Leucine Aminopeptidase (Bovine Lens)

STABILITY AND SIZE OF SUBUNITS*

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SUMMARY

The stability to pH and denaturing agents of crystalline leucine aminopeptidase (bovine lens) (EC 3.4.1.1) is reported. The native enzyme exhibited a molecular weight of 327,000. In 7 M urea below pH 3 and in ≥3.7 M guanidinium chloride below pH 8.5, both leucine aminopeptidase and its reduced and carboxamidomethylated derivative exhibited a molecular weight on equilibrium centrifugation of 54,000 ± 4000 in the presence or absence of mercaptoethanol. A similar value (57,000) for the subunit of the enzyme or its carboxamidomethylated derivative was found on gel electrophoresis after incubation in 0.1 to 1.2 % sodium dodecyl sulfate in the presence of mercaptoethanol.

Culminating several years of investigation of the peptidases of bovine eye lenses (1-3), Gläscher and Hanson (4) and Hansen, Gläscher, and Kirschke (5) isolated a leucine aminopeptidase (EC 3.4.1.1) in a crystalline and highly purified form by a procedure which was elegant in its simplicity. The bovine lens leucine aminopeptidase proved to have many properties in common with the swine kidney cytoplasmic leucine aminopeptidase which had been extensively studied by Spackman, Smith, and Brown (9), Smith and Spackman (7), and Smith and Hill (8). These (5, 8, 9) include substrate specificity, pH optimum, activation by Mg2+ and Mn2+, cross-reaction with antibodies, and an apparent molecular weight of about 326,000. Because of these similarities we were interested in determining whether the crystalline enzyme isolated from the lens would exhibit an action on insulin similar to that reported by Hill and Smith (10) and Smith, Hill, and Borman (11) using a variety of techniques including gel chromatography in sodium dodecyl sulfate (15), electron microscopy (16), and sedimentation velocity and diffusion studies in sodium dodecyl sulfate and urea (17), have concluded that the molecule is made up of 10 subunits with molecular weights of 32,600. In contrast, the studies reported here which were performed on the CAM-derivative as well as the crystalline enzyme and which involved sedimentation velocity and equilibrium studies in a variety of denaturing solvents and gel electrophoresis in sodium dodecyl sulfate are all consistent with a subunit molecular weight of about 54,000, indicating a hexameric structure for the native enzyme. Weber and Osborn (18), using a sample of the crystalline enzyme prepared in this laboratory, found a subunit size of 53,000 for leucine aminopeptidase in their studies on the subunit size of 40 proteins by gel electrophoresis in sodium dodecyl sulfate.

EXPERIMENTAL PROCEDURE

Materials

Leucine aminopeptidase was isolated in crystalline form from bovine lenses (12) according to the procedure of Hanson et al. (5). The crystalline enzyme was stored in the ammonium sulfate-containing mother liquor at 4°C.

CAM-leucine aminopeptidase was prepared from the crystalline enzyme (40 mg) to which was added 6 ml of 0.1 M Tris at pH 8.0, 0.1 ml of 1 M EDTA, and 3.60 g of urea. Under a variety of conditions we found both zinc insulin and zinc-free insulin to be largely resistant to attack by the crystalline leucine aminopeptidase from bovine lenses (12). Either the two enzymes exhibit different specificities towards proteins or the results obtained with the less pure swine kidney enzyme preparation used by Smith, Hill, and Borman (11) must be attributed to a contaminant. The latter explanation seems the most probable in view of the conflicting results obtained by Hill and Smith (10) in 1958 as compared with those of Frater, Light, and Smith (14) in 1965 on the action of kidney leucine aminopeptidase on mercuripapain.

