Leucine Aminopeptidase (Bovine Lens)

EFFECT OF pH ON THE RELATIVE BINDING OF ZN^{2+} AND Mg^{2+} TO AND ON ACTIVATION OF THE ENZYME*

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Incubation of leucine aminopeptidase (bovine lens) (EC 3.4.1.1) with various concentrations of Mg^{2+} at various pH values in 1 M KCl and 0.155 M trimethylamine-HCl at 37°C confirms that Mg^{2+} competes with Zn^{2+} for binding at only 1 site per 54,000 dalton subunit. The ratio of the apparent association constants (K_{Mg}/K_{Zn}) at this site (site 1) was estimated to be 20,720 at pH 8.16, 10,570 at pH 8.44, 3,590 at pH 8.78, and 660 at pH 9.14. The decrease in values of K_{Mg} with increasing pH in the activation of leucine aminopeptidase by Mg^{2+} is attributed to the lowering of the free Zn^{2+} concentration relative to that of free Mg^{2+} caused by the formation of ZnOH^{+} and Zn(OH)_{2} complexes with increasing OH^{-} concentration. When corrections are made for the binding of Zn^{2+} by OH^{-} ions, the pH-independent ratio of association constants (K_{Zn}/K_{Mg} = K_{Zn,Mg}^{sites}) for the relative binding of Zn^{2+} and Mg^{2+} at site 1 of leucine aminopeptidase is 29,800. From the effect of pH on the relative binding constant, a value (β_{Mg}) for the product of the two stepwise association constants for the formation of ZnOH^{+} and Zn(OH)_{2} was estimated to be 4.42 \times 10^{10} M^{-1} at 37°C. Values of K_{Zn} at pH 7.5 and 30°C with L-leucine p-nitroanilide as substrate in the presence of 0.01 M NaHCO_{3} are 4.13 and 2.01 mM for the zinc-zinc and magnesium-zinc enzymes, respectively. Values for V_{max} are 0.2 and 2.49 μmol/min/mg, respectively.

The discovery that Mg^{2+} activates leucine aminopeptidase (EC 3.4.1.1) was made in 1936 by Johnson et al. (2). Since then, a number of investigations have shown that Mn^{2+} is also an activator (3), that the activation is time-dependent (4, 5), and that activation may be attributed to the binding of the metal ion to the protein (6, 7). The latter conclusion, based on the observation that specific activity increased in the presence of increasing free Mg^{2+} or Mn^{2+} concentrations (6, 7), was criticized by Malmström and Rosenberg (8) because direct measurements of bound metals were not made and the possibility that Mg^{2+} or Mn^{2+} might compete with another bound metal was not considered. These criticisms became important when it was found that preparations of leucine aminopeptidase contained Zn^{2+} (9, 10). The criteria of Vallee (11) for the establishment of leucine aminopeptidase as a zinc metalloenzyme were demonstrated in this laboratory (12, 13) by showing that a stoichiometric amount of Zn^{2+} could be removed and reinstated with a concomitant loss and restoration of full enzymic activity. Furthermore, the latter reports indicated that 2 g atoms of Zn^{2+} were bound per 54,000 g of enzyme subunit (12 g atoms/324,000 g of oligomer) in agree-

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buffer anion complex) or the ionization constants of the enzyme ligands. Both of these constants are difficult to determine as the metal hydroxides are usually too complex and insoluble to make stability constant measurements and many metalloenzymes are not stable to procedures for the titration of their metal-binding ionizable groups.

Using an experimental approach similar to that of Carpenter and Vahl (13), this investigation measured the relative binding constant of the enzyme for Zn$^{2+}$ and Mg$^{2+}$ as a function of pH. These relative binding constants differed by a factor of 45-fold in the pH range of 8.16 to 9.14. However, when they were corrected for the effect of OH$^{-}$ on the formation of complexes with Zn$^{2+}$ ions, using the equation of Coleman and Vallee (19), the corrected relative binding constants were equal and independent of pH. These results strongly suggest that the pH effect in the activation of leucine aminopeptidase by Mg$^{2+}$ or Mn$^{2+}$ is due to a competition between the enzyme and OH$^{-}$ for free Zn$^{2+}$ ions rather than to a change in the ionization state of the enzyme.

