Articles

Kinetic Mechanism of the β-Lactam Synthetase of *Streptomyces clavuligerus*†

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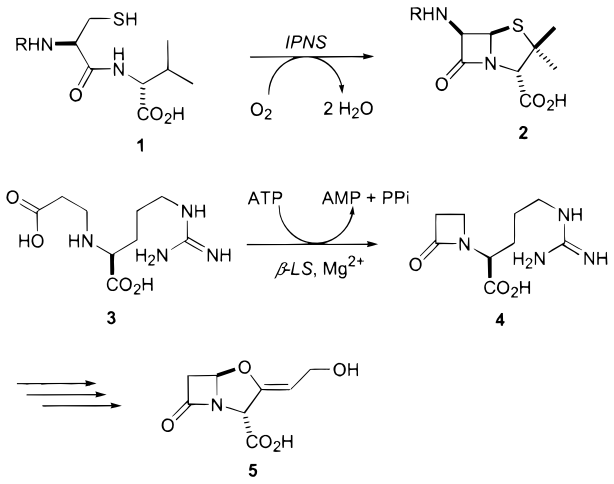
Received March 29, 2000; Revised Manuscript Received July 10, 2000

ABSTRACT: *Streptomyces clavuligerus* β-lactam synthetase (β-LS) was recently demonstrated to catalyze an early step in clavulanic acid biosynthesis, the ATP/Mg^{2+}-dependent intramolecular closure of the β-amino acid N^2-(carboxyethyl)-L-arginine (CEA) to the monocyclic β-lactam deoxyguanidinoproclavaminic acid (DGPC). Here we investigate the steady-state kinetic mechanism of the β-LS-catalyzed reaction to better understand this unprecedented secondary metabolic enzyme. Initial velocity patterns were consistent with a sequential ordered bi-ter kinetic mechanism. Product inhibition studies with PP_i and DGPC demonstrated competitive inhibition versus their cognate substrates ATP and CEA, respectively, and noncompetitive inhibition against their noncognate substrates. To clarify the order of substrate binding, the truncated substrate analogue N^2-(carboxymethyl)-L-arginine was synthesized and demonstrated uncompetitive inhibition versus ATP and competitive patterns versus CEA. These data are consistent with ordered substrate binding, with ATP binding first, an abortive enzyme−DGPC complex, and PP_i released as the last product. The pH dependence of *V* and *V/K* was determined and suggests that residues with a p*K* of 6.5 and 9.3 must be ionized for optimal activity. These observations were considered in the context of investigations of the homologous primary metabolic enzyme asparagine synthetase B, and a chemical mechanism is proposed that is consistent with the kinetic mechanism.

Formation of the four-membered ring characteristic of the β-lactam antibiotics is a central mechanistic question in natural product biosynthesis. Two solutions to this problem have been identified to date. The bicyclic nucleus of penicillin (**2**) is generated by a single protein, isopenicillin N synthase (IPNS),[1] which catalyzes the sequential oxidative cyclization of tripeptide **1** in the presence of ferrous ion (*1*, *2*) (Scheme 1). This unique process is driven thermodynamically by the overall reduction of dioxygen to two molecules of water. Mechanistically related iron/oxygen chemistry is carried out by clavaminate synthase (*3*, *4*) in the biosynthesis of the structurally similar β-lactamase inhibitor clavulanic acid (**5**) (*5*). However, the β-lactam ring itself is formed in a manner entirely distinct from penicillin. In this case, the intramolecular closure of N^2-(carboxyethyl)-L-arginine (**3**, CEA) to deoxyguanidinoproclavaminic acid (**4**, DGPC) was recently shown to be catalyzed by an ATP/Mg^{2+}-dependent β-lactam synthetase (β-LS) (*6*).

Scheme 1



$$\text{CEA} + \text{ATP} \overset{\beta\text{-LS},Mg^{2+}}{\rightleftharpoons} \text{DGPC} + \text{AMP} + PP_i \quad (1)$$

Although this process is formally that of an intramolecular peptide synthetase, amino acid sequence analysis suggests that β-LS is in fact evolutionarily related (33% identity, 49% similarity) to the primary metabolic enzyme asparagine synthetase B (AS-B) (*7*). AS-B catalyzes *inter*molecular

† This work supported by a grant (AI 14937) from the National Institutes of Health, DHHS.

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[1] Abbreviations: CEA, N^2-(carboxyethyl)-L-arginine; DGPC, deoxyguanidinoproclavaminic acid; MOPS, 2-(2-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; HOMOPIPES, homo-1,4-piperazinediethanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CMA, N^2-(carboxymethyl)-L-arginine; IPNS, isopenicillin N synthase; β-LS, β-lactam synthetase; AS-B, asparagine synthetase B.

10.1021/bi000709i CCC: $19.00
Published on Web 08/19/2000

transfer of the γ-amide nitrogen of glutamine to the side-chain carboxylate of aspartate in an ATP/Mg^{2+}-dependent process. Kinetic studies (*8*, *9*) and recent X-ray crystallographic analysis of the AS-B structure (*10*) indicate that its catalytic cycle is similar to the class II glutamine amidotransferase (GAT) family of enzymes. An N-terminal glutaminase domain generates free ammonia, which is channeled within AS-B to a C-terminal aspartate adenylation domain. β-LS shows high homology to AS-B in both its N- and its C-terminal domains. While both β-LS and AS-B catalyze the adenylation of a bound β-amino acid, they otherwise perform quite different chemistry, and it is not clear why such extensive full-length sequence similarity is retained.

