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Spectral and Kinetic Studies of Metal-Substituted *Aeromonas* Aminopeptidase: Nonidentical, Interacting Metal-Binding Sites†

John M. Prescott*
Institute of Occupational Medicine, College of Medicine, Texas A&M University, College Station, Texas 77843
Fred W. Wagner
Department of Agricultural Biochemistry, University of Nebraska, Lincoln, Nebraska 68583
Barton Holmquist and Bert L. Vallee
Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, Boston, Massachusetts 02115
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ABSTRACT: Apoenzyme prepared by removal of the 2 mol of Zn^{2+}/mol from *Aeromonas* aminopeptidase is inactive. Addition of Zn^{2+} reactivates it completely, and reconstitution with Co^{2+}, Ni^{2+}, or Cu^{2+} results in a 5.0-, 9.8-, and 10-fold more active enzyme than native aminopeptidase, respectively. Equilibrium dialysis and spectral titration experiments with Co^{2+} confirm the stoichiometry of 2 mol of metal/mol. The addition of only 1 mol of metal/mol completely restores activity characteristic of the particular metal. Interaction between the two sites, however, causes hyperactivation; thus, addition of 1 mol of Zn^{2+}/mol subsequent to 1 mol of Co^{2+}, Ni^{2+}, or Cu^{2+} per mole increases activity 3.2-, 42-, or 59-fold, respectively. The cobalt absorption spectrum has a peak at 527 nm with a molar absorptivity of 53 M^{-1} cm^{-1} for 1 mol of cobalt/mol, which increases to 82 M^{-1} cm^{-1} for a second cobalt atom and is unchanged by further addition of Co^{2+}. Circular dichroic (CD) and magnetic CD spectra indicate that the first Co^{2+} binding site is tetrahedral-like and that the second is octahedral-like. Stoichiometric quantities of 1-butylboronic acid, a transition-state analogue inhibitor of the enzyme [Baker, J. O., & Prescott, J. M. (1983) *Biochemistry 22*, 5322], profoundly affects absorption, CD, and MCD spectra, but *n*-valeramide, a substrate analogue inhibitor, has no effect. These findings suggest that the tetrahedral-like site is catalytic and the other octahedral-like site is regulatory or structural.

Aeromonas aminopeptidase, isolated from culture filtrates of *Aeromonas proteolytica*, is a zinc metalloenzyme (Prescott & Wilkes, 1966) and contains 2 mol of zinc/mol (Prescott et al., 1971). The apoenzyme is inactive, but reconstitution with Zn^{2+} restores full activity, and its substitution by Co^{2+}, Ni^{2+}, or Cu^{2+} substantially increases activity over that of the native enzyme. Moreover, the addition of 1 mol of Zn^{2+}/mol to aminopeptidase reconstituted with 1 mol of either Cu^{2+} or Ni^{2+} per mole results in hyperactivation of *Aeromonas* aminopeptidase (Prescott et al., 1983). We have now examined the kinetics and spectral consequences of metal ion substitutions at the active site of cobalt *Aeromonas* aminopeptidase, using cobalt as a chromophoric probe, to monitor the interaction of the two metal binding sites with different metal permutations.

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Experimental Procedures

Materials. Tricine[1] and Hepes were obtained from both

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Calbiochem and Sigma Chemical Co.; L-leucine-*p*-nitroanilide and L-alanine-*p*-nitroanilide also were bought from Sigma; 1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid, 1-butylboronic acid, and metal salts (Johnson-Matthey Specpure grade) were purchased from Aldrich Chemical Co.

Enzyme. Aeromonas aminopeptidase (EC 3.4.11.10), isolated from culture filtrates of *Aeromonas proteolytica* by the procedures of Prescott & Wilkes (1976), was generously provided by S. H. Wilkes as a solution that had been dialyzed against 1 mM Tricine, pH 8.0, containing 0.1 mM $ZnSO_4$ and 0.2 M KCl. It was stored frozen until needed. Enzyme concentrations were measured by absorbance, with the value ϵ_{278nm} = 41 800 M^{-1} cm^{-1} (Prescott et al., 1971). The standard assay of enzyme activity was that described by Prescott & Wilkes (1976), as modified by Baker et al. (1983), in which the zero-order hydrolysis of 0.5 mM L-leucine-*p*-nitroanilide (in 20 mM Tricine buffer, pH 8.0, containing 0.2 M KCl and 0.1 mM $ZnSO_4$) is measured at 25 °C. Assays of the apoenzyme and of reconstituted holoenzyme were run under first-order conditions with L-alanine-*p*-nitroanilide (37.5–50 μM) in 50 mM Hepes buffer, pH 7.5. For both substrates, the value $\Delta\epsilon_{405}$ = 10 800 M^{-1} cm^{-1} (Tuppy et al., 1962) was used to calculate the extent of hydrolysis, which was measured by the increase in absorbance at 405 nm in a Varian Model 219, a Gilford Model 222, or a Gilford response spectrophotometer.

Preparation of Apoaminopeptidase. Holoenzyme, at a concentration of 50 μM or higher, was dialyzed at 5 °C against at least three changes of 1 mM 1,10-phenanthroline at pH 7.5 in metal-free 20 mM Tricine or 50 mM Hepes. Each change of 1,10-phenanthroline was 15–25 times the volume of the enzyme, and the total time of dialysis against the complexing agent was 18–30 h. Residual 1,10-phenanthroline was removed by dialysis until it was undetectable in the solution outside the dialysis bag by absorbance readings at 327 nm. Ordinarily, this entailed five to seven changes of buffer, each of which was no more than 50 times the volume of the enzyme sample. Apoenzyme was analyzed for zinc content by atomic absorption and was assayed for residual activity with L-alanine-*p*-nitroanilide.