Having a supply of the crystalline leucine aminopeptidase at hand as well as experience in its isolation from bovine lenses, we decided to investigate some of its chemical, enzymatic, and physical properties. In view of the large molecular weight (326,000) reported for the lens enzyme, it might be expected to be composed of subunits. Indeed, Kretschmer (15, 16) and Kretschmer and Hanson (17), using a variety of techniques including gel chromatography in sodium dodecyl sulfate (15), electron microscopy (16), and sedimentation velocity and diffusion studies in sodium dodecyl sulfate and urea (17), have concluded that the molecule is made up of 10 subunits with molecular weights of 32,600. In contrast, the studies reported here which were performed on the CAM-derivative as well as the crystalline enzyme and which involved sedimentation velocity and equilibrium studies in a variety of denaturing solvents and gel electrophoresis in sodium dodecyl sulfate are all consistent with a subunit molecular weight of about 54,000, indicating a hexameric structure for the native enzyme. Weber and Osborn (18), using a sample of the crystalline enzyme prepared in this laboratory, found a subunit size of 53,000 for leucine aminopeptidase in their studies on the subunit size of 40 proteins by gel electrophoresis in sodium dodecyl sulfate.

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against 0.01 M sodium carbonate buffer (pH 9.5) at 10,589 rpm and 13°. B, concentration dependence of the molecular weight (MW) of leucine aminopeptidase at pH 9.5.

FIG. 1. A, equilibrium centrifugation of native leucine aminopeptidase (0.25 mg per ml) according to the method of Yphantis (20) in 0.01 M sodium carbonate buffer (pH 9.5) at 10,589 rpm and 13°. B, concentration dependence of the molecular weight (MW) of leucine aminopeptidase at pH 9.5.

Methods

Activation during Purification of Enzyme—This was performed by heating for 3 hours at 40° in 1 mM manganese dichloride, 20 mM Tris at pH 9.0. For most other experiments the enzyme was activated with 10 mM magnesium sulfate in 20 mM Tris at pH 9.0.

Assay—These were performed by following the decrease in absorbation in the ultraviolet upon hydrolysis of the amide bond of \( L \)-leucinamide. A Cary model 15 recording spectrophotometer was used to follow with time the decrease in absorbation at either 238 nm (0.05 M substrate) or 225 nm (0.025 M substrate).

Sedimentation Velocity and Equilibrium Experiments—These were performed in a Spinco model E analytical ultracentrifuge equipped with a phase plate schlieren diaphragm, a Rayleigh interference optical system, and a rotor temperature indicator control unit. Additional measurements were made on a centrifuge with ultraviolet absorption optics and a photoelectric scanner recording system. Enzyme samples were dialyzed for 16 hours or more before analysis against at least 1000 volumes of the appropriate buffer.

For samples of protein concentration \( \geq 2 \) mg per ml, sedimentation coefficients were determined at the noted temperatures and rotor speeds with the schlieren optical system, using single or double sector cells. Refractive index gradients were recorded on Kodak metallographic plates and measured with a Nikon model 6C microcomparator with rotational stage. Sedimentation coefficients were calculated from the rate of movement of the maximum of the gradient and converted to values of \( s_{20,w} \) with solvent viscosities and densities from the International Critical Tables of Kawahara and Tanford (19). Samples \(< 2 \) mg per ml were analyzed with scanner optics; the movement of protein was calculated from the changes in position of the half-concentration level of the integral curve.

In velocity experiments with \( \geq 2 \) species present, each species was identified by its sedimentation coefficient; relative proportions were determined by photographing the images and tracing the peak areas with a planimeter. Areas were corrected for radial dilution, but not for Johnston-Ogston effects.

Molecular Weights—These were determined by the meniscus depletion method of Yphantis (20). Enzyme samples at three concentrations (0.1 to 0.8 mg per ml) were sedimented at appropriate rotor speeds in a 12-mm, 6-channel Kel-F Yphantis cell. Concentration gradients were recorded on Kodak type II-G spectroscopic plates through Rayleigh interference optics. After overnight centrifugation pictures were taken at 1- to 2-hour intervals to check for the attainment of equilibrium.

The partial specific volume for native leucine aminopeptidase was calculated to be 0.74 from the amino acid composition (12) according to the method of Cohn and Edsall (21), except that a value of 0.63 cc was used for cystine (22). In high concentrations of guanidine and urea the partial specific volume was assumed to decrease 1 to 2% (23).

