Kinetic Mechanism of *Escherichia coli* Asparagine Synthetase B†

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Received May 7, 1998; Revised Manuscript Received July 9, 1998

ABSTRACT: *Escherichia coli* asparagine synthetase B (AS-B) catalyzes the synthesis of asparagine from aspartate, glutamine, and ATP. A combination of kinetic, isotopic-labeling, and stoichiometry studies have been performed to define the nature of nitrogen transfer mediated by AS-B. The results of initial rate studies were consistent with initial binding and hydrolysis of glutamine to glutamate plus enzyme-bound ammonia. The initial velocity results were equally consistent with initial binding of ATP and aspartate prior to glutamine binding. However, product inhibition studies were only consistent with the latter pathway. Moreover, isotope-trapping studies confirmed that the enzyme−ATP−aspartate complex was kinetically competent. Studies using ^{18}O-labeled aspartate were consistent with formation of a β-aspartyl-AMP intermediate, and stoichiometry studies revealed that 1 equiv of this intermediate formed on the enzyme in the absence of a nitrogen source. Taken together, our results are most consistent with initial formation of β -aspartyl-AMP intermediate prior to glutamine binding. This sequence leaves open many possibilities for the chemical mechanism of nitrogen transfer.

Escherichia coli asparagine synthetase B (AS-B)[1] is one of two key biosynthetic enzymes involved in asparagine production in this organism. This enzyme has served as a model system for the human enzyme, which plays a key role in asparagine resistance in cases of acute lymphoblastic leukemia (ALL) (*1*). In the physiologically relevant reaction, AS-B catalyzes the transfer of the amide nitrogen of glutamine to the side chain of aspartate in an ATP-dependent reaction (Scheme 1). In vitro, the enzyme can also catalyze

Scheme 1

$$\text{Asp} + \text{Gln} + \text{ATP} \rightleftharpoons \text{Asn} + \text{Glu} + \text{AMP} + \text{PPi} \quad (1)$$

$$\text{Asp} + \text{NH}_3 + \text{ATP} \rightleftharpoons \text{Asn} + \text{AMP} + \text{PPi} \quad (2)$$

$$\text{Gln} + \text{H}_2\text{O} \rightleftharpoons \text{Glu} + \text{NH}_3 \quad (3)$$

an analogous reaction in which free ammonia acts as the nitrogen source. Finally, in the absence of aspartate, the enzyme can also act as a glutaminase, hydrolyzing glutamine to glutamate and ammonia.

Asparagine synthesis by AS-B involves formal hydrolysis of glutamine, a reaction common to other members of the class II glutamine amidotransferase (GAT) subfamily of enzymes (*2*). Because nearly all of these enzymes can utilize free ammonia in the absence of glutamine and all members of the subfamily possess an N-terminal cysteine residue that is essential for the catalytic activity (*3*−*5*), a common mechanism has been proposed in which enzyme-bound ammonia is the key intermediate (*6*). By analogy with cysteine proteases, the side chain of Cys-1 is proposed to attack the γ-carbonyl of bound glutamine to produce a covalent thioester intermediate and ammonia, which is used for enzyme-mediated transfer to a second substrate. Before this transfer occurs, the thioester is hydrolyzed to glutamate, which is released prior to binding of the other substrate(s). One difficulty with this proposed mechanism is the requirement that the enzyme completely sequester free ammonia to prevent wasteful hydrolysis of glutamine. Moreover, the enzyme must also control the ionization state of ammonia under conditions where the ammonium cation would be heavily favored. A hydrophobic tunnel connecting the glutaminase domain with a synthetase domain was recently reported in the structure for phosphoribosylpyrophosphate amidotransferase (GPA), another member of the class II subfamily (*7*). It has been proposed that this channel solves the problems inherent in preventing ammonia loss from the enzyme. However, other mechanisms for nitrogen transfer that do not involve free ammonia have also been proposed (*8*, *9*). Here, we describe a series of studies designed to circumscribe more closely the range of mechanistic possibilities for nitrogen transfer in *E. coli* AS-B.

The kinetic mechanism places key limits on the range of allowable chemical mechanisms for an enzymatic reaction. Milman et al. (*10*) concluded that mouse pancreas asparagine synthetase followed a kinetic mechanism in which glutamine

† This work was supported by a grant (CA 28725) from the National Cancer Institute, National Institutes of Health, DHHS.

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[1] Abbreviations: AS, asparagine synthetase; AS-B, asparagine synthetase B; ALL, acute lymphoblastic leukeima; GAT, glutamine amide transfer; GPA, glutamine 5′-phosphoribosylpyrophosphate amidotransferase; LGH, L-glutamic acid γ-monohydroxamate; TCA, 2,2,2-trichloroacetic acid; SA, specific activity.

S0006-2960(98)01058-7 CCC: $15.00
Published on Web 09/05/1998

was the first substrate on, glutamate was released, then ATP and aspartate bound. This kinetic mechanism is only consistent with ammonia as the nitrogen donor. However, the distinction between this and alternate mechanisms was based on a single product inhibition pattern. We have, therefore, determined the kinetic mechanism of *E. coli* AS-B and supplemented these results with data from stoichiometry, isotopic-labeling, and isotope-trapping studies. While our results are consistent with mechanisms involving ammonia as required intermediate in asparagine synthesis, they also conclusively demonstrate that glutamine hydrolysis is not an obligate first step in productive turnover. This allows for species other than free ammonia to act as the nitrogen donor.

EXPERIMENTAL PROCEDURES

General purification and expression of AS-B is described elsewhere (*4*). The Bio-Rad (Hercules, CA) protein assay kit was used to determine protein concentration. A standard curve was made using a known concentration of AS-B.

Initial Velocity Studies. Affinity constants (K_M) for AS-B substrates were determined by incubating purified AS-B in reaction mixtures in which one substrate was held constant, but nonsaturating, while the other two were varied. All assays contained 100 mM Tris-HCl, pH 8.0, 8.0 mM $MgCl_2$, and 0.043 nmol of the enzyme in a total volume of 160 μL. When ATP, aspartate or glutamine were varied, the concentration ranges used were 0.05−0.9 mM, 0.1−0.8 mM, and 0.1−0.9 mM, respectively. When ATP, aspartate, or glutamine was held constant, the concentration used was 1 mM. The initial velocity of each reaction was determined spectrophotometrically by following the production of pyrophosphate during asparagine synthesis using pyrophosphate reagent (Sigma Chemical Co., Technical Bulletin no. BI-100). Each assay was performed two to four times and the averages are presented. Kinetic constants were obtained by nonlinear regression using equations derived from the full kinetic expression using the software program Prism (Graph Pad, San Diego CA).

