PRELIMINARY NOTES

BBA 61208

Multiple forms of L-asparaginase

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) which possesses antileukemic properties in man, has been crystallized from extracts of *Escherichia coli* B[1,2]. Minor components of the crystalline enzyme have been detected by electrophoresis on acrylamide gels and sedimentation equilibrium analysis, however, only a single band is detected in gels which were subjected to electrophoresis in the presence of 7 M urea. The present communication provides evidence that the minor components in the crystalline preparation are indeed the multiple forms of L-asparaginase. The conditions under which the multiple forms of the enzyme are formed are also reported.

Fig. 1 illustrates the pattern of enzyme activity observed when a crystalline preparation (specific activity, 285 I.U./mg solid) of *E. coli* B L-asparaginase was subjected to electrophoresis on a 5% acrylamide gel at pH 8.5. Enzyme activity was

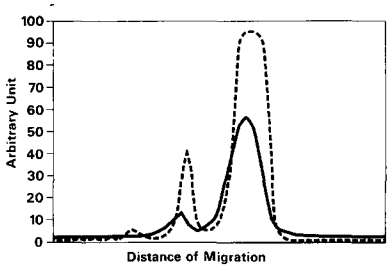


Fig. 1. Electrophoresis of *E. coli* B L-asparaginase on 5% acrylamide gel. The enzyme (specific activity, 285 I.U./mg of solid; 50 μg in 10 μl of 30% sucrose containing 0.05 M Tris–glycine buffer, pH 8.3) was applied to the top of 6 mm × 60 mm 5% acrylamide gel (pH 8.5) prepared according to the method of *Davis*[3] with the upper gel omitted. Electrophoresis was performed at room temperature at a constant current of 2 mA per gel column for 75 min. The buffer was 0.05 M Tris–glycine, pH 8.3. Protein was stained with Coomassie blue. Quantitation of the protein pattern (— — —) was obtained by scanning the stained gel with a Densicord densitometer (Photovolt-Corp., New York). Enzyme activity was detected by the following procedure: a duplicate unstained gel was sliced into 30 fractions which were incubated separately in 1 ml of reaction mixture containing 0.025 M Tris buffer, pH 8.5, and 0.01 M L-asparagine at 37° for 1 h. The ammonia released was determined by direct Nesslerization (———), and an arbitrary unit equals 2 μmoles of ammonia.

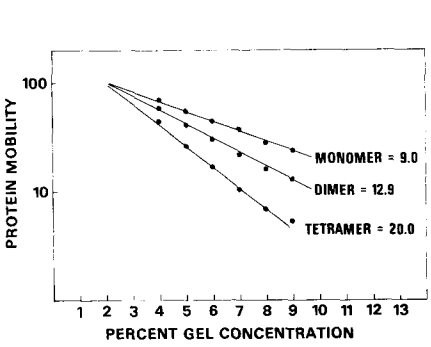
detected by placing the sliced gel fractions in a reaction mixture containing L-asparagine and then measuring the ammonia released by direct Nesslerization. Two separate enzymatic components were observed. By integration of the areas under the peaks in Fig. 1, the major band was calculated to contain 90% of the total activity. The protein profile superimposed readily onto the enzyme activity.

Examination of a number of electrophoretograms revealed that the number of minor bands varied depending on the amount of enzyme applied onto the gels. With a 50-μg sample, three distinct bands were detected by protein staining. The distances migrated by these bands were related logarithmatically. One can suggest from the

results that these minor bands are multiple forms of native L-asparaginase. Support for this hypothesis is provided by the fact that upon electrophoresis on cellulose acetate membranes, only a single zone was obtained over a wide pH range[4].

In light of these results, experiments were undertaken to prove that these multiple forms were a size isomer family of protein. A size isomer family of proteins (*e.g.* bovine serum albumin polymers) may be distinguished from a charge isomer family of proteins (*e.g.* lactate dehydrogenase isoenzymes) utilizing disc gel electrophoresis[5]. When the log of protein mobility relative to the dye front is plotted *vs.* acrylamide gel concentration, size isomeric proteins give a family of nonparallel lines extrapolating to a common point in the vicinity of 2% gel concentration. In contrast, charge isomeric proteins give a family of parallel lines. The isomeric forms of crystalline L-asparaginase were separated by electrophoresis on gels with concentration of 4–9% acrylamide. A plot of log protein mobility *vs.* gel concentrations for these three species of L-asparaginase yielded three nonparallel lines extrapolating to a common point at 2% gel concentration (Fig. 2).

The slope of the lines in the above plot (Fig. 2) is also related to molecular weight. HEDRICK AND SMITH[5] have established the molecular weight–slope relationship utilizing seventeen well characterized proteins as standards. Using these results, the molecular weights of three forms of L-asparaginase were estimated to be 130 000, 270 000, and 520 000 which would correspond to monomer, dimer, and tetramer of L-asparaginase. The molecular weight of the predominating monomer has been found to be 133 000 by sedimentation equilibrium studies[6] in good agreement with results of other workers[10,12].



