Evaluation of the Kinetic Mechanism of *Escherichia coli* Uridine Diphosphate-*N*-acetylmuramate:L-Alanine Ligase†

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ABSTRACT: Initial velocity methods were used to probe the kinetic mechanism of *Escherichia coli* uridine diphosphate-*N*-acetylmuramate:L-alanine ligase (UNAM:L-Ala ligase). When the activity (in the forward direction) versus substrate concentration data were plotted in double-reciprocal form, all line patterns were intersecting. The best fit of these data was to the equation for an ordered mechanism with the following parameters: k_{cat}, 1000 ± 100 min^{-1}; K_{ma}, 210 ± 40 μM; K_{mb}, 84 ± 20 μM; K_{mc}, 70 ± 15 μM; K_{ia}, 180 ± 50 μM; K_{ib}, 68 ± 24 μM. Initial velocity line patterns were also determined when the concentration of one substrate was varied at different fixed concentrations of a second substrate while the third substrate was held at a concentration more than 100 times its K_m value. Reciprocal plots of data collected with either ATP or L-alanine present at more than 100 times their K_m values resulted in intersecting line patterns. Data collected with UNAM present at 100 times its K_m value gave a set of parallel lines. These data are consistent with UNAM binding as the second substrate in an ordered mechanism. ADP, uridine diphosphate-*N*-acetylmuramoyl-L-alanine (UNAMA), and phosphate were tested as product inhibitors versus substrates. None of the products were competitive inhibitors versus L-alanine or UNAM, while the only observed competitive inhibition was ADP versus ATP. These results are consistent with an ordered kinetic mechanism wherein ATP binds first, UNAM binds second, and ADP is the last product released. Rapid quench experiments were performed in the presence of all three substrates or in the presence of ATP and UNAM. The production of acid-labile phosphate as a function of time is characterized by a burst phase followed by a slower linear phase with the rate close to k_{cat} in the presence of all three substrates. Only a burst phase was observed for the time course of the reaction in the presence of ATP and UNAM. In both cases, the burst rate was identical. These observations are consistent with L-alanine being the third substrate to bind in a sequential mechanism involving a putative acyl−phosphate intermediate.

Uridine diphosphate-*N*-acetylmuramate:L-alanine ligase (EC 6.3.2.8, UNAM:L-Ala ligase or *Mur*C gene product)[1] belongs to a family of enzymes involved in bacterial cell wall biosynthesis (van Heijenoort, 1995). This enzyme is responsible for catalyzing the formation of the amide bond between UNAM and L-alanine (Figure 1). Since it catalyzes one of the essential steps in cell wall biosynthesis in both Gram-negative and -positive bacteria, an understanding of its catalytic mechanism may be of pharmaceutical significance (van Heijenoort, 1995).

The catalytic mechanisms of several enzymes in the *Mur* pathway have been studied extensively. Mechanistic studies have been reported for the enzymes involved in bacterial D-alanine metabolism (Wright & Walsh, 1992), the *Mur*A gene product (Marquardt *et al.*, 1994), and the *Mur*B gene product (Benson *et al.*, 1995; Dhalla *et al.*, 1995). Relatively little has been published on the mechanism of the *Mur*C gene product, UNAM:L-Ala ligase. It was reported that during the course of the reaction a single [^{18}O]oxygen was transferred from UDP-*N*-acetyl[^{18}O]muramate to the orthophosphate. This observation is strongly suggestive of an activated acyl−phosphate intermediate (Falk *et al.*, 1996). Several papers have been published reporting the effect of pH, ionic strength, metal concentration, and buffer composition on activity (Liger *et al.*, 1995; Gubler *et al.*, 1996; Emanuele *et al.*, 1996). Recently, optimal assay conditions for initial velocity studies were reported (Emanuele *et al.*, 1996).

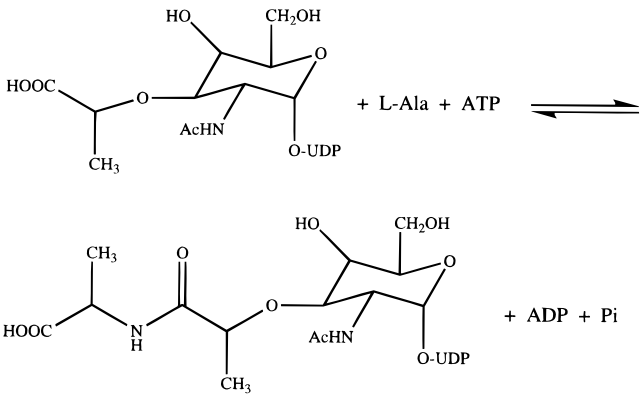

FIGURE 1: Reaction catalyzed by UDP-*N*-acetylmuramate:L-alanine ligase.

† The initial velocity and product inhibition data were presented at the Joint Annual Meeting of American Society for Biochemistry and Molecular Biology, American Society for Investigative Pathology, and The American Association of Immunologists in New Orleans, LA, June 2−6, 1996.

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[1] Abbreviations: UNAM, uridine diphosphate-*N*-acetylmuramate; UNAMA, uridine diphosphate-*N*-acetylmuramoyl-L-alanine; ADP, adenosine diphosphate; ATP, adenosine 5′-triphosphate; DTT, dithiothreitol.

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However, to date no detailed study of this enzyme's kinetic mechanism has been published.

Essential to understanding the chemical mechanism of an enzyme is an understanding of its steady state kinetic mechanism (Viola & Cleland, 1982; Cleland, 1986). While the kinetic mechanism cannot prove the chemical mechanism, no chemical mechanism inconsistent with the kinetic mechanism can be valid. Initial velocity data collected as a function of varying substrate concentrations can give significant insights into the kinetic mechanism of a given enzyme (Segel, 1975; Viola & Cleland, 1982). Product inhibition patterns are also extremely useful in determining the order of substrate addition and product release (Cleland, 1986). In this study, a combination of these techniques was used to derive a kinetic mechanism which is consistent with the available data.