Competitive Inhibitor Studies. The velocities for each experiment were measured spectrophotometrically as described above. Each initial velocity is the average of two parallel experiments. All assays contained 100 mM Tris-HCl, pH 8.0, 10.0 mM $MgCl_2$, and 0.030 nmol of the enzyme in a total volume of 160 μL. When ATP, aspartate, or glutamine was varied, the concentration ranges used were 0.05−0.6 mM, 0.1−1.1 mM, and 0.1−0.9 mM, respectively. When ATP, aspartate, or glutamine was held constant, the concentrations used were 0.5, 1, and 1 mM, respectively. Inhibitors, when added, were varied as follows: AMP-PNP, 0−4.0 mM, β-methyl aspartate, 0−3.0 mM; and L-glutamic acid γ-methyl ester, 0−5 mM.

^{18}O Transfer Studies. Aspartate labeled with ^{18}O at all positions was synthesized by incubating 0.059 g of aspartate in 700 μL of 95% $H_2{}^{18}O$ at pH ∼1 using concentrated HCl, then heating to 80 °C for 14 days in a sealed tube. The water was removed to produce a final concentration of 2.5 M, then the pH was adjusted to pH 8. The solution was frozen until needed.

AS-B (1.2 nmol) was incubated with 10 mM glutamine, 10 mM [^{18}O]aspartate, 10 mM ATP, 15 mM $MgCl_2$, and 100 mM Tris-HCl, pH 8, in a volume of 1 mL for varying times. The reactions were terminated by the addition of TCA to a final concentration of 4%, then filtered. The solutions were centrifuged, and 0.2 mL of 99.9% D_2O, 0.045 g of glycine, and 0.0287 g of EDTA were added to 0.7 mL of the supernatants. The pH of each reaction was adjusted to 9.5 and the total volume to 1 mL.

^{31}P-NMR spectra were recorded at 121 MHz with broadband proton decoupling on a Varian VXR-300 NMR spectrometer at 25 °C. Five hundred scans with an acquisition time of 3.003 s were accumulated. Chemical shifts were referenced with respect to the β-phosphorus of ATP.

ATP−PPi Isotope Exchange Studies. Reactions contained 85 mM Tris, pH 7.0, 8 mM $MgCl_2$, and the specified substrates in a final volume of 200 μL. AS-B, (0.006−0.030 nmol) was added, and the mixture was incubated for a given amount of time (15−120 min). The reaction was then terminated by adding TCA (final concentration 10%), then it was centrifuged to remove precipitated protein. A portion (250 μL) of the supernatant was removed and added to 60 mg of Norit A. To this was added 750 μL of 0.1 M NaOAc, then the mixture was vortexed and centrifuged. The supernatant was removed and placed in a scintillation vial and the amount of radioactivity was determined. The Norit A pellet was washed with 1 mL of 0.05 M acetate buffer, pH 4.5, containing 0.1 M PPi. This wash was repeated three times with 0.05 M acetate buffer, followed by one time with water. The Norit A was then resuspended in 1 mL of 1 M HCl, heated to 100 °C for 20 min, and 510 μL was transferred to a scintillation tube. The radioactivity associated with this fraction was determined.

Determination of the Rate of ATP Hydrolysis in the Absence of a Nitrogen Source. The hydrolysis of ATP in the absence of a nitrogen source was investigated by combining 0.1 mM [2,8-^3H]ATP (1×10^6 cpm/nmol), 5 mM aspartate, 100 mM Tris-HCl, pH 7.0, and 8 mM $MgCl_2$ in a total volume of 75 μL. The mixture was prewarmed to 37 °C, and the reaction was started by the addition of 0.58 nmol of AS-B. At given time points, 10 μL of the mixture was withdrawn and the reaction was terminated by the addition of 0.6 μL of 65% TCA. The pH was then neutralized by adding of 5 μL of 3 M Tris-HCl pH 11, prior to analysis by paper chromatography.

Stoichiometry of Asparagine and Glutamate Formation. The amount of glutamate produced was determined by the use of glutamate dehydrogenase in the presence of NAD^+ (*11*). Reaction mixtures (500 μL) contained 100 mM Tris-HCl, pH 8, and 8.0 mM $MgCl_2$ with a variable concentration of glutamine between 0.1 and 20 mM and fixed concentrations of ATP and aspartate. Reactions were initiated by adding purified AS-B (0.012 nmol) and these were incubated for 10 min before being terminated by the addition of 20 μL of 1 N acetic acid. A 150 μL portion of the reaction mixture was added to 350 μL of the coupling reagent (300 mM glycine, 250 mM hydrazine, pH 9, 1 mM ADP, 1.6 mM NAD^+, and 2.2 units of glutamate dehydrogenase) and this was incubated for 10 min at room temperature. The solution absorbance was measured at 340 nm and the amount of glutamate produced in the reaction was determined from a standard curve. To determine the amount of PPi produced, 200 μL of the reaction mixture was added to 100 μL of pyrophosphate reagent (Sigma Chemical Co., Technical

Bulletin no. BI-100) and 200 μL of H_2O. The reactions were incubated for 5 min, and the amount of PPi produced was determined spectrophotmetrically from a standard curve.

Isotope Trapping Using [^{3}H]ATP in the Pulse. Pulse solutions containing 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, and 0.5 mM [2−8-^{3}H]ATP (200mCi/mmol) were prewarmed to 37 °C. When a nitrogen source was included in the pulse, 10 mM glutamine was used for the glutamine-dependent reaction and 13.3 mM NH_4Cl was used for the ammonia dependent reaction. When aspartate was present in the pulse, it was added at 10 mM. The pulse was initiated by the addition of 1.79 nmol of AS-B in a final volume of 75 μL. Reactions were equilibrated for 10 s, then 25 μL of a chase solution was added, and this was incubated for an additional 3 s before the reactions were terminated by adding TCA to a final concentration of 4%. The final concentration were 50 mM Tris-HCl, pH 8.0, 25 mM ATP, 30 mM $MgCl_2$, 10 mM glutamine or 10 mM NH_4Cl, and 10 mM aspartate. Reactions were then neutralized with Tris-HCl and analyzed using DE81 paper and 1/50 saturated solution of ammonium acetate, pH 2.75, as a solvent. The papers were run in a closed system for 3 h, then the radioactivity associated with ATP, ADP, and AMP was determined using a Beckman scintillation counter, model no. LS60001C.

Isotope Trapping Using [^{14}C]Aspartate in the Pulse. Pulse solutions containing 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, and 0.5 mM aspartate (40 mCi/mmol) were prewarmed to 37 °C. When a nitrogen source was included in the pulse, 10 mM glutamine was used. When ATP was present in the pulse, it was added at a final concentration of 5 mM. The pulse was initiated by the addition of 0.78 nmol of AS-B in a final volume of 50 μL. Reactions were equilibrated for 10 s, then 50 μL of a chase solution was added, and the mixture was incubated for an additional 3 s before it was terminated by adding TCA to a final concentration of 4%. The final concentrations of reagents were 50 mM Tris-HCl, pH 8.0, 25 mM ATP, 30 mM $MgCl_2$, 10 mM glutamine, and 10 mM aspartate. Reactions were then neutralized with Tris-HCl and analyzed using DE81 paper. The chromatography paper was developed for 8 h using H_2O as a solvent.

