Metal Binding Stoichiometry and Mechanism of Metal Ion Modulation of the Activity of Porcine Kidney Leucine Aminopeptidase†

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ABSTRACT: Porcine kidney leucine aminopeptidase has been obtained from commercial sources as an inhomogeneous preparation with variable metal content and purified by affinity chromatography over L-leucylglycyl-AH-Sepharose. Treatment with Zn^{2+} followed by gel filtration restores the Zn^{2+} content of the native enzyme, which is 6 mol of Zn^{2+} per hexamer, each of which is located at a single catalytic binding site per subunit. The activity of the native enzyme is modulated by incubation with divalent metal ions; it is activated by Mn^{2+} and Mg^{2+} and inhibited by Ni^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, and Cd^{2+}. These metals modulate the activity by binding to a separate site on each subunit, referred to as the regulatory site. Binding of these metals at the regulatory site alters the activity of the enzyme by changing k_{cat}, leaving K_M unaltered. The number and nature of the metal binding sites of porcine kidney leucine aminopeptidase are very similar to those of the enzyme from bovine lens.

Leucine aminopeptidase (LAP)[1] (EC 3.4.11.1) is one of a broad class of zinc metallopeptidases that is involved in the metabolism of peptides and proteins (Himmelhoch, 1970; Delange & Smith, 1971). Zinc metallopeptidases are usually subdivided into the endopeptidases, such as thermolysin, the C-terminal exopeptidases, such as carboxypeptidases A and B, and the N-terminal peptidases, such as leucine aminopeptidase. From the former two classes of enzymes, thermolysin and carboxypeptidase A have been extensively studied with respect to both their crystal structures and their kinetic properties in solution, and a substantial body of data pertaining to their mechanism of action is in hand. However, in spite of the fact that the aminopeptidases are probably the most widespread of the three classes, very little mechanistic information has been obtained for these enzymes.

The best studied N-terminal metallopeptidase is leucine aminopeptidase from bovine lens. The native enzyme has a molecular weight of 320 000 and is composed of six identical subunits, each of which has two metal binding sites that are occupied by Zn^{2+} (Melbye & Carpenter, 1971; Carpenter & Vahl, 1973). Thus, the native enzyme can be represented $[(LAP)Zn_6Zn_6]$, where (LAP) denotes the hexameric apoenzyme and the brackets indicate the firm binding of metal ions to the two distinct sites of each subunit.[1] The enzyme is activated by incubation with either Mg^{2+} or Mn^{2+}, and Carpenter & Vahl (1973) have demonstrated that this is due to the replacement of one Zn^{2+} per subunit, located at the regulatory site,[2] by these metals. Thus, activation with Mg^{2+} or Mn^{2+} yields $[(LAP)]Zn_6Mg_6]$ and $[(LAP)Zn_6Mn_6]$, respectively. Removal of the Zn^{2+} at the other site results in a total loss of activity which is regained by reconstitution with Zn^{2+}, but not with Mg^{2+} or Mn^{2+} (Carpenter & Vahl, 1973; Thompson & Carpenter, 1976). Hence, this site is referred to as the catalytic site.[3]

While the metal binding properties of bovine lens leucine aminopeptidase have received careful attention, studies of its mechanism of action have not been carried out, and the enzyme is not readily available. In view of the need for detailed studies of the N-terminal metallopeptidases, we have now undertaken a thorough study of the catalytic mechanism of porcine kidney cytosol leucine aminopeptidase. Porcine kidney is a rich source of leucine aminopeptidase, and the enzyme is available commercially in gram quantities.

Interestingly, the porcine enzyme was the first N-terminal metallopeptidase to be studied in any detail and is similar in many ways to that from bovine lens. Smith & Spackman (1955) showed that the enzyme is activated by Mg^{2+} and Mn^{2+} and speculated that these metal ions are bound at the active site. Himmelhoch (1969) subsequently demonstrated that the native enzyme is a zinc metalloenzyme containing between 4 and 6 mol per 300 000 g of protein and no other metals in stoichiometrically significant quantities. Since the porcine enzyme is a hexamer with a molecular weight of 320 000 (Shen & Melius, 1977), this would suggest that there is one metal binding site per subunit and that the native enzyme can be represented $[(LAP)Zn_6]$, where the Zn^{2+} resides at the catalytic site and there is no regulatory site. Himmelhoch also reported that the mechanism of Mn^{2+} activation is by replacement of this single Zn^{2+} per subunit by Mn^{2+} to give $[(LAP)Mn_6]$, in sharp contrast to the case for the enzyme from bovine lens.

In the present study, the metal binding stoichiometry of the porcine kidney enzyme, the mechanism of activation by Mg^{2+} and Mn^{2+}, and the mechanism of inhibition by Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} have been investigated. We find that this enzyme differs from the bovine lens enzyme in that it binds firmly only 1 mol of Zn^{2+} per subunit, in agreement with the

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[1] Abbreviations used: LAP, leucine aminopeptidase; $[(LAP)M_xM_y]$, metalloleucine aminopeptidase where (LAP) represents the hexameric apoenzyme and the brackets indicate the binding of x mol of metal atom, M, at the "catalytic site" and y mol of metal atom, M, at the "regulatory site", respectively, where the terms catalytic and regulatory are defined in the text; $[(LAP)M_x—]$, enzyme with no metal atoms bound at the regulatory site; Tris, tris(hydroxymethyl)aminomethane; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Caps, 3-(cyclohexylamino)propanesulfonic acid.

[2] This site has also been referred to as the "activation site" (Carpenter & Vahl, 1973); however, the more general term "regulatory site" is preferred since binding of other metal ions at this site may cause inhibition. The term regulatory site does not necessarily imply that binding of metal ions to this site is a means used to regulate the activity of the enzyme in vivo.

[3] This site has also been referred to as the "specificity site" (Carpenter & Vahl, 1973) and the "structural site" (Thompson & Carpenter, 1976); however, since the removal of Zn^{2+} at this site results in complete loss of activity, the term "catalytic site" is more appropriate.

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results of Himmelhoch. However, the modulation of the activity of the native enzyme brought about by divalent metal ions is shown to be due to binding at a second "regulatory" site. Thus, the activation by Mg^{2+} and Mn^{2+} is due to the binding of one additional mole of these metals per subunit, rather than by replacement of the catalytic Zn^{2+}.

Materials and Methods

Leucine aminopeptidase was obtained from Sigma Chemical Co. (type III-CP, lot no. 070F8090, 119C8050, and 110F8010) as a chromatographically purified $(NH_4)_2SO_4$ suspension containing 5 mM $MgCl_2$. Unless stated otherwise, this material was dialyzed against 5 mM Tris-HCl, pH 8, at 4 °C for 2 days to remove salts and unbound metal ions and lyophilized before further use. L-Leucine-*p*-nitroanilide and L-leucylglycine were purchased from Sigma Chemical Co., and AH-Sepharose was obtained from Pharmacia.