MATERIALS AND METHODS

Reagents and Materials. Pyruvate kinase and lactate dehydrogenase were from Boehringer Mannheim. Cation exchange columns (SCX benzenesulfonic acid) were from Varian. [^{14}C]L-alanine (150 mCi/mmol) was purchased from New England Nuclear. [γ-^{32}P]ATP (3000 Ci/mmol) was also purchased from New England Nuclear and was purified prior to use according to Lewis and Villafranca (1989). Scintillation fluid (EcoLite) was from ICN. All other reagents, unless specified, were from Sigma and were of the highest reagent purity available.

Preparation of UDP-N-acetylmuramate:L-Alanine Ligase, UDP-N-acetylmuramate, and UDP-N-acetylmuramoyl-L-alanine. The recombinant enzyme was expressed and purified as previously published (Jin *et al.*, 1996; Falk *et al*., 1996). The synthesis and purification of UNAM and UNAMA were by methods described earlier (Jin *et al.*, 1996; Emanuele *et al.*, 1996).

Activity Assay. Enzyme concentration was estimated by measuring the absorbance at 280 nm using an extinction coefficient of 27 000 cm^{-1} M^{-1} (Emanuele *et al.*, 1996). Enzyme activity was measured using either a continuous assay (Jin *et al.*, 1996) or an end point assay (Emanuele *et al.*, 1996). Briefly, in the continuous assay, the rate of ADP formation is followed by measuring the rate of NADH oxidation at 340 nm; 1 mL of assay mixture contained 100 mM Tris, pH 8.0, 0.2 mM phospho(enol)pyruvate, 0.38 mM NADH, 25 mM $(NH_4)_2SO_4$, 20 mM $MgCl_2$, 2.5 mM β-mercaptoethanol, 1 mM DTT, 70 μg of pyruvate kinase, and 70 μg of lactate dehydrogenase, as well as appropriate amounts of UNAM, ATP, and L-alanine. End point assays, which measure the biosynthetic reaction directly, contained, other than appropriate amounts of UNAM, ATP, and L-alanine, 100 mM Tris-HCl, pH 8.0, 25 mM $(NH_4)_2SO_4$, 2.5 mM β-mercaptoethanol, 1 mM DTT, and 20 mM $MgCl_2$ unless noted otherwise. A 100 μL aliquot of an end point reaction mixture was quenched by vigorous mixing with 20 μL of glacial acetic acid. Unreacted [^{14}C]L-alanine was separated from labeled UNAMA by cation chromatography using SCX columns (benzenesulfonic acid). All radioactive samples were counted for 3 min on a Beckman model LS5000TD liquid scintillation counter. All assays were initiated by the addition of UNAM:L-Ala ligase at a concentration low enough to give a linear signal for at least 1 min. One unit of activity corresponds to 1 μmol of ADP or UNAMA formed/h.

Initial Velocity Data. Data were collected using a 4 $\times$ 4 $\times$ 4 matrix of substrate concentrations; substrate concentrations used were as follows: ATP at 1300, 190, 80, and 60 μM; UNAM at 400, 76, 35, and 25 μM; L-alanine at 400, 76, 34, and 25 μM. Varied substrate concentrations ranged from 0.5 to 10 times their published K_m values. Published K_m values for ATP, UNAM, and L-alanine were 130 $\pm$ 8, 44 $\pm$ 3, and 48 $\pm$ 6 μM, respectively (Emanuele *et al.*, 1996). Amount of enzyme used for the assays was between 1.0 and 6.2 μg in 1 mL of assay mixture. Data were also collected under conditions wherein one substrate was fixed at a concentration more than 100 times its published K_m value. Concentrations of fixed substrate used in these assays were ATP at 13.8 mM, UNAM at 5.85 mM, and L-alanine at 10 mM. Varied substrate concentrations were the same as those used at the subsaturating conditions. Amount of enzyme used for these assays was between 0.6 and 3.1 μg in 1 mL of assay mixture.

Product Inhibition Data. Activity was determined as a function of substrate concentration in the presence of individual products with the end point assay. Activity was measured at 10 different concentrations of the varied substrate ranging from 0.25 to 10 times its published K_m value. Fixed substrates were held at concentrations of 20 times their published K_m values or higher. Assays without added phosphate contained 20 mM $MgCl_2$. Assays with added phosphate contained manganese at a 1:1 stoichiometry with ATP; no $MgCl_2$ was added to these reactions. Activity versus ATP concentration was measured at four concentrations of UNAMA (0, 0.15, 0.30, and 0.60 mM) and at three concentrations of ADP (0, 45, and 180 μM). Activity versus UNAM concentration was measured at four concentrations of UNAMA (0, 0.15, 0.30, and 0.60 mM), at three concentrations of ADP (0, 1.0, and 4.6 mM), and at three concentrations of potassium phosphate (0.0, 110, and 210 mM). Activity versus L-alanine concentration was measured at four concentrations of UNAMA (0, 0.25, 0.50, and 1.0 mM), at three concentrations of ADP (0, 6.4, and 13 mM), and at three concentrations of potassium phosphate (0, 110, and 213 mM). Amount of enzyme used for these assays was between 0.5 and 1.5 μg in 350 μL of assay mixture.

