The Relationship of Extrinsic and Intrinsic Metal Ions to the Specificity of a Dipeptidase from *Escherichia coli* B†

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ABSTRACT: A dipeptidase, 90% pure, from *Escherichia coli* B is shown in this study to be a Zn-metalloenzyme containing 2.0 ± 0.1 g-atoms of Zn/mol of enzyme of two indistinguishable subunits. The action of the enzyme is rapidly inhibited by the chelator *o*-phenanthroline but not by its isomeric nonchelator, *m*-phenanthroline. Inhibition by EDTA is time dependent and the enzyme is immediately reactivated by addition of Zn^{2+} or Mn^{2+}. The addition of Mn^{2+} or Co^{2+} changes the substrate specificity of the enzyme. When substrates are rapidly hydrolyzed by the enzyme, the addition of either metal causes an inhibition of the reaction. For other substrates, the more slowly hydrolysis occurs in the absence of metals, the greater the activating effect of Co^{2+} or Mn^{2+} on the reaction. Mn^{2+} prevents and Co^{2+} increases the inhibition by high concentrations of substrate. Substrates not detectably hydrolyzed in the absence of metal, such as Ala-Ile, Asp-Gly, and D-Leu-Gly, are split at appreciable rates when Co^{2+} is present. The activation by Mn^{2+} is accompanied by an increase in K_m, while that by Co^{2+} occurs with no change in K_m. Long-term incubation of the dipeptidase with Co^{2+} results in no change in the rate of Ala-Gly hydrolysis, progressive activation of Ala-Ile splitting, and loss of 50% of Zn content of the enzyme. Reductions in lag time at the start of the hydrolytic reactions suggest that Co^{2+} induces a conformational change in the dipeptidase that facilitates the change in substrate specificity. Kinetic data suggest the existence of two binding sites on the enzyme for Co^{2+} and two binding sites for Mn^{2+}, at least one of which is distinct from the Co^{2+} sites. Presumably, the common site represents the Zn site. It is suggested that a single dipeptidase is responsible for the hydrolysis of most dipeptides including Gly-Gly and Pro-Gly, substrates that were formerly thought to be hydrolyzed by separate enzymes.

A number of exopeptidases have been found to be Zn-metalloenzymes (carboxypeptidase, Vallee and Neurath, 1954; leucine aminopeptidase, Himmelhoch, 1969; renal particulate dipeptidase, Campbell *et al.*, 1966) and we have previously (Hayman and Patterson, 1971) reported that a dipeptidase isolated from ELD mouse ascites tumor cells belongs to this class of enzymes. In the present paper, we shall present evidence that a dipeptidase purified (Patterson *et al.*, 1973) from a prokaryotic source, *E. coli* B, is also a Zn-metalloenzyme.

In addition, this paper will include data on the effect of addition of Co^{2+} or Mn^{2+} to this bacterial dipeptidase. The consequent marked changes in substrate specificity imply that these metals exert different effects on the dipeptidase conformation. These results suggest that a single dipeptidase may hydrolyze a wider variety of dipeptides than formerly assumed (as reviewed by Smith, 1951, 1960).

Materials and Methods

The dipeptides were purchased from Schwarz/Mann, Sigma, and Cyclo; Sephadex G-25 was from Pharmacia; Chelex was from Bio-Rad; fluorescamine was from Hoffmann-La Roche; 1,10-phenanthroline (*o*-phenanthroline) was from Fisher; EDTA was from Baker; 1,7-phenanthroline (*m*-phenanthroline) was a gift from Dr. Barbara Nevaldine, Syracuse University. Metal chlorides were Baker analytical grade. Metal solutions used for standards in atomic absorption spectrophotometry were certified for this purpose by Fisher.

The 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). Most of the operations were carried out in a scrupulously dust-free room in which a clean air hood was run continuously. Glass distilled water (greater than 10^6 ohms resistance) was used for all solutions. Under these circumstances, it was found that passage of solutions (*e.g.*, phosphate buffer) through Chelex made no difference in the Zn content (<0.05 μM) as determined by atomic absorption.

Methods of preparation of the enzyme to at least 90% purity (specific activity, 4000 I.U./mg) have been reported (Patterson *et al.*, 1973).

Assays of enzyme activity were carried out by a modification (Hayman and Patterson, 1971; Patterson *et al.*, 1973) of the method of Schmitt and Siebert (1961). In this assay, the loss of peptide bond absorbance (230 or 235 nm) as the dipeptides are hydrolyzed (30°) is recorded by a Gilford spectrophotometer. Velocities of hydrolysis, v, ($-\Delta A_{230 \text{ or } 235 \text{ nm}}$ min^{-1}) were measured from a straight line drawn through the linear portions of the recording. The rate of hydrolysis of a 12.5 mM Ala-Gly solution was used as a standard of enzyme activity in all experiments. Substrate solutions were adjusted to pH 8.3 with Kolthoff phosphate-borate-NaOH buffers (Britton, 1942) containing 0.25 M sucrose for maintenance of enzyme stability. Maximum velocities from Lineweaver-Burk plots were calculated relative to that with Ala-Gly and converted to molecular activities [V_M/(E), mol of substrate hydrolyzed at 40° sec^{-1} mol of enzyme^{-1}] by use of previously determined (Hayman and Patterson, 1971; Patterson *et al.*, 1973) molecular

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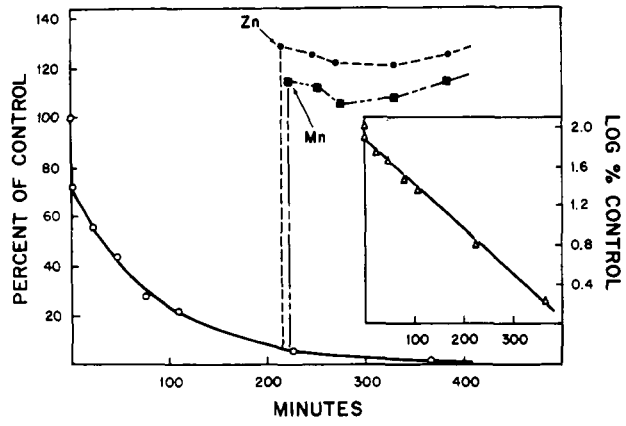

FIGURE 1: The time-dependent inactivation of the bacterial dipeptidase by EDTA and instantaneous reactivation by Zn^{2+} and Mn^{2+}. At the times indicated, the hydrolysis of Ala-Gly (12.5 mM) was assayed in controls without EDTA and solutions with EDTA. The enzyme-EDTA solution (○) was 2.5 μM in dipeptidase and 1.0 mM in EDTA, and the Zn^{2+} (●) or Mn^{2+} (■) (added at the times indicated by dashed lines) in the enzyme-EDTA mixture was 1.5 mM. The insert is a first-order plot of the loss of activity. The $t_{1/2}$ for EDTA inhibition is 70 min.

