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Proteolytic Specificity and Cobalt Exchange of Hemorrhagic Toxin *e*, a Zinc Protease Isolated from the Venom of the Western Diamondback Rattlesnake (*Crotalus atrox*)†

Jon Bragi Bjarnason* and Jay William Fox

ABSTRACT: Hemorrhagic toxin *e*, a zinc protease causing hemorrhage, was isolated from the venom of the western diamondback rattlesnake (*Crotalus atrox*) by a previously published procedure [Bjarnason, J. B., & Tu, A. T. (1978) *Biochemistry 17*, 3395–3404]. The proteolytic specificity of hemorrhagic toxin *e* was investigated by using the oxidized A and B chains of bovine insulin. The enzyme cleaves the Ala_{14}–Leu_{15} bond of insulin B chain very rapidly and the Ser_9–His_{10} and Asn_3–Gln_4 of the same chain more slowly. The enzyme cleaves Tyr_{14}–Gln_{15} of insulin A chain rapidly and Ala_6–Ser_9 of the same chain more slowly. The cleavage of insulin A chain by hemorrhagic toxin *e* was inhibited by ethylenediaminetetraacetic acid but not by aprotinin. Hemorrhagic toxin *e* containing cobalt(II) instead of zinc cleaved the A and B chains of insulin at the same sites and with similar rapidity as the native toxin. The cobalt-containing toxin was prepared by direct exchange with dialysis and contained approximately 1 mol of cobalt per mol of toxin but was devoid of zinc. No structural changes were observed accompanying the metal exchange by using circular dichroism (CD) and ultraviolet spectroscopy as structural probes, whereas considerable structural changes had occurred upon simple removal of zinc from the toxin, i.e., formation of the apoenzyme. The absorption spectrum of the cobalt hemorrhagic toxin *e* in the visible region had a maximum at 505 nm (170 cm^{-1} M^{-1}), and the CD spectrum in the visible region had a minimum at 480 nm (−3200 deg cm^2 $dmol^{-1}$) indicative of a distorted tetrahedral complex with oxygen and nitrogen ligands. Cobalt hemorrhagic toxin *e* was both hemorrhagic and proteolytic to a similar extent as the native toxin.

Five hemorrhagic toxins from the venom of *Crotalus atrox* have been characterized as metalloproteases containing zinc in the native form (Bjarnason & Tu, 1978). Proteolytic activities of each of the hemorrhagic toxins on the general protease substrates dimethylcasein and dimethylhemoglobin were measured by reacting 2,4,6-trinitrobenzenesulfonic acid (TNBS)[1] with the newly generated amino groups. It was furthermore demonstrated that the hemorrhagic activity of hemorrhagic toxin *e*, one of the five hemorrhagic toxins, correlates with its proteolytic activity and with its zinc content.

Although there has been considerable confusion as to the proteolytic activities of the hemorrhagic toxins from the venoms of Japanese snakes, probably due to the use of the casein–trichloroacetic acid precipitation method as a criteria of proteolytic activity, some of them have been demonstrated to act on isolated basement membrane, releasing protein and

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; HT-*e*, hemorrhagic toxin *e*; TIU, trypsin inhibitor units.

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carbohydrate fragments (Ohsaka et al., 1973).

Electron microscopic studies on the action of hemorrhagic toxins from *C. atrox* (Ownby et al., 1978) and toxins from the venom of the Japanese snake Habu, *Trimeresurus flavoviridis* (Tsuchiya et al., 1974), suggest that the mechanism of hemorrhage could be due to lysis of the endothelial cells of the microcirculatory system or digestion of the connective tissue, collagens and basement membrane, surrounding the endothelial cells, or both.

Thus, the primary event of snake venom induced hemorrhage could be disruption of the pericellular collagen basement membrane and other connective tissue collagens by proteolytic toxins with high specificities toward peptide bonds of the connective tissue proteins. It is therefore important to know what kind of proteolytic specificities these hemorrhagic toxins possess and in which manner, if at all, they act on potential connective tissue substrates.

Since the discovery of the catalytically essential zinc in the hemorrhagic toxins of *C. atrox* venom (Bjarnason & Tu, 1978), other hemorrhagic toxins have also been found to contain zinc. The metal is likely involved either in substrate binding or in catalysis, or both. In an effort to further our understanding of the catalytic action of the hemorrhagic toxins and to delineate the mechanism of hemorrhage, it is of interest to investigate the functional and structural consequences of removing the zinc metal from the toxins as well as substituting other metals for the native zinc metal.

The purpose of this study was to investigate the consequences of substituting cobalt(II) for zinc in hemorrhagic toxin *e*, to observe the spectral properties of the cobalt(II)–hemorrhagic toxin *e* complex, and to determine the peptide bond specificity of hemorrhagic toxin *e* and cobalt(II) hemorrhagic toxin *e* on the oxidized chains of bovine insulin.

Materials and Methods

Lyophilized crude venom was purchased from Miami Serpentarium, Miami, FL. Hemorrhagic toxin *e* was isolated from the crude venom by the previously published procedure (Bjarnason & Tu, 1978) with Tris–glycinate disc gel and $NaDodSO_4$ disc gel electrophoresis as the criteria of purity. The oxidized A and B chains of bovine insulin, aprotinin, acrylamide, Tris base, and $NaDodSO_4$ were obtained from Sigma Chemical Co. Zinc and cobalt standards, EDTA (ethylenediaminetetraacetic acid), and Spectrapor dialysis tubing were purchased from Fisher Chemical Co. All other materials used were of analytical grade.