To further our understanding of *Streptomyces clavuligerus* β-lactam synthetase, we have conducted studies to characterize the catalytic cycle of monocylic β-lactam formation mediated by this enzyme. Efficient purification of recombinant β-LS has permitted extensive kinetic investigation of the mechanism. These initial velocity studies, together with inhibition and substrate analogue experiments, have enabled us to propose a detailed mechanism for β-lactam formation.

EXPERIMENTAL PROCEDURES

Protein Purification: Cell Growth. The preparation of recombinant β-LS in *E. coli* BL21(DE3) harboring pBOB1 has been previously described (*6*). Cells were grown at 37 °C in 300 rpm shaken flasks, induced at an OD of 0.6 by adding IPTG to a final concentration of 2 mM, and incubated for 7 h before harvest. All purification steps were carried out at 0−4 °C. Typically 42 g of cell paste (from 12 L fermentation) was resuspended in 300 mL of lysis buffer containing Tris·HCl (50 mM) pH 8, EDTA (2 mM), and PMSF (1 mM). Cells were disrupted by French Press (1 pass), and DNA was precipitated by addition of KCl and $MnCl_2$ to final concentrations of 100 and 30 mM, respectively, and removed by centrifugation (27000*g*). Protein was precipitated by addition of solid ammonium sulfate to 35% and then 55% saturation, and pelleted by centrifugation (13000*g*). The 35−55% pellet was redissolved in enzyme buffer (50 mM Tris·HCl, pH 7.5, 10% v/v glycerol, 2 mM DTT, 10 μM EDTA), and dialyzed (twice for 6 h) against 2000 mL of enzyme buffer. The dialyzed protein was loaded directly onto a preequilibrated Q-Sepharose column (34 × 4 cm, Sigma). The column was washed with 100 mL of enzyme buffer at 2 mL/min, and eluted with a 1000 mL linear gradient of KCl from 0 to 500 mM. β-LS eluted at ca. 280 mM KCl. Fractions containing β-LS were pooled and concentrated to approximately 4 mL with an Amicon Ultrafiltration cell fitted with a YM-30 membrane. The concentrate was passed over a Blue Sepharose CL-6B column (10 × 4 cm) at 2 mL/min. The initial fractions, containing the desired protein, were pooled and concentrated again on a YM-30 membrane, and β-LS protein concentration was determined by the method of Bradford using the Bio-Rad protein assay (catalog no. 500-006, Bio-Rad, Hercules, CA) and bovine serum albumin as a standard. The final yield of protein was 4 mg/L. A stock solution of β-LS (1.1 mg/mL) was made, aliquoted into 0.5 mL portions, and stored at −20 °C until needed.

Preparation of N^2-Carboxymethyl-L-arginine. (A) N^5-Cbz-N^2-(2-benzyloxymethyl)-p-bromobenzyl-L-ornithine. To a stirred solution of N^5-Cbz-*p*-bromobenzyl-L-ornithine (1 g, 2.2 mmol) in DMF (7 mL) were added benzyl bromoacetate (0.605 g, 2.2 mmol) and K_2CO_3 (0.61 g, 4.4 mmol). The reaction mixture was stirred for 5 h, diluted with 200 mL of EtOAc, extracted twice with water, dried (Na_2SO_4), and concentrated in vacuo. The product was purified by flash chromatography (silica 15 × 4 cm, 50% EtOAc/C_6H_{12}) to yield the title compound as a clear oil (1.0 g, 1.71 mmol, 75% yield): TLC R_f (50% EtOAc, cyclohexane) = 0.47; ^{1}H NMR ($CDCl_3$) δ 7.09 (m, 4H), 7.27 (m, 10H) 5.05 (s, 2H), 5.00 (s, 2H), 4.99 (s, 2H), 4.84 (m, 1H), 3.38 (A of ABq, J = 17.4 Hz, 1H), 3.30 (B of ABq, J = 31.4 Hz, 1H), 3.25 (t, J = 6.1 Hz, 2H), 3.10 (dd, J = 6.4, 12.5 Hz, 2H), 1.94 (bs, 1H), 1.69−1.45 (m, 4H); ^{13}C ($CDCl_3$) δ 173.9, 171.5, 156.3, 136.5, 135.4, 134.5, 131.8, 130.0, 128.6, 128.5, 128.4, 128.1, 122.5, 66.6, 66.6, 65.8, 60.3, 49.1, 40.6, 30.2, 26.1; IR (cm^{-1}) 3349, 3090, 3062, 3034, 2951, 1735, 1524, 1491; MS (*m*/*z*) 583.1 $(M+H)^+$; HRMS calcd for $C_{29}H_{31}BrN_2O_6$ 582.1365, found 583.1441 $(M+H)^+$; $[\alpha]_D$ −8.1 (*c* 1.0, CH_2Cl_2).