All assays were carried out spectrophotometrically with L-leucine-*p*-nitroanilide as a substrate by continuously monitoring the appearance of *p*-nitroaniline at 405 nm with a Varian Model 219 spectrophotometer. Initial velocities (v) were calculated from the slope of the absorbance change during the first 10% of hydrolysis and converted into units of moles per liter per minute by using $\epsilon_{405} = 9900\ M^{-1}\ cm^{-1}$ for *p*-nitroaniline (Royer & Andrews, 1973). These rates were converted to molar activity (v/E_0, min^{-1}) by dividing by the enzyme concentration (E_0), assuming a hexameric molecular weight of 320 000. Unless stated otherwise, all assays were carried out at 22 °C in 10 mM Tris-HCl and 1 M KCl, pH 8.0, at a substrate concentration of 1 mM. The concentrations of L-leucine-*p*-nitroanilide and leucine aminopeptidase hexamers were determined spectrophotometrically by using $\epsilon_{320} = 1.39 \times 10^4$ and $\epsilon_{280} = 4.0 \times 10^5\ M^{-1}\ cm^{-1}$, respectively.

The molar extinction coefficient of the enzyme at 280 nm, ϵ_{280}, was determined as described by Carpenter & Vahl (1973). A sample of enzyme was dialyzed exhaustively against metal-free water at pH 7. A portion was diluted into a cuvette, and the absorption spectrum was recorded from 350 to 250 nm. Other portions were pipetted into predryed and preweighed glass shells and placed in a desiccator containing P_2O_5 to remove the water. The shells were weighed and returned to the desiccator, which was evacuated and heated to 100 °C. After 1 h, the desiccator was cooled and the weight of the shells measured as a function of time after opening. Extrapolation to the time of opening gives the relevant weight. This process was repeated until the weight of each shell did not decrease on further heating, this limiting value being the dry weight. The protein concentration was also determined by three other methods. The biuret (Gornall et al., 1949), Lowry (Lowry et al., 1951), and Bio-Rad dye binding assay (Bradford, 1976) methods were used with both bovine serum albumin and bovine γ-globulin as standards.

All work was carried out under metal-free conditions, and extreme care was taken to prevent contamination from adventitious metal ions. Failure to do so causes marked reductions in the activity of the enzyme. Thus, reagent-grade water with a resistivity of 18 $M\Omega\ cm^{-1}$ was prepared with a Millipore Milli-Q system and used in all experiments. All buffer and salt solutions were treated with Chelex 100 resin; solutions of L-leucine-*p*-nitroanilide were extracted with dithizone in carbon tetrachloride. Dialysis tubing was cleansed by repeated washing in warm metal-free water. Only plastic labware was used, except for quartz cuvettes, which were rendered metal free by soaking them in dilute metal-free hydrochloric acid. Solutions of all metal ions were made from the Johnson-Matthey spectrographically pure salts.

All metal analyses were carried out by atomic absorption spectroscopy with a Perkin-Elmer Model 5000 instrument equipped with an air–acetylene flame. Calibration curves for all metals were made by using Fisher certified standards.

An L-leucylglycyl-AH-Sepharose affinity resin was prepared as described by Basha and co-workers (Basha et al., 1978). Thus, 5 mL of packed AH-Sepharose was washed extensively with water and added to 10 mL of a 20 mg/mL solution of L-leucylglycine. The pH was adjusted to 4.5, and 100 mg of finely divided 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide was added over a period of 10 min. The pH was maintained at 4.5 for 1 h and then stirred at room temperature for 24 h. The gel was washed extensively with 10 mM Tris-HCl, pH 8.0, before use.

Results

Purification and Zinc Restoration of Commercial Leucine Aminopeptidase. Commercial leucine aminopeptidase is obtained as a suspension in 2.9 M $(NH_4)_2SO_4$ and 0.1 M Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$. When subjected to electrophoresis on sodium dodecyl sulfate–polyacrylamide gels (not shown), a major protein band with a molecular weight of 54 000 is evident, in agreement with the results of Shen & Melius (1977). In addition, numerous lighter bands are present, indicating that this preparation is not homogeneous. After washing with metal-free 2.9 M $(NH_4)_2SO_4$, gel filtration over Sephadex G-25, or extensive dialysis, it was determined that the enzyme contains nonstoichiometric quantities of Zn^{2+}, variable amounts of Mg^{2+}, and no detectable Mn^{2+}. Data for two different lots of Sigma enzyme are shown in Table I. .

We have purified the enzyme by affinity chromatography with L-leucylglycyl-AH-Sepharose by a modification of the procedure of Basha and co-workers (Basha et al., 1978). L-Leucylglycine is a good substrate for leucine aminopeptidase. The kinetic constants for the Mg^{2+}-activated enzyme are $K_M = 1.5$ mM and $k_{cat} = 2300\ min^{-1}$. However, when bound to AH-Sepharose, it can be used at 4 °C as an affinity ligand. The enzyme was applied to the column in 10 mM Tris-HCl, pH 8.0, and elution was initiated with this same buffer. When there was no further change in the absorbance at 280 nm, the remaining protein was eluted as a single asymmetrical peak with a 0–2 M NaCl gradient in the same buffer (Figure 1). The activity of the fractions indicates that the center of this peak contains the purified enzyme. These fractions were pooled and desalted. The capacity of this resin slowly decreases with time, presumably due to hydrolysis of the Leu-Gly bond, but it can be repeatedly recharged by coupling to more ligand.

The enzyme obtained by this procedure is homogeneous on sodium dodecyl sulfate–polyacrylamide gels but still contains less than stoichiometric quantities of Zn^{2+} per hexamer (Table I). Since the native enzyme is thought to bind 6 mol of Zn^{2+} per hexamer, commercial leucine aminopeptidase has presumably lost some of its native metal ions during purification. Very similar results are obtained with enzyme from other commercial sources. The Zn^{2+} content of the enzyme can be restored by incubation of the dialyzed enzyme (Table I) with 0.1 mM $ZnCl_2$ at 37 °C for 2 h followed by chromatography over L-leucylglycyl-AH-Sepharose (Figure 1). After being desalted, this enzyme contains 5.9–6.1 mol of Zn^{2+} per hexamer and only a trace of Mg^{2+}.