Cobalt Hemorrhagic Toxin e. Cobaltous ion was introduced into hemorrhagic toxin *e* by the method of direct exchange with dialysis. An 80 μM hemorrhagic toxin *e* solution (2.0 mg/mL) in 5 mM Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl and 2 mM $CaCl_2$ was dialyzed against a 50-fold volume of 0.2 M cobaltous ion in 0.1 M sodium acetate, pH 5.5, for 36 h at 2–4 °C. The solution was constantly purged with N_2. Excess cobalt was then removed in two separate batches by extensive dialysis against 0.2 M Tris–acetate, pH 7.5, on the one hand and against 5 mM Tris-HCl, pH 8.5, containing 0.1 M NaCl and 2 mM $CaCl_2$ on the other. The former solution was used in spectroscopic studies while the latter was used for activity measurements. Metal ions were analyzed by atomic absorption spectrophotometry on a Beckman atomic absorption modular system.

Assay for Hemorrhagic Activity. Five Swiss Webster mice (20 g) were each injected subcutaneously with a dose of toxin and sacrificed after 6 h. The minimum hemorrhagic dose was defined as the least quantity of venom or toxin per gram of body weight of mouse which caused a hemorrhagic reaction of 5 mm in diameter 6 h after subcutaneous injection.

Assay for Proteolytic Activity. Proteolytic activity was assayed by using dimethylcasein prepared according to Lin et al. (1969) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) to react with the amino groups. The substrate solution, 9.9 mL of dimethylcasein (1 mg/mL) in 0.01 M borate, pH 9.0, containing 0.1 M NaCl and 2 mM $CaCl_2$, was brought to 37 °C in a water bath, and a 0.9-mL blank was withdrawn and incubated in a boiling water bath for 2 min, after which 0.1 mL of HT-*e* was added and the mixture incubated for another 2 min in boiling water. The reaction was started by adding 1 mL of HT-*e* solution to the remaining 9 mL of substrate solution. At intervals, 1-mL aliquots were withdrawn from the digestion mixture, and the digestion was stopped by immersing the aliquot in a boiling water bath for 2 min. To each aliquot of the reaction mixture were added 1 mL of 4% sodium bicarbonate in H_2O and 1 mL of 0.1% TNBS in H_2O. This mixture was incubated for 30 min at 50 °C, and then 1 mL of 10% $NaDodSO_4$ solution and 0.5 mL of 1 N HCl were added to the mixture. The solution was gently mixed and allowed to stand until the foaming subsided. The extinction of the solution at 420 nm was read vs. the blank. A molar extinction coefficient of 6.46×10^3 M^{-1} cm^{-1} (Goldfarb, 1966) for the trinitrophenyl α-amino groups was used to estimate the turnover number.

Spectroscopy. Ultraviolet and visible region spectra were obtained at 25 °C with a Cary Model 118 UV–vis spectrophotometer from Varian Associates. The circular dichroic spectra were obtained with a Jasco Model J-41C spectropolarimeter at 25 °C. The toxins were in a solution of 0.2 M Tris–acetate, pH 7.5.

Specificity Studies. Oxidized insulin A chain, 0.71 mg in 0.675 mL of 10 mM Tris-HCl buffer, pH 8.1, was incubated with 25 μL of HT-*e* (1 mg/mL) at 37 °C. Another such sample of insulin A chain was incubated with 25 μL of Co(II)HT-*e* (0.76 mg/mL). At timed intervals, 75-μL aliquots were withdrawn from the digestion mixture, and the digestion was stopped by adding 10 μL of glacial acetic acid. After the sample was spun, 75 μL of the solution was injected into a Waters C-18 μBondapak column equilibrated with buffer A containing 0.025 M ammonium acetate, pH 6.0. The column was developed with a gradient of 0–100% buffer B containing 0.025 M ammonium acetate and 60% acetonitrile in 60 min with a flow rate of 1.5 mL/min. This was performed with a Beckman 421 HPLC system using peak detection at 214 nm with a Gilson UV detector. Similarly, oxidized B chain, 0.76 mg in 0.675 mL of 10 mM Tris-HCl buffer, pH 8.1, was incubated with 25 μL of HT-*e* (1 mg/mL) at 37 °C. Another such sample of insulin B was incubated with 25 μL of Co(II)HT-*e* (0.76 mg/mL). Peak fractions were collected and evaporated to dryness. Then the fractions were hydrolyzed in 6 N HCl for 24 h at 110 °C and analyzed for amino acids. From the amino acid compositions of the peak fractions and the known sequences of the insulin chains, sites of cleavage were deduced.

Inhibition Studies. Oxidized insulin A chain (1 mg/mL) was incubated with HT-*e* (25 μg/mL) at 37 °C in 50 mM Tris-HCl, pH 8.1, and 25 μL of one of the three following solutions: distilled water, aprotinin (17 TIU/mL), and 10 mM EDTA. Aliquots were withdrawn and analyzed as described above.

Results

Proteolytic Activity. Hemorrhagic toxin *e* was assayed for proteolytic activity with dimethylcasein as substrate and gave a positive response. A series of four different toxin concen-