(B) Disodium N^2-(Carboxymethyl)-L-ornithine. N^5-Cbz-N^2-(2-benzyloxymethyl)-*p*-bromobenzyl-L-ornithine (205 mg, 0.33 mmol) was dissolved in THF/H_2O (3:1, 40 mL) with $NaHCO_3$ (55 mg, 0.66 mmol), and 30% Pd/C (25 mg) was added. The reaction mixture was shaken in a Parr hydrogenator at 50 psi for 40 min, and filtered though a pad of Celite, which was subsequently washed with acetone. The solvents were removed in vacuo; the remaining aqueous layer was washed with CH_2Cl_2, filtered though a 0.45 μm aqueous HPLC filter (Millipore, Bedford, MA), and lyophilized. Crude product contained some inorganic salts: ^{1}H NMR (D_2O) δ (from acetone δ = 2.04), 3.28 (t, J = 6.1 Hz, 1H), 3.31 (A of ABq, J = 16.5 Hz, 1H), 3.23 (B of ABq, J = 16.5 Hz, 1H), 2.85 (t, J = 7.3 Hz, 2H), 1.45−1.8 (m, 4H); ^{13}C (D_2O) δ (from acetone δ = 30.89), 176.9, 174.8, 62.5, 49.8, 39.6, 28.4, 32.8; IR (cm^{-1}) 3436, 2978(s), 1619, 1585, 1407; HRMS calcd for $C_7H_{12}N_2Na_2O_4$ 234.0592, found 235.0681 $(M+H)^+$; $[\alpha]_D$ +7 (*c* 2.0, H_2O).

(C) N^2-(Carboxymethyl)-L-arginine. To a solution of disodium N^2-(2-carboxymethyl)-L-ornithine (77 mg, 0.33 mmol) dissolved in H_2O (3 mL) were added aminoiminosulfonic acid (49 mg, 0.39 mmol) and K_2CO_3 (59 mg, 0.42 mmol) (*11*). After being stirred for 15 h uncovered, the mixture was lyophilized. The residue was resuspended in 4 mL of 0.2 M HOAc and applied to a 20 mL, preequilibrated Varian SCX ion exchange column. The column was rinsed with 0.2 M HOAc (2 × 10 mL) and water (1 × 10 mL), and the product was eluted with 0.2 M pyridine (5 × 10 mL). Fractions containing product were identified by Sakaguchi analysis (*6*), pooled, and lyophilized to yield the title compound (68 mg, 0.29 mmol, 88% yield): ^{1}H NMR δ (D_2O) 3.50 (dd, J = 5.5 Hz, J = 6.7 Hz, 1H), 3.40 (A of ABq, J = 16.2 Hz, 1H), 3.34 (B of ABq, J = 16.2 Hz, 1H), 3.02 (t, J = 7.0 Hz, 2H), 1.4−1.9 (m, 4 H); ^{13}C (D_2O) δ 174.3, 172.1, 157.7, 62.7, 49.1, 41.4, 27.5, 24.8; IR (cm^{-1}) 3377, 3189, 1625, 1396; HRMS calcd for $C_8H_{16}N_4O_4$ 232.1171, found 233.1253 $(M+H)^+$; $[\alpha]_D$ +14.7 (*c* 1, H_2O).

Assays. The rate of CEA cyclization was measured by monitoring the formation of AMP using a coupled enzyme assay adapted from a procedure for its quantitative determination (*12*). The AMP assay mix, made immediately prior to use, contained 113 mM Tris, pH 7.5, 0.54 mM NADH, 0.81 mM PEP, 0.15 mM ATP, 10 units/15 units of rabbit muscle pyruvate kinase/lactate dehydrogenase enzymes

(Sigma PK/LDH solution, St. Louis, MO), and 110 units of rabbit muscle myokinase. The production of AMP by β-LS was quantified as follows. A quenched 30 μL β-LS reaction mixture was mixed with 90 μL of AMP assay mix, and A_{340} was monitored until ΔA_{340}/min stabilized at less than 0.01/min (generally 90 s). All UV assays were carried out in a 10 mm path length quartz Ultramicro cuvette (Sigma) at 25 °C in a total volume of 120 μL. The assay was calibrated using stock solutions of authentic AMP, allowing extrapolation of the obtained A_{340} value to β-LS enzyme activity.

Rates of CEA cyclization were also measured by HPLC analysis. After appropriate reaction times, 20 μL of the reaction mixture was injected onto a Phenomenex (Torrence, CA) Prodigy 5 μm ODS(3) column (250 × 4.6 mm) with 50 mM pH 7 sodium phosphate buffer as eluent (1 mL/min). DGPC resolved with a retention time of 13−15 min under these conditions, and was quantified by integration of the chromatogram. Integrals were calibrated by comparison to an authentic DGPC reference.

Steady-State Kinetics. (A) Initial Velocity Studies. The β-LS reaction was examined by initial velocity measurements. The amount of enzyme used in each assay was selected to be small enough that the velocity remained linear for at least 15 min. ATP solutions were made fresh in 300 mM PIPES, 30 mM $MgCl_2$, pH 7.5. Assay mixtures were assembled by combining equal volumes of the ATP solution and appropriate CEA concentrations, and reactions were initiated by the addition of an equal volume of stock enzyme (1.069 mg/mL, 19.6 μM). Thus, a total reaction volume of 30 μL contained 100 mM PIPES, 10 mM $MgCl_2$, pH 7.5, and 353 μg/mL β-LS. Rates were acquired spectrophotometrically as described above as single time points at 10 min. Each velocity determination is the average of at least three measurements. A background measurement consisting of ATP and enzyme only was subtracted from all experiments, to compensate for contaminating AMP. The activity of the enzyme, expressed as micromoles per minute, was calculated by extrapolating AMP concentration from a standard curve.

