The Effect of Mn^{2+} and Co^{2+} on the Activities of a Zinc Metallodipeptidase from a Mouse Ascites Tumor†

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ABSTRACT: Kinetic studies of the effect of addition of Co^{2+} or Mn^{2+} to a highly purified dipeptidase from Ehrlich–Lettré mouse ascites tumor cells show that these metals specifically activate the hydrolyses of certain classes of dipeptides. This enzyme was previously (S. Hayman and E. K. Patterson, 1971, *J. Biol. Chem. 246,* 660) reported to be a Zn-metalloenzyme. It is now shown that Zn is the only metal that can partially restore the activity of the EDTA-inhibited dipeptidase in cleaving Ala-Gly. Addition of Co^{2+} increases the V_{max} of N-terminal Gly-dipeptides with increase in K_m while addition of Mn^{2+} primarily activates the hydrolysis of Pro-Gly, again with increases in both V_{max} and K_m. Prior incubation (5 min, 30°) of the dipeptidase with the appropriate metal ions causes decrease in initial lag time in the Co^{2+}-activated hydrolysis of Gly-Gly and the Mn^{2+}-activated hydrolysis of Pro-Gly. Long-term (6–19 hr, 0°) incubation of the enzyme with Co^{2+} results in loss of activity toward Ala-Gly with a concomitant 13-fold increase in the rate of Gly-Gly hydrolysis and loss of 70% of the Zn^{2+} from the dipeptidase; these effects can be partially reversed by addition of Zn^{2+}. In contrast, long-term incubation of the enzyme with Mn^{2+} results in no loss of Zn^{2+} and a twofold increase in activity toward Pro-Gly. One affinity constant of 1.4 μM for Co^{2+} and two constants of 0.23 and 27 μM for Mn^{2+} were determined by kinetic experiments. Comparison of the properties of this tumor enzyme with a dipeptidase purified in our laboratory from *Escherichia coli* B, and with mammalian dipeptidases highly purified by others, shows remarkable similarities in molecular weights and molecular activities toward the preferred substrates but in the case of bacterial dipeptidase, differences in substrate specificities and in the effect of metal ions.

It has long been known that the enzymatic hydrolysis of many dipeptides is activated by added Co^{2+} or Mn^{2+} (as reviewed by Smith, 1951, 1960). In more recent times, dipeptidases have been found to be Zn-metalloenzymes (Campbell et al., 1966; Hayman and Patterson, 1971; Hayman et al., 1974). The study of activation of purified dipeptidases by added Mn^{2+} and Co^{2+}, however, has not been generally pursued. We (Hayman et al., 1974) reported data on a highly purified bacterial dipeptidase from *Escherichia coli* B that showed that (1) addition of Co^{2+} or Mn^{2+} changed the substrate specificity of the enzyme; and (2) incubation of the enzyme with Co^{2+} decreased the Zn^{2+} content of the dipeptidase. Kinetic data suggested the existence of two binding sites on the enzyme for Co^{2+} and two for Mn^{2+}; one may represent the Zn site, the other appears to be distinct for the other two metal ions. A similar study of the effect of Mn^{2+} and Co^{2+} added to a dipeptidase purified from Ehrlich–Lettré ascites tumor cells (Hayman and Patterson, 1971) is given in this paper. We have previously (Hayman and Patterson, 1971) reported that this tumor dipeptidase contains 1 g-atom of Zn/mol of enzyme of molecular weight (Sephadex gel filtration) 85,000 ± 5000.

Materials and Methods

The dipeptides were purchased from Schwarz/Mann, Sigma, and Cyclo; Sephadex was from Pharmacia; fluorescamine was from Hoffmann-La Roche; EDTA and metal chlorides were Baker analytical grade. Metal solutions used for standards in atomic absorption spectrophotometry were certified for this purpose by Fisher.

Usual precautions to avoid metal ion contamination were followed. In particular, Sephadex G-25 was routinely suspended in 1 mM EDTA and then exhaustively washed with 0.02 M K_2PO_4 buffer (pH 8.2). Operations were carried out in a dust-proof room with a clean air hood running continuously. Glass-distilled water (greater than 10^6 ohms resistance) was used for all solutions.

Methods of preparation of the enzyme have been reported (Hayman and Patterson, 1971; Hayman et al., 1974). In brief, the assay method used was a modification of the spectrophotometric method of Schmitt and Siebert (1961). This method permitted recording of the course of the reaction (30°). Velocities of hydrolysis, v ($-\Delta A_{230}$ min^{-1}), were measured from a straight line drawn through the linear portion of the recording. Times of lag are the times until the observed rates become linear. Maximum velocities from Lineweaver–Burk plots were calculated relative to that of Ala-Gly (12.5 mM) hydrolysis run as a standard of enzyme activity in all experiments. The velocities were converted to molecular activities [$V_M/(E)$, mol of substrate hydrolyzed at 40° sec^{-1} mol of $enzyme^{-1}$] by use of a previously determined (Hayman and Patterson, 1971; Patterson et al., 1973) molecular weight (85,000 ± 5000 by Sephadex G-150 gel filtration) and specific activity (40°).