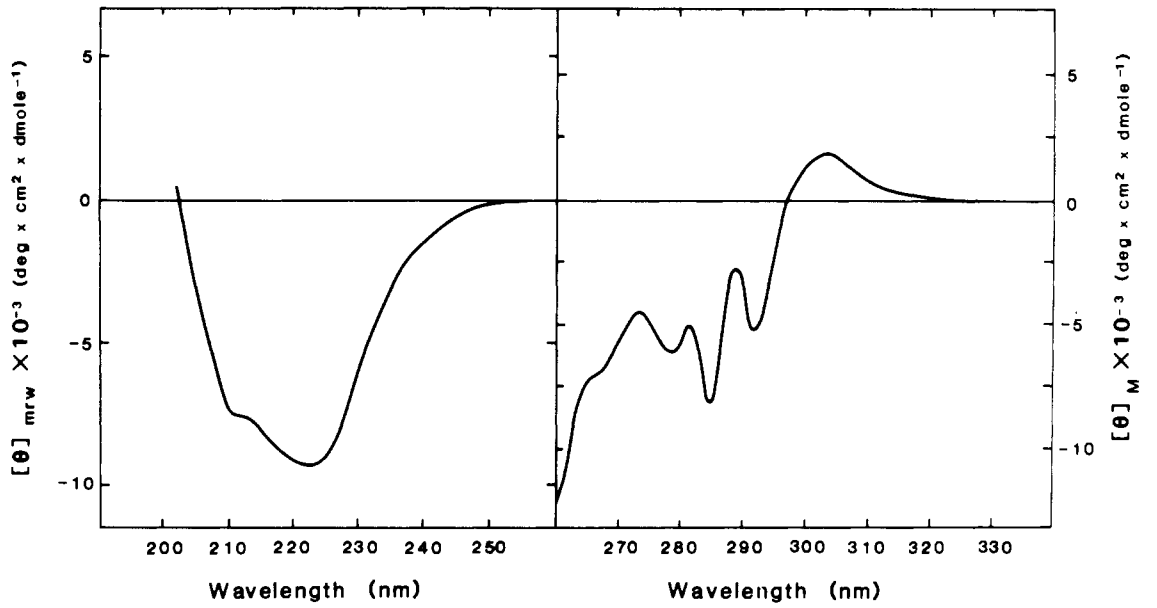

FIGURE 1: Circular dichroic spectra of cobaltous hemorrhagic toxin *e* in the aromatic region (right) and in the peptide region (left). Molar ellipticities are based on a molecular weight of 25 000, and mean residue ellipticities are based on a mean residue weight of 115.

trations were used which gave a family of curves. These results were interpreted in terms of peptide bond cleavage by the use of a molar extinction coefficient for the newly formed trinitrophenyl α-amino groups of 6.43×10^3 M^{-1} cm^{-1} (Goldfarb, 1966). The initial velocities estimated on this basis were plotted against the molar toxin concentration, resulting in a linear plot. The slope of the linear plot was 13 min^{-1}, suggesting that the turnover number of peptide bond cleavage in dimethylcasein by hemorrhagic toxin *e* is approximately 13 peptide bonds per minute for the peptide bonds that are cleaved the fastest.

Cobalt Exchange. When the toxin was dialyzed against cobaltous ion, cobalt hemorrhagic toxin *e* was formed containing 1.1 mol of cobalt per mol of the toxin and no measurable zinc. Previous attempts to incorporate cobalt into the apotoxin were unsuccessful. The cobalt-containing toxin was found to possess both hemorrhagic and proteolytic activities and to a similar degree as the native toxin, although slightly less. The minimum hemorrhagic dose of Co(II)HT-*e* was found to be 1.2 μg as compared to 1.0 μg for the native toxin. The cobalt-containing toxin was estimated to have a turnover number of 10 min^{-1} whereas the turnover number for the native toxin is 13 min^{-1}.

Spectroscopy. The UV spectra of hemorrhagic toxin *e* and cobalt hemorrhagic toxin *e* appear to be identical, with shoulders at 291 and 284 nm and a peak at 278 nm. Likewise, the CD spectra of the cobalt-containing toxin in the aromatic and peptide regions (Figure 1) appear totally identical with the previously published spectra of the native toxin (Bjarnason & Tu, 1978). There thus appears to be no change in structure of hemorrhagic toxin *e* accompanying the metal exchange. This is in stark contrast to the considerable structural changes observed with the same spectroscopic methods accompanying production of the apotoxin by zinc removal. Thus, zinc removal causes large structural perturbations whereas replacing zinc with cobalt is accomplished without observable structural changes.

The absorption and CD spectra of the complex of cobaltous ion and hemorrhagic toxin *e* in the visible region can be seen in Figure 2. The absorption spectrum has a peak at 505 nm with in ϵ value of 170 M^{-1} cm^{-1}. There also appears to be a shoulder at 550 nm and another peak at about 600 nm. The

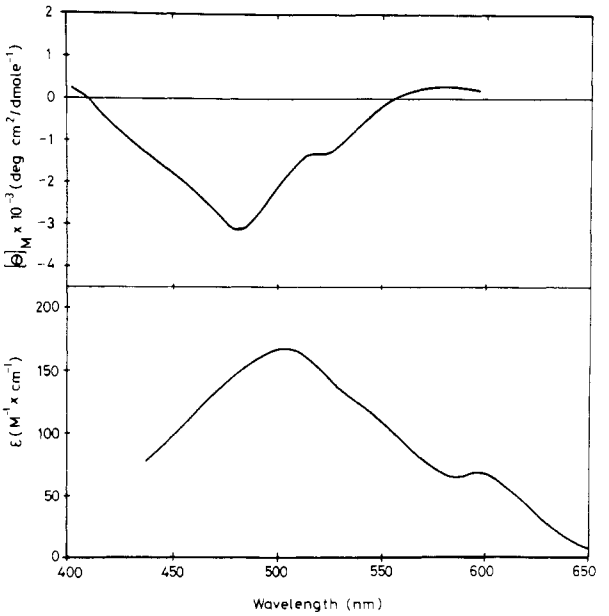

FIGURE 2: Absorption spectrum (bottom) and circular dichroic spectrum (top) of cobaltous hemorrhagic toxin *e* in the visible region.

CD spectrum shows minima at 480 and 520 nm. These spectra resemble those of some other cobalt–enzyme complexes thought to possess distorted tetrahedral structures although we have not come across spectra of striking similarity to those shown here.

