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GLUTAMINASE ACTIVITY OF L-ASPARAGINE AMIDOHYDROLASE*

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Abstract—Relatively high concentrations of 6-diazo-5-oxo-norleucine (DON) and azaserine, potent specific inhibitors of many enzymes using glutamine as substrate, do not have an appreciable effect on the activity of *Escherechia coli* L-asparagine amidohydrolase (EC 3.5.1.1) when either asparagine or glutamine is used as substrate. Glutamine acts as a competitive inhibitor when asparagine hydrolysis is measured, and asparagine inhibits glutamine hydrolysis. These observations, together with the fact that no one has been able to separate these two activities of the *E. coli* enzyme, point strongly to the presence of a single enzymatic site. However, the two activities have different pH dependencies. The glutaminase activity shows a 6-fold increase from pH 5 to pH 8·7; the asparaginase activity is almost constant over this pH range.

The measured Michaelis constants and maximum velocities for the glutaminase and asparaginase activities at pH 8·7 are as follows: $K_m^G = 1{\cdot}3 \times 10^{-2}$ M; $K_m^A = 1{\cdot}3 \times 10^{-3}$M; $V_{max}^G = 0{\cdot}080$ μmoles per min per i.u. of asparaginase activity; $V_{max}^A = 1{\cdot}1$ μmoles per min per i.u.The inhibition dissociation constant for glutamine acting as inhibitor of asparaginase in $1{\cdot}2 \times 10^{-2}$.

IT HAS been recently shown that the blood levels of free asparagine and glutamine vary in parallel in patients undergoing courses of therapy with *Escherechia coli* L-asparagine amidohydrolase (EC 3.5.1.1) preparations.[1] The residual glutaminase activity in these L-asparaginase preparations could account for this effect. Extensive purification of this enzyme did not result in the separation of these activities;[2] the glutaminase activity could not be reduced to below 2 per cent of the asparaginase activity. Since this glutaminase activity resulted in rather profound changes in patients' blood glutamine levels in cases where the therapeutic rationale called for reduction of asparagine levels only, a study of the nature of this secondary enzymatic activity was indicated.

EXPERIMENTAL

Materials

The *E. coli* L-asparagine amidohydrolase (EC 3.5.1.1) and the *E. coli* L-glutamine amidohydrolase (EC 3.5.1.2) were purchased from the Worthington Biochemical Corp., Freehold, N.J. The L-asparagine amidohydrolase had a specific activity of ca. 40 i.u./mg.† The L-asparagine-4-^{14}C and L-glutamine-U-^{14}C were obtained from

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† Enzyme activities are stated in international units; 1 i.u. represents the reaction of 1 μmole of substrate/min at a 0·01 M substrate concentration.

Calbiochem, Los Angeles, Calif. The DEAE paper (diethylaminoethyl cellulose) used was Whatman DE 51.

Radioactive counting

The ^{14}C was determined by liquid scintillation. Aqueous solutions (up to 0·5 ml) were dissolved in 15 ml Diotol scintillant.[3] Papers were suspended in a toluene scintillant which consisted of 5·00 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)benzene (dimethyl POPOP) per liter of toluene.

Asparaginase assays

(A) *For samples containing no interfering substances such as glutamine or 6-diazo-5-oxo-norleucine (DON).* Each assay sample was set up so as to contain approximately $\frac{1}{6}$ of an enzyme unit* in 2·0 ml of a solution which was 0·01 M with respect to L-asparagine, 0·05 M with respect to Tris-HCl buffer at pH 8·8, and containing 12·5 mg per ml of bovine serum albumin. Aliquots were removed after 15 and 30 min of incubation at 37°. The reaction was stopped by the addition of $\frac{1}{5}$ vol. of 20% trichloroacetic acid and rapid chilling. The precipitated protein was removed by centrifugation and the ammonia determined by the direct addition of $\frac{1}{9}$ vol. of Nessler's reagent to a suitably diluted aliquot of the supernatant solution.[4] If the resultant Nessler-ammonia color is read at 420 mμ, the aliquot can contain up to 1 μmole NH_3 per 3 ml final volume.