(B) Inhibitor Studies. Initial velocities were measured as described above. Each data point was the average of at least two measurements. Inhibitor concentrations were varied versus CEA concentrations as follows: DGPC, 0−1.1 mM; AMP, 0−11 mM; PP_i, 0−2.5 mM; CMA, 0−0.5 mM. Inhibitor concentrations were varied versus ATP concentrations as follows: DGPC, 0−4 mM; AMP, 0−5.6 mM; PP_i, 0−3 mM; CMA, 0−5.6 mM.

(C) pH Studies. A stock solution of β-LS was dialyzed into a solution containing 5 mM PIPES, pH 7.0, 10% glycerol, 2 mM DTT, and 10 μM EDTA. Assay buffer solutions contained 30 mM $MgCl_2$ and 150 mM MOPS (pH 6.0−6.5), PIPES (pH 6.75−7.5), HOMOPIPES (pH 7.7−8.5), or CHES (pH 9.0−9.5). ATP was dissolved in assay buffer directly before use. In pH studies, final CEA concentrations were varied between 0.087 and 2.416 mM. Initial rates were measured as described above.

(D) Data Analysis. The nomenclature for mechanistic determinations is that of Cleland (*13, 14*). Data were fitted by regression analysis using Graphit (Erithacus Software Ltd., Middlesex, U.K.) to the appropriate initial velocity equation (listed below), to obtain kinetic constants. The Michaelis−Menten equation was used to evaluate standard errors on sets of initial velocities. For each rate curve, standard errors were generally less than 10% using the mean values for each data set. When CEA was varied in the presence of fixed levels of ATP, data were fitted to eqs 2 and 3 for sequential and rapid equilibrium random mechanisms, respectively:

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{K_{ia}K_b}{K_aB}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{K_b}{B}\right) \quad (2)$$

$$\frac{1}{v} = \frac{\alpha K_a}{V}\left(1 + \frac{K_b}{B}\right)\left(\frac{1}{A}\right) + \frac{1}{V}\left(1 + \frac{\alpha K_b}{B}\right) \quad (3)$$

where A and B are the concentration of substrates, K_a and K_b are the Michaelis constants for A and B, respectively, K_{ia} is the dissociation constant of A from the E·A complex, and α is the factor by which binding of one substrate alters the binding of the other. The effects of pH on enzymatic activity were treated by the method of Dixon (*15*). To obtain V and K values at each pH, velocities were plotted as the average of at least three data points and fit to the Michaelis−Menten equation. V/K and V vs pH data were fit to eqs 4 and 5, respectively, which describe the reduction in rate associated with a single protonation or deprotonation of substrate or enzyme.

$$\log(V/K) = \log(V/K)_0 - \log(1 + [H^+]/K_1^E + K_2^E/[H^+]) \quad (4)$$

$$\log V = \log V_0 - \log(1 + [H^+]/K_1^{ES} + K_2^{E\,S}/[H^+]) \quad (5)$$

$(V/K)_0$ and V_0 are the values obtained at optimum protonation, K_1^E and K_2^E represent the apparent molecular dissociation constants for the free enzyme, and K_1^{ES} and K_2^{ES} represent those for the enzyme−substrate complex.

Mechanistic determinations were made by considering the standard errors associated with the fit to each equation. The data were fit to equations describing competitive, noncompetitive, and uncompetitive inhibition, respectively:

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (6)$$

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \quad (7)$$

$$v = \frac{VA}{K_a + A(1 + I/K_{ii})} \quad (8)$$

The parameters are the following: v, initial velocity; V, maximum velocity; A and B, concentration of substrates; I, inhibitor concentration; K_a and K_b, Michaelis constants; K_{is} and K_i, slope and intercept inhibition constants, respectively.

Pyrophosphate Exchange. Reaction mixtures of 70 μL, containing final concentrations of 86 mM PIPES, pH 7.5, 8.6 mM $MgCl_2$, 2 mM CEA, 4.3 mM ATP, and 0.14 mM PP_i with 0.2 μCi of [^{32}P]PP_i (14 Ci/mmol), were initiated by addition of 5.2 nM β-LS. After 30 min incubation at room temperature, the assays were terminated by the addition of 900 μL of cold 1% (w/v) activated charcoal (Mallinckrodt, USP grade) in 3% (v/v) perchloric acid. The slurry was filtered through glass filters (2.4 cm, G-4, Fischer Scientific, Pittsburgh, PA), which were washed successively with 10 mL of 0.2 M sodium phosphate (pH 8.0), 4 mL of dH_2O,

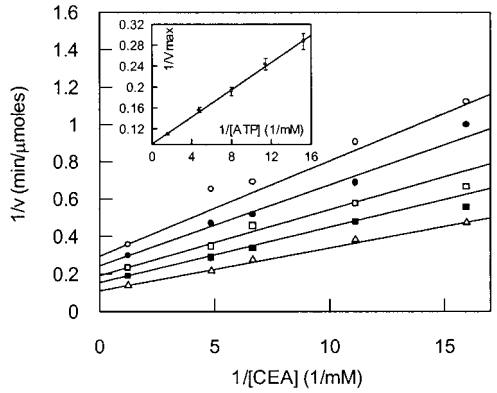

FIGURE 1: Initial velocity plots with CEA as the variable substrate. Total reaction volumes of 30 μL contained β-LS, 100 mM PIPES, and 10 mM $MgCl_2$, pH 7.5. ATP concentrations from top to bottom were: 65.6, 87.2, 132, 206, 626 μM. Experimental points are fit to eq 2. β-LS enzyme, 10.7 μg per cuvette. The inset shows the replot of the *y*-intercept versus ATP concentration with the standard deviations of fitting *V* to eq 2 for each ATP concentration. SD was less than 15% in all cases.

