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THE STABILITY OF L-ASPARAGINASE WITH RESPECT TO PROTEIN DENATURANTS

DAVID A. COONEY AND RUTH D. DAVIS

Laboratory of Toxicology, National Cancer Institute, National Institutes of Health, Bethesda, Md., 20014 (U.S.A.)

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SUMMARY

1. Under certain conditions, L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) from *Escherichia coli* resists denaturation by heat and acid.

2. Tryptone broth protects L-asparaginase from thermal inactivation; but L-asparagine, at saturating concentrations, accelerates the thermal denaturation of the enzyme.

3. It is suggested that heat might offer a valuable tool in the preliminary purification of this oncolytic enzyme.

4. In view of the stability of L-asparaginase, if meaningful measurements of its substrates are to be made in the presence of the enzyme, an instantaneous inactivator should be used.

INTRODUCTION

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) from *Escherichia coli* (ECII) is a useful agent in the treatment of Acute Lymphocytic Leukemia of childhood[1]. The enzyme has been presumed to exert its oncolytic effect by removing L-asparagine from plasma, thus depriving tumor cells which are unable themselves to synthesize this amino acid[2]. During therapy, L-asparagine in the plasma has been reported to fall to undetectable levels[3]; moreover, in some cases the depression of plasmatic L-asparagine has been protracted[4]. In preparing samples for measurements of L-asparagine, boiling or acid have generally been used to precipitate protein. We wish now to report that L-asparaginase is quite thermostable and that residual amidohydrolase activity persists even in the neutralized supernatants of perchloric, trichloroacetic and picric acid extracts of the enzyme.

MATERIALS AND METHODS

L-Asparaginase ECII (specific activity greater than 300 I.U./mg protein) prepared by the Merck Co. was used in these studies. The enzyme was reconstituted with water to a concentration of from 100–2000 I.U./ml; mannitol, present as an aid

to lyophilization was not removed prior to denaturation. In certain experiments, a solution of 20 mM L-asparagine was used to reconstitute the enzyme immediately prior to heating. Samples were boiled in screw-capped glass vessels. At the designated time, aliquots were removed, iced and assayed immediately. In addition to the coupled spectrophotometric assay for L-aspartic acid[5], the hydrolysis of 5-diazo-4-oxo-L-norvaline (DONV, NSC-117,613, procured from Park Davis and Co.) was used for the estimation of L-asparaginase[6,7]. This alternate substrate was especially valuable when L-asparagine had been present in the reaction mixtures, in which case high concentrations of enzymatically produced ammonia and L-aspartic acid would have yielded inordinately high blank values in the other assay methods. Because it was necessary to dilute the boiled enzyme prior to assay, the final concentrations of L-asparagine and ammonia were sub-inhibitory; (L-asparagine is a competitive inhibitor of the hydrolysis of 5-diazo-4-oxo-L-norvaline[7]; ammonia is an inhibitor of the hydrolysis of L-asparagine[8]).

RESULTS

Table I presents the time-course of the denaturation of L-asparaginase by immersion in boiling water. Even after 25 min at 100°, substantial amidohydrolytic activity persists. When high concentrations of L-asparagine are present, the decrease in activity is accelerated. It is important to note that aqueous solutions of L-asparaginase boiled alone remain clear or go very faintly opalescent; on the other hand, when boiled in the presence of L-asparagine, its principal substrate, the enzyme flocculates and settles out as a tan precipitate.

Table II presents the residual activities of samples of L-asparaginase exposed to acid denaturants. Detectable and significant activity persists after exposure to all three acids; but 7% perchloric acid was the poorest denaturant. Even exposure of the enzyme to heat as well as acid, in that order, failed to denature it fully.

In studies with picric acid, repeated extractions with ether failed to extract

TABLE I

INACTIVATION OF L-ASPARAGINASE BY HEAT

L-Asparaginase, 1000 I.U./ml of water or L-asparagine (pH 7) was immersed in a bath of boiling water. At the times indicated aliquots were removed, iced and assayed for L-asparaginase by monitoring the decomposition of 5-diazo-4-oxo-L-norvaline (in 0.1 M Tris, pH 8.0) spectrophotometrically at 274 nm.