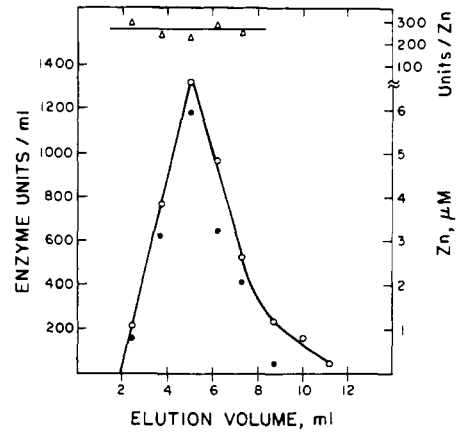

FIGURE 2: Chromatogram of the bacterial dipeptidase at the final purification step, hydroxylapatite. Enzyme-containing fractions eluted from a DEAE-cellulose column were pooled, concentrated against 0.01 M Na_2PO_4 (pH 7.5), and applied to a column of Bio-Gel HTP hydroxylapatite equilibrated with the above buffer solution. The dipeptidase came off the column with the breakthrough peak. The purification factor in the peak tubes was greater than fourfold and the enzyme was calculated to be at least 88% pure by acrylamide gel electrophoresis (Patterson *et al.*, 1973). The specific activity of the dipeptidase was 3800 at the peak. Hydrolysis of Ala-Gly (50 mM) in the presence of 3 mM $MnCl_2$ (○) is expressed in enzyme units (μmol of substrate hydrolyzed/min) per ml. Zn, μM (●), was determined by the carbon rod method of atomic absorption. Units/Zn are represented by Δ. The error in determination of the low level of Zn at the 8.7 ml of elution volume was too great to include this point in the ratios given.

weights and specific activities at 40°. Protein (>0.1 mg/ml) was assayed by a modified (Hayman and Patterson, 1971) method of Nayyar and Glick (1954). Lower concentrations (0.01–0.10 mg/ml) of protein were determined by the fluorescamine method of Bohlen *et al.* (1973). The purified enzyme protein was used as the standard. To 10 μl of enzyme solution, 50 μl of the fluorescamine reagent in dioxane was added with immediate rapid mixing and then 150 μl of 0.05 M K_2PO_4 buffer (pH 8.12) was added and mixing repeated. The fluorescence was measured in a Farrand ratio fluorimeter exactly 120 sec after addition of the reagent. The filters used were Corning 7-51 (primary) and 3-72 (secondary).

Atomic absorption with a carbon rod atomizer (Model 61, Varian-Techtron) was used for determination of the metal content of the enzyme preparations (Amos *et al.*, 1971). The small sample size (0.5–1.0 μl) and high sensitivity made this the method of choice for conservation of material. For accuracy, it was necessary to use standards closely bracketing the samples in metal content and to carry out determinations in at least triplicate. The high sensitivity of the method for Zn necessitated the following precautions to avoid artifacts: all standard solutions and enzyme preparations were kept in plastic tubes that had been soaked in 1 N HCl and thoroughly rinsed with water of greater than 10^6 ohms resistance; plastic syringe tips for injection of samples into the rod were thoroughly (ten times) rinsed with glass-distilled water before use. Standard flame absorption was used on some samples to check the results of the carbon rod assays. In all cases, the values obtained by the two methods agreed.

Results

Effect of Chelators. The bacterial dipeptidase, like the tumor dipeptidase (Hayman and Patterson, 1971), is inhibited by *o*-phenanthroline and not by the isomeric nonchelator *m*-phenanthroline. With 0.05 μM enzyme, 40% inhibition was produced in 45 sec by 50 μM *o*-phenanthroline. In contrast, the inhibition of activity toward Ala-Gly by EDTA is time dependent (Figure 1) and logarithmic after a sharp initial activity drop, the extent of which increases with increasing EDTA concentration. The rate of inactivation by EDTA is maximal at approximately 1 mM EDTA. Addition of either Zn^{2+} or Mn^{2+}, in concentrations in slight excess over the EDTA concentration, produces an instantaneous recovery of activity to greater than control levels; Mg^{2+} is less effective.

Metal Content of the Highly Purified Dipeptidase. Atomic absorption analyses for Zn and Mn were done by both flame and carbon rod methods. The *Escherichia coli* dipeptidase was shown to contain 2.0 ± 0.1 mol of Zn/mol of enzyme of molecular weight 100,000. This enzyme (Patterson *et al.*, 1973) contains two indistinguishable subunits. The content of Mn^{2+} was very low, 0.03 g-atoms/mol of enzyme. The fact that the Zn content followed the peak of enzyme activity toward Ala-Gly (Figure 2) at the final stage of purification also implies that Zn is an integral part of the enzyme.

Effect of Added Metal Ions. Mg^{2+}, Ba^{2+}, Fe^{2+}, Ca^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, and Cu^{2+}, at concentrations of 10^{-5}–10^{-3} M, inhibit to various degrees the hydrolysis of Ala-Gly (50 mM) by the bacterial dipeptidase. However, 10^{-3} mM Mn^{2+} activates the reaction about fivefold, and 10^{-5} mM Mn^{2+} about twofold. The velocity of hydrolysis of 25 mM Ala-Gly increases with increasing Mn^{2+} concentration until a plateau is reached at 80 μM Mn^{2+}. Replacement of phosphate by a buffer which does not complex with metal ions, *e.g.*, 10 mM Hepes,[1] makes no significant difference except that lags (*vide infra*) are pronounced at the start of the reaction in the presence of Hepes. The effect of Mn^{2+} (80 μM) on the hydrolysis of Ala-Gly at lower and noninhibitory substrate concentrations is shown in the double reciprocal plot of Figure 3a. This metal is an activator at substrate concentrations over 6 mM but at lower concen-

[1] Abbreviation used is: Hepes; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