Determination of the ATP K_d Using [^{3}H]ATP. Pulse solutions containing 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, and a variable amount of [^{14}C]ATP (4.4−44.2 μM) were prewarmed to 37 °C. The pulse was initiated by the addition of 0.5 nmol of AS-B in a final volume of 25 μL. Reactions were equilibrated for 10 s, then 25 μL of a chase solution was added and the mixture was incubated for an additional 3 s before it was terminated by adding TCA to a final concentration of 4%. The final reagent concentrations were 50 mM Tris-HCl, pH 8.0, 25 mM ATP, 30 mM $MgCl_2$, 10 mM glutamine, and 10 mM aspartate. Control reactions were performed in which the nonradiolabeled aspartate was added to the pulse in the appropriate concentration and the radiolabeled ATP was added to the chase solution. Reactions were neutralized with Tris-HCl and analyzed using paper chromatography as described above.

Determination of the Aspartate K_d Using [^{14}C]Aspartate. Pulse solutions containing 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 5.0 mM ATP, and varying amounts of [^{14}C]aspartate (0.05−0.5 mM) were prewarmed to 37 °C. The pulse was initiated by the addition of 1.04 nmol of AS-B in a final volume of 50 μL. Reactions were equilibrated for 10 s, then 50 μL of a chase solution was added, and the mixture was incubated for an additional 3 s before it was terminated by adding TCA to a final concentration of 4%. The final reagent concentrations were 50 mM Tris-HCl, pH 8.0, 20 mM ATP, 22 mM MgCl2, 10 mM glutamine, and 10 mM aspartate. Control reactions were performed in which the nonradiolabeled aspartate was added to the pulse in the appropriate concentration, and the radiolabeled aspartate was added to the chase solution. Reactions were neutralized with Tris-HCl and analyzed using paper chromatography as described above.

Product Inhibition Studies. Inhibition constants (K_{is} and K_{ii}) for AS-B were determined by incubating AS-B in a reaction mixture with two substrates present at saturating concentrations and one substrate whose concentration was varied in the presence of several fixed concentrations of inhibitor. The concentration of the substrates for AS-B when held constant were 5 mM ATP, 10 mM glutamine, and 10 mM aspartate. The concentrations of substrates when varied were 0.3−1.3 mM aspartate, 0.3−1.3 mM glutamine, and 0.075−0.5 mM ATP. Products when added were varied as follows: 0−200 mM L-glutamate, 0−30 mM AMP, 0−0.25 mM Asn, and 0−1.5 mM PPi. All assays contained 100 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, and 0.043 nmol of purified enzyme in a total volume of 160 μL. The initial velocity of each reaction was determined spectrophotometrically, and data was analyzed as described above except for the case of PPi. For these reactions, the velocities were measured by monitoring the conversion of aspartate to asparagine using HPLC amino acid analysis. Reactions contained 100 mM Tris-HCl, pH 7.0, to aid in PPi solubility. The concentration of $MgCl_2$ was kept constant at 8 mM. Reaction mixtures were prewarmed to 37 °C, then they were started by the addition of 0.0088 nmol of AS-B in a total volume of 100 μL, incubated for 10 min at 37 °C, then terminated with 4% TCA. Reactions were filtered, and a 10 μL aliquot was analyzed by HPLC amino acid analysis on a 420A derivatizer, 130A separation system (Applied Biosystems).

RESULTS

Initial Velocity Studies. We used the method of Frieden (*12*), in which one substrate was held at a nonsaturating concentration while the other two substrates were varied as in a bireactant system. This method can distinguish among many terreactant schemes and allows one to evaluate the kinetic parameters from secondary replots of the data. Initial velocity patterns for the glutamine-dependent reaction of AS-B were determined for all three of the substrate pairs. The patterns for the double reciprocal plots along with the Michaelis constants (K_m) for the substrates are listed in Table 1. Plots of $1/v$ versus 1/[ATP] or 1/[glutamine] at fixed aspartate concentrations and $1/v$ versus 1/[aspartate] or 1/[glutamine] at fixed ATP concentrations were parallel, characteristic of a ping-pong mechanism in which a product is released before subsequent substrates combine with the enzyme. By contrast, intersecting double reciprocal plots were observed from plots of $1/v$ versus 1/[ATP] or 1/[aspartate] at fixed glutamine concentrations. This indicates that the addition of these two substrates is sequential. Two kinetic mechanisms that are consistent with these patterns are shown in Scheme 2.

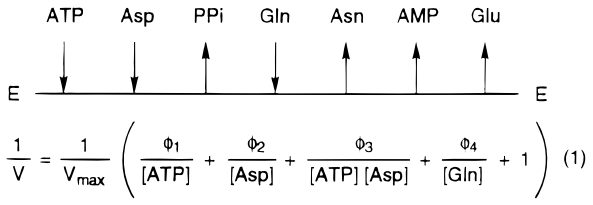
Table 1: Kinetic Constants and Patterns Obtained for AS-B Substrates

			Michaelis constants				
				aspartate		ATP	
substrate pair	fixed substrate	pattern	glutamine, K_{Mi} (mM)	K_{Ms} (mM)	K_{Mi} (mM)	K_{Ms} (mM)	K_{Mi} (mM)
Gln-Asp	ATP (1 mM)	P[b]	0.18 ± 0.031		0.051 ± 0.13		
Gln-ATP	Asp (1 mM)	P	0.013 ± 0.022				0.038 ± 0.09
ATP-Asp	Gln (1 mM)	I		0.20 ± 0.027	0.45 ± 0.067	0.053 ± 0.0035	0.14 ± 0.0036
	(20 mM)	I				0.034 ± 0.0013	0.20 ± 0.014

[a] Experimental conditions: pH 8.0, 37 °C; details given in the Experimental Procedures. [b] P = parallel, I = intersecting.