Protein greater than 0.15 mg/ml was assayed by a modification (Hayman and Patterson, 1971) of the method of Nayyar and Glick (1954) and from 0.01 to 0.15 mg/ml by the fluorescamine method of Bohlen et al. (1973). For details, see Hayman et al. (1974) and Patterson (1975).

Atomic absorption with a carbon rod atomizer (Model 61, Varian-Techtron) was used for determination of the Zn

† From the Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111. *Received May 15, 1975.* Supported by U.S. Public Health Service Grants CA-03102, CA-06927, and RR-05539, and an appropriation from the Commonwealth of Pennsylvania.

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Table I: The Effect of Added Mn^{2+} or Co^{2+} on the Hydrolysis of Dipeptides by the Tumor Dipeptidase.[a]

Metal Addition	$V_M/[E]$ (sec^{-1}) ($\times 10^{-3}$)			K_m (mM)		
	0	Mn^{2+}	Co^{2+}	0	Mn^{2+}	Co^{2+}
Gly-Ile	6.5	6.5	22	5.7	4.1	5.0
Gly-Ala	3.5	5.4	13	22	22	42
CH_3Gly-Ala	(0.005)	(0.016)	0.87	(2.5)	(2.2)	7.9
Gly-Asn	3.2	2.7	3.0	16	15	15
Gly-Asp	0.015	0.014	0.29	7.1	2.4	83
Gly-Ser	0.94	1.9	1.8	12	19	16
Gly-Gly	0.30	0.64	1.5	24	40	30
Gly-Lys	0.48	0.81	0.90	5.6	12	10
Pro-Gly	0.11	1.1	0.40	4.1	21	3.7
Ala-Ile	22	21	20	1.8	1.0	1.8
Ala-Gly	5.0	4.6	4.3	2.4	2.2	2.2
Ser-Gly	1.4	1.3	1.0	6.1	6.0	6.0
Phe-Ala	0.82	0.90	0.91	0.64	0.87	0.74

[a] K_m and V_M were determined from Lineweaver–Burk plots. The V_M data were converted to molecular activities as described under the Materials and Methods section. To 150 μl of substrate solution in phosphate–borate NaOH buffers (pH 8.3) containing 0.25 M sucrose was added 1 μl of H_2O, $MnCl_2$, or $CoCl_2$ sufficient to give 80 and 100 μM metal, respectively, in the final reaction mixture. After 5 min of warming in a cuvet (2 × 10 mm), 5 μl of enzyme solution (in 0.02 M KPO_4–0.25 M sucrose (pH 8.2)) was added. After 15 sec of rapid mixing with the micropipet, the reaction was recorded at 230 nm, using a Gilford spectrophotometer. The peptides consisted of L-amino acid residues. Except for the values in parentheses the errors of the determinations were $\leq \pm 10\%$. The errors for CH_3Gly-Ala were $\pm 100\%$ and those for CH_3Gly-Ala-Mn^{2+} were $\pm 11\%$ (K_m) and $\pm 14\%$ (V_M).

content of the enzyme preparations (Hayman et al., 1974). Checks of samples by standard flame absorption showed excellent agreement with the carbon rod results. The 100-fold less sensitivity for Co than for Zn precluded determination of Co by the carbon rod method.

Results

The Effect of Addition of Co^{2+} or Mn^{2+} to Reaction Mixtures. Mn^{2+} and Co^{2+} were chosen for detailed study of their effect on the dipeptidase because we had shown previously (Hayman and Patterson, 1971) that at millimolar concentrations, Co^{2+} activated the hydrolyses of Gly-Ser, Gly-Gly, and Gly-Thr and that Mn^{2+} activated hydrolyses of Pro-Gly, Hyp-Gly, and Gly-Gly. The hydrolysis of Ala-Gly was inhibited to varying degrees by 12 metals tested at concentrations of 10^{-7}–10^{-2} M. Since these experiments were carried out at only one substrate concentration, 50 mM, which is often inhibitory to the enzymatic reaction, it seemed desirable to determine the effect of the metals at different substrate concentrations.