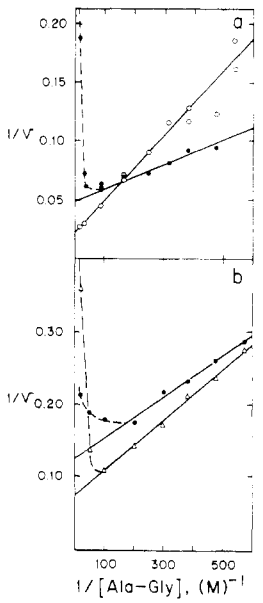

FIGURE 3: Lineweaver-Burk plots showing the effect of addition of Mn^{2+} (a) and Co^{2+} (b) on the rate of hydrolysis of Ala-Gly by the *E. coli* dipeptidase. To 150 μl of Ala-Gly, made up to pH 8.3 in phosphate-borate buffer-0.25 M sucrose, was added 1 μl of H_2O (●), $MnCl_2$ (○), or $CoCl_2$ (△), sufficient to give a concentration of 80 and 100 μM, respectively, in the final reaction mixture. After 5 min of warming in the cuvet to 30°, 5 μl of enzyme solution, diluted in the above buffer, was added. After 15 sec of rapid mixing, the reaction was recorded at 230 nm using a Gilford spectrophotometer. The units of velocity are $v = -\Delta A_{230}$ nm, $min^{-1} \times 300$.

trations is an inhibitor of enzyme activity. The activation is due in part apparently to the prevention of substrate inhibition. As seen from Table I, the overall kinetic effect of Mn^{2+} is to increase the K_m of Ala-Gly sixfold and the V_M twofold.

Table I summarizes the effect of addition of Mn^{2+} to the reaction mixtures when the hydrolyses of a variety of substrates by the bacterial dipeptidase were studied. The substrates are listed in order of their rates of hydrolysis without added metal. In general, the addition of Mn^{2+} increases both V_M and K_m, the effect being greater for substrates hydrolyzed slowly without added metal ions (Patterson *et al.*, 1973). With the substrates (Phe-Ala, Met-Gly) hydrolyzed by this enzyme at the highest maximum velocities (Table I), Mn^{2+} has no effect on V_{max} but increases the K_m about threefold. As is the case with Ala-Gly, inhibition by high substrate concentrations is eliminated or reduced when Mn^{2+} is present. Hydrolysis of Gly-Gly with added Mn^{2+} does not obey Michaelis-Menten kinetics; Lineweaver-Burk plots intersect the coordinates close to the origin suggesting very large (>300 mM) Michaelis constants and maximum velocities (>100). Ala-Ile which in the absence of added metal is scarcely hydrolyzed and is a linear competitive inhibitor (K_I, 52 μM) of Ala-Gly hydrolysis, is split at a measurable rate in the presence of Mn^{2+}. These specific effects of Mn^{2+} differ from those of Co^{2+}, as seen below.

Although Co^{2+} has long been known to stimulate the hydrolysis of Gly-Gly by dipeptidases (Smith, 1951), this metal has also been found (Cordonnier, 1966; Hayman and Patterson, 1971) to activate the hydrolysis of a variety of other substrates (at 50 mM) by this class of enzymes. The effect on the kinetic constants for selected substrates of addition of Co^{2+} to the reaction mixtures is shown in Table I. Co^{2+} inhibits the hydrolysis of the best substrate (Phe-Ala) and activates that of other substrates, the activation being greater (up to 60-fold with Lys-Gly, >60-fold with Ala-Ile) the slower the hydrolysis without metal. In general, the inhibition by high concentrations of substrate is decreased in the presence of Co^{2+}. However, as seen from Figure 3b, the Lineweaver-Burk plots of Ala-Gly hydrolysis in the presence and absence of Co^{2+}, this metal is an activator except at 50 mM substrate where inhibition is increased. It is notable that dipeptides hydrolyzed exceedingly slowly, or not at all, by the bacterial dipeptidase in the absence of metal ions are split in the presence of Co^{2+}. Examples are CH_3Gly-

TABLE I: The Effect of Added Mn^{2+} and Co^{2+} on the Hydrolysis of Dipeptides by the Bacterial Dipeptidase.[a]

	V_M/(E) (sec^{-1}) ($\times 10^{-3}$)			K_m (mM)		
Metal Addition	0	Mn^{2+}	Co^{2+}	0	Mn^{2+}	Co^{2+}
Phe-Ala	15	15	10	0.48	1.2	0.92
Met-Gly	11	11	18	1.0	3.2	2.6
Ala-Gly	11	25	20	2.1	13	4.9
Gly-Lys	11		22	1.2		1.6
Gly-Lys[b]	5.8	15		0.71	6.0	
Ala-Nle	8.3	11	11	0.41	1.9	0.82
Gly-Gly	4.2		19	15		6.2
Gly-Ala	4.1	17	9.6	1.1	13	1.1
Ser-Gly	2.8	8.7	9.3	2.8	15	3.2
Pro-Gly	0.36	1.1	3.3	2.5	8.2	2.1
Lys-Gly	0.14	0.96	8.6	1.7	8.8	1.5
CH_3Gly-Ala	(0.08)	0.10	2.0	(1.0)	1.1	1.0
Ala-Ile	<0.08	0.38	5.3		0.78	0.60

[a] K_m and V_M were determined from Lineweaver-Burk plots. The V_M data were converted to molecular activities as described in the Materials and Methods section. To 150 μl of substrate solution was added 1 μl of H_2O, $MnCl_2$, or $CoCl_2$ sufficient to give 80 and 100 μM, respectively, in the final reaction mixture. After 5 min of warming in the cuvet to 30°, 5 μl of enzyme solution was added. After 15 sec of rapid mixing, the reaction was recorded at 230 nm using a Gilford spectrophotometer. The peptides consisted of L-amino acid residues. Values in parentheses are less accurate than the others. [b] This experiment was carried out with a different sample of Gly-Lys from the one used in the experiment with Co^{2+}.

Ala, Ala-Ile, Asp-Gly, and strikingly D-Leu-Gly but not Gly-D-Leu. The last three substrates are not included in the table since the Co^{2+}-stimulated hydrolyses of Asp-Gly and D-Leu-Gly are too slow [$V_M/(E) = <0.1$] for accurate measurement. The 25-fold activation by Co^{2+} of CH_3Gly-Ala hydrolysis (no hydrolysis without enzyme) as compared with the twofold increase in rate of Gly-Ala hydrolysis is noteworthy. Thus, the effect of Co^{2+} is to change the entire substrate specificity of the dipeptidase.

The effect on K_m of Co^{2+} addition is small except in the case of good substrates (Phe-Ala, Met-Gly, Ala-Gly, Ala-Nle) where K_m is increased about twofold. The case of Gly-Gly hydrolysis is exceptional in that K_m is reduced more than twofold by Co^{2+} addition.