Since asparagine does hydrolyze in the very alkaline Nessler's solution, the optical density of the Nessler's color continues to increase. However, the rate of optical density increase is essentially linear and slow enough (0·003 μmole/min/3 ml of 0·0028 M asparagine) that reading at precise times after Nesslerization and comparison with blanks containing asparagine and no enzyme effectively cope with this problem. As an additional check, tubes were routinely read at three or four known time intervals up to 50 min after the addition of the Nessler's reagent, and the linear plot of optical density with time was extrapolated back to zero time.

(B) *For samples containing DON or other interfering substances.* DON, in the concentrations used, interferes with the direct Nesslerization by causing turbidity. The following equivalent method was used to measure the NH_3 produced when DON was used. Aliquots of the reaction mixture as described in Part A, above, were rapidly chilled and adjusted to pH 7 with 1 N acetic acid using phenol red as an internal indicator, diluted with about 4 vol. of water and transferred to a microcolumn of Permutit with the help of 0·5 ml of wash water. Operation of the column is described below. Since these eluates were essentially free of asparagine, DON, etc., the resulting Nessler-NH_3 colors were quite stable and could be read at any time after 10 min.

(C) *For kinetic studies in which asparaginase activity was measured in the presence of glutamine.* In these studies, ^{14}C-labeled asparagine (0·1 μc/ml) was used. The total reaction volume was 0·5 ml. The reaction was stopped by rapid cooling and the addition of enough solid picric acid to saturate the solution. After the precipitate was removed by centrifugation, the aspartic acid was separated from the asparagine on DEAE paper by a rapid method based on a technique suggested by Knight.[5] An aliquot (0·05 ml) of the supernatant was streaked on the starting line 3 in. from one end of a 1·5 × 12 in. strip of DEAE paper. The paper was immediately placed in a descending chromatography rack (without drying) and allowed to develop with

0·01 M acetate buffer, pH 4·9. In the "chromatocab" used in this laboratory, the solvent front reached the end of the paper in 2 hr. Development was continued for an additional hour. The paper was then carefully removed from the chromatocab, allowed to dry thoroughly in air, and cut into appropriate segments for ^{14}C analysis. The aspartic acid was found just below the picric acid band, 0·75 to 2·5 in. from the origin; the asparagine runs ahead and was found in the segment from 3 to 5·5 in. from the origin.

Permutit microcolumns

These columns were prepared from 9 in. disposable Pasteur pipettes. A tiny cotton plug was positioned in the capillary about 2 cm below the throat of the capillary. The pipette was filled with water and the rate of flow was noted. Only those tubes with flow rates that could be adjusted to about 1 ml per min were used. Sufficient dry Permutit (prepared and washed according to Folin and Bell[6]) was added to the water-filled tube to make a resin bed about 1·5 mm in diameter and 2 cm high. The resin was allowed to settle and the water level was permitted to fall into the resin. The sample (containing up to 1 μmole NH_3) was allowed to percolate through the resin bed and the column was then washed with three 1-ml portions of water. The outside of the column was rinsed and dried and the column was placed in a test tube with the lower end of the capillary immersed in 1·0 ml water. A 0·3-ml amount of 1 N NaOH was allowed to flow into the resin bed. After 10 min, this was followed with 1·4 ml water. When the water level reached the resin, the column was removed and the eluate (2·7 ml) Nesslerized by the addition of 0·3 ml of the reagent.

Glutaminase assays

(A) *For samples containing no asparagine or asparaginase activity.* (1) With the *E. coli* L-asparagine amidohydrolase (EC 3.5.1.1). Conditions for this assay were identical with those indicated in the section on asparaginase assays, Part B, except that 0·01 M L-glutamine replaced the L-asparagine and each assay sample contained 2·5 L-asparaginase units of enzyme per ml. The rate of destruction of glutamine in a Nesslerized solution is so great (over 0·02 μmoles/min/3 ml of 0·0028 M glutamine) that the extrapolation back to zero time may be subject to serious error. In these experiments the glutamine was separated from the NH_3 by a Permutit column before Nesslerization.

(2) With *E. coli* L-glutamine amidohydrolase (EC 3.5.1.2) as enzyme. This enzyme has a pH optimum near pH 5.[7] Hence the final incubation mixture (2·0 ml) with this enzyme contained 0·08 ml of 0·125 M acetate buffer, pH 4·9, instead of the alkaline Tris buffer used with the asparaginase.

