Studies on the Mechanism of the Glutamine-dependent Reaction Catalyzed by Asparagine Synthetase from Mouse Pancreas*

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Initial velocity and product inhibition studies were conducted with the glutamine-dependent reaction of asparagine synthetase from mouse pancreas. Double reciprocal plots of glutamine versus either aspartate or ATP were parallel, while aspartate versus ATP gave intersecting patterns. These patterns are indicative of a hybrid ping-pong mechanism consisting of a glutaminase partial reaction and a sequential catalysis involving aspartate and ATP. Inhibition patterns of the four products, glutamate, AMP, PPi, and asparagine, versus each of the three substrates are consistent with a hybrid Uni Uni Bi Ter Ping Pong Theorell-Chance mechanism where the glutaminase reaction occurs first and aspartate binds to the enzyme before ATP in the sequential segment. PPi is the first product released in the Theorell-Chance reaction, which is followed by the ordered release of AMP and asparagine. Product inhibition patterns also indicate the formation of E·NH2·Asn and E·NH2·Asp·AMP abortive complexes. Although an amide site (for glutamine and asparagine), presumably responsible for the glutaminase reaction, an acid site (for glutamate and aspartate), and a nucleotide site are involved in the overall catalysis, the "two-site" ping-pong mechanism is incompatible with the experimentally observed product inhibition patterns.

Mammalian asparagine synthetase has been partially purified from tumors resistant to treatment with asparaginase (1, 2) and from normal mouse pancreas (3). The enzyme catalyzes the reaction by which asparagine is synthesized from aspartate and glutamine (or ammonia) in the presence of ATP and Mg++. The mechanism of the reaction catalyzed by the enzyme from tumor has been characterized to various degrees. For example, Patterson and Orr (1) and Horowitz and Meister (2) observed stolichiometric amounts of AMP and PPi produced in the reactions catalyzed by the synthetases from the Novikoff hepatoma and RADA1 leukemia, respectively, as well as the formation of β-aspartylhydroxamate when hydroxylamine was substituted for glutamine. In addition, Horowitz and Meister (2) were able to measure the synthesis of [32P]ATP by the enzyme from leukemic cells from [32P]PPi, MgCl2, and ATP and from tumor has been characterized to various degrees. For example, Patterson and Orr (1) and Horowitz and Meister (2) observed stolichiometric amounts of AMP and PPi produced in the reactions catalyzed by the synthetases from the Novikoff hepatoma and RADA1 leukemia, respectively, as well as the formation of β-aspartylhydroxamate when hydroxylamine was substituted for glutamine. In addition, Horowitz and Meister (2) were able to measure the synthesis of [32P]ATP by the enzyme from leukemic cells from [32P]PPi, MgCl2, and ATP.

*This work is in part from a dissertation by Harry A. Milman which was presented to the Department of Pharmacology, The Graduate School of Arts and Sciences, The George Washington University, in partial fulfillment of the requirements for the Ph.D. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Materials

Enzymes—Asparagine synthetase (EC 6.3.1.1) from mouse pancreas was purified according to the procedures described by Milman and Cooney (3). The enzyme was enriched approximately 700-fold over the homogenate and had a specific activity of 0.3 IU/mg of protein. Asparaginase (EC 3.5.1.1) from Escherichia coli (340 IU/mg of protein) was purified at the Merck Institute for Therapeutic Research, West Point, PA and provided by the Drug Research and Development Branch of the National Cancer Institute. Glutamate dehydrogenase (EC 1.4.1.2) was purchased from purchased from a commercial source.

Radiochemicals—[14C]Aspartate ([14C]Aspartate [specific radioactivity, 12.9 to 17.4 μCi/μmol]) was purchased from New England Nuclear Corp., Boston, MA. [U-14C]Glutamine (specific radioactivity, 40 to 57.3 μCi/μmol) was purchased from Amersham/Searle Corp., Arlington Heights, IL.

Chemicals—Asparagine was purchased from Schwarz/Mann, Or-
angeburg, NY. Glutamine and α-ketoglutarate were obtained from Calbiochem, Gaithersburg, MD. ATP, AMP, and glutamate were the products of Sigma Chemical Co., St. Louis, MO. All other chemicals were obtained from Fisher Scientific Co., Silver Spring, MD.