**EXPERIMENTAL PROCEDURE**

**Materials**

L-Leucine p-nitroanilide (free base) was obtained from Sigma Chemical Co. ZnO and MgSO$_4$, used as spectroscopic standards, were the high purity salts from Johnson-Matthey and Co., Ltd. Tris(ethanolamine)-HCl was from Eastman Organic Chemicals while N-ethylmorpholine and 1,10-phenanthroline were obtained from Aldrich Chemical Co. The N-ethylmorpholine was distilled before use. Crystalline leucine aminopeptidase was prepared from bovine lens according to the procedure of Hanson et al. (20) as modified by Mahoney (17). All glass and plasticware was cleaned with concentrated HNO$_3$/H$_2$SO$_4$ to remove ultraviolet and visible absorbances were made with a Cary model 15 absorption spectrophotometer equipped with a deuterium background corrector and a recorder readout.

Solutions of leucine aminopeptidase containing approximately 20 mg/ml were prepared from the crystalline enzyme by the method of Carpenter and Vahl (13) in 0.2 M N-ethylmorpholine-HCl at pH 7.5. These solutions were stored with an added drop of toluene in plastic vials at 4°C for periods of up to 3 months with complete stability.

Enzyme concentrations were determined by measuring the absorbance at 280 nm using an $E_{280}$ of 10 as reported by Carpenter and Vahl (13), corresponding to a molar extinction coefficient of 5.4 x 10$^4$ M$^{-1}$ cm$^{-1}$ for a 54,000-dalton subunit.

Specific activity was measured by observing the increase in absorbance with time at 405 nm upon the hydrolysis of L-leucine p-nitroanilide at 30°C (22, 23). Assay solutions consisted of 2.0 ml of 17 mM substrate, 0.01 M NaHCO$_3$, and 1 M KCl in 0.2 M N-ethylmorpholine-HCl at pH 7.5. Enzyme concentrations were between 5 and 10 $\mu$g/ml in the assay solution. Specific activities are in micromoles/min/mg and are calculated from the difference in molar extinction coefficients of 9,000 M$^{-1}$ cm$^{-1}$ between p-nitroaniline and leucine-p-nitroanilide at 405 nm at pH 7.5 (24). The value of 9.000 M$^{-1}$ cm$^{-1}$ is unchanged between pH 5.0 and 10.5 (25). Michaelis-Menten parameters are reported for similar conditions with the exception that the enzyme concentration was 20 $\mu$g/ml for the zinc-zinc leucine aminopeptidase.

**Measurement of Bound Metals**—Approximately 1 ml of sample was applied to a 24-ml column (1 x 30 cm) of Bio-Gel P-6 equilibrated in 0.2 M N-ethylmorpholine-HCl at pH 7.5 and eluted with a flow rate of 2 ml/min. Fractions of approximately 1 ml were collected in polypropylene vials until 30 ml had been eluted. The enzyme concentration of each fraction was measured before determining its zinc and magnesium concentrations. The values for bound metals in g atoms per 54,000 g of leucine aminopeptidase were calculated from the average of the bound zinc and magnesium in the several fractions comprising the enzyme peak.

**Incubation with Magnesium Chloride at Various pH Values**—Solutions of leucine aminopeptidase at 0.9 mg/ml were incubated for 10 hours at 37°C in 0.155 M trimethylamine-HCl at pH 9.14 with 1 M KCl and various MgCl$_2$ concentrations between 1.62 and 46.5 mM. Activity assays were performed at pH 7.5 at 0, 3, and 6 hours. At 10 hours a 200-ml aliquot was removed from each sample and set aside for total metal determination. The remainder of the solution was used for the determination of the pH enzyme concentration by absorption at 280 nm, activity, and bound metals. The 200-ml samples for total metal determination were diluted to make the zinc and magnesium concentrations approximately 0.006 mm before measurement. This procedure was repeated for solutions at pH 8.78, 8.44, and 8.16 with respective MgCl$_2$ concentration ranges of 1.5 to 46.5 mm, 6.23 to 43.1 mm, and 8.62 to 42.2 mm.

Manganese-zinc leucine aminopeptidase used in determining the Michaelis-Menten parameters was prepared by incubating zinc-zinc leucine aminopeptidase (0.8 mg/ml) at 37°C for 12 hours in 0.2 M trimethylamine-HCl at pH 9.2 with 0.05 M MgCl$_2$ and 1 M KCl.

**Activation with Magnesium at pH 7.5 in Presence of 1,10-Phenan-throline—Zinc-zinc leucine aminopeptidase (1.0 mg/ml) was incubated at 37°C in 0.2 M N-ethylmorpholine-HCl at pH 7.5 with 1 M KCl, 0.01 M MgCl$_2$, and various concentrations of 1,10-phenanthroline between 0.005 and 1 mm. Aliquots were removed for assay of specific activity in 2.5 ml of 2 mm leucine p-nitroanilide assay solution not containing bicarbonate at 0, 1, 2, 4, 6, 7, and 10 hours. After 10 hours the samples were eluted through the P-6 columns and the eluates were measured for bound metals.