The increase in the Zn^{2+} content of the enzyme during the course of the treatments described above correlates with its activity (Table I). When assayed without prior activation with Mg^{2+}, the activity of the enzyme appears to decrease as the Zn^{2+} content increases. However, this is due to the fact that the purification scheme also reduces the amount of Mg^{2+} in

Table I: Metal Content and Activities of Commerical Leucine Aminopeptidase after Various Treatments

treatment[a]	lot no.	metal content[b] (mol/hexamer) Zn	Mg	activity, v/E_0 (min^{-1})	activity after Mg^{2+} activation,[c] v/E_0 (min^{-1})
washing[d]	119C8050	1.2	80		
	110F8010	3.6	35	84	84
gel filtration[e]	119C8050	1.9	1.7		
	110F8010	4.2	3.5	66	90
dialysis[f]	119C8050	2.2	4.6		42
	110F8010	4.5	3.1	60	96
affinity chromatography[g]	119C8050	2.7	1.5		55
	110F8010	4.8	2.0	18	102
0.1 mM $ZnCl_2$ + affinity chromatography[h]	119C8050	5.9	0.4	13	126
	110F8010	6.1	0.3	12	126

[a] The enzyme used was Sigma Type III-CP leucine aminopeptidase, obtained as a suspension in 2.9 M $(NH_4)_2SO_4$, 0.1 M Tris, and 5 mM $MgCl_2$, pH 8.0. The starting material for these treatments is the solid obtained after centrifugation. Very similar results were obtained with other lots of Sigma enzyme and with enzyme from other commercial sources. [b] Metal concentrations were determined by atomic absorption spectroscopy, and the hexamer concentration was determined from the absorbance at 280 nm by using $\epsilon_{280} = 4.0 \times 10^5$ M^{-1} cm^{-1} (see Table II). A molecular weight of 320 000 was used in all calculations. No detectable amounts of Mn were found in any of the samples. [c] Activated for 4 h at 37 °C in 10 mM Tris-HCl, pH 8.8, containing 5 mM $MgCl_2$ and assayed at 22 °C in 10 mM Tris-HCl and 1 M KCl, pH 8.0, containing 5 mM $MgCl_2$ at a substrate concentration of 1 mM. [d] Washed with metal-free 2.9 M $(NH_4)_2SO_4$ and 10 mM Tris-HCl, pH 8.0. [e] Chromatographed over Sephadex G-25 in 10 mM Tris-HCl, pH 8.0. [f] Dialyzed against a 100-fold excess of 10 mM Tris-HCl, pH 8.0, which was changed 3 times daily for 2 days. [g] Dialyzed enzyme was chromatographed over an L-leucylglycyl-AH-Sepharose affinity column (see Figure 1). [h] Dialyzed enzyme was incubated with 0.1 mM $ZnCl_2$ for 2 h at 37 °C and chromatographed over the L-leucylglycyl-AH-Sepharose column (Figure 1). The peak fractions from the NaCl gradient were lyophilized and desalted over Sephadex G-25.

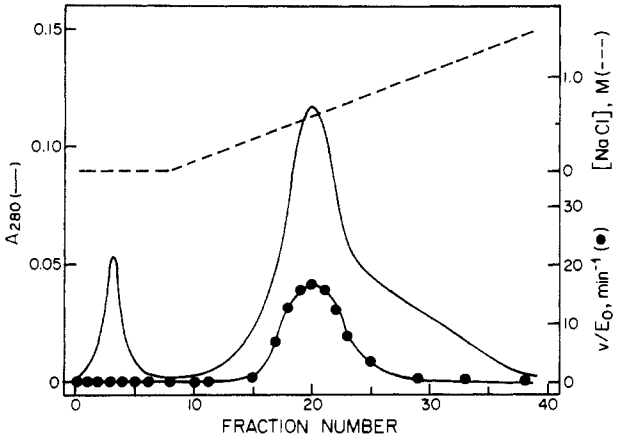

FIGURE 1: Purification of leucine aminopeptidase by affinity chromatography on L-leucylglycyl-AH-Sepharose. Enzyme was pretreated with 10 mM Tris-HCl, pH 8.0, containing 0.1 mM $ZnCl_2$ and applied to the column (diameter, 0.9 cm; height, 8.5 cm). Elution was initiated with 10 mM Tris-HCl, pH 8.0, and then with a 0–2 M NaCl gradient in the same buffer. The flow rate was 0.9 mL/min and the fraction volume 3.4 mL. All of the chromatographic procedures were carried out at 4 °C.

the regulatory site. Since at the different stages of purification the enzyme contains variable amounts of both Zn^{2+} and Mg^{2+} at the catalytic and regulatory sites, respectively, it is appropriate to compare the activities of the fully Mg^{2+}-activated enzymes. This levels out the effect of Mg^{2+} activation on the activity. The activities obtained after Mg^{2+} activation (conditions for the activation are described below) increase in proportion to the Zn^{2+} content of the enzyme. The Zn^{2+} content does not increase beyond 6 mol/hexamer, indicating that the additional Zn^{2+} is bound at the active site and is not bound adventitiously. Hence, treatment of commercial leucine aminopeptidase with 0.1 mM $ZnCl_2$ followed by affinity chromatography restores the native metal content of the enzyme, which is denoted [(LAP)Zn_6—].[1]

Determination of the Molar Extinction Coefficient. The calculation of the metal content of the enzyme presented above is based on an enzyme concentration determined spectrophotometrically with the molar extinction coefficient, ϵ_{280}. This value was obtained from the ultraviolet absorption spectrum of a solution of purified [(LAP)Zn_6—] whose weight percent

Table II: Extinction Coefficients of Leucine Aminopeptidase Obtained by Various Methods

method	standard	$\epsilon_{280} \times 10^{-5}$, M^{-1} cm^{-1}
biuret[a]	bovine albumin	3.2
	bovine γ-globulin	3.6
Lowry[b]	bovine albumin	2.7
	bovine γ-globulin	3.7
Bradford[c]	bovine albumin	3.2
	bovine γ-globulin	2.1
dry weight		4.0

[a] After the procedure of Gornall et al. (1949). [b] After the procedure of Lowry et al. (1951). [c] After the procedure of Bradford (1976).

protein was determined by dry weight analysis. With the assumption of a molecular weight of 320 000, ϵ_{280} is calculated to be 4.0×10^5 M^{-1} cm^{-1}. This is substantially higher than the value of 2.7×10^5 M^{-1} cm^{-1} used by other workers (Bryce & Rabin, 1964). For comparison, values of ϵ_{280} based on three other methods of protein concentration determination are listed in Table II. The Lowry and biuret methods give reasonable agreement when bovine γ-globulin is used as a standard; however, the other methods give substantially lower results.

Activation and Assay Conditions. In order to investigate the relationship between the metal content of leucine aminopeptidase and its activity, it is essential that optimum conditions be established for activating and assaying the enzyme. Initially, the activation conditions for Mn^{2+} and Mg^{2+} described by Smith & Spackman (1955) were used; however, these proved not to be optimal for the enzyme used in our studies. Incubation of [(LAP)Zn_6—] at 37 °C with 10 mM Tris-HCl, pH 8.0, containing 5 mM $MnCl_2$ or with 10 mM Tris-HCl, pH 8.8, containing 5 mM $MgCl_2$ produces a time-dependent activation (Figure 2). Incubation with $MnCl_2$ at pH values above 8.0 produces a brown solution due to oxidation of Mn^{2+}. The assays were carried out at 22 °C in 10 mM Tris-HCl and 1 M KCl, pH 8.0, in the presence of 5 mM $MnCl_2$ or $MgCl_2$, respectively. Failure to include the same concentration of activating metal ion in the assay always results in lower activities. After 4 h, the molar activities of the Mn^{2+}- and Mg^{2+}-activated enzymes level off at 168 and 126 min^{-1}, respectively. The rate of activation is unchanged