High-Performance Liquid Chromatography of Cleavage Products. The disappearance of the insulin A chain and insulin B chain peaks and the generation of specific proteolytic fragments were observed in the HPLC experiments after incubation with hemorrhagic toxin *e* and cobalt hemorrhagic toxin *e* for periods ranging from 30 min to 24 h. After a 30-min incubation time, there was clear evidence of cleavage at more than one point on the insulin B chain. The intact B chain had all but disappeared, and cleavage at a secondary site was already evident. Peaks due to cleavages at a tertiary site start appearing after about 2–4 h of incubation, albeit at a very slow rate. After 1 h of incubation of the A chain with hemorrhagic toxin *e*, about 70% of the insulin A chain peak

Table I: Amino Acid Composition of Cleavage Fragments from the B Chain of Insulin[a]

	peptide B1		peptide B2		peptide B4		peptide B5		peptide B6	
amino acid	theor	exptl	theor	exptl	theor	exptl	theor	exptl	theor	exptl
Lys	1	1.27								
His			2	1.41	1	0.83	1	0.66		
Arg	1	0.93								
Cys	1	0.75	1	0.62	1	0.66	1	0.66		
Asp			1	1.41	1	1.04			1	1.19
Thr	1	1.03								
Ser			1	1.37	1	1.08	1	1.09		
Glu	1	1.35	2	2.39	1	1.22	1	1.18		
Pro	1	1.02								
Gly	2	2.21	1	1.90	1	1.42	1	1.46		
Ala	1	1.02	1	1.16						
Val	1	0.91	2	1.57	1	0.96				
Met									1	0.92
Ile										
Leu	2	1.92	2	1.78	1	1.04	1	0.95		
Tyr	2	1.65								
Phe	2	1.68	1	0.82	1	0.75	1	0.88		

[a] Hydrolysis time of 24 h. Where no values are shown, actual amounts present were negligible.

Table II: Amino Acid Composition of Cleavage Fragments from the A Chain of Insulin[a]

amino	peptide A1		peptide A2		peptide A3		peptide A4	
acid	theor	exptl	theor	exptl	theor	exptl	theor	exptl
Lys								
His								
Arg								
Cys	1	0.88	3	2.55	1	0.84	2	1.49
Asp	2	2.07						
Thr								
Ser			2	1.77	2	1.86		
Glu	2	2.20	2	2.17			2	2.18
Pro								
Gly			1	1.17			1	1.45
Ala			1	1.06			1	1.02
Val			2	1.79	1	1.08	1	0.90
Met								
Ile			1	0.77			1	0.79
Leu	1	1.02	1	1.06	1	1.10		
Tyr	1	0.96	1	0.95	1	1.06		
Phe								

[a] Hydrolysis time of 24 h. Where no values are shown, actual amounts present were negligible.

had disappeared, and cleavage at a secondary site was already evident by the appearance of peaks due to peptide A3 and A4 (Figures 3 and 4).

Analysis of Cleavage Products. Peak fractions from the HPLC separation were collected, dried in a stream of nitrogen gas, hydrolyzed under vacuum with 6 N HCl for 24 h at 110 °C, and analyzed for amino acid composition. The amino acid compositions of the separated peptides are reported in Tables I and II. Peaks due to the undigested material were also treated in this manner and thus confirmed to be due to insulin A chain and insulin B chain, although not reported in Tables I and II.

From Table I, it is evident that peptide B1 is the carboxyl-terminal half of the insulin B chain, i.e., Leu_{15}–Ala_{30}, and peptide B2 is the amino-terminal half of the B chain, i.e., Phe_1–Ala_{14} (see also Figure 3). Early appearance of these peptides is evidence of cleavage at the primary site on the insulin B chain, that is, the Ala_{14}–Leu_{15} bond. Peptide B4 is the amino-terminal nonapeptide Phe_1–Ser_9 and testifies to the secondary site of cleavage on the B chain. Peaks due to peptide B3 (His_{10}–Ala_{15}) or cleavage products thereof could not be located in the HPLC experiment. From the amino acid compositions of peptides B5 and B6, it is clear that peptide B6 is the amino-terminal tripeptide part of the B chain and peptide B5 is the hexapeptide Gln_4–Ser_9 pointing to a tertiary site of cleavage on the B chain. The amino acid compositions of peptides formed by digestion of the insulin A chain with hemorrhagic toxin *e* and cobalt hemorrhagic toxin *e* are shown in Table II. The peptides appearing first are referred to as A1 and A2. Peptide A1 constitutes the carboxyl heptapeptide Gln_{15}–Asn_{21} while peptide A2 is composed of the rest of the A chain, i.e., the Gly_1–Tyr_{14} peptide. Peptides A3 and A4, which are formed by a cleavage at a secondary site, are composed of the hexapeptide Ser_9–Tyr_{14} and the amino-terminal octapeptide Gly_1–Ala_8, respectively.

Table III: Activities of the Two Forms of Hemorrhagic Toxin *e*

	form of toxin	
activity	holo HT-*e*	cobaltous HT-*e*
turnover of hydrolysis of dimethylcasein (min^{-1})	13	10
min hemorrhagic dose (μg of toxin/g of mouse)	1.0	1.2
turnover of cleavage of Tyr_{14}–Gln_{15} bond (min^{-1})	3.6	2.6
turnover of cleavage of Ala_8–Ser_9 bond (min^{-1})	0.46	0.46

Sites of Cleavage. The sites of cleavage of hemorrhagic toxin *e* and the cobalt-substituted toxin on the oxidized A and B chains of insulin can be seen in Figure 3, determined in accordance with the amino acid compositions of the isolated cleavage products as described above. The order of cleavage at the various sites was assigned according to the order of appearance of the different cleavage products. Thus, the most rapid cleavage on the two insulin chains occurred at the Ala_{14}–Leu_{15} bond on the insulin B chain. A secondary site of cleavage on the B chain was observed at Ser_9–His_{10}, and a tertiary and very slow cleavage occurred at Asn_3–Gln_4. The primary site of cleavage on the insulin A chain is the Tyr_{14}–Gln_{15} bond with a secondary site of cleavage at the Ala_8–Ser_9 bond. The difference in the rates of cleavage at the different sites was estimated to be approximately an order of magnitude.