(B) *For kinetic studies in which glutaminase activity is measured in the presence of asparagine and asparaginase activity.* These experiments were carried out in the same manner as the kinetic studies of asparaginase activity (Part C, above). Labeled glutamine (0·1 μc/ml) was used. Glutamic acid and glutamine have essentially the same R_f values on DEAE paper as aspartic acid and asparagine respectively.

RESULTS AND DISCUSSION

The *E. coli* L-asparagine amidohydrolase preparation used corresponds to the EC-2 L-asparaginase of Campbell *et al.*,[2] who were unable to separate the glutaminase and

asparaginase activities of this preparation through several different purification steps. Figure 1 shows the effect of pH on the asparaginase and glutaminase activites of the L-asparagine amidohydrolase. The pH optimum for the glutaminase activity of this enzyme is above 8, quite different and distinct from that of the *E. coli* L-glutamine amidohydrolase activity.[7] It can also be seen that the asparaginase activity of this same enzyme has a considerably different pH dependency from that of the glutaminase activity.

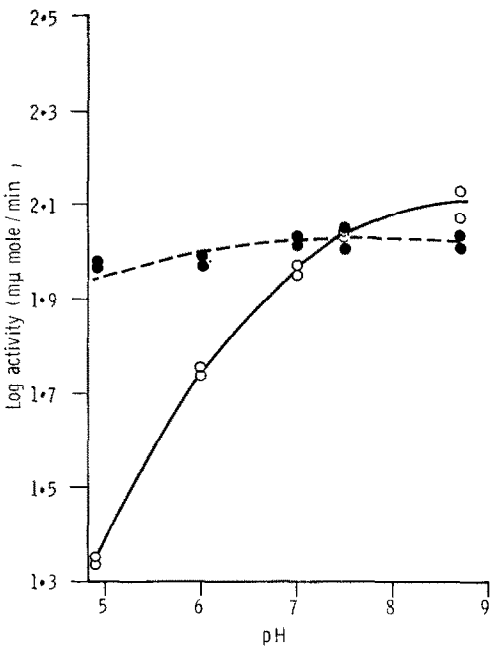

FIG. 1. Effect of pH on the asparaginase and glutaminase activities of *E. coli* L-asparagine amidohydrolase. The solid line represents the log of the glutaminase activity of 3·15 i.u./ml of *E. coli* L-asparagine amidohydrolase at a 0·01 M glutamine concentration. The dashed line represents the log of the asparaginase activity of 0·105 i.u./ml of the enzyme at a 0·01M asparagine concentration. The final concentration of all buffers was 0·05 M. Buffers used: sodium acetate at pH 4·9; sodium phosphate at pH 6·0 and pH 7·0; Tris-HCl at pH 7·5 and pH 8·7.

It has been shown by numerous investigators[8–10] that azaserine and DON inhibit the activity of enzymes for which glutamine is a substrate. Handschumacher *et al.*[10] have recently shown that DON has no effect on guinea pig asparagine amidohydrolase, an enzyme devoid of glutaminase activity. Since these small molecules are thought to bind to the glutamine receptor site of enzymes by virtue of their structural similarity to glutamine, it was felt that these inhibitors might be able to distinguish between glutamine and asparagine receptor sites if they were different. Table 1 summarizes the results of a number of experiments with these inhibitors. Azaserine has no apparent effect on either the asparaginase or glutaminase activities of this enzyme. Preincubation of the enzyme with a relatively high concentration of the inhibitor also had no

apparent effect. DON had a slight inhibitory effect, about equal for both activities. This 15–20 per cent inhibition is in contrast to the extensive inhibition produced by the asparagine analog, 5-diazo-4-oxo-norvaline,[10] and the 84 per cent inhibition produced by the same concentration of DON on the activity of the L-glutamine amidohydrolase (Table 1). From these experiments it would appear that a single enzymatic site is involved for both the asparaginase and the glutaminase activities of the L-asparagine amidohydrolase.