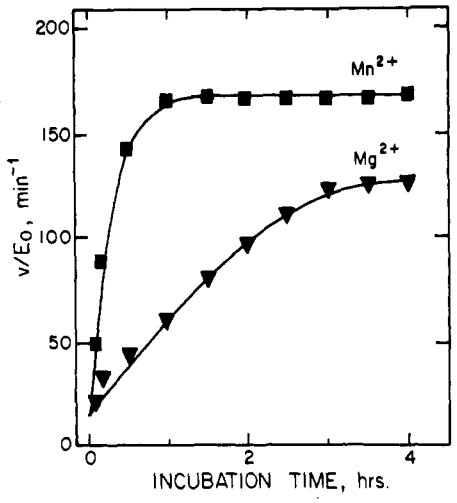

FIGURE 2: Time course of the activation of leucine aminopeptidase with Mn^{2+} and Mg^{2+}. [(LAP)Zn_6—] prepared by affinity chromatography was incubated at 37 °C at a concentration of 0.2 mg/mL with 10 mM Tris-HCl containing 5 mM $MnCl_2$ at pH 8.0 or 5 mM $MgCl_2$ at pH 8.8. Activities were measured as a function of time at 22 °C in 10 mM Tris-HCl and 1 M KCl, pH 8.0, containing 5 mM each of the appropriate metal ion.

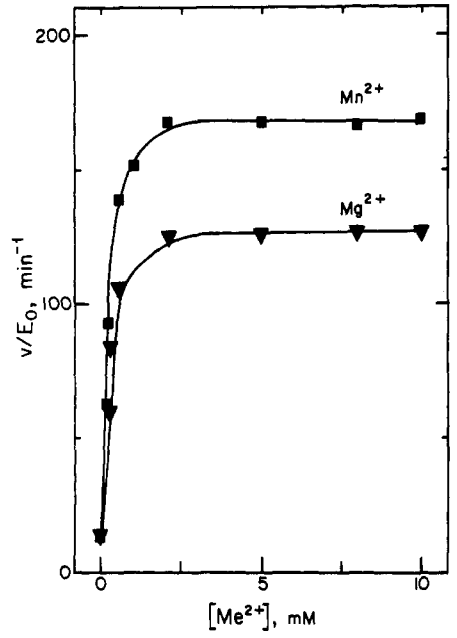

FIGURE 3: Effect of the concentration of Mn^{2+} and Mg^{2+} on the activity of leucine aminopeptidase. [(LAP)Zn_6—] prepared by affinity chromatography was incubated at 37 °C for 4 h in 10 mM Tris-HCl containing various concentrations of $MnCl_2$ and $MgCl_2$ at pH 8.0 and 8.8, respectively. Assays were carried out at 22 °C in 10 mM Tris-HCl and 1 M KCl, pH 8.0, containing the same concentration of $MnCl_2$ or $MgCl_2$ as in the incubation solution.

by including 1 mM 2-mercaptoethanol in the incubation mixture; 1 mM dithiothreitol does not alter the rate of activation either, but reduces the final activity obtained.

The effect of the concentration of $MnCl_2$ and $MgCl_2$ in the incubation solution on the activity reached after 4 h of incubation is shown in Figure 3. At a concentration of about 3 mM, the activity levels off for both metals to the same values reached in Figure 2. Collectively, these results establish that the best standard conditions for the activation of leucine aminopeptidase by $MnCl_2$ and $MgCl_2$ are incubation for 4 h at 37 °C with 10 mM Tris-HCl containing 5 mM $MnCl_2$ at pH 8.0 and 5 mM $MgCl_2$ at pH 8.8, respectively.

The influence of ionic strength on the activities of [(LAP)Zn_6—] and the Mn^{2+}- and Mg^{2+}-activated enzymes

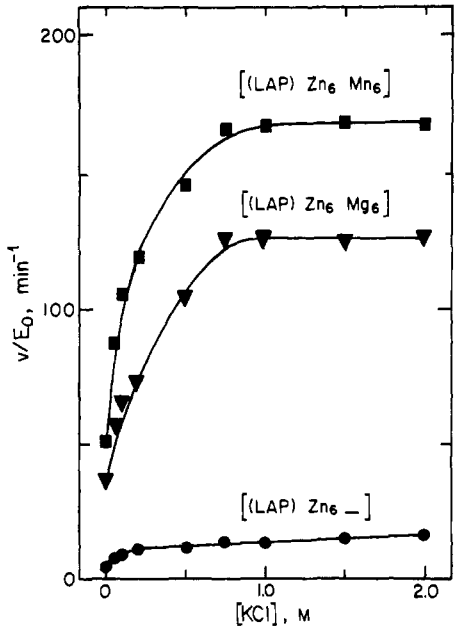

FIGURE 4: Effect of KCl concentration on the activities of metalloleucine aminopeptidases. [(LAP)Zn_6—] was prepared by affinity chromatography and converted into [(LAP)Zn_6Mn_6] and [(LAP)-Zn_6Mg_6] by incubation at 37 °C with 5 mM $MnCl_2$ and $MgCl_2$, respectively. Assays were carried out at 22 °C in 10 mM Tris-HCl, pH 8.0, containing 5 mM each of the appropriate metal ion and the indicated concentration of KCl.

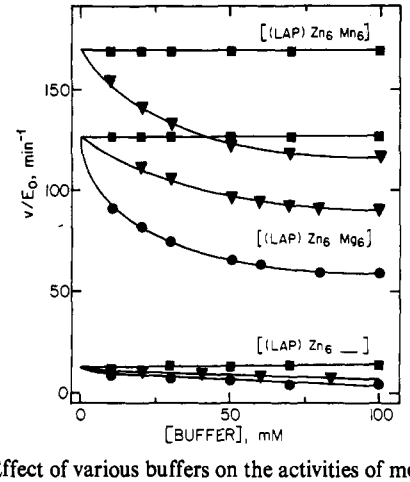

FIGURE 5: Effect of various buffers on the activities of metalloleucine aminopeptidases. [(LAP)Zn_6—] was prepared by affinity chromatography and converted into [(LAP)Zn_6Mn_6] and [(LAP)Zn_6Mg_6] by incubation at 37 °C with 5 mM $MnCl_2$ and $MgCl_2$, respectively. Assays were carried out at 22 °C in 1 M KCl, pH 8.0, containing 5 mM each of the appropriate metal ion and the indicated concentration of (■) Tris, Hepes (results identical for each), (▼) Tricine, and (●) phosphate buffers. Data for [(LAP)Zn_6Mn_6] in phosphate buffer could not be obtained due to precipitation of $MnPO_4$.

was investigated by varying the KCl concentration in the assay mixture. Increasing the KCl concentration up to about 1 M markedly activates all three species. This activation levels off above this value and is essentially constant up to 2 M (Figure 4). The effect of various buffers was also examined to identify those buffers that did not inhibit any of the three species. Tris and Hepes do not inhibit at concentrations as high as 100 mM, while Tricine and phosphate inhibit markedly in this range (Figure 5).