Preparation of Metalloaminopeptidases. Enzyme preparations containing either 2 mol/mol of a single metal or 1 mol/mol each of two metals were prepared by adding the desired metal ions to apoenzyme in 50 mM Hepes buffer, pH 7.5. For mixed-metal preparations, the first metal was added, the solution was mixed, and the preparation was allowed to equilibrate for 1 h. The second metal ion was then added, and the preparation was again allowed to stand for 1 h before being used or frozen for storage.

Other Methods. All buffers were extracted with 0.01% dithizone in CCl_4 (Fisher C-570, 99 mol % purity minimum) to eliminate adventitious metal ions; residual dithizone was removed by extraction with metal-free CCl_4, which was eliminated by aspirating air through the solutions. All operations involving apoenzyme were carried out in plasticware or in glassware washed with 20% nitric acid and then rinsed with deionized water. Dialysis tubing was prepared by heating it to 70 °C in deionized water and then decanting the wash water. At least four such treatments were performed before the dialysis tubing was stored at 5 °C.

Table I: Kinetic Values of Metalloaminopeptidases Formed by Sequential Additions of Metal Ions to Apoenzyme[a]

	order of addition		k_{cat}/K_m ($\times 10^4$ M^{-1} min^{-1})[b]
	first	second	
apoenzyme			0.17
native enzyme			2.1
reactivated enzyme	Zn^{2+}	Zn^{2+}	1.7
	Co^{2+}	Co^{2+}	10.5
	Cu^{2+}	Cu^{2+}	21.0
	Ni^{2+}	Ni^{2+}	20.5
	Zn^{2+}	Ni^{2+}	6.7
	Ni^{2+}	Zn^{2+}	89
	Zn^{2+}	Co^{2+}	1.9
	Co^{2+}	Zn^{2+}	8.7
	Zn^{2+}	Cu^{2+}	5.8
	Cu^{2+}	Zn^{2+}	124

[a] Metalloenzymes were prepared as described under Experimental Procedures. [b] Substrate was L-alanine-*p*-nitroanilide, 43 μM.

Metal analyses were performed on either a Perkin-Elmer Model 2280 atomic absorption spectrophotometer with an acetylene–air flame or a Varian Model AA-1475 with a Model GTA-95 graphite tube atomizer. Absorption spectra were obtained and analyzed with modified Varian programs. Molar absorptivity, ϵ, is expressed as M^{-1} cm^{-1}. CD and MCD spectra were measured in a Cary Model 61 spectropolarimeter equipped with a Varian V4145 superconducting magnet. Absorption spectra were made in quartz cells of 1-cm light path, masked so as to ensure absorption by sample only, containing 0.2 mL of apoenzyme to which Co^{2+} and any other solutions to be tested were added in volumes of 10 μL or less. Each experiment was begun by scanning and recording the spectrum of apoaminopeptidase, which was subsequently subtracted from the spectra that resulted from the addition of metal ions or inhibitors.

The stoichiometry of cobalt binding by the apoenzyme was determined by dialysis in metal-free 10 mM Hepes buffer adjusted to pH 6.53; a value below neutrality was used to prevent oxidation of Co^{2+}. Dialysis cells (Technilab Instruments, 1-mL capacity) were washed in 20% nitric acid and rinsed in deionized water. Both halves of the cells, assembled with membranes cut from dialysis tubing, were rendered metal free by washing with 0.25–0.5 mL of 1 mM 8-hydroxyquinoline-5-sulfonic acid. Apoaminopeptidase (0.2 mL, 0.25 mM) and 0.04 mL of 10 mM Hepes buffer, pH 6.53, containing $CoSO_4$ at concentrations that would supply 1, 2, and 10 mol of Co^{2+}/mol, were placed in one chamber of the cell. The other chamber received 0.24 mL of the 10 mM Hepes buffer, and the cells were sealed with Parafilm. The dialysis cells were shaken at 250 rev/min on a New Brunswick rotary shaker (Model 62) at room temperature. Samples taken at intervals over a period of 213 h were analyzed for cobalt; zinc also was analyzed at the beginning and end of the experiment. After 51 h, each compartment was made 0.1 M in KCl to quench the Donnan membrane effect.

Results

Aeromonas aminopeptidase, when dialyzed against 1,10-phenanthroline, followed by metal-free buffer, concurrently loses both zinc and activity until, ultimately, less than 6% of either remains (Prescott et al., 1983). The apoenzyme is stable for long periods. The addition of Zn^{2+} to samples frozen for 1.5 years resulted in full reactivation, and storage of the reconstituted enzyme for a week at 5 °C did not diminish activity.

[1] Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; BuBA, 1-butylboronic acid; 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.

