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Human Liver Aminopeptidase. Role of Metal Ions in Mechanism of Action†

Charles W. Garner, Jr., and Francis J. Behal*

ABSTRACT: Human liver aminopeptidase is activated 2.4-fold by Co^{2+} which binds in the uncompetitive or coupling fashion. The large dissociation constant (K_a = 50 μM) indicates that the enzyme-Co^{2+} complex dissociates readily, thus preventing the isolation of the intact complex. The enzyme also contains 8.3 ± 1.5 nmol of zinc/mg of protein (*i.e.*, 1 mol of Zn/122,000 ± 20,000 g of protein) and is inhibited by several chelators and complexing agents. Aminopeptidase is inhibited by ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (an EDTA analog) and by 1,10-phenanthroline each in the competitive manner with K_i = 0.012 and 0.028 mM, respectively. Sulfide also inhibits but with kinetics of the mixed type, K_i = 0.076 mM. Inhibition by these and other agents is reversible by removal of the agent through dialysis or gel filtration, by dilution, or by titration with divalet metal ions. The presence of divalent transition metal ions also prevents chelator inhibition. When aminopeptidase is treated at 60° with EDTA, the inhibition is no longer reversible and there is a concomitant loss of zinc. The data suggest that the enzyme forms a cobalt metalenzyme complex and is a zinc metalloenzyme, zinc being located near the substrate binding site.

Enzymes which catalyze the hydrolysis of aminoacyl-β-naphthylamides have been found in numerous human tissues (Behal *et al.*, 1965; Panveliwalla and Moss, 1966; Hopsu-Havu and Makinen, 1967; Marks *et al.*, 1968) and in serum, especially during hepatobiliary disease (Goldbarg and Rutenburg, 1958). Many of the aminopeptidases have been called "arylamidases" after the suggestion of Patterson *et al.* (1963), but amino acids are cleaved from the N-terminal end of numerous substrates having no aryl group, including simple dipeptides.

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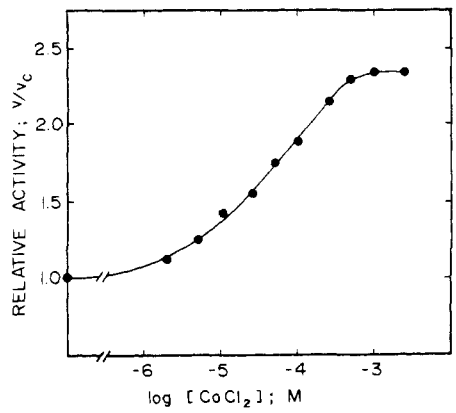

FIGURE 1: Stimulation of aminopeptidase by Co^{2+}. The amount of β-naphthylamine formed in the presence of varying amounts of $CoCl_2$ was determined by diazotization. Imidazole-HCl, 0.05 M, pH 7.0, was used as buffer in place of potassium phosphate.

An aminopeptidase which catalyzes the rapid hydrolysis of alanyl-β-naphthylamide has been isolated and purified from human liver; this enzyme has been shown to be distinctly different from the classical leucine aminopeptidase (Behal *et al.*, 1966). More recently, Little and Behal (1971) and Starnes and Behal (1974) have reported that this human liver aminopeptidase has a molecular weight of 235,000 in solutions of low ionic strength. The composition and some of the physical properties of this enzyme have been reported in the preceding article. Another characteristic of this aminopeptidase (or arylamidase) is that Co^{2+} ions increase the activity of the enzyme while EDTA and other chelating agents are inhibitory.

This paper is concerned with an investigation of the interaction of various metal ion chelators and complexing agents with human liver aminopeptidase in an effort to determine how metal ions might be involved in its mechanism of action.

Experimental Section

Materials and Methods. Human liver aminopeptidase was prepared essentially by the procedure of Little (1970) as modified by Starnes and Behal (1974). The enzyme is stable for long periods at 4 or −20°; however, some loss of activity occurred upon dialysis against distilled water for 4 days at 4°. A_{280} of a 1.0 mg/ml of solution was 1.75. Imidazole, 1,10-phenanthroline, 8-hydroxyquinoline, and 2,2′-bipyridine were obtained from Eastman; EDTA[1] and *N*-(1-naphthyl)ethylenediamine dihydrochloride, from Fisher Scientific Co.; 2,9-dimethyl-1,10-phenanthroline, from G. Fredrick Smith Chemical Co., Dowex chelating resin, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid (EGTA), from Sigma; L-alanyl-β-naphthylamide, recrystallized from water–ethanol, from Mann Research Laboratories; other reagents were obtained from commercial sources. Water was supplied to the laboratory as deionized and distilled and was redistilled in an all-glass still. Water was shown to be free of metal ions by treatment with dithizone–CCl_4. Plastic test tubes and pipets were used in place of glass and were free of metal contamination by the dithizone test.

