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Kinetic Parameters of Metal-Substituted Leucine Aminopeptidase from Bovine Lens†

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ABSTRACT: Leucine aminopeptidase (LAP) is a protease requiring two divalent metal cations per subunit for activity. Zn^{2+}, Mg^{2+}, and Co^{2+} metal-substituted forms of LAP have been prepared and investigated kinetically. Substitution of metal into the two binding sites independently resulted in the preparation of $Zn^{2+}Zn^{2+}$, $Mg^{2+}Zn^{2+}$, $Co^{2+}Co^{2+}$, $Zn^{2+}Co^{2+}$, $Mg^{2+}Co^{2+}$, and $Co^{2+}Zn^{2+}$ LAP derivatives that were characterized by atomic absorption spectrophotometry. Kinetic analysis of the metal-substituted enzymes indicated that site 1 (fast exchanging) metal substitution results in a K_m decrease in the relative order $Zn^{2+} > Mg^{2+} > Co^{2+}$. Similar comparisons for the site 2 metal (slow exchanging) involved only Zn^{2+} and Co^{2+}, since only these metals have been shown to compete effectively for this site. Substitution of these two metals into site 2 revealed a K_m decrease in the order $Zn^{2+} > Co^{2+}$. It was suggested previously [e.g., Thompson, G. A., & Carpenter, F. H. (1976) *J. Biol. Chem. 251*, 1618-1624] that the fast-exchanging site 1 metal predominantly effects k_{cat} while the slow-exchanging metal in site 2 exerts effects exclusively on K_m. The present study, the first direct comparison of K_m change resulting from metal substitution into both sites, clearly indicates that both metal sites exert significant effects on K_m. In addition, the data suggest a more complex interaction between the two bound metals than previously suspected.

Leucine aminopeptidase (LAP)[1] (EC 3.4.11.1) is an exopeptidase that catalyzes the hydrolysis of amino-terminal peptide bonds (Smith & Hill, 1960; Hanson & Frohne, 1977). Though leucyl peptides are especially favored substrates, as implied by the trivial name of this enzyme, substantial rates of hydrolysis are seen for other amino acids. LAP's have been found in many tissues and organs, and a loss in their activity is associated with several pathogenic disorders (Devi, 1963; Uete et al., 1974; Swanson & Truesdale, 1974; Hahn et al., 1976; Van Heyningen & Trayhurn, 1976).

Bovine lens leucine aminopeptidase has a molecular weight of 324000 and contains six identical subunits (Melbye & Carpenter, 1971; Carpenter & Vahl, 1973). Each 54000-dalton subunit contains two independent, nonidentical metal binding sites that display different affinities for divalent metal cations (Bottger et al., 1968; Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a,b). The metal ion in site 1 (fast-exchanging site) is in equilibrium with other divalent cations in solution and is therefore easily replaced by incu-

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[1] Abbreviations: LAP, leucine aminopeptidase (bovine lens); Leu-NH_2, L-leucine amide; LpA, L-leucyl-*p*-anisidine; LpNA, L-leucine-*p*-nitroanilide; NEM, *N*-ethylmorpholine; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; ZnZn LAP, native leucine aminopeptidase with Zn^{2+} in both metal binding sites; MgZn LAP, leucine aminopeptidase with Mg^{2+} in site 1 (fast-exchanging site) and Zn^{2+} in site 2 (slow-exchanging site); CoCo LAP, leucine aminopeptidase with Co^{2+} in both binding sites; MgCo LAP, leucine aminopeptidase with Mg^{2+} in site 1 and Co^{2+} in site 2; ZnCo LAP, leucine aminopeptidase with Zn^{2+} in site 1 and Co^{2+} in site 2; CoZn LAP, leucine aminopeptidase with Co^{2+} in site 1 and Zn^{2+} in site 2.

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bation. The metal in site 2 (slow-exchanging site) is unavailable for metal exchange under conditions that would allow exchange into site 1 (Thompson & Carpenter, 1976a,b). In order to have enzyme activity, both binding sites must be occupied by metal cations (Carpenter & Vahl, 1973). The native enzyme contains 2 Zn^{2+} ions/subunit (Vahl & Carpenter, 1971; Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a,b; Hanson & Frohne, 1977).