Presence of Only One Enzyme in the Preparations. The question arises as to whether all these activities are carried out by a single enzyme since a homogeneous dipeptidase has not yet been achieved; however, the best preparation was at the least 90% pure (Patterson *et al.*, 1973). It was found that the enzymatic activities toward Gly-Gly(Co^{2+}), Met-Gly, Gly-Lys, Pro-Gly (Mn^{2+}), and Ala-Gly occurred in the same ratios throughout purification, followed the same purfication peaks after different types of chromatography and decayed at the same rate on exposure of the dipeptidase to 30 or 40°. Another indication that only one enzyme is involved in the hydrolysis of Ala-Gly and Gly-Gly is the effect of inhibitors on the reactions. The K_I of the linear competitive inhibitor D-Leu-Gly of Gly-Gly hydrolysis (no metal activator) is 0.4 mM, the same as the previously determined (Patterson *et al.*, 1973) value for the inhibition of Ala-Gly hydrolysis. The K_I for a competitive inhibitor should be independent of the reaction inhibited if a common active site is present.

Removal of Zn from the Dipeptidase by Co^{2+}, Mn^{2+}, or EDTA Treatment. Since Mn^{2+} and Co^{2+} replace the Zn in carboxypeptidase (Coleman and Vallee, 1961), it seemed reasonable that this mechanism might account for the effects of these metal ions on a dipeptidase. A sample of highly purified (90% pure) dipeptidase was treated with Co^{2+} for 19 hr at 0° (Table II) and filtered through Sephadex. About 50% of the Zn in the enzyme was lost with essentially no effect on the rate of hydrolysis of Ala-Gly but with activation of Ala-Ile hydrolysis. When a similar experiment was carried out with Mn^{2+} (Table II), the drop in Zn content appeared to be smaller, but this difference is of questionable significance because of large experimental errors. The activity toward Ala-Gly remained essentially unchanged and that toward Ala-Ile increased after Mn^{2+} treatment of the enzyme but dropped after G-25 filtration. In order to facilitate Mn^{2+} replacement of Zn^{2+}, the enzyme was treated with 3 mM EDTA, then filtered through Sephadex G-25 equilibrated with 100 μM Mn^{2+}. As seen from Table II, the enzyme activity was completely inhibited by the chelator, but after filtration and exposure to Mn^{2+} the activity toward Ala-Gly recovered to the control value and some activity toward Ala-Ile could also be measured. The loss of about three-quarters of the Zn^{2+} along with recovery of activity presumably indicated replacement of the Zn^{2+} by Mn^{2+}. That additional sites on the enzyme for metal binding may exist is suggested by the following experiments.

Effect on Lag Time of Prior Incubation of the Dipeptidase with Co^{2+} and Mn^{2+}. When metal ions are added to reaction mixtures, activations of substrate hydrolyses occur after initial lags. Such lags, which we operationally define as the time until the observed rate becomes linear, are considered by Frieden (1970) to indicate a slow conformational change in the enzyme. Lags also occur under inhibitory conditions (*e.g.*, lags increase with increasing substrate concentration). Factors which have been found to have no ef-

TABLE II: The Effect on Zn^{2+} Content and Enzymatic Activity of Long-Term Incubation of the Dipeptidase with Co^{2+}, Mn^{2+}, or EDTA.[a]

Treatment	Hydrolysis of Ala-Gly (v/mol of enzyme)	Hydrolysis of Ala-Ile (v/mol of enzyme)	Zn^{2+} (g-atoms/mol of enzyme)
(1) Control	5.1	<0.01	2.0
(2) Co^{2+}	5.3	1.0	
(3) G-25-Co^{2+}	5.3 ± 0.5	4.2 ± 0.2	0.91 ± 0.12
(1) Control	3.8	<0.01	2.2
(2) Mn^{2+}	3.5	0.28	
(3) G-25-Mn^{2+}	3.9 ± 0.6	0.07 ± 0.03	1.4 ± 0.3
(1) Control	4.3	<0.01	2.0
(2) EDTA (3 mM)	<0.01	<0.01	
(3) G-25-Mn^{2+}	4.9 ± 0.2	0.11[b]	0.53 ± 0.07

[a] The three experiments were carried out on different samples of purified enzyme. In the first of these experiments, 0.5 ml of a solution of dipeptidase 2.9 μM (control) was made 100 μM in Co^{2+} and held for 19 hr at 0° (Co^{2+}) before passage through Sephadex G-25 equilibrated with K_2PO_4 buffer (pH 8.3), 100 μM in $CoCl_2$. In the second experiment, Mn^{2+} was substituted for Co^{2+}. In the third experiment, the enzyme solution was made 3 mM in EDTA, held for 18 hr at 0°, and then passed through Sephadex G-25 equilibrated with phosphate buffer, 100 μM in Mn^{2+}. Velocities of hydrolysis of 12.5 mM substrates were measured spectrophotometrically at 230 nm, Zn was analyzed by atomic absorption, and protein by the bromsulfalein and fluorescamine methods. Means and average deviations of the means of assays on 5–6 peak fractions after G-25 filtration are given. Since the protein levels were 0.02–0.09 mg/ml and the Zn levels were 0.2–0.9 μM, the errors of the assays were appreciable and therefore the errors of the ratios of Zn/mol of enzyme were as high as ±20% in some cases. [b] Only a single determination on the peak fraction was carried out.

TABLE III: The Effect of Prior Incubation of the Bacterial Dipeptidase with Co^{2+} or Mn^{2+} on the Lag in the Rate of Hydrolysis of Gly-Gly and Pro-Gly.[a]

Substrate	Metal	Preassay Treatment	v (ΔA_{230}/min × 300)	Lag (min)
Gly-Gly	Co^{2+}	(1) None	16.4 ± 0.2	1.7 ± 0.4
		(2) Buffer	15.6 ± 0.3	2.1 ± 0.1
		(3) Metal	18.9 ± 0.4	0.070 ± 0.004
Pro-Gly	Co^{2+}	(1) None	8.5 ± 0.3	8.0 ± 0.8
		(2) Buffer	8.6 ± 0.4	5.6 ± 1.1
		(3) Metal	11.3 ± 0.4	0.4 ± 0.3
	Mn^{2+}	(1) None	4.3 ± 0.2	13.7 ± 0.3
		(2) Buffer	5.3 ± 1.0	29 ± 10
		(3) Metal	4.1 ± 0.0	13.5 ± 0.2