Assay of Aminopeptidase. Routinely, peptidase activity was determined according to a procedure similar to that of a Goldbarg and Rutenburg (1958). Enzyme, 0.2–0.5 μg, was added to a solution at 0° containing 0.05 M potassium phosphate (pH 6.86) and 1.0 mM L-alanyl-β-naphthylamide in 1.0 ml in a 10-ml polyallomer centrifuge tube and incubated at 37° for 20 min. In cases where Co^{2+}, EDTA, or EGTA were added to the reaction mixture, the enzyme was incubated with the metal or chelator at 50° for 10 min in a volume of 0.90 ml prior to the addition of alanyl-β-naphthylamide. The amount of β-naphthylamine liberated was measured by addition of 0.5 ml of 2.5 M trichloroacetic acid followed by 0.5 ml of 0.1% sodium nitrite. After 5–7 min, 0.5 ml of 0.5% ammonium sulfamate was added followed by the addition of 1.25 ml of 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 95% ethanol. The solution was thoroughly mixed after each addition. The blue dye which formed was measured after 15 min at 580 nm. The specific activity of freshly prepared enzyme is expressed as micromoles of β-naphthylamine released per min per mg of protein, and was typically 60. Kinetic assays were usually performed by measuring the continuous appearance of β-naphthylamine at 340 nm. The 1.0-ml reaction mixture, containing 0.6 μg of enzyme, was adjusted by certain additions or dilutions as specified. The measurement was made at 37.0° in the thermostated cell holder of a Beckman Acta III spectrophotometer adjusted to read full recorder scale of 0–0.1 absorption unit.

Analysis for Metal Content. Metal analyses of aminopeptidases were accomplished by atomic absorption using a Perkin-Elmer atomic absorption spectrophotometer, Model 303. Some samples were dialyzed overnight against 0.1 mM EDTA, followed by dialysis for 2 days against several changes of distilled water. Other samples were passed through a small column of Dowex chelating resin to remove free metal ions just prior to analysis. Analyses were performed on solutions containing approximately 1.0 mg/ml. Each analysis required 1–2 ml. Standard solutions contained 0.5–1.0 μg of Zn/ml (from $ZnCl_2$).

Results

Stimulation of Aminopeptidase by Co^{2+}. Human liver aminopeptidase was stimulated 2.4-fold by Co^{2+} as shown in Figure 1. Maximum reproducible stimulation occurred when the enzyme was heated with Co^{2+} at 50° for 10 min prior to the incubation at 37° with the substrate alanyl-β-naphthylamide. Heating the enzyme in the absence of Co^{2+} also gave consistently high Co^{2+} stimulation. The stimulatory effect was limited to Co^{2+}. Other metal ions, including Zn^{2+}, had no effect at lower concentrations ($<10^{-4}$ M) and were toxic at higher concentrations ($>10^{-4}$ M). Alkaline earth metals had no effect. A plot of the data in Figure 1 as $1/v$ *vs.* $1/[Co^{2+}]$ (not shown) gave an apparent $K_m(Co^{2+}) = 50\ \mu M$.

To test whether Co^{2+} was interacting with a sulfhydryl group of the protein, the enzyme was treated at 37° for 1 hr with several sulfhydryl-modifying reagents: 5,5′-dithiobis(2-nitrobenzoic acid), 5 mM; iodoacetate, 5 mM; iodoacetamide, 5 mM; *p*-chloromercuribenzoate, 1 mM; and *N*-ethylmaleimide, 10 mM. Neither the activity in the absence of added metal ion, nor the Co^{2+} stimulation nor the inhibition by Cu^{2+}, one of the most toxic metal ions, was affected by treatment with these reagents.

The stimulation by Co^{2+} was found to be of the uncompetitive or coupling type. Shown in Figure 2 is a plot of v *vs.* $v/[S]$ in the presence and absence of 0.5 mM Co^{2+}. The apparent K_m and V_{max} without Co^{2+} were 0.156 mM and 2.45 nmol/min, respectively. With Co^{2+} K_m was raised to 0.376 mM and V_{max} to 5.92 nmol/min.

Inhibition by Chelators and Metal Complexing Agents. Aminopeptidase was sensitive to numerous chelating and com-

[1] Abbreviations used are: EDTA, ethylenediamine-*N,N′*-tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; OP, 1,10-phenanthroline; v, enze reaction velocity; v_c, enzyme reaction velocity of a control.