As seen from Table I, dipeptides containing N-terminal Gly are the only substrates whose hydrolyses are appreciably activated by Co^{2+} (100 μM) while Mn^{2+} (80 μM) enhances the V_{max} of some of these dipeptides but is an effective activator of only Pro-Gly hydrolysis. These metal concentrations were found to be optimal for activation. In general, with neutral dipeptides the activation by Co^{2+} is greater, the smaller the C-terminal R group. The effect of charge is seen in the comparison of the hydrolyses of Gly-Asn and Gly-Asp, where the cleavage of the former dipeptide is not influenced by Co^{2+} addition whereas the slow rate of hydrolysis of the latter is increased almost 20-fold. When a hydrogen of the amino group of the dipeptide is replaced by a methyl group (CH_3Gly-Ala), the barely measurable hydrolysis is activated as much as 100-fold by Co^{2+} addition. In contrast to findings with the bacterial dipeptidase (Hayman et al., 1974), no splitting of D-Leu-Gly in the presence of Co^{2+} was observed with the tumor enzyme. In the cleavage of Pro-Gly, Co^{2+} increases the rate of hydrolysis threefold and Mn^{2+} tenfold. A small effect of Mn^{2+} is seen in the increase in rates of cleavage of Gly-Gly, Gly-Ser, Gly-Lys, and CH_3Gly-Ala (about twofold activation). The rate increases caused by Co^{2+} or Mn^{2+} addition are accompanied in most cases by an increase in K_m. These increases vary from less than twofold to greater than tenfold (Gly-Asp, Co^{2+}). Examples of dipeptides (other than Pro-Gly) containing N-terminal groups other than Gly are given at the bottom of Table I. Their V_{max} and K_m values are not affected significantly by addition of either Co^{2+} or Mn^{2+}.

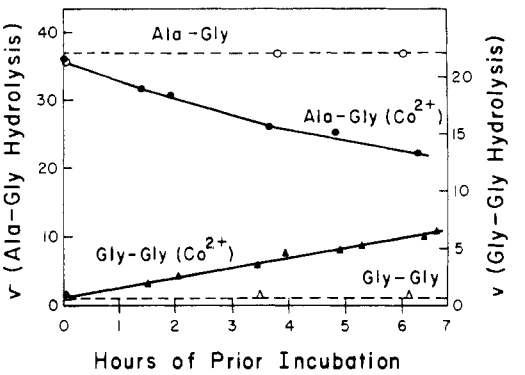

FIGURE 1: The effect of prior incubation (0°) of the dipeptidase with Co^{2+} (10 μM) on the hydrolysis of 12.5 mM Ala-Gly and Gly-Gly. At the indicated times aliquots of dipeptidase solutions (6 μM) in 0.05 M phosphate–borate buffer (pH 8.3) containing 0.25 M sucrose with (●, ▲) or without Co^{2+} (○, △) were added to prewarmed substrate (Ala-Gly, ○; Gly-Gly, △) and the reaction was followed by recording at 230 nm in a Gilford spectrophotometer. The units of velocity are $v = -\Delta A_{230}\ min^{-1} \times 300$.

Since Mn^{2+} abolished inhibition by high substrate concentrations in hydrolysis of Ala-Gly by the bacterial dipeptidase (Hayman et al., 1974), this effect was investigated with the tumor dipeptidase. It was found that at inhibitory substrate concentrations (20–50 mM), addition of either Co^{2+} or Mn^{2+} increases substrate inhibition in the hydrolyses of Ala-Gly or Ala-Ile whereas substrate inhibition by Ser-Gly is decreased by addition of these metal ions.

Effect of Prior Incubation of the Dipeptidase with Co^{2+} or Mn^{2+}. Initial lag phases were observed during the activation of the hydrolyses of Gly-Gly by Co^{2+} and Pro-Gly by Mn^{2+}. Such lags also occurred under inhibitory conditions as in the case of excess substrate or the presence of other enzyme inhibitors. Therefore, the effect of prior incubation of the dipeptidase with Co^{2+} or Mn^{2+} was studied with the above substrates; 5 μl of cold dipeptidase solution was added to 80 μl of phosphate–borate buffer (30°) (pH 8.3) and after 1 μl of $CoCl_2$ or $MnCl_2$ was added, the solutions were held at 30° for 5, 10, and 15 min before the reaction was started by addition of 80 μl of substrate (30°). Controls consisted of (1) prior incubation of metal and substrate before enzyme addition, and (2) prior incubation of enzyme and buffer before addition of metal solution and warmed substrate. In all cases the final concentrations of enzyme, substrate (12.5 mM), and Co^{2+} (93 μM) or Mn^{2+} (49 μM) were constant. Velocities and lag times were measured from the recordings. Essentially identical effects resulted from pretreatments lasting from 5 to 15 min. In no case was an increase in velocity over the controls seen, but a definite de-

Table II: The Effect on Zn^{2+} Content and Enzymatic Activity of the Tumor Dipeptidase of Long-Term Incubation (0°) with Co^{2+} or Mn^{2+}.[a]

	Hydrolysis of (V/mol of enzyme)			Zn^{2+} (g-atoms/mol of Enzyme)
	Ala-Gly	Gly-Gly	Pro-Gly	
(1) Control	10.4	0.10		1.0
(2) G-25-Co^{2+}	1.02 ± 0.06	1.2 ± 0.2		0.29 ± 0.03
(1) Control	5.1		0.11	1.0
(2) G-25-Mn^{2+}	3.2 ± 0.2		0.17 ± 0.02	1.1 ± 0.2

