Schwartz, A., Entman, M. L., Kanike, K., Lane, L. K., Van Winkle, B., & Bornet, E. P. (1976) *Biochim. Biophys. Acta 426*, 57.
Sharma, R., Tam, S. W., Waisman, D. M., & Wang, J. H. (1980) *J. Biol. Chem. 255*, 11102.
Shimazu, T., & Amakawa, A. (1975) *Biochim. Biophys. Acta 385*, 242.
Soderling, T. R., & Park, C. P. (1974) *Adv. Cyclic Nucleotide Res. 4*, 283.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237.
Sutherland, E. W., & Rall, T. W. (1960) *Pharmacol. Rev. 12*, 265.
Tada, M., Kirchberger, M. A., Repke, D. I., & Katz, A. M. (1974) *J. Biol. Chem. 249*, 6174.
Thomas, J. A., Schlender, K. U., & Larner, J. (1968) *Anal. Biochem. 25*, 486.
Vandenheede, J. R., Keppens, S., & DeWulf, H. (1976) *FEBS Lett. 61*, 213.
Walsh, D. A., Krebs, E. G., Reimann, E. M., Brostrom, M. A., Corbin, J. D., Hickenbottom, J. P., Soderling, T. R., & Perkins, J. P. (1970) *Adv. Biochem. Psychopharmacol. 3*, 265.
Walsh, D. A., Perkins, J. P., Brostrom, C. O., Ho, E. S., & Krebs, E. G. (1971) *J. Biol. Chem. 246*, 1968.
Wang, J. H., & Graves, D. J. (1964) *Biochemistry 3*, 1437.
Wang, J. H., Stull, J. T., Huang, T.-S., & Krebs, E. G. (1976) *J. Biol. Chem. 251*, 4521.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.

Kinetic Mechanism of Beef Pancreatic L-Asparagine Synthetase†

Rodney S. Markin, Craig A. Luehr, and Sheldon M. Schuster*

ABSTRACT: The kinetic mechanism of bovine pancreatic asparagine synthetase was deduced from initial velocity studies and product inhibition studies of both the glutamine-dependent and ammonia-dependent reactions. For the glutamine-dependent pathway, parallel lines were observed in the double reciprocal plot of $1/V$ vs. 1/[glutamine] at varied aspartate concentrations, and in the plot of $1/V$ vs. 1/[ATP] at varied aspartate concentrations. Intersecting lines were found for the plot of $1/V$ vs. 1/[ATP] at varied glutamine concentrations. Product inhibition patterns, including dual inhibitor studies for measuring the synergistic effects of multiproduct inhibition, were used to support an ordered bi-uni-uni-ter ping-pong mechanism. Glutamine and ATP sequentially bind, followed by the release of glutamate and the addition of aspartate. Pyrophosphate, AMP, and asparagine are then sequentially released. When the ammonia-dependent reaction was studied, it was found that the mechanism was significantly different. NH_3 bound first followed by a random addition of ATP and aspartate. Pyrophosphate, AMP, and asparagine were then sequentially released as in the glutamine-utilizing mechanism. From these studies, a comprehensive mechanism has been proposed through which either glutamine or NH_3 can provide nitrogen for asparagine production from aspartate.

Asparagine, although one of the first amino acids to be discovered, is one of the last amino acids to have its metabolism and biosynthesis carefully examined. The metabolic importance of asparagine is becoming more apparent. Asparagine has been shown along with glutamine to be able to cross the blood–brain barriers (Tower et al., 1963); it is also required by the developing embryo for normal brain development (Newburg, 1976), and several different tumor lines have been shown to require exogenous asparagine for growth (Broome, 1968).

Ravel et al. (1962) were the first to demonstrate the presence of asparagine synthetase. This enzyme, derived from *Lactobacillus arabinosus*, was shown to produce asparagine from aspartate in the presence of ammonia, ATP, and magnesium. Later studies demonstrated the presence of asparagine synthetase in mammalian systems (Patterson & Orr, 1967). The mammalian enzyme requires the presence of ATP and Mg^{2+}; however, it utilizes glutamine in addition to ammonia to transform aspartate to asparagine (Levintow, 1957).

Recent reports from our laboratory have described the existence of three different molecular forms of asparagine synthetase from rat liver, all differing in molecular weight. These three forms have been shown to interconvert possibly as the result of some type of control system. The appearance of these three forms of asparagine synthetase has also been shown to be dependent upon dietary and circulatory asparagine levels (Markin & Schuster, 1979; R. S. Markin and S. M. Schuster, unpublished experiments).

Cedar & Schwartz (1969) have described the kinetic mechanism of asparagine synthetase derived from *Escherichia coli* to be bi-uni-uni-bi ping-pong. Recently, Milman et al. (1980) reported a uni-uni-bi-ter ping-pong Theorell–Chance kinetic mechanism for the glutamine-dependent reaction of mouse pancreatic asparagine synthetase. The data presented here elucidate the kinetic mechanism of bovine pancreatic asparagine synthetase in the presence of either NH_3 or glutamine.

Materials and Methods

Materials

DL-[4-^{14}C]Aspartate and all other scintillation supplies were obtained from Research Products International (Elk Grove Village, IL). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) in the highest quality available. The radioactive aspartate was diluted in 0.4 M aspartate to

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an activity of 2.5×10^5 cpm/μmol.