In another experiment, zinc-zinc enzyme (1.0 mg/ml) was incubated at 37°C in 0.2 M N-ethylmorpholine-HCl at pH 7.5 with 1 M KCl, 0.05 mM 1,10-phenanthroline, and various MgCl$_2$ concentrations between 0.04 and 4 mm. Aliquots were removed for specific activity assays in 2.5 ml of a 2 mm leucine p-nitroanilide assay solution not containing bicarbonate at 0, 1, 2, 4, 6, and 8 hours. After 8 hours the samples were eluted through the P-6 columns and the eluates were measured for bound metals.

**Treatment of Data—Michaelis-Menten parameters were calculated using the computer program HYPERB (26, 27). The $K_m$ values were corrected by multiplication by 0.455, the fraction of the substrate calculated to be in the deprotonated state at pH 7.5 using a measured value of 7.88 for the $pK_a$ of L-leucine p-nitroanilide. This correction accounts for the fact that leucine aminopeptidase binds only substoichiometric amounts of the substrate with deprotonated a-amino groups (28, 29).

All other constants were calculated with a Control Data Corp. CDC-6400 computer which was programmed to find the least squares fit to the data to a given theoretical equation (30).

The previous work of Carpenter and Vahl (13) as well as the present investigation indicated that there are two metal binding sites per 54,000-g subunit. The total g atoms of metal bound to each enzyme subunit, designated as $\tau$, is composed of the g atoms of metal per subunit bound at site 1 (referred to as the "activation" site), designated as $\gamma$, plus the g atoms of metal per subunit bound at site 2 (referred to as the "structural" site), designated as $\delta$, so that

$$\tau = \gamma + \delta = 2.$$  

(1)

References to a specific metal ion is made by the use of subscripts so that

$$r_{zn} = r_{zn} + r_{zn}$$  

(2)

means that the total g atoms of zinc bound per subunit ($r_{zn}$) is equal to that bound at site 1, $r_{zn}$, plus that bound at site 2, $r_{zn}$. Similarly, the total g atoms of magnesium bound per enzyme subunit ($r_{mg}$) is equal to that bound at site 1, $r_{mg}$, plus that bound at site 2, $r_{mg}$.

$$r_{mg} = r_{mg} + r_{mg}.$$  

(3)

However, under the conditions of exchange used by Carpenter and Vahl (13) as well as those used in this report, Mg$^{2+}$ competes with Zn$^{2+}$ for only one of the sites (the activation site) on the enzyme. Thus,

$$r_{zn} = r_{mg}.$$  

(4)

In addition, under these conditions, it was assumed that the two
binding sites were saturated with metal and that when the measured sum of the g atoms of Zn\(^{2+}\) and Mg\(^{2+}\) was less than 2.0, that some of the less tightly bound Mg\(^{2+}\) had been removed from the enzyme during the separation of free and bound metals by the column procedure. This assumption was based on the consideration that the sum of the bound metals was found to be 2.0 when the enzyme was incubated with no magnesium and was present only as the zinc-zinc enzyme, but generally was found to be progressively less than 2.0 as the amount of bound magnesium increased. As a consequence of this treatment,

\[ R_{\text{Me}} = 2 - R_{z} - 1 - R_{zn} \quad (6) \]

The concentration of free magnesium in each sample was calculated by the following formula,

\[ [\text{Mg}^{2+}]_{f} = [\text{Mg}^{2+}]_{t} - (2 - R_{zn})[E] \quad (6) \]

where \([\text{Mg}^{2+}]_{t}\), \([\text{Mg}^{2+}]_{f}\), and \([E]\) are the molar concentrations of free magnesium, total magnesium, and total enzyme subunits, respectively.

Although the free zinc in solution can be determined by an equation similar to Equation 6,

\[ [\text{Zn}^{2+}]_{f} = [\text{Zn}^{2+}]_{t} - r_{zn}[E] \quad (7) \]

where \([\text{Zn}^{2+}]_{t}\) and \([\text{Zn}^{2+}]_{f}\) are the molar concentrations of free and total zinc, the procedure suffers from inaccuracies deriving from the determination by atomic absorption of small differences between two measured values of similar magnitude. The compounding of errors arising from making two measurements by atomic absorption can be avoided by using a measurement of the bound zinc coupled with a determination of enzyme concentration by the following formula,

\[ [\text{Zn}^{2+}]_{f} = (2 - R_{zn})[E] \quad (8) \]

This procedure is validated by the fact that the contribution to the zinc ion concentration of the solution by replacement of zinc from the enzyme by magnesium (approximately 10\(^{-5}\) mM) is much greater than the concentration of contaminating free zinc ions in the buffer solutions (approximately 10\(^{-3}\) mM).