Rapid Quench Experiments. The rapid quench experiments were performed at 37 °C with a KinTek Corp. model RQF-3 quench flow apparatus. The reactions were typically executed by loading the enzyme into one sample loop and substrates into the other. Final concentrations of the reactants upon mixing in a volume of 67 μL were 46 μM UNAM:L-Ala ligase, 100 mM Tris, pH 8, 20 mM $MgCl_2$, 25 mM $(NH_4)_2SO_4$, 5 mM β-mercaptoethanol, 100 000$-$250 000 cpm [γ-^{32}P]ATP, 1.5 mM ATP, 0.55 mM UNAM, and 0.59 mM L-alanine (if present). The reactions were quenched using 0.5 M HCl and then neutralized with 1 M Tris containing 4 N KOH. [^{32}P]Phosphate was isolated as described by Johnson (1986). The radioactivity of each sample was determined by the Cerenkov method, by counting on the tritium channel in the absence of scintillation fluid. The concentration of phosphate was then calculated by normalizing the data relative to the ATP concentration of the original sample (Johnson, 1986).

Data Analysis. The nomenclature for steady state kinetics used in this paper is that of Cleland. All fits of experimental data to equations were global fits and were done in real space. Graphs in double-reciprocal form were created with Igor

(WaveMetrics, Lake Oswego, OR), using the fit equation in reciprocal form and parameters calculated from real space fits of the data. Initial velocity data collected when the concentrations of all three substrates were varied were fit to numerous ter reactant mechanistic models including ordered, bi bi uni uni, bi uni uni bi, uni uni bi bi, and hexa uni ping pong mechanisms (Segel, 1975). The equation for the ordered mechanism is

$$\frac{v}{V_{max}} = \frac{[A][B][C]}{K_{ia}K_{ib}K_{mc} + K_{ib}K_{mc}[A] + K_{ia}K_{mb}[C] + K_{mc}[A][B] + K_{mb}[A][C] + K_{ma}[B][C] + [A][B][C]} \quad (1)$$

in which v is the observed reaction rate, V_{max} is the maximum rate, [A], [B], and [C] are individual substrate concentrations, K_{ma}, K_{mb}, and K_{mc} are individual Michaelis constants, and K_{ia} and K_{ib} are the dissociation constants for substrates A and B, respectively. Steady state data collected when the concentrations of two substrates were varied in the presence of saturating levels of the third substrate were fit to either eq 2 or 3 (Segel, 1975):

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_{ia}K_{mb} + K_{mb}[A] + K_{ma}[B] + [A][B]} \quad (2)$$

$$\frac{v}{V_{max}} = \frac{[A][B]}{K_{mb}[A] + K_{ma}[B] + [A][B]} \quad (3)$$

Fits of data to the steady state mechanistic models and to eqs 2 and 3 were done 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%. All inhibition data were fit to either eq 4, 5, or 6:

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{(I)}{K_{is}}\right)\frac{1}{[S]} + \frac{1}{V_{max}} \quad (4)$$

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_{ii}}\right) \quad (5)$$

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_{is}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_{ii}}\right) \quad (6)$$

in which K_m is the Michaelis constant, [S] and [I] are the substrate and inhibitor concentrations, respectively, and K_{is} and K_{ii} are the slope inhibition and intercept inhibition constants, respectively. Fits of data to eqs 4−6 were done using *KinetAsyst* software (IntelliKinetics, Princeton, NJ). The best fit was taken to be the one which had the lowest σ value with the fewest number of variables and the smallest relative error in each parameter.

Rapid quench data were fit, by using *KaleidaGraph* (version 3.04, Abelbeck Software), to either eq 7 or 8:

$$[Pi] = \alpha[E]_t[1 - \exp(-k_b t)] \quad (7)$$

$$[Pi] = \alpha[E]_t[1 - \exp(-k_b t)] + Lt \quad (8)$$

Table 1: Global Fitting Analysis of the Forward Initial Velocity Data[a]

mechanism model	variance of fit
ordered[b]	3.0×10^2
bi bi uni uni[c]	6.8×10^2
bi uni uni bi[d]	6.8×10^2
uni uni bi bi[e]	7.6×10^2
hexa uni ping pong[f]	1.2×10^3

[a] Obtained from *NonLin for Macintosh* with confidence probability and variance threshold set at 67% and 0.0001, respectively. [b] Equation IX-262 on p 700 (Segel, 1975). [c] Equation IX-305 on p 726 (Segel, 1975). [d] Equation IX-233 on p 687 (Segel, 1975). [e] Equation IX-307 on p 727 (Segel, 1975). [f] Equation IX-315 on p 730 (Segel, 1975).

in which [Pi] is the phosphate concentration, $[E]_t$ is the concentration of UNAM:L-Ala ligase, α is the unitless burst amplitude, k_b is the observed single-exponential burst rate, L is the observed linear rate, and t is the reaction time.

RESULTS

Initial Velocity Line Patterns. Three separate sets of initial velocity data were collected under identical conditions. These data were fit globally, using individual data sets, all three data sets, or an average of all three data sets, to numerous mechanisms including ordered, bi bi uni uni, bi uni uni bi, uni uni bi bi, and hexa uni ping pong. The variance of the global fits of all three sets of data to these mechanisms is shown in Table 1. It is evident that in all cases, attempts to fit these data to equations describing kinetic mechanisms other than the ordered mechanism resulted in fits with significantly higher variance of fit. The best fit of the data was to the ordered mechanism. A typical subset of data and fit are shown in Figure 2. For clarity, data shown are average values of all three data sets represented in reciprocal space. The lines were drawn using parameters calculated from a fit of all three unaveraged data sets to eq 1. The parameter values from a global fit of these data to eq 1 were as follows: turnover rate (k_{cat}), 1000 ± 100 min^{-1}; K_{ma}, 210 ± 40 μM; K_{mb}, 84 ± 20 μM; K_{mc}, 70 ± 15 μM; K_{ia}, 180 ± 50 μM; K_{ib}, 68 ± 24 μM.

