanine. This makes it possible to measure the amount of ^{14}C-UNAMA formed at less than 15 s, when typically less than 10% of the substrates were consumed, and at initial substrate concentrations equal to approximately 25% of their K_m values.

Kinetic data ought to be collected under conditions wherein the enzyme and substrates are stable and activity is consistently high. Factors affecting enzyme activity and stability include: buffer composition, pH, and ionic strength. Inappropriate buffers may interfere with activity assays by reacting with substrates or by reacting with or binding to the enzyme being assayed. The selection of an appropriate set of buffers is critical when activity is to be determined over a broad range of pH values (Allison & Purich, 1979). If different buffers must be used over the pH range of interest they should be chosen so that at overlapping pH values they exhibit essentially the same effect on enzyme activity. When assays were run at the same pH values, both Tris-HCl and BTP gave virtually identical k_{cat} and K_m values. On the other hand, activity in 0.1 M carbonate/bicarbonate buffer was dramatically different from activity measured in Tris-HCl at the same pH (Liger et al., 1995).

The k_{cat} value observed in this study was in good agreement with the value of 928 min^{-1} obtained at pH 8.6 (Liger et al., 1995). This is reflective of the fact that k_{cat} values were unchanged over the pH range of 8.0–10. Liger et al. reported K_m values of 450, 100, 20 μM for ATP, UNAM and L-Ala, respectively (Liger et al., 1995). The difference between the value reported in this study and that reported by Liger et al. may reflect the fact that K_m values for ATP increase over the pH range 8–10. Though similar K_m values at pH 8.5 for ATP, UNAM, and L-Ala were observed by Gubler et al. (1996), the k_{cat} value reported in that study, 411 min^{-1} at pH 8.5, was about half the value observed in this study, 980 ± 30 min^{-1}. This may be due to difference in how the enzyme was expressed and purified. It is also possible that the aspartic acid to asparagine mutation introduced at position 2 of the *Mur*C polypeptide may have affected k_{cat} (M. Gubler, pers. commun.). This mutation is not present in the protein used by Liger et al. (1995) or in the protein used in the study reported here; k_{cat} values reported in both of these studies are significantly higher than those reported by Gubler et al. (1996).

The metal ion requirement of the ligase reaction was studied under initial velocity condition. At a concentration of 1 mM, $CoCl_2$ could support activity albeit only at 30% of the activity measured with $MnCl_2$ and only at 67% of the activity measured with $MgCl_2$ under the same conditions. When the concentration of $CoCl_2$ was increased to 40 mM, no activity could be detected. None of the other metals tested at either 1 or 40 mM levels were able to support activity. This behavior was similar to that exhibited by the *S. aureus* enzyme (Mizuno et al., 1973). The effect of various divalent cations on activity was also examined in the presence of saturating levels of substrates. Under these conditions, 2.1 mM concentrations of Mg^{2+}, Mn^{2+}, or Co^{2+} had similar effects on activity. When these metal ions were present at the 40 mM levels differences in activity were apparent; activity measured with Mg^{2+} was twice that measured with Mn^{2+} and 6.5-fold higher than activity measured with Co^{2+}.

The effect of ionic strength on activity observed in this study differs somewhat from previously published results. All four salts tested here had little effect on activity up to a concentration of 75 mM. Above the 100 mM level, $(NH_4)SO_2$ appeared to reduce activity by about 20%. As the concentration of NaCl increased to 120 mM, there was a 20% increase in activity. The *S. aureus* form of the enzyme was strongly activated by both ammonium and potassium, showing a maximal activity at 80 mM NH_4^+ (Mizuno et al., 1973). The effect of salt reported here was also different from the result on *E. coli* enzyme reported previously. Gubler et al. report that ammonium sulfate concentrations between 5 and 25 mM stimulated activity by 15% while concentrations of potassium and sodium above 100 mM suppressed activity (Gubler et al., 1996). Although we cannot explain these differences, it is worthy noting that the k_{cat} value of 980 min^{-1} from this study at pH 8.0 with or without 25 mM ammonium sulfate is comparable to the value of 928 min^{-1}, which was obtained at pH 8.5 without ammonium sulfate (Liger et al., 1995). Both values are higher than the k_{cat} value of 411 min^{-1}, which was obtained at pH 9.0 with 25 mM ammonium sulfate (Gubler et al., 1996). It is also worthy commenting that in both studies, 25 mM ammonium sulfate was routinely added to all assays except otherwise noted.

