Asparagine Biosynthesis by the Novikoff Hepatoma

ISOLATION, PURIFICATION, PROPERTY, AND MECHANISM STUDIES OF THE ENZYME SYSTEM

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SUMMARY

An enzyme system that catalyzes the conversion of L-aspartate-14C to asparagine-14C in the presence of glutamine, ATP, and Mg++ has been purified 108-fold from a homogenate of the Novikoff hepatoma. ATP is converted to adenosine 5'-phosphate and inorganic pyrophosphate, and substitution of hydroxylamine for glutamine yields β-aspartyl-hydroxamate. The data suggest that the synthesis of asparagine in mammalian tissues proceeds through the intermediate β-aspartyladenylate.

A recent report from this laboratory described a cell-free assay system for asparagine biosynthesis in mammalian tissues (1). In contrast to cells showing a nutritional requirement for L-asparagine in culture and adult rat liver, cells not requiring the amino acid showed high levels of enzyme activity. Recently, Arfin (2) reported an asparagine-synthesizing system that required both the mitochondrial and supernatant fraction in embryonic chick livers. Enzyme activity was lost by the 19th day of chick development. Levintow (3) has reported that the amide 15N of glutamine was the amide donor, and several authors have reported on the carbon source of asparagine in mammalian tissue (4, 5). Although a number of studies have been carried out in plants and bacteria (6), no reports could be found in the literature relating to the mechanism of asparagine biosynthesis in the higher organisms.

In the present report, the isolation and purification of an asparagine-synthesizing enzyme system from Novikoff hepatoma, as well as studies on the properties and mechanisms of action of the enzyme system, are described and compared with those found in bacteria (7, 8).

EXPERIMENTAL PROCEDURE

Materials—L-Aspartic acid-14C (uniformly labeled) was obtained from Isotopes, Inc., and further purified by methods previously described (9); β-aspartylhydroxamic acid was obtained from Dr. Gordon Skinner, North Texas State College; L-isoglutamine, D-glutamine, and pyridoxal phosphate monohydrate were obtained from Calbiochem; 5-diazo-4-oxo-L-norvaline was obtained from Dr. R. E. Handschumacher of Yale University School of Medicine.

Enzyme Assay—The standard incubation mixture for measurement of formation of asparagine contained L-aspartic acid-14C, 2 μmoles (1 x 10^4 cpm per μmole); L-glutamine, 20 μmoles; potassium ATP, 8 μmoles; MgCl2, 8 μmoles; Tris buffer, pH 8.0, 100 μmoles; and a rate-limiting amount of enzyme in a total volume of 1.0 ml. It was incubated for 30 min in a 37° water bath. The reaction was stopped by the addition of 1 ml of cold 0.8 M perchloric acid, and the asparagine was isolated from the supernatant by an ion exchange method previously described (9). The number of micromoles of asparagine synthesized was calculated from the total radioactivity obtained after isolation. Because of the limited amount of enzyme available, the more sensitive radiometric assay method was chosen over the hydroxamate method normally used in studies of this type.

Analyses—Methods for identifying the final product of the reaction as asparagine have been described (1). Net synthesis of asparagine was demonstrated by increasing the standard incubation mixture 3-fold, extending the incubation time to 1 hour, and comparing the product, both before and after removal of aspartic acid-14C, with authentic asparagine by ascending paper chromatography in a water-saturated phenol solvent system and by electrophoresis at pH 4.7 in K-102 buffer (Kensco model 50 electrophoresis apparatus). Only those ninhydrin-positive spots that moved with the same Rf as authentic asparagine and aspartic acid (in instances when aspartic acid was not removed prior to chromatography) were found to contain radioactivity. Glutamate was identified as a reaction product, when glutamine was used as the amide donor, by electrophoresis and the column procedure (1).