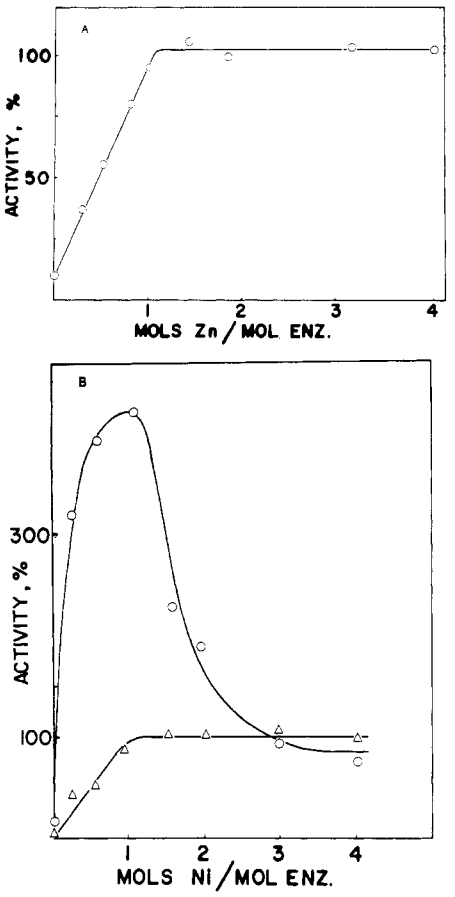

FIGURE 1: (A) Activity titration of apoaminopeptidase with Zn^{2+} ions. Apoenzyme (8.5×10^{-7} M) was incubated for 1 h at room temperature in 50 mM Hepes, pH 7.5, with Zn^{2+} ions at the molar ratios indicated. Reactions were started by adding sufficient L-alanine-*p*-nitroanilide to yield a concentration of 43 μM in the reaction mixture. Activities are expressed relative to that of the sample containing 2 mol/mol. (B) Reactivation of apoaminopeptidase by stoichiometric additions of Ni^{2+} (△) and by Ni^{2+} followed by Zn^{2+} (○). The apoenzyme (1.2×10^{-6} M) in 50 mM Hepes, pH 7.5, was equilibrated for 1 h with Ni^{2+} in the ratios shown, and reactions were started by the addition of substrate as above. After the initial velocity of each reaction was apparent, 1 equiv of Zn^{2+} was added, and the new velocity was determined.

Reactivation of Apoaminopeptidase. Zn^{2+}, Co^{2+}, Cu^{2+}, or Ni^{2+} instantaneously reactivates apoaminopeptidase (Table I), but Mg^{2+}, Mn^{2+}, and Ca^{2+} do not. Slight activity, 22%, was restored with Cd^{2+}. Zinc titration of the apoenzyme increases activity incrementally until 1 mol of Zn^{2+}/mol has been added; Zn^{2+} in excess of that does not effect activity further (Figure 1A). Figures 1B and 2 show analogous Ni^{2+} and Co^{2+} titrations. Thus, though the native enzyme contains 2 mol of Zn^{2+}/mol, only 1 mol/mol is required to restore full activity. Similarly, only 1 mol of Co^{2+}, Ni^{2+}, or Cu^{2+} per mole is required to restore maximal activity for each of these metals.

The activities that result from addition to the apoenzyme of 1 mol of either Cu^{2+} (Prescott et al., 1983) or Ni^{2+} per mole, followed by 1 mol of Zn^{2+}/mol, are greater than those due to the presence of only a single mole of these metal ions per mole (Figure 1B). When 2 or more mol of Cu^{2+} or Ni^{2+} per mole is added to the apoenzyme before the addition of Zn^{2+}, hyperactivation does not occur, indicating that both metal-binding sites are occupied by the first metal and that Zn^{2+} does not displace the metal in either site during the course of the reaction. Reversal of the order of addition, i.e., 1 mol of Zn^{2+}/mol before Cu^{2+} or Ni^{2+}, results in initial velocities that are substantially lower than those when Cu^{2+} or Ni^{2+} is the ion added first. The consequences of sequential Co^{2+} and Zn^{2+} additions are similar although less in degree. In all these instances, the extent of hyperactivation depends on the order of metal ion addition. Table I also compares the k_{cat}/K_m values measured under first-order conditions of various metal-substituted aminopeptidases, prepared by the addition of stoichiometric quantities of metal ions to apoenzyme for all combinations of Zn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+}.

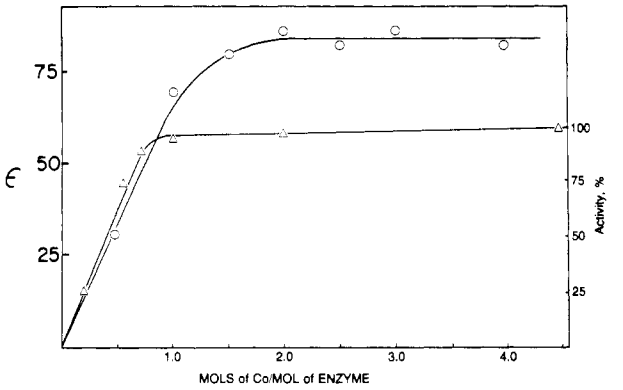

FIGURE 2: Spectroscopic (○) and activity (△) titrations of apoaminopeptidase by Co^{2+}. In the spectroscopic titration, aliquots of Co^{2+} were added sequentially to apoenzyme (2.39×10^{-4} M), and the visible region of the spectrum was scanned after each addition. Corrections were made for absorption by unbound Co^{2+} ($\epsilon = 4.8$ M^{-1} cm^{-1}) above 2 mol/mol. The left-hand ordinate is the molar absorptivity at 527 nm. Enzyme activity was titrated by mixing apoenzyme (1×10^{-6} M) and substrate (43 μM L-alanine-*p*-nitroanilide) and then adding Co^{2+} to yield the molar ratios shown. Activities (right-hand ordinate) are expressed as percent of the activity of the enzyme fully reconstituted with Co^{2+}.