V_{max} values decrease with a unit slope of one when a group with a pK_a value of 6.1 ± 0.1 was protonated. Since V_{max} values can be limited by any unimolecular step on the catalytic pathway (Cleland, 1986), the assignment of a pK_a value to a specific base requires a more detailed analysis of the enzyme mechanism than is reported here.

In a separate study, it was shown that L-alanine is the last substrate to bind in an ordered ter-ter mechanism (Emanuele et al., 1996). For multi-substrate enzymes with a sequential mechanism, only the last substrate to bind has a V_{max}/K_m value, which represents the rate of reaction from binding of that substrate through the first irreversible step (Cleland, 1986). This study demonstrates that protonation of a group with an apparent pK_a value of 7.9 ± 0.4 resulted in a drop of $(V_{max}/K_m)_{L\text{-}Ala}$ values. Since the pK_a of this $(V_{max}/K_m)_{L\text{-}Ala}$ versus pH profiles are, in fact, pK_a values for the enzyme substrate complex (Cleland, 1979), this pK_a value may be that of a group on the enzyme's surface that is involved in abstracting a proton from the amino group of L-alanine. Protonation of this group results in a drop in $(V_{max}/K_m)_{L\text{-}Ala}$ values. This ap-

parent pK_a value may also be that of the substrate itself. The pK_a of L-alanine in aqueous solution is 9.87 (Dawson et al., 1986), which is significantly higher than the value determined in this $(V_{max}/K_m)_{L\text{-}Ala}$ versus pH profile. Since catalysis takes place at the active site of an enzyme and not in bulk solvent, the pK_a value of the substrate may be different in this environment than it is in aqueous solution. It should be pointed out that pK_a values determined from kinetic data are apparent pK_a value and may differ markedly from their intrinsic values (Cleland, 1986). L-Alanine may be a sticky substrate, i.e., once bound, it may partition forward to form product more readily than it dissociates from the enzyme (Cleland, 1986). The pK_a values measured with sticky substrates are often shifted outwards from the intrinsic values. In either case, a deprotonated substrate amino group is consistent with the need for a lone pair of electrons on the amino group being necessary for nucleophilic attack on the putative acyl phosphate intermediate. Clearly, more work is required to determine the identity of the group titrating with the apparent pK_a value of 7.9 ± 0.4.

The enzyme can hydrolyze ATP in the absence of L-alanine but only in the presence of UNAM. The rate of this activity was dependent upon the concentration of both ATP and UNAM. Values of k_{cat}, $(k_{cat}/K_m)_{ATP}$, and $(k_{cat}/K_m)_{UNAM}$ were 3.6%, 21%, and fivefold the values measured for these parameters in the presence of saturating levels of L-alanine. While the rate of ATPase activity observed in the absence of L-alanine is too slow to be on the catalytic pathway of the biosynthetic reaction, it is unclear if the slow rate of turnover observed in the absence of L-alanine is due to the slow rate of ADP formation or to the slow rate of ADP release. Published isotope exchange experiments carried out at saturating levels of L-alanine showed that [^{18}O]oxygen of the carboxyl group of UDP-*N*-acetyl[^{18}O]muramate is transferred to the orthophosphate, consistent with the formation of UDP-*N*-acetylmuramyl-phosphoryl intermediate (Falk et al., 1996). If the observed ATPase activity is due to the slow release of ADP, it may have resulted from non-productive hydrolysis of such an intermediate. The question of the rate of ADP formation versus the rate of ADP release is being addressed by a series of rapid quench experiments.

Clearly, L-alanine with a k_{cat}/K_m value of 20 ± 6 min^{-1} μM^{-1} is the preferred substrate. Values of k_{cat}/K_m determined for other substrates range from values of 0.0055 ± 0.0016 min^{-1} μM^{-1} measured with N(2-aminopropionyl)piperidine to values of 6.4 ± 0.6 min^{-1} μM^{-1} measured with H-Ala-O-tBu. In general, k_{cat}/K_m values correlate well with the structural similarity of the compounds to L-alanine. Both glycine and L-serine have been shown to be substrates for both the *S. aureus* and *E. coli* enzymes (Mizuno et al., 1973; Liger et al., 1991). While the rate of the ligase activity was not measured, evidence that these substrates did form novel products does confirm that the compounds tested both promote ATP hydrolysis and are substrates for the ligase reaction.