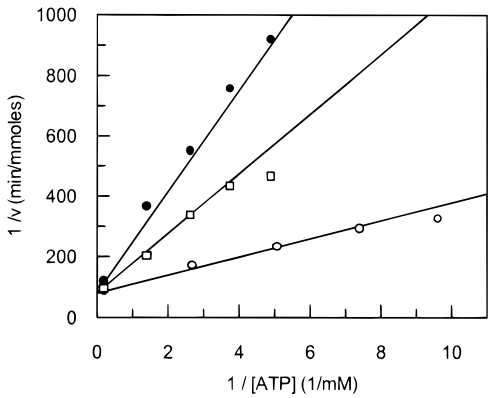

FIGURE 2: Product inhibition patterns of PP_i with ATP as variable substrate. Each velocity is the average of two data points. The basic system is as shown in Figure 1. Concentrations of PP_i: ○, none; □, 0.5 mM; ●, 1 mM; β-LS enzyme, 10.7 μg per cuvette.

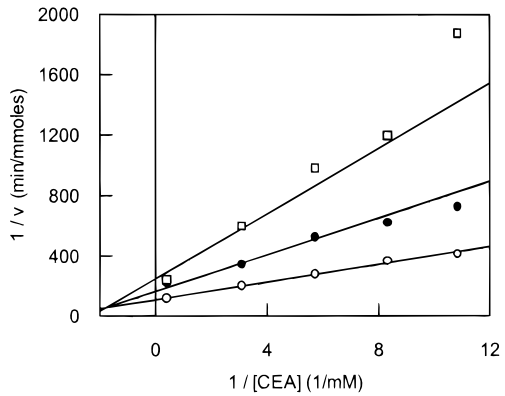

FIGURE 3: Product inhibition patterns of PP_i with CEA as variable substrate. Each velocity is the average of two data points. The basic system is as shown in Figure 1. Concentrations of PP_i: ○, none; ●, 1.0 mM; □, 2.5 mM. β-LS enzyme, 10.7 μg per cuvette.

and 1 mL of ethanol, and air-dried. The filters were mixed with 5 mL of scintillation fluid (OptiFluor, Packard Instrument Co., Meridan, CT), and counted on a Beckman LS-5801 scintillation counter. Each reaction was performed in duplicate.

RESULTS

Owing to low levels of β-LS expression in *E. coli*, an efficient purification protocol was developed. β-LS precipitated in a narrow range of ammonium sulfate concentration and, following Q-Sepharose anion exchange purification, was substantially pure. Filtration through Blue-Sepharose CL-6B advantageously removed remaining impurities and resulted in protein which was homogeneous by SDS−PAGE gel electrophoresis.

The absence of unique chromophores in the substrates or products necessitated the development of a sensitive assay for product formation. To this end, a procedure for the enzymatic determination of AMP in biological tissues (*12*) was modified to follow the β-LS reaction. The validity of the AMP assay method was verified by HPLC analysis, which measured product DGPC formation directly by integration. Both experiments produced identical progress curves, and linear regression of these plots yielded comparable K_m and V_{max} values within experimental error.

Initial Velocity Studies. Initial velocity patterns for the forward β-LS reaction were obtained for both CEA and ATP as variable substrates. Substrate concentrations were selected such that one set of assays would provide reciprocal plots versus either CEA or ATP. The data fit well to eq 2, as illustrated for varied [CEA] in Figure 1, suggesting an ordered sequential bi-ter mechanism. Both substrates for β-LS showed initial velocity patterns intersecting to the left of the vertical axis, signifying sequential substrate binding prior to release of products, and below the horizontal axis, indicating K_a is greater than K_{ia}. The Michaelis constants derived for CEA (K_a) and ATP (K_b) were 220 ± 21 and 153 ± 18 μM, respectively. Likewise, the dissociation constants for CEA and ATP were found to be K_{ia} = 153 ± 15 μM and K_{ib} = 106 ± 20 μM, respectively. However, a similar pattern of initial velocity curves might be obtained from the rapid-equilibrium random case, in which the order of substrate binding is random. Although the standard error of fitting initial velocity data to the random equation (eq 3) was generally worse (random K_a = 153 ± 26 μM), further experiments were needed to distinguish an ordered from a random mechanism.

Product Inhibition. Substrate binding order can be elucidated or confirmed with product inhibition experiments. To this end, analysis of β-LS was aided by the earlier studies on pigeon liver malic enzyme, which catalyzes the reversible decarboxylation of malate though a sequential bi-ter kinetic mechanism, and for which rate equations for initial velocity studies and product inhibition have been derived (*16*). Representative product inhibition plots are shown in Figures 2 and 3. When CEA was the variable substrate, DGPC and pyrophosphate produced competitive and noncompetitive plots, respectively. When ATP was the variable substrate, DGPC demonstrated noncompetitive inhibition, whereas PP_i was a competitive inhibitor. AMP inhibition experiments had to be assayed by HPLC, due to interference from the AMP assay. However, the AMP results were ambiguous due to the fact that AMP is only moderately inhibitory toward CEA and ATP (ca. 4 and 15 mM, respectively) and such high levels of AMP eclipsed product DGPC peaks on the chromatogram, making integral values unreliable.

