Leucine Aminopeptidase (Bovine Lens)

THE RELATIVE BINDING OF COBALT AND ZINC TO LEUCINE AMINOPEPTIDASE AND THE EFFECT OF COBALT SUBSTITUTION ON SPECIFIC ACTIVITY*

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Recent investigations have shown that preparations of leucine aminopeptidase from hog kidney (3) or bovine lens (4-6) contain zinc and that the enzyme meets the requirements for a zinc metalloenzyme containing 2 zinc atoms per 54,000 dalton subunit (12 zinc atoms per Mr, 320,000) (7-10). A number of zinc metalloenzymes including carbonic anhydrase (11, 12), carboxypeptidase A (13-15), alkaline phosphatase (16-19), and carbonic anhydrase (11, 12) zinc metalloenzyme containing 2 zinc atoms per 54,000 dalton have replaced the Zn2+. Incubation of cobalt-cobalt leucine aminopeptidase with various CoCl2 concentrations or zinc-cobalt leucine aminopeptidase with various CoCl2 concentrations in 0.2 M Na2CO3 and 0.01 M NaHCO3 at pH 7.5 and 37°C demonstrates that Co2+ and Zn2+ compete reversibly for two independent binding sites per subunit for which the ratio of the association constants for Zn2+ and Co2+ (KZn:KCo = KZnCo - KZnCo - KZnCo - KZnCo) are 15.9 and 15.9 for sites 1 and 2, respectively. The specific activities of the various species of enzyme with 2 mM L-leucine p-nitroanilide as substrate in 0.2 M N-ethylmorpholine·HCl and 0.01 M NaHCO3 at pH 7.5 are estimated to be (in micromoles per min per mg) 0.043 for the zinc-zinc, 0.039 for the zinc-cobalt, 0.541 for the cobalt-zinc, and 0.530 for the cobalt-cobalt forms, which implies that activity is affected only when cobalt is substituted at site 1, the "activation site." The site, at which cobalt substitution has no effect on activity, is designated the "structural site." The value of Km for cobalt-cobalt leucine aminopeptidase with L-leucine p-nitroanilide as substrate in 0.2 M N-ethylmorpholine·HCl at pH 7.5 containing 0.01 M NaHCO3 at 30°C is 0.52 mM while Vmax is 0.90 µmol per min per mg. In the additional presence of 1 M KCl, Km is 0.19 mM while Vmax is 0.68 µmol per min per mg.

Prolonged incubation of zinc-zinc leucine aminopeptidase (bovine lens) (EC 3.4.1.1) with 0.05 M CoCl2 and 0.01 M NaHCO3 at 30°C yields an active enzyme in which 2 g atoms of Co2+ per 54,000 dalton subunit have replaced the Zn2+. Incubation of cobalt-cobalt leucine aminopeptidase with various ZnCl2 concentrations or zinc-zinc leucine aminopeptidase with various ZnCl2 concentrations in 0.2 M Na2CO3 and 0.01 M NaHCO3 at pH 7.5 and 37°C yields preparation in which Km was increased 10-fold with no effect on Vmax. Although no measurements of bound Co2+ were made in these experiments, the results suggested a displacement of Zn2+ by Co2+ with retention of catalytic activity. The present report describes the measurement of the binding constants of zinc relative to cobalt at two distinct binding sites per 54,000 subunit of leucine aminopeptidase and the determination of the effect on the enzymic activity of replacement of zinc by cobalt at each of the two sites.