Time of immersion in boiling water (min)	*% Residual activity (boiled in water)*	*% Residual activity (boiled in 0.02 M L-asparagine)*
0	100	100
4	68	15
8	53	15
12	41	15
14	37	15
16	31	15
20	25	10
24	16	10

TABLE II

THE PARTIAL RESISTANCE OF L-ASPARAGINASE (ECII) TO ACID DENATURANTS

200 μl of L-asparaginase, 100 I.U./ml, were shaken for 15 min with 200 μl of denaturant at the concentration indicated. A precipitate formed. The mixtures were then extracted five times with 2.5 vol. of water-saturated ether and brought to pH 8.0 with 200 μl of 0.5 M Tris. The perchloric acid extracts were neutralized with 200 μl of a saturated solution of $KHCO_3$. 20 μl of the supernatant from these neutralized suspensions were incubated for 1 h with 0.1 M Tris at pH 8, containing 10 μmoles of L-asparagine per ml. The reaction was stopped by immersion of the capped reaction vessels in vigorously boiling water for 30 min. The L-aspartic acid formed was measured spectrophotometrically.

Denaturant	*Concentration of denaturant added*	*Normalized initial activity* (I.U./ml)*	*Final activity (I.U./ml)*	*% Residual activity*
Picric acid	satd. soln.**	333	14.5	4.35
	0.5 satd. soln.	333	17.5	5.25
	0.25 satd. soln.	333	19.5	5.85
Trichloroacetic acid	3 M	333	1.5	0.45
	1 M	333	13.0	3.9
	0.5 M	333	18.5	5.55
Perchloric acid	7%	333	100.0	30.0
Boiling and perchloric acid	Boiling for 9 min, followed by exposure to 7% perchloric acid for 15 min	333	10.0	3.0

* Corrected for dilutions during sample work-up.
** After five extractions with ether, the aqueous phase was deep yellow.

picric acid completely from the aqueous phase. Since active enzyme was detected in this phase, it is likely that the L-asparaginase had been picrylated to an extent compatible with the preservation of catalytic activity; on the other hand, the deep-yellow precipitated enzyme exhibited little residual activity.

Since roughly 70% of the starting activity of L-asparaginase remained after a 4-min immersion in boiling water, whereas most other proteins are denatured under these conditions, we considered that a brief exposure to heat of cultures of disrupted *E. coli* might offer a valuable one-step preliminary purification of the enzyme. Since our cultures of *E. coli* B produced little L-asparaginase, an artificial enrichment technique was used to study this point. The organisms in tryptone broth were harvested during logarithmic growth and disrupted in a Polytron mechanical-sonic homogenizer at top speed for a net homogenization time of 1 min. Purified ECII was added and the enriched cultures, in a 500-ml erlenmeyer flask, were immersed in a boiling-water bath. Thermal equilibrium in this system was prompt. The results of this study are given in Table III. Under these conditions, L-asparaginase was dramatically thermostable. Whether it can be thermally purified from contaminating enzymatic activities —as ribonuclease and acetyl phosphatase can[9]—must await tests with productive cultures. Nevertheless, the present experiments indicate the feasibility of a one-step thermal purification of ECII.

The thermostability of ECII prompted us to examine its catalytic efficiency at elevated temperatures. Once again the activity of the enzyme was measured by the

TABLE III

THE THERMAL STABILITY OF ECII L-ASPARAGINASE IN CULTURES OF *E. coli* B

E. coli B (ATCC 11303) was brought to logarithmic growth in tryptone broth, and disrupted on ice with the Brinkman Polytron (mechanical-sonic) homogenizer at top speed for 6 bursts of 10 sec each. To 50 ml of the homogenate in a 500-ml stoppered erlenmeyer flask 2000 I.U. of purified L-asparaginase (ECII) were added. A zero-time sample was taken and the flask was immersed in a bath of boiling water. The flask was swirled throughout its immersion. At the times designated, 1 ml of the mixture was removed, iced and assayed for residual L-asparaginase both by the hydrolysis of L-asparagine and the hydrolysis of 5-diazo-4-oxo-L-norvaline. Beginning at 4 min of immersion, and progressing until the close of the experiment, the reaction mixture exhibited progressive opacification.

