Modified Activity of *Aeromonas* Aminopeptidase: Metal Ion Substitutions and Role of Substrates†

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ABSTRACT: *Aeromonas* aminopeptidase contains two nonidentical metal binding sites that have been shown by both spectroscopy and kinetics to be capable of interacting with one another [Prescott, J. M., Wagner, F. W., Holmquist, B., & Vallee, B. L. (1985) *Biochemistry 24*, 5350–5356]. The effects of metal ion substitutions on the susceptibility of the *p*-nitroanilides of L-alanine, L-valine, and L-leucine—substrates that are hydrolyzed at widely differing rates by native *Aeromonas* aminopeptidase—were studied by determining values of k_{cat} and K_m for the 16 metalloenzymes that result from all possible combinations of Zn^{2+}, Co^{2+}, Ni^{2+}, and Cu^{2+} in each of the two sites. The different combinations of metal ions and substrates yield a broad range in kinetic values; k_{cat} varies by more than 1800-fold, K_m by 3000-fold, and k_{cat}/K_m ratios by more than 10000. L-Leucine-*p*-nitroanilide is by far the most susceptible of the three substrates, and the hyperactivation previously observed with aminopeptidase containing either Ni^{2+} or Cu^{2+} in the first binding site and Zn^{2+} in the second site occurs only with the two poorer substrates, L-alanine-*p*-nitroanilide and L-valine-*p*-nitroanilide. Although the enzyme with Zn^{2+} in both sites hydrolyzes the substrates with N-terminal alanine and valine poorly, it is extremely effective toward L-leucine-*p*-nitroanilide. Neither metal binding site can be identified as controlling either K_m or k_{cat}; both parameters are influenced by the identity of the metal ions, by the site each occupies, and, most strongly, by the substrate. The presence of Zn^{2+} in the first site generally results in high K_m values in comparison with the other metalloenzymes and produces high k_{cat} values toward both substrates with branched side chains, whereas Cu^{2+} in the first site yields low K_m values with the two poorer substrates. A time dependence of activation occurs with metalloenzymes that have Cu^{2+} in the first site and another metal ion in the second binding site, but was not observed for any other combination of ions tested.

Metalloenzymes constitute one of the great classes of proteinases and are of particular importance among the aminopeptidases, the best known of which are oligomeric molecules of considerable size (McDonald & Barrett, 1986). In contrast to the more prominent mammalian aminopeptidases, *Aeromonas* aminopeptidase consists of a single peptide of 29500 daltons (Prescott et al., 1971; Prescott & Wilkes, 1976) that is not activated by the addition of Mg^{2+} or Mn^{2+} ions. The enzyme is accompanied through the isolation procedure by 2 mol of Zn^{2+} ions/mol, removal of which by 1,10-phenanthroline yields enzymatically inactive apoenzyme that is readily reactivated by Zn^{2+} (Prescott et al., 1983). In addition to zinc, the divalent ions of cobalt, copper, and nickel are capable of restoring activity to apoaminopeptidase and, in fact, produce activity toward L-alanine-*p*-nitroanilide (Ala*p*NA)[1] higher than that of the native enzyme (Prescott et al., 1983, 1985). Titration of apoenzyme with any one of these metal ions revealed that essentially full activity was restored by 1 mol of metal ion/mol of enzyme; the addition of a second mole of the same metal ion did not discernibly affect enzymatic activity (Prescott et al., 1983). Equilibrium dialysis and spectral titration experiments confirmed that *Aeromonas* aminopeptidase does, indeed, bind 2 mol of metal ions/mol, and we suggested that one of the metal binding sites was noncatalytic and might be structural or regulatory (Prescott et al., 1985). The influence of the second metal binding site was revealed, however, by experiments in which two different metal ions were used; when 1 equiv of Zn^{2+} was added to aminopeptidase that had been reconstituted with 1 mol of either Cu^{2+} or Ni^{2+}, the result was a hyperactivation that yielded k_{cat}/K_m ratios as much as 2 orders of magnitude greater than that of native enzyme (Prescott et al., 1983, 1985). In contrast, reversal of the order of addition (1 mol of Zn^{2+}, followed by 1 mol of Ni^{2+} or Cu^{2+}) produced activities less than one-tenth those observed when Ni^{2+} or Cu^{2+} was added before Zn^{2+}. These experiments demonstrated that profound kinetic effects ensue from variations in the order of addition of two different metal ions to *Aeromonas* apoaminopeptidase; the first site to be filled is the one that is essential to catalysis, but occupancy of the second site produces far-reaching effects.