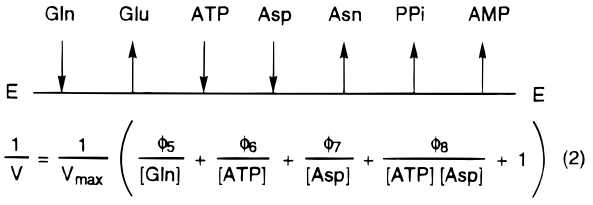
Scheme 2

Mechanism A:



$$\frac{1}{V} = \frac{1}{V_{max}}\left(\frac{\Phi_1}{[ATP]} + \frac{\Phi_2}{[Asp]} + \frac{\Phi_3}{[ATP]\,[Asp]} + \frac{\Phi_4}{[Gln]} + 1\right) \quad (1)$$

Mechanism B:



$$\frac{1}{V} = \frac{1}{V_{max}}\left(\frac{\Phi_5}{[Gln]} + \frac{\Phi_6}{[ATP]} + \frac{\Phi_7}{[Asp]} + \frac{\Phi_8}{[ATP]\,[Asp]} + 1\right) \quad (2)$$

Initial velocity equations were derived for both mechanism A (bi−uni−uni) and mechanism B (uni−uni−bi), according to the method of Huang (*13*). Equations 1 and 2 in Scheme 2 have been simplified and are presented for mechanisms A and B, respectively.

Equation 1 in Scheme 2 predicts that varying the concentration of glutamine will affect only the intercept of the $1/v$ vs 1/ [ATP] or 1/[aspartate] plot, which would result in parallel lines, characteristic of a ping-pong mechanism. Equation 1 in Scheme 2 also predicts that varying the concentration of ATP and aspartate will affect both the slope and the intercept of the $1/v$ vs 1/[aspartate] and vs 1/[ATP] plots. This would result in intersecting lines, which suggests that the addition of ATP and aspartate are sequential. Similarly, eq 2 in Scheme 2 predicts that changes in glutamine concentration will affect only the intercept of the $1/v$ vs 1/[ATP] and 1/[aspartate] plots, whereas varying the levels of ATP and aspartate will affect both the slope and intercept of the $1/v$ vs 1/[aspartate] and 1/[ATP] plots resulting in intersecting lines. In short, the initial rate studies agree with the theoretical results predicted from both eqs 1 and 2 in Scheme 2.

A key prediction of both mechanisms A and B is that altering the rate constants associated with glutamine consumption will not affect those associated with ATP and aspartate. L-Glutamic acid γ-monohydroxamate (LGH) can substitute for glutamine, and its hydroxylamine moiety can be transferred to aspartate (Figure 1) (*14*). Experiments with this alternate substrate confirmed the prediction. For example, when the aspartate concentration was fixed at 10 mM and the ATP concentration was varied in the presence of either 0.5 mM glutamine or LGH, the slope of the $1/v$ vs 1/[ATP] plot was identical within experimental error (Figure 1). Complementary experiments in which either the aspartate concentration was held constant at 1 mM or the concentrations of glutamine and LGH were held constant at 10 mM were also performed. In all cases, the lines were parallel within experimental error. Unfortunately, these substrate analogue experiments do not distinguish between mechanisms A and B since both make identical predictions.

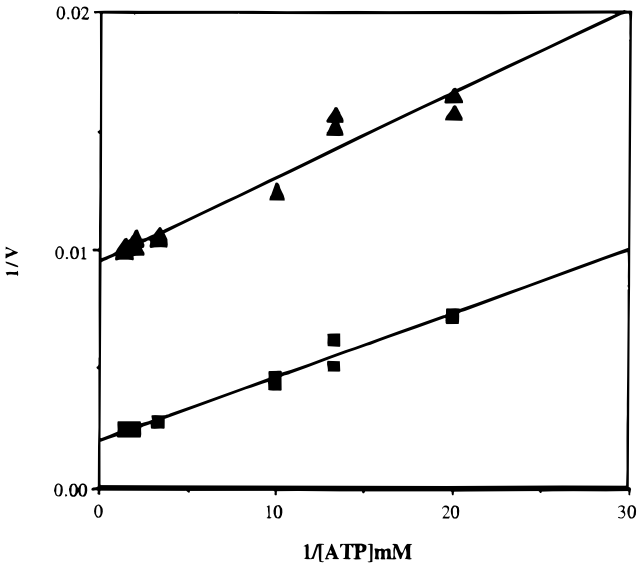


FIGURE 1: Effect of a glutamine analogue (L-glutamic acid γ-hydroxamate; LGH) on the kinetic parameters associated with ATP and aspartate. In both cases, the concentration of asparate was fixed at 10 mM and that of ATP was varied. The slopes obtained when 0.50 mM L-glutamine [(■) $(2.4 \times 10^{-4}) \pm (1.28 \times 10^{-5})$] and 0.50 mM LGH [(▲) $(3.1 \times 10^{-4}) \pm (2.55 \times 10^{-5})$] were used as nitrogen donors are nearly equivalent.

Use of Competitive Inhibitors To Study Substrate-Binding Order. Competitive inhibitors were also used to study the substrate-binding order according to the method of Fromm (*15*). The substrate analogues, AMP-PNP, β-methyl aspartate, and L-glutamic acid γ-methyl ester (Figure 2), were found to be competitive inhibitors of ATP, aspartate, and glutamine, respectively (Table 2). Initial velocity studies in the presence of AMP-PNP demonstrated competitive inhibition with respect to ATP, noncompetitive inhibition with respect to aspartate, and uncompetitive inhibition with respect to glutamine. β-Methyl aspartate was found to be competitive with respect to aspartate and noncompetitive with respect to ATP and glutamine. L-Glutamic acid γ-methyl ester was found to be competitive with glutamine, uncompetitive with respect to ATP, and noncompetitive with respect to aspartate. The rate equations for a competitive inhibitor with respect to each substrate were derived for both mechanisms A and

Table 2: Inhibition Patterns for AS-B Using Substrate Analogs[a]

inhibitor	substrate	predicted pattern	observed pattern	K_{is} (mM)	K_{ii} (mM)
AMP-PNP	ATP	C[b]	C	0.91	
	aspartate	NC	NC	5.2	4.5
	glutamine	UC	UC		3.2
β-methyl	ATP	NC	NC	8.15	15
aspartate	aspartate	C	C	18	>50
	glutamine	UC	NC	93	16.5
L-glutamic	ATP	UC	UC		10.4
acid γ-methyl	aspartate	UC	?		
ester	glutamine	C	C	6.6	3.2

[a] When equations are derived for mechanism A, where A = ATP, B = aspartate, and C = glutamine, and AMP-PNP is competitive with respect to A, $1/v = 1/v_{max}\{K_a/[A](1 + I/K_i) + K_b/[B] + K_{ia}K_b/[A][B](1 + I/K_i) + K_c/v_{max}[C]\}$; β-methyl aspartate is competitive with respect to B; $1/v = 1/v_{max}\{K_a/[A] + K_b/[B](1 + I/K_i) + K_{ia}K_b/[A][B](1 + I/K_i) + K_c/v_{max}[C]\}$; L-glutamic acid γ-methyl ester is competitive with respect to C; $1/v = 1/v_{max}\{K_a/[A] + K_b/[B] + K_{ia}K_b/[A][B] + K_c/[C](1 + I/K_i)\}$. When the same procedure is used to derive the equations for inhibition constants from mechanism B, the same predicted patterns are obtained. [b] C = competitive inhibition, UC = uncompetitive inhibition, NC = noncompetitive inhibition.