Inhibition of UNAM:L-Ala ligase by L-alanine analogs was first observed for the enzymes from both *B. subtilis* and *B. creus* (Hishinuma et al., 1970, 1971). Inhibition by some of these compounds was also demonstrated with the *E. coli* enzyme but under conditions that did not allow the investigators to specify the type of inhibition (Liger et al., 1995; Falk et al., 1996; Gubler et al., 1996). In this study inhibition patterns were determined for β-alanine, β-CN-L-alanine, and L-vinylglycine under initial velocity conditions. The fact that all tested compounds are competitive inhibitors versus L-alanine demonstrates that these compounds do bind to the same form of the enzyme as does L-alanine, the physiological substrate.

In summary, an efficient assay method has been developed to measure the amount of UNAMA formed when, typically, less than 10% of the substrates were consumed, and at substrate concentrations of about 25% of their K_m values. Optimal conditions for assays under initial velocity conditions have been determined. UNAM:L-Ala ligase is stable enough to be studied at 37 °C over the pH range of 5.6 to 10.1. Suitable assay buffers include both Bis-Tris Propane and Tris-HCl. Enzyme activity is dependent upon the presence of either Mn^{2+} or Mg^{2+}. There is an L-alanine independent ATPase activity with the activity of only 3.6% of the biosynthetic activity measured at 37 °C at pH 8.0. The enzyme can use a number of L-alanine analogs as substrates, and appears to be relatively tolerant to structural differences from L-alanine, the physiological substrate. A number of L-alanine analogs have been shown to form novel UNAMA analogs. Finally, it is hoped that the kinetic and crystallographic work reported here can facilitate the determination of the structure and mechanism of this scientifically interesting and physiologically important enzyme.

Materials and methods

Reagents and materials

Reverse phase columns (Hi-Pore 318 preparative 250 × 21.5 cm and analytical 250 × 4.6 cm) were from Bio-Rad. Pyruvate kinase and lactate dehydrogenase were from Boehringer Manneheim. Cation exchange columns (SCX benzenesulfonic acid) were from Varian. Microcon micro-concentrators were from Amicon. Radiolabeled L-alanine, L-[[^{14}C(U)]-$^{14}CH_3{}^{14}CH(NH_2)^{14}COOH$ was purchased from NEN. H-Ala-OtBu, H-Ala-OMe, H-Ala-OBzl, H-Ala-OEt, H-Ala-NH_2, H-Ala-N-MeOH, 2-amino-N-butyric acid, DL-Propargylglycine, L-vinylglycine, β-cyano-L-Ala, β-Cl-L-Ala, and β-alanine were from Bachem Bioscience Inc. L-Ala-ethylester was from Aldrich. Scintillation fluid (EcoLite) was from ICN. All other reagents, unless specified, were from Sigma and were of the highest reagent purity available.

Expression and purification of UDP-N-acetylmuramate: L-alanine ligase

The enzyme was expressed and purified as reported previously (Falk et al., 1996; Jin et al., 1996).

Synthesis of UDP-N-Acetylmuramate and synthesis and purification of UDP-N-acetylmuramate-L-alanine

The synthesis and purification of UDP-*N*-acetylmuramate was as previously reported (Jin et al., 1996). The product of UNAM:L-Ala ligase was prepared by the enzymatic conversion of UNAM to UNAMA. Typical 80 mL reaction mixtures contained the following: 100 mM Tris, pH 8.0, 40 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1.0 mM DTT, 2.5 mM β-mercaptoethanol, 20 mM L-alanine, 15.3 mM UNAM, 20 mM ATP (ATP was added in small aliquots approximately every 12 h). A total of 1000 units of *Mur*C fusion protein was added, half at the beginning of the reaction and half after 24 h. One unit of ligase activity corresponds to 1 μmol of UNAMA formed per hour. Reactions were run at 25 °C and pH was held in the range of 8.0–8.5 by the addition of either KOH or HCl. The progress of the reaction was monitored by HPLC. Protein was removed from small aliquots of reaction mixture by centrifugation at 14,000 rpm for 25 min in a Microcon 10, micro-