TABLE 1. EFFECT OF AZASERINE AND DON ON AMIDOHYDROLASE ACTIVITIES OF *E. coli* L-ASPARAGINE AMIDOHYDROLASE

DON				Azaserine			
DON				Azaserine			
Preinc.* (mM)	Assay† (mM)	Asparaginase activity‡ (units)	Glutaminase activity§ (units)	Preinc.* (mM)	Assay† (mM)	Asparaginase activity‖ (units)	Glutaminase activity§ (units)
0	0	0·0716	0·0787	0	0	0·0956	0·0615
3·5	2·9	0·0605(85%)	0·0612(78%)	22	2·9	0·0931(97%)	0·069(112%)
3·5	0	0·0676(94%)	0·0663(84%)	22	0	0·0941(98%)	0·064(104%)
0	2·9	0·0584(82%)	0·0659(84%)	0	2·9	0·0922(96%)	0·068(111%)
				0	0		0·0566
				0	16		0·054(96%)
E. coli L-glutamine amidohydrolase							
0	0		0·091¶				
0	2·9		0·015(16%)				

* The enzyme (150 units/ml) was preincubated for 10 min at 37° with 2 vol. of bovine serum albumin (25 mg BSA/ml of 0·85% NaCl) solution and 1 vol. of inhibitor dissolved in 0·1 M Tris, pH 7·5. The enzyme was suitably diluted in 50% BSA solution for assay.

† The inhibitor was added to the assay mixture at the start of the assay incubation.

‡ Ammonia produced (μmoles per minute) by ca. 0·083 unit of asparaginase/ml of 0·01 M asparagine, 0·05 M with respect to Tris-HCl buffer, pH 8·8, and containing 12·5 mg/ml BSA. Incubation for 15- and 30-min intervals. Ammonia was determined by Asparaginase, Method B (see Experimental). Percentages in parentheses are percentages of the uninhibited controls at the start of the series.

§ Ammonia produced (μmoles per minute) by ca. 2·5 units of asparaginase/ml of 0·01 M glutamine under conditions specified in footnote ‡ above. Ammonia was determined by Glutaminase, Method A, 1 (see Experimental).

‖ See footnote ‡. Ammonia determined by Asparaginase, Method A (see Experimental).

¶ Ammonia produced (μmoles per minute) by ca. 0·1 unit of glutaminase/ml of 0·01 M glutamine, 0·05 M acetate buffer, pH 4·9, and containing 12·5 mg/ml BSA. Incubation for 15- and 30-min intervals. Ammonia was determined by Glutaminase, Method A, 2 (see Experimental).

If these substrates are competing for the same enzymatic site, then each should inhibit the reaction of the other. To test this, asparaginase assays with varying amounts of asparagine were carried out in the presence of several concentrations of glutamine. Figure 2 is a Lineweaver–Burk[11] plot of the results of this experiment. The Michaelis constant for the asparaginase activity, K_m^A, is $1{\cdot}3 \times 10^{-3}$ with a V_{max}^A of 1·3 μmoles per min per i.u. of enzyme. The three lines fit the experimental data and meet in a point on the ordinate axis, as would be expected in a competitive inhibition. The inhibition dissociation constant, K_i^G, has a value of $1{\cdot}2 \times 10^{-2}$. The reverse experiment, the inhibition of glutaminase activity by asparagine, gave experimental data

that could not be similarly analyzed, since the concentration of the inhibitory compound, asparagine, decreased appreciably during the time needed to obtain statistically significant quantities of glutamate from glutamine. Since the rate at which asparagine is hydrolyzed is dependent on both the glutamine and asparagine concentrations, the inhibitor concentration effective during the experimental period used for rate determination would be different at differing substrate concentrations, even when the