[a] To 500 μl of enzyme solution in 0.02 M KPO_4–0.25 M sucrose (pH 8.3), 1 μl of 50 mM $CoCl_2$ or $MnCl_2$ was added to give 100 μM Co^{2+} or Mn^{2+} in the enzyme solution. After 19 hr at 0°, the solution was filtered through a Sephadex G-25 column (0.9 × 11.5 cm) equilibrated with 0.02 M KPO_4 (pH 8.3) and saturated with the given metal ions and the protein-containing fractions were collected. The velocity of hydrolysis of Ala-Gly (12.5 mM) and Gly-Gly (12.5 mM), in the case of Co^{2+} treatment, and that of Ala-Gly and Pro-Gly (10 mM), in the case of Mn^{2+} treatment, was measured (1) before addition of metal ions (control) and (2) after Sephadex G-25 filtration. Velocities are expressed as the decrease in absorbancy at Δ_{230nm} per min times the scale expansion, 300. Protein in the control was assayed by the bromosulfalein method and that in the fractions by the fluorescamine method. Zn was determined by carbon rod atomic absorption. The means and average deviations of four peak fractions are listed for the G-25 values.

Table III: Partial Recovery of Native Enzyme by Treatment of Co^{2+}-Incubated Dipeptidase with Zn^{2+}.[a]

	Hydrolysis of			
	Ala-Gly		Gly-Gly	
Hr	V/mol of Enzyme	Lag (min)	V/mol of Enzyme	Lag (min)
0	0.21	15	0.55	1.4
3.5	0.81	4.0	0.073	4.4
Control	10.4	1.2	0.10	11

[a] After Sephadex G-25 filtration of the dipeptidase exposed to $CoCl_2$ (100 μM) for 19 hr (0°) (Table II), a fraction containing 0.27 μM Zn^{2+}, 100 μM Co^{2+}, and 1.1 μM dipeptidase was made 11.0 μM in Zn^{2+} and the activity measured toward Ala-Gly (12.5 mM) and Gly-Gly (12.5 mM) at intervals of incubation (0°) after Zn^{2+} addition. The lags at the start of the reactions were also estimated.

crease in lag time (13.1 ± 1.4–8 ± 1 min) was observed in the case of Gly-Gly hydrolysis after prior incubation of the enzyme with Co^{2+}. The decrease in lag (3.3 ± 0.6–2.0 ± 0.4 min) was less but still significant with Mn^{2+} and Pro-Gly. Prior incubation of the enzyme with Co^{2+} had no measurable effect on Pro-Gly hydrolysis by the tumor enzyme. Longer periods (6 hr) of preincubation (0 or 26°) of the dipeptidase with Mn^{2+} (250 μM) caused no change in either velocity of hydrolysis of Pro-Gly or in lag time. In addition, the Mn^{2+}-activated Pro-Gly hydrolysis was exceptional in that the decrease in lag time seen with decreasing concentrations of other substrates did not occur.

The effect of long-term (7 hr) prior incubation (0°) of the dipeptidase with Co^{2+} on the hydrolysis of Ala-Gly and Gly-Gly is shown in Figure 1. Under these conditions, the enzyme is stable as seen from the data on hydrolysis of both substrates without Co^{2+}. When the enzyme was incubated with a slight molar excess of Co^{2+}, the velocity of hydrolysis of Ala-Gly slowly decreased and that of Gly-Gly increased at a linear rate. In similar experiments with Ala-Ile or Val-Ile as substrate, the decrease in velocity of hydrolysis paralleled that of Ala-Gly. These results are similar to those of Capobianco and Vescia (1967) who studied a yeast dipeptidase with Gly-Gly and Leu-Gly as substrates.

Removal of Zn from the Dipeptidase by Incubation with Co^{2+} or Mn^{2+}. Since Mn^{2+} and Co^{2+} replace Zn^{2+} in carboxypeptidase (Coleman and Vallee, 1961), another exopeptidase, it seemed possible that this mechanism could account for the results with the tumor dipeptidase. Samples of purified dipeptidase (specific activity 2100, 0.33 mg/ml, 3.9 μM) in 0.02 M KPO_4–0.25 M sucrose (pH 8.3) were made 100 μM in either Co^{2+} or Mn^{2+}, kept at 0° for 19 hr, and filtered through Sephadex G-25 equilibrated with either 100 μM Co^{2+} or Mn^{2+} in 0.02 M K_2PO_4 buffer (pH 8.3). As seen from Table II, treatment with Co^{2+} resulted in loss of 71% of the Zn^{2+} content of the enzyme and of 90% of the activity toward Ala-Gly while the activity toward Gly-Gly increased 13-fold. The material available was insufficient for Co^{2+} determination. On the other hand, treatment of the dipeptidase with Mn^{2+} resulted in no measurable loss of Zn^{2+} but loss of 37% of the activity toward Ala-Gly while the activity toward Pro-Gly increased 50%. Control experiments showed that exposure of this dilute dipeptidase preparation to 0° for 19 hr resulted in loss of 18% of the activity toward Ala-Gly.

