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ONE HUNDRED FOLD INCREASED ACTIVITY OF *AEROMONAS* AMINOPEPTIDASE BY SEQUENTIAL SUBSTITUTIONS WITH Ni(II) OR Cu(II) FOLLOWED BY ZINC

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SUMMARY: Full substitution of Cu(II) or Ni(II) for the two g-atom zinc in *Aeromonas* aminopeptidase hyperactivates the enzyme 6.5 and 25 fold respectively. Even greater enhancements of activity can be achieved with mixed metal substitutions. Thus, apoenzyme reactivated by first adding one g-atom zinc followed by one g-atom of either Cu(II) or Ni(II) is 15 and 22 times more active than the native enzyme. Reversing the order, i.e. by first adding either one g-atom Cu(II) or Ni(II) followed by one g-atom zinc, activates the enzyme nearly 100 fold. The order of metal addition is critical and suggests the existence of two non-identical metal sites, each with a different function.

INTRODUCTION: Among the aminopeptidases known to be zinc metalloenzymes (1-7) some contain two and others one g-atom zinc per monomer (3, 4, 8). However, much more is known about the mechanism of action of "one-zinc" enzymes, such as thermolysin and carboxypeptidase A, than is understood about "two-zinc" enzymes. For this and other reasons, the role of zinc and other metals in the action of aminopeptidases has recently come under scrutiny in several laboratories (9-11). *Aeromonas* aminopeptidase (EC 3.4.11.10) is an unusually stable microbial protease of simple structure and consists of a single peptide chain of 29,500 daltons (8, 12). Moreover, like the large hexameric leucine aminopeptidases, the native enzyme contains two g-atom zinc (8). Recently, we have probed the active site of *Aeromonas* aminopeptidase by means of chemical modification (13, 14) and with

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inhibitors (15). The results underscored the desirability of elucidating the role of metals in the activity of this enzyme. Both total and partial substitution of copper or nickel for zinc reveals remarkable activity responses: partial substitution can result in specific activities as much as 100-fold greater than that of the native enzyme.

MATERIALS AND METHODS: Aeromonas aminopeptidase was prepared by the procedure of Prescott & Wilkes (12). Activities were measured at 25° C by spectrophotometric determination of the rate of hydrolysis of L-alanine-p-nitroanilide under first order conditions using 3.75×10^{-5} M substrate in 50 mM Hepes, pH 7.5, and an assay volume of either 0.5 or 1.0 ml. Initial velocities were calculated from the spectrophotometer (Varian 219, Gilford 222 or Zeiss PMQII) recordings at 405 nm; some reactions were permitted to go to completion for graphic calculations of first order rate constants. The value $\varepsilon = 10{,}800\ M^{-1}\ cm^{-1}$ (16) was used to calculate the concentration of p-nitroaniline liberated. Contamination of apoenzyme by adventitious ions was minimized by extraction of buffers with dithizone in CCl_4 and by the use of plastic-ware or acid cleaned glassware. Assays were routinely performed in plastic spectrophotometer cuvettes. Zinc analyses were made on a Perkin Elmer Model 2280 atomic absorption spectrophotometer. All metal ions used were Johnson-Matthey "Specpure"; dipicolinic acid was from Sigma Chemical Co., and 1,10-phenanthroline was from Aldrich Chemical Co. Dialysis tubing ("Spectrapore", Spectrum Medical Industries) was washed in deionized water at 70°C several times before use. Enzyme concentrations were estimated from the value $\varepsilon_{280} = 41{,}760\ M^{-1}\ cm^{-1}$.

RESULTS AND DISCUSSION: The enzyme used in the present studies, freed of adventitious metal ions by passage through a column of Sephadex G-25 equilibrated in metal-free Tris, 0.1 M, pH 7.3, contained 1.73 g-atom zinc per mol, consistent with our earlier report (8) that the enzyme contains 2 g-atom zinc per mol. Both 1,10-phenanthroline and dipicolinic acid, chelating agents frequently used to remove zinc from zinc metalloenzymes, completely and reversibly inactivate the Aeromonas aminopeptidase, presumably by removing this metal (Figure 1). 1,10-Phenanthroline was selected to prepare apoenzyme since it inhibits the enzyme instantaneously and more effectively than dipicolinic acid which inhibits it in a time dependent manner (Figure 1).