Methods

Initial Velocity Studies—Initial velocity studies were conducted by measuring the synthesis of [4-14C]asparagine (7). A 5-μl enzyme (4 × 10^{-5} IU) solution was used to initiate the reaction. The assay was linear with respect to time for up to 60 min under our experimental conditions. All reactions were conducted in triplicate in a 15-μl reaction volume in Eppendorf 1500-μl centrifuge vessels at 37°C for 30 min. The reactions were terminated by heating the closed vessels for 5 min at 95°C. Internal blanks were constituted as asparaginase as described (7).

When [4-14C]aspartate was the fixed substrate (0.337 to 3.8 mM), the concentration of glutamine (0.312 to 10 mM) was varied, while ATP (10 mM) was held constant. When ATP was the fixed substrate (0.05 to 5 mM), the concentration of glutamine was varied as above while [4-14C]aspartate (0.95 mM) was held constant, or the concentration of glutamine (0.05 to 5 mM) was varied, while ATP (10 mM) was held constant. When ATP was the fixed substrate (0.337 to 3.8 mM), the concentration of glutamine was varied as described (7).

RESULTS

Initial Rate Patterns—Initial velocity patterns of the glutamine-dependent activity of asparagine synthetase were determined for all three substrate pairs. Parallel double reciprocal plots were observed with glutamine versus either aspartate or ATP (Figs. 1 and 2), whereas the double reciprocal plot for aspartate versus ATP was intersecting (Fig. 3). This

FIG. 3. Double reciprocal plots of initial velocity studies of aspartic acid at various fixed levels of ATP (•, 0.1 mM; 0, 0.25 mM; 0, 0.5 mM; 0, 5 mM). Eo/v, nanomoles of asparagine synthesized/mg of protein/h. Values are the means (±3%) of triplicate determinations. The experimental conditions are described under "Methods." Aspartic acid is expressed in terms of molarity.

TABLE I

Kinetic constants for asparagine synthetase from mouse pancreas

<table>
<thead>
<tr>
<th>Substrate pair</th>
<th>Glutamine</th>
<th>Aspartate</th>
<th>ATP</th>
<th>Specific velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kᵢ₀</td>
<td>Kᵢᵣ</td>
<td>Kᵣᵢ</td>
<td>µmol/h/mg protein</td>
</tr>
<tr>
<td>Gln-Asp</td>
<td>1.4</td>
<td>1.3</td>
<td>0.61</td>
<td>15</td>
</tr>
<tr>
<td>Gln-ATP</td>
<td>1.9</td>
<td>1.0</td>
<td>0.47</td>
<td>60.60</td>
</tr>
<tr>
<td>Asp-ATP</td>
<td>1.6</td>
<td>0.26</td>
<td>0.37</td>
<td>0.58</td>
</tr>
</tbody>
</table>

Mean 1.7 1.3 0.37 0.59 18

a Calculated from inhibition patterns of glutamate versus glutamine according to Equation 2.

Product Inhibition Studies—Product inhibition studies were conducted by measuring the syntheses of [4-14C]asparagine (7). When the concentration of one substrate is varied, the remaining substrates are held at fixed concentrations: ATP, 10 mM; MgCl₂, 50 mM; [4-14C]-aspartate, 0.655 mM; and glutamine, 16 mM.

Data Analysis—All initial rate and product inhibition data were treated by double reciprocal plots, and Eadie-Hofstee plots were used to confirm the observed patterns. Initial velocity data were analyzed according to Equation 1 given under "Discussion." When glutamine (A) and aspartate (B) were the substrate pair, ATP (C) was saturating, which permitted the calculation of Kᵢ₀, Kᵢᵣ, and Kᵣᵢ, specific velocity. When aspartate (B) and ATP (C) were the substrate pair, glutamine (A) was saturating, making possible the determination of Kᵢ₀, Kᵢᵣ, and Kᵣᵢ. Product inhibition data were treated according to Equation 2 given under "Discussion." The kinetic parameters obtained from initial rate studies were substituted into appropriate equations to calculate various inhibition constants.