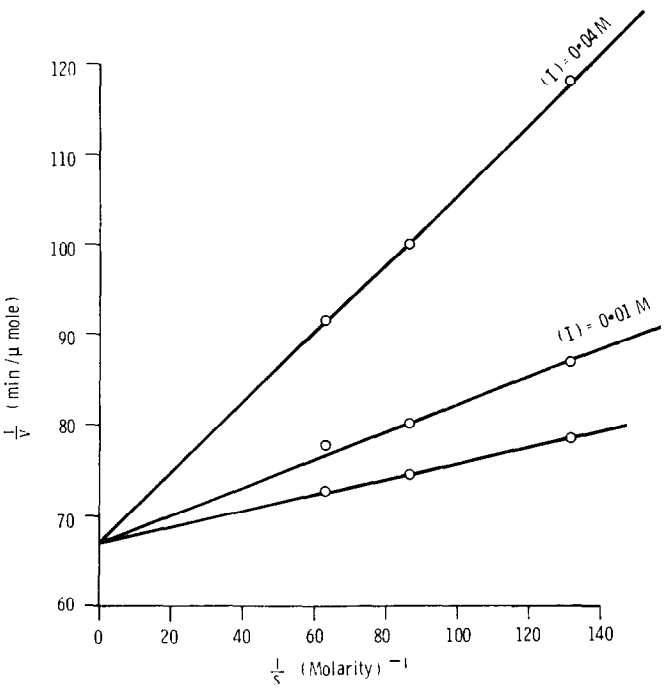

FIG. 2. Competitive inhibition of the asparaginase activity of 0·0132 i.u./ml of L-asparagine amidohydrolase by two concentrations of glutamine. (See Experimental.)

initial concentrations were the same. From the data in Table 2, it is apparent that asparagine inhibits the glutaminase activity of this enzyme, although no definitive statement can be made as to the competitive nature of this inhibition.

A Lineweaver–Burk plot[11] of the data for the uninhibited glutaminase activity indicated a K_m^G of $1{\cdot}3 \times 10^{-2}$ M and a V_{max} of 0·080 μmoles per min per i.u. of asparaginase activity.

The experimental data in these studies are in agreement with the thesis that both substrates are hydrolyzed at the same enzymatic site. The overall similarity of asparagine and glutamine, particularly in terms of the number and kinds of chemically active groups and their measured pK values, would indicate that the observed differences in their pH dependency curves are due to differences in the ionic environment on the enzyme for the two substrates rather than to differences in the ionization of the substrates themselves. If, indeed, there is only one active site, the glutaminase curve would seem to indicate that glutamine hydrolysis requires the removal of an additional

proton, possibly from an imidazolium or ammonium grouping at or near the attachment site. This is not too difficult to imagine. If the amide end of the molecule is fixed at the "hydrolytic site," the alpha carboxyl or the alpha amino groups (or both groups) of the longer glutamine molecule might be incorrectly attracted or repelled by a positively charged group just beyond the asparagine attachment site.

TABLE 2. INHIBITION OF THE GLUTAMINASE ACTIVITY OF *E. coli* L-ASPARAGINE AMIDOHYDROLASE BY ASPARAGINE*

Asparagine concn (mM)	Glutamine concn (mM)		
	6·67	10	20
0	0·037†	0·045	0·059
2	0·019	0·028	0·035
5	0·008	0·012	0·017

* Assay mixture: 1·32 i.u./ml of *E. coli* L-asparagine amidohydrolase; 0·05 M Tris-HCl buffer, pH 8·8; 0·1 μc/ml L-glutamine-U-^{14}C at the indicated concentrations; L-asparagine at the indicated concentrations. Samples (0·5 ml) were incubated at 37° for 5- and 15-min intervals and the glutamic acid produced was determined as described in Glutaminase, Method B, Experimental.

† Rate of glutamic acid production (μmoles/min) during the first 5 min of incubation.

If the two enzymatic activities of the L-asparagine amidohydrolase are attributable to one active site, the therapeutic use of this enzyme must involve both reactions, even when the blood level of glutamine appears normal.[1] Although the glutaminase activity is much less than the asparaginase activity, the glutamine concentration in serum is far higher and side reactions such as the destruction of glutamine and the increase in free ammonia levels are of potential significance during prolonged courses of asparaginase therapy.

Note added in proof—Recently, Rubinow and Lebowitz[12] have derived a simple time-dependent extension of Michaelis-Menten theory for an enzyme–substrate–inhibitor system in which two substrates, one reacting much more readily than the other, compete for a single enzymatic site. Our data for the inhibition of glutaminase activity by L-asparagine and asparaginase activity by L-glutamine appear to be in accord with this theoretical model.

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