Methods

Preparation of Enzyme. Fresh bovine pancreas was obtained from the American Stores Packing Co. (Lincoln, NE) and immediately cooled to 4 °C, allowing all fatty substances to solidify. After all fat, ducts, and connective tissues were removed, the cold pancreas was chopped into small pieces, diluted to 4 times its original volume with the homogenization buffer [50 mM Tris-HCl, 1.0 mM dithiothreitol (DTT), and 0.5 mM EDTA at pH 7.8], and homogenized in a Waring blender at high speed. The homogenate was then centrifuged at 9000 rpm in a GS-3 (Sorvall) rotor for 30 min. The supernatant was recovered, passed through cheesecloth, and centrifuged at 34 000 rpm in a type 35 (Beckman) rotor for 90 min. The resulting supernatant was then fractionated with ammonium sulfate at 4 °C. The proteins which precipitated between 30% and 70% saturation were recovered by centrifugation and resuspended in the homogenization buffer. This suspension was then stored frozen and used as asparagine synthetase extract. This frozen extract retained 75–100% of its asparagine synthetase activity after 2 weeks and 70–80% of its initial activity after 3 months. Before use, the frozen extract was quickly thawed and desalted on a Sephadex G-25 column (1.5 × 38 cm) equilibrated with the homogenization buffer. The asparagine synthetase was eluted from the column, collected, and diluted to the desired concentration and immediately used. A specific activity of 5–10 nmol min^{-1} mg^{-1} was obtained after this preparation was desalted on a Sephadex G-25 column when assayed under the following conditions at 37 °C: 83 mM Tris-HCl, pH 8.0, 17 mM aspartate, 10 mM ATP, 10 mM glutamine, 12 mM $MgCl_2$. Protein was determined by a biuret procedure (Layne, 1957) using bovine serum albumin as a standard. This preparation of pancreatic asparagine synthetase was shown to have a molecular weight of 110 000 by exclusion chromatography on Sephadex G-100 (C. A. Luehr and S. M. Schuster, unpublished observations). Asparagine synthetase activity was also eluted in a single peak from the Sephadex G-100 column. Thus, under the conditions used, this sample was found not to interconvert as did the rat liver asparagine synthetase.

Enzyme Assays. Asparagine synthetase activity was assayed by the method of Luehr & Schuster (1980). All velocities were determined by measuring the production of [4-^{14}C]asparagine from 4-^{14}C-labeled aspartate. The assay mixture was composed of the following: 83.3 mM Tris-HCl (50 L), pH 8.0, asparagine synthetase extract (350 μL), and varying amounts of ATP, AMP, PP_i, aspartate, asparagine, glutamate, glutamine, and NH_4Cl (all at pH 8.0) as indicated. $MgCl_2$ concentrations were determined so as to provide 2 mM excess above the ATP, AMP, and PP_i used. The total reaction mixture was maintained at 600 μL. The reaction mixture was incubated at 37 °C in a shaking water bath for 15 min and then immediately terminated by pipetting 0.5 mL into 1.0 mL of sodium acetate (1.2 M, pH 5.5) in a boiling H_2O bath. One half of this solution was put into a scintillation vial, and 1.0 mL containing 500 μmol of pyridoxal and 5 μmol of $Al_2(SO_4)_3$ was added. These vials were then incubated at 75–80 °C for 30 min in a well-vented oven after which 100 μL of 5 N HCl was added. 3a70B scintillation coctail (10 mL) was added, and the samples were counted. All variations were performed in triplicate. All velocities were found to be at a pseudo-first-order rate within this time range, and all activity measured was found to be asparaginase sensitive.

Data Handling. The data collected were evaluated by averaging the counts from each of the three samples and then subtracting the average of the three corresponding zero time samples. All velocities were then reported as millimoles of asparagine produced per minute of assay time per milligram of protein.

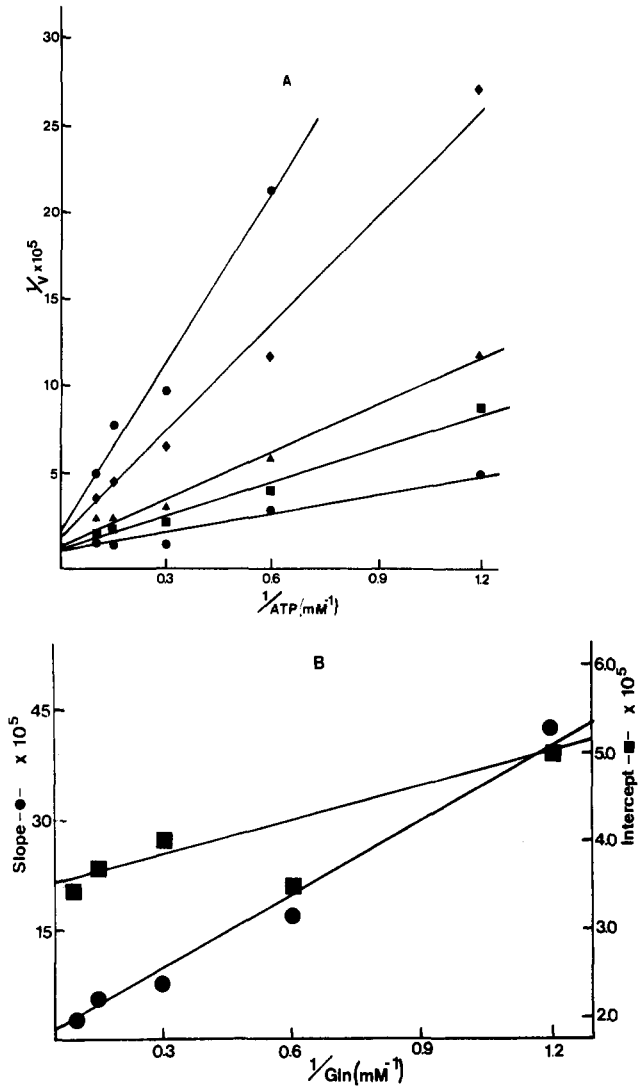


FIGURE 1: (A) Double reciprocal plot of initial velocity studies of ATP (0.84, 1.67, 3.33, 6.3, and 10.3 mM) at various fixed levels (mM) of glutamine: (●) 0.84; (♦) 1.67; (▲) 3.33; (■) 6.33; (●) 10.33. Initial velocity is reported as millimoles of asparagine per milligram of protein per minute. Aspartate was saturating (16.67 mM), and all products were absent. (B) Replot of the reciprocal-fixed variable substrate glutamine vs. the slope (●) and intercept (■) from (A).

