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

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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 stoichiometric 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 ß-aspartylhydroxamate when hydroxyamine 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 aspartyl-ß-adenylate, indicating that the synthesis of asparagine by the enzyme from tumor may proceed by means of an intermediate, aspartyl-ß-adenylate. These same authors also reported that the enzyme catalyzes an aspartate-dependent ATP ÆPPi exchange.

Patterson and Orr (1) also showed that, with the enzyme from the Novikoff hepatoma, at saturating levels of glutamine, no enhancement of the synthetase activity by NH4Cl occurred, and that the glutamine- and NH4Cl-dependent activities were purified. The authors concluded, therefore, that the enzyme utilizes both ammonia donors at the same active site. Horowitz and Meister (2), on the other hand, observed that the synthetase from the RADA1 leukemia exhibits a glutaminase activity which is independent of the presence of other substrates but dependent on the concentration of chloride ions.

On the basis of initial velocity and partial product inhibition studies, Chou (4) suggested that the reaction catalyzed by asparagine synthetase from the 6C3HED-RG1 tumor is ping-pong. In this reaction, glutamine is suggested to be the first substrate to add to the enzyme, while glutamate is the first product to be released. ATP and PPi are the second substrate-product pair, and aspartate is the third substrate, with AMP and asparagine as third and fourth products released.

Previously, we reported on the presence of asparagine synthetase in high specific activity in normal pancreas (5). The enzyme has been further purified and shown to be free of asparaginase, ATPase, and aspartate-t-RNA synthetase activities (6) which may interfere with kinetic experiments. It was felt to be important, therefore, to study the kinetic behavior of the enzyme from pancreas in order to compare it with that of the tumoral enzyme and to gain insight into its mode of action.

EXPERIMENTAL PROCEDURES

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 decarboxylase from E. coli (EC 4.1.1.15; specific activity, 50 IU/mg of protein) was purified by the method of Shikata and Schwart (6). Glutamate dehydrogenase (EC 1.4.1.2; specific activity, 45 IU/mg of protein) was purchased from Boehringer Mannheim Corp., New York, NY.

Radiochemicals—[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 the product of 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 × 10−6 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-pl 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 with 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 [4-14C]aspartate (0.95 mM) was held constant. When 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 concentra-

![Fig. 1](http://example.com/fig1.png)

**Fig. 1.** Double reciprocal plots of initial velocity studies of glutamine at various fixed levels of aspartic acid (□, 0.357 mM; ○, 0.657 mM; ■, 1.9 mM; ●, 3.8 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.” Glutamine is expressed in terms of molarity.

![Fig. 2](http://example.com/fig2.png)

**Fig. 2.** Double reciprocal plots of initial velocity studies of glutamine at various fixed levels of ATP (□, 0.05 mM; ○, 0.1 mM; ▽, 0.5 mM; ●, 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.” Glutamine is expressed in terms of molarity.

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 ATP (Figs. 1 and 2), whereas the double reciprocal plot for glutamine 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; ○, 0.25 mM; ▽, 0.5 mM; ●, 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 1**

Kinetic constants for asparagine synthetase from mouse pancreas

<table>
<thead>
<tr>
<th>Substrate pair</th>
<th>Glutamine</th>
<th>Aspartic acid</th>
<th>ATP</th>
<th>Specific velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K&lt;sub&gt;x&lt;/sub&gt;</td>
<td>K&lt;sub&gt;y&lt;/sub&gt;</td>
<td>K&lt;sub&gt;b&lt;/sub&gt;</td>
<td>K&lt;sub&gt;e&lt;/sub&gt;</td>
</tr>
<tr>
<td>Gln-Asp</td>
<td>1.4</td>
<td>1.3</td>
<td>0.61</td>
<td>mM</td>
</tr>
<tr>
<td>Gln-ATP</td>
<td>1.9</td>
<td>1.0</td>
<td>0.47</td>
<td>0.60</td>
</tr>
<tr>
<td>Asp-ATP</td>
<td>1.6</td>
<td>0.26</td>
<td>0.58</td>
<td>0.59</td>
</tr>
<tr>
<td>Mean</td>
<td>1.7</td>
<td>1.3</td>
<td>0.37</td>
<td>0.59</td>
</tr>
</tbody>
</table>

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

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 glutamine versus ATP was intersecting (Fig. 3). This
Kinetic Studies of Pancreatic Asparagine Synthetase

latter plot is different from that reported by Chou (4) for the enzyme from a tumor source in which parallel plots were observed. Although a different mechanism may be operative for that enzyme, the relatively short range of substrate concentration employed by Chou's studies may explain the apparent parallel plots. Intersecting patterns were also reported by Cedar and Schwartz (8) for the enzyme from E. coli. The Michaelis constants \( K_m \) are listed in Table I. The \( K_m \) values for the same substrate obtained from various experiments are in good agreement.

**Product Inhibition Studies**—To delineate the kinetic mechanism, all the inhibition patterns involving the four products versus each of the three substrates were carried out. The observed inhibition patterns are summarized in Table II. Three inhibition patterns which are central to the proposed mechanism are shown in Figs. 4 to 6. They are PP, versus ATP, competitive (Fig. 4); AMP versus ATP, noncompetitive (Fig. 5); and asparagine versus glutamine, noncompetitive (Fig. 6). Glutamate and AMP are poor inhibitors, whereas PP, and asparagine are good inhibitors.