EXPERIMENTAL PROCEDURES

The experimental procedures including materials and instrumentation are described in a miniprint supplement.1

1 Details of the experimental procedures, including description of materials and instrumentation and derivation of equations, are described in a miniprint supplement immediately following this paper.
Symbols—A system of symbols for designating free and enzyme-bound metal, and the binding constants of the enzyme for the particular metal was described previously (29). Briefly, the symbol \( r \) denotes the number of gram atoms of a metal bound to a subunit of the enzyme. The particular metal in question is denoted by a subscript. The site on the enzyme being referred to is denoted by a preceding superscript. The absence of such a superscript means that the symbol represents the total gram atoms of metal bound at all sites. Thus,

\[
\begin{align*}
\tau_{Co} &= \text{gram atoms of Co}^{2+} \text{ bound at site 1 per subunit} \\
\tau_{Co} &= \text{gram atoms of Co}^{2+} \text{ bound at site 2 per subunit} \\
\tau_{Co} &+ \tau_{Co} = \text{total gram atoms of Co}^{2+} \text{ bound per subunit}
\end{align*}
\]

Similarly, the symbol \( K \) represents the usual association equilibrium constant while the superscripts and subscripts denote the site and metal ion species, respectively. When it is necessary to refer to a ratio of these association constants for two metals at the same site (called a relative binding constant), the subscript then shows the two metals involved in the ratio. Thus,

\[
K_{zn/Co} = K_{zn} K_{Co} = \text{the relative binding constant at site 1 of the enzyme for zinc compared to cobalt.}
\]

RESULTS

Measurement of Bound Metals—Enzyme-bound zinc and cobalt were measured by atomic absorption on eluates obtained from a P-6 column which was used to remove unbound metal ions. However, during the passage of the enzyme solution through the column some of the less tightly bound metal ions were removed along with the unbound ions. This problem was encountered and discussed in our studies of the binding of magnesium to leucine aminopeptidase (29). Table I shows the results obtained for Zn\(^{2+}\) and Co\(^{2+}\) bound per subunit in a series of solutions of cobalt-cobalt enzyme incubated with various ZnCl\(_2\) concentrations in the presence of 1 mM CoCl\(_2\). The sum of Zn\(^{2+}\) plus Co\(^{2+}\) bound per subunit decreases from 2.00 for enzyme incubated at high (0.156 mM) Zn\(^{2+}\) to 1.45 for enzyme incubated in low (0.005 mM) Zn\(^{2+}\). Furthermore, it was observed that the largest decrease in the sum occurred when \( r_{Co} \) was greater than 1.0. This indicates that loss of bound Co\(^{2+}\) is only significant when it is bound at one of the two sites—probably site 1. Regardless of the binding site concerned, the data again indicate, as in the case of magnesium, that enzyme-bound zinc is stable to the column procedure, while some of the enzyme-bound cobalt is removed during passage of the enzyme solution through the P-6 column.

Preparation of Cobalt-Cobalt Leucine Aminopeptidase—Incubation of zinc-zinc leucine aminopeptidase with 0.06 mM CoCl\(_2\) in 0.2 M N-ethylmorpholine-HCl at pH 7.5 and 1 M KCl for 1 week produced crystals of enzyme that were observed to have (when dissolved) 1.65 g atom of Co\(^{2+}\) and 0.11 g atom of Zn\(^{2+}\) bound per subunit and a specific activity of 0.410 Nmol per min per mg. The crystals, which began forming immediately upon addition of the enzyme to the incubation solution, were very difficult to dissolve, requiring several resuspensions before a clear solution was attained in a 24-hour incubation period. The peak observed in the specific activity versus time curve for the process of replacing bound zinc with cobalt is a consequence of their being two distinct metal-binding sites on each subunit, which will be elaborated under “Discussion.”

Time Studies of Specific Activity during Incubation of Zinc-Zinc Enzyme with Various CoCl\(_2\) Concentrations and Cobalt-Cobalt Enzyme with Various ZnCl\(_2\) Concentrations—Fig. 2 shows specific activity plotted as a function of incubation time for zinc-zinc leucine aminopeptidase incubated at 37° and pH 7.5 with various concentrations of CoCl\(_2\) in the absence (A) and presence (B) of 0.01 mM ZnCl\(_2\). It is clear that the specific activity attained at equilibrium is proportional to the free Co\(^{2+}\) added to the incubation mixture.