The theoretical equation for calculating the correlation of specific activity with magnesium bound was that from Carpenter and Vahl (13)

\[ a = (2 - r_{zn})(m_{z} - 22) + z^{*} \quad (9) \]

where \(a\) is the measured specific activity, and \(m_{z}\) and \(z^{*}\) are the specific activities of the magnesium-zinc and zinc-zinc forms of the enzyme, respectively.

**Ratio of Association Constants**—Klotz et al. (31) have presented an equation describing the binding of two species of metal ion competing for the same binding site on a protein, neglecting electrostatic interactions. Using this equation, the binding of zinc to leucine aminopeptidase in the presence of competing magnesium can be expressed as follows:

\[ r_{zn} = \frac{K_{zn/Mg}[Zn^{2+}]_{f}/[Mg^{2+}]_{f}}{1 + K_{zn/Mg}[Zn^{2+}]_{f}/[Mg^{2+}]_{f}} \quad (13) \]

where \(K_{zn/Mg}\) is equal to \(K_{zn}/K_{Mg}\). Given the observed values of \(r_{zn}\) and \([Zn^{2+}]_{f}/[Mg^{2+}]_{f}\), Equation 13 was used to calculate a value for \(K_{zn/Mg}\) at each pH by a computer program (30).

**Correction for Formation of Zn(OH)\(_{2}\)**—It has been proposed that competition of OH\(^{-}\) with leucine aminopeptidase for free Zn\(^{2+}\) in the solution is responsible for the observed pH dependence of Mg\(^{2+}\) activation of this enzyme. A treatment which compensates for competition of buffer anions with a protein for various metal ions has been presented by Coleman and Vallae (19) for carboxypeptidase. Their method involves the correction of a measured apparent association constant (K) by the equation

\[ K' = K(1 + \beta_{n}(\text{pH}^n)) \quad (14) \]

where \(K'\) is the corrected constant, \(K\) is the molar concentration of the competing anion, and \(\beta_{n}\) is the product of \(n\) stepwise constants for the nth complex.

The competing anion in the buffer solutions used here was OH\(^{-}\)—Data from Sillen and Martell (35) indicates that while the OH\(^{-}\) concentration is not high enough for it to be an effective magnesium-binding agent in the pH range of these experiments, it is important in the calculation of the true free zinc concentration.

According to Fulton and Swinehart (14), the primary species of zinc in solution between pH 6.0 and 8.0 at 25\(^\circ\)C are Zn\(^{2+}\), Zn(OH)\(^{+}\), and Zn(OH)\(_{2}\). In terms of a simple treatment as stepwise associations, the formation of these species can be written as

\[ \text{Zn}^{2+} + \text{OH}^{-} \rightarrow \text{Zn(OH)}^{+} \quad K_{1} \]

\[ \text{Zn(OH)}^{+} + \text{OH}^{-} \rightarrow \text{Zn(OH)}_{2}^{-} \quad K_{2} \]

The sum of these equations is

\[ \text{Zn}^{2+} + 2\text{OH}^{-} \rightarrow \text{Zn(OH)}_{2}^{-} \quad K_{1}K_{2} = \beta_{2} \]

Since \(K_{1}K_{2}\) is the product of two stepwise association constants, \(n\) in Equation 14 is 2 and \(K_{zn/Mg}\) becomes \(\beta_{1}\). Assuming that the concentrations of these species are still much greater than that of Zn(OH)\(_{2}\) at 37\(^\circ\)C in this pH range, Equation 14 as it applies to the present work can be written

\[ K'_{zn/Mg} = K_{zn/Mg}(1 + \beta_{1}(\text{pH}^{1})) \]

By the definition of \(K_{zn/Mg}\), this becomes

\[ K'_{zn/Mg} = K_{zn/Mg}(1 + \beta_{2}(\text{pH}^{2})) \quad (15) \]

Substituting the expression \(10^{\text{pH} - 13.651}\) for (OH\(^{-}\)) and rearranging yields

\[ K_{zn/Mg} = \frac{K'_{zn/Mg}}{1 + \beta_{2}(10^{\text{pH} - 13.651})} \quad (18) \]

where 13.651 is the negative logarithm of the ionization constant of water at 37\(^\circ\)C (35).