Initial velocity data were collected with one of the substrates held at a concentration more than 100 times its published K_m value. The best fit of the data collected in the presence of 13 mM ATP was to eq 2 describing an intersecting line pattern (Figure 3A). Kinetic constants from this fit were as follows: k_{cat}, 1300 ± 100 min^{-1}; K_a, 36 ± 7 μM; K_b, 44 ± 8 μM; K_{ia}, 110 ± 30 μM. The best fit of data collected when UNAM was held at 5 mM was to eq 3 describing a parallel line pattern (Figure 3B). Values from this fit were as follows: k_{cat}, 1100 ± 100 min^{-1}; K_a, 130 ± 20 μM; K_b, 50 ± 8 μM. The best fit of the data collected in the presence of 10 mM L-alanine was to eq 2 describing an intersecting line pattern (Figure 3C). Values from this fit were as follows: k_{cat}, 1400 ± 100 min^{-1}; K_a, 120 ± 20 μM; K_b, 73 ± 12 μM; K_{ia}, 240 ± 60 μM.

Product Inhibition. Product inhibition data are summarized in Table 2. Fixed substrates were held at concentrations of 20−100 times their published K_m values. Experiments were repeated at different fixed substrate concentrations than those reported in Table 2, and the observed inhibition patterns were qualitatively the same. Versus all three substrates, UNAMA affected both the slope and intercept terms in double-reciprocal plots (data not shown). The best

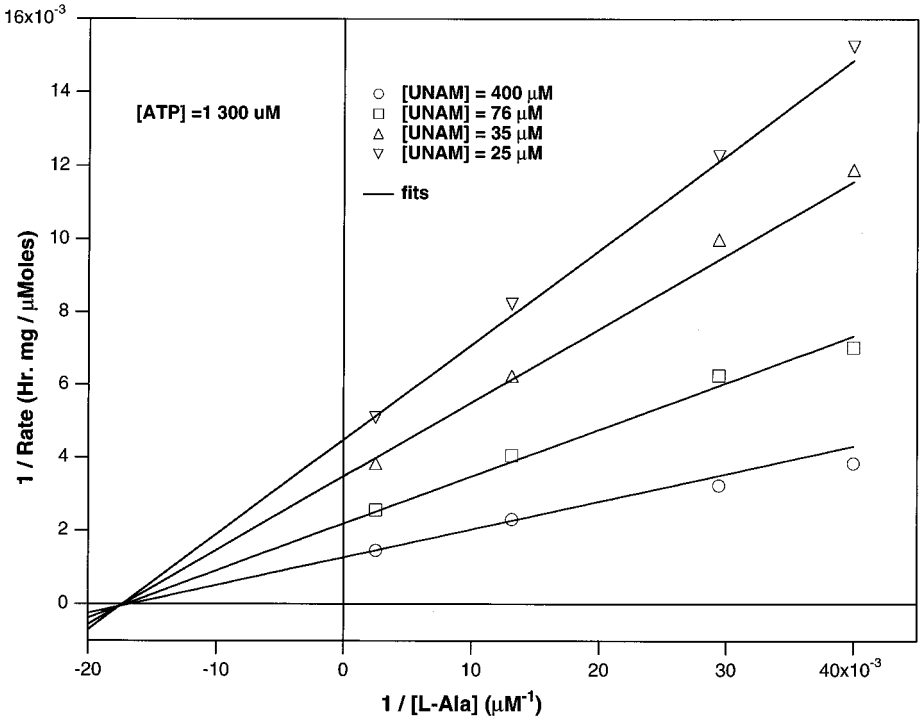


FIGURE 2: Representative initial velocity kinetic data presented in reciprocal form. Data points represent the average of three data sets. The lines belong to a subset of the global fits of all three data sets to eq 1. Data collected while varying L-alanine concentration with 1300 μM ATP present at the following concentrations of UNAM: 400 μM (○), 76 μM (□), 35 μM (△), 25 μM (▽).

fit of product inhibition data collected with UNAMA versus all three substrates was to eq 6 for noncompetitive inhibition. ADP was a noncompetitive inhibitor versus either UNAM or L-alanine; inhibition data collected with ADP versus either UNAMA or L-alanine fit best to eq 6. The best fit of ADP versus ATP data was to eq 4. ADP was a competitive inhibitor versus ATP with a K_{is} value of 14 ± 3 μM. Phosphate was an uncompetitive inhibitor versus L-alanine; the best fit of these data was to eq 5. Phosphate was a noncompetitive inhibitor versus UNAM; the best fit of these data was to eq 6. Replots of data collected when MnATP concentration was varied in the presence of high concentrations of phosphate were not linear. All attempts to fit these data to models describing multiple binding of inhibitor molecules to the same form of the enzyme were unsatisfactory (Cleland, 1986, 1979).