In the past there has been a great deal of interest in metal substitution with different LAP's [e.g., Johnson et al. (1936), Berger & Johnson (1939), Thompson & Carpenter (1976a,b), and Van Wart & Lin (1981)]. Initially, investigators discovered that incubation of porcine kidney and porcine intestinal mucosa LAP's with Mg^{2+} or Mn^{2+} resulted in significant increases in enzymatic activity (Johnson et al., 1936; Berger & Johnson, 1939; Smith & Bergmann, 1941, 1944; Smith, 1946; Smith & Spackman, 1955). In the case of the bovine lens enzyme, this activation was shown to result from metal exchange into site 1 (Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a,b). Later attempts were undertaken to exchange both bound metals with Ca^{2+}, Mg^{2+}, and Mn^{2+} [e.g., Carpenter & Vahl (1973) and Thompson & Carpenter (1976a,b)]. The resulting enzyme derivatives did not bind these metals stoichiometrically, and they had little enzymatic activity. For these reasons, site 2 was considered the zinc-specific, catalytic site; only Zn^{2+} seemed to function well there. Site 1 was termed the activation site since Mg^{2+} or Mn^{2+} substitution into this site was stoichiometric and resulted in activation (Carpenter & Vahl, 1973).

A current hypothesis suggests that in bovine lens LAP the activation site metal ion is involved in conformational modifications that predominantly affect k_{cat}, while the site 2 metal is involved in substrate binding and affects K_m (Thompson & Carpenter, 1976a,b). However, no conclusive evidence has been presented that directly compares kinetic effects resulting from metal exchange into both sites independently. Earlier studies in this area were hindered since only Zn^{2+} was found to bind effectively in site 2. Later, studies by Thompson & Carpenter (1976a,b) showed that Co^{2+} could bind stoichiometrically and reversibly into both sites under certain conditions. In this paper, procedures are outlined for the preparation of the following metal-substituted LAP's: MgZn, CoCo, MgCo, CoZn, and ZnCo. These metal-substituted enzymes were studied kinetically with LpNA, LpA, and Leu-NH_2 as substrates. This is the first definitive comparison of kinetic effects resulting from metal substitution into both sites independently.

Experimental Procedures

Leu-NH_2 and LpNA were obtained from Sigma. LpA was synthesized as outlined by Taylor et al. (1981). NEM, purchased from Fluka, was vacuum distilled and eluted through a Chelex 100 column before use. Sodium-form Chelex 100 resin, 100–200 mesh, was purchased from Bio-Rad. As spectroscopic standards, zinc powder, magnesium powder, and cobalt powder were obtained from Alpha Products, General Chemical Co., and Aldrich Chemical Co., respectively. Ultrapure $(NH_4)_2SO_4$ was obtained from Schwarz/Mann. Dialysis membranes from Union Carbide Corporation were 1 cm in diameter and demetallized through the following procedure. The tubing was heated to 80 °C in 0.02 M EDTA/0.01 M $KHCO_3$ for 30 min and then washed with deionized water. This step was repeated; then, the tubing was heated to 80 °C in deionized water for 30 min (Vahl, 1970). Glassware and plasticware were rendered metal free by soaking them in a 1/1 solution of H_2SO_4/HNO_3 for 24 h followed by a thorough washing with deionized water (Thiers, 1957). House distilled water was further purified by distillation and treatment with a mixed-bed resin [Bio-Rad AG 501-X8(D)] until the conductivity was below 0.5 $\mu\Omega^{-1}$. All centrifugation was done at 4 °C in a Sorvall RC-5B centrifuge equipped with either a GS-3 or a SS-34 rotor.

Isolation of LAP. ZnZn leucine aminopeptidase was isolated from bovine lens by a procedure based on Hanson's et al. (1965), later modified by Melbye (1970) and most recently amended in this paper. It was found that several changes in Melbye's purification scheme resulted in a procedure that was less tedious and gave significantly greater yields. This isolation procedure is outlined below.

Fresh bovine lenses were added to a 0.85% sodium chloride solution prechilled to 4 °C. The volume of saline solution to be used was determined with the equation: (grams of lenses × 100)/12 = milliliters of 0.85% saline. Typically, a batch (200 lenses) weighed about 400 g and required 3500 mL of saline solution. The lenses were stirred in this solution at 4 °C for 48–72 h. During this period, the lenses dissolved, except for a small number of inner cores, which were discarded.