The effect of pH on the activities of [(LAP)Zn_6—] and the Mn^{2+}- and Mg^{2+}-activated enzymes was investigated by using five separate, but overlapping, buffers: Mes (5.5–6.5); Hepes (6.5–7.5); Tris (7.5–8.5); borate (8.5–9.5); and Caps (9.5–11.0). All three pH profiles (Figure 6) consist of smooth,

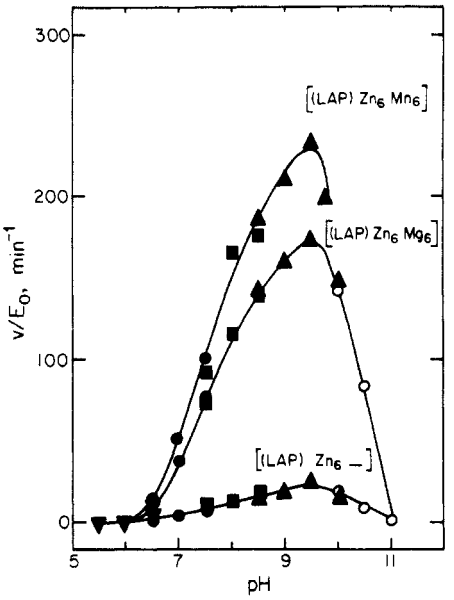

FIGURE 6: Effect of pH on the activities of metalloleucine aminopeptidases. [(LAP)Zn_6—] was prepared by affinity chromatography and converted into [(LAP)Zn_6Mn_6] and [(LAP)Zn_6Mg_6] by incubation at 37 °C with 5 mM $MnCl_2$ and $MgCl_2$, respectively. Assays were carried out at 22 °C in 10 mM buffer and 1 M KCl containing 5 mM each of the appropriate metal ion at the indicated pH in (▼) Mes, (●) Hepes, (■) Tris, (▲) borate, and (○) Caps buffers. The activity of [(LAP)Zn_6Mn_6] was not obtained above pH 9.75 because of Mn^{2+} oxidation.

bell-shaped curves. The enzymes are virtually inactive at pH 6 and have maximal rates near pH 9.5. Data for the Mn^{2+}-activated enzyme are difficult to obtain at high pH values due to Mn^{2+} oxidation, and reliable rates could not be measured above pH 9.75. The combined results of Figures 4–6 suggest that the best conditions for routinely assaying all three species are in 10 mM Tris-HCl and 1 M KCl, pH 8.0, containing, if pertinent, 5 mM of the activating metal ion.

Mechanism of Activation by Mn^{2+} and Mg^{2+}. The native form of leucine aminopeptidase contains approximately 6.0 mol of Zn^{2+} per hexamer (Table I). After activation with Mn^{2+} and Mg^{2+}, samples of the enzyme were gel filtered over Sephadex G-25, and the metal content was measured by atomic absorption spectroscopy. Control experiments demonstrated that this column completely separates bound and unbound metals. The activated enzymes retain, within experimental error, the same Zn^{2+} content as before activation; in addition, they acquire approximately 6 mol per hexamer of the activating metal ion, confirming the presence of a second, "regulatory" metal binding site per subunit (Table III). Thus, the Mn^{2+}- and Mg^{2+}-activated enzymes are designated as [(LAP)Zn_6Mn_6] and [(LAP)Zn_6Mg_6], respectively.

The effect of activation by Mg^{2+} and Mn^{2+} on the kinetic constants of the enzyme has been examined. Double-reciprocal plots were obtained for each species, and k_{cat} and K_M values were calculated by assuming that there are six active sites per hexamer. Within experimental error, the K_M values of [(LAP)Zn_6—], [(LAP)Zn_6Mn_6], and [(LAP)Zn_6Mg_6] are all 1.0 mM. The disparate activities of the three species are due to their different values of k_{cat}, which are 4.2, 42, and 55 min^{-1}, respectively. Thus, Mn^{2+} and Mg^{2+} activate [(LAP)Zn_6—] by binding to a previously unoccupied regulatory site in each subunit and increasing k_{cat}.

Mechanism of Inhibition by Other Divalent Metal Ions. [(LAP)Zn_6—] is inhibited by other divalent metal ions, including Zn^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, and Cd^{2+}. The time course of inhibition by 0.1 mM concentrations of these ions is shown

Table III: Metal Content and Kinetic Parameters for Leucine Aminopeptidase with Different Metals in the Regulatory Site

enzyme[a]	metal content (mol/hexamer)[b]		kinetic parameters[c]	
	x	y	k_{cat} (min^{-1})	K_M (mM)
[(LAP)Zn_x–]	6.1	0	4.2	1.0
[(LAP)Zn_xMn_y]	6.1	6.4	55	1.0
[(LAP)Zn_xMg_y]	5.8	6.2	42	1.0
[(LAP)Zn_xNi_y]	6.1	5.8	3.0	0.9
[(LAP)Zn_xCu_y]	6.2	6.1	2.3	1.0
[(LAP)Zn_xZn_y]	5.9	*d*	2.0	1.0
[(LAP)Zn_xHg_y]	5.9	*e*	*f*	*f*

[a] Prepared by incubation of [(LAP)Zn_6–] with the appropriate metal ion (Figures 2 and 7) followed by gel filtration over Sephadex G-25. [b] Metal concentrations were determined by atomic absorption spectroscopy, and the hexamer concentration was determined from the absorbance at 280 nm by using $\epsilon_{280} = 4.0 \times 10^5$ M^{-1} cm^{-1}. A molecular weight of 320 000 was used in all calculations. [c] Assays were carried out in the presence of excess (5 mM for Mn^{2+} and Mg^{2+} and 0.1 mM for Zn^{2+}, Ni^{2+}, and Cu^{2+}) regulatory metal ion. k_{cat} values were calculated by assuming that there are six active sites per hexamer. [d] No metal ions are found in this site after gel filtration, but y is assumed to be 6 on the basis of metal competition studies. [e] Not measured due to the poor sensitivity of determination of Hg^{2+} by atomic absorption. [f] Not measured since the enzyme is inactive.