When PPi determinations were made, one aliquot of the neutralized perchloric acid supernatant was removed and subjected to the method of Flynn, Jones, and Lipmann (10). It was necessary to include 10 μmoles of KF in the incubation mixture containing enzyme not subjected to the final purification to obtain stoichiometric quantities of PPi. Another aliquot was used for the assay of asparagine. A third aliquot was used to isolate and measure AMP by a modification of the method described by Hurlbert et al. (11). The sample was embedded on...
a column (250 × 15 mm) of Dowex 1 × 10 resin (200 to 400 mesh, formate cycle) and eluted with an asymptotic gradient of formic acid (0 to 4 M). The eluted fractions (9.5 ml) were read at 260 μm on a Beckman spectrophotometer (model DU) and compared with authentic AMP for quantitative determination.

Preparation of Enzyme—Novikoff hepatomas were transplanted and grown for 6 days in the peritoneal cavity of 8 female Holtzman rats. The tumors were removed and cleaned of connective and bloody tissues, and the resultant tissue (90 g) was homogenized in 2 volumes of cold Medium A (12) in a Waring Blender for 2 min. The homogenate (270 ml) was centrifuged for 10 min at 20,000 × g in a model HR-2 International centrifuge. To the supernatant (171 ml) were added 17.1 ml of a 10% solution of streptomycin sulfate, and the mixture was stirred for 30 min in an ice bath. The precipitate was removed by centrifugation and discarded. To the supernatant (130 ml) were added 90 ml of a cold saturated ammonium sulfate solution. After stirring for 30 min in the cold, the precipitate was removed by centrifugation and dissolved in Medium D (19). This fraction contained 18.7% of the enzyme activity and was discarded. To the supernatant were added 20 ml of cold saturated ammonium sulfate solution; the solutions were stirred for 30 min, and the precipitate was collected by centrifugation. This fraction was dissolved in 15 ml of Medium B, and calcium phosphate gel (2 mg per mg of protein) was added; the mixture was stirred for 30 min, and the gel was removed by centrifugation. To the gel supernatant (14 ml) were added 9 ml of a cold saturated ammonium sulfate solution; the mixture was stirred, and the precipitate was removed by centrifugation. Addition of 1 ml of cold saturated solution of ammonium sulfate to the resultant supernatant yielded a precipitate, which was then dissolved in 1 ml of Medium B and passed over a column (1.5 × 15 cm) of Sephadex G-100, equilibrated and eluted with Medium B. The final enzyme solution (0.383 mg of protein per ml) was eluted immediately after the void volume of the column (8.5 ml). The steps of purification and the related purification are shown in Table I. A final purification of 108-fold with a 2.9% yield was obtained. This fraction could be stored frozen for several

TABLE I

<table>
<thead>
<tr>
<th>Enzyme purification procedure</th>
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<tr>
<td>The reaction mixture contained L-aspartate-14C, 2 μmoles (10⁴ cpm per μmole); potassium ATP, 8 μmoles; L-glutamine, 20 μmoles; Tris buffer, 100 μmoles; MgCl₂, 8 μmoles; and a rate-limiting amount of enzyme protein in a final volume of 1 ml at a final pH of 8.0. The reaction was incubated for 30 min at 37°, and the product was isolated as described in “Experimental Procedure.” The specific activity is expressed as micromoles of asparagine per mg of protein per 30 min.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
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<td>100</td>
<td>1.0</td>
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<td>20.1</td>
</tr>
<tr>
<td>Calcium phosphate supernatant</td>
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<td>0.667</td>
<td>29</td>
<td>26.9</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, No. 2</td>
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<td>Sephadex G-100</td>
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<td>0.4</td>
<td>2.68</td>
<td>3.9</td>
<td>108.3</td>
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FIG. 1. Response curve and Lineweaver-Burk plot of L-aspartic acid for asparagine biosynthesis. The reaction mixture contained L-glutamine, 20 μmoles; potassium ATP, 8 μmoles; MgCl₂, 8 μmoles; Tris buffer, pH 8.0, 100 μmoles; and 19 μg of enzyme protein. Incubation and product isolation are described in “Experimental Procedure.”