Substituting the expression for \(K'_{zn/Mg}\) from Equation 18 into Equation 15 gives

\[ r_{zn} = \frac{K_{zn/Mg}[Zn^{2+}]_{f}/[Mg^{2+}]_{f}}{1 + K_{zn/Mg}[Zn^{2+}]_{f}/[Mg^{2+}]_{f}} \quad (19) \]

This equation was incorporated into the computer program (30) which was used to calculate the values of \(r_{zn}\) and \(K_{zn/Mg}\) from the observed values of \([Zn^{2+}]_{f}/[Mg^{2+}]_{f}\) and pH for the combined experiments at pH 8.16, 8.44, 8.78, and 9.14.
Leucine Aminopeptidase: Effect of pH on Binding of Metal Ion

RESULTS

Measurement of Bound Metals—An elution diagram (Fig. 1) for the separation of free and bound metals shows that a 24-ml column of Bio-Gel P-6 completely separates 0.05 mM MgCl₂ from a 1-ml sample of the enzyme. Ultraviolet absorbance measurements at 280 nm on the eluates indicate that Fractions 6 to 10 contain the enzyme. Associated with the enzyme peak are peaks in the atomic absorption measurements of Zn²⁺ and Mg²⁺, corresponding to the enzyme-bound forms of these metals. The free Mg²⁺ peak is very large, extending from Fractions 12 to 31. No free zinc peak was observed, probably because the column dilutes the small amount of free zinc present to a level below the detection limit of atomic absorption using the Böling burner.

Table 1 shows the results obtained from measuring the Zn²⁺ and Mg²⁺ bound per subunit for the series of solutions incubated at pH 9.14. The sum of the Zn²⁺ plus Mg²⁺ bound per subunit decreases from 2.0 for enzyme incubated in the absence of added MgCl₂ to 1.8 for enzyme incubated in 46.5 mM MgCl₂. In general, the more Mg²⁺ bound to the enzyme, the greater the difference between 2.0 and the sum of the Mg²⁺ plus Zn²⁺ bound per enzyme subunit. These results indicate that enzyme-bound zinc is completely stable to the column procedure used to remove unbound metal ions but that a portion of the enzyme-bound magnesium is removed by passage of the enzyme solution through the P-6 column.

As a consequence of these observations and the assumption that the two metal sites on each enzyme subunit were completely saturated before the enzyme solution was placed on the P-6 column, the g atoms of Mg²⁺ bound per subunit (rₘg) was calculated as being equal to 2 minus the measured value for bound zinc (2 - rₘz).

Specific Activity of Magnesium-Zinc Leucine Aminopeptidase—Fig. 2 shows the change in specific activity with incubation time at 37°C for various pH values and MgCl₂ concentrations. Equilibrium was reached, as judged by activity measurements, at 10 hours at which time the g atoms of zinc bound per subunit were determined by the column method. The relation between specific activity and bound Mg²⁺ or Zn²⁺ is shown in Fig. 3. Extrapolation of the line defined by the least squares fit of the data to the intercept at 1 g atom per subunit of either Mg²⁺ or Zn²⁺ yields a specific activity for the 1-magnesium/1-zinc enzyme of 2.35 ± 0.03 μmol/min/mg in the standard assay using leucine p-nitroanilide at pH 7.5.

Determination of Ratio of Association Constants (Kₖ₃M₄₄) as Function of pH—Fig. 4 illustrates the g atoms of zinc bound per subunit (rₘz) at site 1 as a function of [ZnCl₂]/[MgCl₂] at various pH values plotted in the reciprocal form used for graphical calculation of relative binding constants (36). From the data shown in Fig. 4, two major observations can be extracted. First, the ordinate intercept of the plot gives a value for the reciprocal of the number of binding sites involved. In this case, it is clear that Mg²⁺ is competing with Zn²⁺ for only one site on each enzyme subunit in the pH range studied. Second, the value of the slope, where only one class of site is involved, is equal to the number of binding sites divided by the relative binding constant. Since the number of sites is equal to unity, the slope is the reciprocal of the relative binding constant and is seen to vary with pH.
Leucine Aminopeptidase: Effect of pH on Binding of Metal Ion

The specific activity of leucine aminopeptidase (LAP) versus $r_{Mg^2+}$ and $r_{Zn^{2+}}$ in the number of $Zn^{2+}$ and $Mg^{2+}$ ions bound per 54,000-dalton subunit, respectively. The different symbols represent data gathered at pH 8.16 ($\bullet$), 8.44 ($\square$), 8.78 ($\triangle$), and 9.14 ($\circ$). The line is the best least squares fit of the data according to Equation 9.

The value of $K_{Zn,Mg}$, the ratio of the apparent association constants of the enzyme for $Zn^{2+}$ to that for $Mg^{2+}$, decreases as the pH is increased, which indicates that it is easier for $Mg^{2+}$ to replace $Zn^{2+}$ at the activation site at high pH than at low pH. This result is consistent with the reports on the effect of pH on the activation of leucine aminopeptidase by either $Mg^{2+}$ or $Mn^{2+}$ (16, 37).