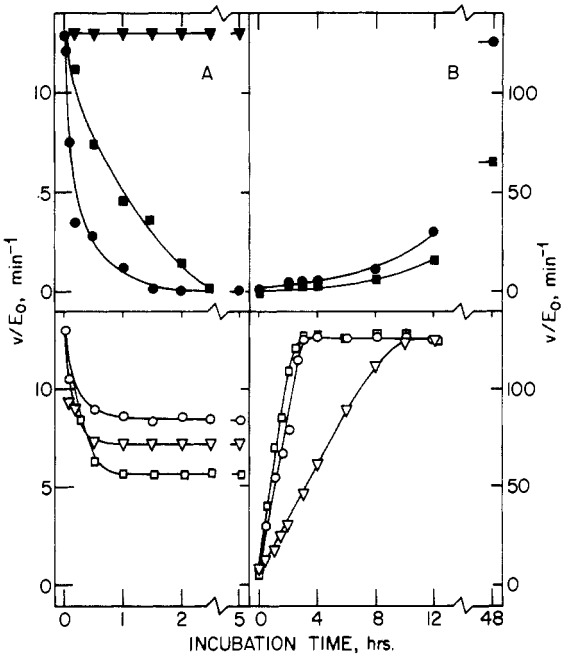

FIGURE 7: Time course of the inhibition of [(LAP)Zn_6—] by various divalent metal ions and the reversal of the inhibition by Mg^{2+}. (A) [(LAP)Zn_6–] prepared by affinity chromatography was incubated at 37 °C with 10 mM Tris-HCl, pH 8.0, in separate tubes containing 0.1 mM each of (▼) $CaCl_2$, (■) $CdSO_4$, (●) $HgCl_2$, (○) $NiCl_2$, (▽) $CuSO_4$, and (□) $ZnCl_2$, respectively. Activities (left ordinate) were measured as a function of time at 22 °C in 10 mM Tris-HCl and 1 M KCl, pH 8.0, containing 0.1 mM each of the appropriate metal ion. (B) After 5 h, $MgCl_2$ was added to all solutions to a concentration of 5 mM, and the activities (right ordinate) were measured in the same manner, except that the 0.1 mM metal ions in the assay were replaced by 5 mM Mg^{2+}.

in Figure 7. Zn^{2+}, Ni^{2+}, and Cu^{2+} cause partial inhibition, which levels off after 1 h, while Cd^{2+} and Hg^{2+} inhibit completely within 3 h. Ca^{2+} does not alter the activity of the enzyme. After 5 h, the incubation solutions were gel filtered, the protein fractions were concentrated, and the metal content of the enzyme was measured by atomic absorption spectroscopy.

Incubation with Ca^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, and Hg^{2+} does not alter the Zn^{2+} content of the native enzyme, which is in the 5.8–6.2 mol per hexamer range for all samples (Table III). In contrast, the Zn^{2+} content of the enzyme incubated with Cd^{2+} fell to 3.1 mol per hexamer, and 2.2 mol of Cd^{2+} became bound, suggesting that partial exchange of Cd^{2+} for the Zn^{2+} at the catalytic site has occurred. After gel filtration, the enzymes incubated with Cu^{2+} and Ni^{2+} retain approximately 6 mol of these metals per hexamer. In contrast, no Ca^{2+} or additional Zn^{2+} beyond the 6 mol of catalytic Zn^{2+} is found after incubation with these metals. Unfortunately, the sensitivity of detection for Hg^{2+} by atomic absorption spectroscopy is too low to permit its concentration in the enzyme incubated with this metal to be determined.

The kinetic constants for the enzyme in the presence of 0.1 mM Zn^{2+}, Ni^{2+}, and Cu^{2+} have been determined and are listed in Table III. Within experimental error, all three enzymes have a value for K_M of about 1 mM, the same value as that for [(LAP)Zn_6—], [(LAP)Zn_6Mn_6], and [(LAP)Zn_6Mg_6]. The inhibition of the enzyme in the presence of Zn^{2+}, Ni^{2+}, and Cu^{2+} is due to lower values of k_{cat}. These results strongly suggest that the mechanism of inhibition of [(LAP)Zn_6—] by Zn^{2+}, Ni^{2+}, and Cu^{2+} and of activation by Mn^{2+} and Mg^{2+} is the same and is due to the binding of these metal ions to be a regulatory site present on each subunit. Inhibition by Hg^{2+} and Cd^{2+} probably follows the same mechanism, except that Cd^{2+} slowly exchanges with the catalytic Zn^{2+}. Ca^{2+} does not affect the activity of the enzyme nor does its presence at a concentration of 10 mM prevent inhibition or activation by these other metals. Hence, it apparently does not bind to the regulatory site.

For examination of whether all of the metal ions that inhibit and activate the enzyme bind to the same site, a series of experiments has been carried out involving the sequential addition of different ions to solutions containing [(LAP)-Zn_6—]. The results of one series of experiments are illustrated in Figure 7. In separate experiments, [(LAP)Zn_6—] was incubated for 5 h with solutions containing 0.1 mM Cd^{2+}, Hg^{2+}, Zn^{2+}, Ni^{2+}, and Cu^{2+}, to give the inhibition characteristic of each of these metal ions. Then the solutions were brought to a concentration of 5 mM in Mg^{2+}. Within 12 h, the activities of the enzymes treated with Zn^{2+}, Ni^{2+}, and Cu^{2+} increased to the value of [(LAP)Zn_6Mg_6]. The Hg^{2+}- and Cd^{2+}-treated enzymes also regained activity, but at a much slower rate. After 2 days, the activity of the Hg^{2+}-treated enzyme also leveled off at the value of [(LAP)Zn_6Mg_6], but the activity of the Cd^{2+}-treated enzyme only increased to about half of this value.

Subsequent to gel filtration, metal analysis of all of these samples, except the Cd^{2+}-treated enzyme, showed that they contained approximately 6 mol per hexamer of both Zn^{2+} and Mg^{2+}. The Cd^{2+}-treated sample contained approximately 3.1 and 2.2 mol per hexamer of Zn^{2+} and Cd^{2+}, respectively, the same as before treatment; in addition, it also contained approximately 6 mol per hexamer of Mg^{2+}. These results strongly imply that Mg^{2+} binds at the same site as these other metals. The failure of the Cd^{2+}-treated enzyme to reactivate beyond about half of the activity of [(LAP)Zn_6Mg_6] is probably due to the fact that only about half of the catalytic sites are occupied by Zn^{2+}. Apparently, the enzyme is not active when Cd^{2+} is at the active site. Similar experiments have been carried out with other pairs of metal ions, and the results (not shown) parallel those shown in Figure 7, supporting the hypothesis that Zn^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}, Cd^{2+}, Mn^{2+}, and Mg^{2+} all modulate the activity of [(LAP)Zn_6—] by binding to a single regulatory site per subunit.

Discussion

Using the criteria established by Vallee (1960), Himmelhoch has shown quite clearly that porcine kidney leucine aminopeptidase is a zinc metalloenzyme. Smith & Spackman (1955) have shown that the enzyme is activated by Mn^{2+} and Mg^{2+} and inhibited by Zn^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+}. However, the precise stoichiometry of binding of Zn^{2+} to the native enzyme and the mechanism by which its activity is raised and lowered by divalent transition metal ions have not heretofore been firmly established.

Himmelhoch found that native enzyme contains between 4 and 6 mol of Zn^{2+} per 300 000 g of protein. Since the enzyme has a molecular weight of approximately 320 000 (Spackman et al., 1955; Himmelhoch & Peterson, 1968; Shen & Melius, 1977) and a subunit molecular weight of 54 000, it appears that the native enzyme is a hexamer that contains 0.67–1.0 mol of Zn^{2+} per subunit. However, the accuracy of these figures relies heavily on the method used to determine the concentration of protein, and no details were given in Himmelhoch's study. Hence, we have measured the Zn^{2+} content of commercial samples of the enzyme that have been purified by affinity chromatography by using a protein concentration determined by accurate dry weight analysis.