[a] In all cases, the substrate was warmed at 30° for 5 min. Preassay treatment of the enzyme was carried out in three different ways: (1) the dipeptidase solution was left at 0°; (2) 5 μl of enzyme solution was added to 80 μl of phosphate–borate buffer (pH 8.3) and held at 30° for 5–15 min; (3) the same as (2) but 1 μl was added of $CoCl_2$ or $MnCl_2$ of a concentration sufficient to give 100 μM Co^{2+} or 80 μM Mn^{2+} in the final reaction mixture. The reactions were started in case (1) by adding 5 μl of cold enzyme to 180 μl of substrate to which metal solution had been added; (2) by adding 1 μl of metal solution followed by 80 μl of substrate; and (3) by adding 80 μl of substrate. In all cases, the final substrate concentration was 12.5 mM, the metal concentration as given, and the enzyme concentration was also kept constant. Since in most cases there was no significant difference in 5, 10, or 15 min of preassay treatment, the mean and average deviation of the velocities and lags are given.

fect on the duration of the lag were: (1) high ionic strength (0.2 μ); and (2) preincubation of the enzyme with substrate at 0° for 4 hr. In order to learn whether the lags could be reduced by prior incubation of the enzyme with metal ions, the hydrolyses of Pro-Gly and Gly-Gly were studied (Table III) after incubation of the enzyme with Co^{2+} or Mn^{2+}. Co^{2+} has a similar effect on the hydrolysis of both substrates, an increase in rate over the controls, and near abolition of the lag. In contrast, prior incubation of the dipeptidase with Mn^{2+} is ineffective either in reducing the protracted lag of Pro-Gly hydrolysis or in increasing the velocity. Longer periods (6 hr) of incubation (0 or 26°) of the dipeptidase with 80 μM Mn^{2+} had no effect on the hydrolyses of Pro-Gly or Ala-Ile. Therefore, Co^{2+}, but not Mn^{2+}, is effective in reducing the lag duration.

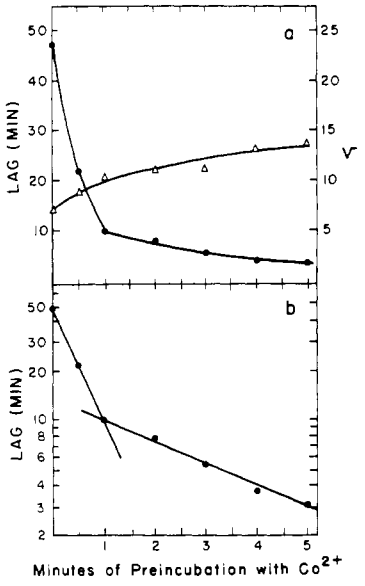

FIGURE 4: The effect of prior incubation (25°) with Co^{2+} of the bacterial dipeptidase on velocity and lag with Ala-Ile as substrate; 5 μl of enzyme solution was added to 75 μl of phosphate-borate-sucrose buffer (pH 8.3) in a cuvet and 1 μl of a $CoCl_2$ solution sufficient to give 200 μM was stirred in. After the given times at 25°, 75 μl of an Ala-Ile solution (12.8 mM in the above buffer at 25°) was added and the reaction at 25° recorded at 230 nm. The final concentrations during the hydrolytic reaction were 6.4 mM Ala-Ile and 100 μM Co^{2+}. For the zero time values, 5 μl of enzyme solution was added to a mixture of all the other components at 25°. In the top portion (a) the velocities (Δ) and lag (●) are plotted linearly *vs.* time whereas in the bottom portion (b), the lag duration is plotted on a logarithmic scale.

Since the reductions in lag caused by prior incubation with Co^{2+} (Table III) appeared to be complete in 5 min, it was necessary to measure this process at shorter times to be able to estimate the rate. The hydrolysis of Ala-Ile, which is Co^{2+} activated, was chosen as a model reaction because of the prolonged lags that have been observed at relatively low substrate concentrations. In order to permit detailed analysis, the experiment was carried out at 25° (Figure 4) and it is apparent that there are at least two first-order steps in the activation with half-times of 0.43 and 2.0 min. The activation process, which required 47 min without prior incubation of the enzyme with Co^{2+}, was 80% complete after only 2 min of treatment of enzyme with Co^{2+} prior to substrate addition. The velocity of the reaction, which doubles over the 5-min period of preincubation, follows a smooth curve. The lags with this Co^{2+}-activated hydrolysis, like those observed with other substrates, are shorter at lower substrate concentrations. However, a similar two-step process with comparable half-times was observed when 3.2 mM Ala-Ile was the substrate. With another substrate, Pro-Gly (10 mM), lag times (25°) could also be plotted as two first-order rates with half-times of 1.1 and 5.4 min. From these data, it appears that Co^{2+} can bind to the enzyme in the absence of substrate; the two-step lag reduction process may mean that this metal binds to more than one site.

Affinity of Co^{2+} and Mn^{2+} for the Dipeptidase. Although binding studies have not been carried out, some information on affinity of Co^{2+} and Mn^{2+} for the enzyme has been obtained from kinetic experiments. As seen from Figure 5, the reciprocal plot of increase in velocity *vs.* free Co^{2+} concentration at varied concentrations of Gly-Gly, a

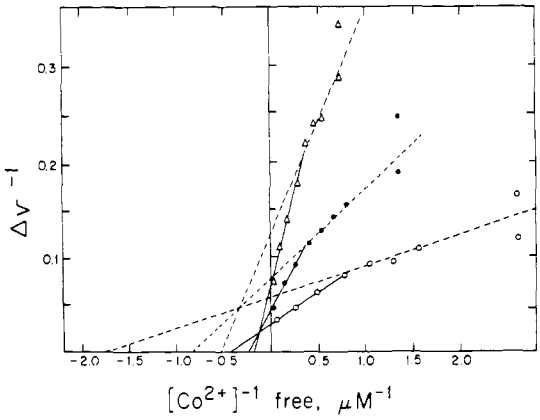

FIGURE 5: Double reciprocal plot of increase in velocity of hydrolysis of Gly-Gly *vs.* concentration of free Co^{2+}. The assays were carried out as described in the legend of Table I except that the $CoCl_2$ concentration was varied from 1 to 170 μM. The Δv values were calculated by subtraction of 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 $-\Delta A_{230}$ nm, $min^{-1} \times 300$. 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, 5.1 mM; (△) 2.5 mM; (○) 12.5 mM. K_A's (8 and 3 μM) were obtained from the triple intersections of straight lines drawn through the points (Mildvan and Cohn, 1965).

