Lecine Aminopeptidase (Bovine Lens)

MECHANISM OF ACTIVATION BY Mg^{2+} AND Mn^{2+} OF THE ZINC METALLOENZYME, AMINO ACID COMPOSITION, AND SULFHYDRYL CONTENT*

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SUMMARY

Spark emission and atomic absorption spectroscopy of crystalline leucine aminopeptidase (bovine lens) (EC 3.4.1.1) shows the presence of 2 zinc atoms per subunit molecular weight of 54,000 (12 zinc atoms per oligomer of 320,000). Removal of zinc by dialysis yields a zinc-free product with no enzymatic activity which upon readdition of Zn^{2+}, regains full activity with the concomitant binding of 2 zinc atoms per subunit. Addition of Cu^{2+}, Ca^{2+}, Mg^{2+}, or Mn^{2+} to the zinc-free enzyme does not result in stoichiometric binding of these ions or in significant regain in activity. Incubation of the enzyme containing 2 zinc atoms with various concentrations of Zn^{2+} and Mg^{2+} or Zn^{2+} and Mn^{2+} shows (a) that there is a competition between the ions for one binding site, (b) that the ratio of dissociation constants for this site is $K_{Mg}:K_{Zn} = 150$ at pH 9.5 and $K_{Mn}:K_{Zn} = 35$ at pH 8.5, and (c) that a maximum of 1 mole of Mg^{2+} or Mn^{2+} is bound per 54,000 g of leucine aminopeptidase. The results suggest that leucine aminopeptidase has two metal binding sites: a specificity site that must be occupied by zinc in order to have an active enzyme and an activation site which can be occupied by Zn^{2+}, Mg^{2+}, or Mn^{2+} to give zinc-zinc, zinc-magnesium, or zinc-manganese enzymes with different specific activities. The nature of the ion occupying the activation site has a pronounced effect on the maximum velocity of the reaction and a minor effect on the Michaelis constant.

A new amino acid analysis of the lens leucine aminopeptidase is presented which accounts for all of the total nitrogen and dry weight of the enzyme in terms of amino acid residues and which indicates that the enzyme contains eight histidines per 54,000-dalton subunit. Six of these are in the sulfhydryl form, but only one of these is available for reaction in the native enzyme. This reactive sulfhydryl is not needed for enzymatic activity. On a dry weight basis the absorbance of a 1% solution of leucine aminopeptidase at neutral pH in a 1-cm cuvette at 280 nm is 10. The partial specific volume as calculated from the amino acid composition is 0.74.

The activation of leucine aminopeptidase (EC 3.4.1.1) with certain metal ions has been known since 1936 (2) and has been the subject of a number of investigations (3–5) including one by Smith (6) who in 1946 presented evidence that binding of the metal ion to the protein was responsible for the activation by Mg^{2+} and Mn^{2+}. Recently, and during the course of this investigation, Himmelhoch (7), working with hog kidney enzyme, and Ketthann and Hanson (8), working with the lens enzyme, reported the presence of zinc in leucine aminopeptidase. The fact that the zinc could be replaced by dialysis against Cd^{2+} to give an inactive enzyme which in turn could be reactivated by dialysis against Zn^{2+} strongly suggested that Zn^{2+} was an essential component of the enzyme (7).

The present report is a continuation of the characterization of the crystalline leucine aminopeptidase isolated from bovine lens tissue by the procedure of Hanson et al. (9). In contrast with previous studies (10–12) which indicated that the lens enzyme was a decamer with a subunit size of 32,000 daltons, our results have indicated that it is a hexamer with a subunit size of 54,000 daltons (13, 14). The present report is concerned with the number of zinc binding sites per subunit, the mechanism of activation by metal ions, the amino acid composition, the number and reactivity of the sulfhydryl groups, and certain physical properties of the enzyme.

EXPERIMENTAL PROCEDURE

Materials

L-Leucinamide-HCl was obtained from Mann Research Laboratories, L-leucine from Nutritional Biochemicals Corp., DTNB and 2-thioethanol from Aldrich Chemical Co., Tris-HCl from Sigma Chemicals, and sodium dodecyl sulfate and 1,10-phenanthroline from Eastman Organic Chemicals. Guanidine hydrochloride (Matheson, Coleman and Bell) was treated with charcoal in boiling methanol, the hot mixture was filtered, and the product was crystallized by cooling the solution. Iodoacetamide (K and K Laboratories) was recrystallized just before use from ethyl acetate. MgSO_4, MnSO_4, ZnO, CuO, and CaCO_3 were obtained as high purity metal salts produced by Johnson, Matthey and Co., Ltd., for spectroscopic standards. Nitrogen gas was the prepurified grade from Matheson Co. Dialysis tubing (9/4 inch) was heated for 30 min at 80° in a solution that was 0.01 M both in EDTA and KHCO_3 and then washed well.

* The abbreviation used is: DTNB, 5,5′dithiobis(2-nitrobenzoic acid).
with deionized water. This step was repeated, and finally the tubing was heated at 80° in deionized water, washed in deionized water, and stored in water at 4°. All glassware and plasticware used in metal ion experiments was cleaned according to the method of Thiers (15).

Crystalline leucine aminopeptidase was prepared from bovine lenses according to the procedure of Hanson et al. (9, 16).

Methods

Amino acid analyses were performed on a Beckman-Spinco model 120B instrument equipped with a digital integrator (Infotronics Corp.), long path length cuvettes (12-mm), and an expanded range card at a buffer flow rate of 70 ml per hour at 53° (17, 18). Determinations of pH were made on a Radiometer PHM 40 pH meter. Measurements of ultraviolet absorbance were conducted on a Cary model 15 recording spectrophotometer. Whenever maintenance of pH was required, a Radiometer type TTTc pH-stat was used. Kjeldahl and Dumas nitrogen determinations were performed by the Microchemical Laboratory of the Chemistry Department at the University of California, Berkeley. Spectroscopic analyses by spark emission were performed by Mr. George Shalimoff of the Lawrence Radiation Laboratory at Berkeley. Atomic absorption analyses were performed on a Perkin-Elmer model 303 instrument, equipped with a recorder read-out, using a three-slot burner head, and an air-acetylene flame.