Initial Rate Patterns—Initial velocity patterns of the glutamine-dependent activity of asparagine synthetase were determined for all three substrate pairs. Parallel double reciprocal plots were observed with glutamine versus either aspartate or ATP (Figs. 1 and 2), whereas the double reciprocal plot for aspartate versus ATP was intersecting (Fig. 3). This
Kinetic Studies of Pancreatic Asparagine Synthetase

The overall glutamine-dependent reaction catalyzed by asparagine synthetase from mouse pancreas is as follows:

\[ \text{L-Glu + L-Asp + ATP + H}_2\text{O} \rightarrow \text{Mg}^{2+} \]

Two partial reactions have been demonstrated (3, 9). The first one is a glutaminase reaction which has also been found in enzymes from various tumoral sources (1, 2):

\[ \text{L-Gln} + \text{H}_2\text{O} \rightarrow \text{L-Glu} + \text{NH}_3 \]

Unlike the enzyme from leukemic cells, the glutaminase activity is not dependent on Cl\(^-\) ions\(^1\) and a 1:1 stoichiometry has been established for the amount of glutamate formed and aspartate consumed (3). Previous studies (10) also showed that the glutaminase and asparagine synthetase activities co-migrated on polyacrylamide gels. These observations indicate that the glutaminase activity is an intrinsic property of asparagine synthetase. It should be pointed out that several glutamine amidotransferases, e.g. anthranilate synthase, carbamoylphosphate synthetase, CTP synthetase, and formylglyco-

\(^1\) NaCl, 1 to 10 mM, did not stimulate or inhibit the glutaminase activity of asparagine synthetase (H. A. Milman and D. A. Cooney, unpublished observations).

![Fig. 5. Inhibition patterns of AMP versus ATP. Concentration of AMP: •, none; ○, 30 mM; □, 40 mM. E\(_{\text{v}}\)/v, nanomoles of asparagine synthesized/mg of protein/h. Values are the means (±3%) of triplicate determinations. The experimental conditions are described under "Methods." ATP is expressed in terms of molarity.](http://www.jbc.org)

![Fig. 6. Inhibition patterns of asparagine versus glutamine. Concentration of asparagine: •, none; □, 3.5 mM; △, 7 mM. E\(_{\text{v}}\)/v, nanomoles of asparagine synthesized/mg of protein/h. Values are the means (±3%) of triplicate determinations. The experimental conditions are described under "Methods." Glutamine is expressed in terms of molarity.](http://www.jbc.org)

![Fig. 4. Inhibition patterns of PP vs ATP. Concentration of PP: •, none; □, 3 mM; ○, 6 mM. E\(_{\text{v}}\)/v, nanomoles of asparagine synthesized/mg of protein/h. Values are the means (±3%) of triplicate determinations. The experimental conditions are described under "Methods." ATP is expressed in terms of molarity.](http://www.jbc.org)
cineamidine ribonucleotide synthetase, have been found to exhibit glutaminase activity. In each case, stoichiometry was achieved between the formation of glutamate and the other products.

The second partial reaction is an aspartate-dependent ATP pyrophosphatase activity as evidenced by the ATP→PP, exchange (9). This reaction has been previously described by Horowitz and Meister (2) for the enzyme from leukemic cells:  

\[ \text{Asp} + \text{Mg}^{2+} + \text{ATP} \rightarrow \text{AMP} + \text{PP}_i \]

This activity is not due to the presence of contaminating ATP pyrophosphatase since no exchange was observed in the absence of aspartate. Nor was it due to contamination by aspartate-t-RNA synthetase, whose activity was not found in our enzyme preparation (3). In addition, a competitive inhibitor of aspartate, aminomalonic acid, can substitute for aspartate to allow the exchange to proceed (9). These observations are consistent with initial velocity patterns reported here. The parallel plots for the glutamine-aspartate and glutamine-ATP pairs indicate a hybrid ping-pong mechanism with the release of a product intervening between the glutaminase reaction and the bimolecular reaction involving aspartate and ATP. The intersecting patterns observed with ATP versus aspartate, together with the aspartate-dependent ATP→PP, exchange, are indicative of a sequential reaction for these two substrates.

The observed product inhibition patterns are used to differentiate various possibilities. First, we examine the possibility that the sequential reaction takes place before the glutaminase reaction. This type of mechanism has been proposed for the asparagine synthetase from *E. coli* (8). Since asparyl-β-adenylyl has been implicated as a reaction intermediate (2, 8), PP, is the most probable product to be released prior to the addition of glutamine. The experimentally observed uncompetitive inhibition of PP, versus glutamine, however, is contrary to such a mechanism in which competitive inhibition of PP, versus glutamine is the expected pattern. It is therefore concluded that the glutaminase reaction must occur prior to the sequential reaction of aspartate and ATP.