Preparation of Apoenzyme. The results were essentially identical for six preparations of apoenzyme made by the same general procedure. In a typical experiment native Aeromonas aminopeptidase, 2.3×10^{-4} M, was dialyzed for 29 h against 3 changes of 15 volumes of 2 mM

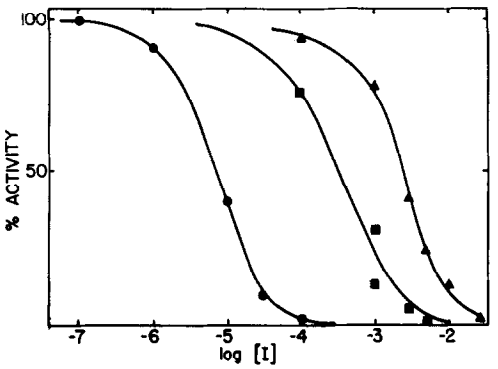

Figure 1: Inhibition of Aeromonas aminopeptidase by metal-chelating reagents. Concentration-dependence of inhibition by: 1,10-phenanthroline (●) and by dipicolinic acid at zero (▲) and 17-28 min (■) incubation. In the experiments with 1,10-phenanthroline, samples of enzyme (3.26×10^{-9} M) were allowed to stand for 30 min in the concentrations of reagent shown. The enzyme concentration in the dipicolinic acid experiments was 1.43×10^{-9} M. First-order assays were started by adding L-leucine-p-nitroanilide to yield a final concentration of 5×10^{-6} M in 20 mM Tricine, pH 7.5.

1,10-phenanthroline in 50 mM Tricine containing 50 mM KCl, pH 7.5. The 1,10-phenanthroline was removed by dialysis against seven changes of 50 mM Hepes, pH 7.5 with 15 volumes/change over a 48 h period. The specific activity of the six apoenzyme preparations varied from 3 to 9% of that of native aminopeptidase. The zinc content averaged 0.12 g-atom per mol of protein, i.e. approximately 6% of the zinc of the native enzyme. The stock preparation used for the experiments shown in Table I was 1.35×10^{-4} M with respect to protein and 6.9×10^{-6} M with respect to zinc. The apoenzyme remains stable in the frozen state and can be reactivated fully by the addition of zinc.

Reactivation of Apoaminopeptidase by Metal Ions:

Cu(II), Ni(II), and Co(II) all reactivate the apoenzyme to a much greater extent than zinc, which restores native activity. Cd(II) restores ~20% of the activity, but Mn(II), Ca(II), and Mg(II) have no effect at all. The apoenzyme was titrated with Zn(II), Cu(II), Ni(II) and Co(II) to determine the requirements for restoration of maximal activity (Figure 2). With Zn(II), Co(II) and Ni(II), addition of only one g-atom of metal results in maximal activity, which is different for each metal. However, with Cu(II) maximal activity is achieved only in the presence of excess Cu(II),

TABLE I: Effects of the Order of Addition of Metal Ions on the Activity of Reactivated Aeromonas Apoaminopeptidase[a]

Sequential Additions to Apoenzyme		k_{cat}/K_m[b]	Fold Activation[c]
First Metal	Second Metal	($min^{-1} M^{-1} \times 10^{-4}$)	
Native Enzyme		1.3	1
Cu^{2+}	Cu^{2+}	8.4	6.5
Cu^{2+}	Zn^{2+}	127	98
Zn^{2+}	Cu^{2+}	8.2	6.3
Ni^{2+}	Ni^{2+}	31.9	25
Ni^{2+}	Zn^{2+}	114	88
Zn^{2+}	Ni^{2+}	5.1	3.9
Co^{2+}	Co^{2+}	10	7.7
Co^{2+}	Zn^{2+}	11.1	8.5
Zn^{2+}	Co^{2+}	5.4	4.2

[a] Preparations were made by adding apoenzyme and one g-atom of the first metal to 50 mM Hepes (pH 7.55), mixing and equilibrating for 1 h at room temperature. One g-atom of the second metal was then added, mixed, and an additional 1 h of equilibration time allowed.
[b] Determined by first-order kinetics with 3.75×10^{-5} M L-alanine-p-nitroanilide substrate. Values obtained graphically for k observed were divided by $[E_o]$ to obtain k_{cat}/K_m.
[c] Ratio of k_{cat}/K_m for the metal reconstituted enzyme to that of the native enzyme.

the activity when one g-atom is present being two thirds of its maximum (Figure 2).