B (see Table 2 footnote), and although there was one disagreement (β-methyl aspartate vs glutamine), the data were in good agreement with the predicted patterns.

^{18}O Transfer Studies. While it has been widely assumed that asparagine synthesis involves a β-aspartyl-AMP intermediate, the existence of this species has never been directly demonstrated for the case of *E. coli* AS-B. When AS-B was incubated with ^{18}O-labeled aspartate in the presence of ATP and glutamine, two peaks were observed for AMP, at 24.692 and 24.670 ppm, along with a single peak for free phosphate (23.583 ppm) (Figure 3). Free phosphate was seen rather than pyrophosphate due to the presence of a small amount of contaminating pyrophosphatase activity. The presence of two peaks for AMP, separated by 0.022 ppm, indicated that ^{18}O was transferred from aspartate to AMP and not to phosphate (*16*, *17*). Addition of 2 mM unlabeled AMP increased the size of the downfield peak, confirming the assignment.(Figure 3).

If β-aspartyl-AMP is an intermediate in asparagine synthesis, it should be possible to observe exchange of labeled PPi into ATP. A variety of different reaction conditions were tested in order to identify an exchange reaction in AS-B, but without success (conditions listed in Supporting Information). Surprisingly, conditions that were successfully used for *E. coli* asparagine synthetase A (AS-A) failed to give detectable ATP/PPi exchange for AS-B (*18*).

Stoichiometry of Aspartyl-AMP Intermediate. Taken together, the above results suggest that aspartyl-AMP formation must be effectively irreversible. To test this hypothesis, radiolabeled ATP and aspartate were mixed with 0.58 nmol of AS-B in the absence of a nitrogen source, and the reaction was terminated after various time intervals. Clearly, the amount of extrapolated product does not pass through the origin (Figure 4). The burst height (0.41 nmol) is comparable to the amount of AS-B present (0.59 nmol), indicating that approximately 1 equiv of PPi is formed/nmol of AS-B. After this rapid formation of the intermediate, it can undergo very slow rate of ATP hydrolysis in the absence of a nitrogen source. This rate, 2.2 nmol/min/mg AS-B, is approximately 1500-fold slower than the rate of breakdown in the presence of a nitrogen source, and most likely corresponds to adventitious hydrolysis of the intermediate. To ensure that the breakdown rate was not due to contaminating ammonia, HPLC amino acid analysis was performed on duplicate samples. No asparagine was observed under these conditions where 0.035 nmol (0.06 equiv) would have been detectable.

Stoichiometry of Asparagine and Glutamate Formation. We and others have shown that glutamate and asparagine are not formed in a 1:1 ratio [unpublished data (*19*)], which would indicate that the glutamine-consuming and asparagine-producing reactions are not completely coupled. To understand the relevance of the glutaminase reaction to the asparagine synthesis reaction, a study was performed in which the stoichiometry of the products was measured under varying conditions. Under all circumstances, the amount of asparagine produced was equal to the amount of pyrophosphate and AMP produced (data not shown). Thus, coupling between glutaminase and synthetase reactions was studied by comparing pyrophosphate and glutamate production. Several conditions were used to learn how glutamate production was coupled to asparagine formation. In the first experiment, glutamine was varied between 0.2 and 20 mM in the presence of nonsaturating ATP and aspartate (Figure 5). Even at low glutamine concentrations (0.2 mM), the glutamate/PPi ratio is >1.5:1. This ratio steadily increased with increasing glutamine concentration and finally leveled out at a ratio of approximately 4 nmol of glutamate produced for every 1 nmol of PPi produced. Since the enzyme activity appeared to be extremely uncoupled under these conditions, the concentrations of ATP and aspartate were varied to determine if a set of conditions could be found where the reaction was coupled. This was unsuccessful. In no case was the glutamate:PPi ratio equal to 1. The smallest degree of uncoupling occurred when glutamine was low (0.2 mM), ATP was nonsaturating, and aspartate was saturating.

Isotope Trapping. Since the results from the initial velocity experiments could not differentiate between mechanism A and B, isotope-trapping methods were performed to obtain information about the order of addition of the substrates in the AS-B reaction. First, [^{3}H]ATP, Mg^{2+}, and AS-B were added in the pulse solution, then a chase solution containing a 50-fold excess of unlabeled ATP and saturating concentrations of aspartate, glutamine, and Mg^{2+} was added. The reaction was then quenched, and the products were analyzed (Table 3). When [^{3}H]ATP was present in the pulse solution, 0.78 equiv of [^{3}H]AMP was formed/nmol of AS-B. The presence of 10 mM glutamine in the pulse had no effect on the amount of radiolabled ATP trapped; however, the presence of 10 mM aspartate increased the amount of ATP trapped to stoichiometric levels (Table 3). An analogous series of reactions in which ammonia was substituted for glutamine in the pulse gave similar results (Table 3). Control experiments were performed in which the labeled ATP was added to the chase solution. The amount of AMP produced was used in the determination of the background activity. Control experiments were also performed in which one of the substrates in the chase were omitted, so that the overall reaction was not allowed to proceed. The results presented here support a mechanism whereby ATP can bind competently to the enzyme prior to the addition of glutamine or aspartate.

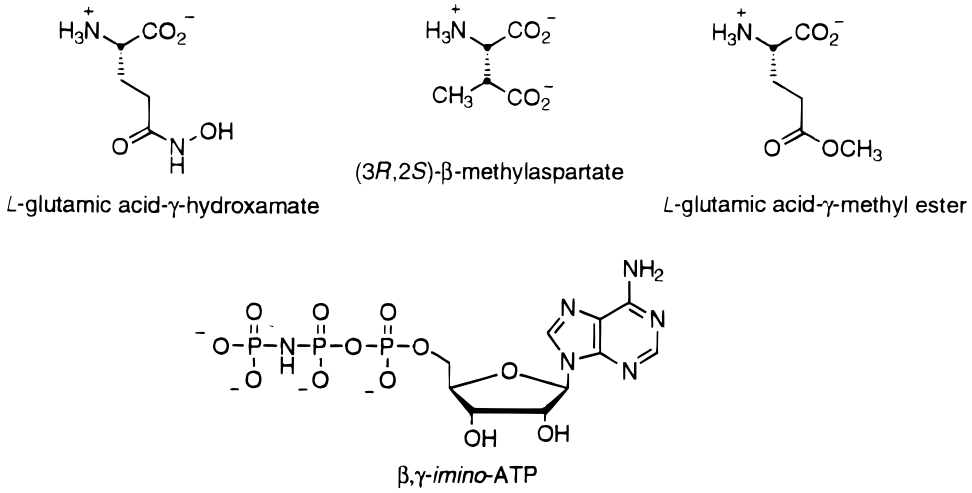


FIGURE 2: Structures of alternate substrate and inhibitors used in this study.