Determination of Amino Acid Composition, Molar Extinction Coefficient, and Nitrogen Content

A solution containing approximately 17 mg per ml of leucine aminopeptidase was prepared by dissolving the crystals which had been centrifuged out of the ammonium sulfate mother liquor from the crystallization in 0.1 M Tris at pH 8.0. The solution was clarified by centrifugation, followed by filtration through a 0.45 µ Millipore filter. An aliquot (3 ml) was dialyzed successively at 4° against 3 liters of potassium phosphate buffers at pH 8.0 of the following composition for the times indicated: 0.01 M (16 hours), 0.001 M (24 hours), 0.001 M (48 hours). The contents of the dialysis sacs were diluted to 5 ml with the final buffer to give a solution containing approximately 10 mg per ml. Aliquots of this solution as well as equal aliquots of the final dialyze (as blanks) were subject to the following analyses. Total nitrogen content was determined by the Kjeldahl method. Total zinc was determined by atomic absorption spectroscopy. The ultraviolet absorption spectrum from 240 to 400 nm was measured on a 1:20 dilution in 0.05 M potassium phosphate at pH 7.0. Dry weight determinations were performed on 1.00-ml aliquots contained in thin glass shells from which the solvent was removed over P2O5 at room temperature, followed by 4 hours drying over P2O5 in a vacuum drying pistol at 100°. After cooling to room temperature, the shells were placed on one pan of a double pan microbalance in which the other pan contained an appropriate tare vessel and the weight was recorded as a function of time. Linear extrapolation to the time of opening the pistol gave the measured weight. The dry weight of the protein was calculated as the difference in the zero time weights of the enzyme and buffer samples. Tryptophan was determined from the tyrosine to tryptophan ratio as proposed by Bencze and Schmid (19) by making a 1:13 dilution in 0.1 N NaOH and recording the spectrum from 260 to 360 nm. Amino acid analyses were conducted on 0.1-ml aliquots which were hydrolyzed in 6 N HCl in evacuated, sealed tubes at 120° for 6, 11, and 24 hours. Unless otherwise indicated the results given in Table I were calculated from the average of the 6-, 11-, and 24-hour hydrolysates.

Several analyses were performed on secondary solutions where the concentration of enzyme was determined from the alanine content. Tyrosine was measured in amino acid hydrolysates performed in 6 N HCl in the presence of 0.1% phenol at 120° for 6 hours (20). Half-cystine was determined as cysteic acid by the method of Moore (21) as reported by Blackburn (22). The performic acid-oxidized protein was hydrolyzed in 6 N HCl in evacuated tubes at 110° for 18 hours. The recovery of cysteic acid was assumed to be 94% (22). Carboxamidomethylated-leucine aminopeptidase was prepared from the reduced enzyme as described by Melbye and Carpenter (13). The carboxymethylcysteine was determined in hydrolysates of the protein conducted in 6 N HCl in evacuated tubes for 6 hours at 120°.

Determination of Sulfhydryl Groups

By DTNB—Solutions were prepared as so to contain: 0.01 M EDTA; 0.1 M Tris at pH 8.0; either 5 M guanidinium chloride or 0.5% sodium dodecyl sulfate or no denaturing agent; and as the last addition, 0.54 mg per ml of leucine aminopeptidase. Immediately upon the addition of the enzyme, 0.75-ml aliquots were treated with 0.025 ml of 0.01 M DTNB in 0.05 M potassium phosphate at pH 7.0, and the absorbance at 412 nm was recorded with time against an appropriate blank containing everything except the enzyme. After the development of the maximum color, the downward sloping line was extrapolated back to zero time to determine the concentration of the thiol using the molar extinction coefficient of 1.36 × 104 M-1 cm-1 (23).

By Iodoacetamide—In the dark at room temperature, solutions of leucine aminopeptidase at 1 mg per ml in 0.01 M EDTA, 0.1 M Tris at pH 8.0, and either 5 M guanidinium chloride or 0.5% sodium dodecyl sulfate or no denaturing agent were treated with iodoacetamide at 0.05 M. At various times 0.5-ml aliquots were mixed with 2 ml of 10% trichloroacetic acid. The precipitated protein was collected by centrifugation, washed successively with 10% trichloroacetic acid, ethanol, and ether, and dried. The dry protein was hydrolyzed in 6 N HCl in evacuated, sealed tubes at 120° for 6 hours. The carboxymethylcysteine in the hydrolysates was determined by amino acid analysis and related to the alanine content of the sample. In the experiment where no denaturating agent was present, 0.05-ml aliquots were removed at various times and subjected to activity determinations by the spectrophotometric procedure.

Leucine Aminopeptidase Activity on Leucinamide

Spectrophotometric Assay—In the standard assay the change in absorbance at 238 nm (24) upon the hydrolysis of leucinamide (0.025 µ) in 0.1 M borate at pH 9.25 and 30° was followed with time. The concentration of the enzyme in the assay solution depended on the form of the enzyme and varied from about 10 µg per ml for the zinc-zinc enzyme to 1 µg per ml for the zinc-manganese enzyme. Enzyme concentration was calculated from the molar extinction coefficient at 280 nm of 5.4 × 104 M-1 cm-1 for the monomer with molecular weight of 54,000. The activity is expressed as ΔA238 per 100 s per mg or as micromoles per min per mg. The latter was calculated from the difference in the molar extinction coefficient between leucinamide and leucine at 238 nm in the borate buffer of 14.3 cm-1 µmol per ml for assays performed in a 1-cm cuvette containing 2.5 ml of solution yields the relationship: (ΔA238 per 100 s per mg) × 105 equals micromoles per min per mg. Similar conditions were
used for the determination of the Michaelis-Menten parameters except that the leucinamide concentration was varied from 0.004 to 0.02 M.

Amino Acid Assay—At four to six time intervals after the addition of enzyme, 0.05-ml aliquots of a reaction mixture composed of 0.025 M leucinamide in 0.1 M borate at pH 9.25 and 30° were removed and added to 2.0 ml of 0.2 M citrate buffer at pH 3.0. These solutions were analyzed for their content of free leucine on the column, 9 × 50 mm, of the amino acid analyzer which had been equipped with a sample injector valve (Chromatotronix Corp.). The column was equilibrated and developed with the standard second buffer (0.2 M citrate, pH 4.25) at 70 ml per hour and 58°. Samples were injected at 10 min intervals with the first peak of leucine emerging at about 53 min. With repeated injections there was a retardation of the leucine peak because of the passage of the acidic sample buffer. Up to 12 samples containing about 0.1 amole of leucine (and an equivalent amount of ammonia) could be injected without regeneration of the column. The specific activity was calculated from the rate of production of leucine, expressed as micromoles per min per mg.

Removal of Unbound Metals from Leucine Aminopeptidase Solutions

Unbound Zn²⁺, Mg²⁺, and Mn²⁺ were removed from leucine aminopeptidase solutions by passing the solutions through Beckman type 50A resin. Before use, the resin was washed with 10% KOH, deionized water, 4 N HCl, and deionized water. The resin was placed in a column and small portions of 1 ml of the desired composition and pH were passed through the resin until the effluent had the same pH as the starting buffer. Then the resin was washed with 0.01 M buffer (either Tris at pH 8.0 or 8.5 or potassium carbonate at pH 9.5) until the effluent had the same conductivity as the starting buffer. The resin was stored as a 50% suspension in the 0.01 M buffer.