Although addition of one g-atom of each of the metals restores maximal or near maximal enzymatic activity characteristic for each, the results

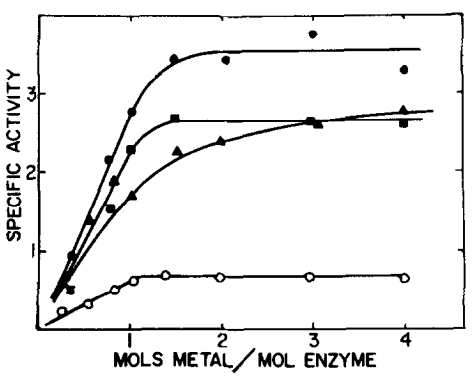

Figure 2: Reactivation of apoaminopeptidase with divalent metals. Apoaminopeptidase (6.75×10^{-7} M) was equilibrated with the metal in 50 mM Hepes, pH 7.5, for 1 h before initiating reaction by the addition of L-alanine-p-nitroanilide to a final concentration of 3.75×10^{-5} M and recording initial velocities. Activities are expressed as mols of product per minute per mol enzyme. ○, Zn(II); ■, Co(II); ▲, Cu(II); ●, Ni(II).

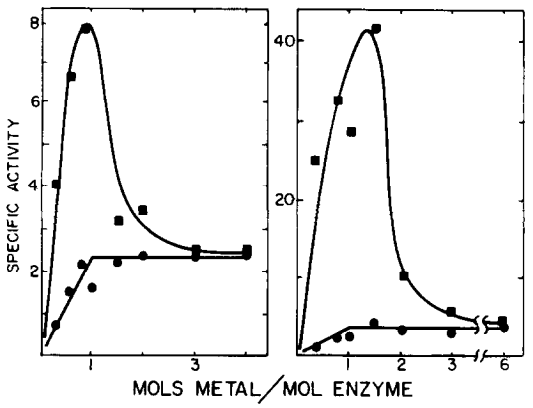

Figure 3: Reactivation of apoaminopeptidase by stoichiometric combinations of Zn(II) with Cu(II) and Ni(II). The apoenzyme (6.75×10^{-7} M) was equilibrated at room temperature for 1 h with the metals at the molar excess shown before the reactions were started by addition of substrate. Reactions were performed with 3.75×10^{-5} M L-alanine-p-nitroanilide in 50 mM Hepes, pH 7.5. After the initial velocity of each reaction was apparent (●), one equivalent of Zn(II) was added and the new rate was determined (■). Left panel: ●, Cu(II) only; ■, Cu(II) plus 1 equivalent of Zn(II). Right panel: ●, Ni(II) only; ■, Ni(II) plus 1 equivalent of Zn(II). Activities expressed in Figure 2.

differ strikingly when they are added in combination with zinc. When the apoenzyme is first preequilibrated with one equivalent of Cu(II) or Ni(II), subsequent addition of one equivalent of zinc increases activity dramatically, far exceeding that brought about by any one of these metals alone (Figure 3). However, when two or more equivalents of Cu(II) or Ni(II) are present, the addition of zinc does not increase activity (Figure 3). This suggests that the apoaminopeptidase requires no more than two equivalents of metal ion either for the restoration and/or hyperactivation of enzymatic function. In contrast to the truly remarkable increases in activity which result from adding zinc to enzyme containing Ni(II) or Cu(II), the addition of zinc to enzyme containing Co(II) stimulates activity only to a minor extent.