As the crude extract was stirred at 0 °C, $ZnSO_4$ (0.20 M) was added at a rate of 20–25 drops/min to make a final concentration of 0.006 M in $ZnSO_4$. It is important to maintain a pH between 7.2 and 7.4 (with 1 M NaOH). Following $ZnSO_4$ addition, the milky white solution was centrifuged at 9000 rpm for 15 min, and the pellet was discarded. The $ZnSO_4$ supernatant solution was quickly brought to 54 °C in an 80 °C water bath and then placed into a 54 °C bath for exactly 15 min. The resulting suspension was centrifuged as before and the pellet discarded.

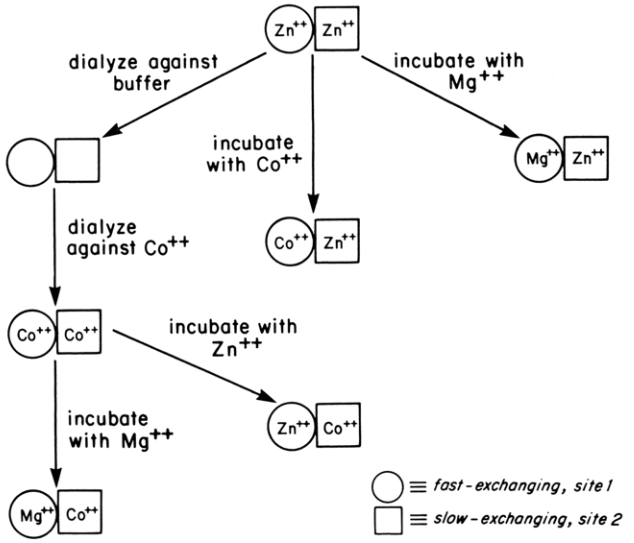
The resultant supernatant solution was vacuum filtered through a large sintered glass funnel, and enzyme-grade $(NH_4)_2SO_4$ was added in small increments until the final concentration of the solution was 340 mg of $(NH_4)_2SO_4$/mL. Then the pH of the solution was adjusted to 7, and 2–3 drops of toluene were added to inhibit bacterial growth. The vessel was sealed and allowed to remain undisturbed at 4 °C for 7 days.

Following this period the LAP precipitate was resuspended by gently swirling the flask and centrifuged for 20 min at 11 000 rpm. Repeated pooling of pellets and recentrifugation was done until all of the LAP precipitate was collected in one small centrifuge tube. This procedure gives greater yields than does filtration. The LAP pellet was washed and finally dissolved in 0.1 M Tris, pH 8. Typical preparations yielded 70 mg of protein, which appeared homogeneous by 7.5% polyacrylamide gel electrophoresis on both native and denatured enzymes.

Preparation of Metal-Substituted Enzyme Derivatives. Scheme I outlines the preparation procedure for the metal-substituted LAP's.

(1) MgZn LAP. A 10-μL aliquot containing 120 μg of ZnZn LAP was combined with 125 μL of 80 mM $MgCl_2$, 50 μL of 0.5 M Na_2CO_3, pH 9.5, and 0.965 mL of distilled water. This mixture was allowed to incubate in a stoppered test tube at 37 °C for 4 h with occasional mixing. Unbound Mg^{2+} and Zn^{2+} were removed by using Chelex 100 resin (1 × 18.5 cm) equilibrated and eluted with 0.2 M NEM, pH 7.5. The elution profile was determined by absorbance at 280 nm. Protein concentration was determined by the Bradford protein assay (Bradford, 1976) and from the relationship $\epsilon_{280}^{1\%} = 10$ for LAP (Vahl, 1970). Protein-containing fractions were assayed immediately for activity (using LpNA; Thompson, 1974) and analyzed for Mg^{2+}, Zn^{2+}, and Co^{2+} on a Perkin-Elmer Model

Scheme I: Metal Substitution in Leucine Aminopeptidase



372 atomic abosorption spectrophotometer equipped with a deuterium background corrector, an air–acetylene flame, and a HGA 2200 graphite furnace.

(*2*) *CoZn LAP.* A 250-μL aliquot containing 1500 μg of ZnZn LAP was dialyzed against 2 L of 1 M KCl and 0.2 M NEM, pH 7.5, for 6 h. A 100-μL portion of the dialyzed ZnZn LAP was combined with 0.1 mL of distilled water and 0.8 mL of a buffer containing 0.25 M NEM, 1.25 M KCl, and 1.25 mM $CoCl_2$ at pH 7.5. This mixture was incubated at 37 °C with occasional mixing for 3 h. The specific activity was checked every half hour, and the incubation mixture was removed and placed in an ice bath when the rapid increase in activity was completed. Unbound metals were removed by the same procedure as described for the MgZn derivative. Fractions containing LAP were immediately assayed for protein concentration and enzymatic activity and analyzed for bound metals as before.