concentrator. Samples were analyzed on an Hewlett Packard 1090 HPLC using a Bio-Rad HiPore R318 C-18 column (250 × 4.6 mm). The column was run at a flow rate of 2.25 mL/min at pH 3.3 in 200 mM ammonium formate. Absorbance was monitored at 262 nm. Typical injections were 5 to 25 μL of 1:100 diluted material. The retention times for ATP, ADP, UNAM, and UNAMA were 1.4, 1.5, 2.6, and 4.0 min, respectively. After 84 h, the reaction mixture was filtered over a YM 10 membrane at 4 °C using an Amicon ultrafiltration cell to remove protein and stop the reaction. The filtrate was diluted to 120 mL with water. Five milliliter injections were made onto a preparative HiPore R318 HPLC column (250 × 21.5 mm) run on a BioCAD Perfusion Chromatography Workstation (PerSeptive Biosystems, Cambridge, MA). The column was equilibrated with 50 mM ammonium formate, pH 3.3, and washed for 50 min at a flow rate of 10 mL min^{-1}. Five milliliter column fractions were collected. Fractions rich in pure UNAMA began eluting at 12 min. Purity was established by analytical HPLC. Fractions judged to be pure were pooled and concentrated by roto-vapping under vacuum at 35 °C. The volume of the concentrate was again adjusted to 120 mL. This material was desalted by injecting 5 mL aliquots onto the preparative C-18 column equilibrated in water. Excess ammonium formate was removed by washing the column with water at a flow rate of 5 mL/min. Both absorbance at 262 and conductivity were monitored. Five milliliters fractions were collected. Retention times for ammonium formate and UNAMA were 1.0 and 5.0 min, respectively. Fractions rich in pure UNAMA were pooled and concentrated by roto-vapping under vacuum at 35 °C. The material was stored at −20 °C. Analysis by negative ion mass spectrum gave a molecular weight of 750. The concentration of UNAMA was estimated using an extinction coefficient of 10 100 cm^{-1} M^{-1} at 262 nm.

Determination of protein concentration

The protein concentration was determined by a modification of the Edelhoch method (Pace et al., 1995). Using this approach the extinction coefficient at 280 nm is 27,000 M^{-1} cm^{-1}. This value agrees well with the value published previously (Jin et al., 1996).

Activity assay

Enzyme activity was measured either with a continuous assay or with an endpoint assay. Activity can be monitored continuously by following the rate of ADP formation (Jin et al., 1996). One unit of activity corresponds to 1 μmol ADP formed per hour. The activity for the biosynthetic reaction can be measured directly with an endpoint assay described below. Appropriate amounts of UNAM, [^{14}C]-L-alanine and ATP were incubated at 37 °C in 100 mM Tris HCl, pH 8.0, 2.5 mM β-mercaptoethanol, 20 mM $MgCl_2$, and 25 mM $(NH_4)_2SO_4$. Reactions were initiated by the addition of catalytic amounts of enzyme. Before the addition of enzyme and at 15, 30, and 45 s after the addition of enzyme, 100 μL samples of the reaction mixture were taken. Samples were immediately mixed with 20 μL of glacial acetic acid and stored at 4 °C. Remaining [^{14}C]-L-alanine was separated from [^{14}C]-UNAMA on SCX columns run under 25 kPa of vacuum. Columns were prepared for use by first rinsing with one column volume (1.5 mL) of methanol and then with two column volumes of equilibration buffer (50 mM ammonium formate at pH 3.5). Quenched reaction samples were supplemented with 100 μL of equilibration buffer. Two hundred microliter aliquots of this material was loaded directly unto the columns. Columns were rinsed twice with 1.5 mL of 50 mM ammonium formate at pH 3.5. The filtrate was collected in 20 mL scintillation vials and dissolved in 10 mL of scintillation fluid. Whenever the concentration of [^{14}C]-L-alanine was changed a blank sample was run to access chromatographic efficiency. All radioactive samples were counted for three minutes on a Beckman model LS5000TD liquid scintillation counter.

Metal ion specificity

Activity was measured at pH 8.0 and at 37 °C using the endpoint assay in 100 mM Tris, pH 8.0, 25 mM $(NH_4)_2SO_4$, 1 mM DTT, 2.5 mM β-mercaptoethanol, 0.3 mM UNAM, 0.65 mM Tris-ATP, and 0.3 mM L-alanine supplemented with either 1 mM or 40 mM of the metal chloride salt. A reaction with no metal was also run to determine what effect, if any, trace metals in the assay mixture might have on activity. Assays were also run using the following substrate concentrations; 600 μM UNAM, 600 μM L-Ala, 2.0 mM ATP at both 2.1 mM and 40 mM concentrations of the metal chloride salts.