Polyacrylamide Gels—These were prepared according to the method of Davis (24). Runs were made at a constant current of 5 to 7 ma per tube until the marker dye was within 0.5 cm of the gel bottom. Protein bands were visualized by staining with aniline blue-black or Coomassie blue. Leucine aminopeptidase activity was revealed by incubation of the gels with \( L \)-leucyl-L-naphthylamide (25).

Electrophoresis—In the presence of sodium dodecyl sulfate, electrophoresis was performed on 10% gels according to the method of Shapiro, Vinuela, and Maizel (26). Samples were previously incubated for 3 hours at 37° with 0.5% sodium dodecyl sulfate \( \pm 1\% \) mercaptoethanol in pH 7.1 sodium phosphate.

RESULTS

Homogeneity of Leucine Aminopeptidase—Homogeneity was indicated by the results of sedimentation velocity studies in which only a single peak was detected which exhibited an \( s_{20,0.0} \) of 12.9 S at infinite dilution (12). Determination of the molecular weight by the Yphantis procedure (20) gave values which were dependent on protein concentration. Extrapolation of the values to infinite dilution gave a molecular weight of 327,000 \( \pm \) 10,000 (Fig. 1). Essentially the same values were obtained in 10 mM magnesium sulfate or in 10 mM EDTA. In disc gel electrophoresis (24) most preparations exhibited
only one protein band (12). Occasional preparations exhibited a second, very faint, slower moving band. In these cases both bands were stained in the chromogenic assay of Felgenhauer and Glenner (25) using L-leucyl-β-naphthylamide as the substrate (12).

Stability to pH as well as Activatability of Leucine Aminopeptidase—This is shown in Fig. 2. The pH optimum for activation with Mg2+ occurred at about pH 9.0 (Fig. 2, Curve b). The enzyme was relatively stable between pH values 6 to 11 and also largely retained its ability to be activated by Mg2+ (at pH 8.5) over the same range. Below pH 5 and above pH 11 the enzyme was rapidly inactivated (Fig. 2, Curve a) and also lost its ability to be activated by Mg2+ at pH 8.5 (Fig. 2, Curve c). If the enzyme was first activated at pH 8.5 with Mg2+ and then exposed to various pH values, the stability was about the same (Fig. 2, Curve d) as that of the unactivated enzyme. These results are of interest to the present studies in that they indicate an essentially irreversible denaturation of the enzyme at pH values below 5.

Effect of Urea on Enzymatic Activity of Magnesium-activated Leucine Aminopeptidase at pH 9—This is shown in Fig. 3. There is an initial drop of about 50%, in the apparent activity in the first few hours of exposure to 7 M urea and then a very gradual decrease in activity in the ensuing days. Sedimentation velocity studies were performed on a solution of leucine aminopeptidase at pH 9.4 (5 mg per ml) immediately after the addition of solid urea to give a concentration of 7 M. The enzyme contained in the urea solution sedimented somewhat slower ($s_{20,w} = 9.8$ S) than the control enzyme (no urea) and exhibited a raised baseline in the area behind the main peak. The latter can be attributed either to a small amount of dissociation into subunits or to base line discrepancies brought about by the lack of equilibration of the enzyme solution and the buffer blank, or to both. After removal of the urea by dialysis, the urea-treated and control enzyme solutions exhibited the same sedimentation behavior with an $s_{20,w}$ value of 12.1 S (12). Also, although the enzymatic activity of the enzyme contained in the 7 M urea solution was about 40% of the control, after removal of the urea by dialysis, the solution exhibited the full activity of the untreated control. These results indicated that at pH values around 9, the enzyme was largely resistant to dissociation in 7 M urea, and further, that the short term effects of urea on the activity of the enzyme were reversible. However, if the enzyme were subjected to prolonged dialysis with 7 M urea at pH 9.4, a small amount of slower moving component was found on centrifugation. Repeating this procedure at decreasing pH values increased the amount of slow moving material until at pH values below 3 only the slow moving component was present. At intermediate pH values (about 6.0), three peaks could be discerned on centrifugation in 7 M urea (12). The sedimentation coefficient of the slow moving material was dependent on concentration and ionic strength. In 0.04 M glycine buffer (pH 2.7), which was 0.25 M in sodium chloride, the $s_{20,w}$ values extrapolated to 2.06 S at infinite dilution. Molecular weight determination on the subunit in 7 M urea and glycine buffer (0.01 M) at pH 2.7 by the equilibrium method of Yphantis (20) gave a value of 55,000 ± 4000 (Fig. 4A). The addition of 0.1 M mercaptoethanol to the solution had no effect on the molecular weight (Table I).