These data were then plotted in the form of either a double reciprocal plot or a Dixon plot depending upon the type of experiment performed. The slopes and intercepts of the primary plots were determined by using standard nonweighted linear least-squares methods.

Results

For determination of the order of addition of the substrates in the glutamine-dependent asparagine synthetase catalyzed reaction, all of the substrate's (glutamine, aspartate, and ATP) concentrations were varied in pairs with the third fixed and saturating in the absence of any products. The plot of $1/V$ vs. 1/[ATP] at varied glutamine concentrations (Figure 1) shows intersecting lines. This was verified by replotting the slope and intercept (Figure 1B), which indicates that the enzyme forms to which each of these substrates bind are reversibly connected by one or more steps. The plot of $1/V$ vs. 1/[glutamine] at varied aspartate concentrations (Figure 2) revealed parallel lines as did the plot of $1/V$ vs. 1/[ATP] at

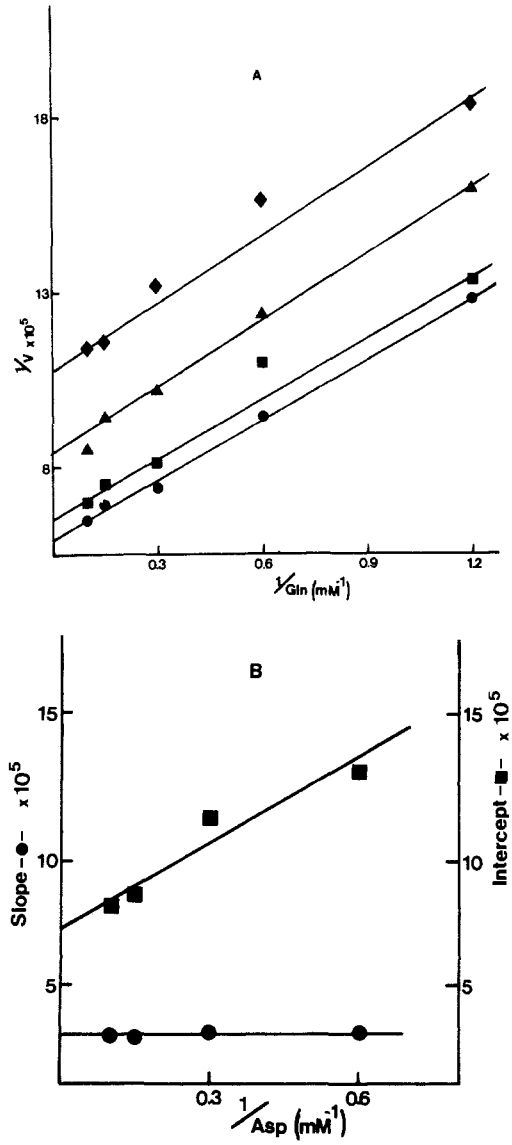


FIGURE 2: (A) Double reciprocal plot of initial velocity studies of glutamine (0.84, 1.67, 3.33, 6.3, and 10.3 mM) at various fixed levels (mM) of aspartate: (♦) 1.67; (▲) 3.33; (■) 6.66; (●) 10.0. Initial velocity is reported as millimoles of asparagine per milligram of protein per minute. ATP was saturating (10.2 mM), and all products were absent. (B) Replot of the reciprocal-fixed variable substrate aspartate vs. the slope (●) and intercept (■) from (A).

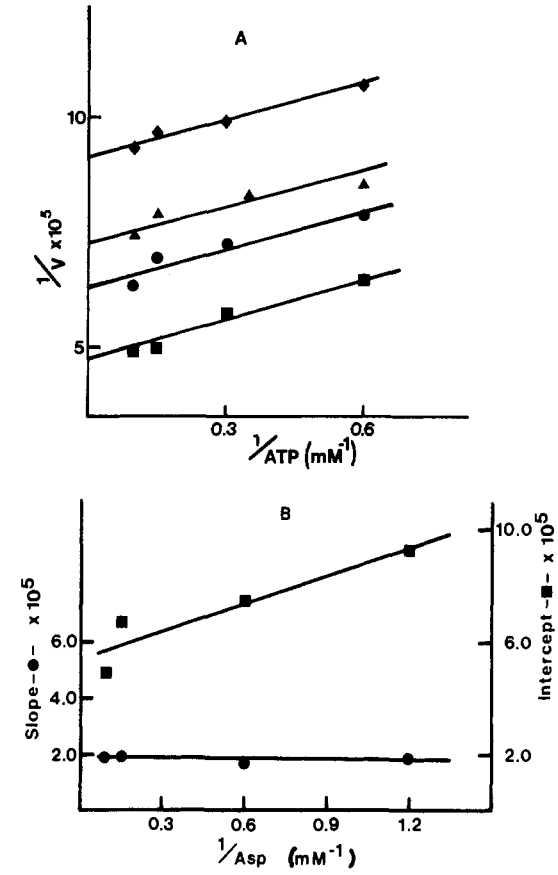


FIGURE 3: (A) Double reciprocal plot of initial velocity studies of ATP (1.67, 3.33, 6.73, and 10.3 mM) at various fixed levels (mM) of aspartate: (♦) 0.84; (▲) 1.67; (●) 6.67; (■) 10.3. Initial velocity is reported as millimoles of asparagine per milligram of protein per minute. Glutamine was saturating (16.67 mM), and all products were absent. (B) Replot of the reciprocal-fixed variable substrate aspartate vs. the slope (●) and intercept (■) from (A).

varied aspartate concentrations (Figure 3). These patterns were also verified by replotting the slopes and intercepts (Figures 2B and 3B, respectively). These data indicate that the enzyme forms that each of these substrate sets bind are irreversibly connected by one or more steps (in the absence of products), or indicate that a random addition of glutamine and ATP occurs. The irreversibility can result from either the release of a product or the addition of a saturating substrate occurring between the addition of the two varied substrates.