These observations on hybrid enzymes containing two different metal ions had included only those combinations possible with zinc and each of the other three ions capable of activating the aminopeptidase, and it was therefore deemed desirable to extend the kinetic evaluations to include all combinations of the four active ions. Moreover, the results cited above had been obtained with Ala*p*NA as the substrate, but pronounced differences in the susceptibilities of substrates containing N-terminal L-leucine, L-valine, and L-alanine to hydrolysis by native *Aeromonas* aminopeptidase are known to exist (Wagner et al., 1972). Therefore, we undertook a determination of the

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[1] Abbreviations: Ala*p*NA, Val*p*NA, and Leu*p*NA, *p*-nitroanilides of L-alanine, L-valine, and L-leucine, respectively; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; [(AAP)X-], *Aeromonas* aminopeptidase in which (AAP) represents the apoenzyme and the brackets signify the addition of 1 mol of metal ion/mol of enzyme; [(AAP)XY], binding of 2 mol/mol where X represents the first and Y the second metal ion added to apoenzyme.

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Table I: Kinetic Values of Metalloaminopeptidase with the Same Metal Ion in Both Binding Sites

	AlapNA			ValpNA			LeupNA		
enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})
[(AAP)ZnZn]	17.3	1000	1.73	10.6	25	42.4	4720	23	20520
[(AAP)CoCo]	44.8	435	10.3	7.3	11	66.4	1360	4	34000
[(AAP)NiNi]	53.6	137	39.1	5.3	0.9	589	784	10	7840
[(AAP)CuCu]	5.4	36	15.0	2.6	1	260	240	13	1850

extent to which the kinetics of hydrolysis of substrates containing these three N-terminal residues could be altered by changes in the metal ions provided to the apoenzyme and by the binding sites they occupied. The values for k_{cat}/K_m reported in our previous work were obtained by determining first-order rate constants, k_{obsd}, and calculating k_{cat}/K_m from the relationship $k_{cat}/K_m = k_{obsd}/[E]$. The present study entailed separate measurements of the kinetic parameters for each metalloenzyme on the three substrates with the intent that kinetic changes might be attributable primarily to K_m, to the catalytic step(s), or to a combination of effects.

Experimental Procedures

Materials. AlapNA and LeupNA were obtained from Sigma, and ValpNA was from Bachem; Hepes ("Ultrol" grade) was purchased from Calbiochem Behring, and all metal ions were Johnson Matthey "Puratronic" or Fisher certified atomic absorption standards. Aldrich Chemical was the source of 1,10-phenanthroline, and metal-free pipet tips were purchased from Bio-Rad.

Procedures for Metal Ion Work. Adventitious metal ions were removed from buffers by extraction with 0.01% dithizone in CCl_4 (Fisher C-570, 99 mol % purity minimum); the dithizone that remained was removed by extraction with metal-free CCl_4, and air was drawn through the solution until residual CCl_4 was eliminated. All dilutions of enzyme and substrates were made with extracted buffers, and metal ion contamination was further reduced by rinsing each item of glassware, plasticware (including pipet tips), and spectrophotometer cuvettes in 20% nitric acid and then with deionized water. Dialysis tubing was prepared for use as previously described (Prescott et al., 1985).