In these experiments the order of addition of ions to the apoenzyme is critically important: the hydrolytic rates which result from addition of Ni(II) or Cu(II) to enzyme containing one equivalent of zinc are considerably less than those shown in Figure 3. This dependence of activity upon the order of metal addition suggests strongly that the functional

potential of the two metal sites is not the same. This was examined by varying the order of addition of all possible combinations of Zn(II) with Cu(II), Ni(II) and Co(II). The first order rate constant for each preparation was determined and k_{cat}/K_m ratios were calculated (Table I). When zinc is added first, followed by any of the other three metals, the k_{cat}/K_M values are about four to six times greater than that of the native enzyme. The addition of zinc to enzyme containing one equivalent of either nickel or copper, however, yielded preparations with k_{cat}/K_m values nearly 100 times the activity of the native Aeromonas aminopeptidase toward L-alanine-p-nitroanilide.

The most striking results of these experiments are: (i) the nearly 100 fold increased activity on addition of zinc to enzyme that contains one equivalent of Cu(II) or Ni(II) and (ii) the 6.5 and 25 fold increase in activity resulting from the substitution of copper and nickel, respectively for zinc. Neither carboxypeptidase A nor thermolysin, the zinc metalloproteases which have been studied most intensively, are active towards peptides when zinc is replaced by copper (17, 18). To our knowledge this is the first example of a very active, copper substituted metalloprotease.

The activity of the nickel, cobalt and copper Aeromonas aminopeptidase is considerably higher than that of the zinc enzyme, when substituted either totally or partially. Figure 3 and Table I illustrate the critical importance of the order of metal addition. The data strongly suggest that the metals bind specifically to two characteristic but non-identical sites, each exercising a different function. These features of the enzyme and the detailed role of the metal in substrate specificity are under investigation.

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REFERENCES

1. Prescott, J. M. and Wilkes, S. H. (1966) Archives Biochem. Biophys. 117, 328-336.
2. Himmelhoch, S.R. (1969) Archives Biochem. Biophys. 134, 597-602.
3. Kettmann, U. and Hanson, H. (1970) FEBS Lett. 10, 17-20.
4. Carpenter, F. H. and Vahl, J. M. (1973) J. Biol. Chem. 248, 294-304.
5. Garner, C. W. and Behal, F. J. (1974) Biochemistry 13, 3227-3233.
6. Rodriguez-Absi, J. and Prescott, J. M. (1978) Arch. Biochem. Biophys. 186, 383-391.
7. Wagner, F. W., Ray, L. E., Ajabnoor, M. A., Ziemba, P. E. and Hall, R. L. (1979) Arch. Biochem. Biophys. 197, 63-72.
8. Prescott, J. M., Wilkes, S. H., Wagner, F. W. and Wilson, K. J. (1971) J. Biol. Chem. 246, 1756-1764.
9. Van Wart, H. E. and Lin, S. H. (1981) Biochemistry 20, 5682-5689.
10. Chan, W. W.-C., Dennis, P., Demmer, W. and Brand, K. (1982) J. Biol. Chem. 257, 7955-7957.
11. Taylor, A., Sawan, S. and James, T. L. (1982) J. Biol. Chem. 257, 11571-11576.
12. Prescott, J. M. and Wilkes, S. H. (1976) Methods Enzymol. 45, 530-543.
13. Mäkinen, K. K., Mäkinen, P-L., Wilkes, S. H., Bayliss, M. E. and Prescott, J. M. (1982) J. Biol. Chem. 257, 1765-1772.
14. Mäkinen, K. K., Mäkinen, P.-L., Wilkes, S. H., Bayliss, M. E. and Prescott, J. M. (1982) Eur. J. Biochem. 128, 257-265.
15. Baker, J. O., Wilkes, S. H., Bayliss, M. E. and Prescott, J. M. (1983) Biochemistry, 22, 2098-2103.
16. Tuppy, H., Weisbauer, U. and Wintersberger, E. (1962) Z. Physiol. Chem. 329, 278-288.
17. Holmquist, B. and Vallee, B. L. (1973) J. Biol. Chem. 249, 4601-4607.
18. Vallee, B. L. (1964) Fed. Proc. 23, 8-17.