Kinetics of Cleavage. An attempt was made to quantitate the rates of the various activities of hemorrhagic toxin *e* and cobalt hemorrhagic toxin *e*. The results are summarized in Table III. Under the experimental conditions used, the rate

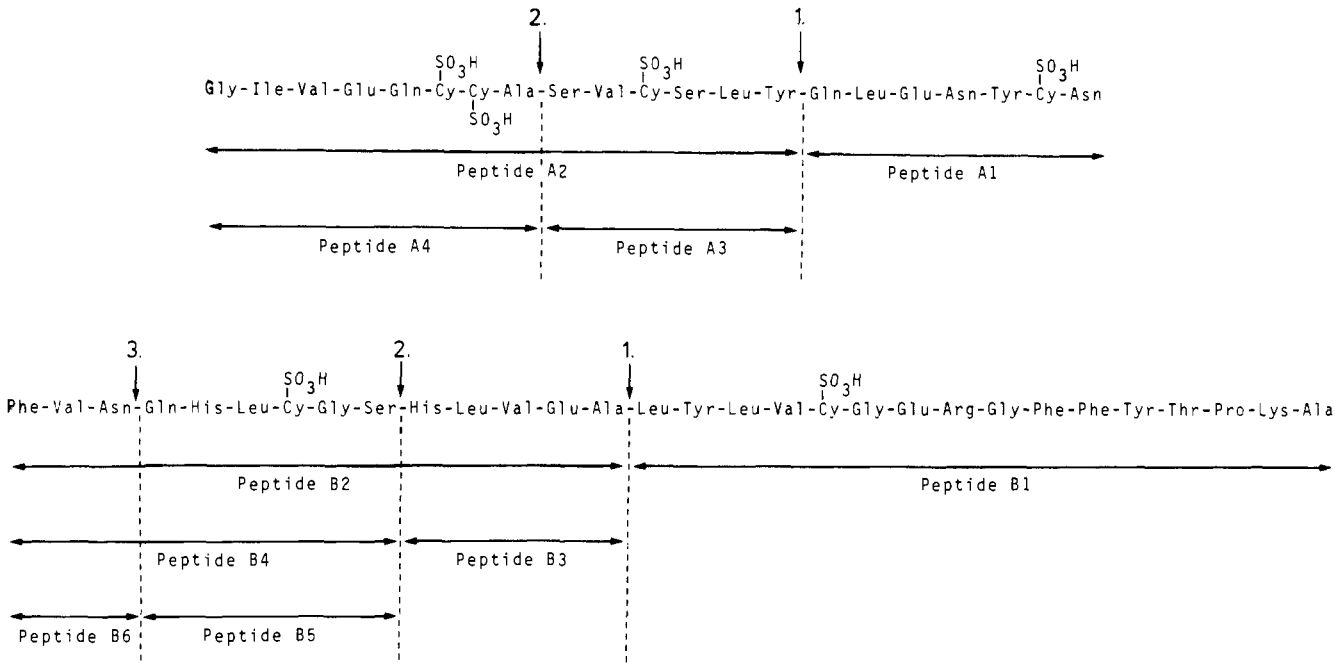

FIGURE 3: Sites of cleavage of the oxidized A and B chains of insulin by hemorrhagic toxin *e* and cobalt hemorrhagic toxin *e* and assignment of cleaved peptides. Numbers indicate sequence of cleavage.

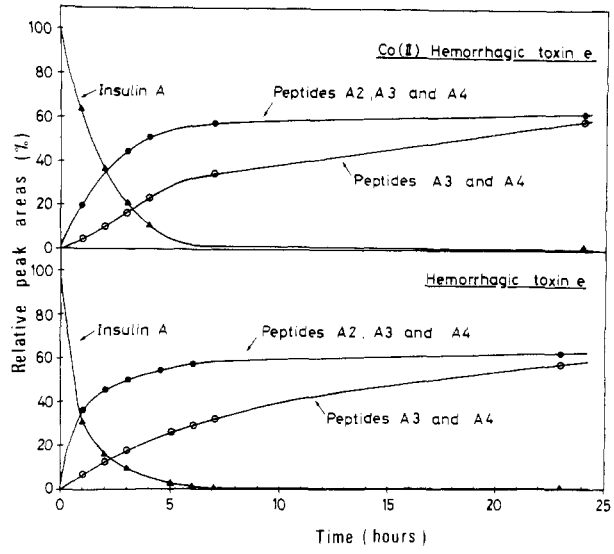

FIGURE 4: Rate of cleavage at the two sites on the oxidized A chain of insulin by hemorrhagic toxin *e* (bottom) and cobaltous hemorrhagic toxin *e* (top) expressed in terms of the relative peak areas of the insulin A chain peak, the sum of the relative peak areas of the peptides A_2, A_3, and A_4, and the sum of the relative peak areas of peptides A_3 and A_4 peaks from the HPLC experiment as a function of time.

of cleavage of the primary site on the insulin B chain was too fast to allow reliable estimates of cleavage rates to be made. However, it seems appropriate to state that a turnover of cleavage of at least 4 min^{-1} was observed. The primary site of cleavage on the insulin A chain is the Tyr_{14}–Gln_{15} bond, and the secondary site of cleavage is the Ala_8–Ser_9 bond. Figure 4 shows the relative peak areas and sums of relative peak areas for various peptides formed during insulin A digestion, allowing estimates of the rates of cleavage at the two sites to be made. The appearance of peptides A2, A3, and A4 represents rates of cleavage at the primary site while the appearance of peptides A3 and A4 represents rates of cleavage at the secondary site, both on a comparable scale of reference. The turnover of cleavage for the primary site was on this basis estimated to be 3.6 min^{-1} for HT-*e* and 2.6 min^{-1} for Co(II)HT-*e*, but for the secondary sites, the turnover of cleavage was estimated to be 0.46 min^{-1} for both forms of the toxin.