Enzyme. Aeromonas aminopeptidase (EC 3.4.11.10) was isolated from culture filtrates of *Aeromonas proteolytica* by the procedure of Prescott and Wilkes (1976), and from it, apoenzyme was prepared (Prescott et al., 1985) by dialysis against 2 mM 1,10-phenanthroline to remove Zn^{2+} from the enzyme, followed by extensive dialysis against extracted 10 mM Hepes buffer, pH 7.5, to remove the 1,10-phenanthroline. Enzyme preparations containing the desired combinations of metal ions were made by additions of the metals to apoenzyme on the basis previously shown (Prescott et al., 1985), that the order of addition governs whether each is bound to the first or second site. Thus, the substitution of one metal ion for another did not involve the displacement of one ion by dialysis or prolonged exposure to an excess of another ion (Thompson & Carpenter, 1976; Allen et al., 1983). The concentration of apoenzyme was determined from its absorbance at 287.5 nm by using the value $\epsilon = 41\,800$ M^{-1} cm^{-1} (Prescott et al., 1971); the precise volume of the first metal ion solution needed to supply 1 mol of metal ion/mol of apoenzyme was added from acid-cleaned, metal-free pipet tips, and after mixing, the second metal was then added. Preparations containing all combinations of the four active metal ions in each of the two binding sites were made by this procedure; at least three individual prepartions were made for each combination. Repeated experiments showed the kinetic properties of [(AAP)ZnZn] made from apoenzyme to be indistinguishable from those of the native enzyme as isolated. In the present experiments, therefore, [(AAP)ZnZn] made by additions of Zn^{2+} to apoenzyme was used to provide a direct comparison with all other metalloenzymes, and it is referred to interchangeably as native aminopeptidase or [(AAP)ZnZn].

Kinetic Experiments. Duplicate assays of each substituted metalloaminopeptidase at five to eight substrate concentrations were run in 1-mL volumes in 10 mM Hepes, pH 7.5, in a Perkin-Elmer Lambda 3B or Zeiss PMQ II spectrophotometer. Values for K_m and k_{cat} were determined from Lineweaver–Burk plots.

Results

We recently reported that metal exchange does not appear to occur during the time course of kinetic experiments such as those reported here (Prescott et al., 1985). This was confirmed in the present investigation, which revealed that preparations could be frozen, thawed, and used again with essentially identical results. As was the case in our previous studies, no evidence was found to suggest that the addition of the second metal ion displaced the first which, in the case of Zn^{2+}, has a $K_{diss} \simeq 1.5 \times 10^{-10}$ M (Baker & Prescott, 1985). Values for k_{cat} and K_m were determined for each of the 16 metalloaminopeptidases with the goal of detecting differences of approximately 25% or more in k_{cat} and 2-fold in K_m. Overall, very large variations in kinetic values were found between the different substrates and metal ion combinations; k_{cat} varied by more than 1800-fold, K_m by more than 3000-fold, and k_{cat}/K_m ratios ranged over more than 4 orders of magnitude.

The overriding factor governing activity is the identity of the substrate. Although the substitution of Ni^{2+} or Cu^{2+} for Zn^{2+} in the first metal binding site greatly increases activity toward AlapNA and ValpNA, no combination of metal ions in the two binding sites alters the general order of susceptibility of substrates from that of the native enzyme, which is LeupNA $\gg$ ValpNA > AlapNA; in fact, only one exception to this order of susceptibility was observed (see below). These wide variations in kinetic values are illustrated by the data in Table I, in which a comparison is shown of the four metalloenzyme preparations that contain the same metal ion in both binding sites. Our earlier studies showed that substituting Co^{2+}, Ni^{2+}, or Cu^{2+} for both Zn^{2+} ions of the native aminopeptidase results in greater activities toward AlapNA (Prescott et al., 1983, 1985). The data in Table I show that the increase in k_{cat}/K_m ratios toward AlapNA (compared with [(AAP)ZnZn]) is due to lowered K_m values for all three enzymes as well as from increases in k_{cat} with [(AAP)NiNi] and [(AAP)CoCo]. Replacing the 2 mol of Zn^{2+} with 2 mol of another metal ion also yields greater activities toward ValpNA, but for each such metalloenzyme, the increase in k_{cat}/K_m relative to that of [(AAP)ZnZn] comes primarily from lowered K_m values. With LeupNA as the substrate, however, only [(AAP)CoCo] produces greater activity than the native enzyme. In this enzyme–substrate combination, both k_{cat} and K_m are lower than the corresponding values for [(AAP)ZnZn], but K_m undergoes

Table II: Kinetic Values of Mixed Metal Ion Aminopeptidases with Zn^{2+} in either the First or the Second Binding Site

	AlapNA			ValpNA			LeupNA		
enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})
[(AAP)ZnCo]	21.2	1250	1.7	10.5	22	47.7	2166	14	15470
[(AAP)CoZn]	52.2	870	6	9.5	7	135	1990	8	24880
[(AAP)ZnNi]	27	286	9.44	14.6	20	73	4200	23	18260
[(AAP)NiZn]	312	213	146	14.8	0.4	3700	248	16	1550
[(AAP)ZnCu]	14.2	740	1.92	12.4	29	42.8	3270	13	25150
[(AAP)CuZn]	61.5	25	246	10.5	3	350	557	15	3710
[(AAP)ZnZn]	17.3	1000	1.73	10.6	25	42.4	4720	23	20520