For removal of unbound divalent metals, 3 ml of the resin suspension were placed on a 3-ml coarse sintered glass funnel, and suction was applied to drain the buffer to the top of the resin. A 0.5- to 1-ml aliquot of the metal-containing sample was then passed through the column, followed by several washes with 0.5 ml of the 0.01 M buffer. Controls were run with buffers containing the highest concentration of divalent ions to make sure of their removal by the column.

Removal and Readdition of Zinc to Leucine Aminopeptidase

All dialyses were conducted at room temperature under nitrogen in solutions that had been purged with nitrogen for several hours before use. Leucine aminopeptidase (8.0 mg in 1.2 ml of starting buffer) was placed in a dialysis sac and dialyzed first for 6 hours against 1 liter of buffer composed of 2 mM 1,10-phenanthroline, 0.115 M NaCl, 0.1 M L-leucine, and 0.25 M Tris, all at pH 8.0, and then twice for 2 hours each against 1 liter of buffer composed of 0.115 M NaCl, 0.1 M L-leucine, and 0.25 M Tris at pH 8.0 to which 0.5 ml of 2-thiotoehanol was added just before use. The dialysis sac was opened and the contents were diluted to 2 ml with the final dialysate. The solution was divided into two 1-ml portions. To one portion was added 0.05 ml of 0.1 M ZnCl₂ (or other metal salt), followed by dialysis for two 2-hour periods at room temperature against 1-liter portions of 0.01 M ZnCl₂ (or other metal salts) in 0.01 M Tris at pH 8.0. The other portion, without metal ion addition, was similarly dialyzed against 0.01 M Tris at pH 8.0. The resulting solutions were analyzed for enzymatic activity and for bound metals after removal of unbound metal ions by the ion exchange column procedure.

Competition between Mg²⁺ and Zn²⁺ at pH 8.5

Solutions of leucine aminopeptidase were incubated for 2 hours at 37° in 0.01 M potassium carbonate at pH 9.5 under three conditions. (a) No added Zn²⁺ and variable Mg²⁺. Enzyme at 0.81 mg per ml (with no added Zn²⁺) was incubated with various concentrations of Mg²⁺ from 0.5 to 10 mM. (b) Constant added Zn²⁺ and variable Mg²⁺. Enzyme at 0.81 mg per ml was incubated in the presence of 0.04 mM added Zn²⁺ and various concentrations of Mg²⁺ from 0.5 to 10 mM. (c) Constant Mg²⁺ and variable Zn²⁺. Enzyme at 0.54 mg per ml (or 0.108 mg per ml in order to lower the concentration of total Zn²⁺) was incubated in 10 mM Mg²⁺ and the total concentration of Zn²⁺ was varied from 0.002 to 0.15 mM. Unbound metal ions were removed from 1-ml aliquots of the incubation mixtures by the ion exchange procedure in which the columns had been equilibrated with 0.01 M potassium carbonate buffer at pH 9.5. The eluates were immediately analyzed for enzyme activity by the spectrophotometric method, and later for protein concentration by absorbance at 280 nm and for divalent metal ions by atomic absorption spectroscopy.

Competition between Mn²⁺ and Zn²⁺ at pH 8.5

Solutions of leucine aminopeptidase at 0.54 mg per ml were incubated for 30 min at 37° in 0.01 M Tris at pH 8.5 in the presence and absence of 0.02 mM added Zn²⁺ and with varying Mn²⁺ from 0.1 to 2 mM. Removal of unbound metals was performed on resin equilibrated with 0.01 M Tris at pH 8.5. Analyses of the eluates were conducted as for the Mg²⁺ experiments.

Treatment of Data

The following derivations, which are abstracted from a more detailed presentation (25), are based on four considerations. (a) There are two metal binding sites for Zn²⁺ per 54,000-dalton subunit of leucine aminopeptidase. This is supported by the analyses on the isolated enzyme. (b) Under the conditions in which the experiments on metal ion competition were conducted, the Zn²⁺ in one site (the Zn²⁺-specific site) does not exchange with other metals. The Zn²⁺ in the other site (the activation site) is in slow equilibrium with free Zn²⁺ and other divalent metal ions. This is supported by the observation that upon incubating with high concentrations of Mg²⁺ or Mn²⁺, the isolated enzyme contains as a maximum 1 activating metal ion (Me²⁺) and 1 Zn²⁺ per 54,000 daltons. (c) Under the experimental conditions where the concentration of the activating metal ion (Me²⁺) was relatively high as compared with the concentration of the enzyme, the affinity of the "activation site" for Me²⁺ is strong enough so that the concentration of the enzyme with no metal bound in the activation site can be ignored. In other words the total concentration of the enzyme (E₀) is equal to the sum of the concentrations of the zinc-zinc enzyme (E₀) and the zinc-metal enzyme (E₀). (d) The binding of the different metals to the activation site results in enzyme species with different specific activities. This is supported by the experimental data which indicate different specific activities for the Zn²⁺, Mg²⁺, or Mn²⁺-activated enzyme.

Combining Statements e and d in a mathematical form yields the following equation

\[ a = (m - z)E₀/E + z \]  

(1)
where \( a \) is the observed specific activity, \( m \) is the specific activity of the zinc-metal enzyme, \( E_n \) is the concentration of the zinc-metal enzyme, and \( E_t \) is the total concentration of enzyme. The ratio, \( E_m/E_t \), is experimentally determinable as the number of activation metal ions (Me\(^{2+}\)) per 54,000-dalton subunit. According to this expression there should be a straight line relationship between the observed specific activity (\( a \)) and the number of activating metal ions bound to the enzyme. The slope of the line should yield the increase in specific activity due to the activating ion (\( m - z \)) and the intercept should yield the specific activity of the zinc enzyme (\( z \)).

When leucine aminopeptidase is mixed with Zn\(^{2+}\) and other activating ions (Me\(^{2+}\)), the dissociation constants for the binding of the ions to the activation site can be expressed as

\[
K_{Zn} = E_f Z_f/E_t
\]  
(2)

and

\[
K_{Me} = E_f M_f/E_n
\]

where \( E_f \) is the concentration of free enzyme, \( Z_f \) is the concentration of free Zn\(^{2+}\), \( E_s \) is the concentration of the zinc-zinc enzyme, \( M_f \) is the concentration of free Me\(^{2+}\), and \( E_m \) is the concentration of the zinc-metal enzyme. By experimental design, the total concentration of Me\(^{2+}\) in solution can be made much larger than \( E_f \), so that \( M_f \) can be replaced with \( M_t \), the total concentration of Me\(^{2+}\) in solution. However, because of the low solubility of Zn\(^{2+}\) at pH 8.5 to 9.5, it is not possible to make a similar substitution for \( Z_f \).