(*3*) *CoCo LAP.* The stock ZnZn LAP was diluted to about 1 mg/mL in 0.2 M NEM, pH 6.75. Aliquots (3 mL) of this dilution were placed into demetallized dialysis bags and dialyzed against 1 L of 1 M KCl and 0.2 M NEM, pH 6.75, for 6 h at 4 °C. The bags were then transferred to another flask containing 2 L of 50 mM $CoCl_2$, 1 M KCl, and 0.2 M NEM, pH 6.75, and dialyzed at 37 °C for 24 h. The precipitated CoCo LAP was collected by centrifugation at 9000 rpm for 5 min. The pellet was washed and finally dissolved in 0.2 M NEM, pH 7.50. Unbound metals were removed by a technique similar to that described by Vahl (1970). Enough glass wool was placed into a drying tube to support a small polypropylene collecting tube. The small-diameter end of the drying tube was connected to a vacuum line, and the upper end was fitted with a rubber adapter. The rubber adapter was small enough to create a snug fit around the end of a Bio-Rad standard Econo-column (0.7 × 10 cm) that contained 3 mL of Dowex 50 W-X8 resin equilibrated in 0.2 M NEM, pH 7.5. This apparatus is detailed in Figure 1.

The column was opened and allowed to drain until no more buffer eluted, at which time the buffer level was about 1 mm below the top of the resin. A 0.5-mL aliquot of approximately 2 mg/mL CoCo LAP was added to the top of the column and allowed to run into the resin. The column was then manually placed onto the rubber adapter on the drying tube so that the solution could be slowly drawn through under mild vacuum. The column was rinsed with 0.5 mL of buffer. The CoCo LAP (in the collecting tube) was immediately assayed for protein

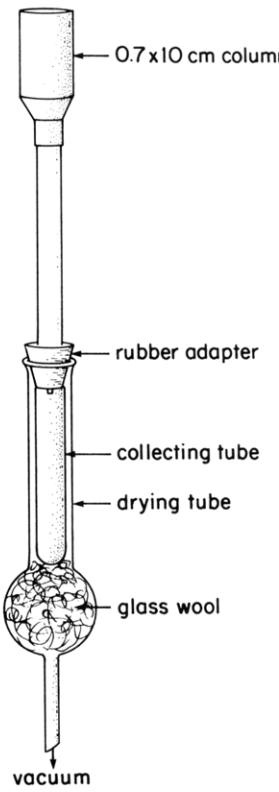


FIGURE 1: Vacuum-assisted column apparatus used to remove unbound metals from the CoCo LAP analogue. A similar apparatus was used to remove unbound metals from the MgCo LAP analogue; a 0.7 × 15 cm column with 6 mL of Dowex 50 W-X8 resin was used in this case.

concentration and enzymatic activity and analyzed for Zn^{2+}, Mg^{2+}, and Co^{2+} as before.

(*4*) *ZnCo LAP.* A 300-μL aliquot containing 480 μg of CoCo LAP was dialyzed against 2 L of an aqueous pH 7 buffer containing 1 M KCl and 0.2 M NEM for 6 h. A 200-μL portion of the dialyzed CoCo LAP was combined with 0.8 mL of a buffer containing 1.25 mM $ZnCl_2$ and 0.25 M NEM, pH 7.0, and incubated at 37 °C for 3 h. The specific activity was checked every half hour, and incubation was terminated when the rapid decrease in activity was completed. Unbound Co^{2+} and Zn^{2+} were removed by the same procedure as described for the MgZn enzyme. Fractions containing LAP were assayed immediately for activity and protein concentration and analyzed for bound metals as before.