It is proposed that this effect of pH on the $Mg^{2+}$ activation of the enzyme can be attributed on one hand to a competition between $Zn^{2+}$ and $Mg^{2+}$ for one site on the enzyme, and on the other hand, by a competition between the enzyme and $OH^-$ ion for the free $Zn^{2+}$ in solution. At high pH values the $OH^-$ effectively competes with the enzyme for zinc and lowers the free $Zn^{2+}$ concentration of the solution enough so that the less tightly bound $Mg^{2+}$ (which forms much less stable hydroxides than zinc) can effectively compete with lowered $Zn^{2+}$ for one site on the enzyme.

Correction of the Relative Binding Constant ($K_{Zn,Mg}$) for Formation of $Zn(OH)_2$—The measurements of $r_{Zn}$ and $[Zn^{2+}]/[Mg^{2+}]$ at each pH were fitted by the least squares method to Equation 19 and yielded a value of 29,800 for the corrected relative binding constant ($K_{Zn,Mg}$) and $4.42 \times 10^{-16} \text{ M}^{-1}$ for $\beta_p$, the product of the stepwise association constants of $Zn^{2+}$ and $OH^-$. Putting these values into Equation 18 yields the sigmoid curve in Fig. 5 representing the relation between the apparent relative binding constant ($K_{Zn,Mg}$) and pH. The points along this curve are the individual values of $K_{Zn,Mg}$ at each pH that were derived in the preceding section. The point of inflection (arrow) at pH 8.33 represents the pH at which one-half of the total nonenzymic zinc in solution forms a complex with $OH^-$. The upper curve in Fig. 5 represents the pH-independent value of $K_{Zn,Mg}$ of 29,800. The points along the line are individual values of this constant that were calculated from Equation 18 using the values of the apparent relative binding constants ($K_{Zn,Mg}$) derived in the preceding section (plotted along the lower curve) and the fitted value of $\beta_p$. The comparison of these two curves demonstrates clearly that correction of the data for the formation of $Zn(OH)_2$ yields a relative binding constant that is independent of pH. In other words, the phenomenon of pH-dependent $Mg^{2+}$ activation of leucine aminopeptidase is a result of the lowered concentration of free zinc caused by the formation of zinc hydroxides.

Activation of Leucine Aminopeptidase at pH 7.5 with Magnesium in Presence of 1,10-Phenanthroline—In order to show that other $Zn^{2+}$-specific complexing agents will produce the same effect on $Mg^{2+}$ activation of leucine aminopeptidase (O) or $K_{Zn,Mg}$, the relative binding constant for $Zn^{2+}$ compared to $Mg^{2+}$ at site 1 on leucine aminopeptidase corrected for $Zn(OH)_2$ formation ($\square$) versus pH. The lines are the best least squares fit of the data according to Equation 18. The point of inflection (arrow) at pH 8.33 represents the pH at which one-half of the total nonenzymic zinc in solution forms a complex with $OH^-$. The graph shows that up to a concentration of 0.05 mM increasing concentrations of the complexing agent enhanced activation, while further increases resulted in the eventual destruction of activity. Analysis of the samples for bound metals (Fig. 6A, inset) shows that $Mg^{2+}$ bound per subunit increases in correspondence with rising activity, while those samples which had lost activity at high $Mg^{2+}$ concentration had nearly all of their bound metals removed. In addition, the latter samples appeared to have nearly equal amounts of bound $Mg^{2+}$ and $Zn^{2+}$, leading to...
The value of $V_{\text{max}}$ is increased from 0.20 ± 0.09 to 2.49 ± 0.83 μmol/min/mg in going from the zinc-zinc to the magnesium-zinc enzyme.

**DISCUSSION**

**Measurement of Bound Metals**—In previous reports from this laboratory (13) free divalent ions were separated from enzyme-bound metals by passage of the solution through a short column of sulfonated polystyrene resin (Beckman type 50A). The enzyme was in contact with the resin for less than 30 s, during which time there was complete removal of free divalent ion with virtually no effect on the bound Mg$^{2+}$ and Zn$^{2+}$. In the present experiments, incubation with divalent ions was performed for the most part in the presence of 1 mM KC1. Since this quantity of salt in the eluate resulted in considerable interference with the measurement of Zn$^{2+}$ and Mg$^{2+}$ by atomic absorption spectrophotometry, it was necessary to use a technique that would largely remove these monovalent ions along with the unbound divalent metal ions. Several Sephadex and Bio-Gel resins were investigated in order to determine an effective separation of bound from unbound ions in a short exposure time. As shown in Fig. 1 the use of a Bio-Gel P-6 column (1 × 30 cm) gave a good separation of bound from unbound ions in a 1-ml sample when operated at a flow rate of 2 ml/min. Under these conditions the enzyme is exposed to the gel resin for about 4 min. Even though bound zinc was completely retained by the enzyme under these conditions, the longer exposure time (4 min for the Bio-Gel P-6 versus 30 s for the sulfonated polystyrene) resulted in some removal of bound magnesium.