Inhibition Studies. The cleavage of oxidized insulin A chain by hemorrhagic toxin *e* was totally inhibited by EDTA but unaffected by aprotinin. This is in good agreement with our previous finding that EDTA and 1,10-phenanthroline were found to inhibit both the hemorrhagic and caseinolytic activities of hemorrhagic toxin *e* and demonstrates further that the toxin is indeed a metalloprotease.

Discussion

The occurrence of hemorrhage is one of the most striking consequences of the parenteral injection of many snake venoms, especially crotalid and viperid venoms. In the less severe cases, hemorrhage is limited to cutaneous and subcutaneous tissues at the site of injection. In severe cases, hemorrhage may even spread to cover a large portion of the involved extremity, affecting the muscle tissue. Bleeding in several organs such as the brain, heart, lungs and kidneys is often encountered (Ohsaka, 1979). The most outstanding local effects of rattlesnake poisoning are edema, hemorrhage, and myonecrosis. In cases of severe poisoning, these effects can lead to complete destruction and subsequent dysfunction or loss of an extremity. In the United States, the main problem in rattlesnake bite cases is local tissue damage owing to the inability of polyvalent Crotalidae antivenom to prevent these lesions (Ownby, 1981).

Since crotalid venoms are strongly hemorrhagic and also posses proteolytic activity, it was thought as early as 1930 (Houssay, 1930) that venom proteases were responsible for inducing hemorrhage. In certain cases, some isolated proteases from snake venom were found to contain hemorrhagic activity (Oshima et al., 1968), but in other cases, and hemorrhagic activity of a venom could be separated from the proteolytic activity by using casein as substrate (Takahashi & Ohsaka, 1970b). Furthermore, a few nonvenom proteases such as bacterial collagenases, elastases, and thermolysis have been found to possess hemorrhagic activity while other more general proteases do not induce hemorrhage (Takahashi & Ohsaka, 1970b; Bjarnason, 1978; Ohsaka, 1979). There are also many reports of proteases isolated from snake venom that do not

possess hemorrhagic activity (Ohsima et al., 1968; Takahashi & Ohsaka, 1970s; Fox & Bjarnason, 1982; Hong, 1982). There is no reason to assume that all proteases from snake venom or other sources possess hemorrhagic activity, even though all hemorrhagic toxins may be proteolytic. There is, however, reason to ask whether hemorrhagic toxins contain proteolytic activity toward unusual substrates such as the peptide bonds of connective tissue proteins. For example, the nature of hemorrhage is such that it appears to demand the lysis of connective tissue, and indeed, such a lysis appears to take place, judging from electron microscopic studies (Tsuchiya et al., 1974; Ownby et al., 1978). Bacterial collagenases cause hemorrhage, and hemorrhagic venom components have been found to cause lysis of basement membrane. Many of the proteolytic hemorrhagic toxins, isolated to date, have low activities toward general protease substrates.

For the past 50 years, one of the central issues in the study of snake venom induced hemorrhage has been whether the hemorrhagic toxins are proteolytic enzymes or not. This question has evaded the efforts of researchers in the field for several reasons. The purification of the hemorrhagic components of venoms has proven to be difficult due to the complexity of the venoms, demanding techniques only available in the last decade or so. Some investigators have only used conventional substrates such as casein and less sensitive assay methods such as trichloroacetic acid precipitation followed by measurement of soluble peptides as described by Anson (1938) and Kunitz (1946). If a protease has low activity or specificity toward these substrates, the activity may not be detected at all with these methods. Unfortunately, some researchers have come to the conclusion that certain hemorrhagic venom components are not proteolytic at all if they have shown a negative protease response with these assay methods.

The hemorrhagic components of snake venom have been termed hemorrhagic principles (Ohsaka et al., 1960), hemorrhagins (Grotto et al., 1967), hemorrhagic factors (Mandelbaum et al., 1975), and hemorrhagic toxins (Bjarnason & Tu, 1978). There are now reports of 19 hemorrhagic venom components isolated from 6 species of snakes and characterized to such a degree as to allow comparisons of their properties to be made (Table IV). It is apparent from this comparison that there are two classes of hemorrhagic venom components: high molecular weight (60 000–90 000) and high activity toxins such as HT-*a* from *C. atrox*, HR-I and HR-II from *Agkistrodon halys blomhoffi*, HR-1A and HR-1B from *T. flavoviridis*, and the hemorrhagins from *Vipera palistinae* and low moleular weight (22 000–26 000) and relatively low activity toxins such as HT-*b*, *-c*, *-d*, and *-e* from *C. atrox*, Ac_1 proteinase and Aa hemorrhagins from *A. acutus*, and HR-2a and HR-2b from *T. flavoviridis*. Of the 19 hemorrhagic toxins purified so far, proteolytic activity has not been detected in 4 of them. The other 15 appear to be metal-dependent proteolytic enzymes. Six of the toxins have been found to contain zinc in a ratio of approximately 1 mol of zinc/mol of toxin. Both hemorrhagic and proteolytic activities of these toxins are dependent on the zinc content. Unfortunately, the metal composition of the other 13 toxins has not been measured, but solutions of most of the toxins have been found to be stabilized by the presence of calcium ions. It therefore seems apparent that venom-induced hemorrhage is, in most cases, caused by some type of proteolytic action, probably on the connective tissue, but whether all the hemorrhagic toxins are proteolytic enzymes is not yet clear. It is of course conceivable that some toxins induce hemorrhage by a different mechanism than proteolysis, such as by another type of lytic action or in an