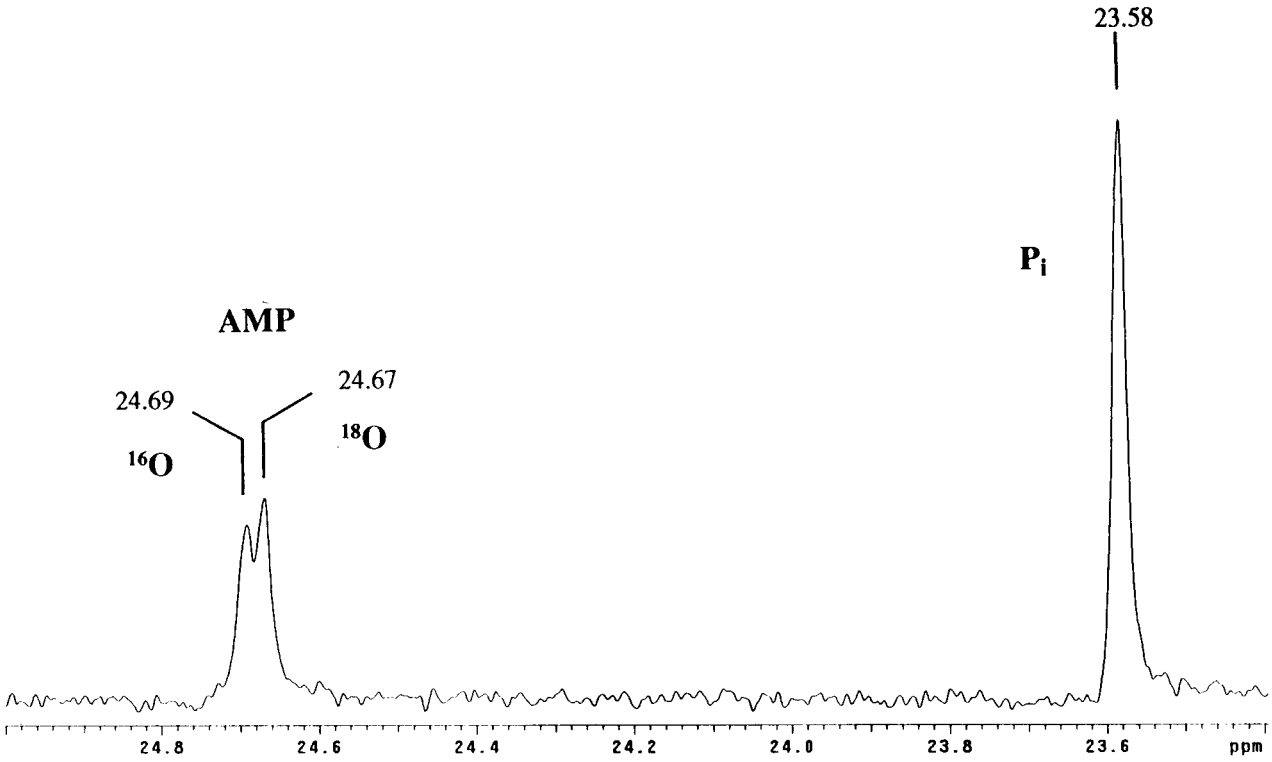


FIGURE 3: ^{31}P-NMR spectrum of the reaction mixture obtained from ^{18}O-labeled aspartate. Detailed conditions are given in the Experimental Procedures. Nominal chemical shifts for AMP and P_i were determined by comparison to authentic materials. Peaks for ^{16}O- and ^{18}O-labeled AMP (separated by 0.022 ppm) are indicated. No ^{18}O-labeled P_i was observed.

These studies were then repeated using [^{14}C]aspartate in the pulse. Very little [^{14}C]asparagine was produced when either labeled aspartate or the combination of labeled aspartate and glutamine was present in the pulse solution (Table 4). By contrast, a substantial amount of [^{14}C]-asparagine was produced when labeled aspartate and 5.0 mM ATP were present in the pulse. The results of these trapping experiments indicate that aspartate cannot bind to free enzyme in a catalytically competent manner, but it can bind to the E−ATP complex. Moreover, the combination of aspartate and glutamine cannot form a catalytically competent complex.

The K_d values for ATP and the binding of aspartate to the AS-B−ATP complex were determined. Scatchard plots yielded ATP and aspartate K_d values of 19 ± 2.5 μM and K_d of 47 ± 4.4 μM, respectively (figures available in Supporting Information). The value obtained for the ATP K_d is consistent with that found by protection studies employing FSBA as an inhibitor (*20*). These studies also revealed that only one molecule of ATP and aspartate is bound to the enzyme, since extrapolation of the line leads to n values for ATP and aspartate of 1.1 ± 0.1 and 1.0 ± 0.1, respectively.

Product Inhibition Studies. Product inhibition studies were undertaken to try to understand better the kinetic mechanism of AS-B and to gain insight into the regulation of the enzyme activity byproduct levels. The results of these studies along with the inhibition constants obtained are shown in Table 5. The concentration of reactants was held at saturating levels, and product concentrations used in these studies are outlined

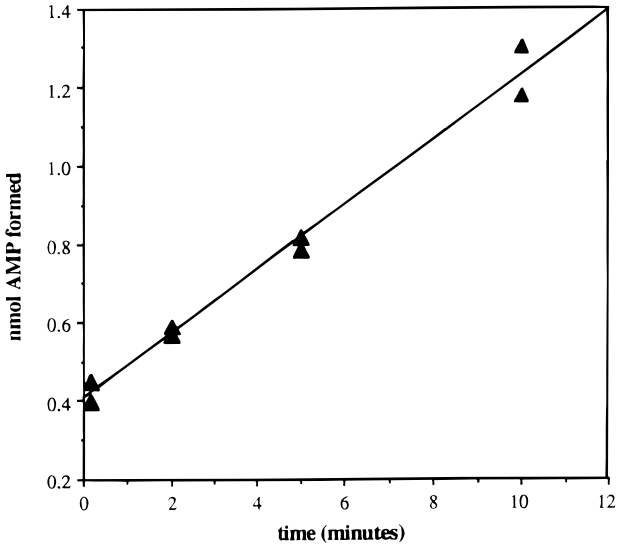


FIGURE 4: AMP formation in the absence of a nitrogen source. Enzyme (0.58 nmoles) was mixed with saturating levels of ^{3}H-labeled ATP and aspartate and the amount of AMP formed was determined. The extrapolated *y*-intercept is 0.41 ± 0.02 nmol.