Dividing the expressions in Equation 2 and substituting \( M_f \) for \( M_t \) gives the equation

\[
K_{Zn}/K_{Me} = Z_f E_m/E_n M_f E_t
\]  
(3)

Substituting the equivalent expression \((E_t - E_m)\) for \( E_f \) and rearranging yields

\[
E_m/E_t = (M_f/Z_f)/(M_f/Z_f + K_{Me}/K_{Zn}) = (a - z)/(m - z)
\]  
(4)

The last equivalent found in Equation 4 derives from the relationship found in Equation 1. Equation 4 is in the form of a rectangular hyperbola and could be used to calculate the ratio of dissociation constants \((K_{Me}/K_{Zn})\), if the experiments could be performed at a low enough enzyme concentration so that \( Z_f \) could be replaced by the total Zn\(^{2+}\) concentration (\( Z_t \)). Unfortunately the sensitivity of the atomic absorption metal assay will not allow for accurate determination of the bound activation ion at concentrations of the enzyme low enough to make this valid.

However, substituting for \( Z_f \) in Equation 4 the equivalent expression \((Z_t - E_f)\) and rearranging gives the quadratic equation

\[
(E_m)^2 + (Z_t - E_f + K^* M_t) E_m - K^* E_t M_f = 0
\]  
(5)

where \( K^* \) is equal to \( K_{Zn}/K_{Me} \). In this equation all of the terms are known or measurable except the ratio of dissociation constants \((K^*)\). The equation may be solved for \( E_m \) by means of the quadratic formula to give

\[
2E_m = E_t - Z_t - K^* M_f
\]

\[
\pm \sqrt{\left[Z_t - E_f + K^* M_t\right]^2 + 4K^* E_t M_f}\]

As the total enzyme concentration \(E_t\) approaches zero, the concentration of enzyme containing the activating metal ion \(E_m\) must also approach zero. This is true for Equation 6 only for the positive form of the radical which, therefore, is the only root which needs to be considered in this discussion.

There is a disadvantage in attempting to use Equation 6 directly to determine the ratio of dissociation constants \((K^*)\) from the experimental data. Since the total concentrations of zinc and activating metal ion (\( Z_t \) and \( M_t \)) appear as separate terms in Equation 6, rather than as a ratio as in Equation 4, and since the total concentration of enzyme \(E_t\) is also present as a separate term in Equation 6, it is difficult to compare the data obtained from different experiments. To avoid this problem a solution for \( K^* \) was derived from a reiteration process involving Equations 4 and 6.

First an estimate of \( K^* \) was obtained from a set of experimental data by using a computer program (HYPERB) (25, 26) for the solution of Equation 4. The estimated \( K^* \) value was then placed in Equations 4 and 6, and both equations were solved for the ratio \( E_m/E_t \) for each experimental set of \( E_t, M_t, \) and \( Z_t \). The \( E_m/E_t \) ratio obtained from Equation 4 was divided by the ratio obtained from Equation 6 to give a correction factor for each data point. Each of the data points of the experiment was then multiplied by its correction factor, and a new estimate of \( K^* \) was determined by using the corrected data points in the HYPERB solution for Equation 4. The process was repeated until the values for \( K^* \) remained constant within experimental error. In essence this process corrects the observed metal binding and specific activity data to what would have been observed at very low enzyme concentrations with the same experimental concentrations of zinc and activating metal ion.

**RESULTS**

Molar Extinction Coefficient and Nitrogen Content—By a combination of the data from the dry weight determinations with the ultraviolet absorption spectrum at pH 7.0, a molar extinction coefficient at 280 nm of \( 0.4 \times 10^4 \, m^{-1} \, cm^{-1} \) was calculated for the subunit of 54,000 daltons. In more useful terms a neutral solution of 1 mg per ml of leucine aminopeptidase in a 1-cm cuvette gave an absorbance of 1.00 at 280 nm, or \( E_{280}^\text{cm} = (280 \, nm) = 10.0 \). The average of two independent determinations of dry weight and nitrogen content gave a value of 16.97% nitrogen.

Metal Content—Samples of leucine aminopeptidase at about 5 mg per ml in 0.1 M Tris at pH 8.0 were dialyzed overnight at 4° against 0.001 M Tris at pH 8.0 and analyzed for metals by spark emission spectroscopy. The only metals that were detected in amounts estimated to be greater than 0.1 residue per 54,000 g of leucine aminopeptidase were zinc, magnesium, calcium, and copper. An accurate determination of these metals was made by atomic absorption spectroscopy with the proper lamp for each metal with the following results (for two preparations), expressed as gram atoms of metal per 54,000 g of leucine aminopeptidase: zinc (1.9, 2.0); magnesium (0.02); calcium (0.05, 0.2); and copper (0.03, 0.1). Samples of leucine aminopeptidase which had been exhaustively dialyzed for dry weight and amino acid analyses as described in the experimental section were also analyzed for Zn\(^{2+}\) and gave values of 1.6 and 1.5 g atoms per 54,000 g of leucine aminopeptidase.

Amino Acid Composition—The results of two independent amino acid analyses of leucine aminopeptidase are shown in Table I. The results are expressed as a percentage of total nitrogen or percentage of dry weight as determined on that particular sample. The recovery of nitrogen was 97.5 and 103% while the recovery of dry weight as amino acid residues (including zinc) was 100.5 and 100.6%. Of those amino acids which occur
### Table I

**Amino acid analysis of leucine aminopeptidase**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amino acid N as % total N</th>
<th>Amino acid res. wt. as % dry wt.</th>
<th>No. of residues per 8 histidines</th>
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<tbody>
<tr>
<td></td>
<td>(1)</td>
<td>(2)</td>
<td>(1)</td>
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<tr>
<td>Lys</td>
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<td><strong>102.98</strong></td>
<td><strong>100.51</strong></td>
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</table>

*Extrapolated to zero time and blank value subtracted.

- Extrapolated to zero time.
- Determined as cysteic acid.
- Value for 24-hour hydrolysis.
- Value for 24-hour hydrolysis plus alloisoleucine.

Determination of carboxymethylcysteine in carboxamidomethylated protein yielded 7.56 residues per 54,000 daltons. The methionine sulfone content of the performic acid-oxidized protein gave 10.8 and 10.6 residues per subunit as compared to values of 11.0 and 10.8 obtained from direct measurement of methionine (Table I). Tryptophan was determined by the method of Beneze and Schmid (19) which measures the tyrosine to tryptophan ratio by the absorption of the protein in alkaline solution. Leucine aminopeptidase dissolved in 0.5 N NaOH with or without added 0.5% sodium dodecyl sulfate yielded tyrosine to tryptophan ratios from 1.01 to 1.05.