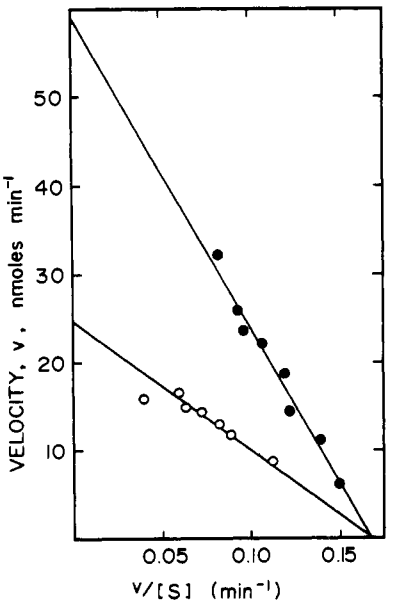

FIGURE 2: Kinetics of Co^{2+} stimulation of aminopeptidase. The amount of β-naphthylamine formed with varying amounts of substrate was determined by measuring the increase in absorption at 340 nm. Reaction mixtures contained Co^{2+}, 0.5 mM (●—●) or no Co^{2+} (○—○).

plexing agents as shown in Table I. The sensitivity to EDTA was pronounced at the level of 10^{-6}–10^{-7} M, but the exact level for 50% inhibition was somewhat variable due, presumably, to variable levels of trace metal ion contaminants. The expression of EDTA inhibition was variable, especially with aged preparations. In some instances EDTA concentrations as high as 0.1 M were not inhibitory. It was later observed that incubation of the enzyme with EDTA at elevated temperatures (*e.g.*, 50° for 10 min) resulted in consistent inhibition. Heat treatments without EDTA had no effect on enzymatic activity. Extensive dialysis or passage through Sephadex G-25 was required for removal of EDTA; removal resulted in restoration of the original level of activity.

Several inorganic metal ion complexing agents also were inhibitory at low concentrations including cyanide, sulfide, and azide. Borate and citrate were not inhibitory.

TABLE I: Inhibition by Chelators and Metal Complexing Agents.[a]

Agent Added	Concn (mM)	Rel Activity Remaining (v/v_c)
None		1.00
EDTA	0.001	0.02
EGTA	1.0	0.04
1,10-Phenanthroline	0.5	0.01
8-Hydroxyquinoline	2.0	0.16
2,2′-Bipyridine	5.0	0.71
2,9-Dimethyl-1,10-phenanthroline	2.0	0.48
Na_2S	1.0	0.12
NaN_3	10	0.08
KCN	10	0.16
Ammonium oxalate	50	0.29

[a] EDTA and EGTA were incubated with aminopeptidase prior to addition of substrate. The amount of β-naphthylamine formed was measured by diazotization.

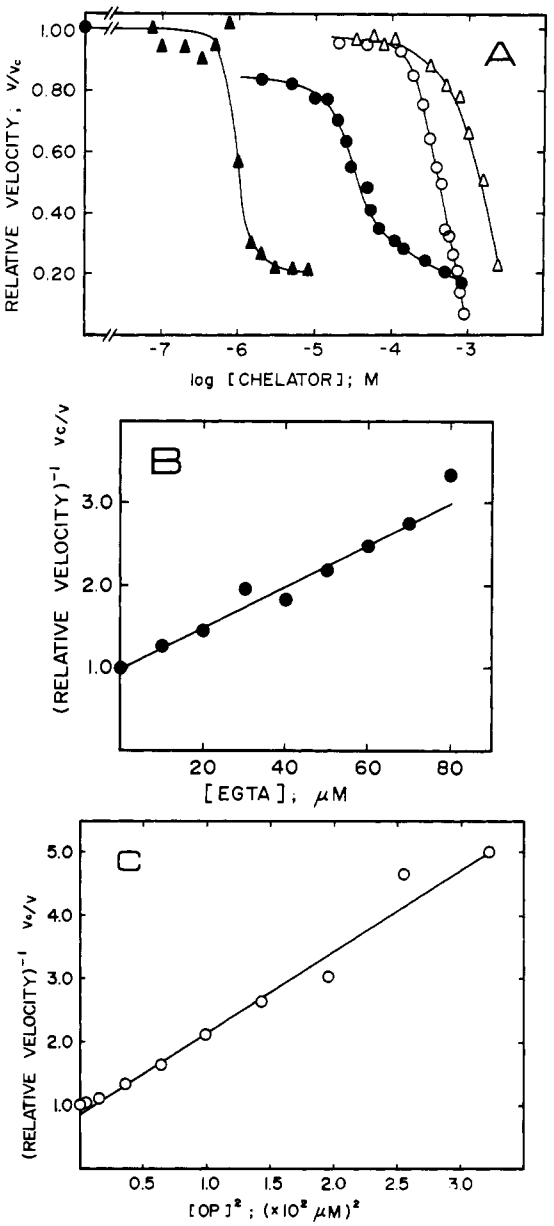

FIGURE 3: Inhibition of aminopeptidase by chelators. In A, relative activity (v/v_c) is expressed as a function of log inhibitor concentration: EDTA (▲—▲), EGTA (●—●), OP (○—○), and 8-hydroxyquinoline (△—△). In B, the data in A for EGTA were plotted as $1/v$ *vs.* [EGTA]. In C, the data in A for OP were plotted as $1/v$ *vs.* $[OP]^2$. β-Naphthylamine liberated was detected by diazotization.