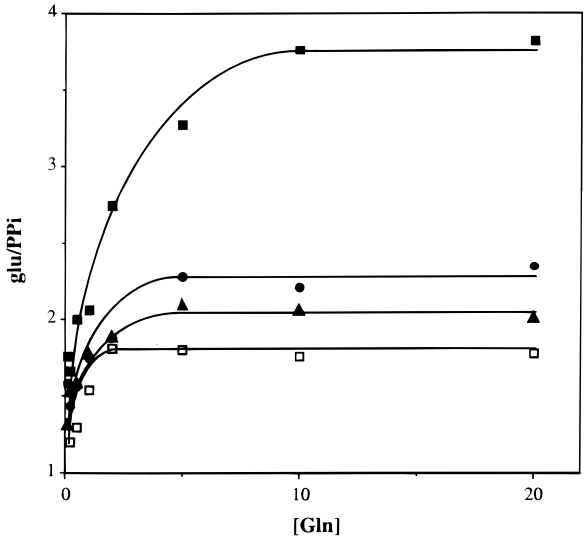


FIGURE 5: Stoichiometry of asparagine formation versus glutamine consumption. Detailed conditions are given in the Experimental Procedures. Both ATP and aspartate concentrations were fixed at the indicated levels and the glutamine concentration was varied, 0.5 mM ATP and 1.0 mM aspartate (■); 5.0 mM ATP and 1.0 mM aspartate (●); 5.0 mM ATP and 10 mM aspartate (□); 0.5 mM ATP and 10 mM aspartate (▲). The ratio of the velocities of glutamine consumption and PP_i formation was determined.

Table 3: Isotope Trapping Using [^{3}H]ATP in the Pulse and Detection of [^{3}H]AMP[a]

nitrogen source	ATP	ATP plus nitrogen source	ATP plus Asp
Gln	0.78 ± 0.10	0.78 ± 0.021	1.12 ± 0.065
ammonia	0.73 ± 0.15	0.54 ± 0.065	1.05 ± 0.17

[a] Data are presented as nanomoles of *AMP formed of enzyme.

in the Materials and Methods. Most of the patterns obtained were obviously competitive, noncompetitive, or uncompetitive; however, some patterns could not be distinguished. For example, pyrophosphate vs aspartate was shown to have an intersecting pattern very close to the *y*-axis. Whether this was competitive or noncompetitive could not be determined.

Table 4: Isotope Trapping Using [^{14}C]Aspartate in the Pulse with Detection of [^{14}C]Asparagine[a]

nitrogen source	Asp	Asp plus Gln	ATP plus Asp
Gln	0.12 ± 0.032	0.11 ± 0.078	0.76 ± 0.030

[a] Data are presented as nanomoles of *asparagine formed per nanomole of enzyme.

Table 5: Product Inhibition Patterns and Inhibition Constants for AS-B

varied substrate	product inhibitor	inhibition pattern	inhibition constants K_{is}	inhibition constants K_{ii}
ATP	PPi	NC[a]	0.25 ± 0.025	1.06 ± 0.16
aspartate	PPi			
glutamine	PPi			
ATP	asparagine	NC	0.93 ± 0.19	0.13 ± 0.06
aspartate	asparagine	NC	0.33 ± 0.07	0.20 ± 0.03
glutamine	asparagine			
ATP	AMP	NC	nl	28 ± 9
aspartate	AMP	UC		21 ± 7
glutamine	AMP	UC		29 ± 8
ATP	glutamate	NC	333 ± 38	542 ± 280
aspartate	glutamate	NC	179 ± 55	159 ± 52
glutamine	glutamate	UC		372 ± 101

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

Also when examining inhibition patterns for PPi vs glutamine, a definitive pattern could not be obtained. When asparagine was used as the inhibitor with respect to glutamine, a similar problem was encountered. The double reciprocal plot appeared to intersect just left of the *y*-axis. When higher asparagine concentrations were used (0.25 mM) the lines intersected further away from the *y*-axis. Whether this pattern was competitive or noncompetitive remains questionable. Both PPi and asparagine had inhibition constants in the mid to high micromolar range. AMP and glutamate were shown to be very poor inhibitors of the AS-B reaction, with inhibition constants approximately 30 mM for AMP and greater than 200 mM for glutamate. The patterns described for these two inhibitors were unequivocal and are presented in Table 5.

DISCUSSION

Our goal is to understand the mechanism of nitrogen transfer in *E. coli* asparagine synthetase B. Since this enzyme possesses significant glutaminase activity and it can utilize free ammonia in the synthesis of asparagine, it has been assumed by analogy to GPA (*6*) that the mechanism involves initial hydrolysis of glutamine followed by attack of enzyme bound ammonia on an aspartyl-AMP intermediate. Such a mechanism is intuitively appealing and well precedented from studies of other GAT enzymes. Determining the substrate-binding order can provide support for this type of mechanistic proposal. For example, an ordered kinetic mechanism in which glutamine binding is followed by glutamate release prior to aspartate and ATP binding (mechanism B, Scheme 2) would only be consistent with nitrogen transfer involving free ammonia. However, we note that not all the results obtained for *E. coli* AS-B are consistent with this type of mechanism. For example, the differing ^{15}N kinetic isotope effects found for the glutaminase and aspar-

agine synthetase reactions of *E. coli* AS-B are difficult to reconcile with obligatory glutamine hydrolysis prior to nitrogen transfer (*21*). These and other observations have compelled us to examine other possible nitrogen-transfer mechanisms.

The initial velocity patterns for *E. coli* AS-B are consistent with those presented by Milman et al. (*10*) and Hongo and Sato (*22*) for asparagine synthetases from mouse pancreas and mouse liver, respectively. The substrate-binding patterns were consistent with a mechanism involving initial glutamine hydrolysis (mechanism B, Scheme 2); however, they were equally consistent with an ordered mechanism in which ATP and aspartate binding are followed by an irreversible step prior to glutamine association with the enzyme (mechanism A, Scheme 2). Inhibition patterns for substrate analogues provided further support for the two possible kinetic mechanisms. The noncompetitive inhibition observed for glutamine vs β-methyl aspartate is likely the result of weak binding to the glutamine site.

While it has long been assumed that the *E. coli* AS-B mechanism involves an aspartyl-AMP intermediate, this has never been directly demonstrated. The observed transfer of ^{18}O from aspartate to AMP is most easily explained by a covalent β-aspartyl-AMP intermediate. In addition, the absence of ^{18}O transfer to Pi strongly argues against a β-phosphorylaspartate intermediate. We then probed the question of whether the aspartyl-AMP intermediate could form in the absence of a nitrogen source. Clearly, 1 equiv of PPi was formed prior to the addition of a nitrogen source (Figure 4). This intermediate appears to be sequestered from H_2O, although some breakdown does occur (a rate approximately 1500 times slower than the rate in the presence of a nitrogen source). The burst of PPi indicates that a step after the formation of the intermediate is rate limiting when no nitrogen source is available.