**Ratio of Association Constants ($K_{Zn,Mg}$)**—The values obtained for the ratios of apparent association constants at various pH values are in good agreement with that obtained by Carpenter and Vahl (13) at pH 9.5. Using the values of $\beta_{Mg}$ and $K_{Zn,Mg}$ reported here in Equation 18, the predicted apparent relative binding constant ($K_{Zn,Mg}$) at pH 9.5 is 134, extremely close to the value of 132 reported by Carpenter and Vahl (13).

Problems associated with obtaining these ratios and their corrected values are numerous. From a practical point of view, the most critical problem is the measurement of pH. Equations 18 and 19 show that values of $K_{Zn,Mg}$ are extremely sensitive to pH as this parameter occurs in an exponent. Thus, an error of 0.05 pH unit can affect $K_{Zn,Mg}$ by as much as 20%.

Another critical factor is the measurement of metal. Although atomic absorption is an extremely sensitive method, and is perhaps also the most precise for quantitative purposes, it is still subject to at least 5% standard error unless impractical numbers of determinations are performed. Therefore, all measurements made by this technique are subject to at least this level of error. It was for this reason that, instead of determining the free zinc concentration from the difference corrected values are numerous. From a practical point of view, the most critical problem is the measurement of pH. Equations 18 and 19 show that values of $K_{Zn,Mg}$ are extremely sensitive to pH as this parameter occurs in an exponent. Thus, an error of 0.05 pH unit can affect $K_{Zn,Mg}$ by as much as 20%.

Another critical factor is the measurement of metal. Although atomic absorption is an extremely sensitive method, and is perhaps also the most precise for quantitative purposes, it is still subject to at least 5% standard error unless impractical numbers of determinations are performed. Therefore, all measurements made by this technique are subject to at least this level of error. It was for this reason that, instead of determining the free zinc concentration from the difference between two atomic absorption measurements of similar magnitude (total minus bound zinc), a protein concentration measurement, which has much higher precision, was substituted for one of the atomic absorption measurements. This procedure was not used for the determination of free magnesium concentration. In this case the atomic absorption measurements used to calculate the free Mg$^{2+}$ concentration differed from one another by at least 1 order of magnitude. Consequently the error involved in the two measurements was insignificant as compared with their difference.

No determinations of the relative binding constants at pH
values lower than 8.16 are reported. In order to obtain measurable amounts of Mg^{2+} bound to the enzyme (>0.15 g atom per subunit) at these or lower pH values, MgCl_2 concentrations greater than 0.05 M must be employed. Such concentrations brought about a slow crystallization of the enzyme with resulting uncertainties in the attainment of equilibrium and in the measurement of bound metals. Although it was not possible to determine the relative binding constant at pH values lower than 8.16, it was possible to demonstrate that the enzyme could be at least partially activated by Mg^{2+} at pH values as low as 7.5. Incubation of the zinc-zinc enzyme with 0.5 M MgCl_2 at pH 7.5 led to the formation of crystals which contained at least 0.63 g atom of Mg^{2+}/subunit and possessed an enhanced specific activity (1.61 μmol/min/mg) commensurate with the magnesium content (39). These results indicate that magnesium can replace zinc at the activation site at pH values around neutrality if one can obtain a high enough ratio of magnesium to zinc ions to compete for the site. Attempts to drive this displacement to completion by performing the incubation in dialysis sacks which were placed in large volumes of zinc-free MgCl_2 solutions were only partially successful. Under such conditions zinc ions were also removed from the structural site (site 2), leading to inactivation and precipitation of a large portion of the enzyme (38).

Another comment is that the treatment proposed in this paper ignores electrostatic interactions (38). Since the measurements involved determining the ratio of binding constants rather than individual constants, electrostatic effects, which should be approximately equal for Mg^{2+} and Zn^{2+}, should cancel out in the ratio determination.