Time of immersion in boiling water (min)	*% of activity remaining*	
	Hydrolysis of L-asparagine	*Hydrolysis of 5-diazo-4-oxo-L-norvaline*
0	100	100
2	98	84
4	100	84
6	102	91
8	88	84
10	90	80
12	78	72
14	72	68

TABLE IV

THE CATALYTIC EFFICIENCY OF L-ASPARAGINASE AT ELEVATED TEMPERATURES

In screw-capped tubes, 10 ml of 0.01 M L-asparagine, in 0.05 M Tris (pH 8) were brought to the temperatures indicated. L-Asparaginase, 0.35 I.U. of ECII, was added and the mixtures incubated at the temperatures indicated for 30 min. Then 500 μl of 60% perchloric acid were added to terminate the reaction by acidification. Residual L-asparaginase was destroyed by immersing the reaction vessels for 20 min in a bath of boiling water. The acidified mixtures were neutralized with a saturated solution of $KHCO_3$. The concentration of L-aspartic acid in the clear supernatants was measured spectrophotometrically. Under these conditions there is a very considerable "blank" hydrolysis of L-asparagine. Since this blank was consistent, it could reasonably be subtracted from the experimental measurements. The hydrolysis of 5-diazo-4-oxo-L-norvaline at various elevated temperatures was monitored in the thermo-controlled cell compartment of the Beckman DB spectrophotometer. Corrections have been made for the non-enzymatic breakdown of the substrate, where appropriate. At 77° the A274 of 5-diazo-4-oxo-L-norvaline was 90% of its A274 at 37°; no corrections for this diminished extinction have been made.

Temp.	*Relative velocity*		*Time to reduction of rate by 50% (min)*	*Time to reduction of rate by 100% (min)*
	Hydrolysis of L-asparagine (% of the velocity at 37°)	*Hydrolysis of 5-diazo-4-oxo-L-norvaline (% of the initial velocity at 37°)*		
37°	100	100	≃25	>120
57°	160	148	≃ 7.5	45
77°	90	80	≃ 2.5	5
97°	0	—	—	—

hydrolysis of both 5-diazo-4-oxo-L-norvaline and L-asparagine. The results of this study are given in Table IV. At 57° the velocity of hydrolysis of both substrates was augmented by about 50%. However, at 77° the velocity of hydrolysis fell below the velocity at 37°; and at 97° L-asparagine was not detectably hydrolyzed. It is interesting that the inactivation of L-asparaginase by 5-diazo-4-oxo-L-norvaline was markedly accelerated at 77°; upon cooling the reaction vessels to 37°, no residual hydrolytic activity was measurable. Doubtless, two opposite phenomena were involved in these studies at elevated temperatures: fractional inactivation of the enzyme, which retarded the reaction velocity, and increased molecular motion, which accelerated the reaction velocity.

DISCUSSION

We initially observed the thermal stability of L-asparaginase during the preparation of the enzyme for digestion with pronase. In order to achieve meaningful values for L-asparaginyl residues in L-asparaginase, we realized that it was necessary to denature the enzyme thoroughly. However, 4 boiling periods of 20 min each were required to eliminate all amidohydrolase. Moreover, although many enzymes are protected against denaturation by the presence of their substrates, L-asparagine appeared to accelerate the destruction of L-asparaginase by heat. The possibility that this effect was a nonspecific one has not been ruled out. The thermostability of L-asparaginase is somewhat surprising since most enzymes—even those present in thermophilic bacteria—are denatured rapidly at the temperature of boiling water[10].

In view of the striking stability of L-asparaginase to protein precipitants, if meaningful measurements of plasma L-asparagine are to be made during or after therapy with the enzyme, a prompt and total inactivator must be found. 5-Diazo-4-oxo-L-norvaline has been used for this purpose by BROOME[2]; but it is itself readily hydrolyzed by the enzyme so that the rate and extent of inactivation are limited. The search for an instantaneous denaturant is therefore continuing.

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