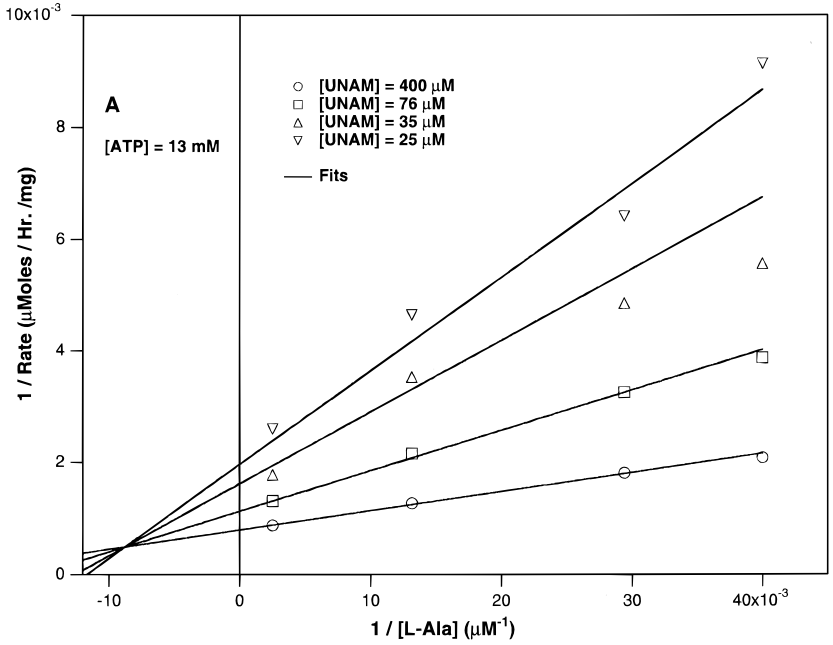
Rapid Quench. Rapid quench experiments were carried out in the presence of all three substrates or in the presence of ATP and UNAM. The results of the experiments are shown in Figure 4. The production of acid-labile phosphate as a function of time is characterized by a burst phase followed by a slower linear phase with the rate close to k_{cat} in the presence of all three substrates. The kinetic constants obtained from the fit of the data to eq 8 are as follows: $\alpha = 0.56 \pm 0.05$, $k_b = 110 \pm 20$ s^{-1}, and $L = 580 \pm 30$ μM s^{-1}. The ratio of the linear rate over the enzyme concentration is 13 ± 1 s^{-1}, which is close to the k_{cat} value of 15 ± 1 s^{-1} calculated from steady state kinetic data. Only a burst phase was observed for the time course of the reaction in the presence of ATP and UNAM. The best fit of this data was to eq 7 with the following kinetic constants: $\alpha = 0.60 \pm 0.03$ and $k_b = 100 \pm 10$ s^{-1}. It should be noted that burst rate constant was identical in both the presence and absence of L-alanine. Similar results were also obtained when the experiments were performed at 20 °C (Jin *et al.*, unpublished results).

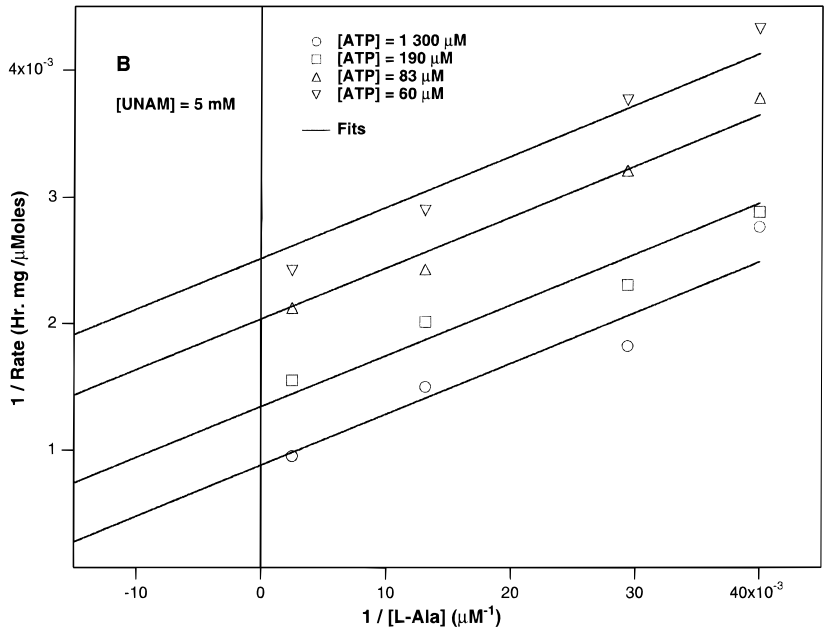
DISCUSSION

The order, if any, of substrate addition is an important question which must be addressed in trying to determine the kinetic mechanism of a multisubstrate enzyme. Steady state kinetics is the usual approach used to address this question (Cleland, 1986) and is one of the methods employed in the present report.

The kinetic data show that initial velocity line patterns were all intersecting when determined at substrate concentrations as high as 10 times their published K_m values. The best global fit of the data collected was to eq 1, the equation for an ordered addition of substrates. None of the line patterns were parallel; hence there is no kinetically irreversible step between the binding of any of the substrates. All attempts to fit these data to equations which describe different plausible kinetic mechanisms resulted in markedly poorer fits. For example, a fit of these data to an equation describing a product release step between the binding of any two substrates resulted in a value of variance that was at least 2 times as large as that determined for the ordered mechanism.

From a fit of initial velocity data such as the one just described here, it is not possible to establish the order of substrate binding or product release. One technique which is used to determine the order of substrate binding to an enzyme that uses multiple substrates is to determine the effect of extremely high concentrations of substrates on initial velocity line patterns (Viola & Cleland, 1982). Very high levels of a given substrate (more than 100 times its K_m value) make its binding kinetically irreversible, which has the effect of introducing an irreversible step into the mechanism. The presence of an irreversible step between the binding of any two substrates in an ordered mechanism results in a parallel