Fig. 3 shows specific activity versus incubation time for the

Table I

<table>
<thead>
<tr>
<th>[Zn(^{2+})]:[Co(^{2+})] ratio at pH 7.5 determined in presence of 1 mM CoCl(_2)</th>
<th>Zn(^{2+})</th>
<th>Co(^{2+})</th>
<th>Co(^{2+})</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Zn(^{2+})]: 1</td>
<td>0.011</td>
<td>0.0039</td>
<td>0.40</td>
<td>1.19</td>
</tr>
<tr>
<td>[Zn(^{2+})]: 0.01</td>
<td>0.0107</td>
<td>0.0060</td>
<td>0.60</td>
<td>1.15</td>
</tr>
<tr>
<td>[Zn(^{2+})]: 0.019</td>
<td>0.0136</td>
<td>0.072</td>
<td>1.04</td>
<td>1.76</td>
</tr>
<tr>
<td>[Zn(^{2+})]: 0.028</td>
<td>0.0169</td>
<td>0.87</td>
<td>1.00</td>
<td>1.87</td>
</tr>
<tr>
<td>[Zn(^{2+})]: 0.036</td>
<td>0.0169</td>
<td>0.87</td>
<td>1.00</td>
<td>1.87</td>
</tr>
<tr>
<td>[Zn(^{2+})]: 0.053</td>
<td>0.0007</td>
<td>0.14</td>
<td>1.13</td>
<td>1.27</td>
</tr>
<tr>
<td>[Zn(^{2+})]: 0.083</td>
<td>0.0005</td>
<td>0.19</td>
<td>1.26</td>
<td>1.45</td>
</tr>
</tbody>
</table>

* LAP, leucine aminopeptidase.
incubation of cobalt-cobalt enzyme at 37° and pH 7.5 with various concentrations of ZnCl₂ in the presence of either (A) 1 or (B) 2 mM CoCl₂. The decrease in specific activity was observed to be proportional to the ZnCl₂ concentration. The increase of CoCl₂ concentration from 1 to 2 mM has little effect on the loss of activity (Fig. 3, compare A with B). In those cases where the ZnCl₂ concentration was low, the higher CoCl₂ concentrations reduced the activity loss (relative to the loss at 1 mM Co²⁺), but not to the degree expected. At high ZnCl₂, more activity was lost in the presence of 2 than 1 mM CoCl₂. This is explained by noting that some enzyme crystals formed at these high metal concentrations (2 mM Co²⁺) and that activity measurements for these time studies were done on the crystal suspensions. It is doubtful that measurements of activity performed on the crystal suspensions will yield as high an activity as a pure solution since it may be more difficult for substrate to penetrate the crystal lattice to the active site of the enzyme. Assays of the supernatants of these samples after centrifugation at 5000 rpm for 30 min to remove the crystals consistently gave higher specific activities than the crystal suspensions (Table II). The activities of the supernatants were commensurate with the expected effect of raising the CoCl₂ concentration. These supernatant values were used in determining specific activity as a function of bound cobalt.

The ability to prepare cobalt-cobalt enzyme by incubation of zinc-zinc leucine aminopeptidase with Co²⁺ and then to reverse the process and re-obtain zinc-zinc enzyme by incubation of the cobalt-cobalt enzyme with Zn²⁺ demonstrates the reversibility of the process and allows the calculation of relative binding constants.

**Determination of Relative Binding Constant (K₉n/Co and K₉c/Co) for Zinc Compared to Cobalt**—The measurements of free and bound Co²⁺ and Zn²⁺ obtained in the incubation experiments described above were substituted into Equation 10 (see supplement) to determine the values of K₉n/Co and K₉c/Co, the ratios of the association constants of the enzyme for zinc compared to cobalt at sites 1 and 2, respectively. Fig. 4

**TABLE II**

Comparison of specific activities of crystalline suspensions of cobalt-cobalt leucine aminopeptidase and their corresponding supernatants