Rate equations for three sequential Bi Ter mechanisms are also derived, and the product inhibition patterns predicted for each case (Table III) are compared with the experimental results. The ordered and the rapid equilibrium random mechanisms are ruled out because of the predicted noncompetitive and uncompetitive inhibition of PP, versus ATP, respectively, which are contrary to the observed competitive inhibition (Fig. 4). The competitive nature of this inhibition has also been reported by Horowitz and Meister (2) for the enzyme from leukemic cells. The Theorell-Chance mechanism, on the other hand, predicts that PP, is competitive with ATP. As shown in Table III, however, there are disagreements between the predicted patterns and experimental results for AMP versus ATP and asparagine versus all three substrates. These discrepancies indicate the existence of abortive complexes. With the formation of E·NN, Asn and E·NN, Asp·AMP complexes, the predicted inhibition patterns are now in complete agreement with the observed patterns listed in Table II. The complete mechanism is described in Scheme 1.

\[
\begin{align*}
\text{Gln} & \quad \text{Glu} & \quad \text{Asp} & \quad \text{ATP} & \quad \text{PP}_i & \quad \text{AMP} & \quad \text{Asn} & \quad \text{E} \\
\downarrow & \quad \uparrow & \quad \downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow & \quad \downarrow \\
\text{E·NN} & \quad \text{Asp} & \quad \text{AMP} & \quad \text{E} \\
\end{align*}
\]

**Scheme 1**

The reciprocal initial velocity equation for Scheme 1 is given by Equation 1:

\[
\frac{1}{v} = \frac{1}{V} \left( 1 + \frac{K_a}{A} + \frac{K_p}{P} + \frac{K_c}{C} + \frac{K_nK_p}{BC} \right)
\]

where A = glutamine, B = aspartate, and C = ATP. The initial rate patterns (Figs. 1 to 3) are consistent with Equation 1.

The product inhibition equation (Equation 2), omitting terms involving the simultaneous presence of two or more products, has the expression:

\[
\frac{1}{v} = \frac{1}{V} \left( 1 + \frac{K_a}{A} + \frac{K_p}{P} + \frac{K_c}{C} + \frac{K_nK_p}{BC} \right)
\]

where P = glutamate, Q = PP, R = AMP, and S = asparagine.

The noncompetitive inhibition of asparagine versus glutamine reported in Table II is apparently different from prior observations from other laboratories (2, 4). However, as can be seen from Fig. 5, the intersection point of the double reciprocal plot is only slightly to the left of the ordinate (Edid-Hofstee plots were used to verify the noncompetitive inhibition), and the plot could easily be diagnosed as compet-

### Table III

**Product inhibition patterns predicted for various Uni·Uni Bi Ter ping-pong mechanisms**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Theorell-Chance Bi Ter</th>
<th>+ Abortive complexes*</th>
<th>Predicted inhibition pattern*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln</td>
<td>Glu</td>
<td>NC</td>
<td>NC</td>
<td>Ordered Bi Ter</td>
</tr>
<tr>
<td>Asp</td>
<td>Glu</td>
<td>C</td>
<td>C</td>
<td>Rapid equilibrium Bi Ter</td>
</tr>
<tr>
<td>ATP</td>
<td>Glu</td>
<td>NC</td>
<td>NC</td>
<td>Two-site ping pong</td>
</tr>
<tr>
<td>Gln</td>
<td>PP</td>
<td>UC</td>
<td>UC</td>
<td>+ Abortive complexes*</td>
</tr>
<tr>
<td>Asp</td>
<td>PP</td>
<td>NC</td>
<td>NC</td>
<td></td>
</tr>
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<td>C</td>
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<td>Asn</td>
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<tr>
<td>Asp</td>
<td>Asn</td>
<td>UC</td>
<td>UC</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>ATP</td>
<td>UC</td>
<td>UC</td>
<td></td>
</tr>
</tbody>
</table>

*NC, noncompetitive; C, competitive; UC, uncompetitive.

*With E·NN, Asn and E·NN, Asp·AMP complexes.

*Rate equations derived by the method of Cha (11).

*With E·Asp·PP, E·Asp·AMP, E·Glu·ATP, and E·Asn·ATP complexes; also, asparagine is capable of binding to the glutamine site.
itive inhibition in an expanded plot. The noncompetitive inhibition observed with asparagine versus ATP or aspartate indicates that asparagine must be capable of binding to the aspartate site, otherwise uncompetitive inhibition would have occurred.