When evaluating the ammonia-dependent asparagine synthetase reaction, it was found that the plots of $1/V$ vs. $1/[NH_4Cl]$ at varied aspartate concentrations (at saturating ATP) and $1/V$ vs. $1/[NH_4Cl]$ at ATP concentrations (with saturating aspartate) resulted in parallel lines (Figures 4 and 5, respectively). This indicates either a random addition of ATP and aspartate or the release of a product between their additions.

For further clarification of the order of substrate addition and product release, the product inhibition of the initial velocity was studied for the glutamine-dependent asparagine synthetase

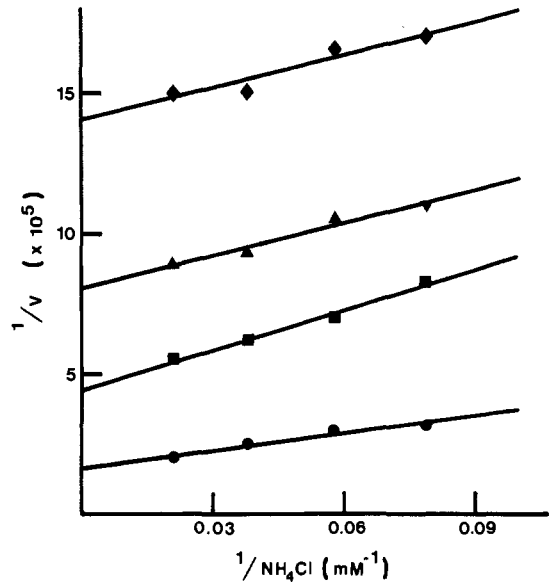


FIGURE 4: Double reciprocal plot of initial velocity studies of NH_4Cl (0.013, 0.017, 0.026, and 0.048 M) at various fixed levels (mM) of aspartate: (♦) 0.8; (▲) 1.6; (■) 3.3; (●) 16.7. Initial velocity is reported as millimoles of asparagine per milligram of protein per minute. ATP was saturating (10 mM), and all products were absent.

reaction. The plot of $1/V$ vs. 1/[glutamine] at varied asparagine concentrations resulted in competitive inhibition (Figure 6) at saturating ATP and aspartate. This means that asparagine and glutamine must bind to the same enzyme form,

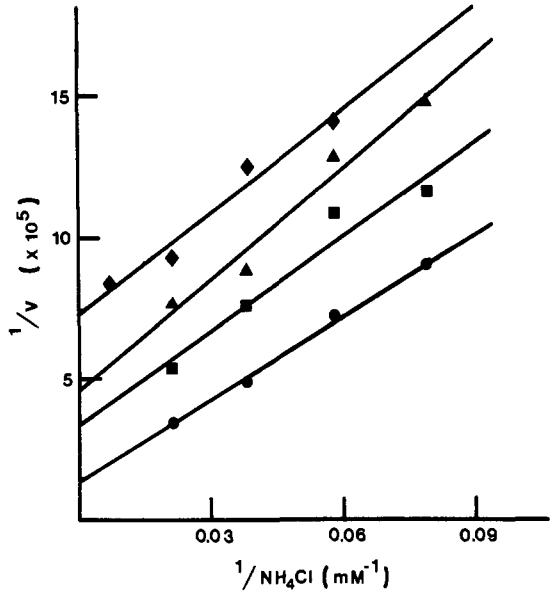
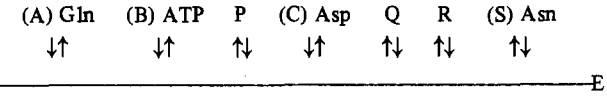


FIGURE 5: Double reciprocal plot of initial velocity studies of NH_4Cl (0.013, 0.017, 0.026, 0.048, and 0.143 M) at various fixed levels (mM) of ATP: (♦) 0.5; (▲) 0.7; (■) 2.0; (●) 13.3. Initial velocity is reported as millimoles of asparagine per milligram of protein per minute. Aspartate was saturating (13.3 mM), and all products were absent.

indicating that asparagine is released before glutamine binds. This verifies that asparagine is the last product released and glutamine is the first substrate to add. The observed competitive inhibition by asparagine with respect to glutamine eliminates the possibility of a random addition mechanism. If the addition were random, the inhibition by asparagine with respect to glutamine would be noncompetitive. The following partial mechanism was therefore adopted as a working hypothesis:



The identity of products P, Q, and R cannot be stated as yet. Although the data to this point do not indicate that Q and R release at the positions shown, the following results verify that these are indeed the correct placements.

Using the method of King & Altman (1956), we derived the steady-state initial velocity rate equations from the proposed mechanism, assuming products P, Q, and R were not present (eq 1 and 2). Equation 1 predicts that substrate C

$$\frac{1}{V} = \frac{1}{A}\left(\frac{1}{B} + 1\right) + \left(\frac{1}{B} + 1 + \frac{1}{C}\right) \quad (1)$$

$$\frac{1}{V} = \frac{1}{B}\left(\frac{1}{A} + 1\right) + \left(\frac{1}{A} + 1 + \frac{1}{C}\right) \quad (2)$$

(aspartate) affects only the intercept of the $1/V$ vs. $1/A$ (glutamine) plot which would result in parallel lines. Similarly we see in eq 2 that substrate C (aspartate) affects only the intercept of the $1/V$ vs. $1/B$ (ATP) plot, also parallel lines. However, eq 2 also demonstrates that substrate A (glutamine) affects both the slope and intercept of the $1/V$ vs. $1/B$ (ATP) plot which results in intersecting lines. When we add the terms containing S (asparagine) to the derived rate equation, only the slope term of $1/V$ vs. $1/A$ (glutamine) is altered, thus predicting competitive inhibition. Substrate A is the only substrate for which product S competitively inhibits even at saturating ATP and aspartate concentrations. All of these analyses agree with our data, verifying that asparagine is the last product released and glutamine is the mandatory first substrate to bind.