A description of the inhibition by several chelators is shown in Figure 3A as a function of changing concentrations. In order to gain information concerning the stoichiometry of the interaction of chelators with the enzyme, the data in Figure 3A were replotted in Figure 3B and C as $1/v$ *vs.* [I] or $1/v$ *vs.* $[I]^2$. Linear plots were obtained for EGTA when plotted as $[EGTA]^1$ and for 1,10-phenanthroline when plotted as $[OP]^2$, suggesting that one molecule of EGTA was bound to the enzyme while two molecules of 1,10-phenanthroline were bound.

The kinetics of the inhibition by 1,10-phenanthroline are shown in Figure 4. The data, plotted as v *vs.* $v/[S]$, are of the competitive type. K_i was calculated to be 0.028 mM. Similar data shown in Figure 5 indicate competitive inhibition also for EGTA. Its K_i was 0.012 mM. Sodium sulfide showed mixed ki-

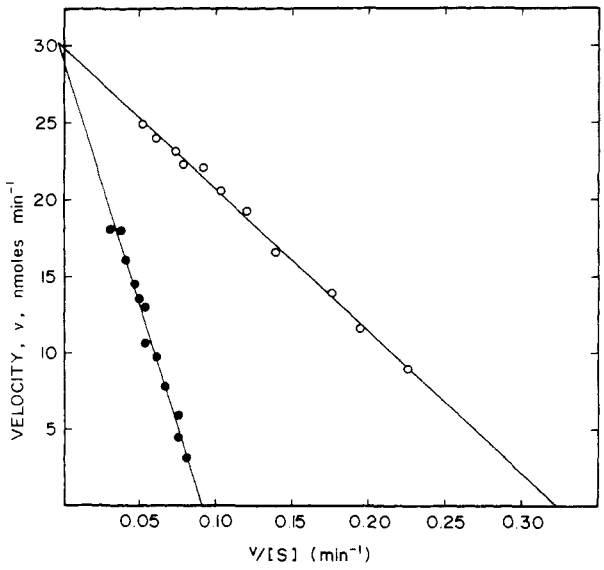

FIGURE 4: Kinetics of 1,10-phenanthroline inhibition. The inhibition by chelator was determined as a function of changing substrate concentrations (mM) by measuring the rate of formation of β-naphthylamine at 340 nm. Reaction mixtures contained 1,10-phenanthroline, 0.072 mM (●—●), or no chelator (O--O).

netics (Figure 6), that is, features of both competitive and noncompetitive patterns. The K_i for sulfide was 0.076 mM. Sodium cyanide (date not shown) inhibited with kinetics similar to sodium sulfide.

Reversal of Chelator Inhibition by Dilution. Inhibition by all chelators and complexing agents tested was reversed by dilution of the chelator to a concentration at which it was not toxic, as described in Table II. In experiment 1, EDTA was added to a concentration of 30 μM to enzyme and buffer in a volume of 100 μl. After an incubation at 50° for 10 min, the volume was increased to 1.0 ml with buffer, substrate and, where indicated, EDTA to maintain the same concentration as in the 0.1-ml incubation. When EDTA was maintained at 30 μM, there remained only 7% of the activity of the control. When the EDTA concentration was lowered from 30 to 3.0 μM by dilution, the activity was restored to 94% of the control. Similar data were obtained for all other agents tested.

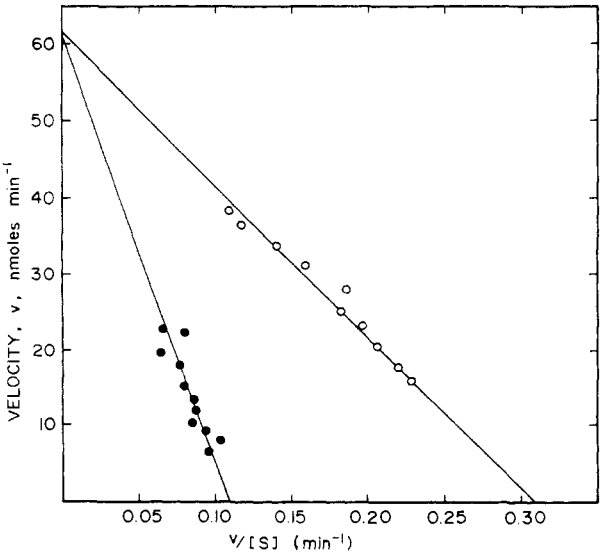

FIGURE 5: Kinetics of EGTA inhibition of aminopeptidase. The inhibition as a function of changing substrate concentrations (mM) and measured by diazotization. The reaction was performed at 50° for 20 min. Reaction mixtures containing EGTA (0.033 mM) are shown with (● —●); those without with (O—O).