Partial Recovery of Native Enzyme by Treatment of the Co^{2+}-Incubated Dipeptidase with Zn^{2+}. As seen from Table III, addition of Zn^{2+} for 3.5 hr at 0° to a frozen and thawed fraction of the dipeptidase preparation which had been treated with Co^{2+} and run through Sephadex G-25 (Table II), resulted in a fourfold increase in activity toward Ala-Gly accompanied by a 7.5-fold decrease in activity toward Gly-Gly. The relatively low rates compared with the control may be due to instability of the Co^{2+}-treated (or relatively Zn free) enzyme to freezing and thawing. The durations of initial lags also reversed toward those of the native Zn-enzyme; that of Ala-Gly hydrolysis decreased whereas that of Gly-Gly hydrolysis increased. Further exposure of the preparations to Zn resulted in decreased activity toward Ala-Gly probably caused by denaturation of the dilute enzyme preparations.

Partial Reversal by Zn^{2+} of the EDTA-Inhibited Enzyme. A dipeptidase (1 μM) preparation was 60% inhibited (as measured by hydrolyses of Ala-Gly, 12.5 mM) by exposure at 0° to 10 μM EDTA for 6 hr and then made 12 μM in Zn^{2+}. Reactivation was slow and incomplete; 2 hr after Zn addition 15% of the initial activity toward Ala-Gly was recovered. In another experiment, 40% of the activity reappeared 5 hr after Zn treatment. In a similar experiment addition of $MnCl_2$ (14 μM) had no effect, while $CoCl_2$ (12 μM) or increased concentrations (27 μM) of $ZnCl_2$ caused even greater inhibition of the EDTA-treated dipeptidase. Incubation of the enzyme with 13 μM Zn^{2+} and 10 μM EDTA resulted in no inhibition of activity toward Ala-Gly whereas incubation with 13 μM Co^{2+} and 10 μM EDTA caused an initial (2 hr) pseudo-first-order loss of activity that was 1.7 times as rapid as that resulting from incubation with the same concentration of EDTA alone; however, the residual activities after 4-hr incubation were 42% for enzyme in the presence of EDTA and Co^{2+} and only 29% of the control values for enzyme treated with EDTA alone.

In order to determine whether inhibition by EDTA resulted from removal of an essential metal from the dipeptidase or from interaction of EDTA with the enzyme, dipeptidase–EDTA mixtures were passed through a Sephadex G-25 column which had been equilibrated with 0.02 M

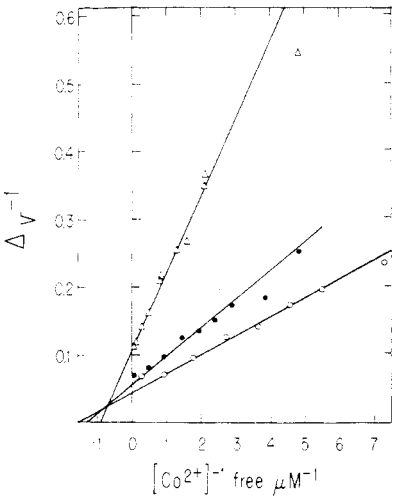

FIGURE 2: Double reciprocal plot of increase in velocity of enzymatic hydrolysis of Gly-Gly vs. concentration of free Co^{2+}. The assays were carried out as described in the legend of Table I except that the $CoCl_2$ concentration was varied from 2.5 to 100 μM. The Δv values were calculated by subtraction of the velocity at a given substrate concentration in the absence of Co^{2+} from that obtained at the stated Co^{2+} concentration. The units of v are $-\Delta A_{230}$ $min^{-1} \times 300$. Free Co^{2+} was calculated for each concentration of Gly-Gly from the stability constant (log K_1 = 3.49, Bjerrum et al., 1958) of Gly-Gly-Co^{2+} at pH 8.2. Gly-Gly: △, 12.6 mM; ●, 25.3 mM; ○, 50.6 mM. A K_A of 1.4 μM is obtained from the triple intersection of straight lines drawn through the points (Mildvan and Cohn, 1965).