Table IV: Comparative Properties of Hemorrhagic Toxins Isolated to Date

family:	Crotalidae				Crotalidae	Crotalidae			Crotalidae	Crotalidae					Viperaidae
genus:	*Crotalus*				*Agkistrodon*	*Agkistrodon*			*Agkistrodon*	*Bothrops*					*Vipera*
species:	*atrox*				*acutus*	*acutus*			*halys*	*jararaca*					*palistinae*
subspecies:									*blomhoffi*						
	hemorrhagic toxin[d]				hemorrhagic toxin Ac_1 proteinase	Aa-hemorrhagic			hemorrhagic factor	hemorrhagic factor HF_2	hemorrhagic principle				hemorrhagin
	a	*b*	*c, d*	*e*		AaHI	AaHII	AaHIII	HR-I	HR-II	HR-1A	HR-1B	HR-2a	HR-2b	HR1	HR2	HR3
mol wt	68 000	24 000	24 000	25 700	24 500	22 000	22 000	22 000	85 000	95 000	60 000	60 000	ND[c]	ND	60 000	60 000	60 000
zinc content	0.99	0.82	0.86	1.03	1.15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
inhibited by EDTA[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
proteolytic activity[b]	+	+	+	+	+	+	+	+	–	+	+	+[e]	–	–	+	+	–
isoelectric point	acidic	basic	acidic	5.6	4.7	acidic	acidic	basic	4.70	4.18	ND	4.4	4.4	ND	ND	basic	weakly acidic	strongly acidic
ref	Bjarnason & Tu (1978)				Nikai et al. (1982)	Xu et al. (1981)			Oshima et al. (1972)	Oshima et al. (1968)	Mandelbaum et al. (1975)	Omori-Satoh & Sahahiro (1979)		Takahashi & Ohsaka (1970b)	Ovadia (1978)		

[a] Inhibition of hemorrhagic activity by EDTA is indicated by +. [b] Presence of proteolytic activity is indicated by +; nondetection of proteolytic activity is indicated by –. [c] ND means not determined. [d] All data presented here for hemorrhagic toxin *c* is identical with the data for hemorrhagic toxin *d*. [e] Almost free from proteolytic activity (Omori-Satoh & Sadahiro, 1979).

indirect manner such as by activation of proteolytic enzymes already present in the system. The observation that the noncaseinolytic HR-2a and HR-2b from *T. flavoviridis* digest basement membrane (Ohsaka et al., 1973) suggests a mechanism of direct lytic action.

It has previously been demonstrated that the five hemorrhagic toxins isolated from the venom of *C. atrox* are zinc proteases and that the structure and dual activity of hemorrhagic toxin *e* are directly related to the zinc content. A large number of metalloenzymes are now known to contain zinc, a diamagnetic metal whose complexes do not exhibit visible absorption spectra. These properties limit the suitability of zinc as a probe for the environment of the metal ion at the active sites of such enzymes. In contrast, cobalt is paramagnetic and gives rise to visible absorption spectra and therefore can be a good environmental probe. In complex ions, cobalt is known to share with zinc the ability to accept unusual coordination environments, and further, cobalt can readily replace zinc to form functionally active derivatives of a large number of zinc enzymes (Simpson et al., 1971; Chlebowski & Coleman, 1976).

When compared to the absorption spectra of the three types of cobalt(II) complex ions, i.e., octahedral, tetrahedral, and trigonal bipyramidal, most commonly observed, the band positions, separations, and degree of complexity for the spectrum of cobalt hemorrhagic toxin *e* bear less resemblance to octahedral and trigonal-bipyramidal systems. Rather, the spectrum of cobalt hemorrhagic toxin *e* resembles those reported for distorted tetrahedral complexes with oxygen and nitrogen ligands. The intensities of the bands in these spectra are intermediate between those of the spectra of regular tetrahedral complexes and those normally associated with the spectra of octahedral complexes (Cotton & Soderberg, 1962; Carlin, 1965; O'Connor et al., 1968; Pignolet et al., 1969; Vallee & Holmquist, 1980).

The asymmetry of metal binding implicit in the absorption spectrum of cobalt hemorrhagic toxin *e* has been observed for a number of other cobalt metalloenzymes (Chlebowski & Coleman, 1976). The absorption spectrum of cobalt hemorrhagic toxin *e* is similar to those of cobalt yeast aldolase (Simpson et al., 1971), cobalt carboxypeptidase (Latt & Vallee, 1971), and cobalt thermolysin (Holmquist & Vallee, 1974), possibly reflecting common features of their active-site environments.

In Table V, a comparison is made of the proteolytic specificity on the oxidized B chain of insulin of hemorrhagic toxin *e* and other hemorrhagic toxins and proteases from snake venom as well as with two nonsnake venom proteases, thermolysis and elastase, which are known to induce hemorrhage. It is of interest to note that most of the snake venom proteases have a very narrow specificity for this substrate, cleaving only three, four, or five bonds on the chain. One cleavage site on the B chain is common to all the proteases except protease A from *Bothrops jararaca* venom. They all cleave at the Ala_{14}–Leu_{15} bond, and many also cleave at the His_{10}–Leu_{11} bond. Interestingly, some of the proteases, such as hemorrhagic toxin *a* and hemorrhagic factor 2, that cleave the His_{10}–Leu_{11} bond do not cleave the His_5–Leu_6 bond. On the other hand, protease *a* from *A. halys blomhoffi* venom cleaves the His_5–Leu_6 bond but not the His_{10}–Leu_{11} bond.