TABLE II

<table>
<thead>
<tr>
<th>Varied substrate</th>
<th>Product inhibitor</th>
<th>Inhibition pattern*</th>
<th>Inhibition constants*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln</td>
<td>Glu</td>
<td>NC</td>
<td>( K_p = 93 )</td>
</tr>
<tr>
<td>Asp</td>
<td>Glu</td>
<td>C</td>
<td>( K_p = 60 )</td>
</tr>
<tr>
<td>ATP</td>
<td>Glu</td>
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<td>( K_p = 54 )</td>
</tr>
<tr>
<td>Gln</td>
<td>PP,</td>
<td>UC</td>
<td>( K_p = 0.39 )</td>
</tr>
<tr>
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<td>PP,</td>
<td>NC</td>
<td>( K_p = 0.15 )</td>
</tr>
<tr>
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<td>PP,</td>
<td>C</td>
<td>( K_p = 0.30 )</td>
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<td>AMP</td>
<td>UC</td>
<td>( K_p = 32 )</td>
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<td>AMP</td>
<td>UC</td>
<td>( K_p = 30 )</td>
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<td>( K_p = 10 )</td>
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<tr>
<td>Gln</td>
<td>Asn</td>
<td>NC</td>
<td>( K_p = 0.50 )</td>
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<td>Asp</td>
<td>Asn</td>
<td>NC</td>
<td>( K_p = 0.33 )</td>
</tr>
<tr>
<td>ATP</td>
<td>Asn</td>
<td>NC</td>
<td>( K_p = 0.21 )</td>
</tr>
</tbody>
</table>

* Symbols used: NC, noncompetitive; C, competitive; UC, uncompetitive.

* All the constants are as defined in Equation 2. \( K_p \) and \( K_o \) are the dissociation constants for AMP (for E-NH₂-Asp-AMP) and for asparagine (for E-NH₂-Asn). Mean values of kinetic parameters presented in Table I are used in the calculation of these constants.

**DISCUSSION**

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

\[ L\text{-Gln} + L\text{-Asp} + ATP + H_2O \xrightarrow{Mg^{2+}} L\text{-Glu} + L\text{-Asn} + AMP + PP, \]

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):

\[ L\text{-Gln} + H_2O \rightarrow L\text{-Glu} + NH_3. \]

Unlike the enzyme from leukemic cells, the glutaminase activity is not dependent on Cl⁻ ions 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 formylglu-
cineamidine ribonucleotide synthetase, have been found to exhibit glutaminase activity. In each case, stoichiometry was reported 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 \( \text{PP}_i \) exchange (9). This reaction has been previously described by Horowitz and Meister (2) for the enzyme from leukemic cells:

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

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-\( \text{r-RNA synthetase}, \) 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 \( \text{PP}_i \) 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-\( \beta \)-adénylate has been implicated as a reaction intermediate (2, 8), \( \text{PP}_i \) is the most probable product to be released prior to the addition of glutamine. The experimentally observed uncompetitive inhibition of \( \text{PP}_i \) versus glutamine, however, is contrary to such a mechanism in which competitive inhibition of \( \text{PP}_i \) 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 \( \text{PP}_i \) 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 \( \text{PP}_i \) 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 \( E\text{-NH}_2\text{-Asp} \) and \( E\text{-NH}_2\text{-Asp-AMP} \) 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.

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

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

where A = glutamate, 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:

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

where P = glutamate, Q = PP\(_i\), 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 (Endic-Hofstee plots were used to verify the noncompetitive inhibition), and the plot could easily be diagnosed as compet-

### Table III

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Theorell-Chance Bi Ter</th>
<th>+ Abortive complexes</th>
<th>Ordered Bi Ter</th>
<th>Rapid equilibrium Bi Ter</th>
<th>Two-site ping pong</th>
<th>+ Abortive complexes</th>
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<tbody>
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<td>Gln</td>
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<td>NC</td>
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<td>UC</td>
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</tr>
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</table>

\(^*\) NC, noncompetitive; C, competitive; UC, uncompetitive.

\( ^* \) With \( E\text{-NH}_2\text{-Asp} \) and \( E\text{-NH}_2\text{-Asp-AMP} \) complexes.

\( ^\dagger \) Rate equations derived by the method of Cha (11).

\( ^\ddagger \) With \( E\text{-Asp-PP}_i \), \( E\text{-Asp-AMP} \), \( E\text{-Glut-ATP} \), and \( E\text{-Asn-ATP} \) 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 asparagine 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 NH₃⁺ or 20 mM NH₂⁺ 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 NH₂⁺ is present, it should be able to combine with the E-NH₂ complex. The implication is that there are not only distinctive amide and acid sites but also a transfer mechanism which delivers the NH₂⁺ 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 glutamine was not 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 PP₃, 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. 5) 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 Uni Bi Ter Ping Pong Theorrell-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. (6) 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 amidaion 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

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