(*5*) *MgCo LAP.* A 2.25-mL aliquot of CoCo LAP (approximately 2 mg/mL), adjusted to pH 9.5, was combined with 6.5 mL of distilled water, 1.15 mL of 80 mM $MgCl_2$, and 0.45 mL of 0.5 M Na_2CO_3, pH 9.5, and incubated at 37 °C for 6 h. As before, the activity was checked every half hour, and the incubation was terminated when the rise in activity tapered off. Following incubation, the MgCo LAP was placed into a 10-mL Dia-flo ultrafiltration apparatus equipped with an XM101A filter (molecular weight cutoff = 100000). N_2 was applied at 12.5 psi and the LAP concentrated to 2.5 mg/mL. Unbound metals were removed with a vacuum-assisted column apparatus similar to that described for the CoCo LAP derivative except that Dowex 50 W-X8 resin (0.7 × 15 cm) equilibrated in 0.2 M NEM, pH 7.5, was used. A 0.5-mL aliquot of the MgCo LAP was added to the column. The postcolumn MgCo LAP was immediately assayed for protein and activity and analyzed for bound metals as before.

Kinetics. Kinetic analysis was performed on all six metal-substituted LAP derivatives with LpNA ($\Delta\epsilon_{405}$ = 9900 M^{-1}

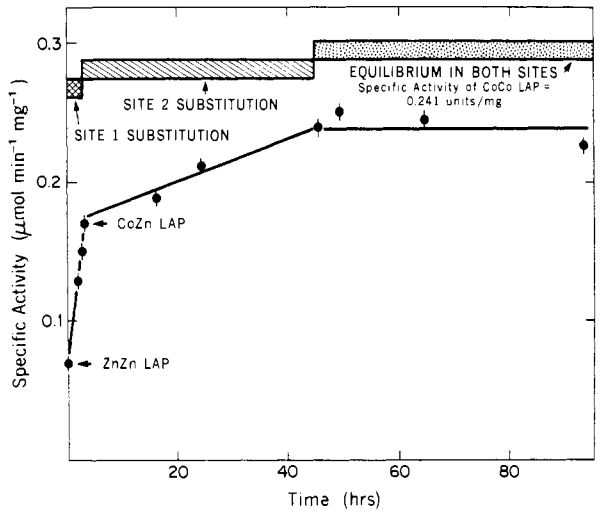


FIGURE 2: Specific activity vs. time plot when 0.50 mg/mL ZnZn LAP (1.91 atoms of Zn^{2+}/subunit) is incubated at 37 °C with 1 mM $CoCl_2$, 1 M KCl, and 0.2 M NEM at pH 7.5. Before incubation, the stock 6 mg/mL ZnZn LAP was dialyzed vs. 0.2 M NEM/1 M KCl, pH 7.5, until excess Zn^{2+} was removed as evidenced by atomic absorption. The graph illustrates the time necessary for site 1 (3 h) and site 2 (45 h) metal exchange.

cm^{-1}), LpA ($\Delta\epsilon_{300}$ = 1850 M^{-1} cm^{-1}), and Leu-NH_2 ($\Delta\epsilon_{238}$ = −14.3 M^{-1} cm^{-1}) as substrates. All kinetic measurements were done at 30 °C in a substrate buffer containing 0.01 M $NaHCO_3$ and 0.2 M NEM, pH 7.5. A pH of 7.5 was chosen (rather than an optimum at ~9) to allow increased solubility of substrates and metal ions. Substrate concentrations were at least 4-fold greater and less than K_m with the exception of the LpNA substrate where in two cases (ZnZn LAP and MgZn LAP) solubility was limiting. When necessary, dilutions of concentrated stock enzyme were done in a buffer that contained an appropriate concentration of metal ions; this was done to avoid loss of bound metals. It was found that the bound metal ions did not dissociate appreciably during the few minutes required for the kinetic assays. On occasion, the substrate buffers were prepared with appropriate concentrations of metal ions, and the kinetic results were the same as when excess metal ions were absent. The substrate hydrolysis reactions were followed spectrophotometrically when possible, using a Gilford Model 252 spectrophotometer equipped with a 2451-A cuvette automatic positioner and a 6051 chart recorder. For the Leu-NH_2 substrate, the small change in extinction made necessary the use of amino acid analysis to follow hydrolysis for the CoCo LAP and MgCo LAP analogues. Kinetic parameters K_m (in mM) and k_{cat} (in μmol min^{-1} mg^{-1}) were determined with the HYPERB computer program (Hanson et al., 1967).

Results and Discussion

Metal-Substituted LAP Preparation. The preparation of the CoCo-substituted enzyme from the native form was straightforward, involving extensive dialysis against high concentrations of $CoCl_2$. This resulted in complete exchange in both binding sites. The metal-substituted enzymes that contained a different divalent ion at each of the two sites (MgZn, MgCo, CoZn, and ZnCo) were possible to prepare because of the large difference in relative binding between the two sites. The preparation of the MgZn form from the ZnZn enzyme and the MgCo form from the CoCo enzyme resulted in homogeneous products since magnesium binds only to site 1 (Carpenter & Vahl, 1973).