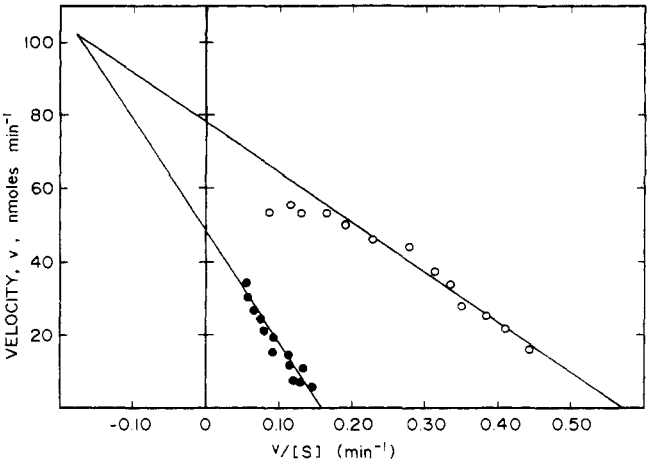

FIGURE 6: Kinetics of inhibition of aminopeptidase by sodium sulfide. Activity in the presence of 0.20 mM sodium sulfide (●—●) and in its absence (O—O) was determined by measuring the increase in absorption at 340 nm.

Prevention and Reversal of Chelator Inhibition by Metal Ions. When the addition of a fivefold excess of divalent transition metal ion preceded the addition of either 1,10-phenanthroline or EGTA, the expected inhibition by the chelator was prevented, as shown in Table III. The addition of Ca^{2+} or Mg^{2+} did not prevent inhibition by the subsequent addition of chelator, since calcium and magnesium form comparatively weak complexes with these chelators. When the order of addition was reversed, the addition of a fivefold excess of transition metal ion restored the activity to the level attained with metal ion alone. Calcium and magnesium ions did not restore activity.

Irreversible Inactivation by Heat in the Presence of EDTA. Incubation of aminopeptidase with EDTA or 1,10-phenanthro-

TABLE II: Reversal of Chelator Inhibition by Dilution.[a]

Agent Added	Concn of Agent in 1st Incubation (mM)	Concn of Agent in 2nd Incubation (mM)	Rel Rate of Hydrolysis (v/v_o)
Expt 1			
None			(1.00)
EDTA	0.03	0.03	0.07
EDTA	0.03	0.003	0.94
EDTA	0.003	0.003	1.04
Expt 2			
None			(1.00)
o-Phenanthroline	0.4	0.4	0.31
o-Phenanthroline	0.4	0.04	0.95
o-Phenanthroline	0.04	0.04	0.91
Expt 3			
None			(1.00)
Na_2S	1.0	1.0	0.04
Na_2S	1.0	0.1	0.87
Na_2S	0.1	0.1	0.91

[a] The first incubation was in a volume of 0.1 ml and was at 50° for 20 min. Each reaction mixture was cooled and increased in volume to 1.0 ml with buffer, substrate, and, where indicated, chelator. After an incubation at 37° for 20 min, the amount of β-naphthylamine formed was measured by diazotization.

TABLE III: Prevention and Reversal of Chelator Inhibition by Metal Ion.[a]

Metal Ion Added	Rel Activity with Metal Ion Alone	Prevention of Chelator Inhibition by Metal Ion: Metal Ion Followed by OP, Rel Activity	Prevention of Chelator Inhibition by Metal Ion: Metal Ion Followed by OP, Inhibition by OP (%)	Prevention of Chelator Inhibition by Metal Ion: Metal Ion Followed by EGTA, Rel Activity	Prevention of Chelator Inhibition by Metal Ion: Metal Ion Followed by EGTA, Inhibition by EGTA (%)	Reversal of Chelator Inhibition by Metal Ion: OP Followed by Metal Ion, Rel Activity	Reversal of Chelator Inhibition by Metal Ion: OP Followed by Metal Ion, Reversal by Metal Ion (%)	Reversal of Chelator Inhibition by Metal Ion: EGTA Followed by Metal Ion, Rel Activity	Reversal of Chelator Inhibition by Metal Ion: EGTA Followed by Metal Ion, Reversal by Metal Ion (%)
None	1.00	0.12	88	0.03	97	0.12	12	0.03	3
Co	1.85	1.92	−4	1.81	2	2.05	110	1.96	106
Cu	0.63	0.62	2	0.64	−2	0.60	95	0.68	108
Ni	0.53	0.54	−2	0.52	2	0.53	100	0.43	81
Zn	0.23	0.28	−22	0.22	4	0.25	109	0.21	91
Mg	0.96	0.09	91	0.02	98	0.12	12	0.01	1
Ca	0.96	0.09	91	0.05	95	0.12	12	0.07	7