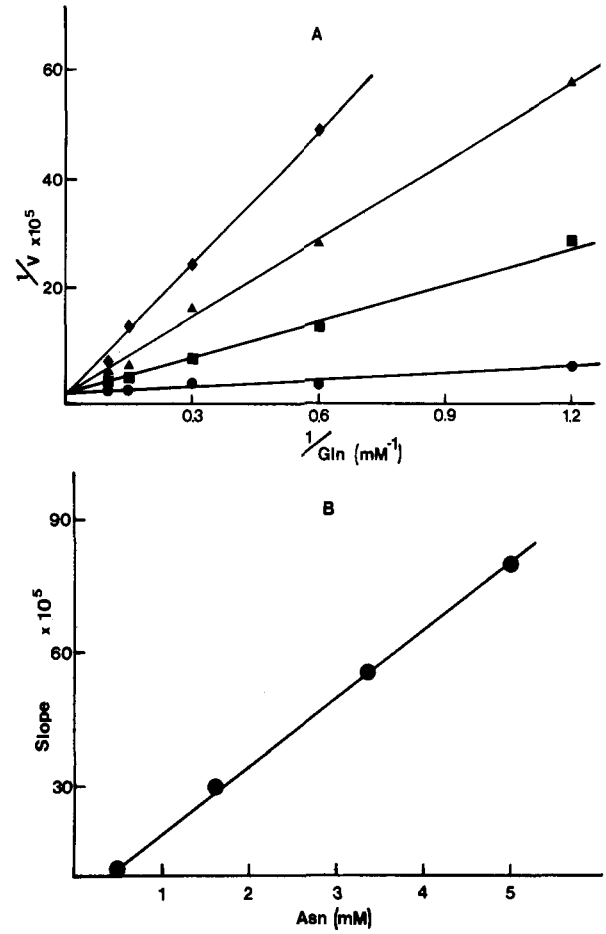


FIGURE 6: (A) Double reciprocal plot of initial velocity and studies of glutamine (0.84, 1.68, 3.33, 6.67, and 10.3 mM) at various asparagine levels (mM): (♦) 5.0; (▲) 3.3; (■) 1.6; (●) 0.5. Initial velocity is reported as millimoles of asparagine produced per milligram of protein per minute. Aspartate (16.67 mM) and ATP (10.3 mM) were saturating, and pyrophosphate, AMP, and glutamate were absent. The values for the intercept of each line were evaluated by a nonweighted linear least-squares method. All four of the lines were found to intersect at the same point within standard error limits. (B) Replot of asparagine concentration vs. the slope from (A).

Table I: Absence of Inhibition by the Products of Asparagine Synthetase[a]

addition to assay mixture	cpm over background
no addition	10 450 ± 99
Glu (15 mM)	10 550 ± 106
AMP (15 mM)	10 010 ± 101
AMP and Glu (15 mM each)	10 125 ± 94

[a] Assays were performed in triplicate as described under Materials and Methods. The concentrations of substrates were 10 mM ATP, 14 mM Asp, 14 mM Gln, and 12 mM $MgCl_2$.

The order of release of PP_i, AMP, and glutamate was then elucidated. Equation 3 predicts that product P should not inhibit with respect to ATP at saturating aspartate levels.

$$\frac{1}{V} = \frac{1}{[\text{ATP}]}\left(\frac{1}{[\text{Gln}]} + 1 + \frac{P}{[\text{Gln}][\text{Asp}]} + \frac{P}{[\text{Asp}]}\right) + \left(\frac{1}{[\text{Gln}]} + 1 + \frac{1}{[\text{Asp}]} + \frac{P}{[\text{Asp}]}\right) \quad (3)$$

However, at less than saturating aspartate levels, product P should be noncompetitive with respect to ATP. Glutamate was found to have no inhibitory effect at saturating aspartate

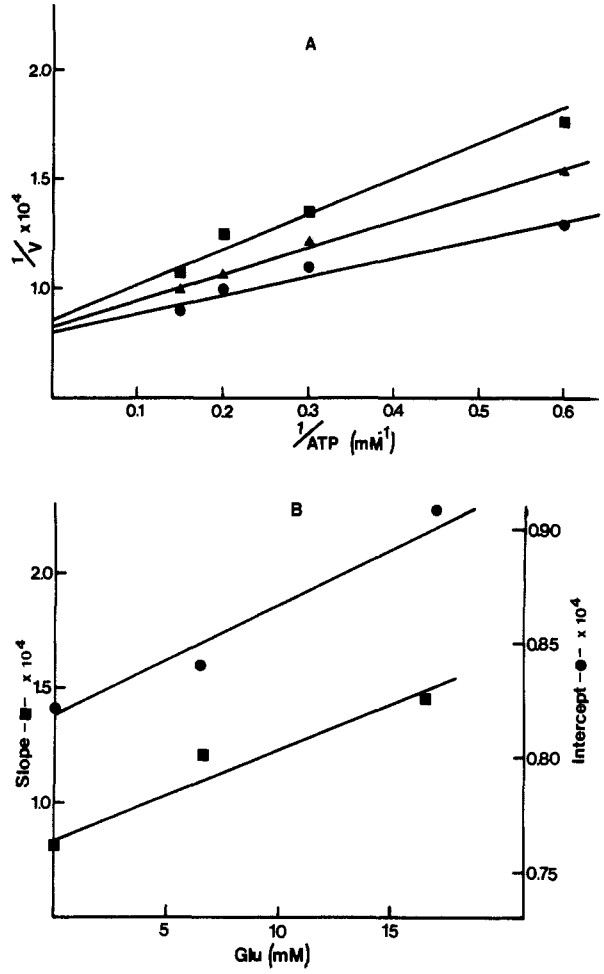


FIGURE 7: (A) Double reciprocal plot of initial velocity studies of ATP (1.67, 3.33, 5.0, and 6.25 mM) at various fixed levels of glutamate: (■) 16.67 M; (▲) 6.67 mM; (●) 0.0 mM. Initial velocity is reported as millimoles of asparagine per milligram of protein per minute. Glutamine (16.67 mM) was saturating, but aspartate (3.33 mM) was not. Pyrophosphate, AMP, and asparagine were absent. (B) Replot of glutamate concentration vs. the slope (■) and intercept (●) from (A).