FIG. 2. The inhibition of asparagine biosynthesis by L-asparagine. The reaction mixture contained L-aspartic acid-14C, 2 μmoles (10⁴ cpm per μmole); potassium ATP, 8 μmoles; L-glutamine, 20 μmoles; Tris buffer, 100 μmoles; MgCl₂, 7 μmoles; and 19 μg of enzyme protein. Velocity is expressed as micromoles of asparagine formed per mg of protein per 30 min.
months without appreciable loss (less than 0.1%) of activity. The final purified preparation was used in all studies reported.

RESULTS

The synthesis of asparagine was found to increase linearly with time, up to 2 hours, and with protein up to 0.1 mg per ml. The response of the enzyme system to aspartic acid is shown in Fig. 1. The enzyme was essentially saturated with 2.0 mM aspartic acid, and a Lineweaver-Burk plot (inset, Fig. 1) revealed a $K_m$ of $5.58 \times 10^{-3}$ M. To further characterize product formed, the effects of L-asparagine on the enzyme system were studied. As the Lineweaver-Burk plot in Fig. 2 shows, increasing concentrations of L-asparagine inhibited the formation of asparagine by the enzyme system. When 1.0 or 3.0 mM of 5-diazo-4-oxo-L-norvaline, an analogue of L-asparagine (14), were added, 22.9% and 37.9% inhibition, respectively, occurred. This compares with 57.1% and 87.9% inhibition when L-asparagine was added at the same levels.

The response curves of L-glutamine and ammonium chloride for asparagine synthesis are shown in Fig. 3. As can be seen, ammonium chloride could substitute for L-glutamine, but the enzyme system had a greater affinity for the latter. A Lineweaver-Burk plot of L-glutamine (inset, Fig. 3) yielded a $K_m$ of $1.1 \times 10^{-3}$ M. The $K_m$ for ammonium chloride was calculated to be $120 \times 10^{-3}$ M. However, as Meister (6) has pointed out, with other enzymes which will utilize both substrates as amide donors, the $K_m$ differences would be much less if calculated on the basis of un-ionized ammonia rather than total salt added. The pH-activity curve with glutamine as a substrate exhibited a broad optimum from pH 6.6 to 8.0, whereas when ammonium chloride was the substrate a linear increase with increasing pH from 6.0 to 8.6 was observed (Fig. 4). This would further suggest that ammonia rather than ammonium ion is the reactive species. The effects of varying concentrations of ammonium chloride and glutamine in combination are shown in Table II.

While at the lower concentrations the effects were not strictly additive, some enhancement by ammonium chloride was observed. At saturating levels of L-glutamine, however, no enhancement by ammonium chloride occurred. From these data, and since no separation of activities occurred during purification, it would appear that the same enzyme site is used for both substrates.

Glutamine synthetase was absent from the enzyme preparation, since the addition of 20 mM of glutamate to an incubation mixture containing 20 mM of ammonium chloride did not enhance asparagine synthesis. Furthermore no glutamine-14C

TABLE II

Supplemental effects of ammonium chloride on L-glutamine requirements

The reaction mixture, containing L-aspartate-14C, 2 mM (105 cpm per mM); potassium ATP, 8 mM; MgCl2, 8 mM; and 19 mM of enzyme protein, in a total volume of 1.0 ml at pH 8.0, was incubated for 30 min at 37°C. The asparagine was isolated as described in "Experimental Procedure."
could be detected as a product when glutamate-$^{14}$C (uniformly labeled) and ammonium chloride were substituted for glutamine and aspartic acid-$^{14}$C in the standard incubation mixture.

Other possible amide donors such as L-isoglutamine, n-glutamine, and l-glutamate at a 20-μmole level were inactive. Hydroxylamine (20 μmoles) or pyridoxal phosphate (20 μmoles), a proposed cofactor for amide nitrogen transfer reactions (15), were also ineffective when added to the standard incubation, but the addition of 20 μmoles of azaserine inhibited the reaction 31.0%.