Observed product inhibition patterns (summarized in Table 1) do not conform to an ideal ordered system. In an ordered bi-ter mechanism, the second product released is surrounded by irreversible product release steps, and is predicted to

Table 1: Inhibition Patterns for β-LS Products and Substrate Analogue

inhibitor	variable substrate	inhibition pattern	inhibition constants (μM) K_{is}	K_{ii}
DGPC	CEA	C[a]	0.28 ± 0.06	
DGPC	ATP	NC	5.45 ± 0.89	8.55 ± 0.90
PP_i	CEA	NC	0.95 ± 0.26	1.90 ± 0.38
PP_i	ATP	C		0.22 ± 0.02
CMA	CEA	C	0.16 ± 0.03	
CMA	ATP	UC		2.84 ± 0.15

[a] C = competitive inhibition, UC = uncompetitive inhibition, NC = noncompetitive inhibition.

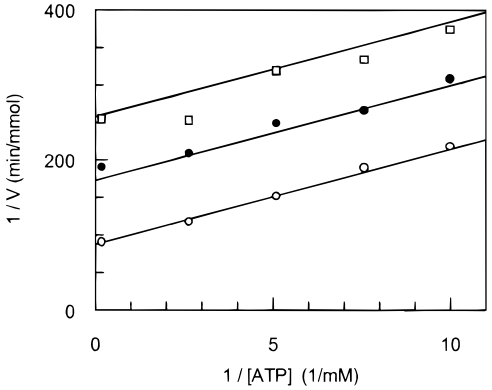

FIGURE 4: Inhibition by alternate substrate CMA with ATP as the variable substrate. Each velocity is the average of two data points. [CEA] = 7.2 mM. Concentrations of CMA: ○, none; ●, 2.8 mM; □, 5.6 mM. β-LS enzyme, 10.7 μg per cuvette.

demonstrate uncompetitive inhibition. The presence of multiple competitive patterns and the absence of uncompetitive inhibition with β-LS indicate either an ordered mechanism with abortive enzyme−product complex formation or a random mechanism. Abortive complex formation with PP_i and DGPC would not be surprising in β-LS, because these are functionally similar and sterically smaller than their corresponding substrates.

Dead End Inhibition. The substrate analogue carboxymethylarginine (CMA) was synthesized and used to study the order, if any, of substrate binding, and the binding properties of the active site. Due to prohibitively high energy barriers in aziridinone formation, CMA was predicted to be a dead end inhibitor of β-LS, competitive with CEA. Indeed, β-LS was shown to be completely incapable of catalyzing the release of pyrophosphate in the presence of CMA and ATP (data not shown). CMA resulted in strong competitive inhibition versus CEA ($K_i = 160 \pm 30$ μM), with an inhibition constant comparable to that of the Michaelis constant of the natural substrate, and uncompetitive inhibition ($K_i = 2800 \pm 150$ μM) versus ATP (Figure 4). This uncompetitive pattern implied that CMA binds more tightly to E·ATP than to free enzyme. Slopes versus [ATP] are unaffected because CMA binds after ATP. Assuming CMA binds in an analogous fashion to CEA, this result supports a mechanism involving ordered substrate binding. The β-lactam product DGPC and CMA both demonstrate competitive binding versus CEA as substrate, with binding constants of the same order of magnitude as the K_a of CEA. Thus, the principal binding interactions appear to be with the guanidino and/or α-carboxyl group functionalities in the substrate.

pH Dependence of V and V/K. Initial velocity plots of variable [CEA] at various pH values were prepared. A series of buffers was selected in order to test a broad range of pH. Special care was used in buffer selection due to the fact that metal-complexing buffers such as Tris·HCl buffers are known to catalyze the degradation of β-lactams (*17*). Control experiments, in which β-LS was incubated at pH 9.25 and 6.25 for 10 min, adjusted to neutral pH, and assayed for activity, demonstrated no loss in activity, and, therefore, assured the continued stability of the enzyme under this range of assay conditions. pH profiles for β-LS-catalyzed β-lactam formation are shown in Figure 5. According to eqs 4 and 5, the variation of log V and log(V/K) with pH defines log V and log(V/K), respectively, for a slow, nonsticky substrate (*15*). Log V drops off at the pK value of catalytic groups in β-LS when both substrates are present with a slope of +1 and −1, corresponding to single protonation and deprotonation events, respectively. log (V/K) profiles give pK values for the E·ATP complex. Fitting of data to these equations resulted in calculated values of $pK^{ES} = 6.5 \pm 0.1$ and 9.3 ± 0.1 and pK^{E} values of 6.8 ± 0.1 and 9.0 ± 0.1.

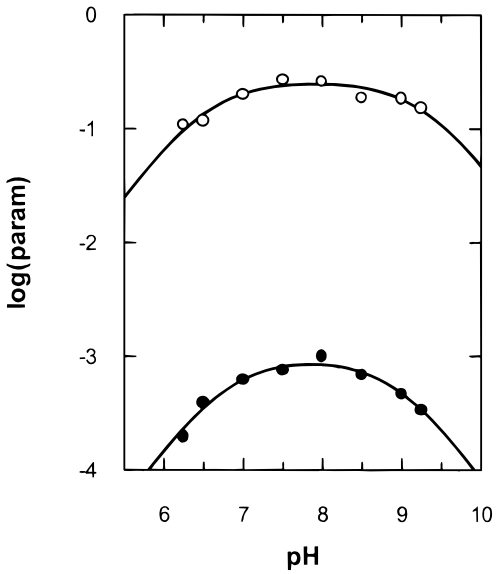

FIGURE 5: pH dependence of the kinetic parameters for β-lactam synthetase. (○) log V and (●) log(V/K) versus pH, fit to eqs 4 and 5, respectively. Velocities were scaled to fit the pH profiles on the same graph. V and K were determined by fitting initial velocity curves to eq 2. The standard deviation of V was less than 10% in all cases, and the standard deviation of K_m was less than 15%, except for pH 6.25 and 9.25 which were 21% and 27%, respectively.