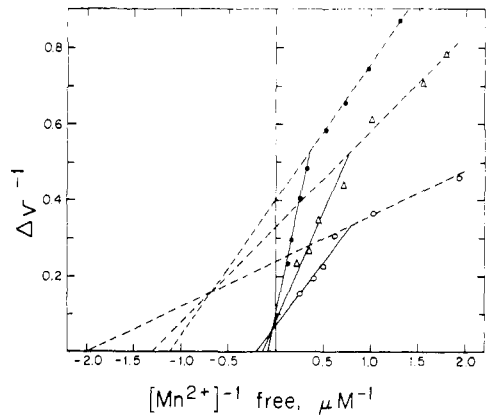

FIGURE 6: Double reciprocal plot of increase in velocity of hydrolysis of Pro-Gly *vs.* concentration of free Co^{2+}. The assays, Δv values, and calculations of free Mn^{2+} were carried out as described for Figure 5. It was assumed that for Pro-Gly-Mn^{2+} log K_1 was 2.3, an average value based on 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; (△) 8.2 mM; (○) 10.1 mM. K_A's of 1.5 and 24 μM were obtained from the triple points.

K_A of 8 μM may be determined (Mildvan and Cohn, 1965) at the intersection of lines drawn through points at high Co^{2+} concentrations and a second K_A, 3 μM, from straight lines through the points at low Co^{2+}, suggesting two Co^{2+} binding sites. From similar experiments carried out with Ala-Ile as substrate, again two K_A's of about the above magnitudes could be obtained.

Figure 6 shows the results of experiments with Mn^{2+} concentration varied and concentrations of the substrate, Pro-Gly, bracketing the K_m (8.2 mM). Again the plots each consist of two straight lines which when extrapolated give two K_A's, about 20 and 1 μM, indicating the enzyme has two sites of different affinity for Mn^{2+}.

Competition between Added Metal Ions. In order to study competition between metal ions, various combinations of Zn^{2+}, Mn^{2+}, and Co^{2+} were added to reaction mixtures containing highly purified enzyme solutions and hydrolyses of Ala-Gly and Al-Ile were measured. As seen in Table IV, the hydrolysis of Ala-Gly was significantly activated only by Co^{2+}, and the presence of stoichiometric amounts of Zn^{2+} completely inhibited this activation whereas Mn^{2+} only slightly decreased the activation. As seen before, the hydrolysis of Ala-Ile was strongly activated by Co^{2+}, and this activation was also inhibited by Zn^{2+}. While Mn^{2+} caused some activation of Ala-Ile hydrolysis, the highest velocity was obtained with the combination of Co^{2+} plus Mn^{2+}. Results identical with these were obtained when the same metal competition experiments were carried out on enzyme from which three-quarters of the Zn had been removed (Table II, third experiment). The above experiments suggest that Co^{2+} and Mn^{2+} may bind at separate sites possibly on different subunits and that Zn^{2+} competes more effectively with Co^{2+} than with Mn^{2+}.

Discussion

Evidence that the native bacterial dipeptidase is a Zn-metalloenzyme falls into three categories: (1) metal chelators such as EDTA and *o*-phenanthroline inhibit the enzymatic activity toward Ala-Gly; the nonchelating isomer *m*-phenanthroline has no effect; (2) the highly purified enzyme contains 2 g-atoms of Zn/mol of enzyme of two indistinguishable subunits; (3) Zn content and enzymatic activity toward Ala-Gly are in constant ratio around the chromatographic peak at the last stage of purification (Figure 3).

That at least one Zn in the bacterial dipeptidase may be replaceable by Co^{2+} or Mn^{2+} (Table II) seems highly probable. In addition, the apparently instantaneous reversibility of EDTA inhibition of Ala-Gly hydrolysis by Zn^{2+} or Mn^{2+} implies that these metals could rapidly replace the lost Zn with no change in the rate of hydrolysis. By suitable treatment with chelators, it may be possible to obtain an apoenzyme from which could be prepared a Mn^{2+}- or Co^{2+}-dipeptidase analogous to the Mn^{2+}- and Co^{2+}-carboxypeptidase (Piras and Vallee, 1967).

The main actions of Co^{2+} and Mn^{2+} are of three types: displacement of Zn from the native enzyme, activation or inhibition of enzymic hydrolysis of specific substrates, and

TABLE IV: The Effect of Competition between Added Metal Ions on the Velocity of Hydrolysis of Ala-Gly and Ala-Ile by the Dipeptidase.[a]

Metals (100 μM each)	Substrate: Ala-Gly v ($\Delta A_{230\ nm}$, $min^{-1} \times 300$)	Substrate: Ala-Ile v ($\Delta A_{230\ nm}$, $min^{-1} \times 300$)
0	21.5	0.4
Zn^{2+}	17.1	0.1
Mn^{2+}	22.2	1.5
Co^{2+}	33.2	25.2
Zn^{2+} + Mn^{2+}	14.6	0.2
Zn^{2+} + Co^{2+}	17.6	3.5
Co^{2+} + Mn^{2+}	28.6	29.4

[a] The maximum noninhibitory substrate concentrations (Ala-Gly, 12.5 mM; Ala-Ile, 2.5 mM) were used; 5 μl of enzyme (1.2 μM) solution was added to a cuvet at 30° containing 156 μl of substrate solution and 1 μl of solution of the stated metal chlorides sufficient to give 100 μM of each metal ion. The average deviation of the velocity values is ±0.2.

prevention or enhancement of substrate inhibition of enzyme activity. Comparison of addition of Co^{2+} and Mn^{2+} shows that these two metals differ significantly in their effects. Mn^{2+} is generally a less effective activator than Co^{2+} of the hydrolysis of "poor" substrates, consistently increases K_m values, and markedly reduces substrate inhibition, and only partially removes Zn from the enzyme. Co^{2+} appears to replace one enzyme–Zn^{2+}, reduces substrate inhibition in only a few cases and increases it in many more instances, and spectacularly activates the hydrolyses of peptides that are not normally substrates. This activation is accompanied by no increase in K_m (Table I).

It is evident from the kinetic binding experiments (Figures 5 and 6) that Mn^{2+} and Co^{2+} each bind to the enzyme at two sites of different affinities. Moreover, when present together (Table IV), Mn^{2+} and Co^{2+} act synergistically in activating Ala-Ile hydrolysis, implying that there may be specific Co^{2+} and Mn^{2+} activation sites on the enzyme. Zn^{2+}, which abolishes the Co^{2+} activation and decreases the Mn^{2+} activation, may bind with high affinity to a Co^{2+} site but perhaps binds with low affinity to the Mn^{2+} site. Possibly the lower K_A values (Figures 5 and 6) reflect the relative affinities of the enzyme for the extrinsic metals and Zn and the higher values refer to combination of these metals at the Mn^{2+} or Co^{2+} activation sites on the enzyme. Also, the two-step process in lag reduction in Ala-Ile hydrolysis by Co^{2+} (Figure 4) suggests that the fast step may be attributed to loose binding of the metal to the enzyme and the slow step to displacement of native Zn^{2+}.