The question as to whether or not factors other than OH\(^{-}\) could explain the pH activation phenomenon warrants further discussion. In order to avoid complications from ions which chelate metals and whose concentrations change with pH, such as ammonia, carbonate, or Tris, buffers were prepared from either N-ethylmorpholine or trimethylamine, both of which show little tendency to bind divalent ions (33). This left OH\(^{-}\) as the primary component of the system which could bind metals and whose concentration would change with pH. The possibility proposed by Melbve (17) that ionization of functional groups on the protein are involved seems unlikely. Functional groups are better chelators at high pH as it is the tree electrons in the deprotonated form that participate in the metal-ligand association, or in other words, at high pH the H\(^{+}\) concentration is low enough to allow the metal ions to compete for association with the ligand anion. In addition, the functional groups present in proteins which associate with zinc and magnesium all bind zinc more strongly than magnesium (18). To propose that the ionization of a functional group on the protein at increased pH causes a stronger association with Mg^{2+} compared to Zn^{2+} goes against all previous reports in this field. In most likelihood, the reverse is true, namely, that increased pH causes an even stronger association with Zn^{2+} compared to Mg^{2+}.

The theoretical treatment used to calculate the effect of [OH\(^{-}\)] on the concentration of free Zn^{2+} in solution is based on the work of Fulton and Swinehart (34), who determined that, at pH values around 9 the predominant species of zinc at 25\(^{\circ}\) are Zn^{2+}, ZnOH\(^{+}\), and Zn(OH)_2. It is assumed that these same equilibria are predominant at 37\(^{\circ}\). Fulton and Swinehart (34) determined the constants for the following two equilibria at 25\(^{\circ}\):

\[
\begin{align*}
\text{Zn(OH)}_2(s) & \rightleftharpoons \text{Zn(OH)}_3; \quad K_{s2} \\
\text{Zn(OH)}_3(s) & \rightleftharpoons \text{Zn}^{2+} + 2\text{OH}^-; \quad K_{sp}
\end{align*}
\]

and reported values of \(K_{s2}\) equal to \(4 \times 10^{-6}\) M and \(K_{sp}\) equal to \(7 \times 10^{-14}\) M. Rearrangement and addition of these two equations gives

\[
\text{Zn}^{2+} + 2\text{OH}^- \rightleftharpoons \text{Zn(OH)}_3; \quad K_{s2}/K_{sp}
\]

where \(K_{s2}/K_{sp}\) is equal to \(\beta_2\) of Equation 15. The value of \(\beta_2\) at 25\(^{\circ}\) of 5.73 \times 10^{-11} \text{ M}^{-4}, calculated from the Fulton and Swinehart constants, can be compared with the value reported here for \(\beta_2\) of 4.42 \times 10^{-11} \text{ M}^{-4}, calculated from the binding data obtained at 37\(^{\circ}\). Although these values differ by a factor of 10, the difference is not out of line with what might be expected from the effect of temperature on the stepwise stability constants of Zn^{2+} with other ligands such as ethylenediamine (39, 40). In addition, Perrin (41) has reported a value of 2.4 \times 10^{-11} \text{ M} for the equilibrium constant of the reaction

\[
\text{Zn}^{2+} + \text{H}_2\text{O} \rightleftharpoons \text{ZnOH}^+ + \text{H}^+
\]

at 36\(^{\circ}\). If this constant is divided by the ionization constant of water at 35\(^{\circ}\), a new constant results, which is \(K_{s}\), the first stepwise constant for the association of Zn^{2+} and OH\(^{-}\) at 36\(^{\circ}\). Doing this, the value of \(K_1\) is found to be 2.40 \times 10^{13} \text{ M}^{-1}. If \(\beta_2\) equals 4.42 \times 10^{13} \text{ M}^{-2}, then \(K_{s}\), the second stepwise association constant, must be equal to 1.84 \times 10^{12} \text{ M}^{-1}. Thus, \(K_1\) and \(K_{s}\) are of equal magnitude, in agreement with the general observation that the n stepwise association constants between a metal with a high coordination number and univalent anions are usually of similar magnitude (42).

Michaelis-Menten Parameters—Since different assay conditions were used in this investigation than in previous work, new measurements were made of \(K_m\) and \(V_{max}\) for both the magnesium-zinc and zinc-zinc enzymes. These results agree with previous observations (13, 23, 43) in that \(K_m\) is not changed significantly by substitution of Mg^{2+} for Zn^{2+}, while \(V_{max}\) is increased approximately 12-fold. The actual values of \(K_m\) for the zinc-zinc and magnesium-zinc enzymes (4.13 and 2.87 M respectively) are somewhat higher than those reported by Lauch et al. (23). However, this difference may be attributed to different assay conditions plus the limitations imposed by the low solubility of the substrate. Because of the low substrate solubility, \(K_m\) values reported here as well as in Lauch et al. (23) are estimated from a small range of substrate concentrations, all of which are below \(K_m\), and consequently are subject to considerable error.

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