Table II: Cobalt Binding by *Aeromonas* Apoaminopeptidase during Extended Dialysis

Co^{2+} added (mol/mol)	Co^{2+} and Zn^{2+} ions bound (mol/mol) at a dialysis time (h) of[a]					
	0	21	44	68	164	213
1	0.95 (0.25)	1.20	0.91	0.88	0.83 (0.37)	0.77
2	1.58 (0.24)	1.96	1.79	1.55	1.66 (0.30)	1.59
10	7.18 (0.33)	4.64	5.04[b]	2.06	1.75 (0.38)	1.67

[a] At each time shown, samples were removed from both sides of each dialysis cell and analyzed for Co^{2+}. Concentrations of bound metals shown represent the differences in metal concentrations in the enzyme compartment and the buffer compartment of each cell. Enzyme concentration was 2.05×10^{-4} M. Zinc was analyzed at zero and 164 h; moles of zinc per mole of enzyme are shown by the numbers in parentheses. [b] After 51 h of dialysis, the solution in each cell compartment was made 0.1 M in KCl to damp the Donnan membrane effect. The results of this addition are evident from a comparison of the Co^{2+} values for the 10 equivalent samples before KCl was added with those after the ionic strength was increased.

Spectra. The apoenzyme binds 2 mol of cobalt/mol even when the metal ion is in 10-fold excess (Table II) confirming the existence of the two metal-binding sites (Prescott et al., 1971). Spectrophotometric titration of apoenzyme with increments of Co^{2+}, accompanied by scanning of the visible absorption spectrum,[2] confirms this stoichiometry (Figure 2). One mole of Co^{2+} per mole results in [(AAP)Co-] ($\epsilon_{527} = 53$ M^{-1} cm^{-1}) while 2 mol of Co^{2+}/mol increases ϵ_{527} to 82 M^{-1}

[2] The choice of buffers proved important to the spectral experiments in which cobalt was added to apoenzyme. In an initial experiment with the apoenzyme in Tricine buffer, 50 mM, pH 8.0, the addition of Co^{2+} failed to generate a detectable absorption spectrum. The ability of Tricine to form complexes with metal ions (Vieles et al., 1972) suggested a reason for the failure and prompted the use of Hepes buffer, which proved successful. Subsequently, Hepes was used in both spectral and kinetic experiments.

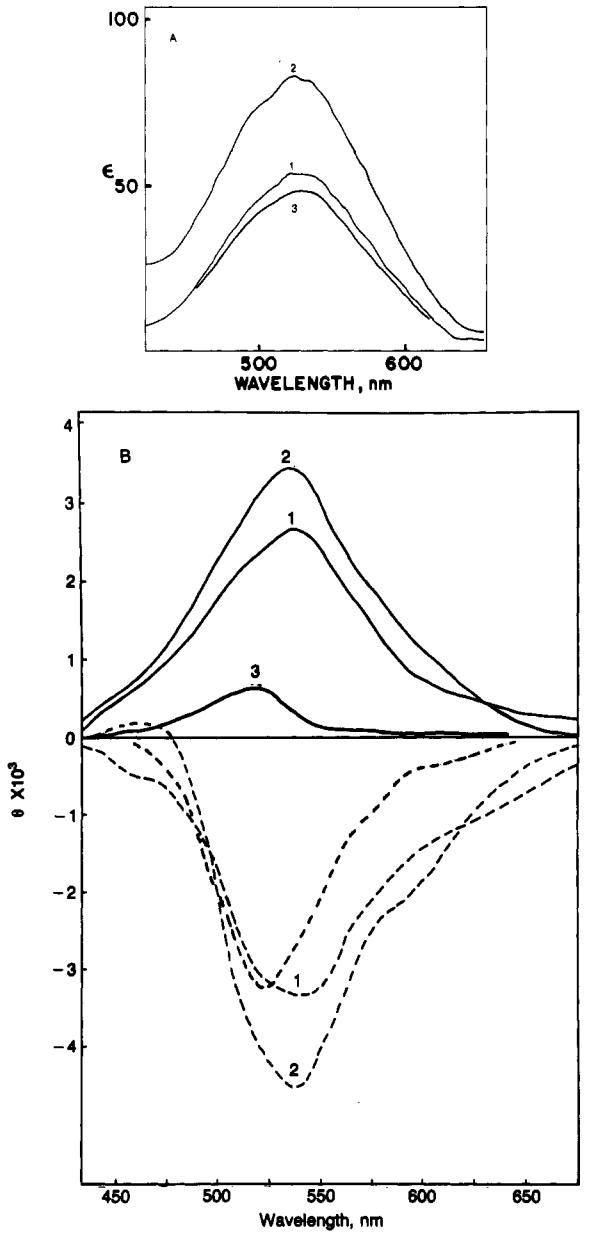

FIGURE 3: Electronic spectra of cobalt aminopeptidases. (A) Absorption spectra of [(AAP)Co-] (1), [(AAP)CoCo] (2), and [(AAP)CoZn] (3). (B) Corresponding CD (—) and MCD (--) spectra measured at 4 T. The MCD spectra have been corrected for CD. Enzyme concentrations: 2.64×10^{-4} M in (A) and 1.56×10^{-4} M in (B).

cm^{-1} to form [(AAP)CoCo] (Figure 3A). More than 2 mol of Co^{2+}/mol does not alter the spectrum further (Figure 2).