In spite of the many similarities of cleavage specificity of these proteases, no two have exactly the same cleavage patterns. Hemorrhagic toxin *e* is similar to hemorrhagic factor HR-1 (also called proteinase *b*), proteinase *a*, and elastase in its cleavage of the Asn_3–Gln_4 bond. Hemorrhagic factor 2

Table V: Sites of Cleavage of Proteolytic Hemorrhagic Toxins and Other Venom and Nonvenom Proteases on the Oxidized B Chains of Insulin

	Phe–Val–Asn–Gln–His–Leu–Cys–Gly–Ser–His–Leu–Val–Gly–Ala–Leu–Tyr–Leu–Val–Cys(SO_3^-)–Gly–Gln–Arg–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala
hemorrhagic toxin *e* (*C. atrox*)[a]	↑ ↑ ↑ ↑ ↑
hemorrhagic toxin *a* (*C. atrox*)[b]	↑ ↑ ↑ ↑
hemorrhagic factor 2 (*B. jararaca*)[c]	↑ ↑ ↑ ↑ ↑
HR-1, proteinase *b* (*A. halys blomhoffi*)[d]	↑ ↑ ↑ ↑ ↑
proteinase *a* (*A. halys blomhoffi*)[d]	↑ ↑ ↑ ↑ ↑
proteinase *c* (*A. halys blomhoffi*)[d]	↑ ↑ ↑ ↑ ↑
α-protease, partially pure (*C. atrox*)[b]	↑ ↑ ↑ ↑ ↑
H_2 protease (*T. flavoviridis*)[f]	↑ ↑ ↑ ↑ ↑
proteinase II (*C. adamanteus*)[g]	↑ ↑ ↑ ↑ ↑ ↑ ↑
peptidase *a* (*A. piscivorus leucostoma*)[h]	↑ ↑ ↑ ↑ ↑ ↑ ↑
thermolysin[i]	↑ ↑ ↑ ↑ ↑
elastase[j]	↑ ↑ ↑ ↑ ↑ ↑ ↑
protease *a* (*B. jararaca*)[k]	↑ ↑ ↑

[a] This study. [b] Tu et al. (1981). [c] Mandelbaum et al. (1975). [d] Oshima et al. (1968). [e] Pfleiderer & Krauss (1965). [f] Takahashi & Ohsaka (1970a). [g] Kurecki et al. (1978). [h] Spiekerman et al. (1973). [i] Matsubara et al. (1966). [j] Narayanan & Anwar (1969). [k] Mandelbaum et al. (1975).

cleaves all the same bonds as hemorrhagic toxin *a* in addition to which it also cleaves the Phe_{25}–Phe_{26} bond. Hemorrhagic toxin *a* is also similar to α-protease from *C. atrox*, proteinase II from *Crotalus adamanteus*, and thermolysis in its cleavage of both the His_{10}–Leu_{11} bond and the Tyr_{16}–Leu_{17} bond. The only difference in cleavage pattern between α-protease and hemorrhagic toxin *a* is that α-protease cleaves at the His_5–Leu_6 bond whereas the toxin does not. Other cleavage sites are identical. It must, however, be kept in mind that α-protease is not a pure enzyme but rather a partially purified protease fraction (Pfleiderer & Krauss, 1965) and could well include hemorrhagic toxin *a*. Unfortunately, the notion that α-protease is a pure enzyme is found in the literature and has been assigned the EC number 3.4.24.1 (Enzyme Nomenclature, 1979; Dixon & Webb, 1979; Iwanaga & Suzuki, 1979). Proteinase II from *C. adamanteus* has not been tested for hemorrhagic activity unless it is identical with collagenase II from *C. adamanteus* previously reported on by Kurecki & Laskoweki (1978), in which case it is hemorrhagic. Careful scrutiny of the literature suggests that this might be the case.

It is noteworthy to observe the similarities of cleavage patterns between the two low molecular weight hemorrhagic components HT-*e* and HR-1 on the one hand and the two high molecular weight hemorrhagic components HT-*a* and hemorrhagic factor 2 on the other. This is indeed suggestive of a pattern, but data on cleavage patterns of more hemorrhagic venom components are needed before conclusions concerning classifications can be drawn.

It is becoming increasingly evident that the venom of *C. atrox* contains a multitude of proteolytic enzymes. In addition to the five hemorrhagic toxins, there are reports of five proteolytic, anticomplementary factors (Man & Minta, 1977; Minta & Man, 1980), one nonhemorrhagic collagenase (Hong, 1982), three partially purified general proteases [the so-called α-, β-, and γ-proteases (Pfleiderer & Sumyk, 1961; Zwilling & Pfleiderer, 1967)], two fibrinolytic enzymes (Bajawa et al., 1981), and two kallikrein-like enzymes with arginine esterase activity (Fox & Bjarnason, 1982). All these proteases appear to be distinct from each other, judging from the available data, if the partially purified α-, β-, and γ-protease fractions are discounted.

Recent results from our laboratory have demonstrated the ability of hemorrhagic toxin *e* to cleave certain chains of several collagen types. The α2 chain of type I collagen, the α1, α3, and α2 chains of type V collagen, and the 1α, 2α, and 3α chains of pericellular collagen all showed various degrees of digestion by HT-*e*. Furthermore, fibronectin was extensively digested by HT-*e*. From examination of the digestion products from the various collagen types, it appears that HT-*e* acts in a rather specific manner on certain of the collagen chains. These data corroborate our hypothesis that these hemorrhagic toxins probably act by disruption of certain collagen structures in capillaries. Also, these data indicate that the toxins can act as collagenases and need not necessarily serve as activating proteases of endogenous collagenases at the site of envenomation.

We have thus demonstrated in the present investigation that cobaltous ion can be exchanged for zinc in the native toxin without detectable changes in the protein conformation and with only minor quantitative changes in function. Both forms of the toxins cleave the oxidized chains of insulin in an identical manner. This cleavage pattern is unique and different from any other protease studied to date.

Registry No. *Crotalus atrox* metalloproteinase, 86161-99-1; Co, 7440-48-4; Zn, 7440-66-6.

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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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