Leucine aminopeptidase obtained from commercial sources is not homogeneous and is contaminated with large quantities of Mg^{2+}. After extensive dialysis and chromatography over L-leucylglycyl-AH-Sepharose, the enzyme runs as a single band on sodium dodecyl sulfate–polyacrylamide gels with a molecular weight of approximately 54 000 but still contains less than stoichiometric quantities of Zn^{2+}. Since the enzyme is isolated, purified, and stored in the presence of millimolar concentrations of Mg^{2+} (Himmelhoch, 1970), it is possible that the Zn^{2+} residing at the active site of the native enzyme is slowly replaced by Mg^{2+}. During the course of various treatments, including gel filtration, dialysis, and affinity chromatography, the Zn^{2+} content of the enzyme increases from 1.2 to 2.7 and from 3.6 to 4.8 mol per hexamer, respectively, for the two lots of enzyme (Table I). This is probably due either to the loss of impurities that contributed to the absorbance at 280 nm or to the acquisition of trace quantities of Zn^{2+} from the environment, or both. Incubation of the dialyzed enzyme with 0.1 mM $ZnCl_2$ for 2 h at 37 °C followed by affinity chromatography yields an enzyme containing strictly 6 mol of Zn^{2+} per hexamer and only a trace of Mg^{2+}. This treatment, therefore, restores the commercial enzyme to its native form, designated [(LAP)Zn_6—].

The purification and restoration of the Zn^{2+} content of the enzyme dramatically alter its activity. When assayed without prior activation with Mg^{2+}, the crude commercial enzyme has a higher activity than that of the purified enzyme. This is due to the fact that the crude enzyme has Mg^{2+} bound at the regulatory site, which activates it. If the enzyme is assayed after Mg^{2+} activation at every stage of purification, the activity increases in direct proportion to the Zn^{2+} content, indicating that Zn^{2+} is, indeed, a catalytic metal. Thus, commercial enzyme has partial occupancy of the catalytic sites with Zn^{2+} and full occupancy of the regulatory sites with Mg^{2+}.

The metal stoichiometries discussed above were calculated by using a molar extinction coefficient for the enzyme determined by dry weight analysis, giving ϵ_{280} value of 4.0×10^5 M^{-1} cm^{-1}. Apparently, this is the first accurate determination of this number. Bryce & Rabin (1964) used $\epsilon_{280} = 2.7 \times 10^5$ M^{-1} cm^{-1}, which is based on the data of Spackman et al. (1955), who did not report any details regarding the method

of their protein determination. For comparison with the dry weight determination, the protein concentration was measured by three colorimetric methods. The ϵ_{280} values obtained by these methods with bovine serum albumin and γ-globulin as standards show a considerable spread (Table II) and are all lower than the value obtained by dry weight analysis. Since the gravimetric method is clearly the most accurate, we have used it to calculate ϵ_{280}. The colorimetric methods all have the serious drawback that they require a standard whose color response matches that of the unknown. For example, the Lowry method gives reliable results only if the standard and unknown both contain similar amounts of tyrosine and tryptophan. The wide discrepancies between the results obtained with these methods underscore the importance of basing metal stoichiometries on a protein concentration obtained only by the more accurate gravimetric technique.

We have explored the optimum conditions for activating [(LAP)Zn_6—] to form [(LAP)Zn_6Mn_6] or [(LAP)Zn_6Mg_6], and for assaying all three of these species. In our initial studies, the activation conditions described by Spackman and coworkers (Spackman et al., 1955) were employed. Thus, the enzyme was incubated at pH 8.0 in the presence of 1 mM $MnCl_2$ or 4 mM $MgCl_2$ for 15 min at 40 °C. These authors report an approximately 2-fold increase in activity. However, we have found that the activation of [(LAP)Zn_6—] by $MnCl_2$ and $MgCl_2$ under similar conditions is much slower (Figure 2), results in a 10- and 13-fold increase in activity, respectively, and requires a higher concentration of $MnCl_2$ (Figure 3). Since these authors used enzyme that was purified by precipitation with 10 mM $MgCl_2$, it seems certain that they were working with a preparation already predominantly in the [(LAP)Zn_6Mg_6] form. This accounts for the low degree of activation found by these authors and for the fact that less stringent conditions were needed to achieve full activation. Hence, to prepare metalloleucine aminopeptidases of well-defined stoichiometry with Zn^{2+} in the catalytic site and different metal ions in the regulatory site, it is essential that purified [(LAP)Zn_6—] enzyme be prepared first by pretreatment with $ZnCl_2$ followed by affinity chromatography and then converted into [(LAP)Zn_6M_6] by incubation with the metal under conditions shown to achieve complete binding.

Since the effects of ionic strength and buffers on the activities of [(LAP)Zn_6—], [(LAP)Zn_6Mn_6], and [(LAP)-Zn_6Mg_6] have not been investigated previously, we have examined the effects of these parameters as well as that of pH to find assay conditions compatible with all three species. The enzymes are not inhibited by either Tris or Hepes buffers but are markedly inhibited by Tricine and phosphate. Tricine is known to chelate metal ions much better than Tris and Hepes (Good et al., 1966); hence, it may inhibit the enzyme by interacting with the metal at either the catalytic or the regulatory sites. Phosphate buffer is known to inhibit many metalloenzymes for the same reason (O'Sullivan & Smithers, 1979).

All three species are markedly activated by KCl. We have also investigated the influence of other alkali metal salts on the activity of the enzyme (S. H. Lin and H. E. Van Wart, unpublished experiments), and it is clear that the activity observed can be raised or lowered and is markedly dependent on the particular cation and anion. Hence, the increase in activity brought about by KCl is apparently not due solely to the increase in the ionic strength of the solution. The basis for these salts effects is presently under investigation and is thought to involve alterations in the tertiary or quaternary structure of the enzyme. For routine assays, it is recommended that these be carried out in 1 M KCl to maintain a constant ionic strength without inhibiting the enzyme. In order to prevent dissociation of the metal ion in the regulatory site during the rate measurement, the assay must be carried out in the presence of the appropriate metal ion. Even though the pH optimum of all three species is about 9.5, routine assays are best carried out at pH 8.0 to prevent the oxidation of Mn^{2+} that occurs at high pH. Finally, it is essential that extreme precaution to be taken to exclude adventitious metal ions, since failure to do so leads to inhibition.

In addition to being activated by Mn^{2+} and Mg^{2+}, [(LAP)Zn_6—] is inhibited by Zn^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, and Hg^{2+}. Metal analysis of the enzyme after incubation with these metal ions shows that the common mechanism for the modulation of the activity of the native enzyme by these metal ions is binding at a single regulatory site per subunit. Thus, activation of [(LAP)Zn_6—] by Mn^{2+} or Mg^{2+} or inhibition of Cu^{2+} and Ni^{2+} followed by gel filtration yields species containing 6 mol of the modulating metal ion in addition to 6 mol of Zn^{2+}. Inhibition by Zn^{2+}, Cd^{2+}, and Hg^{2+} presumably occurs by this same mechanism, except that these metal ions do not bind tightly enough to the regulatory site to remain bound during gel filtration. The fact that all of these metal ions can reverse the effects on the activity of any other strongly supports the idea that they all exert their influence on the enzyme by binding at a common regulatory site. Furthermore, the kinetic parameters for all these metalloleucine aminopeptidases reveal the common theme that the activity modulation is achieved by altering k_{cat}, leaving K_M unchanged.