Sedimentation of Leucine Aminopeptidase—Sedimentation in guanidinium chloride solutions ($>3.7$ M) over the pH range from 2.5 to 8.5 revealed only a single peak with an $s_{20,w}$ of 19. Molecular weight determinations in the presence of 0.1 M mercaptoethanol over a pH range of 2.5 to 8.5 gave an average value of 53,000 (±4000) (Fig. 4B, Table I). The enzyme was inactive in guanidinium chloride solutions ($>3.7$ M).

Gel Electrophoresis—This was performed in the presence of sodium dodecyl sulfate (26) on enzyme which had been incubated...
FIG. 4. Equilibrium centrifugation of leucine aminopeptidase (0.4 mg per ml) at 24,630 rpm and 20° in (A) 7 M urea, 0.01 M glycine (pH 2.7); (B) 3.7 M guanidinium chloride, 0.01 M glycine (pH 2.7), 0.10 M β-mercaptoethanol. MW, molecular weight.

FIG. 5. Gel electrophoresis in sodium dodecyl sulfate of leucine aminopeptidase (LAP), CAM-leucine aminopeptidase (CAM-LAP), and various marker proteins (BSA refers to bovine serum albumin) after incubation in 0.5% sodium dodecyl sulfate, pH 7.1 phosphate buffer for 3 hours. Electrophoresis performed on 10% acrylamide gels, 0.5% sodium dodecyl sulfate and pH 7.1 phosphate buffer.

Table I
Bovine lens leucine aminopeptidase subunit molecular weight

<table>
<thead>
<tr>
<th>Conditions</th>
<th>pH</th>
<th>Mol wt X 10^9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>9.5</td>
<td>327 ± 10^6</td>
</tr>
<tr>
<td>7 M urea</td>
<td>2.7</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>7 M urea, 0.1 M β-mercaptoethanol</td>
<td>2.7</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>3.7 M guanidine hydrochloride, 0.1 M β-mercaptoethanol</td>
<td>8.5</td>
<td>48 ± 5^b</td>
</tr>
<tr>
<td>3.7 M guanidine hydrochloride</td>
<td>2.7</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>3.7 M guanidine hydrochloride</td>
<td>4.2</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>3.7 M guanidine hydrochloride, 0.1 M β-mercaptoethanol</td>
<td>2.7</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>3.7 M guanidine hydrochloride, 0.1 M β-mercaptoethanol</td>
<td>8.0</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>4.85 M guanidine hydrochloride, 0.1 M β-mercaptoethanol</td>
<td>8.0</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>6.0 M guanidine hydrochloride, 0.1 M β-mercaptoethanol</td>
<td>8.0</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>6.0 M guanidine hydrochloride</td>
<td>2.3</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>0.5% sodium dodecyl sulfate ± 1% β-mercaptoethanol</td>
<td>7.1</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>0.5% sodium dodecyl sulfate ± 1% β-mercaptoethanol</td>
<td>(115 ± 12)</td>
<td>58 ± 4</td>
</tr>
</tbody>
</table>

- a The ± values refer to the extremes from the average encountered in at least three and generally six determinations.
- b Determined by the Archibald method (27).
- c CAM-leucine aminopeptidase.

for 3 hours at 37° in the presence of sodium dodecyl sulfate and mercaptoethanol. Most of the material moved in a band with relative mobility corresponding to a molecular weight of 57,000. Two minor bands with relative mobilities of 115,000 (±12,000) and 144,000 (±15,000) were detected (Fig. 5). The amount of material in these minor bands was increased when mercaptoethanol was omitted from the incubation buffer. Variation of the sodium dodecyl sulfate concentration from 0.1 to 1.2% had little effect on the electrophoresis pattern, although staining of the protein was weaker in the higher concentrations of sodium dodecyl sulfate. Reduced and carboxamidomethylated leucine aminopeptidase migrated in the gel electrophoresis in sodium dodecyl sulfate essentially as one component with a mobility corresponding to a molecular weight of 58,000 ± 4000 (Fig. 5). The electrophoresis pattern was not affected by the presence or absence of mercaptoethanol (Table I).