Table III: Kinetic Values of Mixed Metal Ion Aminopeptidases without Zn^{2+}

	AlapNA			ValpNA			LeupNA		
enzyme	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} min^{-1} × 10^{-4})
[(AAP)NiCo]	93.3	122	76.5	8.1	0.9	900	713	6	11880
[(AAP)CoNi]	85.6	333	25.7	9.8	11	89.1	942	5	18840
[(AAP)NiCu]	14.4	114	12.6	4.1	0.8	513	271	13	2090
[(AAP)CuNi]	15.3	64	23.9	3.9	0.6	650	523	21	2490
[(AAP)CoCu]	41.3	80	51.6	5.9	1.0	590	706	4	17650
[(AAP)CuCo]	27.6	39	70.8	3.9	1.0	390	447	6	7450
[(AAP)ZnZn]	17.3	1000	1.73	10.6	25	42.4	4720	23	20520

a greater decrease than k_{cat}. Thus, substitution of Co^{2+} for both Zn^{2+} ions diminishes K_m values for each of the three substrates, and this effect is the most pronounced with LeupNA. Although [(AAP)ZnZn] and [(AAP)CoCo] hydrolyze AlapNA and ValpNA poorly, they are by far the most effective of these four enzymes for cleavage of the most susceptible substrate, LeupNA.

Kinetic Effects of Zn^{2+} in One of the Binding Sites. In Table II are shown the individual kinetic parameters for metalloenzymes that contain Zn^{2+} in combination with each of the other active metal ions, in both the first and second metal binding sites. It is significant that the enhancement of activity over that of the native enzyme, previously observed with [(AAP)NiZn] and [(AAP)CuZn], occurs only with AlapNA and ValpNA. In fact, the activities of [(AAP)NiZn] and [(AAP)CuZn], which are 84- and 142-fold, respectively, more active than [(AAP)ZnZn] toward AlapNA, are only 8% and 18%, respectively, as high as that of [(AAP)ZnZn] with LeupNA, toward which these two mixed-metal enzymes display particularly low k_{cat} values. [(AAP)CoZn], however, shows a greater k_{cat}/K_m ratio than the native enzyme toward all three substrates. The enhanced k_{cat}/K_m ratio toward AlapNA, previously observed with [(AAP)NiZn], is based on a large increase in k_{cat} and a moderate decrease in K_m, but the increase in activity shown by [(AAP)CuZn], relative to the native enzyme, is primarily the result of a 40-fold decrease in K_m. In contrast to the low k_{cat} values they display toward the hydrolysis of AlapNA, however, all four metalloenzymes with Zn^{2+} in the first binding site have high k_{cat} values toward LeupNA, (cf. Tables I–III). In fact, the highest k_{cat} observed in the entire investigation is that of [(AAP)ZnZn] toward LeupNA. The native enzyme has high K_m values toward all three substrates, and in general, the presence of Zn^{2+} in the first binding site results in high K_m values, relative to other metalloenzymes, for each substrate. Reversing the positions of the metal ions in [(AAP)ZnCu] produces a large reduction in K_m toward both AlapNA and ValpNA, but not toward LeupNA, and a similar, but less dramatic, effect results from changing [(AAP)ZnCo] to [(AAP)CoZn].

Mixed-Metal Enzymes without Zn^{2+}. The data in Table III show that all combinations of Ni^{2+}, Cu^{2+}, and Co^{2+} form active enzymes, each of which displays more activity toward AlapNA and ValpNA, and less toward LeupNA, than the native aminopeptidase. Each of these enzymes has a lower K_m than [(AAP)ZnZn] toward all three substrates, and with AlapNA, k_{cat} values for four of the enzymes are greater than that of [(AAP)ZnZn].