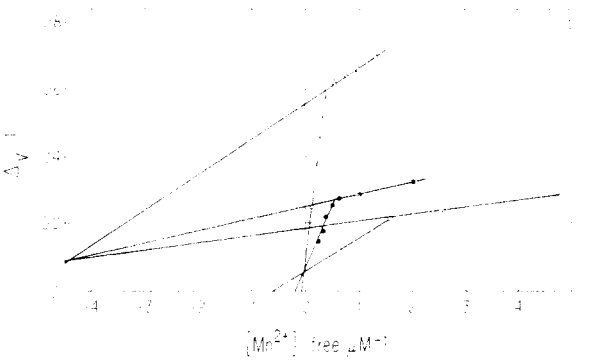

FIGURE 3: Double reciprocal plot of increase in velocity of enzymatic hydrolysis of Pro-Gly vs. concentration of free Mn^{2+}. The assays, Δv values, and calculations of free Mn^{2+} were carried out as described for Figure 2. It was assumed that for Pro-Gly-Mn^{2+} log K_1 was 2.3, an average value based on data on stability constants (Sillén and Martell, 1964) of other dipeptides with Mn^{2+}. The variation in values was small. Pro-Gly: △, 5.1 mM; ●, 10.1 mM; ○, 25.3 mM. K_A's of 0.23 and 27 μM were obtained from the triple points.

KPO_4. The enzyme (3.9 μM) was 96% inhibited by 17-hr treatment with 1.0 mM EDTA and, after passage through Sephadex G-25, 22% of the original activity toward Ala-Gly was recovered and 20% of the Zn content of the native enzyme was retained. Addition of a stoichiometric amount of Zn to the enzyme resulted in only a 10% further recovery of the original activity after 6.5 hr at 2°. This experiment confirmed that Zn was lost by treatment with EDTA and that Zn content was related to enzyme activity toward Ala-Gly.

Affinity of Co^{2+} and Mn^{2+} for the Dipeptidase. Kinetic experiments have given information on the binding of Co^{2+} and Mn^{2+} to the enzyme. As seen from Figure 2, the reciprocal plot of increase in velocity vs. free Co^{2+} concentration at levels of the substrate Gly-Gly bracketing its K_m, a K_A for Co^{2+} of 1.4 μM may be determined (Mildvan and Cohn, 1965) from the intersection of the lines. The identical K_A was found in another experiment in which 8.2, 12.6, 25.3, and 50 mM Gly-Gly were used. Figure 3 shows the same type plot for the effect of Mn^{2+} on Pro-Gly hydrolysis by the tumor dipeptidase. Here, two K_A's are obtained, one from the intersection of lines drawn through points at high Mn^{2+} concentrations (K_A = 0.23 μM), and the other (K_A = 27 μM) from the intersection of straight lines through the points at low Mn^{2+} concentrations. Therefore, the enzyme apparently has only one activating site for Co^{2+} and two activating sites for Mn^{2+} of differing affinities.

Discussion

There are three possible explanations for the effects exerted by extrinsic metal ions on the hydrolysis of substrates by this tumor dipeptidase: (1) that there is more than one dipeptidase present; (2) that the extrinsic metal replaces the native Zn; and (3) that the extrinsic metal is exerting an effect at a site on the enzyme other than the Zn site. Evidence for the presence of only one dipeptidase in the preparation has been previously published (Hayman and Patterson, 1971; Patterson et al., 1973). That Co^{2+} may replace the Zn^{2+} in the native dipeptidase is implied by the loss of Zn^{2+} on incubation with Co^{2+} (Table II). The K_A obtained from kinetic studies shows a tight binding site 1.4 μM (Figure 2), which may possibly refer to binding of Co^{2+} at the Zn^{2+} site. Incubation with Mn^{2+} (Table II), in contrast, caused no loss of Zn from the dipeptidase. On the other hand, two K_A's (Figure 3) of 0.23 and 27 μM were obtained for Mn^{2+} indicating one tight and one loose binding site. These sites on the protein may be separate from each other and from that of Zn^{2+}. It seems likely that Mn^{2+} is exerting its effect at a site or sites on the enzyme other than the Zn^{2+} site.

The mechanisms by which Co^{2+} and Mn^{2+} enhance the rates of substrate hydrolysis by this dipeptidase may be quite different. The fact that activations by both metals are generally accompanied by increases in K_m indicates that K_m is a kinetic parameter rather than a dissociation constant. Studies on model compounds (Buckingham et al., 1974) of the nonenzymatic hydrolysis of peptide bonds by Co^{3+} have given rates (10^{10} acceleration) comparable to those observed enzymatically. The reaction rates are fastest with N-terminal Gly-dipeptides, the rate decreasing as the C-terminal R group becomes larger (Oh and Storm, 1974). The rates with other N-terminal R groups are very slow. Although Co^{3+} is used for the model reaction, it is striking that this order of rates is comparable to that of activation of the enzymatic hydrolyses observed after addition of Co^{2+}, and therefore the mechanism may be similar. But in the case of Mn^{2+}, no such hydrolyses of model compounds, as far as we know, have been reported. The major activation (tenfold) by Mn^{2+} of the hydrolysis of Pro-Gly by the tumor dipeptidase is accompanied by a small decrease in lag which might be thought to indicate the induction of a small conformational change in the enzyme (Frieden, 1970). However, the lag times were similar at all substrate concentrations; this effect contradicts the rules (Bates and Frieden, 1973) for enzyme conformational change. Whatever change that occurs in the enzyme as a result of Mn^{2+} binding may enhance the postulated hydrogen bonding of the substrate N-terminal nitrogen to the enzyme (Patterson et al., 1973). Since in Pro-Gly this nitrogen is in a five-membered ring, positioning may be critical for hydrolysis of