Figure 3B shows the CD and MCD spectra of [(AAP)Co-], [(AAP)CoZn], and [(AAP)CoCo]. The broad, positive CD band of [(AAP)Co-] is centered at 525 nm. The second cobalt atom does not alter the shape of the CD spectrum, but the intensity increases about 1.3-fold, analogous to the absorption spectrum. Similarly, the MCD spectrum of [(AAP)CoCo] is 1.3 times more intense than that of [(AAP)Co-], and both exhibit a major negative band centered near 530 nm and fine structure not readily apparent in the respective CD or absorption spectra. The hybrid enzyme, [(AAP)CoZn], formed by adding Zn^{2+} to [(AAP)Co-], is 4-fold more active than the native zinc enzyme (Table I), but its absorption and MCD spectra give no indication of accompanying changes in cobalt coordination number. Zinc in the second site does perturb the microenvironment of the cobalt atom, however, as shown by a change in the cobalt stereochemistry detected by circular dichroism. Thus, zinc abolishes the large positive CD band associated with the enzyme containing 1 mol of Co^{2+}/mol.

The absorption spectrum of [(AAP)ZnCo], a derivative labeled solely in the second site, is markedly different from that of [(AAP)CoZn]. It exhibits only weak absorptivity ($\epsilon \simeq 20$ M^{-1} cm^{-1}) centered near 500 nm with an MCD spectrum essentially that of octahedral-like cobalt (results not shown).

Effects of Inhibitors on Cobalt Spectra. Both BuBA and *n*-valeramide are reversible inhibitors of *Aeromonas* aminopeptidase (Baker et al., 1983). BuBA is a transition-state analogue inhibitor whereas *n*-valeramide is a substrate analogue inhibitor (Baker & Prescott, 1983). In contrast to *n*-valeramide, which does not perturb the spectrum of [(AAP)Co-] or [(AAP)CoCo] when added up to 22.9 mM ($5.2K_i$), BuBA markedly perturbs both, increasing the molar absorptivity of [(AAP)CoCo] from 82 to 110 M^{-1} cm^{-1} at 527 nm and eliciting shoulders near 480, 500, and 590 nm (Figure 4A). There are also analogous changes in the corresponding CD and MCD spectra (cf. Figures 4B and 3B); transitions hidden in the absorption spectra are resolved (Figure 4B). The MCD and CD spectra of the [(AAP)Co-]·BuBA complex (not shown) are very similar to those of [(AAP)CoCo]·BuBA.

The perturbations by BuBA are primarily a result of its interaction with the metal in the first binding site without apparent contributions from the metal ion in the second site. Thus, addition of Co^{2+} to [(AAP)Zn-]·BuBA (Figure 5) yields a weak absorption spectrum, $\epsilon < 20$ M^{-1} cm^{-1}. It differs qualitatively and quantitatively from that obtained by adding BuBA to either [(AAP)Co-] or [(AAP)CoCo] (Figure 4B) but is similar to that observed when BuBA is added to [(AAP)ZnCo] (Figure 5). Thus BuBA, bound to the first metal (i.e., the spectroscopically silent Zn^{2+} ion), is not available to react with the Co^{2+} ion added subsequently. Like the absorption spectra, the CD and MCD spectra of [(AAP)ZnCo]·BuBA are of very low intensity and virtually undetectable under the conditions employed in Figure 5. Nevertheless, Zn(II) in the second metal site spectrally perturbs the Co(II) occupying the first site in a manner different from that of Co(II) in the second site; the addition of BuBA to [(AAP)CoZn] (Figure 5) yields an absorption spectrum substantially different from that produced by its association with [(AAP)CoCo] (Figure 4A). This effect of the second site on the first again is reflected in the MCD spectra (Figure 5), which show that the complex [(AAP)CoZn]·BuBA differs markedly from [(AAP)CoCo]·BuBA (cf. Figure 4B).

DISCUSSION

The present investigation of the roles of metal ions confirms that *Aeromonas* aminopeptidase contains 2 mol of zinc that can be replaced by other metals, e.g., cobalt. The stoichiometry of Co^{2+} binding, measured by equilibrium dialysis and spectral titration of the apoenzyme, confirms earlier analytical zinc data (Prescott et al., 1971). One and two moles of Co^{2+} per mole is bound when the apoenzyme is exposed to those amounts during dialysis (Table II), and when exposed to 10 mol of Co^{2+}/mol, only 2 mol is bound, demonstrating the absence of nonspecific cobalt binding.

We recently reported that Co^{2+}, Ni^{2+}, and Cu^{2+} each restores enzymatic activity to apoaminopeptidase, inducing a level of activity higher than that of the native zinc enzyme (Prescott et al., 1983). Moreover, the addition of only 1 mol of metal/mol of enzyme restores full activity. The present study shows that the first mole of metal ion per mole preferentially enters the active site, resulting in catalytic activity. It is not surprising that Co^{2+} produces activity exceeding that of Zn^{2+}: this behavior is quite analogous to that previously