The reductions in lag (Figure 4 and Table III) by Co^{2+} binding indicate that this metal may cause a conformational change (possibly quite small) in the dipeptidase (Frieden, 1970; Bates and Frieden, 1973). In accordance with the properties of some hysteretic enzymes, it was observed that in general the lag times decreased (1) with decreasing substrate concentration, and (2) with increasing enzyme concentration. These observations are further evidence for a conformational change.

Speculations about the Co^{2+}-induced alterations in enzyme conformation that result in major changes in substrate specificity can be related to our previous (Patterson *et al.*, 1973) postulation about the shape of the active site of the bacterial dipeptidase; *i.e.*, that this enzyme contains two differing hydrophobic pockets (R_N and R_C) which hold the N-terminal and C-terminal R groups of the substrates, and that the carboxyl group is bound to a positively charged residue as in carboxypeptidase (Lipscomb *et al.*, 1968). The R_N pocket appears to be a more positive determinant of reactivity than the R_C pocket. The change in substrate configuration caused by Co^{2+} chelation may enable the R_N group of D-Leu-Gly to fit into the R_N pocket so that this peptide can be slowly hydrolyzed. In addition, there may occur changes in enzyme conformation as a result of Co^{2+} binding. To account for the greatly enhanced hydrolysis of Ala-Ile, it must be assumed that the R_C pocket opens out to accommodate the large R_C residue. Gly-Gly which has no R groups appears to be poorly bound (K_m, 15 mM) to the enzyme; moreover, inhibition of Ala-Gly hydrolysis by Gly-Gly is uncompetitive (unpublished). In the presence of Co^{2+} (K_m, 6.2), the hydrolysis of this substrate is activated with a shorter lag duration than in the case of Ala-Ile which may mean that a small change in enzyme conformation is somehow sufficient for productive binding of this small substrate. Hydrolysis of Gly-Gly may be promoted by Co^{2+} chelation in a manner similar to hydrolysis of this dipeptide by model compounds, such as Co-trienes (Collman and Buckingham, 1963). Clarification of the validity of these speculations requires studies by physical techniques such as X-ray crystallography and nuclear magnetic resonance relaxation spectroscopy.

The effect of Mn^{2+} on the dipeptidase conformation appears to differ from, and be less than, that of Co^{2+} in that addition of this metal has no observable effect on lag time, causes increases in both V_{max} and K_m, and decrease in substrate inhibition. Because relatively little Zn is displaced by long-term incubation with Mn^{2+}, unless substrate accelerates metal exchange it is unlikely that these short-term effects derive from active-site Zn removal. Therefore, the binding of Mn^{2+} to its activation site on the enzyme probably alters the active site in a way to account for the above effects. Since reduction of inhibitory substrate concentrations by metal chelation would be achieved somewhat better by Co^{2+} as judged by the stability constants of Co^{2+} and Mn^{2+} for dipeptides (Sillén and Martell, 1964), this mechanism cannot account for the specific decrease of substrate inhibition in the presence of Mn^{2+}. The activation of Pro-Gly hydrolysis by Mn^{2+}, as well as that of Gly-Ala, suggests that the change in the enzyme permits enhanced hydrogen bonding of the N-terminal nitrogen to the enzyme. Because Mn^{2+} has no observable effect on lag time, it is possible that any conformational changes that may occur require the presence of substrate. If Mn^{2+} forms a bridge between enzyme and substrate (Mildvan, 1970), the substrate configurations may be rearranged by this chelation to those more suitable for binding to the enzyme.

The bacterial dipeptidase resembles other Zn–metalloenzymes that have subunits; *e.g.*, hog kidney particulate dipeptidases (Campbell *et al.*, 1966), leucine aminopeptidase from bovine lens (Carpenter and Vahl, 1973), and alkaline phosphatase from *E. coli* (Petiteler *et al.*, 1970). The native Zn, 2–4 g-atoms/mol of enzyme, some Zn^{2+} bound more loosely than others, can be replaced by Mn^{2+}, Mg^{2+}, or Co^{2+} with various effects on enzyme activity. In no case, however, are the effects on substrate specificity as great as in the present study of the bacterial dipeptidase.

Precedent for changes in protein conformation caused by binding of metals either at the site of a native metal or at other sites, for example, are concanavalin A (Edelman *et al.*, 1972), glutamine synthetase (Hunt and Ginsberg, 1972), TPN isocitric dehydrogenase (Colman, 1972), inorganic pyrophosphatase (Höhne and Rapoport, 1973), and pyruvate kinase (Kupiecki and Coon, 1960). Therefore, the dipeptidases as well as the closely related carboxypeptidases (Piras and Vallee, 1967) are not unusual in that changes in enzyme conformation with resultant alterations in enzyme specificity can be caused by binding of metal ions.

The results of our studies on the effects of added metal ions on the bacterial dipeptidase bring up the question of the number of specific dipeptidases existing in cells. As reviewed by Smith in 1960, it has been thought that many separate dipeptidases exist and some have been defined tentatively according to the metals that activated the hydrolysis of a given substrate, *e.g.*, Co-Gly-Gly dipeptidase and Mn-Pro-Gly dipeptidase (imino dipeptidase). With the advent and use of methods of purification less injurious to these enzymes, doubt has been cast on the plurality of dipeptidases (Capobianca and Vescia, 1967; Cordonnier, 1966). By use of zonal electrophoresis and DEAE-cellulose chromatography, we (Patterson *et al.*, 1965) separated a maximum of three soluble dipeptidases from rat liver: di-

peptidases hydrolyzing (1) Gly-Pro, Mn^{2+} activated (prolidase highly purified from hog kidney by Davis and Smith, 1957); (2) Ala-Gly, Gly-Leu, Cys-Gly, and Pro-Gly; and possibly (3) Gly-Leu, not Zn^{2+} inhibited. All these activities occurred at the same position after Sephadex G-200 gel filtration with molecular weights in the region of 65,000–80,000. Dipeptidases highly purified from ELD mouse ascites tumor cells and *E. coli* B (Patterson *et al.*, 1973) have been shown to hydrolyze a great variety (45–50) of dipeptide substrates, and in a paper now in preparation we show that added Co^{2+} and Mn^{2+} activate the hydrolyses of Gly-Gly and Pro-Gly, respectively, with evidence for conformational change in the tumor dipeptidase. In this way, only a few dipeptidases may be responsible for hydrolysis of all dipeptides in the cell.