The behavior of the Cd^{2+}-incubated enzyme is somewhat different from that of the others. Like Hg^{2+}, Cd^{2+} binds at the regulatory site, to completely inactivate the enzyme. This inhibition is reversed by Mg^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}. In addition, Cd^{2+} apparently exchanges slowly with the Zn^{2+} in the catalytic site, producing an inactive species whose activity is not restored by incubation with Mg^{2+}. This exchange of Cd^{2+} for Zn^{2+} to produce an inactive enzyme has also been reported by Himmelhoch (1969).

Our proposed mechanism for the modulation of the activity of [(LAP)Zn_6—] by divalent metal ions is very similar to that reported by carpenter & Vahl (1973) for the activation of the bovine lens enzyme by Mn^{2+} and Mg^{2+}. The major difference is that the lens enzyme contains firmly bound Zn^{2+} at both the catalytic and the regulatory sites of each subunit, and, hence, activation by Mn^{2+} or Mg^{2+} requires displacement of the Zn^{2+} in the regulatory site by these metal ions. Our results contrast sharply with those of Himmelhoch (1969), who reported that activation of the porcine kidney enzyme by Mn^{2+} is due to replacement of the catalytic Zn^{2+} by Mn^{2+}. The metal analyses presented here clearly show that, using our incubation conditions, this is not the case for Mn^{2+}, Mg^{2+}, Hg^{2+}, Ni^{2+}, or Cu^{2+}. This does not, of course, rule out the possibility that the catalytic Zn^{2+} of the enzyme is replaced by Mn^{2+} under the prolonged dialysis conditions used by Himmelhoch (1969).

The marked activation and inhibition of native leucine aminopeptidase by divalent metal ions represent a means of regulating the activity of the enzyme under physiological conditions. To say with certainty whether such regulation does indeed occur would require a knowledge of the binding constants for each metal ion as well as of their intracellular concentrations. Since these numbers are not known, no firm conclusion can be drawn. It is of interest that, unlike bovine lens leucine aminopeptidase, the regulatory site of this enzyme does not bind Zn^{2+} strongly. In terms of flexibility of regu-

lation, this confers a decided advantage to the porcine kidney enzyme, since binding of other metal ions does not require displacement of Zn^{2+} from this site. Hence, inhibition or activation would be achieved at lower concentrations of these metal ions, and this modulation may be more likely to have a physiological role.

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References

Basha, S. M. M., Horst, M. N., Bazer, F. W., & Roberts, R. M. (1978) *Arch. Biochem. Biophys. 185*, 174–184.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Bryce, G. F., & Rabin, B. R. (1964) *Biochem. J. 90*, 509–512.
Carpenter, F. H., & Vahl, J. M. (1973) *J. Biol. Chem. 248*, 294–304.
Delange, R. J., & Smith, E. L. (1971) *Enzymes, 3rd Ed. 3*, 81–118.
Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry 5*, 467–477.
Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.
Himmelhoch, S. R. (1969) *Arch. Biochem. Biophys. 134*, 597–602.
Himmelhoch, S. R. (1970) *Methods Enzymol. 19*, 508–513.
Himmelhoch, S. R., & Peterson, E. A. (1968) *Biochemistry 7*, 2085–2092.
Lowry, O., Rosebrough, N., Farr, A., & Randall, R. (1951) *J. Biol. Chem. 193*, 265–275.
Melbye, S. W., & Carpenter, F. H. (1971) *J. Biol. Chem. 246*, 2459–2463.
O'Sullivan, W. J., & Smithers, G. W. (1979) *Methods Enzymol. 63*, 294–336.
Royer, G. P., & Andrews, J. P. (1973) *J. Biol. Chem. 248*, 1807–1812.
Shen, C., & Melius, P. (1977) *Prep. Biochem. 7*, 243–256.
Smith, E. L., & Spackman, D. H. (1955) *J. Biol. Chem. 212*, 271–299.
Spackman, D. H., Smith, E. L., & Brown, D. M. (1955) *J. Biol. Chem. 212*, 255–269.
Thompson, G. A., & Carpenter, F. H. (1976) *J. Biol. Chem. 251*, 1618–1624.
Vallee, B. L. (1960) *Enzymes, 2nd Ed. 3*, 225–276.

Fluorescence Studies on the Lipoprotein Complex of the Fatty Acid Synthetase from the Insect *Ceratitis capitata*†

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ABSTRACT: The fatty acid synthetase complex from the insect *Ceratitis capitata* forms a stable lipoprotein complex. The intrinsic fluorescence of the complex was studied by observing the emission spectra with different excitation wavelengths, both in the native complex and after treatment with sodium cholate and sodium dodecyl sulfate. The excitation spectrum of the native form also was recorded. The fluorescence behavior of the native enzyme showed two families of tryptophan residues. Cholate influenced the fluorescence, suggesting that phospholipids are the conformational support at this level. The two families of fluorescing tryptophan residues were similarly accessible to quenching by acrylamide. Thermal changes in the fluorescence characteristics were observed; warming caused a decrease in the quantum yield as well as a red shift in the emission maximum. The high fluorescence remaining after the thermal transition suggested that the lipid–protein interaction was affected but maintained shielding of the fluorophore by the lipids. Fluorescent probe molecules 1,6-diphenylhexa-1,3,5-triene (DPH) and dansylphosphatidylethanolamine (DPE) also were used. DPH uptake was temperature dependent, with a middle point consistent with the thermal conformation transition, indicating that internal lipids are nonrandomly distributed within the complex. DPE uptake did not reach the saturation of the complex, suggesting that its solubilization sites would be located on the lipoprotein surface.

Native fatty acid synthetase from *Ceratitis capitata* has been described as a lipoprotein (Gavilanes et al., 1979) which requires the lipid component for retaining its conformational properties (Gavilanes et al., 1978, 1979); thus, changes in the lipid content and composition resulted in modification of both secondary structure and enzymatic activity (Gavilanes et al., 1979, 1981). Triacylglycerols are the most abundant lipid class whereas a specific phosphatidylethanolamine dependence of the enzyme activity has been demonstrated (Gavilanes et al., 1979). An attempt to clarify the structural organization of the enzyme complex was carried out by a circular dichroism study of the native complex and after a series of sequential treatments with lipolytic and proteolytic enzymes; it obtained evidence for the shielding of certain polypeptide domains by the lipid environment (Gavilanes et al., 1981). Nevertheless, it is not clear from these experiments whether the phospholipids are interacting specifically with the polypeptide chains or if they are simply playing a structural role important for the complex conformation.

Other information that may give insight as to the complex organization expected is from fluorescence data on this lipid–protein system. The intrinsic protein fluorescence arises from tyrosine and tryptophan residues, which act as nonperturbing hydrophobic probes of their immediate environment. Intrinsic fluorescence offers a sensitive tool for observing in-

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