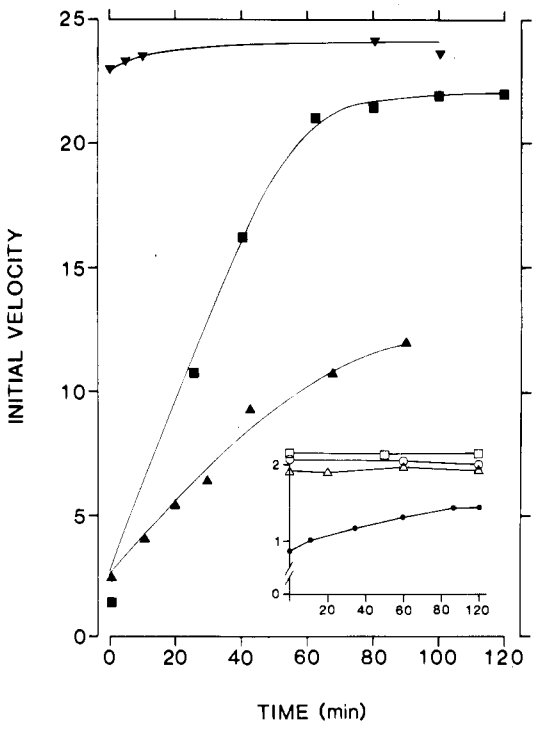

FIGURE 1: Effect of time of incubation and binding site on the activity of metalloaminopeptidases containing Cu^{2+} or Ni^{2+}. The first metal ion was added to apoenzyme, and the solution was mixed; the second metal ion was then added, and samples were assayed for activity at time zero and at intervals thereafter. The ordinate represents initial velocities expressed as micromoles per milliliter per minute × 10^4; the inset provides details for enzymes with low activities. All enzyme concentrations were 3.57 × 10^{-8} M; the substrate was AlapNA at the concentrations shown. (■) [(AAP)CuZn], 0.05 mM; (○) [(AAP)ZnCu], 1 mM; (▲) [(AAP)CuCo], 0.1 mM; (△) [(AAP)-CoCu], 0.1 mM; (□) [(AAP)CuCu], 0.1 mM; (●) [(AAP)CuNi], 0.1 mM; (▼) [(AAP)NiZn], 0.5 mM.

Time Dependence of Activation. Kinetic values for some of the metal-substituted enzymes, particularly [(AAP)CuZn], showed more variation than those of other metalloenzymes,

and it appeared that the variations might reflect a time dependence for the attainment of full activity by this enzyme. The results in Figure 1 show the existence of a time dependence for full activity that is related to the presence of Cu^{2+} in the first binding site, with a different metal ion in the second site. Both [(AAP)CuZn] and [(AAP)CuCo] show marked increases in activity with increasing times of incubation after addition of the second metal ion. This cannot be attributed to metal ion exchange, as both [(AAP)ZnCu] and [(AAP)-CoCu] have much lower activities toward Ala*p*NA than [(AAP)CuZn] and [(AAP)CuCo]; moreover, none of the three enzymes with Cu^{2+} in the second site shows an increase in activity with time of incubation. Even though the activity of [(AAP)CuNi] is lower than that of [(AAP)CuZn] and [(AAP)CuCo], there is still evidence of the time dependence. [(AAP)NiZn], like [(AAP)CuZn], shows a large hyperactivation effect toward Ala*p*NA (Table II), but its activity is not dependent on the time of incubation (Figure 1). The time dependence for activation of [(AAP)CuZn] toward Ala*p*NA occurs also with Val*p*NA and Leu*p*NA substrates, although the magnitude of increase with time is only about 4-fold with the former and 3-fold with the latter (data not shown).

Effects of Side Chain on Kinetic Parameters. The susceptibility of Leu*p*NA to hydrolysis by the various aminopeptidases is by far greater than that of the other two substrates, largely because of higher k_{cat} values; only 4 of the 16 enzymes have k_{cat} values less than 500 min^{-1} toward Leu*p*NA. Values of k_{cat} for all of the enzymes toward Leu*p*NA are much higher than those for the same metalloenzyme acting on either of the other substrates, with a single exception; [(AAP)NiZn] has a slightly higher k_{cat} toward Ala*p*NA than toward Leu*p*NA. Values of k_{cat} toward Val*p*NA are the lowest of the substrates and show the least variation, with a range of only 2.6–14.8 min^{-1}. In contrast, k_{cat} values for Ala*p*NA range from 5.4 to 312 min^{-1} and those for Leu*p*NA from 240 to 4720 min^{-1}. In the hydrolysis of Ala*p*NA, k_{cat} increases when any of the other three metal ions replaced Zn^{2+} in the first binding site, in the general order $Ni^{2+} > Cu^{2+} > Co^{2+}$.