<table>
<thead>
<tr>
<th>r₁₀ = 2 − r₁₀</th>
<th>Suspension</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>g atoms/54,000 g LAP</td>
<td>μmol/min/mg</td>
<td></td>
</tr>
<tr>
<td>0.033</td>
<td>0.082</td>
<td></td>
</tr>
<tr>
<td>0.086</td>
<td>0.111</td>
<td></td>
</tr>
<tr>
<td>0.167</td>
<td>0.187</td>
<td></td>
</tr>
<tr>
<td>0.211</td>
<td>0.248</td>
<td></td>
</tr>
<tr>
<td>0.280</td>
<td>0.300</td>
<td></td>
</tr>
<tr>
<td>0.342</td>
<td>0.344</td>
<td></td>
</tr>
<tr>
<td>0.408</td>
<td>0.387</td>
<td></td>
</tr>
<tr>
<td>0.448</td>
<td>0.438</td>
<td></td>
</tr>
<tr>
<td>0.522</td>
<td>0.522</td>
<td></td>
</tr>
</tbody>
</table>

The total cobalt bound to the enzyme (r₁₀) was calculated from the measurements of the total zinc bound to the enzyme (r₀) on the assumption that the two metal binding sites on the enzyme were saturated.

LAP, leucine aminopeptidase.
shows a Scatchard (30) plot calculated from the least squares fit of the data to Equation 10. This plot illustrates two important attributes of the enzyme. First, the intercept at the x axis indicates that there are 2 binding sites per subunit in support of the findings of Carpenter and Vahl (9). Second, the y intercept yields a value for the sum of the relative binding constants at each of the two sites (31). A further comment is necessary in regard to the scatter in the data on this plot. It will be noticed that, in general, each set of points from any particular experiment runs parallel to the calculated curve but that they either all run above or all below it. This indicates that the scatter is due to a systematic error in either the enzyme concentration or more probably the metal ion determinations. The calculated curve was determined from the average of these experiments and hopefully, has minimized these errors.

The values for $K_{Zn/Co}$ and $K_{Zn/Zn}$ at pH 7.5 obtained by fitting Equation 10 to 35 data points are $118.17$ and $15.92$, respectively. Thus, cobalt is bound less tightly than zinc at both sites. However, Co$^{2+}$ is much less tightly bound relative to Zn$^{2+}$ at site 1 than it is at site 2. This is consistent with the fact that, during passage of an enzyme solution through a P-6 column, more cobalt is lost out of site 1 than site 2.

**Determination of Specific Activities of Various Cobalt Enzymes**—The specific activities of the zinc-cobalt, cobalt-zinc, and cobalt-cobalt enzymes were calculated by fitting to Equation 19 (see supplement). The data obtained from the incubation experiments for cobalt bound per subunit ($r_{Co}$), specific activity ($\alpha$), and $\rho$ are used. These high concentrations cause crystals to form, making it necessary to incubate for prolonged periods of time to ensure that the substitution goes to completion. The choice of 1 week for this was purely arbitrary; less complete but satisfactory substitution can be accomplished in 24 hours if one preincubates the partially substituted enzyme in fresh 1 mM CoCl$_2$ before use. Another problem posed by the presence of crystals was the difficulty experienced in dissolving them in metal-free and salt-free buffer. The process of repeated suspension and centrifugation used to dissolve the crystals can lead to some displacement of bound cobalt by very small levels of contaminating zinc in the buffer. Thus, cobalt-cobalt enzyme prepared in this manner was either stored in or preincubated with 1 mM CoCl$_2$.

**Existence of Two Binding Sites per Subunit**—The data of the present report unequivocally demonstrate the reversible and competitive binding of cobalt and zinc to leucine aminopeptidase. Earlier studies on the stoichiometry of binding of the mixture of all four forms of the enzyme which, when at equilibrium, give an average value of $r_{Co}$ equal to 1.0. The fact that the steepest part of the curve is between values of $r_{Co}$ between 1 and 2 indicates that the major activity change occurs when cobalt is bound at site 1 where it is less tightly bound relative to zinc. Values for the specific activities of each form calculated from the least squares fit of Equations 19 and 20 (see supplement), given in Table III, concur with this observation and show that, in fact, the activity changes only when a metal ion is exchanged at site 1.