The log V and log(V/K) values are relatively pH independent between pH 7 and 9, with maximum activity at about pH 8. Adjoining biosynthetic enzymes in the clavulanic acid biosynthetic cluster proclavaminate amidinohydrolase (PAH) and clavaminate synthase (CS) have pH optima of 9 and 7, respectively, and function optimally in tandem at pH 8 (*18*). The V/K profiles shown in Figure 5 presumably indicate that ionizable groups with pK values of 6.5 and 9.3 must be protonated/deprotonated in the E·ATP complex. Histidine and lysine exhibit side chain pK_a's in these ranges, but local perturbations by ionization of other side chains are also possible. Expected pK_a values for the γ-guanidino and carboxyl functionalities in CEA lie well outside of these ranges. While the α-amino group of CEA may be assigned to the 9.3 value, this protonation state is inconsistent with the chemistry of β-lactam formation. An ammonium ion, however, could be readily visualized to be involved, for example, in substrate binding.

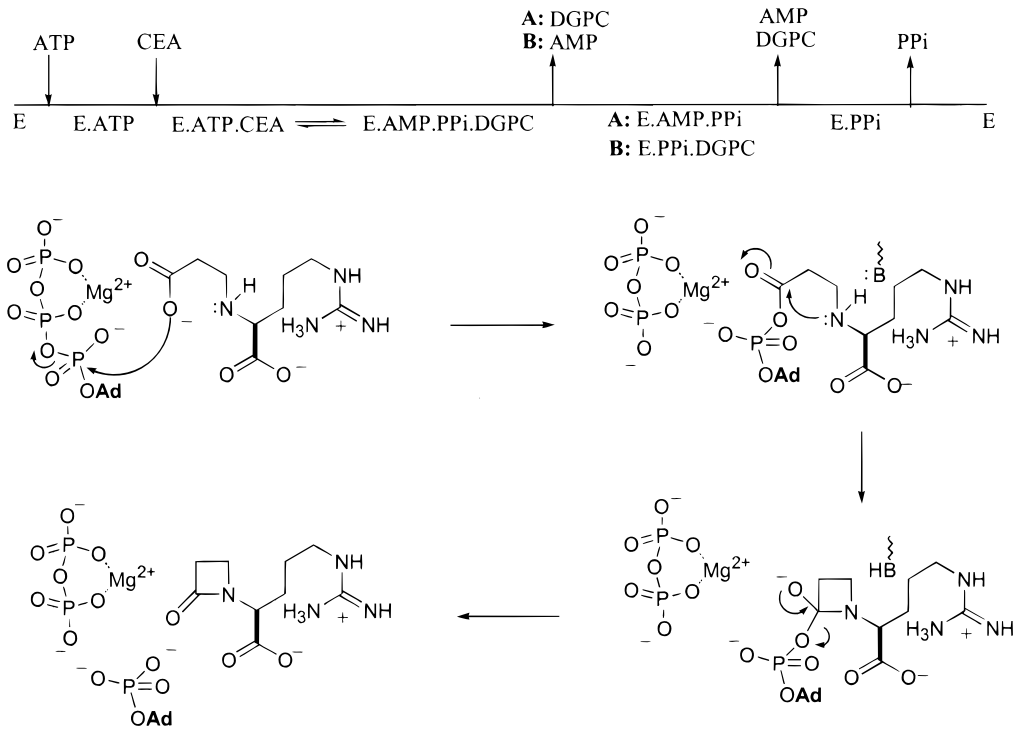

FIGURE 6: Proposed kinetic and hypothetical chemical mechanisms of β-LS. Routes A and B specify two possible orders of product release consistent with kinetic data.

DISCUSSION

β-Lactam synthetase catalyzes intramolecular amide bond formation in CEA by activating this β-amino acid through adenylation with ATP. The simplest kinetic mechanism consistent with the initial velocity studies and inhibition data presented in this work is shown in Figure 6. The order of substrate binding is strongly supported by uncompetitive inhibition exhibited by the substrate analogue CMA. Unfortunately, we were unable to observe the reverse reaction of β-LS, and, therefore, the order of product release cannot be conclusively determined from our data. The competitive inhibition of PP_i vs ATP suggests that PP_i is released last, but the absence of AMP inhibition data prevents rigorous assignment of their order of release. The proposed kinetic mechanism implies that DGPC, which bears structural resemblance to CEA, forms dead-end complexes with β-LS.

A hypothetical chemical mechanism of β-LS consistent with the kinetic mechanism is shown in Figure 6. After ordered binding of ATP and CEA, the β-carboxy group of CEA is adenylated. The carboxyethyl side chain must be held in an eclipsed conformation prior to intramolecular β-lactamization. Cyclization occurs via an oxo-anion intermediate, which may be stabilized by an "oxo-anion hole" (*19*, *20*) similar to class A and C β-lactamases, or by bound Mg^{2+}-PP_i, in a fashion analogous to class B Zn^{2+}-dependent β-lactamases (*21*, *22*). After the formation of DGPC, AMP and PP_i are free to diffuse from the active site.