The effect of $MgCl_2$ on activity was measured using the endpoint assay at both 1 mM and 20 mM concentrations of Tris-ATP. Each assay contained; 1.54 mM UNAM, 1.4 mM L-alanine, 100 mM Tris-HCl, pH 8.0, 50 mM $(NH_4)_2SO_4$, 2.5 mM β-mercaptoethanol, and 1 mM DTT. The effect of $MnCl_2$ concentration on activity was measured over the concentration range 0.025–10 mM at pH 8.0 and 37 °C; substrate concentrations were ATP, 1.0 mM, L-alanine, 1.4 mM, and UNAM, 1.54 mM.

Salt specificity

Activity was measured as a function of salt concentration using the endpoint assay. Reaction mixtures contained 40 mM $MgCl_2$. In the place of 25 mM $(NH_4)_2SO_4$ used in the standard assay one of the following salts was used: NaCl, KCl, NH_4Cl, or $(NH_4)_2SO_4$. All salts were tested over the concentration range, 10–400 mM.

Activity and stability versus pH

Assay buffers used were either Tris-HCl over the pH range 7.3–9.0 or Bis-Tris Propane (BTP), over the pH ranges of 6.0–7.3 and 9.0–10.1. The effect of buffers on activity was assessed by running assays at overlapping pH values. Activities over the pH range 6.8–9.0 were generally measured using the continuous assay. Activities below and above this range were measured using the endpoint assay. When tested at identical pH values, both the continuous and endpoint assays gave, within experimental errors, values identical to each other. Stability was determined by incubating 0.17 μg of enzyme in 1 mL of the appropriate assay buffer. Bis-Tris propane was used at pH 10.1; Tris HCl was used at pH 8.0. Stability at pH 5.6 was accessed by incubating the enzyme in assay buffer made with 50 mM acetic acid, MES, triethanolamine (AMT). Samples held at 37 °C were withdrawn periodically and assayed for activity at pH 8.0 and 37 °C using the coupled enzyme assay.

Kinetic parameters measured with L-alanine analogs

Activity was measured by following the rate of ADP formation using the continuous assay at pH 8 and 37 °C in the presence of 0.5 mM UNAM and 2 mM ATP and varying concentrations of

alanine analogs. All reactions were initiated by the addition of enzyme. Inhibition by these compounds was measured at pH 8.0 and 37 °C using the endpoint assay. The rate of incorporation of ^{14}C L-alanine into UNAMA was measured at 10 different concentrations of L-alanine ranging from 20–500 μM in the presence 0.5 mM UNAM and 2 mM ATP. The assay was repeated in the presence of three different concentrations of each inhibitor tested.

Isolation of reaction products from L-alanine analogs

One milliliter overnight reaction mixtures contained 100 mM Tris-HCl, pH 8.0, 25 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 1.0 mM DTT, 2.5 mM β-mercaptoethanol, 0.5 mM UNAM, 5 mM ATP, and saturating concentrations of L-alanine analogs. Samples containing reaction products were prepared and analyzed using the same conditions for UNAMA. The molecular weight of novel compounds was determined by negative ion mass spectrometry.

Data analysis

Steady-state rate data were fit to Equation 1, and data collected with inhibitors were fit to Equation 2 (Cleland, 1979). Stability data were fit to Equation 3 (Segel, 1976). Data obtained by varying pH were fit to either Equation 4 or 5 (Cleland, 1979). Equation 4 was used to fit rates that decreased with a unit slope at low pH. Equation 5 was used to fit rates that decreased with a unit slope of one at both high and low pH. Fits of the data to Equations 1, 2, 4, and 5 were performed using KinetAsyst (IntelliKinetics, Princeton, NJ). Fits of data to Equation 3 were performed using NonLin for Macintosh, Robelko Software, Carbondale, IL. This is a Macintosh-compatible version of software developed by Johnson and Frasier (1985), modified for use on the Macintosh by R. J. Brenstein. The confidence limits of the optimized parameters were set at 67%.