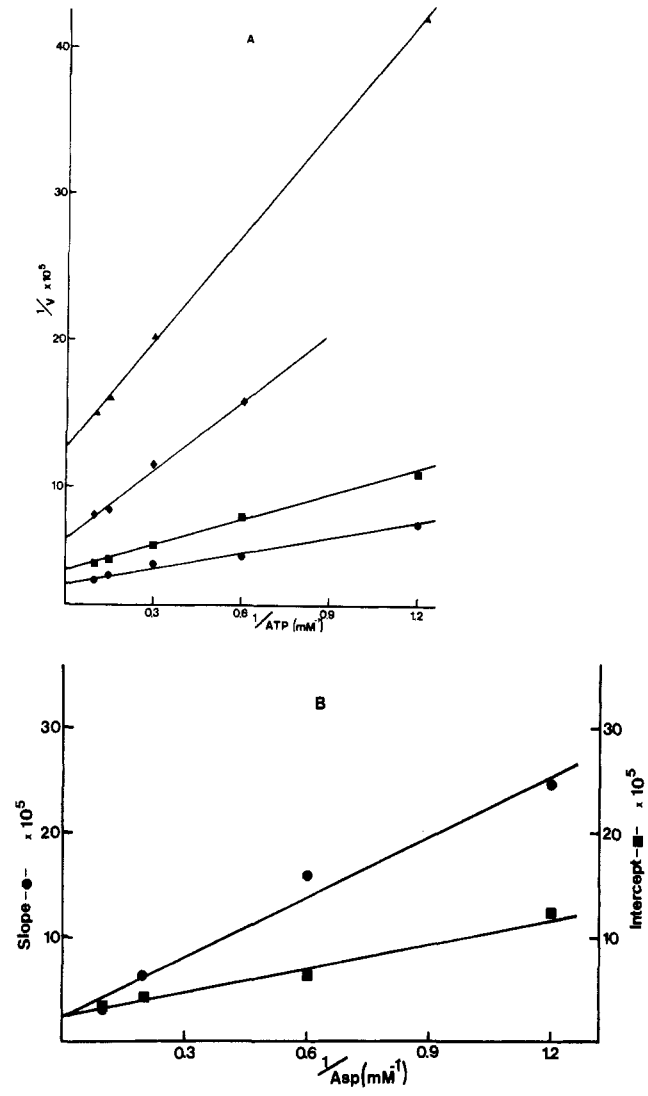


FIGURE 8: (A) Double reciprocal plot of initial and velocity studies of ATP (0.84, 1.67, 3.33, 6.67, and 10.3 mM) at various fixed levels (mM) of aspartate in the presence of glutamate (16.67 mM): (▲) 0.84; (♦) 1.67; (■) 5.0; (●) 10.3. Glutamine was saturating (16.67 mM), and all other products were absent. Initial velocity is reported as millimoles of asparagine per milligram of protein per minute. (B) Replot of the reciprocal concentration of aspartate vs. the slope (●) and intercept (■) from (A).

concentrations (see Table I), but glutamate was noncompetitive with respect to ATP (Figure 7) when aspartate was not saturating. This identifies glutamate as product P.

In order to verify that glutamate was the only product released between the additions of ATP and aspartate, an initial velocity experiment was performed where ATP and aspartate concentrations were varied against each other in the presence of glutamate. If glutamate is the only product released between the ATP and aspartate additions, then a double reciprocal plot of $1/V$ vs. $1/[ATP]$ at various aspartate concentrations will give intersecting lines in the presence of glutamate (see eq 3). If, however, another product release occurs in addition to glutamate between the additions of ATP and asparatate, then the double reciprocal plot will result in parallel lines. Figure 8 shows the results of $1/V$ vs. $1/[ATP]$ at various aspartate concentrations in the presence of glutamate which verifies that only one product (glutamate) is released between the addition of substrates ATP and aspartate.

We are now limited to placing the release steps for pyrophosphate and AMP after the addition of aspartate but before the release of asparagine. However, the order of release as yet needs to be deduced. It was found that neither AMP nor pyrophosphate showed substantial inhibition at saturating substrate levels (Table I). Milman et al. (1980) have shown that AMP is a very weak inhibitor of the asparagine synthetase reaction (K_i = 32 mM). Therefore, at the inhibitory concentrations of AMP, other nonspecific effects (salt effects, metal binding, etc.) could be encountered. These possible experimental problems, however, could be circumvented by the application of dual inhibitor studies. Equation 4, which

$$\frac{1}{V} = \frac{1}{[Gln][ATP]} + \frac{1}{[Gln]} + \frac{1}{[ATP]} + 1 + \frac{1}{[Asp]} + \frac{[S]}{[Gln][ATP]} + \frac{[S]}{[Gln]} + \frac{[O]}{[Asp]} + [Q] \quad (4)$$

was derived from mechanism I (in the absence of product R), shows that the reciprocal of the velocity equation contains terms with product S and product Q separately, but no SQ terms exist. One would then expect that product S (asparagine) and product Q would inhibit in an additive manner. Equation 5 (also derived from mechanism I in the absence of Q), however, has terms containing S (asparaginine), terms containing product R, and terms with the combination of products RS. The inhibition by S (asparagine) and the product (R) should then be greater than the sum of their individual inhibitory effects. In our system, since products Q and R show

$$\frac{1}{V} = \frac{1}{[Gln][ATP]} + \frac{1}{[Gln]} + \frac{1}{[ATP]} + 1 + \frac{1}{[Asp]} + \frac{[S]}{[Gln][ATP]} + \frac{[S]}{[Gln]} + [R] + \frac{[R][S]}{[Gln][ATP]} + \frac{[R][S]}{[Gln]} \quad (5)$$

no detectable inhibition alone, we would expect product Q to have no increased effect on the inhibition by asparagine. However, we would expect product R to enhance the inhibition by asparagine. Figure 9 shows the plot of $1/V$ vs. [asparagine] at various concentrations of AMP and pyrophosphate. It can be seen that the presence of pyrophosphate shows no enhancement of asparagine inhibition, but AMP shows a synergistic effect with asparagine. We now identify product Q as pyrophosphate and product R as AMP in proposed mechanism I. From these data, an ordered bi-uni-uni-ter ping-pong mechanism is proposed for the glutamine-dependent reaction of asparagine synthetase.