The partial specific volume of leucine aminopeptidase was calculated from the amino acid composition by the method of Cohn and Edsall (27) to be 0.74.

in low frequency in leucine aminopeptidase (histidine, half-cystine, tyrosine, and tryptophan), histidine was selected as the one least subject to error upon analysis and was used for the calculation of the amino acid ratios. The studies of Melbye and Carpenter (13) as well as the metal-binding experiments reported below indicate a subunit molecular weight of about 54,000. The use of 8.00 residues of histidine as a reference amino acid yielded a molecular weight, near 54,000 as calculated from the amino acid composition (Table I) and also gave reasonably good whole number ratios for the amino acids occurring with low frequency. A total of 531 amino acid residues was calculated per subunit.

The half-cystine values (8.0 and 7.9) reported in Table I were determined as cysteic acid in the performic acid-oxidized protein. Determination of carboxymethylcysteine in carboxamidomethylated protein yield 7.56 residues per 54,000 daltons. The methionine sulfone content of the performic acid-oxidized protein gave 10.8 and 10.6 residues per subunit as compared to values of 11.0 and 10.8 obtained from direct measurement of methionine (Table I). Tryptophan was determined by the method of Beneze and Schmid (19) which measures the tyrosine to tryptophan ratio by the absorption of the protein in alkaline solution. Leucine aminopeptidase dissolved in 0.5 N NaOH with or without added 0.5% sodium dodecyl sulfate yielded tyrosine to tryptophan ratios from 1.01 to 1.05.

The partial specific volume of leucine aminopeptidase was calculated from the amino acid composition by the method of Cohn and Edsall (27) to be 0.74.
Sulfhydryl Groups—The results of the determination of sulfhydryl groups by reaction with DTNB and by reaction with iodoacetamide in the presence and in the absence of denaturing agents are shown in Table II. Both methods indicate that of the 8 half-cystine residues per leucine aminopeptidase subunit, 6 are present as cysteine and presumably the other two are present as cystine, suggesting the presence of one disulfide bond per subunit. In the absence of denaturing agents, only 1 of the cysteine residues reacts readily with either DTNB or iodoacetamide. As shown in Fig. I, the reactive sulfhydryl is not needed for enzymatic activity.

Removal and Readdition of Zn²⁺—Conditions for the removal and readdition of Zn²⁺ to leucine aminopeptidase, as described in the experimental part with the results shown in Table III, were developed from many empirical experiments. Several factors deserve comment. The rate of removal of Zn²⁺ by dialysis even in the presence of 1,10-phenanthroline was extremely slow at 4°C and was greatly increased at room temperature, especially in the presence of added NaCl. The recovery of enzymatic activity upon readdition of Zn²⁺ to the low metal enzyme was enhanced if all of the dialyzed enzymes were conducted in the presence of leucine and in an inert atmosphere. The presence of 2-thioethanol in the final dialysis buffers increased the recovery of activity. These results suggest that some of the sulfhydryl groups of the enzyme may be exposed and subject to oxidation upon the removal of zinc. They may even be involved in the Zn²⁺ binding. Another important factor in these and subsequent experiments on metal ion binding was the ability to remove unbound metal ions from solution so that the kind and amount of bound ions could be determined by atomic absorption. The success of the ion exchange procedure for the removal of unbound ions without at the same time removing the bound ions probably rests on two factors: the short duration of time in which the enzyme solution was in contact with the resin and the relatively slow rate at which the metal ions exchange with the protein. The slowness of this rate is indicated by the periods of incubation at 37°C of up to 30 min or longer which are needed in order to reach constant activity during activation by Mg²⁺ or Mn²⁺.

As indicated in Table III, removal of zinc yielded an inactive enzyme, which upon readdition of Zn²⁺ produced a fully active enzyme which contained two zincs per 54,000-dalton subunit.

Table II

<table>
<thead>
<tr>
<th>Method and denaturing agent</th>
<th>--SH per 54,000 g LAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTNB</td>
<td></td>
</tr>
<tr>
<td>None (2 hrs)</td>
<td>0.85</td>
</tr>
<tr>
<td>5 M Guanidine HCl (1 min)</td>
<td>5.7</td>
</tr>
<tr>
<td>0.5% Sodium dodecyl sulfate (45 min)</td>
<td>5.9</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.18</td>
</tr>
<tr>
<td>5 M Guanidine HCl</td>
<td>6.2</td>
</tr>
<tr>
<td>0.5% Sodium dodecyl sulfate</td>
<td>6.3</td>
</tr>
<tr>
<td>1 hr</td>
<td>5.0</td>
</tr>
<tr>
<td>2 hrs</td>
<td>6.1</td>
</tr>
<tr>
<td>0.5 hrs</td>
<td>6.3</td>
</tr>
</tbody>
</table>

* After the addition of DTNB the absorbance at 412 nm was followed with time. The time for maximum absorbance is noted in parentheses. This was followed by a slow decrease in absorbance owing to reoxidation of the thiophenol. The downward sloping line was extrapolated back to zero time to yield the reported values.

![Fig. 1](image-url)

**Fig. 1.** A, activity of leucine aminopeptidase against time of treatment with iodoacetamide at pH 8.0 (()). Untreated enzyme as a control (O). B, the number of residues of carboxamidomethylcysteine (CMC) formed versus the time of treatment of leucine aminopeptidase (LAP) at pH 8.0 and room temperature with iodoacetamide.

Table III

<table>
<thead>
<tr>
<th>Sample</th>
<th>Zinc/54,000 g LAP</th>
<th>Specific activity</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Zinc</td>
</tr>
<tr>
<td>Starting material</td>
<td>2.12</td>
<td>39.6</td>
<td>100</td>
</tr>
<tr>
<td>Low metal enzyme</td>
<td>0.082</td>
<td>1.01</td>
<td>3.9</td>
</tr>
<tr>
<td>Reconstituted zinc-zinc enzyme</td>
<td>2.09</td>
<td>38.1</td>
<td>98</td>
</tr>
</tbody>
</table>

* Determined by the amino acid assay on 0.025 m leucinamide in 0.1 M boric acid at pH 9.25 and 30°C.
incubation mixtures were then analyzed for enzyme-bound metals and specific activity. A pH of 9.5 was used for the Mg2+ system as this is the pH for maximum activation (13). A pH of 8.5 was used for the Mn2+ system because this was the highest pH in which the incubation could be performed for 30 min without the development of a brown precipitate (presumably MnO).