$$v = \frac{V_{max}A}{K_m + A} \quad (1)$$

$$v = \frac{V_{max}S}{K_m\left(1 + \frac{I}{K_{is}}\right) + S} \quad (2)$$

$$\log N = -\frac{\lambda}{2.3}t + \log N_0 \quad (3)$$

$$\log Y = \log\left(\frac{C}{1 + \frac{[H^+]}{K_1}}\right) \quad (4)$$

$$\log Y = \log\left(\frac{C}{1 + \frac{K_2}{[H^+]}\frac{[H^+]}{K_1}}\right). \quad (5)$$

Acknowledgments

We would like to thank Drs. Hsu-Tso Ho and Micheal Pucci for providing the cell lines that individually overexpress *Mur*A, *Mur*B, and *Mur*C gene products, Bethanne Warrack for mass spectrometric analysis, Dr. Hannguang Chao for the generous gift of N-(2-aminopropionyl) piperidine, Joseph Yanchunas for technical assistance in the early phase of the project, and Drs. Adil Dhalla and James Robertson for helpful discussions.

References

Allison RD, Purich DL. 1979. Practical considerations in the design of initial velocity enzyme rate assays. *Methods Enzymol 63*:3–22.

Cleland WW. 1979. Statistical analysis of enzyme kinetic data. *Methods Enzymol 63*:103–138.

Cleland WW. 1986. Enzyme kinetics as a tool for determination of enzyme mechanism. In: Bernasconi CF, ed. *Investigation of rates and mechanisms of reactions*. 4th ed, vol. 6, part 1. New York: John Wiley & Sons. pp 791–868.

Dawson MC, Elliott DC, Elliott WH, Jones KM. 1986. *Data for biochemical research*. Oxford, UK: Clarendon Press. pp 4.

Emanuele JJ Jr, Villafranca JJ, Jin H, 1996. Steady-state kinetic mechanism of *Escherichia coli* UDP-*N*-acetylmuramate:L-alanine ligase. *Biochemistry*. Submitted.

Falk PJ, Ervin KM, Volk KS, Ho H-T. 1996. 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. *Biochemistry 35*:1417–1422.

Gubler M, Appoldt Y, Keck W. 1996. Overexpression, purification, and characterization of UDP-*N*-acetylmuramyl:L-alanine ligase from *Escherichia coli*. *J Bacteriol 178*:906–910.

Hishinuma F, Izaki K, Takahashi H. 1971. Inhibition of L-alanine adding enzyme by glycine. *Agric Biol Chem 35*:2050–2058.

Hishinuma F, Izaki K, Takahashi H. 1970. Inhibition of incorporation of L-alanine into uridine-diphospho-N-acetylmuramic acid by glycine. *Agri Biol Chem 34*:655–657.

Jin H, Emanuele JJ Jr, Fairman R, Robertson JG, Hail ME, Ho H-T, Falk PJ, Villafranca JJ. 1996. Structural studies of *Escherichia coli* UDP-*N*-acetylmuramate:L-alanine ligase. *Biochemistry 35*:1423–1431.

Johnson KA. 1992. Transient-state kinetic analysis of enzyme reaction pathways. In: Sigman DS, ed. *The enzymes*. Vol. XX, third ed. New York: Academic Press. pp 1–61.

Johnson ML, Frasier SG. 1985. Nonlinear least-squares analysis. *Methods Enzymol 117*:301–342.

Liger D, Blanot D, van Heijenoort J, 1991. Effect of various alanine analogues on the L-alanine-adding enzyme from *Escherichia coli*. *FEMS Microbiol Lett 80*:111–116.

Liger D, Masson A, Blanot D, van Heijenoort J, Parquet C. 1995. Overproduction, purification and properties of the uridine-diphosphate-*N*-acetylmuramate:L-alanine ligase from *Escherichia coli*. *Eur J Biochem 230*:80–87.

Mizuno Y, Yaegashi M, Ito E. 1973. Purification and properties of uridine diphosphate N-acetylmuramate:L-alanine ligase. *J Biochem 74*:525–538.

Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. 1995. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci 4*:2411–2423.

Segel I. 1976. *Enzyme kinetics*. New York: John Wiley & Sons. pp 39–43.

van Heijenoort J. 1995. In: Ghuysen JM, Hakenbeck R, eds. *Bacterial cell wall*. New York: Elsevier. pp 39–54.