For any given combination of metal ions in the enzyme, K_m values with Ala*p*NA are by far the highest of the three substrates, indicating that the small side chain does not provide a good fit for binding to the active site. Overall, the metalloenzymes show the lowest K_m values toward Val*p*NA, ranging from 0.4 to 29 μM. The metal ion in the first binding site exerts a pronounced influence on K_m values with each substrate, but the identity of the metal producing the lowest K_m varies with the substrate. Enzymes with Cu^{2+} in the first binding site have the lowest K_m values toward Ala*p*NA, ranging from 25 to 64 μM; with Val*p*NA, enzymes with either Ni^{2+} or Cu^{2+} in the first site give low K_m values (0.4–0.9 μM and 0.6–3 μM, respectively). When Leu*p*NA is the substrate, however, the presence of Co^{2+} in the first site produces enzymes with the lowest K_m values (4–8 μM).

Discussion

Our recent finding that *Aeromonas* apoaminopeptidase can be reactivated by the addition of only 1 mol of metal/mol shows that the first mole of metal ion per mole of enzyme preferentially enters the active site; a regulatory role for the second metal binding site was indicating by the hyperactivation resulting from the addition of 1 mol of Zn^{2+} to [(AAP)Ni-] and [(AAP)Cu-]. These experiments utilized Ala*p*Na as the substrate because its low susceptibility to hydrolysis allowed the use of high concentrations of enzyme which, in turn, suppressed the reactivation of apoenzyme by adventitious metal ions. We were curious to determine the factors that influenced the hyperactivation observed with Ala*p*NA and to investigate the kinetic effects of metal ion substitutions on the hydrolysis of more susceptible substrates. It is obvious that *Aeromonas* aminopeptidase can function with Zn^{2+}, Co^{2+}, Ni^{2+}, or Cu^{2+} in either binding site (Table III). The data also show that the control of either k_{cat} or K_m does not reside exclusively in only one of the two metal binding sites or solely in the identity of the metal ion occupying either site. Instead, both k_{cat} and K_m are governed in a complex manner by the metal ions occupying both sites, further confirming interaction between the two sites that was previously shown by both kinetics and spectroscopy (Prescott et al., 1985). A limited number of generalizations can be made, however: (i) the presence of Zn^{2+} in the first site produces high K_m values with all substrates, relative to most other metalloenzymes; (ii) enzymes with Cu^{2+} in the first site have low K_m values toward Ala*p*NA and Val*p*NA; (iii) Zn^{2+} in the first site yields high k_{cat} values toward the two substrates with branched side chains. The latter observation points to another fact; viz., the effect of metal ions is overwhelmingly dependent on the identity of the amino-terminal residue of the substrate. It is noteworthy, for example, that even though Val*p*NA and Leu*p*NA differ by merely a methylene group, k_{cat} values of individual metalloenzymes toward the latter were 60–400-fold greater than those for the former, with the exception of [(AAP)NiZn]. Metal substitutions thus can either stimulate or reduce activity, relative to that of the native enzyme, depending on the identity of the metal, its position, and the substrate. The complexity of the relationships between metal ion, binding site, and substrate is illustrated by the enzymes that contain Zn^{2+} in combination with Ni^{2+} or Cu^{2+}. Reversing the positions of the ions in [(AAP)ZnNi] produces a large increase in k_{cat}/K_m toward Ala*p*NA (by virtue of increased k_{cat}) and toward Val*p*NA (because of a greatly diminished K_m), but with Leu*p*NA, this exchange of metal ions between the two binding sites results in a 17-fold decrease in k_{cat} and thus a lower k_{cat}/K_m ratio. Similar results ensue from changing [(AAP)ZnCu] to [(AAP)CuZn]; activities toward Val*p*NA and Ala*p*NA increase, principally because of lower K_m values, and the activity toward Leu*p*NA decreases as a result of a lower k_{cat}.