According to Equation 1, there should be a linear dependence of the observed specific activity on the number of activating ions bound per subunit. Fig. 2 shows a plot of the data for the number of activating metal ions bound per subunit versus the observed specific activity. It shows the predicted straight line relationship for both Mg2+ and Mn2+. In the Mg2+ experiment, extrapolation of the line to the y-intercept gives a value for the specific activity of the zinc-zinc enzyme of 42 mmoles per min per mg which is identical with that observed by direct measurement on the starting enzyme. In the case of the Mn2+ experiment, extrapolation of the best fitting line (determined by the least square procedure) gives a value (105 mmoles per min per mg) somewhat higher than expected by direct measurement on the zinc-zinc enzyme. This result may reflect the decreased reliability of the data for the Mn2+ experiment because of both a decreased sensitivity in atomic absorption analysis (about 3-fold less sensitive as compared with Zn2+ or Mg2+) and the decreased accuracy of the assay for enzymatic activity. The latter is due to the higher $K_m$ of the zinc-manganese which results in a smaller linear portion of the assay curve.

Table IV shows the results of measurements of bound Zn2+ and Mg2+ in activation experiments involving various ratios of Mg2+:Zn2+. These results show that not more than 1 of the 2 zinc atoms is replaced by Mg2+ even in high concentrations of Mg2+ and further that the sum of the bound Zn2+ and bound Mg2+ approximates 2 g atoms/54,000 g of leucine aminopeptidase for all ratios of activating ions.

Equation 4 expresses the relationship between the number of activating metal ions bound to the enzyme or the increase in specific activity (a-z) as a function of the ratio of the concentration of the activating metal ion to the concentration of free Zn2+. As explained under “Experimental Procedure,” correction of the data to low enzyme concentration allows the use of this equation to calculate the ratio of the dissociation constants ($K_{1a}:K_{1b}$) and the maximum number of activating metal ions that can be bound per subunit. The computer program (HYPERB) (25, 26) was used to solve the equation for the constants, but in order to display the results the corrected data points are presented in a reciprocal plot similar to the Lineweaver-Burk treatment (28) of the Michaelis-Menten equation. Fig. 3 shows a plot of the reciprocal of the number of

Table IV

<table>
<thead>
<tr>
<th>Mg2+:Zn2+ ratio</th>
<th>Zn2+</th>
<th>Mg2+</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:00</td>
<td>1.98</td>
<td>0.04</td>
<td>2.02</td>
</tr>
<tr>
<td>33</td>
<td>1.75</td>
<td>0.32</td>
<td>2.07</td>
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<tr>
<td>67</td>
<td>1.02</td>
<td>0.435</td>
<td>2.05</td>
</tr>
<tr>
<td>167</td>
<td>1.47</td>
<td>0.65</td>
<td>2.12</td>
</tr>
<tr>
<td>333</td>
<td>1.44</td>
<td>0.77</td>
<td>2.21</td>
</tr>
<tr>
<td>667</td>
<td>1.37</td>
<td>0.80</td>
<td>2.17</td>
</tr>
</tbody>
</table>

* No added Mg2+.
activating metal ions bound per subunit as a function of the reciprocal of the ratio of the concentration of activating metal ion to the concentration of Zn$^{2+}$. The points for Mg$^{2+}$ were derived from three types of experiments: no added Zn$^{2+}$ and variable Mg$^{2+}$; added constant Zn$^{2+}$ and variable Mg$^{2+}$; and constant Mg$^{2+}$ and variable Zn$^{2+}$. Solution of the data for Mg$^{2+}$ gave a value of 152 for the ratio of the dissociation constants ($K_{Mg}:K_{Zn}$) and a maximum binding of 0.99 g atom of Mg$^{2+}$ per 54,000 g of enzyme at pH 8.5 in 0.01 M potassium phosphate at 37°. The points for Mn$^{2+}$ are derived from two types of experiments: no added Zn$^{2+}$ and variable Mn$^{2+}$; and constant added Zn$^{2+}$ and variable Mn$^{2+}$. The data for Mn$^{2+}$ gave a value of 40 for $K_{Mg}:K_{Zn}$ and a maximum binding of 0.82 g atom of Mn$^{2+}$ per 54,000 g of enzyme at pH 8.5 in 0.01 M Tris at 37°.

Fig. 4 is a plot of the reciprocal of the increase in specific activity ($a-z$) against the reciprocal of the concentration of the activating metal ions to the concentration of Zn$^{2+}$, after all of the points have been corrected to low enzyme concentration. The value of the specific activity of the zinc-zinc enzyme ($z$) which was used for the calculations was 42 μmoles per min per mg. The data for Mg$^{2+}$ gave a value of 146 for the ratio of the dissociation constants ($K_{Mg}:K_{Zn}$) at pH 9.5 and a specific activity for the zinc-magnesium enzyme (determined at pH 9.25) of 678 μmoles per min per mg. Those for Mn$^{2+}$ gave a value of 31 for $K_{Mg}:K_{Zn}$ at pH 8.5 and a specific activity for the zinc-manganese enzyme of 935 μmoles per min per mg.

In summary, the results indicate that a maximum of one activating metal ion is bound per 54,000 subunit of leucine aminopeptidase, that at pH 9.5 Zn$^{2+}$ is bound to the activation site approximately 150 times more strongly than Mg$^{2+}$, that at pH 8.5 Zn$^{2+}$ is bound about 35 times more strongly than Mn$^{2+}$, and that the ratios of the specific activities in 0.025 M leucinamide, pH 9.25, 30° for the zinc-zinc to zinc-magnesium to zinc-manganese enzymes are 1:16:22.

Michaelis-Menten Parameters—Determination of the Michaelis-Menten parameters was performed by the spectrophotometric assay with varying concentrations of leucinamide. The determinations were complicated by the fact that at high substrate concentration (greater than 0.025 M) there was a "lag period" after the addition of the enzyme before observing the maximum rate of change in the absorption of the solution. This lag period is probably due to a transpeptidation reaction which gives rise to leucylleucinamide rather than hydrolysis (29) and which takes place with very little change in the absorbance of the solution. Because of this, data for calculation of the parameters by the HYPERB computer program were derived only from those experiments where the substrate concentration was below the point where the lag period was observed.