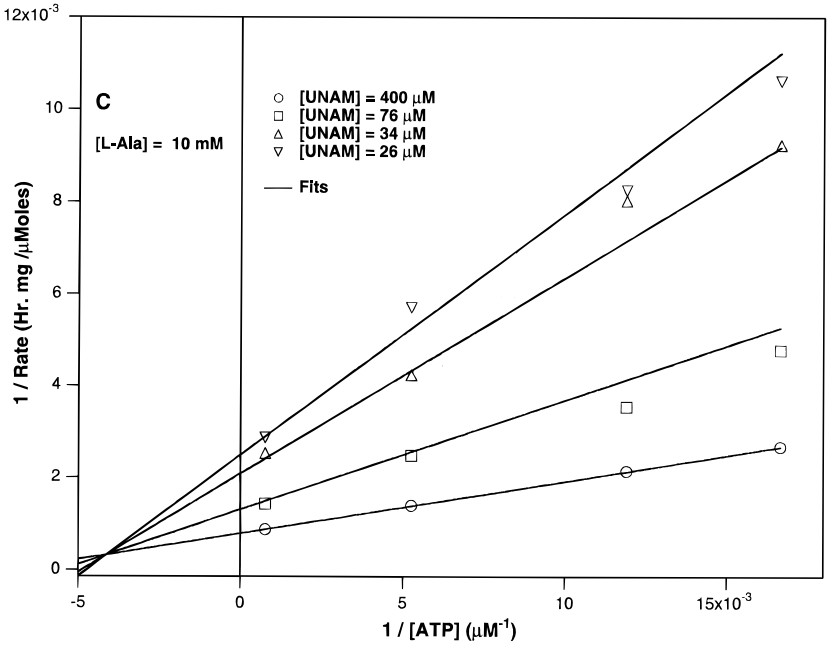


FIGURE 3: Initial velocity data collected with one substrate held at saturating levels. (A) Data collected while varying L-alanine concentrations with 13 mM ATP present at the following concentrations of UNAM: 400 μM (○), 76 μM (□), 35 μM (△), 25 μM (▽). The lines are a global fit of these data to the reciprocal form of eq 2 using parameters given in the text. (B) Data collected while varying L-alanine concentrations with 5 mM UNAM present at the following concentrations of ATP: 1300 μM (○), 930 μM (□), 83 μM (△), 60 μM (▽). The lines are a global fit of these data to the reciprocal form of eq 3 using parameters given in the text. (C) Data collected while varying ATP concentrations with 10 mM L-alanine present at the following concentrations of UNAM: 400 μM (○), 76 μM (□), 34 μM (△), 26 μM (▽). The lines are a global fit of these data to the reciprocal form of eq 2 using parameters given in the text.

Table 2: Product Inhibition Patterns[a]

variable substrate	inhibitor	fixed substrate	inhibition pattern	K_{is} (mM)	K_{ii} (mM)
ATP	UNAMA	UNAM, 1.0 mM; L-Ala, 1.0 mM	NC	0.52 ± 0.09	1.1 ± 0.1
UNAM	UNAMA	MgATP, 2.8 mM; L-Ala, 1.1 mM	NC	0.24 ± 0.08	0.62 ± 0.15
L-Ala	UNAMA	MgATP, 3.0 mM; UNAM, 1.0 mM	NC	1.0 ± 0.5	1.5 ± 0.3
UNAM	phosphate	MnATP, 6.6 mM; L-Ala, 1.1 mM	NC	68 ± 13	340 ± 100
L-Ala	phosphate	MnATP, 4.4 mM; UNAM, 1.0 mM	UC		170 ± 20
ATP	ADP	UNAM, 1.1 mM; L-Ala, 1.1 mM	C	0.014 ± 0.003	
UNAM	ADP	MgATP, 12 mM; L-Ala, 1.1 mM	NC	0.39 ± 0.08	1.0 ± 0.1
L-Ala	ADP	MgATP, 2.0 mM; UNAM, 1.1 mM	NC	6.6 ± 2.1	5.8 ± 0.7

[a] At pH 8.0 and 37 °C. K_{is}, apparent slope inhibition constant; K_{ii}, apparent intercept inhibition constant; C, competitive; NC, noncompetitive; UC, uncompetitive. Other experimental conditions are given in Materials and Methods.

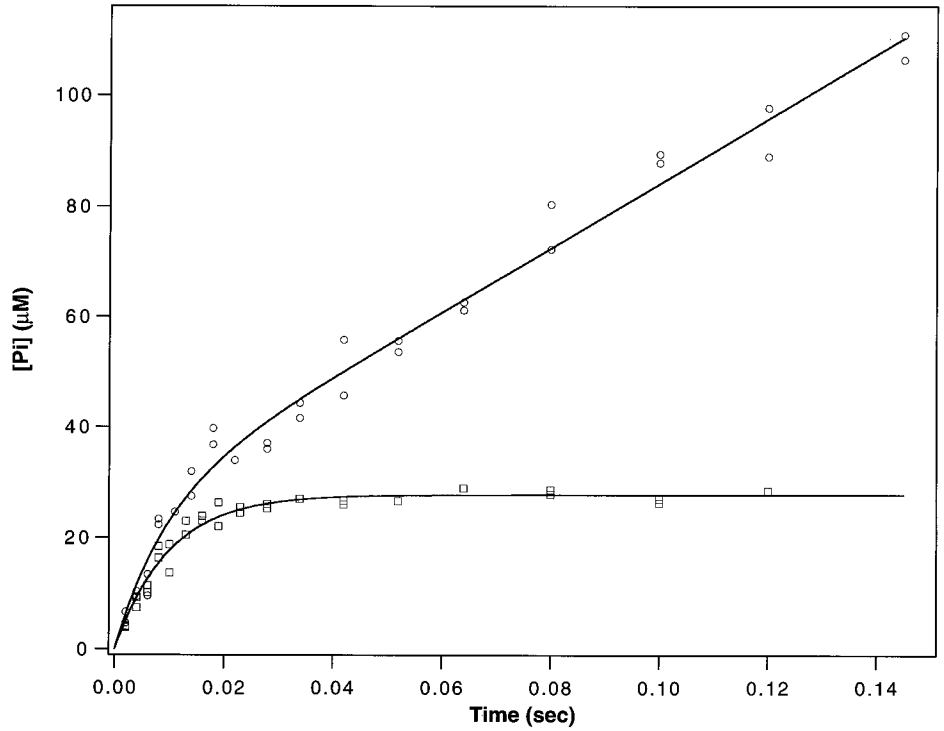


FIGURE 4: Rapid quench experimental data collected at 37 °C when all substrates were present (○) and when ATP and UNAM were present (□). Other experimental conditions are given in Materials and Methods. The lines are fits of the experimental data to eq 7 or 8, as discussed in Results.