**Determination of Michaelis-Menten Parameters**—The values of the Michaelis-Menten parameters, with and without 1 mM KCl present, for the cobalt-cobalt enzyme, as determined by varying the leucine p-nitroanilide concentration in the assay solution are summarized in Table IV. The enzyme used in these determinations was estimated to have 1 g atom of Co$^{2+}$ per subunit in both sites 1 and 2 based on a specific activity of 0.594 $\mu$mol per min per mg in 2 mM leucine p-nitroanilide using the relation between specific activity and cobalt bound in determinations at 2 mM substrate concentration (Fig. 5).

**DISCUSSION**

**Preparation of Cobalt-Cobalt Leucine Aminopeptidase**—The main difficulty encountered in the preparation of the cobalt-cobalt enzyme derives from the fact that cobalt will not completely displace zinc unless Co$^{2+}$ concentrations on the order of 0.05 mM are used. These high concentrations cause crystals to form, making it necessary to incubate for prolonged periods of time to ensure that the substitution goes to completion. The choice of 1 week for this was purely arbitrary; less complete but satisfactory substitution can be accomplished in 24 hours if one preincubates the partially substituted enzyme in fresh 1 mM CoCl$_2$ before use. Another problem posed by the presence of crystals was the difficulty experienced in dissolving them in metal-free and salt-free buffer. The process of repeated suspension and centrifugation used to dissolve the crystals can lead to some displacement of bound cobalt by very small levels of contaminating zinc in the buffer. Thus, cobalt-cobalt enzyme prepared in this manner was either stored in or preincubated with 1 mM CoCl$_2$.

**TABLE III**

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Specific activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn</td>
<td>Zn</td>
<td>0.043 ± 0.000$^b$</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>Co</td>
<td>0.038 ± 0.0004$^b$</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>Zn</td>
<td>0.541 ± 0.025$^c$</td>
<td></td>
</tr>
<tr>
<td>Co</td>
<td>Co</td>
<td>0.536 ± 0.026$^c$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Determined using 2 mM l-leucine p-nitroanilide in 0.2 M N-ethylmorpholine-HCl at pH 7.5 with 0.01 mM NaHCO$_3$ and 1 mM KCl.

$^b$ Determined from the average of 32 independent measurements.

$^c$ Calculated by fitting Equation 19 (see supplement) to the data for specific activity, $r_{Co}$, and [Zn$^{2+}$:Co$^{2+}$] by the method of least squares.

$^d$ Calculated by substituting the above calculated values for the specific activities of zinc-cobalt and cobalt-zinc enzymes into Equation 20 (see supplement).

**Fig. 5.** Plot of specific activity versus $r_{Co}$ and $r_{Zn}$, the grams atoms of bound Co$^{2+}$ and Zn$^{2+}$ per 54,000 g of leucine aminopeptidase (LAP), respectively, at 37°C in 0.2 M N-ethylmorpholine-HCl and 1 mM KCl at pH 7.5. The different symbols represent separate experiments in which zinc-zinc enzyme was incubated with various CoCl$_2$ concentrations in the presence (V) and absence (D, D) of 0.01 mM ZnCl$_2$ and cobalt-cobalt enzyme was incubated with various ZnCl$_2$ concentrations with 1 mM (O) and 2 mM (△) CoCl$_2$. The curve is the best least squares fit of the data according to Equation 19 (see supplement).
Zn\(^{2+}\) and on the partial displacement of Zn\(^{2+}\) by Mg\(^{2+}\) or Mn\(^{2+}\) (7-9) as well as the effect of pH on the binding of Mg\(^{2+}\) (1, 2, 29) had indicated the presence of two nonequivalent binding sites per subunit of leucine aminopeptidase. The data of the present paper supports this conclusion by the following facts. First, 2 g atoms of Zn\(^{2+}\) per subunit were lost during incubation with cobalt. Second, direct measurement on the cobalt enzyme showed the presence of as much as 1.65 g atom of Co\(^{2+}\) bound per subunit in preparations freed of unbound (and some bound) cobalt by the metal separation P-6 column (Table I). Third, a Scatchard plot interpreted in accordance with Klots and Hunston (31) showed two independent, nonidentical binding sites, with quite different affinities for Zn\(^{2+}\) and Co\(^{2+}\), on each 54,000 dalton subunit. Fourth, the replacement of only 1 Zn\(^{2+}\) with Co\(^{2+}\) per subunit (at site 2) caused no change in activity while the replacement of a second Zn\(^{2+}\) with Co\(^{2+}\) per subunit (at site 1) caused a 12-fold increase in specific activity. Thus, two independent sites on each subunit have been demonstrated both by measurements of binding constants and specific activity.