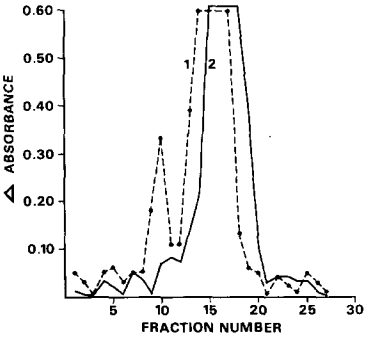


Fig. 2. The effect of different gel concentrations on the mobility of *E. coli* B L-asparaginase polymers. The negative slopes of the lines are noted on the figure.

Fig. 3. Enzymatic activity of L-asparaginase on 5% acrylamide gel. An enzyme solution (25 μl) containing 30% sucrose, 0.025 M Tris–HCl buffer, pH 8.5, and 1.5 I.U. of L-asparaginase was layered on the gels. The procedures for electrophoresis are similar to that described in Fig. 1. A sensitive spectrophotometric method based on an enzymatically coupled oxidation of reduced pyridine nucleotide was used[8] to detect minute quantities of L-asparaginase in this experiment. Sliced gel fractions were added to a reaction mixture (2 ml) containing 50 μmoles of Tris–HCl buffer, pH 8.5, 2 μmoles of α-ketoglutarate, 15 μg of glutamate–oxaloacetate transaminase, 1.2 μmolar units (as defined by Sigma) of malate dehydrogenase, and 0.4 μmoles of β-NADH. The reaction was started by the addition of 1 ml of 0.4 mM L-asparagine and the mixture was incubated at 37° for 25 min. Differences in absorbance at 340 nm taken before and after incubation represented the amount of L-asparagine hydrolyzed by the enzyme. Curve 1, the enzymatic pattern of crystalline L-asparaginase; Curve 2, the enzymatic pattern of crude extract from *E. coli* B.

Attempts to dissociate the polymeric forms into monomers under various conditions, including decreasing the protein concentration to 0.05 mg/ml, lowering pH to 4.0, dialysis against EDTA and 2-mercaptoethanol, and heating at 50° for 15 min, were unsuccessful, in contrast to the results of KIRSCHBAUM *et al.*[12]. The enzyme, however, does dissociate into subunits (mol. wt. 32 500) in nonaqueous solvents such as 4 M guanidine and 7 M urea solutions[7], a finding in agreement with results of other workers[10,12], although the subunit molecular weight reported earlier was considerably lower (19 000–24 000). Upon electrophoresis in gels free of urea, the enzyme reassociated into native monomer but the amount of higher polymeric forms was reduced.

Since multiple forms are not present in the crude extract (Fig. 3), it is likely that isomers are formed during purification. In order to determine the influence of individual purification steps on appearance of these isomers, the enzyme activity patterns of preparations derived from each step were examined on gel electrophoresis. Identical amounts of enzyme were used, although specific activities of these materials varied from 1.5 to 125.0 I.U./mg. No minor band of enzyme activity was detected in fractions after pH 5.0 treatment, $(NH_4)_2SO_4$ precipitation, and first ethanol fractionation in several experiments. Although the enzyme with lower specific activity (58 I.U./mg) did not contain multiple forms of L-asparaginase, the preparation with higher specific activity (125 I.U./mg) contained a minor component identical to that observed in the crystalline preparation. Since both enzyme preparations have been subjected to similar treatments, it is likely that aggregation is not due to use of ethanol. Therefore, aggregation probably occurs during lyophilization of an enzyme solution containing large quantities of L-asparaginase (30–50% of the protein content). L-Asparaginase of different isoelectric point[9,10] and partially deaminated forms of L-asparaginase[11] have been reported, but these charge isomers should not be confused with the size isomers described here.

The Lilly Research Laboratories,
Eli Lilly and Co.,
Indianapolis, Ind. 46206 (U.S.A.)

PETER P. K. HO
ELIZABETH B. MILIKIN

1 P. P. K. HO, B. H. FRANK AND P. J. BURCK, *Science*, 165 (1969) 510.
2 P. P. K. HO, E. B. MILIKIN, J. L. BOBBITT, E. L. GRINNAN, P. J. BURCK AND B. H. FRANK, *J. Biol. Chem.*, submitted.
3 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
4 P. P. K. HO AND E. B. MILIKIN, *Federation Proc.*, 28 (1969) 728.
5 J. L. HEDRICK AND A. SMITH, *Arch. Biochem. Biophys.*, 126 (1968) 155.
6 B. H. FRANK AND A. J. VEROS, *Federation Proc.*, 28 (1969) 728.
7 B. H. FRANK, A. H. PEKAR, A. J. VEROS AND P. P. K. HO, *J. Biol. Chem.*, submitted.
8 R. E. HANDSCHUMACHER, in *Exploitable Molecular Mechanisms and Neoplosia, 22nd Annual Symp. on Fundamental Cancer Research, Houston, Texas, 1968*, Wilkins, Baltimore, 1969, p. 565.
9 J. ROBERTS, G. BURSON AND J. M. HILL, *J. Bacteriol.*, 95 (1968) 2117.
10 H. A. WHELAN AND J. C. WRISTON, JR., *Biochemistry*, 8 (1969) 2386.
11 O. WAGNER, E. IRION, A. ARENS AND A. G. BAUER, *Biochem. Biophys. Res. Commun.*, 37 (1969) 383.
12 J. KIRSCHBAUM, J. C. WRISTON, JR. AND O. T. RATYCH, *Biochim. Biophys. Acta*, 194 (1969) 161.

Received January 13th, 1970