[a] Each reaction mixture (1.0 ml) initially contained enzyme, 0.10 M imidazole-HCl (pH 7.0), and alanyl-β-naphthylamide, 1.0 mM. The appearance of β-naphthylamine was monitored at 340 nm at 37.4°. In the study of the prevention of chelator inhibition by added metal ion, metal ion (0.5 mM) was added 1 min after the initiation of the enzymatic reaction. As soon as the new rate was established (additional 1–5 min), EGTA (0.10 mM) or OP (0.10 mM) was added. Reaction rates, originally recorded as slopes of recorder traces, are reported as relative activity (v/v_c). In the study of reversal of chelator inhibition by metal ion, EGTA (0.10 mM) or OP (0.10 mM) was added after establishment of the enzymatic rate, followed after 1–5 min by the addition of metal ion (0.50 mM). The per cent inhibition by chelator is calculated from the relative activity with metal ion followed by chelator and the relative activity with metal ion alone. The per cent reversal of chelator inhibition is calculated from the relative activity in the presence of chelator followed by metal ion and the relative activity in the presence of metal ion alone. Additions of chelators and metal ions were in 10 μl.

line at temperatures of up to 57° resulted in inactivation which could be reversed by dialysis, gel filtration, dilution, and addition of transition metal ion. However, when the temperature of the incubation was increased to 60° in presence of the chelator, the inhibition became irreversible. In the experiment shown in Figure 7, enzyme was heated for the indicated time with or without EDTA and then assayed in the presence of an excess of Co^{2+}. Under these conditions, permanent inactivation was observed to proceed at rapid rate only in the presence of EDTA. Irreversible inactiation was also observed by incubation at 37° with increasing amounts of guanidinum chloride, increasing amounts of urea, or by raising or lowering the pH; however, the rate of inactivation under each of these conditions was the same in the presence as in the absence of EDTA.

Zinc Content of Aminopeptidase. The zinc content of several preparations of the enzyme was measured by atomic absorption spectroscopy. The best estimate of the zinc content of samples tested was 8.3 ± 1.5 nmol of zinc/mg of protein, shown in Table IV. Heating the enzyme at 60° for 20 min without chelator lowered the zinc content only by 19%. However, heating the enzyme at 60° for 20 min with EDTA lowered the zinc content by 83%. The specific activity of the enzyme dropped by similar amounts. Zinc analysis on a preparation which had been activated by 0.1 mM Co^{2+} and dialyzed against two changes of dilute buffer for 2 days showed the presence of 7.0 nmol of zinc/mg of protein. One preparation of enzyme having a low specific activity due to long storage at −20° also showed a reduced zinc content. Cobalt and iron analyses of one preparation of enzyme gave less than 2 and less than 1 nmol of metal ion/mg of protein, respectively.

Discussion

Human liver aminopeptidase complexes reversibly with Co^{2+} to form a cobalt-enzyme complex with a dissociation constant of 50 μM. The complex is 2.4-fold more active than the enzyme in the absence of Co^{2+}. A 30% increase in activity of human liver aminopeptidase with 0.2 mM Co^{2+} was observed by Smith *et al.* (1965). Based on the kinetics of Co^{2+}-activated hydrolysis, it is possible that Co^{2+} combines only with the enzyme–substrate complex, resulting in an enzyme–

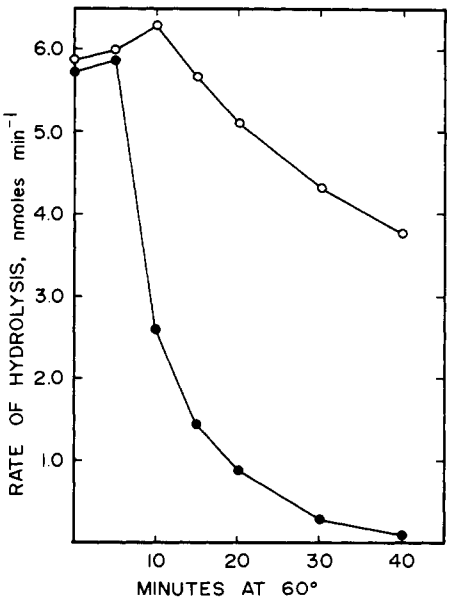

FIGURE 7: Irreversibility of inhibition of aminopeptidase by EDTA at 60°. Enzyme was heated at 60° without substrate in 0.05 M potassium phosphate (pH 6.86) in the presence (●—●) and absence (○—○) of 0.10 mM EDTA. Aliquots (0.90 ml) were withdrawn at the indicated time and placed on ice. At the completion of the 60° treatment, substrate (1.0 mM) and Co^{2+} (0.50 mM) were added, bringing the volume to 1.0 ml. The amount of β-naphthylamine formed during the subsequent incubation at 37° for 20 min was determined by diazotization.