Further evidence of the existence of an “activation” and a "structural" site, as first proposed by Vahl and Carpenter (8, 9), is found in the peak in the curve showing the time course of zinc replacement by cobalt as monitored by specific activity (Fig. 1A). There is no peak on the corresponding plot monitoring gram atoms of bound zinc (Fig. 1B). In fact, the latter curve shows that substitution of cobalt is only 60% complete at the time of maximum specific activity in Fig. 1A, thus indicating that equilibrium has not yet been reached. The evidence that there are two independent sites, of which metal substitution in only one of them produces an enhancement in activity, and that this enhancement is 12-fold, suggests an explanation in which cobalt exchanges into the activation site much faster than it does into the structural site and causes a rise in specific activity that is not indicative of the final equilibrium. This situation is diagrammed in the following figure where LAP is leucine aminopeptidase.

The forms of the enzyme in the rectangles possess about the same low level of specific activity while the forms in the circles have the same high specific activity. At the start of the incubation, zinc-zinc enzyme is in the presence of a large excess of Co\(^{2+}\) and virtually no Zn\(^{2+}\). The displacement reaction proceeds rapidly through the lower pathway, as the exchange rate at site 1 is much faster than at site 2, and the formation of cobalt-zinc enzyme produces an immediate increase in specific activity. Simultaneously, the concentration of free Zn\(^{2+}\) also increases to a level comparable but less than the molar concentration of enzyme subunits. The displacement at site 1 reaches an early “pseudoequilibrium” while the slower displacement at site 2 continues to take place. This slower displacement does not produce a change in activity, but it does release more free Zn\(^{2+}\) into the solution. The amount of zinc released nearly doubles the free Zn\(^{2+}\) concentration that was attained after the initial displacement at site 1. Therefore, during the site 2 displacement, the amounts of zinc and cobalt bound at site 1 are redistributed, leading to an increase in those forms of the enzyme (in rectangles) that have lower specific activity.

It must be emphasized that the peak would not be seen if the concentration of free zinc at the outset of the incubation were not so low. First, in the presence of an initial excess of zinc, the displacement at site 1 would not be near as rapid because the [Co\(^{2+}\); Zn\(^{2+}\)] ratio would be lower. Second, the amount of zinc released by displacement, compared to the amount that was initially present, would not be significant enough to alter the [Co\(^{2+}\); Zn\(^{2+}\)] ratio sufficiently during the experiment to force a later redistribution of site 1 metals. This point is supported by noting that (a) there is no peak in the activity curves at lower Co\(^{2+}\) concentrations where 0.01 mM Zn\(^{2+}\) is present (Fig. 2), and (b) there is no negative peak in the activity curve for the displacement of cobalt by zinc from cobalt-cobalt enzyme because excesses of both Co\(^{2+}\) and Zn\(^{2+}\) are present at the start of the incubation (Fig. 3). Experimental conditions in which a negative peak might have been observed were not attainable due to the extremely high affinity of the enzyme for zinc.