We also examined the mechanism of the production of asparagine when ammonia was used as the nitrogen source. We expected minor changes from the mechanism operative when glutamine was the nitrogen source since the only difference was the lack of glutamate release and NH_3 substituting for glutamine. We found that asparagine was a competitive inhibitor with respect to NH_3 (data not shown) as it was with glutamine, indicating that asparagine was the last product released and NH_3 was the first substrate to add. As was mentioned earlier (Figures 4 and 5), plots of $1/V$ vs. $1/[NH_4Cl]$ (varying ATP concentration) and $1/V$ vs. $1/[NH_4Cl]$ (varying aspartate concentration) (with the third substrate saturating in each case) yielded parallel lines. This indicated an irreversible step between the addition of ATP and NH_3 as well as between aspartate and NH_3. There can be no product released immediately after the addition of NH_3 so one must interpret these data as evidence of the random addition of ATP and Asp after the addition of NH_3. After these three substrates add, the complex appears to release products in the same order as does the glutamine-dependent reaction (data not shown). Thus, the ammonia-dependent reaction of asparagine synthetase appears to be the addition of ammonia followed by the random addition of ATP and aspartate with the products PP_i, AMP, and asparagine being released in the same order as in the glutamine-dependent reaction.

Discussion

This paper has attempted to reconcile both the glutamine-dependent reaction and the ammonia-dependent reaction of asparagine synthetase. The proposed mechanism in Figure 10 combines these two independent reactions of asparagine synthetase. Also included is the glutaminase reaction of asparagine synthetase reported by Horowitz & Meister (1972) and by Milman & Cooney (1979). However, the order of ammonia and glutamate release from the glutaminase reaction is not specified since the kinetics of the glutaminase reaction have not been extensively studied. Milman et al. (1980) have proposed a mechanism for the glutamine-dependent reaction of asparagine synthetase from mouse pancreas that is significantly different from the glutamine-dependent reaction presented in Figure 10. They have proposed a uni-uni-bi-ter ping-pong Theorell–Chance mechanism where glutamine binds first followed by the release of glutamate. Aspartate is the second substrate to bind with the Theorell–Chance step, (ATP on/PP_i off) following. AMP and asparagine are the last products to be released, respectively. Their proposed mechanism also required the addition of two product dead-end inhibition steps (AMP competitive with ATP and asparagine

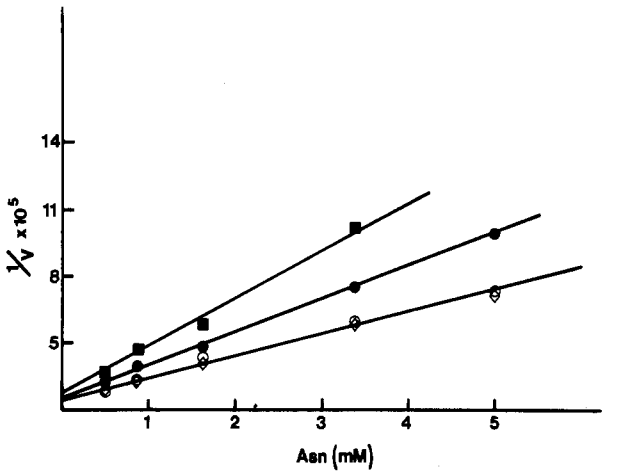


FIGURE 9: Dual inhibitor studies plot of asparagine concentration vs. reciprocal initial velocity with no other product present (◊) and in the presence of 6.67 mM AMP (●), 16.67 M AMP (■), and 8.05 mM pyrophosphate (○). Glutamine (16.67 mM), ATP (10.3 mM), and aspartate (16.67 mM) were all saturating, and the initial velocity is reported as millimoles of asparagine, of protein per minute.

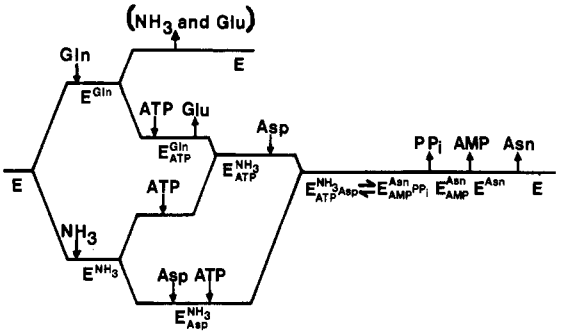


FIGURE 10: Proposed kinetic mechanism of bovine pancreatic asparagine synthetase.

competitive with aspartate) in order to explain their data. Using the 6C3HED-RG1 tumor, Chou (1970) also produced a different mechanism from that of Figure 10 where glutamine bound first followed by the release of glutamate. ATP was the second substrate bound followed by PP_i release; aspartate was the third substrate to bind, and AMP and asparagine were the last products to be released.

There are discrepancies between the data presented by Milman et al. (1980), Chou (1970), and this paper, and any plausible reasons for the differences are not obvious at this time. All three reports do agree that glutamine is the first substrate to bind and that AMP and asparagine are the last products to be released. This paper is the first reported study of the kinetics of the ammonia-dependent reaction of asparagine synthetase. Now the glutamine-dependent reaction can be evaluated in correlation to the random ammonia mechanism.