Table V summarizes the kinetic constants obtained with the three forms of the enzyme. Under the enzyme and metal ion concentrations used in these experiments, it can be calculated from the relative binding constants of the activating metal ions for the enzyme that the Mg$^{2+}$-activated enzyme was 97% in the zinc-magnesium form and the Mn$^{2+}$-activated enzyme was 94% in the zinc-manganese form. The results indicate that activation by either Mg$^{2+}$ or Mn$^{2+}$ substantially increases the maximum velocity as compared with the zinc-zinc form. The effect on the $K_m$ values was less dramatic. The $K_m$ value for the zinc-magnesium form (0.01 M) was somewhat lower than that for the zinc-zinc enzyme (0.02 M) while that of the zinc-manganese form (0.03 M) was somewhat higher. Hanson and Lasch (29), with different but not clearly specified assay conditions (probably pH 8.5 and 38°), found essentially the same $K_m$ values as those reported here for the Mg$^{2+}$- and Mn$^{2+}$-activated enzymes.

**DISCUSSION**

Extinction Coefficient—The value of $5.4 \times 10^4$ M$^{-1}$ cm$^{-1}$ reported here for the molar extinction coefficient, which was determined on a dry weight basis with 54,000 for the subunit molecular weight, agrees exactly with that reported by Kretschmer (30) (corrected to 54,000) who used the Lowry method (31) to determine the protein concentration, but it is 20% higher than that reported by Hanson et al. (9) who used the nitrogen content to estimate the protein concentration. Of the three methods, reference to the dry weight is the most accurate.

**Table V**

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Michaelis constant</th>
<th>Maximum velocity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V$</td>
<td>$V$</td>
</tr>
<tr>
<td></td>
<td>mole/1</td>
<td>μmoles/min/mg</td>
</tr>
<tr>
<td>Zinc-zinc</td>
<td>0.20 ± 0.004</td>
<td>82.2 ± 6.3</td>
</tr>
<tr>
<td>Zinc-magnesium (magnesium-activated at pH 9.5, 37°)</td>
<td>0.010 ± 0.001</td>
<td>605 ± 42</td>
</tr>
<tr>
<td>Zinc-manganese (manganese-activated at pH 8.5, 37°)</td>
<td>0.033 ± 0.011</td>
<td>2040 ± 410</td>
</tr>
</tbody>
</table>

* Activities were measured in 0.1 M borate at pH 9.25 and 30°.

* Error estimates are 95% confidence limits given by the t test of significance and were determined by the computer program HYPERB.
only method that does not involve any secondary assumptions as to relative color value or nitrogen content.

**Amino Acid Composition**—There have been two previous reports on the amino acid composition of lens leucine aminopeptidase (32, 33). Our investigation on this subject was initiated because in the most recent and definitive analysis (33), 5% of the dry weight of the protein was unaccounted for and because analyses for half-cystine determined in this laboratory (13) were in substantial disagreement with the reported values. The two completely independent analyses for the amino acid composition reported in terms of percentage recovery of dry weight (Table I) agree very well and indicate that the total weight can be accounted for as amino acid residues and Zn$^{2+}$.

The agreement between the two analyses expressed as percentage of total nitrogen content was not as good as the analyses based on dry weight. This reflects a greater disagreement in total nitrogen content. However, the average of the results for the nitrogen recovery indicates virtually complete recovery of the total nitrogen in terms of amino acid residues. These recoveries indicate that the enzyme cannot contain more than a fraction of a percentage of nonprotein components. Comparison of the most recent report on the composition of the lens enzyme (33), corrected to our terms of reference of eight histidines per subunit, with our results reveals substantial differences in half-cystine (13 versus 8), isoleucine (27 versus 29), tryptophan (7 versus 10), and amide (51 versus 35), and small differences in tyrosine (9 versus 10), methionine (10 versus 11), and proline (27 versus 26). The other amino acid residues agree quite well.

The value reported here for half-cystine (8 residues) was determined from analysis for cysteic acid in the performic acid-oxidized protein. The methionine sulfone value of 10.8, determined in the same hydrolysate, agreed quite well with the value (11.0) determined for methionine in an acid hydrolysate of the nonoxidized protein. This not only confirms the methionine value but also indicates that our lower value for cysteic acid as compared with the previous work (33) was not due to incomplete oxidation of the protein. Determination of 7.56 residues of carboxymethylcysteine in the reduced and carboxamidomethylated protein further confirms the half-cystine value. The higher value reported here for isoleucine probably reflects the inclusion of alloisoleucine, which amounts to about 5% of the isoleucine in the 24-hour hydrolysate at 130$^\circ$. The lower values reported here for amide content were determined on enzymes that had been exhaustively dialyzed to remove ammonia and include corrections for the ammonia content of the buffers.

Tryptophan is notorious for the difficulties encountered in its determination (34). It was estimated in the present instance by relating it to the tyrosine content from the absorption spectrum in alkaline solution. The previous determination had been performed by reaction of the tryptophan with $N$-bromosuccinimide (33). Resolution of the differences may well have to await the determination of the primary sequence. It has been our experience that analyses for tyrosine are generally low (35) even when performed in the presence of phenol (36). For this reason the values for tyrosine of 9.2 and 9.6 were pushed up to 10 rather than dropped to 9 as the average would have dictated. The differences of one residue between the present and previous analyses (33) for tyrosine, methionine, and proline are perhaps no larger than might be expected from analyses performed in different laboratories.

**Number and Reactivity of Sulphydral Groups**—The data of Frohne and Hanson (37), when recalculated to a subunit of 54,000, indicates a total of 1.7 sulphydryl groups per subunit, whereas the present data (Table II) clearly indicates that six of the eight half-cystines of leucine aminopeptidase are present in the sulphydryl form. This difference is probably due to a failure to use effective denaturing agents or to take adequate precautions to prevent reoxidation of the thiols, or both. In one experiment reported by Frohne and Hanson (37), the enzyme was incubated for 12 hours in the presence of sodium dodecyl sulfate without protection from air oxidation before reaction with DTNB. Considerable oxidation of the sulphydryls could have taken place during this time. The sulphydryl groups of leucine aminopeptidase are rapidly exposed in guanidinium chloride but are much more slowly exposed in sodium dodecyl sulfate (Table II).

Of the six sulphydryl groups in leucine aminopeptidase, only one reacts readily with DTNB or iodoacetamide in the native state. Reaction of this exposed group with iodoacetamide has no deleterious effect on enzyme activity. In this sense leucine aminopeptidase cannot be classified as a "classical" sulphydryl enzyme where reactive sulphydryl groups are closely linked with enzymatic activity (38-40). This does not necessarily mean that the sulphydryl groups are not involved in the catalytic site, but if so it is the "buried" sulphydryls that are important. In addition some sulphydryl groups may be needed for the binding of Zn$^{2+}$ which is essential for the enzyme.