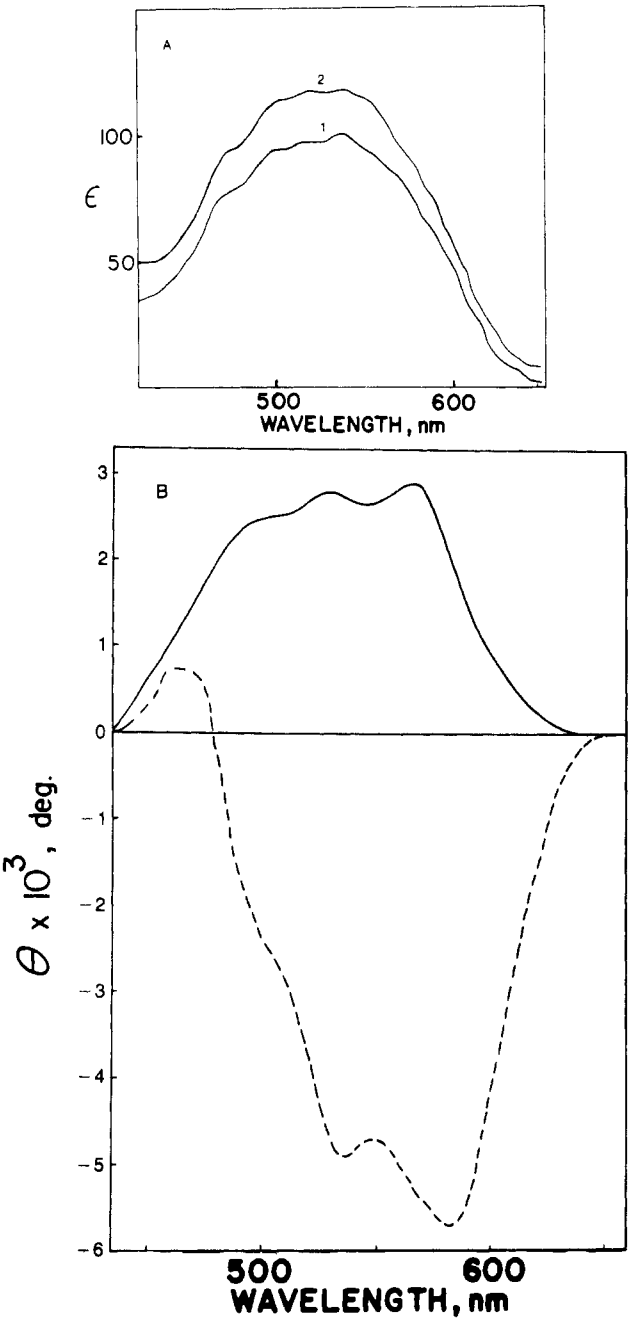

FIGURE 4: Effects of BuBA on the absorption, CD, and MCD spectra of cobalt aminopeptidase. (A) Absorption spectrum of [(AAP)Co-] (1) and [(AAP)CoCo] (2) after the addition of a stoichiometric amount of BuBA. (B) CD (—) and MCD (--) spectra of [(AAP)CoCo]·BuBA formed by addition of 1 equiv of BuBA. Enzyme concentrations: 2.64×10^{-4} M in (A) and 1.56×10^{-4} M in (B).

observed for other proteases, e.g., carboxypeptidase A (Coleman & Vallee, 1961), thermolysin (Holmquist & Vallee, 1974), and angiotensin converting enzyme (Bünning & Riordan, 1981). The degree of activation of *Aeromonas* aminopeptidase by Ni^{2+} is 10 times that achieved with Zn^{2+}. Thermolysin and angiotensin converting enzyme are inactive with this metal, whereas Ni^{2+} reactivates apocarboxypeptidase A virtually to the same extent as Zn^{2+} with respect to peptide hydrolysis (Coleman & Vallee, 1961). Most surprisingly, copper *Aeromonas* aminopeptidase is catalytically active, in fact, more so than the native enzyme; to our knowledge, this is the first example of a catalytically active copper metalloprotease with amidase activity.

The capacity of an enzyme containing 1 mol/mol of a given metal to function catalytically as effectively as that containing

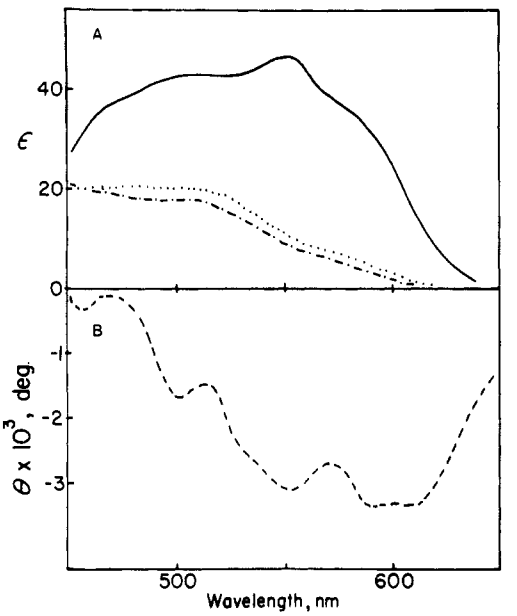

FIGURE 5: (A) Effects of BuBA on the cobalt absorption spectra of the first and second metal binding sites of metalloaminopeptidases: (···) [(AAP)Zn-] after sequential additions of 1 equiv of BuBA, then Co^{2+}; (—) [(AAP)CoZn] after the addition of 1 equiv of BuBA; (-·-) [(AAP)ZnCo] following the addition of 1 equiv of BuBA. (B) (--) MCD spectrum of [(AAP)CoZn] after addition of 1 equiv of BuBA.