The response curve and Lineweaver-Burk plot of ATP for the enzyme system is shown in Fig. 5. A $K_m$ value of 0.11 $\times$ 10$^{-4}$ M was calculated. Substitution of GTP, CTP, or UTP at 20 μmoles for ATP showed only GTP to be slightly active, at 5.6% of the level found with ATP.

The response curve for Mg$^{++}$ and Mn$^{++}$ and the Lineweaver-Burk plot for Mg$^{++}$ are shown in Fig. 6. Although the enzyme system showed an optimum response to Mn$^{++}$ at 2 mM, the activity represented only 22.0% of that observed with Mg$^{++}$ at its optimum concentration. The $K_m$ value for Mg$^{++}$ was calculated as 23.7 $\times$ 10$^{-5}$ M. Although this value appears high in contrast to the $K_m$ values of ATP, it may have resulted because no activity was observed at the 0.5 mM level or less of either cation.

The amounts of AMP and $P_i$ formed and their ratios to the amount of asparagine synthesized are shown in Table III. The stoichiometric amounts of AMP and PPi produced suggested the formation of β-aspartyladenylate. When 100 μmoles of neutralized hydroxylamine were substituted for glutamine in 4 times the standard incubation mixture, β-aspartylhydroxamate was identified as a product. The neutralized perchloric acid supernatant was lyophilized, redissolved in water, and spotted on paper chromatograms. These were developed by the ascending technique with methanol-pyridine-water (3:1:1), water-saturated phenol, or butanol-acetic acid-water (4:1:1) as solvent. Three FeCl$_3$-positive spots were observed on each chromatogram. The spot which had the same $R_F$ as authentic β-aspartylhy-
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The reaction mechanism of the present system appears to be similar to the asparagine synthetase (EC 6.3.1.1) of L. arabinosus (7) and Streptococcus bovis (8). Certain dissimilarities exist, however, in the requirements of the mammalian system and the bacterial systems, as well as a recently described system in embryonic chick liver (2). The primary amide donor in the mammalian system would seem to be L-glutamine. The present data, however, suggest that un-ionized ammonia is also reactive and has an affinity for the same enzyme sites required for the glutamine amide transfer. Glutamine synthetase does not play an intermediate role in the reactivity of ammonia, since none could be detected in the enzyme system. L-Glutamine was the active amide donor in the chick embryo liver system, whereas ammonium ion was not reactive. The reverse has been found in the bacterial systems.

The ATP requirement of the present system, although in common with the findings in the bacterial systems, is contrary to that shown for the chick embryo liver system. In the latter studies an energy source was required, as evidenced by inhibitor studies, but stimulation of asparagine synthesis by added nucleoside triphosphates or by ATP generated in situ did not occur. The present study did not resolve this difference between the two systems.

The Mg++ requirement of the mammalian system can be partially spared (22%) by Mn++. S. bovis asparagine synthetase and the chick embryo liver system require Mg++, and Mn++ cannot be substituted, but Mn++ is 70% more effective than Mg++ with the L. arabinosus enzyme.

A common property of the present system and the two bacterial enzymes is the inhibition of the reaction by its product, L-asparagine. While the inhibition of the S. bovis enzyme was competitive, the mechanism in the present study and in the L. arabinosus enzyme is not. The last two systems, however, do not appear to be strictly noncompetitive. Whether or not this is the control mechanism for asparagine biosynthesis at the cellular level in the mammalian system must await further study. The formation of the L. arabinosus enzyme is repressed by L-asparagine, whereas formation of the S. bovis enzyme is not.

Our studies indicate that varying levels of L-asparagine (0 to 0.3 mM) have no effect on the rate of asparagine synthesis by the JA tumor, a tissue exhibiting no nutritional requirement for L-asparagine in cell culture. Recently, however, Broome and Schwartz (18) reported that asparaginase (EC 3.5.1.1)-resistant mouse lymphoma cells responded to deprivation of exogenous L-asparagine by increasing their rate of asparagine synthesis.

REFERENCES

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