**Zinc Content of Unactivated Leucine Aminopeptidase**—A report in 1968 by Böttger et al. (41) indicated the presence of 1.3 to 2.0 Zn$^{2+}$ per subunit when recalculated to 54,000. On the other hand Himmelhoch in 1969 (7), with hog kidney enzyme, and Kettmann and Hanson in 1970 (8), with bovine lens enzyme, reported values corresponding to 1 g atom of Zn$^{2+}$ per subunit. Rationalization of these differences is possible by assuming that the enzyme has two binding sites for Zn$^{2+}$ of somewhat different affinities. The isolation of the enzyme from bovine lens involves a Zn$^{2+}$ precipitation step and subsequent handling of the enzyme at relatively high protein concentrations, all of which favor retention of Zn$^{2+}$ in the weaker site. Himmelhoch's isolation of the enzyme from hog kidney involved no Zn$^{2+}$ precipitation step and also involved procedures where the enzyme was present in quite dilute solution, all of which favor loss of Zn$^{2+}$ from the weakest site. In addition, the work presented here shows that although one of the two Zn$^{2+}$ is needed for activity, the other can be replaced by other divalent ions which may have inadvertently been the case in the preparations noted above.

**Essentiality of Zinc and Zinc-specific Site**—The data on the removal and readdition of zinc show that the metal-free enzyme is inactive but that activity can be restored upon the addition of Zn$^{2+}$, resulting in the binding of two Zn$^{2+}$ per 54,000 subunit. Addition of other metals, such as Cu$^{2+}$, Ca$^{2+}$, Mg$^{2+}$, or Mn$^{2+}$, to the metal-free enzyme did not result in stoichiometric binding or in significant regain of enzymatic activity. However, upon incubation of the zinc-zinc enzyme with high concentrations of Mg$^{2+}$ or Mn$^{2+}$, one but not both of the zinc atoms was replaced. These results suggest that there is a specific binding site for zinc and that the presence of zinc in this site is needed for enzymatic activity. The essentiality of zinc is also indicated by Himmelhoch's results (7) where it was found (a) that zinc was the natural constituent of the hog kidney enzyme and (b) that the zinc could be slowly replaced by dialysis against Cd$^{2+}$ to give an inactive product, which in turn could be reactivated by dialysis against Zn$^{2+}$. Working with
the lens enzyme, Kettmann and Hanson (8) were also able to displace the zinc by dialysis against Cd2+ to give an inactive product but were not able to reverse the process. Although all of these results indicate that zinc is essential for the action of the enzyme, it is conceivable, in view of the results obtained with other zinc metalloenzymes (42-44), that other untested metal ions (such as Co2+) might be able to replace zinc to give an active enzyme.

Activation Site—Since 1 of the 2 zinc atoms of leucine aminopeptidase can be replaced by incubation with high concentrations of Mg2+ or Mn2+, it is indicated that the enzyme has two different classes of sites for binding zinc: the zinc-specific site (as noted above) and another site, the activation site, where the zinc can be replaced by other metal ions to give products with different specific activities. It is the binding of Mg2+ or Mn2+ to the latter site that accounts for the activation of leucine aminopeptidase first noted in 1936 (2). The presence of two classes of sites for the same metal with different reactivities has also been noted for liver alcohol dehydrogenase (45). The preparations of leucine aminopeptidase investigated by Himmelhoch (7) and also by Kettmann and Hanson (8) contained only one zinc per 54,000 subunit and both showed activation by Mg2+ and Mn2+. This would imply that the activation site does not need to be occupied by zinc in order to isolate an activatable enzyme.

pH Effect on Activation of Leucine Aminopeptidase—The degree to which the specific activity of leucine aminopeptidase can be increased by incubation with Mg2+ or Mn2+ is strongly dependent upon the pH, with the most active enzyme being formed between pH 9 and 10 (9, 13). The possibility that this pH effect can be attributed to ionization of functional groups on the enzyme has been considered by Melbye (16). However, the realization that activation involves a competition between Zn2+ and other metal ions for the activation site suggests an alternate explanation for this pH effect. The concentration of Zn2+ needed for competition with the activating metal ions is quite low as shown by the ratio of their dissociation constants. The over-all solubility of Zn(OH)2 and its hydrates is strongly pH-dependent near pH 8.5 (46). It is possible that the pH dependence of activation of leucine aminopeptidase actually reflects a pH-dependent lowering of the activity of zinc ions which are in competition with the activating metal ions.

Physiological State of Leucine Aminopeptidase—If the pH activation curve (13) reflects the competition between zinc ions and other metal ions for the activation site, then at neutral pH, where there is little activation by other metal ions, the zinc must not be displaced from the activation site. This indicates that the physiological form of the enzyme is the zinc-zine form rather than the zinc-magnesium or zinc-manganese form. Further evidence in support of this statement is afforded by the relative dissociation constants and the relative metal ion concentrations in the lens and kidney tissues. If the total metal ion of these tissues is assumed to exist as free divalent cations, then the concentration of Mg2+ is 10 to 15 times that of Zn2+, which in turn is higher than the concentration of Mn2+ by a factor of 20 (41, 47). At these relative metal ion concentrations and with $K_{Mg}:K_{Zn} = 150$ at pH 9.5 and $K_{Mn}:K_{Zn} = 35$ at pH 8.5, the leucine aminopeptidase would be almost entirely in the zinc zine form, even at pH values where activation is known to take place. Thus, under pH conditions which are near optimum for activation but which are far removed from the physiological pH, the relative concentrations of Mg2+ and Mn2+ versus Zn2+ in the lens and kidney tissues are not high enough to effectively compete for the activation site. It would appear that the Mg2+ and Mn2+ activation of leucine aminopeptidase is a laboratory phenomenon and is unrelated to the physiological function of the enzyme in the tissue.

Subunit Size of Leucine Aminopeptidase—Previous results from this laboratory have indicated a subunit size for leucine aminopeptidase of about 54,000 (13, 14) whereas other workers have reported values of about 32,000 daltons (10-12). The metal binding data yield further information on this question. Although the stoichiometry of binding of zinc to the unactivated enzyme (the zinc-zine enzyme) might infer that the subunit size is 27,000, the results from the Mg2+- and Mn2+-activated enzymes clearly indicate that a maximum of 1 g atom of activating metal ion is bound per 54,000 g of leucine aminopeptidase. This is in good agreement with our previous determinations of subunit size.

Acknowledgments—We are indebted to Jeannine Caulfield Thieme for aid with the amino acid analyses and to Dr. Arthur Wainer for suggesting the ion exchange method for removing unbound metal ions.

REFERENCES

Leucine Aminopeptidase (Bovine Lens): MECHANISM OF ACTIVATION BY MG2+ AND MN2+ OF THE ZINC METALLOENZYME, AMINO ACID COMPOSITION, AND SULFHYDRYL CONTENT
Frederick H. Carpenter and James M. Vahl


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