TABLE IV: Zinc Content of Aminopeptidase.[a]

Expt	Treatment	Zinc content (nmol/mg of protein)
1	none	8.3 ± 1.5
2	60°	6.7
3	60° + EDTA	1.4

[a] Zinc analyses in expt 1 were performed on seven samples from two different preparations of enzyme. Samples were prepared for analysis either by extensive dialysis or by passage through Dowex chelating resin in distilled water. In expt 2 and 3, aminopeptidase (2 mg) was heated with or without EDTA, as described in Figure 6, for 20 min in a volume of 2.0 ml. The samples were then dialyzed overnight against 0.10 mM EDTA and 2 days against several changes of distilled water. Zinc analyses were performed without further treatment.

substrate-Co^{2+} complex which decomposes into products faster than the enzyme-substrate complex alone. Such a mechanism accounts for the observed increase in both K_m and V_{max} although other mechanisms are possible.

The involvement of free sulfhydryl groups in overall catalysis or in the binding of metal ion seems unlikely since treatment of the enzyme with several sulfhydryl-modifying reagents had no observable effects. This is in agreement with the observation of Starnes and Behal (1974) that cysteine is present in this aminopeptidase in very small amounts.

Experiments by Behal *et al.* (1966) and Smith *et al.* (1965) suggested the presence of metal ion based on the inhibition of aminopeptidase by EDTA and subsequent reversal by metal ions, notably Co^{2+}. Atomic absorption analysis of the enzyme for metal content revealed the presence of 8.3 ± 1.5 nmol of zinc/mg of protein. This value gives a weight of 122,000 ± 20,000 g of protein/mol of zinc, which is comparable to the monomeric molecular weight of 118,000 obtained by Starnes and Behal (1974). Since much care was taken to remove all loosely bound metal ion, it is possible that the value for zinc content is low due to the inadvertent inactivation of the enzyme during preparation for analysis. Destruction of activity through excessive aging or heating was observed to lower the zinc content.

The activation of aminopeptidase by Co^{2+} is thought to be through the formation of a metal-enzyme complex rather than through displacement of the zinc ion since zinc analysis on the Co^{2+}-activated enzyme showed 7.0 nmol of Zn/mg of protein. Aminopeptidase then would have metal binding sites for both zinc and cobalt ions, zinc being bound very tightly and cobalt very loosely. Because of the large dissociation constant for cobalt (50 μM) it seems unlikely that the enzyme-cobalt complex could ever be isolated.

Aminopeptidase is inhibited by a variety of chelators and complexing agents. This fact alone provides strong evidence for the involvement of a second metal ion, more tightly bound than Co^{2+}. Kinetic studies with two of the chelators, EGTA and 1,10-phenanthroline, demonstrate competitive inhibition. Likewise, inhibitions by sulfide and cyanide were of the mixed type, *i.e.*, competitive inhibition with an accompanying decrease in V_{max}. These data suggest that these four agents bind at or near the substrate binding site. Vallee and coworkers (Hoch and Vallee, 1956 and Hoch *et al.* 1958) found 1,10-phenanthroline to inhibit yeast alcohol dehydrogenase competitively with the cofactors NAD^+ and NADH while inhibiting noncompetitively with the substrates ethanol and acetaldehyde. They concluded (Williams *et al.*, 1958) that the zinc ion in yeast alcohol dehydrogenase was buried at or near the cofactor binding site.

Inhibition by EDTA and 1,10-phenanthroline is a function of the chelation property of the agents. Metal ions known to complex strongly with these agents not only prevent the expression of inhibition but also reverse the inhibition once it is expressed. In contrast, metal ions which only form weak complexes with these agents were ineffective in preventing or reversing inhibition.

The function of the zinc in aminopeptidase is not understood. An involvement in catalysis or in the binding of substrate are appealing possibilities; however, a requirement of zinc only for the maintenance of tertiary structure is possible.

The zinc ion is bound with sufficient tightness, that is, is removed only by relatively drastic treatment, such as heat treatment at 60° in the presence of EDTA followed by dialysis against EDTA. Under normal conditions, the inhibition by various chelators was freely reversed by dilution of the agent to an ineffective level. Such a reversal would not be expected if the metal ion had been removed by the agent.