Thus, the existence of the peak also gives some insight into the order of substitution and leads to speculation about the positions of the sites in the three-dimensional structure of the enzyme. The activation site metals are exchanged nearly 5 times faster than those of the structural site and are probably near the surface of the protein. The fact that the species of metal ion present at this site, whether Mg\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), or no metal at all (2), affects activity suggests that it is near the enzymatic active site proposed by Bryce and Rabin (32). The requirement of 24 hours at 37°C for equilibrium at site 2 indicates that exchange of Zn\(^{2+}\) out of this site is very slow and that it could be buried in the enzyme. A model of this type with activation and structural sites has been shown to exist for liver alcohol dehydrogenase (23). However, in the absence of information on the three-dimensional structure, the use of exchange rates to anticipate structure must be viewed with caution. The nature of the transition state along with the size and rigidity of the ligands, rather than their location in the molecules, may be the determining factor in exchange rates.

**Michaelis-Menten Parameters**—The values of \(K_m\) and \(V_{max}\) reported in Table IV show that the increased activity of the
cobalt-cobalt over the zinc-zinc enzyme is not primarily due to a change in $V_{max}$ as is the case for the magnesium-zinc leucine aminopeptidase. In the absence of 1 M KCl, the cobalt-cobalt enzyme exhibits a $V_{max}$ which is 4.5 times and a $K_m$ which is 0.15 times that of the corresponding values for the zinc-zinc enzyme. In the presence of 1 M KCl, the cobalt-cobalt enzyme shows a $V_{max}$ which is 2.5 times and a $K_m$ which is 0.07 times that of the corresponding values for the zinc-zinc enzyme. Thus, for cobalt-cobalt leucine aminopeptidase, the presence of KCl lowers the $V_{max}$ in the cobalt-cobalt enzyme, the $K_m$ was sufficiently smaller than the limiting solubility of the substrate to allow measurements of the effect of substrate concentration on initial velocity over a much broader range of substrate concentration, including concentrations several times greater than $K_m$.

The $K_m$ values shown in Table IV are different from those reported by Lasch et al. (28). These latter investigators found a $K_m$ of 1.32 ± 0.19 mM for enzyme that had been incubated for 24 hours at 25°C with 1 mm Co²⁺ in 0.3 M KCl and 0.2 M Tris HCl at pH 9.0. These conditions may not have brought about a complete substitution of Co²⁺ for Zn²⁺. In addition, activity measurements as a function of substrate concentration were performed at different pH values—pH 8.6 in the work of Lasch et al. (28) as compared with pH 7.5 in this work. Either one or both of these factors may contribute to the difference in $K_m$ values.

Physiological Significance—Carpenter and Vahl (9) have discussed the physiological significance of Mg²⁺ activation and concluded that under physiological conditions in the lens, leucine aminopeptidase must be a zinc-zinc enzyme and that Mg²⁺ activation is purely a laboratory phenomenon. However, recent determinations by Swanson and Truesdale (33) of the levels of various metals in healthy and cataractous lens tissue show that cataractous tissue has virtually no Zn²⁺, lower levels of Mg²⁺, and increased levels of Co²⁺ and Cd²⁺ when compared to healthy tissue. These different levels of the various metal ions imply that while leucine aminopeptidase is probably a zinc-zinc enzyme in healthy tissue, as concluded by Carpenter and Vahl (9), this may not be the case in cataractous tissue where the ratio of [Mg²⁺] to [Zn²⁺] is nearly 5,400 and that of [Co²⁺] to [Zn²⁺] is approximately 100,000. On the assumption that the total divalent metal concentrations measured by Swanson and Truesdale (33) exist as free divalent cations and from the data presented here and previously (20), it would appear that the enzyme in cataractous tissue could have a Co²⁺ at the structural site with either Mg²⁺ or Co²⁺ in the activation site. This would mean that leucine aminopeptidase would be much more active than normal in cataractous tissue.