line pattern when activity is measured as the concentration of one of these substrates is varied at a series of fixed concentrations of the other substrate. Very high concentrations of ATP and L-alanine had no effect on the line patterns: they remained intersecting. Initial velocity line patterns observed in the presence of very high concentrations of UNAM were parallel. This is consistent with the obligatory binding of UNAM between the binding of the other two substrates. From this type of analysis, binding order of the other two substrates can not be inferred.

It is worth commenting that a mechanism involving partially or fully random addition of substrates would result in either no or two parallel line patterns in the presence of very high levels of a given substrate. The fact that parallel line patterns were observed only in the presence of very high concentrations of UNAM rules out these mechanisms.

The k_{cat} value obtained from the fit of those data represented in Figure 1 to eq 1 (1000 ± 100 min^{-1}) was in excellent agreement with the previously reported value of 980 ± 40 min^{-1} (Emanuele *et al.*, 1996). The K_m values obtained from fits of data in Figure 2 to eqs 2 or 3 were also in good agreement with the published K_m values which were obtained when the other two substrates were at high levels (at least $10K_m$). At 13 mM ATP or 5 mM UNAM, K_m^{L-Ala} values were 44 ± 8 and 50 ± 8 μM, respectively, which coincide with the published value of 48 ± 6 μM. At 5 mM UNAM or 10 mM L-alanine, K_m^{ATP} values were 130 ± 20 and 120 ± 20 μM, respectively, which agree well with the published value of 130 ± 10 μM. At 13 mM ATP or 10 mM L-alanine, K_m^{UNAM} values were 36 ± 7 and 73 ± 12 μM, respectively, compared to the published value of 44 ± 3 μM. In addition, the dissociation constants for ATP, K_{ia}, and for UNAM, K_{ib}, obtained from the fit of full initial velocity data to eq 1 were in good agreement with the values obtained from partial initial velocity data to eqs 2 or 3. K_{ia} was 180 ± 50 μM from the full initial velocity data and was 240 ± 60 μM when L-alanine concentration was 10 mM. K_{ib} was 68 ± 24 μM from the full initial velocity data and was 110 ± 30 μM when ATP concentration was 13 mM. While K_m values obtained from the global fit to eq 1 were slightly higher than the published values, they did agree qualitatively.

Product inhibition patterns may help to establish the order of substrate binding and product release (Cleland, 1979). If both substrate binding and product release are fully ordered, then only one competitive inhibition pattern will be observed.

Only the last product released and the first substrate to bind will bind to the same form of the enzyme. Other products will be released from (and likewise bind to) forms of the enzyme which are different from the forms which bind substrate. The presence of these products will affect only the intercepts of reciprocal plots of activity versus substrate concentration.

Double-reciprocal plots of initial velocity data collected in the presence of UNAMA were linear. UNAMA is a noncompetitive inhibitor versus all three substrates: the presence of UNAMA affects both the slope and intercept terms. This is consistent with the release of UNAMA from a form of the enzyme which is different from any of the substrate-binding forms. The slope effect observed may be due to dead end inhibition (Cleland, 1986). UNAMA is structurally similar to UNAM and to a lesser extent to ATP. It is not surprising that present in high concentration UNAMA may bind to the enzyme forms which bind either one or both of these compounds resulting in an effect on the slope term.

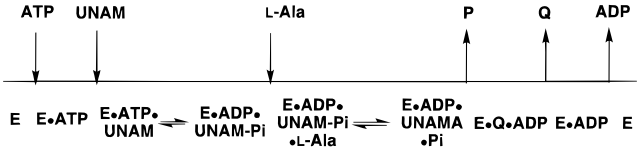
ADP is a noncompetitive inhibitor versus both UNAM and L-alanine. This is consistent with ADP release from a form of the enzyme which is different from the forms that bind either of these substrates. Invoking the same argument used to explain the slope effect observed with UNAMA inhibition, ADP appears to bind to a second from of the enzyme which affects the equilibrium of enzyme forms that bind UNAM and L-alanine. Over the concentration range examined, ADP is a competitive inhibitor versus ATP: there is only a slope effect. Both molecules bind to the free form of the enzyme. This is consistent with ADP being the last product released and ATP being the first substrate to bind.

Attempts to determine product inhibition patterns with phosphate were complicated by the necessity to use high concentrations of phosphate (Liger *et al.*, 1995) and the tendency of $MgCl_2$ and phosphate to form a precipitate. It was, however, possible to assay for activity using manganese ATP (Emanuele *et al.*, 1996). Plots in reciprocal space of activity versus either L-alanine or UNAM concentration were linear. Phosphate is a noncompetitve inhibitor versus UNAM. It is an uncompetitive inhibitor versus L-alanine: the presence of phosphate affects only the intercept term. This is consistent with these two molecules binding to kinetically distinct forms of the enzyme.