The possibility of a two-site ping-pong mechanism, originally proposed by Northrop (12) for transcarboxylase and later extended to other systems (13, 14) was also examined. Several lines of evidence actually suggest that there are separate "amide sites" and "acid sites" on the enzyme. (a) The existence of an amide site which is inaccessible to glutamate or aspartate is evidenced by the fact that, when assayed at a glutamine concentration of 0.087 mM, the glutaminase activity of aspartase synthetase was not hindered by the presence of 1.5 mM aspartate or 4 mM glutamate, whereas 4 mM asparagine completely inhibited this activity (3). In addition, the presence of 20 mM NH4+ or 20 mM NH3 and 4 mM glutamate did not affect the glutaminase activity (3). This indicates that the lack of inhibition by aspartate is not due to its inability to bind to the free enzyme form since, when NH4+ is present, it should be able to combine with the E-NH4 complex. The implication is that there are not only distinctive amide and acid sites but also a transfer mechanism which delivers the NH4 produced at the amide site to another site for asparagine synthesis. (b) It follows that there exists an acid site to which the substrate aspartate binds, but which also has low affinity for glutamate and asparagine. The competitive inhibition of glutamate versus aspartate supports this concept. Glutamine does not appear to bind to this site since substrate inhibition with glutamate is observed. By assuming that the glutaminase reaction takes place at the amide site independent of the events occurring on other sites, the appropriate rate equations can be derived by the method of Cha (11). The predicted product inhibition patterns are given in Table III. Since the two-site ping-pong mechanism is applicable only to rapid equilibrium random treatment, the predicted inhibition patterns for PPI, AMP, and asparagine are identical. Inclusion of various abortive complexes (see Footnote d in Table III) makes it possible to yield inhibition patterns which, with three exceptions, agree with the experimentally observed patterns. Of these three exceptions, the competitive inhibition of AMP versus ATP is most difficult to reconcile with the observed noncompetitive inhibition pattern (Fig. 6) which was also reported by Horowitz and Meister (2) for the enzyme from leukemic cells. With the two-site mechanism, it is possible to generate noncompetitive inhibition for AMP versus ATP only if an E-NH2-ATP-AMP abortive complex exists. Such a complex, however, is unlikely because ATP and AMP presumably occupy the same site. An additional nucleotide binding site would have to be proposed to satisfy this requirement. Consequently, the usual two-site ping-pong model does not seem to be a likely one for asparagine synthetase. Another possibility is that ordered substrate binding or product release may be involved in the two-site ping-pong mechanism. In fact, a recent kinetic study suggested that the glutaminase reaction catalyzed by a separate subunit of carbamoylphosphate synthetase was the cause of the observed parallel double reciprocal plots (15). It is not clear, however, how the two-site ping-pong model can accommodate a steady state ordered mechanism. The rate equation derived for such a case would undoubtedly give rise to higher order terms and would predict nonhyperbolic kinetic behavior for the enzyme.

Based on the product inhibition patterns, therefore, we favor the hybrid Uni Bi Ter Ping Pong Theorell-Chance mechanism as the correct model. The fact that different inhibition constants calculated from various inhibition studies according to Equation 2 (see Table II) are in good agreement with one another lends further support to the proposed mechanism. This model has several distinctive features. (a) Separate amide and acid sites are involved. The significance of having two different sites on this enzyme is probably evolutionary and regulatory rather than mechanistic. Asparagine synthetase from bacterial sources utilizes NH3 exclusively as the amide donor. In contrast, mammalian enzymes preferentially use glutamine as the donor. Conceivably, asparagine synthetase acquires the glutaminase activity, hence the amide site, through evolution so that asparagine can be made in vivo from glutamine instead of NH3, which is both toxic and in short supply. The high affinity asparagine site, on the other hand, permits an effective regulation of the utilization of glutamine by means of product inhibition. (b) The NH3 generated from the glutaminase reaction is, for all practical purposes, not released. In other words, the NH3 generated in situ is very efficiently coupled to the synthesis of asparagine. Whether the NH3 is tightly bound to the enzyme or an enzyme amimation is involved is not known. Although Horowitz and Meister (2) have observed that the formation of glutamate was nearly three times higher than that of asparagine with the enzyme from leukemic cells, it is not the case with the mouse pancreatic enzyme (3). It should be noted that if "leakage" of NH3 occurs to a significant degree, the double reciprocal plots for glutamine versus aspartate or ATP will become intersecting, contrary to the observed parallel plots.

REFERENCES

Studies on the mechanism of the glutamine-dependent reaction catalyzed by asparagine synthetase from mouse pancreas.
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