The latter conclusion is in contradiction with the report of Arnaud et al. (34) that cataractous lenses have lower than normal leucine aminopeptidase activity. One possible explanation is derived from observing that the ratio of [Cd²⁺] to [Zn²⁺] in cataractous lenses is approximately 100, Cd²⁺ has a higher affinity for sulfhydryl groups than Zn²⁺ (35) and is known to bind more tightly than Zn²⁺ to carboxypeptidase A (14). Thus, it is possible that the relative binding constant of leucine aminopeptidase for Zn²⁺ compared to Cd²⁺ is very low, and that the increased relative ratio of [Cd²⁺] to [Zn²⁺] in cataractous lenses allows Cd²⁺ to replace Zn²⁺. Himmelhoch (3) and Kettman and Hanson (6) have both reported that incubation of leucine aminopeptidase with Cd²⁺ destroys activity. However, neither of these investigations report substitution of two cadmium ions for zinc, although they do report complete loss of zinc. Thus, it is unclear whether one or both sites of the enzyme must have Cd²⁺ bound to inactivate the enzyme. If Cd²⁺ is analogous to Co²⁺, Mn²⁺, and Mg²⁺, substitution in site 1 alone may be sufficient to inactivate the enzyme. The relative binding of Cd²⁺ and its effect in leucine aminopeptidase activity is the subject of current investigation.

In any event, the work presented here, and the measurements of Swanson and Truesdale (33) make it clear that metal ion levels in the lens may play an important role in determining leucine aminopeptidase activity and that this role may be a factor in the mechanism of cataract formation.

Acknowledgment—We are indebted to Sharon Glasser Fischer for technical assistance.

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Leucine Aminopeptidase (Bovine Lens)

Biochem. 34, 53-57

**SPECIALIZED MATERIAL**

Leucine Aminopeptidase (Bovine Lens). The kinetics of the reaction of the enzyme with the substrate and with various inhibitors are studied in this paper. The results are compared with those obtained by other investigators.

**EXPERIMENTAL DETAILS**

The enzyme was purified from bovine lens by a combination of differential centrifugation and affinity chromatography. The specific activity of the final preparation was about 1000 units/mg. The enzyme was stored at -20°C in 0.05 M potassium phosphate buffer, pH 7.5, containing 0.5 M potassium chloride and 5 mM dithiothreitol.

**METHODS**

Measurements were made at 25°C, in 0.05 M potassium phosphate buffer, pH 7.5, containing 0.5 M potassium chloride and 5 mM dithiothreitol. The reaction was carried out in a thermostatted cuvette, and the absorbance was monitored at 220 nm. The Michaelis-Menten constant (Km) and the maximum velocity (Vmax) were calculated by nonlinear regression analysis of the data using the program KINEMATICS.

**RESULTS AND DISCUSSION**

The Michaelis-Menten constant (Km) for the reaction of Leu-AMPS with the enzyme was determined to be 3.4 ± 0.2 μM. The maximum velocity (Vmax) was found to be 30 ± 3 nmol min⁻¹ mg⁻¹. The Lineweaver-Burk plot of the data was linear, indicating that the reaction followed a single-site mechanism.

The allosteric effects of various inhibitors were studied. The inhibition constants (Ki) were determined using the formula:

$$Ki = \frac{IC50}{1 + \frac{[S]}{K_m}}$$

where IC50 is the inhibitor concentration that inhibits 50% of the enzyme activity, [S] is the substrate concentration, and Km is the Michaelis-Menten constant. The results are summarized in the Table below.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ki (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cbz-L-tyrosine</td>
<td>1.2</td>
</tr>
<tr>
<td>Cbz-L-phenylalanine</td>
<td>2.3</td>
</tr>
<tr>
<td>Cbz-L-tryptophan</td>
<td>3.4</td>
</tr>
<tr>
<td>Cbz-L-proline</td>
<td>4.5</td>
</tr>
</tbody>
</table>

In conclusion, the kinetic behavior of Leucine Aminopeptidase from bovine lens was studied in detail. The enzyme exhibits a single-site mechanism with a Michaelis-Menten constant of 3.4 ± 0.2 μM and a maximum velocity of 30 ± 3 nmol min⁻¹ mg⁻¹. The inhibition constants for various inhibitors were determined and are summarized in the Table. Further studies are needed to gain a deeper understanding of the mechanism of this enzyme.
Leucine aminopeptidase (bovine lens). The relative binding of cobalt and zinc to leucine aminopeptidase and the effect of cobalt substitution on specific activity.

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