Since the initial velocity pattern data are consistent with the obligatory binding of UNAM between the binding of the other two substrates, it is not expected that any of the products will be a competitive inhibitor versus UNAM. Indeed, that is what was observed. On the other hand, none of the products is a competitive inhibitor versus L-alanine. This is consistent with L-alanine being the third substrate to bind. Given that ADP is the only observed competitive inhibitor versus ATP, even without conclusive data on inhibition by phosphate versus ATP, it can be concluded that ATP is the first substrate to bind. So, the simplest kinetic mechanism consistent with the data presented here is given in Scheme 1. Substrate binding is fully ordered: ATP binds first, UNAM binds second, L-alanine binds last, and the last product released is ADP. The order of phosphate and UNAMA release remains to be determined.

Double-reciprocal plots collected as a function of ATP concentration in the presence of phosphate concentrations high enough to impact activity were not linear. Attempts to fit these data to physically significant models for nonlinear plots (Cleland, 1982) were unsuccessful. Although unlikely, it could be argued that phosphate may still be a competitive inhibitor versus ADP and the release of phosphate and ADP may be random. However, published kinetic mechanisms for D-alanyl-D-alanine ligase in the same biosynthetic pathway (Mullins *et al.*, 1990) and glutamine synthetase (Meek *et al.*, 1982) show that ADP is the last product released. Arguing by analogy to these enzymes, which also hydrolyze ATP to ADP and phosphate and catalyze biosynthetic reactions, ADP is most likely the last product released by UNAM:L-Ala ligase.

Scheme 1: Kinetic Mechanism of Uridine Diphosphate-*N*-acetylmuramate:L-Alanine Ligase



The rapid quench data presented here suggest that L-alanine binds after ATP and UNAM bind. In recent isotope transfer experiments, transfer of ^{18}O label from the carboxyl group of UNAM to inorganic phosphate during turnover was demonstrated (Falk *et al.*, 1996). This means that UNAM:L-Ala ligase catalyzes a reaction proceeding through a putative acyl−phosphate intermediate. The chemical mechanism for this enzyme is therefore very similar to the chemical mechanism for glutamine synthetase. In the case of glutamine synthetase, the biosynthetic reaction can be divided into two chemical steps: the formation of a γ-glutamyl phosphate intermediate and the subsequent displacement of the activated phosphate group by ammonia. The burst formation of acid-labile phosphate in rapid quench experiments was observed in the presence and absence of ammonia and was taken as evidence for the phosphoryl intermediate (Meek *et al.*, 1982). In the rapid quench data presented here, the burst formation of acid-labile phosphate was also observed in the presence and absence of L-alanine. More importantly, the observed burst rate was independent of the presence of L-alanine. This suggests that the putative acyl−phosphate intermediate can form before L-alanine binds.

The proposed sequential mechanism is also consistent with the rapid quench data where the linear phase release of phosphate was only observed in the presence of L-alanine. This suggests that the release of phosphate is after the binding of L-alanine. Chemically, UNAMA can form only after the binding of L-alanine. Therefore, neither phosphate nor UNAMA can be released before L-alanine binds. ADP was detected in the absence of L-alanine, but at a much slower rate than the biosynthetic reaction rate (Emanuele, *et al.*, 1996). In addition, ATP and ADP bind to the same form of the enzyme. Before L-alanine binds, ADP cannot be released at a rate fast enough to be on the biosynthetic reaction pathway. The simplest kinetic mechanism consistent with these observations is a sequential one.

Further evidence for this kinetic mechanism comes from an analysis of substrate affinity for the free form of the enzyme. In an ordered mechanism, the first substrate to bind has the highest affinity for the free form of the enzyme. Affinity capillary electrophoresis (ACE) was used to measure the binding constants (K_d) for the free form of the enzyme.

Changes in migration time were measured as a function of substrate concentration. The K_d values for the three substrates were as follows: 11 ± 1 nM for ATP, 92 ± 6 μM for UNAM, and 1.5 ± 0.4 mM for L-alanine (Abid *et al.*, 1997). The K_d value for UNAM measured with ACE was close to the K_{ib} value for UNAM measured with steady state methods, while the K_d value for ATP measured with ACE was different from the K_{ia} value for ATP measured with steady state methods. Values of K_d measured using ACE were obtained assuming: (1) a monovalent interaction between the protein and the ligand, (2) equilibrium binding between ligand and protein during electrophoresis, and (3) no significant alteration in protein structure due to surface adsorption of protein on the capillary column (Avila *et al.*, 1993). That some assumptions might not be justified in the present case could explain the observed difference. Despite that, when measured with the same method, ATP has the highest affinity for the free form of the enzyme among the three substrates. This is consistent with ATP binding first.

Recently, the order of substrate addition was proposed for *E. coli* UDP-*N*-acetylmurate-tripeptide D-alanyl-D-alanine-adding enzyme (*Mur*F) based on a study using a glutathione *S*-transferase fusion enzyme (Anderson *et al.*, 1996) and was similar to the order of substrate addition proposed here for UNAM:L-Ala ligase. *Mur*C, -D, -E, and -F proteins have been shown to bear significant blocks of homology (Ikeda *et al.*, 1990). It has been suggested that this homology reflects shared regions of macromolecular structure necessary for substrate recognition and/or catalytic function. The similarity in the order of substrate binding for *Mur*C and *Mur*F lends support to this hypothesis.

In conclusion, initial velocity line patterns are consistent with a sequential mechanism with the obligatory binding of UNAM between the binding of ATP and L-alanine. ATP has a significantly higher affinity for the free form of the enzyme than either UNAM or L-alanine, consistent with ATP binding first. Rapid quench data show that phosphate is formed at the active site of the enzyme in the absence of L-alanine but that L-alanine is required for phosphate release at a rate fast enough to be on the catalytic pathway. The simplest kinetic mechanism consistent with these data is ordered sequential, with ATP binding first, UNAM second, L-alanine third, and ADP being the last product released.

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