2 mol/mol of the same metal suggests that the second metal-binding site is noncatalytic and might be structural or regulatory. Significant kinetic changes ensue, however, when two different metal ions are introduced into the two sites, emphasized by the fact that the reactivation of apoaminopeptidase markedly depends on the order of addition of zinc and of another metal. This is illustrated by the experiment shown in Figure 1B, in which the first metal ion was added to apoenzyme and permitted to equilibrate; the reaction was then started by the addition of substrate. After the reaction had continued long enough to permit measurement of the initial rate, the second metal was added and the new rate determined. These studies indicate that stable hybrid enzymes with different metals at the two sites can be prepared merely by varying the order of their addition. Hence, subsequent experiments were performed by adding each metal ion to apoenzyme in the desired order, allowing 1 h of equilibration after each addition, and then adding substrate at $[S] << K_m$ and determining first-order rate constants. The resulting mixed-metal enzyme preparations are remarkably active, up to 2 orders of magnitude greater than that of the native enzyme. We have substantially refined and extended earlier results on the activity of these hybrid enzymes (Prescott et al., 1983) to include preparations of each combination of metals (Table I).

A comparison of the specific activity of [(AAP)ZnNi] and [(AAP)ZnCu] with those of [(AAP)NiZn] and [(AAP)-CuZn] is particularly informative. The marked dependence of activity on the order of addition is best reconciled by postulating specific, nonidentical but interacting metal binding sites that have different functions. The high activities of both [(AAP)CuZn] and [(AAP)NiZn] relative to the native enzyme are due primarily to increased k_{cat} values (unpublished observations). Despite complete reactivation of apoaminopeptidase by the first mole of metal per mole added, substantial interactions between the two metal binding sites are clearly possible, and the second site, while not essential for enzyme activity, can strongly influence the catalytic step. Metal exchange does not appear to occur within the time frame of these

experiments, in that k_{cat}/K_m values determined immediately after metal addition or following up to 1 h of preincubation were identical. Evidently, the metals fill the binding sites virtually instantaneously, and once bound, they exchange slowly, if at all.

The absorption, CD, and MCD spectra of Co(II)-substituted enzymes have been employed extensively to relate structure to the function of metalloenzymes (Vallee & Holmquist, 1980). In the present instance, the spectra allow differentiation and characterization of the two metal sites in relation to their functional role. As Co^{2+} is added to the apoenzyme, binding to the active site results in a large increase in absorbance per cobalt atom from 4.8 M^{-1} cm^{-1} for octahedral $Co(H_2O)_6^{2+}$ to what appears to be a distorted tetrahedral-like geometry (ϵ = 53 M^{-1} cm^{-1}) in the bound state (Vallee & Holmquist, 1980). Both the spectral band shape and intensity of [(AAP)Co-] are very similar to cobalt-substituted thermolysin in which the active site metal is bound in a distorted tetrahedron with two His and a Glu residue as ligands from the protein to the metal (Colman et al., 1972). The absence of a clear transition in the 300–400-nm region in either the absorption or MCD spectra is inconsistent with cysteine sulfhydryl groups as ligands; proteins known to contain Cys as a ligand (e.g., alcohol dehydrogenase, β-lactamase, azurin) typically exhibit intense (ϵ = 1000 M^{-1} cm^{-1}) cobalt-to-sulfur charge-transfer bands in this region (Vallee & Holmquist, 1980). The fact that the intensity and overall line shape of the MCD spectrum of [(AAP)Co-] (Figure 3B) are similar to those of cobalt thermolysin and other known cobalt tetrahedral complexes also confirms the tetrahedral-like assignment of this site.

Occupancy of the second metal-binding site by cobalt to form [(AAP)CoCo] increases the absorptivity to 83 M^{-1} cm^{-1}. The added absorptivity ($\Delta\epsilon$ = 30 M^{-1} cm^{-1}) probably does not solely reflect absorption contributed by the second cobalt atom. Thus, the addition of zinc to [(AAP)Co-] does affect the cobalt absorption spectrum, reducing ϵ_{527} from 53 to 43 M^{-1} cm^{-1}. Also, zinc virtually abolishes the CD signal (Figure 5B). Both effects indicate an altered environment of the first metal site induced by occupancy of the second. It is likely, however, that the second site is octahedral-like on the basis of spectral studies of those derivatives in which cobalt occupies the second site, i.e., in [(AAP)ZnCo] and [(AAP)ZnCo]·BuBA. The absorptivity and magnetic ellipticity of these species are characteristic of octahedral cobalt (Holmquist & Vallee, 1980) in respect to both their low absorptivity, $\epsilon < 10$ M^{-1} cm^{-1}, and their weak MCD spectra.

On the basis of kinetic evidence, Baker & Prescott (1983) recently proposed that BuBA and *n*-valeramide inhibit *Aeromonas* aminopeptidase by different mechanisms. Their results indicate that BuBA functions as a transition-state analogue inhibitor and that *n*-valeramide—even though essentially isosteric and electronically similar to BuBA—is a substrate analogue inhibitor. The spectral perturbations resulting from the interaction of stoichiometric amounts of BuBA but not *n*-valeramide with [(AAP)Co-] and [(AAPCoCo] indicate that these two compounds inhibit by fundamentally different mechanisms. The former alters the shape of the absorption, CD, and MCD spectra and amplifies the magnitude of the absorption and MCD signals by nearly half. This behavior is reminiscent of the effect of inhibitors on cobalt thermolysin in which binding, probably to the metal, produces large increases in spectral intensity to closely resemble model tetrahedral complexes (Vallee & Holmquist, 1980). The fact that BuBA reacts stoichiometrically with the metal in the first site provides further evidence for the critical catalytic role played by the first metal bound. These inhibition studies also signal the interaction between the sites via their CD spectra. The effect of BuBA on [(AAP)CoCo] differs from its effect on [(AAP)CoZn], thus underscoring the effect of occupancy of the second site on the conformation of the first (catalytic) metal binding site.