Comparison of the present studies with those of others on bacterial dipeptidases shows that there are many consistencies and inconsistencies in the field. Payne (1972) concluded from detailed studies of the effect of metal ions on dipeptidases in crude soluble extracts of *E. coli* B that the principal dipeptidase activity resided in a single Co^{2+}-activated enzyme. The extent of Co^{2+} activation varied with the nature of the peptide substrate. Mn^{2+} stimulated the hydrolysis of only a few of the peptides (Gly-Gly, Pro-Gly) tested and to a lesser extent than Co^{2+}. From the additive effects of Co^{2+} and Mn^{2+} activation and the effect of buffers on the hydrolysis, he concluded that separate dipeptidases were activated by Co^{2+} and Mn^{2+}. We feel from the present work with a highly purified bacterial dipeptidase that there is little evidence for more than one enzyme in the preparations and that it is possible that Co^{2+} and Mn^{2+} cause differing conformational changes by binding to separate sites in the same protein in order to account for the effects observed.

Brown (1973) has purified 370-fold a dipeptidase M from *E. coli* B which is homogeneous on acrylamide gel and has a specific activity of 1100 with Met-Ala as substrate. This enzyme is activated by Mn^{2+} at 1 mM substrate concentration. The dipeptidase has two subunits of molecular weight, 47,000–48,000. It is inhibited by EDTA and 100% reactivated by only Mn^{2+} of six divalent cations tested, including Zn^{2+}. The reactivation by Mn^{2+} is time dependent. In contrast to the dipeptidase we (Patterson *et al.*, 1973) purified from the same source, dipeptidase M is inhibited by iodoacetic acid and requires mercaptoethanol for stability and may therefore be a sulfhydryl enzyme. Although Met-Ala is an excellent substrate for both dipeptidases, the substrate specificities differ sufficiently to warrant conclusion that dipeptidase M is not identical with the one we have purified from *E. coli* B.

The dipeptidase purified by Simmonds (1972) from *E. coli* K12 also requires mercaptoethanol for stability. Again N-terminal Met-dipeptides are the best substrates. The enzyme has an absolute requirement for Mn^{2+}. Addition of Co^{2+} gives low levels of activity and no activity is measured with addition of Zn^{2+} or Mg^{2+}; Co^{2+} and Zn^{2+} inhibit the Mn^{2+} activation.

A thermostable (50°) dipeptidase has been highly purified from *Streptococcus thermophilus* (Rabier and Desmazeaud, 1973). This enzyme has a molecular weight of 50,000, a pH optimum of 7.5, is inhibited by metal chelators, and can be partially reactivated by Co^{2+}, Zn^{2+}, Mn^{2+}, Mg^{2+}, and Ca^{2+} in order of effectiveness. N-terminal methionyl dipeptides are hydrolyzed most rapidly. No requirement of SH stabilizers was mentioned.

It appears that the bacterial dipeptidases are enzymes that have many properties in common and the differences observed in enzymes purified in various laboratories may be related to the presence of metal ions in their environment and the conditions (presence of EDTA,[2] metals, etc.) used in purification.

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The Effect of Histidine Modification on the Activity of Dihydrofolate Reductase from a Methotrexate-Resistant Strain of *Escherichia coli* B†

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ABSTRACT: Dihydrofolate reductase from *Escherichia coli* strain MB 1428 contains five histidines. The enzyme has been reacted with ethoxyformic anhydride (EFA) at pH 7.2 and the treated enzyme has been assayed at both pH 7.2 and pH 4.7. The treatment with EFA rapidly ethoxyformylates two histidines; the rest react more slowly. At pH 7.2 there is a rapid 50–60% loss of enzymatic activity corresponding to the modification of the rapidly reacting histidines. When the enzyme modified at pH 7.2 is assayed at pH 4.7, however, there is a loss of 90% of the activity in the same time period. In the presence of dihydrofolate, methotrexate, or folic acid, one rapidly reacting histidine is protected from reaction with EFA at pH 7.2. There is no protection against the loss of activity at pH 7.2, but the folates protect against approximately half the activity loss at pH 4.7. The enzyme from *E. coli* MB 1428 has two nonequivalent NADPH binding sites. The binding of NADPH at the stronger of the two sites also protects one of the rapidly reacting histidines from ethoxyformylation at pH 7.2 and protects almost fully against the loss of activity at pH 7.2 and against approximately half of the activity loss at pH 4.7. When the dihydrofolate reductase is protected by both methotrexate and NADPH approximately 2 molar equiv of histidine are protected from rapid reaction with EFA suggesting that the histidines protected by the folates and NADPH are different. Fluorescence and circular dichroism experiments suggest that the modified enzyme exhibits no loss of binding capacity for NADPH, dihydrofolate, and methotrexate. The K_m's for dihydrofolate and NADPH are unchanged at pH 7.2 when compared to those of the native enzyme, but when the enzyme is modified at pH 7.2, but assayed at pH 4.7 the K_m for NADPH increases relative to that of the native enzyme. The pH–activity profile of the modified enzyme changes upon histidine modification; the pH optimum remains the same.

N-Bromosuccinimide oxidation of dihydrofolate reductase from a methotrexate (amethopterin)-resistant strain of *Escherichia coli* (strain MB 1428) has suggested histidine is involved in the active site (Williams, 1972, 1974). When dihydrofolate reductase is titrated with *N*-bromosuccinimide (NBS[1]) there is an initial loss of 40–50% of the enzymatic activity with no tryptophan oxidation. Amino acid analysis indicates that the initial loss of activity may correspond to the oxidation of one histidine. The partially modified protein, however, shows no loss of capacity to bind reduced nicotinamide adenine dinucleotide phosphate (NADPH), the cofactor involved in dihydrofolate reduction, or methotrexate, a potent inhibitor of the enzyme. Moreover, the modified protein has fluorescence and circular dichroism properties identical with the native enzyme (Williams, 1972, 1974). Further additions of NBS result in the modification of tryptophan residues, and partial loss of ability to bind both NADPH and methotrexate.

Efforts to determine whether substrates and cofactors protect against the initial modification by NBS are complicated because both dihydrofolate and NADPH interact with NBS. The reaction of both of these produces spectral changes in the ultraviolet region used to determine the extent of tryptophan modification, thus it is difficult to perform protection studies.

In order to explore more fully the role that histidine plays in the activity of dihydrofolate reductase it has been necessary to extend the study to a "histidine specific" reagent.

Modification of proteins with ethoxyformic anhydride (EFA) at acidic pH has been shown to be relatively specific

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[1] Abbreviations used are: NBS, *N*-bromosuccinimide; EFA, ethoxyformic anhydride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); N_2phF, fluorodinitrobenzene.