Figure 2 shows the increase in specific activity with time when ZnZn LAP is incubated with 1 mM $CoCl_2$. The rapid rise in activity in the first 3 h represents site 1 substitutiton and the gradual increase in specific activity between 3 and 44 h represents site 2 substitution. It was found that both incubation of the ZnZn enzyme with 1 mM $CoCl_2$ and incubation of the CoCo enzyme with 1 mM $ZnCl_2$ resulted in site 1 substitution in 3 h. Steady-state equilibrium in both sites required >40 h; thus, a 3-h incubation resulted in an enzyme with site 1 substitution essentially complete and the site 2 bound metal only slightly altered.

Table I: Atomic Absorption of Metal-Substituted LAP

enzyme form		metal ions per subunit			
site 1	site 2	Zn^{2+}	Mg^{2+}	Co^{2+}	total
Zn^{2+}	Zn^{2+}	2.08	0.00	0.00	2.08
Mg^{2+}	Zn^{2+}	0.89	0.84	0.00	1.73
Co^{2+}	Co^{2+}	0.03	<0.01	1.91	1.95
Mg^{2+}	Co^{2+}	0.04	1.08	1.04	2.16
Co^{2+}	Zn^{2+}	1.12	0.01	0.78	1.91
Zn^{2+}	Co^{2+}	1.09	0.00	0.94	2.03

The preparation of the CoZn enzyme and the ZnCo enzyme presented some difficulty since both zinc and cobalt compete reversibly for both binding sites. Plots of specific activity vs. time such as Figure 2 (similar results were obtained for the conversion of ZnZn LAP → MgZn LAP, CoCo LAP → MgCo LAP, and CoCo LAP → ZnCo LAP; data not shown) illustrate a rapid substitution (3 h) into site 1 and, for the ZnCo and CoZn derivatives, a gradual (approximately 45 h) exchange into site 2. These data indicate that the site 1 bound metal exchanges with divalent metals in solution ~15-fold faster than does the site 2 metal. Therefore, a 3-h incubation at 37 °C results in an enzyme where site 1 substitution is essentially complete while site 2 is only slightly altered (~5% site 2 substitution). In the cases of the CoZn analogue and the ZnCo analogue, it is not suggested that the preparations resulted in entirely homogeneous enzymes. For both the CoZn enzyme and the ZnCo enzyme, it is expected that small amounts of the CoCo and the ZnZn forms were also present. On the basis of the specific activity vs. time plots (indicating that site 2 substitution requires ~15-fold more time for substitution under the conditions used) and the atomic absorption data for the 3-h time points (Table I), it is estimated that the metal-substituted analogues CoZn and ZnCo contained >90% of the desired metal ions.

The atomic absorption data for all six metal-substituted enzyme forms are presented in Table I. These results are the average of several independent measurements that range from three for the CoZn enzyme to as many as ten for the MgCo enzyme. Many preliminary attempts at the removal of unbound metals resulted in enzyme preparations with less than 2 bound metal ions/subunit and with a corresponding loss in enzymatic activity. Great care was necessary in the selection of a column and resin that would remove only unbound metals since passage of the enzyme solution through the column may cause loss of the more loosely bound metals. In most cases a fast flow rate (45 drops/min) and a mild cation-exchange resin (Chelex 100) in addition to an appropriate column length and sample concentration were sufficient to remove only unbound metals. In the cases of the CoCo and the MgCo derivatives, Dowex 50 W-X8, a strong cation exchanger, was necessary, presumably becuase of the large number of unbound ions. In these cases, the flow rate was increased through the use of a vacuum-assisted apparatus (Figure 1).