It is possible that while only one molecule of EGTA was bound per enzyme molecule, two molecules of 1,10-phenanthroline were bound. However, it is not understood whether this represents the presence of two zinc ions, whether there are two molecules of 1,10-phenanthroline bound to one zinc ion, or whether one of the 1,10-phenanthroline molecules is bound to the zinc while the other is bound at some other site near the substrate binding site. Similarly, yeast alcohol dehydrogenase was shown (Kägi and Vallee, 1960) to bind one 1,10-phenanthroline molecule rapidly and reversibly while a second one binds more slowly and irreversibly. The binding of the second molecule to the yeast enzyme also results in a dissociation of the protein into four subunits.

Zinc metalloenzymes fall into two classes with respect to stability of the zinc-protein complexes. Many enzymes, including hog kidney aminopeptidase (Lehky, 1973), will lose zinc to chelators upon dialysis only to have activity restored by the addition of zinc or other metal ions (Vallee and Wacker, 1970). Others, apparently including human liver aminopeptidase, lose their metal ion irreversibly and frequently under harsh conditions. The activity of amino peptidase which had been inactivated at 60° in the presence of EDTA was not restored by the incubation of the enzyme at 37° with 0.33 mM Zn^{2+}, Co^{2+}, or Mn^{2+} for periods of up to 1 week.

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Human Liver Rhodanese. Nonlinear Kinetic Behavior in a Double Displacement Mechanism†

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ABSTRACT: Rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) has been purified from human liver tissue to apparent monodispersity. Comparison of this enzyme with that isolated from bovine liver showed differences in specific activity, ultraviolet absorption, and kinetic behavior which indicate that the two proteins are not identical. The substrate activation behavior shown by the human but not by the bovine rhodanese with cyanide as sulfur-acceptor substrate has been analyzed in terms of the formation of an isomerizing substituted enzyme.

Many (Wong and Hanes, 1962; Fisher and Hoagland, 1968) but not all (Cleland, 1970) branched single displacement formal mechanisms[1] yield nonlinear double reciprocal plots. In contrast, the only nonlinearity usual in plots for double displacement formal mechanisms has been that resulting from the competitive substrate inhibition characteristic of this form. The rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1) of human liver, however, is a double displacement enzyme that shows substrate activation behavior with cyanide as sulfur-acceptor substrate. Purification of the human enzyme now has provided an opportunity to compare it with the better known bovine rhodanese and to examine the causes of its nonlinear kinetic behavior.

Experimental Procedures

Materials. Normal human liver tissue was obtained from the morgue of Billings Hospital at the University of Chicago and then cubed and frozen. Sodium ethanethiosulfonate was synthesized as described previously (Westley and Heyse, 1971); calcium phosphate for chromatography was prepared by the method of Anacker and Stoy (1958). Sephadex G-100 and DEAE-Sephadex A-50 were purchased from Pharmacia. Distilled water was deionized before use. All other materials used were of the best analytical grades commercially available.

Enzyme Assay. Rhodanese activity was measured as the rate of thiocyanate production in the colorimetric system of Wang and Volini (1968). The units of activity used in this report are defined as micromoles of thiocyanate produced per minute in this assay system at 25°.

Protein Determinations. Biuret methods for macro (Gornall *et al.,* 1949) and micro (Zamenhof, 1957) scale work and absorbance measurements at 280 nm were all used in estimating protein concentrations, as indicated in the Results section.

Purification of Human Liver Rhodanese. Rhodanese was prepared from extracts of human liver tissue by fractionation with ammonium sulfate followed by chromatography on successive columns of DEAE-Sephadex A-50, Sephadex G-100, and calcium phosphate. Table I is a summary of the data from a purification by this procedure. For additional information describing this experiment see the Supplementary Material Available paragraph at the end of the paper.

Steady-State Kinetics. Kinetic assays were carried out at 0° in Tris-acetate buffer (pH 8.5) containing glycine at 1.4 M, ionic strength 0.5, as described previously (Westley and Heyse, 1971). Initial rate data were plotted in double reciprocal form and also processed with a Hewlett-Packard 2000 C digital computer using a BASIC program[2] for least-squares fitting to a hyperbolic function assuming equal variance for the velocities. The same computer was used to generate theoretical curves corresponding to rate equations for postulated mechanisms.

Results

Characterization of Purified Human Liver Rhodanese. All of the protein in final preparations migrated as a single band on polyacrylamide disc gel electrophoresis at pH 8.3. The ultraviolet absorbance at 280 nm of solutions containing 1 mg of protein (biuret method) per ml in cells with a 1.00-cm light path was 1.5 ± 0.1. In contrast, bovine liver rhodanese had a

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[1] Also known as "sequential" mechanisms; double displacement mechanisms are sometimes referred to as "ping-pong."

[2] Written in this laboratory by Dr. S. R. Burstein.