the peptide bond. The greater activation by Mn^{2+} than Co^{2+} of Pro-Gly hydrolysis by the tumor dipeptidase may relate to an activation site on this enzyme for Mn^{2+}. That Co^{2+} activation may also act in the region of binding of the amino group is implied by the great activation of CH_3Gly-Ala hydrolysis by addition of this metal ion. Such activation was observed by E. L. Smith (1948) with crude tissue extracts and attributed to complex formation of Co^{2+} between substrate and enzyme. Since major Co^{2+}-activation of hydrolyses of substrates by this enzyme is confined to that of N-terminal Gly-dipeptides which have a hydrogen in place of the N-terminal R group, and since it has been postulated (Patterson et al., 1973) that the dipeptidase has two hydrophobic pockets to hold the N- and C-terminal R groups, the binding of these substrates would be relatively weak. Apparently, if Co^{2+} replaces the native Zn^{2+}, this may induce a conformational change that enhances binding of N-terminal Gly-dipeptides and reduces the binding of N-terminal Ala-dipeptides (Figure 1). Whether this mechanism involves chelation of Co^{2+} between amino acids in the enzyme protein or between enzyme and substrate remains to be determined. Until the dipeptidases have been studied by other methods such as X-ray crystallography and nuclear or electron magnetic resonance relaxation spectroscopy, such questions cannot be answered.

The partial reactivation of the enzyme by Zn after treatment of the dipeptidase with EDTA with or without Sephadex filtration to remove EDTA has a two part explanation: Zn is specifically required for Ala-Gly hydrolysis (Figure 1, Tables II and III), and the apoenzyme is unstable. The latter is consistent with the previous observation (Hayman and Patterson, 1971) that after prolonged dialysis of the tumor dipeptidase against *o*-phenanthroline and then KPO_4 buffer, no reactivation by Zn^{2+} was observed. The relatively facile restoration of native activity (Table III) resulting from addition of Zn^{2+} to the Co^{2+}-treated enzyme may indicate that the presence of metal is required for maintenance of active enzyme.

The more rapid loss of activity toward Ala-Gly with exposure of the dipeptidase to a slight excess of Co^{2+} over EDTA than with EDTA alone may be caused by the greater accessibility of the Zn^{2+} site to Co^{2+} than to the chelator. Since the stability constants of EDTA–Zn and EDTA–Co are similar (Bjerrum et al., 1958; log K_1 16.3 and 16.1, respectively), free Co^{2+} may replace the Zn^{2+} in the enzyme which would then combine with EDTA releasing more Co^{2+} to replace enzyme Zn^{2+}. Because Co^{2+} may have less affinity for the enzyme than Zn^{2+}, after 4-hr incubation an equilibrium may be reached in which little more Zn^{2+} is removed from the enzyme. On the other hand, in the experiment with EDTA alone, the chelator by this time may be reaching the Zn^{2+} site with greater ease and be causing more inhibition than the EDTA–Co^{2+} mixture in which less free EDTA is available for Zn^{2+} removal.

Comparison of the results of addition of Co^{2+} or Mn^{2+} to the tumor dipeptidase with those obtained (Hayman et al., 1974) with another Zn-metallodipeptidase purified in this laboratory from *E. coli* B point up the necessity of using more than one substrate for such studies. For instance, we know that for optimum Ala-Gly (12.5 mM) hydrolysis by the tumor enzyme Zn^{2+} is required, whereas with the bacterial dipeptidase Mn^{2+} or Co^{2+} can replace Zn^{2+} with no loss in activity. The situation, however, is different when some of the other dipeptides are used as substrates. Addition of Co^{2+} to the bacterial enzyme activates the hydrolysis of a wide variety of substrates so that the more narrow range of substrate specificity of this dipeptidase (Patterson et al., 1973) approaches the broad range of the tumor enzyme. Addition of Mn^{2+} to both enzymes strikingly increases the V_{max} of hydrolysis of Pro-Gly which indicates a similar action of this metal on both enzyme proteins. Two K_A's for Mn^{2+} of comparable magnitude (1.2 and 24 μM, tumor and 0.3 and 27 μM, bacterial) were found for both enzymes whereas only one association constant (1.4 μM) was found for Co^{2+} for the tumor enzyme and two for the bacterial enzyme (3 and 8 μM). The greater effect of Co^{2+} on the latter enzyme may well mean that in addition to replacement at the Zn site, Co^{2+} may act at another site on this dipeptidase. If only one substrate had been used, these actions of the metal ions could not have been even partially understood.