Although we have never directly observed CEA-adenylate, indirect observation of time-dependent AMP and PP_i formation leave little room for alternative explanations. No conditions were found that indicated pyrophosphate exchange in the presence of saturating substrates and β-LS. This suggests that the forward commitment of β-LS, after adenylation of substrate, is very great and that the transformation is effectively irreversible. The homologous primary metabolic enzyme asparagine synthetase B (AS-B) demonstrates similar behavior under PP_i exchange conditions, and ^{18}O exchange from aspartate to AMP has been used to support the intermediate aspartyl adenylate in the formation of asparagine (*8*).

AS-B's form a branch of the widely distributed GAT superfamily of enzymes (*23*). These enzymes, to which β-LS is to a lesser extent homologous, couple the generation of ammonia, derived from the hydrolysis of glutamine, with the catalysis of N−C bond-forming reactions for the biosyntheses of diverse metabolites including amino acids, amino sugars, and ribonucleotides. Structural comparisons among members of this superfamily of proteins reveal two distinct domains: that responsible for glutamine utilization, or "glutamine amide transfer" (GAT); and the domain responsible for synthetase/synthase activity. The glutamine-binding site in AS-B is well resolved in the X-ray structure (*10*), which also reveals a putative hydrophobic tunnel approximately 19 Å from the less well-defined C-terminal synthetase domain, resolved with AMP bound. AS-B is subcategorized as an Ntn amidotransferase (formerly purF, or class II), in which an active site cysteine, invariably located at the N-terminus, attacks glutamine, resulting in a bound thioacyl enzyme intermediate and ammonia. Interestingly, amino acid sequence analysis suggests an evolutionary relationship between β-LS and AS-B. Several regions of conserved sequence are present in both N-terminal and C-terminal regions. However, the invariant N-terminal Cys-1 and Asn-74, essential for glutaminase activity (*24*, *25*), are missing in β-LS.

The relationship between the structure and function of unique secondary metabolic enzymes to the primary metabolic enzymes from which they are derived is poorly understood. The emergence of superfamilies of enzymes, the members of which possess the same fold, but often dissimilar activities, aids in our understanding of this functional divergence [cf. the enoyl hydratase superfamily (*26*)]. The

broad homology of β-LS to AS-B prompts the question: Why is there an apparent evolutionary relationship between β-LS and AS-B rather than, for instance, nonribosomal peptide or t-RNA synthetases? One possible explanation is that the synthetase domain of AS-B, which catalyzes the adenylation of aspartate, is readily adapted for adenylation of carboxyethylarginine. The substrate for β-LS, like aspartate, is a β-amino acid, and an oxo-anion intermediate is requisite in both mechanisms. Furthermore, it is possible that aspartate in AS-B is bound in an eclipsed conformation, similar to the conformation that CEA must be bound in order to resemble the product β-lactam. Schofield has proposed the possibility of cyclization of the adenylated aspartate in AS-B resulting in a β-lactam intermediate (*27*). Proving the existence of such an intermediate, or the eclipsed conformation of aspartate, would provide a compelling explanation for an evolutionary relationship between β-LS and AS-B.

The scope of the β-LS-mediated reaction as a general solution to the biosynthesis of β-lactams is limited only by the free energy of hydrolysis of ATP to AMP and PP_i under physiological conditions. While the value of $\Delta G^{\circ\prime} = -32.2$ kJ/mol (*28*) is commonly cited for this hydrolysis, careful examination of the more recent literature has revealed that the free energy change can be more accurately estimated as $\Delta G^{\circ\prime} = -48.6$ kJ/mol (*29*). The energy required to form a β-lactam ring can be estimated from thermodynamic data from the reverse reaction, the hydrolysis of the bicyclic β-lactams penicillin G and ampicillin. At 37 °C, pH 7, and an ionic strength of 0.25 M, a value of $\Delta G^{\circ\prime} = -5.4$ kJ/mol has been calculated for penicillin G, and $\Delta G^{\circ\prime} = -17.8$ kJ/mol for ampicillin (*30*). Apparently, coupling β-lactam formation with the hydrolysis of ATP is thermodynamically favorable, implying that β-LS-type enzymes could make a wide variety of monocyclic and bicyclic β-lactams.

The highly exothermic oxidative chemistry of IPNS (estimated $\Delta G^{\circ\prime} = -119.6$ kJ/mol) (*31*) is modulated by precise control of substrate and intermediate conformations through numerous steric interactions with the enzyme. In this way, IPNS functions as a template by favoring formation of a single product out of a continuum of possible side-products. (*32*) This product-selective mode of action, typical in enzymatic oxidative chemistry, makes IPNS a highly specific solution to the biosynthesis of fused thiazolidine ring β-lactams. In contrast, *Streptomyces clavuligerus* β-LS catalyzes the discrete intramolecular condensation of a β-amino acid to a monocyclic β-lactam, coupling this process to the hydrolysis of ATP. Enzymes of this type may occur more widely and play an analogous role, for example, in carbapenem biosynthesis (*33*). The power of biosynthetic pathway manipulation by genetic means has been demonstrated in the synthesis of structurally altered natural products (*34*, *35*). In this regard, as a catalyst of a functional group transformation, β-LS may have broader potential application in engineered β-lactam antibiotic biosynthesis.

ACKNOWLEDGMENT

We are grateful to Dr. Ronald L. Koder and Dr. Timothy S. Hitchman for advice on kinetics and for critical comments during preparation of the manuscript.

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BI000709I