Kinetic Data. Table II shows the kinetic results for the six metal-substituted enzymes with the three substrates previously mentioned. We are interested here in comparing the relative

Table II: Kinetic Parameters of Metal-Substituted LAP

enzyme form		L-leucine-*p*-nitroanilide		L-leucyl-*p*-anisidine		L-leucine amide	
site 1	site 2	K_m (mM)	k_{cat} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (μmol min^{-1} mg^{-1})
Zn^{2+}	Zn^{2+}	5.9 ± 0.6	0.13 ± 0.01	6.0 ± 0.8	0.22 ± 0.02	51 ± 8	43 ± 2
Mg^{2+}	Zn^{2+}	2.6 ± 0.5	2.8 ± 0.6	3.5 ± 1.3	6.7 ± 0.6	14 ± 3	400 ± 40
Co^{2+}	Co^{2+}	0.26 ± 0.03	0.26 ± 0.01	0.94 ± 0.07	1.9 ± 0.1	0.35 ± 0.05	59 ± 3
Mg^{2+}	Co^{2+}	0.90 ± 0.10	2.7 ± 0.2	1.4 ± 0.1	5.9 ± 0.1	2.0 ± 0.3	42 ± 5
Co^{2+}	Zn^{2+}	1.2 ± 0.2	0.20 ± 0.01	2.2 ± 0.2	1.1 ± 0.1	20 ± 3	39 ± 2
Zn^{2+}	Co^{2+}	1.1 ± 0.1	0.18 ± 0.01	1.8 ± 0.3	0.35 ± 0.03	3.1 ± 0.5	23 ± 3

effects of Zn^{2+}, Mg^{2+}, and Co^{2+} in site 1 and Co^{2+} and Zn^{2+} in site 2 on kinetic parameters. The behavior of K_m will be discussed first. Several trends consistent throughout the data were observed. The data indicate that metal substitution into site 1 (fast exchanging) results in a K_m decrease in the relative order $Zn^{2+} > Mg^{2+} > Co^{2+}$ and that substitution into site 2 (slow exchanging) results in a K_m decrease in the relative order $Zn^{2+} > Co^{2+}$. With LpNA as an example, several comparisons can be made to illustrate this point. The K_m of MgZn LAP is about half that of ZnZn LAP. Similarly, looking at the ZnZn enzyme and the CoZn enzyme reveals that the latter has a K_m approximately a fifth that of the former. Comparison of the ZnCo form with the CoCo form and the MgCo form with the CoCo form again illustrates that Co^{2+} substitution into site 1 results in the largest decrease in K_m, followed by Mg^{2+} and then by Zn^{2+}. The corresponding comparisons for both the LpA substrate and the Leu-NH_2 substrate lead to the same conclusions. There was one exception to this trend that was observed in the Leu-NH_2 data. In this case the MgZn form gives a lower K_m than does the CoZn form, suggesting that the MgZn enzyme does not follow the pattern. As will be discussed later, the MgZn enzyme proved to be exceptional again when its k_{cat} was studied by Leu-NH_2 kinetics.

By using LpNA as an example, the effects of site 2 metal substitution of Co^{2+} for Zn^{2+} can be seen. A comparison of the ZnZn enzyme with the ZnCo enzyme (~6-fold decrease in K_m), the CoZn enzyme with the CoCo enzyme (~5-fold decrease in K_m), and the MgZn enzyme with the MgCo enzyme (~3-fold decrease in K_m) indicates that Co^{2+} substitution into site 2 results in a decrease in K_m. These data along with those for LpA and Leu-NH_2 illustrate that site 2 substitution results in a K_m decrease in the order $Zn^{2+} > Co^{2+}$.

Comparisons can be made that indicate the magnitude of change in K_m associated with each independent metal binding site. To illustrate this, pairs of LAP analogues were compared, and the K_m change resulting from Co^{2+} substitution into site 1 was compared with that of Co^{2+} substitution into site 2. Since the arylamide substrates behave differently from Leu-NH_2, their data will be discussed separately. LpNA illustrates the behavior of the arylamide substrates. Conversion of the ZnZn analogue to the CoZn analogue results in an approximate 5-fold decrease in K_m. A similar decrease in the magnitude of K_m is seen going from the ZnZn analogue to the ZnCo analogue. Similarly, the 4-fold difference in K_m between the ZnCo form and the CoCo form can be compared to the 4.5-fold difference in K_m between the CoZn form and the CoCo form. These comparisons indicate that the magnitude of change in K_m when substituting Co for Zn is nearly the same for both metal binding sites. The corresponding comparisons for the LpA substrate substantiate this conclusion.