Milman et al. (1980) and Chou (1970) both present mechanisms that start with a glutaminase reaction followed by ordered reactions (that do not agree with each other). One can predict from these models of asparagine synthetase that if ammonia was to replace glutamine, ammonia would bind first followed by an ordered addition of the other substrates. In the mechanism of Chou (1970), ammonia should bind first followed by the addition of ATP, the release of PP_i, and finally the binding of aspartate as the third substrate. From the mechanism of Milman et al. (1980), one would also expect an ordered reaction if ammonia was used, with ammonia being the first substrate and aspartate the second substrate to bind. ATP would be the third substrate bound. Since both of these

proposals clearly expect an ordered mechanism when ammonia is used as well as when glutamine is used as substrate, neither is compatible with the random ATP–aspartate addition to the E–NH_2 complex that we found to occur.

The mechanism of the glutamine-dependent reaction proposed in this report (Figure 10) can be shown to be compatible with a random ammonia-dependent reaction. Figure 10 shows that glutamine and ATP bind before glutamate is released, and aspartate is then the last substrate to bind. Since asparagine synthetase has glutaminase activity, one can postulate that when ATP binds immediately after glutamine binds, it stabilizes the enzyme–ammonia complex, allowing the glutamate to be released without losing the ammonia. If no ATP were present, a glutaminase reaction would occur. The question as to why aspartate does not add before ATP is bound, during the glutamine-dependent asparagine synthetase reaction, has two possible explanations: (1) the presence of glutamate sterically prohibits aspartate from binding until glutamate is released, and (2) if ATP does not bind to the enzyme–glutamine complex, then the ammonia group is lost along with the glutamate. These conditions demand that the substrate addition be ordered when glutamine is the nitrogen source while a random addition can occur when ammonia is the nitrogen source for asparagine synthetase. Thus, both mechanisms proposed in this paper can be shown to be mechanistically compatible.

It is noteworthy that Milman et al. (1980) proposed the same order of release of PP_i, AMP, and asparagine as is proposed in this paper, even though different methods were used. Milman et al. (1980) observed the inhibition by all products with respect to each substrate. In the present study, the use of dual inhibitor studies and selected product inhibition studies greatly decreased the experimentation needed to arrive at the same conclusions. This shows the great potentiality of dual inhibitor studies where the presence of two or more inhibitors produces a multiplicative effect. It has not been cited extensively in the literature, but we feel it has potential for answering numerous important kinetic questions [see, for example, Krull & Schuster (1980)].

References

Broome, J. D. (1968) *J. Exp. Med. 127*, 1055.
Cedar, H., & Schwartz, J. H. (1969) *J. Biol. Chem. 244*, 4122.
Chou, T. C. (1970) Ph.D. Dissertation, University of Michigan, Ann Arbor, MI.
Horowitz, B., & Meister, A. (1972) *J. Biol. Chem. 247*, 6708.
King, E. L., & Altman, C. (1956) *J. Chem. Phys. 60*, 1375.
Krull, K. W., & Schuster, S. M. (1980) *Biochemistry 20*, 1592–1599.
Layne, E. (1957) *Methods Enzymol. 3*, 447.
Levintow, L. (1957) *Science (Washington, D.C.) 126*, 611.
Luehr, C. A., & Schuster, S. M. (1980) *J. Biochem. Biophys. Methods 3*, 151.
Markin, R. S., & Schuster, S. M. (1979) *Biochem. Biophys. Res. Commun. 88*, 583.
Milman, H. A., & Cooney, D. A. (1979) *Biochem. J. 181*, 51.
Milman, H. A., Cooney, D. A., & Huang, C. L. (1980) *J. Biol. Chem. 255*, 1862.
Newburg, D. S. (1976) Ph.D. Dissertation, Boston University, Boston, MA.
Patterson, M. K., Jr., & Orr, G. R. (1967) *Biochem. Biophys. Res. Commun. 26*, 228.
Ravel, J. M., Norton, S. J., Humphreys, J. S., & Shive, W. (1962) *J. Biol. Chem. 237*, 2845.
Tower, D. B., Peters, E. L., & Curtis, W. C. (1963) *J. Biol. Chem. 238*, 983.

Heterogeneity of Bovine Seminal Ribonuclease†

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ABSTRACT: Bovine seminal ribonuclease, a dimeric protein found to be homogeneous by several standard criteria of purity, is heterogeneous when analyzed by ion-exchange chromatography on (carboxymethyl)cellulose. Three increasingly cationic subforms can be separated. The heterogeneity is due to the presence of two types of subunits, α and β, which make up three isoenzymic dimers: α_2, β_2, and $\alpha\beta$. Deamidation reactions can convert the most cationic β_2 subform into the $\alpha\beta$ subform, which in turn can be converted into stable α_2 subform. These conversions involve the hydrolysis of 2 mol of differentially labile amide groups per mol of protein. The ratios α_2:$\alpha\beta$:β_2 are constant in all preparations of seminal ribonuclease tested; they are independent of the purification procedure as well as of the biological source of the enzyme (seminal plasma or seminal vesicles). These results indicate that deamidations occur in vivo before the protein is secreted from the seminal glands. They also suggest that heterogeneity of seminal ribonuclease reflects a physiological need of distinct molecular forms of enzyme or, alternatively, a process which leads to the aging of the protein.

Cases of exhaustively purified proteins, found to be heterogeneous even after they had been defined as homogeneous by standard criteria of purity, are not uncommon. In many cases, heterogeneity was found to be due to different degrees of glycosylation of a unique protein moiety; in other cases, it was evident that deamidation processes, often occurring as artifacts of purification, could generate multiformity in a protein preparation. The results we obtained studying the heterogeneity of bovine seminal ribonuclease provide satisfactory evidence both for deamidation as being the cause of heterogeneity and for its occurrence in vivo before isolation of the protein is undertaken.

Bovine seminal ribonuclease isolated from bull semen (D'Alessio et al., 1972a) or from seminal vescicles (De Prisco et al., 1972) had in fact been found to be homogeneous by several criteria of purity, including polyacrylamide gel electrophoresis, amino acid and ultracentrifuge analyses, and N- and C-terminal group determinations (D'Alessio et al.,

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