The ability of metalloproteases to function with Cu^{2+} in place of Zn^{2+} appears to be rare, having been reported, insofar as we are aware, only for microsomal aminopeptidase (Lehky et al., 1973), *Aeromonas* aminopeptidase (Prescott et al., 1983), and carboxypeptidase A (Schäffer & Auld, 1986). When assayed against the highly susceptible substrate Leu*p*-NA, [(AAP)CuCu] is less than one-tenth as active as native *Aeromonas* aminopeptidase, but it is 8-fold and 6-fold more active toward Ala*p*NA and Val*p*NA, respectively. Moreover, [(AAP)CuZn] is 16 times as active as [(AAP)CuCu] toward Ala*p*NA. With Ala*p*NA and Val*p*NA substrates, the presence of Cu^{2+} in the first binding site produces K_m values much lower than that of [(AAP)ZnZn], as shown in Table II. The metalloenzymes that contain Cu^{2+} in the first site are particularly interesting since they are the only ones found to have a time dependence for activation. This strongly implicates an interaction between the occupants of the two metal binding sites that is dependent on a slow process, perhaps either slow binding of the second metal when Cu^{2+} occupies the first site or a conformational change in the enzyme.

The best characterized aminopeptidases have proved to be zinc metalloenzymes that can bind either 1 or 2 mol of metal per monomer or subunit. Human liver aminopeptidase (Little et al., 1976) and purified enzymes from *Bacillus licheniformis* (Rodríguez-Absi & Prescott, 1978) and *Bacillus subtilis*

(Wagner et al., 1979) are representatives of one-zinc enzymes whereas the hexameric leucine aminopeptidases from bovine lens (Carpenter & Vahl, 1973) and from swine kidney (Van Wart & Lin, 1981) contain two metal binding sites per subunit. *Aeromonas* aminopeptidase also is a two-metal enzyme but differs in several respects from the latter two. Our earlier work has shown that one of the two Zn^{2+} ions in *Aeromonas* aminopeptidase is bound more tightly than the other. When apoaminopeptidase is reactivated by the sequential addition of 2 mol of metal ion, the first ion added is the more tightly bound and reestablishes catalytic activity (Prescott et al., 1983, 1985). It thus is likely that the first metal binding site in *Aeromonas* aminopeptidase is analogous to the site in bovine lens leucine aminopeptidase that Allen et al. (1983) labeled "site 2" or the "slow-exchanging" site and which earlier was referred to as the "specificity site" (Carpenter & Vahl, 1973) and the "structural site" (Thompson & Carpenter, 1976); Van Wart and Lin (1981) have termed this the "catalytic site" in cytosolic leucine aminopeptidase from swine kidney. There seems also to be an analogy between what we have termed the second metal binding site in *Aeromonas* aminopeptidase and site 1, or the "fast-exchanging" site (Allen et al., 1983) or "activation site" (Carpenter & Vahl, 1973), and the "regulatory site" (Van Wart & Lin, 1981) of the mammalian enzymes. The reason for what appears to be an opposite designation of sites with similar functions is that our experiments were commenced by adding metal ions to apoenzyme, so that the more strongly binding site was filled first, whereas the experiments of Allen et al. (1983) were begun by displacement of the ion from the more weakly binding of the two sites. The interactions between the metal ions in the two sites of aminopeptidases are certainly complex, and in view of the results of Allen et al. (1983) with bovine lens leucine aminopeptidase and our experiments described above, it is clear that both k_{cat} and K_m are influenced by the identity of the metal ions, by the site each occupies, and by the fit of the substrate to the active site of each of the 16 metalloenzymes. In this respect, the lens and *Aeromonas* enzymes appear to differ from cytosolic leucine aminopeptidase from swine kidney, since Van Wart and Lin (1981) observed no changes in K_m when different metal ions were placed in the regulatory site of the latter. The degree to which the metal ions in *Aeromonas* aminopeptidase interact with one another, as evidenced by both kinetics and spectroscopy, indicates that the metal binding sites are in close proximity—close enough to one another that each influences both cataysis and binding. This kinetic observation is consistent with the physical evidence presented by Taylor et al. (1982) showing that Mn^{2+} in the "activation" metal binding site of bovine lens leucine aminopeptidase is in very close proximity to the carbonyl oxygen of the peptide bond. Despite the structural and catalytic differences that exist between the two enzymes, it appears that the metal ion in the second binding site of *Aeromonas* aminopeptidase and that in the fast-exchanging site of bovine lens leucine aminopeptidase perform similar functions.

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