Surprisingly, no experimental conditions were identified under which externally added PPi was exchanged into ATP. This is interesting since the AS-A protein from *E. coli* and the AS protein from *L. arabinosus* both exhibit an ATP/PPi exchange reaction which is dependent on the presence of both Mg^{2+} and aspartate, although both of these proteins are exclusively ammonia dependent (*19, 23*). Although this lack of exchange is intriguing in light of the above results, few GAT enzymes exhibit such an exchange. For example, in GPATase, no exchange was seen between PPi and PRPP or glutamate and glutamine (*24*). Similarly, no ATP Pi exchange was seen for xanthylate synthetase (*25*−*27*).

Since the results from the initial velocity experiments could not differentiate between mechanism A and B, isotope-trapping methods were performed to obtain information about the order of addition of the substrates in the AS-B reaction (*28*).

Very little trapping (<15% of radiolabeled aspartate) occurred in the absence of ATP regardless of the presence or absence of a nitrogen source (Table 4). These data suggest that either aspartate cannot bind to the free enzyme in a catalytically competent manner or it dissociates from the binary (E−aspartate*) or ternary complex (E−aspartate*−glutamine) faster than it goes on to form product. On the other hand, when isotope-trapping using [^{14}C]aspartate in the presence of ATP was performed, 76% of the aspartate went on to form radioactive product (Table 4). The ability to trap radioactive aspartate when ATP was included in the pulse solution implies that the noncovalent E−Asp−ATP complex is formed in a catalytically competent manner. Furthermore, the observation that 1 equiv of PPi was produced in the absence of a nitrogen source demonstrates that a kinetically competent aspartyl-AMP intermediate can form prior to glutamine binding. This observation strongly suggests that ATP can bind free enzyme which is then followed by the binding of aspartate. Alternatively, when [^3H]ATP was used in the pulse solution it was shown that 78% of the radiolabeled ATP was trapped in the absence of other substrates (Table 3). Addition of glutamine had no effect on the ability of ATP to be trapped while the addition of aspartate increased the ability of ATP to be trapped to 100% (Table 3). These results are consistent with previously published work in which the addition of ATP and aspartate completely protected the enzyme from inactivation by a covalent affinity analogue, FSBA (*20*). In principle, the relative amounts of trapped product should have been identical when [^3H]ATP plus aspartate or [^{14}C]aspartate plus ATP were present in the pulse. Although both experiments conclusively demonstrated that ATP must bind first, less radioactivity was trapped when using radiolabeled aspartate. One reason these results may differ is due to experimental limitations using radiolabeled isotopes which yielded different concentrations of the reactants. In the case where [^3H]-ATP and aspartate were present in the pulse, their concentrations were 0.5 and 1 mM respectively, whereas when [^{14}C]aspartate and ATP were in the pulse, their levels were 0.5 and 5 mM, respectively. These differences in substrate concentrations might account for the discrepancies in the actual amount of radiolabeled product trapped.

These observations are extremely important since previously published asparagine synthetase mechanisms (*10, 29*) propose that the binding of glutamine must precede the binding of ATP and aspartate. Although our present results do not exclude a mechanism whereby glutamine can bind to the enzyme first, they also allow ATP and aspartate to bind productively and react prior to the addition of glutamine. Isotope trapping with labeled glutamine would in principle allow mechanisms A and B to be distinguished. Unfortunately, because of the rapid glutaminase rate, such an experiment is not possible using manual mixing techniques.

In their studies, both Milman et al. (*10*) and Hongo and Sato (*23*) used product inhibition studies to differentiate between mechanisms A and B. For the mouse pancreas AS, the observed uncompetitive inhibition of PPi vs glutamine was used to rule out mechanism A and favor mechanism B, a uni−uni−bi−ter ping-pong mechanism. Similarly, product inhibition studies for the rat pancreas AS were used as a differentiating factor even though patterns could not be obtained for either glutamate vs glutamine or glutamate vs ATP. In the studies presented here, the rate equation for mechanisms A and B were derived assuming P, Q, R, and S were present, and the product inhibition patterns predicted were compared with the experimental results (Table 6).

There are three product inhibition patterns that can be used to distinguish between mechanisms A and B: glutamine versus glutamate, glutamine versus PPi, and glutamine versus asparagine (Table 6). Unfortunately, we were able to obtain unambiguous results only for glutamine versus glutamate (uncompetitive inhibition). This agrees with the prediction

Table 6: Theoretical Patterns for Mechanism A and Mechanism B

substrate	inhibitor	mechanism A	mechanism B	observed
Gln		**UC**[a,b]	**NC**	UC
asp	Glu	NC	NC	NC
ATP		C	C	NC
Gln		**C**	**UC**	?[c]
asp	PPi	NC	NC	?
ATP		NC	NC	NC
Gln		UC	UC	UC
asp	AMP	UC	UC	UC
ATP		UC	UC	NC
Gln		**NC**	**C**	?
asp	Asn	UC	UC	NC
ATP		UC	UC	NC

[a] Bold terms represent predicted theoretical differences between mechanism A and mechanism B. [b] C = competitive inhibition, UC = uncompetitive inhibition, NC = noncompetitive inhibition. [c] A conclusive pattern could not be obtained.

of mechanism A and is inconsistent with mechanism B. Clearly, there are several discrepancies between the predicted and observed inhibition patterns. These most likely arise from abortive complexes that form readily in the presence of saturating substrate concentrations. Weak binding of aspartate and asparagine to the glutamine/glutamate site is particularly likely to contribute to the formation of these dead-end complexes.

It is interesting that conditions that favor complete coupling of glutamine consumption and asparagine synthesis involve saturating aspartate and subsaturating ATP and glutamine concentrations. Such conditions would favor formation of an enzyme-bound aspartyl-AMP intermediate prior to glutamine binding. Our stoichiometry and isotope-trapping results indicate that such an intermediate formed in the absence of nitrogen is catalytically competent. It is therefore tempting to speculate that formation of this intermediate prior to glutamine binding fully commits the enzyme to asparagine synthesis and that the glutaminase side reaction arises from enzyme that instead forms an initial binary complex with glutamine prior to ATP and aspartate binding.

SUPPORTING INFORMATION AVAILABLE

One table of conditions used for PPi-ATP exchange reaction and two Scatchard analysis figures of (1) the aspartate K_d and (2) the ATP K_d, both using the isotope-trapping method (5 pages). See any current masthead page for ordering information.

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BI981058H