TABLE I
Sedimentation of Reduced and Carboxamidomethylated Leucine Aminopeptidase—Sedimentation, carried out at pH 9.4 in the presence or absence of 3 M urea, revealed that most of the material (85%) retained the sedimentation coefficient of untreated leucine aminopeptidase. A minor portion was dissociated to 2 S subunits. In 6 M guanidinium chloride at pH 2.3, the CAM-leucine aminopeptidase was completely dissociated to 1.7 S units. A molecular weight determination under these conditions yielded a value of 53,000 ± 3000 (Table I).

DISCUSSION
The results of the ability of magnesium to activate the enzyme as a function of pH (Fig. 2) are similar to those previously reported by Hanson et al. (5) for the lens enzyme and by Smith and Spackman (7) for the hog kidney enzyme. In addition our results show that the pH stability of the magnesium-activated enzyme and the unactivated enzyme are about the same: both are fairly stable between pH 6 to 11 but are rapidly inactivated outside this range. Further, the inactivation is not reversed by incubation with Mg²⁺ near the pH optimum for metal ion activation.

In confirmation of the work of Frohne and Hanson (28), our studies performed with urea demonstrate that the enzyme is
obtained. Although there is a rapid drop of about 50% in the specific activity upon dissolving the enzyme in 7 M urea, the enzyme still exhibits considerable activity and this activity is only slowly lost during several days' incubation in urea. When the urea concentration was diminished from 7 M to 0.41 M by dilution followed by immediate assay of the diluted solution, there was a partial regain of activity. However, if the urea was removed slowly by dialysis, full activity was regained. The facts that centrifugation revealed no immediate dissociation of the enzyme in 7 M urea at pH 9 and that full activity was regained upon removal of the urea by dialysis indicate that the decreased specific activity exhibited in 7 M urea is due to a subtle change in the quaternary or tertiary structure of the enzyme.

Dissociation of the enzyme into subunits occurred upon prolonged incubation in 7 M urea at pH 9. The rate of dissociation was increased by lowering the pH, being practically instantaneous at pH values below 3. At low pH, a subunit size of about 54,000 was found by sedimentation equilibrium studies in 7 M urea. Similar values were obtained by sedimentation equilibrium studies in 3.7 M guanidinium chloride at pH values ≤ 8.5. The addition of mercaptoethanol had no effect on the values obtained.

Gel electrophoresis in sodium dodecyl sulfate yielded a subunit size of about 58,000 for the smallest component. In these experiments there was a small amount of higher molecular weight material (115,000) which probably represents dimers owing to disulfide bond formation during electrophoresis (26). The high molecular weight material was absent in experiments using CAM-leucine aminopeptidase.

The above data indicate that the subunit size is about 54,000 and that the native enzyme is a hexamer. This is in contrast to the work of Kretschmer (15, 16) and Kretschmer and Hanson (17) who have proposed a subunit size of 32,800 to 40,500 and a decameric structure for the native enzyme. Of course it is possible that we did not obtain complete dissociation of the molecule under our conditions and that the subunits of 54,000 are composed of two or more smaller units. Although this appears unlikely in view of the severity of treatment to which the enzyme was subjected, even if true it would be difficult to rationalize our results with the earlier work. For example, it would mean that the 54,000 unit was composed of subunits of unequal size (34,000 and 20,000) and that the native enzyme is a dodecamer rather than the decamer proposed from the electron microscope studies (16).

REFERENCES

Leucine Aminopeptidase (Bovine Lens): STABILITY AND SIZE OF SUBUNITS
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