The metalloaminopeptidases that have been characterized appear to fall into the categories of one-metal and two-metal enzymes, the former being represented by human liver aminopeptidase (Gardner & Behal, 1971), aminopeptidase M, (Wacker et al., 1970), and two bacterial enzymes of the genus *Bacillus* (Rodriguez-Absi & Prescott, 1978; Wagner et al., 1979). Carpenter and co-workers have demonstrated the two-metal nature of bovine lens leucine aminopeptidase and did much to define the functions of the metals (Carpenter & Vahl, 1973; Thompson & Carpenter, 1976a,b; Allen et al., 1983), as have Taylor et al. (1982). In contrast to *Aeromonas* aminopeptidase, the bovine lens enzyme requires filling of both metal binding sites as a requisite to activity (Carpenter & Vahl, 1973). Van Wart & Lin (1981) reported that the similar leucine aminopeptidase from porcine kidney cytosol firmly binds only 1 mol of Zn^{2+}/subunit but that a second, regulatory site accomodates 1 additional mol of metal ion/mol of subunit. The binding of Mg^{2+} or Mn^{2+} to this regulatory site accounts for the observed stimulation of activity by these ions (Johnson et al., 1936; Smith & Spackman, 1955). Unlike either of these hexameric enzymes, *Aeromonas* aminopeptidase is not affected by the addition of either Mg^{2+} or Mn^{2+} ions to [(AAP)Zn-]. The results at hand suggest differences in metal function among the various two-metal aminopeptidases that extend beyond their sizes and states of aggregation. The existence of two defined nonexchanging metal sites in the *Aeromonas* enzyme that interact kinetically to such a high degree raises the question of their physical proximity, which is the subject of current investigations.

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Registry No. BuBA, 4426-47-5; Zn, 7440-66-6; Co, 7440-48-4; Ni, 7440-02-0; Cu, 7440-50-8; *Aeromonas proteolytica* aminopeptidase, 37288-67-8; L-alanine-*p*-nitroanilide, 1668-13-9.

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Comparison of the Energetics of the Uncatalyzed and Glutamate Dehydrogenase Catalyzed α-Imino Acid-α-Amino Acid Interconversion†

R. Srinivasan* and Harvey F. Fisher*

Laboratory of Molecular Biochemistry, Department of Biochemistry, University of Kansas School of Medicine, and Veterans Administration Medical Center, Kansas City, Missouri 64128

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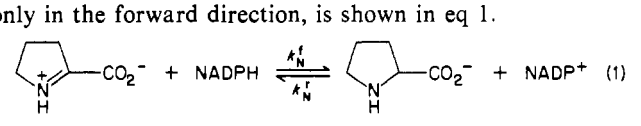
ABSTRACT: The thermodynamic and activation parameters for the reduction of Δ^1-pyrroline-2-carboxylic acid (an α-imino acid) by reduced nicotinamide adenine dinucleotide phosphate (NADPH) are compared with those for the reduction of the same imino acid by the glutamate dehydrogenase–NADPH complex. The enthalpies of activation and standard free energy changes for these two reactions are found to be virtually the same. The catalysis by the enzyme, expressed as the ratio of the reactivity of the enzyme–NADPH complex to that of NADPH itself in reducing the iminium ion, is entirely accounted for by a more favorable entropy of activation with enzyme–NADPH as the reductant. This entropic driving force is large enough to overcome the exergonic formation of the binary complex and still lead to considerable catalysis by glutamate dehydrogenase. Comparison of $\Delta S^{\ddagger}$ and ΔS° values for the reduction of the iminium ion by NADPH suggests that the solvation of the transition state resembles that of the reactants, even though the substituent effects on rate have shown that the hydride transfer from the reduced coenzyme is complete at the transition state [Srinivasan, R., Medary, R. T., Fisher, H. F., Norris, D. J., & Stewart, R. (1982) *J. Am. Chem. Soc. 104*, 807]. The ΔG° and $\Delta S^{\ddagger}/\Delta S^{\circ}$ values for the reduction by the enzyme–NADPH complex indicate that this reaction has a fairly symmetric transition state, the solvation properties of which are intermediate between those of the reactants and those of the products. Both the enzyme-catalyzed model reaction and the transient phase of glutamate reaction involve the hydride transfer step, and in both cases, the enthalpy barriers are larger than the corresponding entropy barriers.

We have previously employed the stable α-imino acid Δ^1-pyrroline-2-carboxylic acid (PCA)[1] as a model for α-iminoglutarate (Srinivasan et al., 1982). Iminoglutarate, in the enzyme-bound state, occurs as a reactive intermediate in the glutamate dehydrogenase catalyzed reductive amination of α-ketoglutarate (Brown et al., 1978; Hochreiter et al., 1972; Fisher & Viswanathan, 1984). The cyclic imino acid is reduced to DL-proline by several 1,4-dihydropyridines including reduced nicotinamide adenine dinucleotide phosphate (NADPH) (Meister et al., 1957; Srinivasan et al., 1982). The nonenzymatic reaction, which proceeds at a measurable speed only in the forward direction, is shown in eq 1.



We have previously reported that the nonenzymatic reaction has the following features (Srinivasan et al., 1982): (1) the iminium ion is the active oxidant, (2) the rates for the reduction

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* Address correspondence to the Veterans Administration Medical Center.

[1] Abbreviations: PCA, Δ^1-pyrroline-2-carboxylic acid; E, glutamate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Tris, tris(hydroxymethyl)aminomethane.