Comparison of the properties of dipeptidases purified in this laboratory with those of mammalian dipeptidases purified by others such as the hog kidney plasma membrane dipeptidase (Campbell et al., 1966; Campbell, 1970; Armstrong et al., 1974; Ferren et al, 1975) and dipeptidases from intestinal mucosa of the monkey (Das and Radhakrishnan, 1972) and the pig (Norén et al., 1971, 1973; Norén and Sjöström, 1974; Norén, 1974) reveals many similarities in these enzymes. Molecular weights vary from 85,000 to 107,000, and subunit weights are 53,000 (bacterial) and 51,000 (pig intestinal mucosa). The kidney membrane porcine dipeptidase has a complex subunit structure (Ferren et al., 1975); it consists of four subunits, two of 28,000, one of 20,000, and one of 18,000. This result suggests a possible structural explanation for the multiple bands seen in our preliminary unpublished results of sodium dodecyl sulfate acrylamide gel electrophoresis of the tumor dipeptidase. This latter enzyme, however, contains 1 g-atom of Zn/mol of enzyme whereas the hog kidney dipeptidase (Campbell et al., 1966), like the bacterial dipeptidase (Hayman et al., 1974), contains 2 g-atoms of Zn/mol of enzyme. Zn^{2+} has been 80% removed from the hog kidney dipeptidase (Armstrong et al., 1974) and replaced by Co^{2+} which resulted in a 41% decrease in V_{max} and no change in K_m of the substrate glycyldehydrophenylalanine. The molecular activities (mol of substrate hydrolyzed sec^{-1} mol of $enzyme^{-1}$, 40°) of the dipeptidases with their preferred substrates vary only from 1.6 to 2.3 $\times 10^4$, the latter value being obtained with the tumor enzyme with Ala-Ile as substrate (Hayman and Patterson, 1971). Comparison of substrate specificities is difficult since different sets of dipeptides have been utilized in the various laboratories. It is clear, however, that the mammalian dipeptidases hydrolyze a broad range of dipeptides with a possible preference for N-terminal Ala-dipeptides.

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Stoichiometry, ATP/2e Values, and Energy Requirements for Reactions Catalyzed by Nitrogenase from *Azotobacter vinelandii*†

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ABSTRACT: The stoichiometry of the nitrogenase ATP-dependent H_2 evolution and acetylene reduction reactions using $S_2O_4^{2-}$ as an electron source was studied by various techniques. For each mole of $S_2O_4^{2-}$ oxidized to $2SO_3^{2-}$ by the enzyme-catalyzed reactions at 25° and pH 8, 1 mol of H_2 (1 mol of ethylene for acetylene reduction) and two protons are produced. Under these conditions, 4.5 mol of ATP was hydrolyzed to ADP and inorganic phosphate for each $S_2O_4^{2-}$ oxidized. ATP/$S_2O_4^{2-}$ (ATP/2e) values determined at 5° intervals from 10 to 35° were found to go through a minimum at 20°. This effect is explained in terms of possible enzyme structure modifications. Calorimetric measurements for the enzyme-catalyzed H_2 evolution and acetylene reduction reactions gave ΔH values of −32.4 and −75.1 kcal/mol of $S_2O_4^{2-}$, respectively.

The nitrogen reducing enzyme, nitrogenase, from various biological sources is capable of reducing N_2 to ammonia when supplied with adenosine 5′-triphosphate (ATP), Mg^{2+}, and a source of low potential electrons. Other substrates (Burris, 1971; Murray and Smith, 1968; Hardy and Burns, 1968) including H^+ (H_2O) are reduced under the same conditions with apparently the same ATP requirement (Hadfield and Bulen, 1969) per reducing equivalent transferred by the enzyme. The amount of ATP required to transfer two electrons, the ATP/2e value, has been a much sought after quantity but reported results still indicate some disagreement. Most reported ATP/2e values fall in the range 4–6 at 25–30° but extreme values of from 2 to 18 have been reported (Hardy and Knight, 1966; Kelly, 1969; Jeng et al., 1970).

The function of ATP is not known but its energy content is likely to be released in some controlled way in satisfying the activation requirements of N_2. Consequently, the amount of ATP utilized for electron transfer (ATP/2e) is an essential quantity in determining the total energy necessary for enzymatic N_2 reduction. Furthermore, the ATP/2e value determined under various conditions of temperature, pH, etc., may provide insight into the purpose of the ATP requirement as well as the mechanism for its utilization by nitrogenase.

As a prelude to determining both the energetics of nitrogenase action and the energy requirements of each of the nitrogenase component proteins, it was necessary to determine precisely the ATP/2e value, the overall stoichiometry, and the thermodynamic states of each reactant and product. The results of these experiments and the energy changes accompanying them are the subject of this report.

Experimental Section

Materials. Adenosine 5′-triphosphate (ATP) and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid

† Contribution No. 543 from the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio 45387. *Received May 2, 1975.*

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