The situation for Leu-NH_2 is somewhat different than that with the arylamide substrates. The K_m change resulting from Co^{2+} substitution into site 1 compared with that of Co^{2+} substitution into site 2 clearly indicates that the site 2 metal exerts a greater effect on substrate binding. Going from the ZnZn form to the CoZn form (~2.6-fold decrease in K_m) compared with the conversion of the ZnZn form to the ZnCo form (~16.5-fold decrease in K_m) reveals that site 2 has a 6.4-fold greater effect on K_m. Similarly, the change in K_m (~9-fold) when going from the ZnCo enzyme to the CoCo enzyme compared with the change in K_m (~56-fold) from the CoZn enzyme to the CoCo enzyme illustrates that the site 2 metal has a 6.3-fold greater effect on K_m than does the site 1 metal. Thus, in both series of comparisons, site 2 shows an approximate 6-fold greater effect on K_m than does site 1.

The behavior of k_{cat} with metal substitution is more complex than that of K_m. For both arylamide substrates, the data indicate that Co^{2+} substitution into site 1 or site 2 results in a slight activation and that Mg^{2+} suubstitution into site 1 results in substantial activation. With LpNA as an example, conversion of ZnZn LAP to CoZn LAP (~2-fold increase in k_{cat}), ZnCo LAP to CoCo LAP (~1.5-fold increase in k_{cat}), ZnZn LAP to MgZn LAP (~20-fold increase in k_{cat}), CoCo LAP to MgCo LAP (~10-fold increase in k_{cat}), ZnZn LAP to ZnCo LAP (~1.5-fold increase in k_{cat}), and CoZn LAP to CoCo LAP (~1.3-fold increase in k_{cat}) illustrates the behavior of k_{cat} as mentioned above. Similar comparisons with the LpA substrate give similar activations for Mg^{2+} in site 1 and slightly greater activations when Co^{2+} is substituted for Zn^{2+} in site 1.

The data on k_{cat} suggest that (1) Co^{2+} substitution into either of the binding sites results in a small activation and (2) site 1 exerts a slightly greater effect on k_{cat} than does site 2. However, the differences in k_{cat} values are not conclusive since they are small relative to the combined standard errors associated with k_{cat} values. We cannot therefore make any definitive conclusions regarding the behavior of k_{cat} when Co^{2+} is substituted for Zn^{2+}.

With Leu-NH_2 as substrate, the effects of metal substitution on k_{cat} are extremely complex, possibly suggesting some type of interaction between the metal binding sites. Here, the k_{cat} values for all the enzyme derivatives are similar with the exception of the MgZn derivative. Mg^{2+} substitution into site 1 of the ZnZn enzyme (producing the MgZn form) results in an approximate 10-fold increase in k_{cat}. Mg^{2+} substitution into the CoCo enzyme (producing the MgCo form), in contrast, gives a slightly decreased k_{cat} value. This represents the first case where Mg^{2+} substitution into site 1 has not resulted in an increased K_{cat}. This suggests that site 1 Mg^{2+} activation is not absolute but is dependent on the metal present in site 2. On the basis of k_{cat} values for the Leu-NH_2 substrate, the metal-substituted enzyme forms can be ranked in order of decreasing k_{cat} as follows: MgZn (k_{cat} = 396 ± 36), CoCo (k_{cat} = 59.7 ± 3.0), ZnZn (k_{cat} = 43.2 ± 1.9), MgCo (k_{cat} = 41.7 ± 5.1), CoZn (k_{cat} = 38.8 ± 1.7), and ZnCo (k_{cat} = 22.8 ± 2.7). These data indicate that k_{cat} is not determined exclusively by one site but is dependent on the metal bound in

both sites and suggests some form of interaction between the two.

The kinetic results clearly illustrate that each of the metal binding sites exert significant effects on K_m and k_{cat}. This finding is contrary to previous suggestions [e.g., Thompson & Carpenter (1976a,b)] that K_m is affected by site 2 only and that site 1 effects k_{cat}. Previous to this study, however, direct experimental comparison of kinetic parameters resulting from site 2 metal exchange was not attempted.

The data indicate a more complex interaction between the bound metal than previously believed. The interaction between the bound metals can be seen in several places in the data. The observation from the arylamide substrates showing equivalent K_m effects from both binding sites suggests that the two bound metals are close. Also, data from the Leu-NH_2 substrate comparing the CoCo enzyme with the MgCo enzyme show that Mg^{2+} does not always activate but that its effect depends on the metal bound in site 2. In the case of the MgZn enzyme with the Leu-NH_2 substrate, the k_{cat} value is significantly higher than all others, suggesting that the Mg^{2+}(site 1)–Zn^{2+}(site 2) combination yields the highest hydrolysis rates.

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