Purification and Properties of a Mouse Ascites Tumor Dipeptidase, a Metalloenzyme*

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SUMMARY

A dipeptidase that hydrolyzes L-Ala-Gly and a wide spectrum of other L-α-dipeptides has been purified 800-fold from the soluble fraction of Ehrlich-Lepté mouse ascites tumor cells. The highest specific activity (micromoles of dipeptide hydrolyzed at 40° per min per mg of protein) achieved was 2,600 with Ala-Gly, the substrate with which purification was followed. With the best substrate, Ala-Ile, this specific activity was equivalent to a molecular activity (moles of dipeptide hydrolyzed at maximum velocity at 40° per min per mole of enzyme) of 2 × 10⁶. Instability of the enzyme at this high activity prevented determination of homogeneity; a sample of molecular activity of 1 × 10⁶ (Ala-Gly specific activity, 1,360) was estimated to be 50% pure by acrylamide gel electrophoresis. Studies with metal chelators indicate that the dipeptidase is a metalloenzyme. For instance, α-phenanthroline completely inhibited the enzyme at this high activity prevented determination of homogeneity; a sample of molecular activity of 1 × 10⁶ (Ala-Gly specific activity, 1,360) was estimated to be 50% pure by acrylamide gel electrophoresis. Studies with metal chelators indicate that the dipeptidase is a metalloenzyme. For instance, α-phenanthroline completely inhibited the enzyme activity (50% at 0.1 µM), whereas m-phenanthroline had no effect. Although atomic absorption analyses showed a correlation of zinc content with enzyme activity in the final chromatographic step and a value of 0.9 ± 0.1 g atom of zinc per mole of enzyme of specific activity 2,600 (Ala-Gly), it has not been confirmed that the dipeptidase is a zinc metalloenzyme. The enzyme was separated from leucine aminopeptidase, prolidase, and tripeptidase by Sephadex G-150 filtration and, by comparison with standard proteins, was shown to have a molecular weight of 85,000 ± 5,000. The dipeptidase hydrolyzes only L-α-dipeptides with a free amino and carboxyl group. Kinetic studies of 15 dipeptides have shown the Kₘ values to vary between 0.4 and 22 mM and the relative Vₘₐₓ values (Ala-Gly standard, Vₘₐₓ = 1) to vary from 5.3 (Ala-Ile) to 0.01 (Gly-Gly). Inhibition by high substrate concentrations (3 to 50 mM) was observed in cases where the Kₘ was low. Peptides with small NH₂-terminal and bulky nonpolar COOH-terminal R groups were preferred. The hydrolyses of the relatively poor substrates, Pro-Gly and Gly-Gly, were activated by mm Mn²⁺ and Cu²⁺, respectively, whereas that of Ala-Gly was inhibited by both these metals as well as by 10 other metals. No evidence was obtained that these hydrolyses were carried out by separate enzymes: the enzyme peaks were congruent, the ratios of activities were constant throughout purification, and the activities toward the three substrates decayed at the same rate on exposure to 30° for periods up to 24 hours.

Although many proteolytic enzymes have been studied intensively during recent years, the dipeptidases have received little attention, probably because of their lability. This class of enzymes hydrolyzes only dipeptides with a free amino and carboxyl group (3, 4). It has long been known that dipeptidase occurs in all cells studied; most notably, the enzymes that hydrolyze Ala-Gly are found in highest activity in rapidly growing and protein-synthesizing cells (5, 6). The Ala-Gly dipeptidase has been shown to increase in activity along with the active protoplasm (5), but to decrease in mature cells and cells that are breaking down (5-7). Simmonds (8), from her studies of peptidase activity in log and stationary phase Escherichia coli K-12 cells, has postulated that the intracellular crypticity of peptidase activity is controlled by metal ions. The function of the dipeptidases has been clarified by the work of Coffey and de Duve (9), who have shown that proteins are hydrolyzed to small peptides (mainly dipeptides) by liver lysosomal enzymes and that soluble dipeptidases split the dipeptides into amino acids which can then be used for protein synthesis.

Several stable dipeptidases have been highly purified from hog kidney (4, 10-15) and E. col (16), but these occur in most cells in low activity compared to the unstable enzyme that hydrolyzes Ala-Gly (4, 17, 18). One renal particulate dipeptidase has been crystallized and is a zinc metalloenzyme (18). In addition, Cordonnier (19) has isolated a dipeptidase from yeast that hydrolyzes Ala-Gly as well as a variety of other dipeptides. The present paper describes progress in the purification (800-fold) of a relatively labile soluble dipeptidase from a rich mammalian source, a mouse ascites tumor. This enzyme hydrolyzes Ala-Gly and a variety of other dipeptides and has been shown to be a metalloenzyme.

EXPERIMENTAL PROCEDURE

Materials and Methods

Chemicals—The peptides were purchased from Mann with the exception of Ala-Ile and Ala-Leu, which were obtained from Sigma. The dehydropeptides were a gift from Dr. Benedict...
Campbell of the University of Missouri. Crystallized bovine serum albumin, five times crystallized egg albumin, and mouse albumin, Fraction V, were all purchased from Pentex. Goat antibody to mouse serum was produced by Hyland Laboratories, Los Angeles, California. Cytochrome c (Kellin-Hartree) was purchased from Boehringer, Mannheim. Bromosalphenalin was obtained from Hyson, Westcott, and Dunning, Inc., Baltimore, Maryland. Chemicals for disc electrophoresis were supplied by Chemical Industrial Corporation, Bethesda, Maryland. Dansyl-bovine serum albumin (20) was synthesized by Judith Liebowitz of this Institute. The ammonium sulfate and sucrose, special enzyme grade, were manufactured by Mann, and analytical grade chemicals and glass distilled water (greater than 10^6-ohm resistance) were used for all solutions. DEAE-Cellulose (microgranular DE 32) was obtained from the Whatman Company, Reeve Angel, Clifton, New Jersey, Distributors, and Sephadex G-150 and Ficoll from Pharmacia. Nevaldine, Syracuse University. Chelex 100 was from BioRad Laboratories and Coomassie brilliant blue, R-250, from Colab Laboratories, Inc., Chicago Heights, Illinois.

**General Conditions**—The following precautions were taken to reduce metal ion contamination. All operations were carried out in a relatively dust-free room in glassware which had been chromic acid-cleaned and boiled in water of greater than 10^6-ohm resistance. All operations were carried out in a relatively dust-free room in glassware which had been chromic acid-cleaned and boiled in water of greater than 10^6-ohm resistance. The enzyme preparations were stored frozen in plastic tubes which had been cleaned in dilute Acelonox, rinsed in distilled water, 0.1 N HCl, and glass-distilled water. Exposure of solutions to room air was kept to a minimum.

Cell filtration and chromatography were performed at 5°C in a Gilson cold box fraction collector, by using timed collection and recording 280 nm absorbance on an Esterline-Angus recorder. Flow was maintained constant by a Sigmamotor pump. Since it was found that the enzyme was more stable to freezing (-30°C) in the presence of sucrose, sufficient powdered sucrose to give a final concentration of about 0.25 M was added to the collection tubes. Material was conserved by use of microchemical methods of assay.

**Peptidases**—The dipeptidase purification was followed by measuring the rate of hydrolysis at 40°C of the substrate L-Ala-Gly (50 mM, pH 8.3). The ninhydrin method of Matheson and Tattrie (21) was modified for this substrate concentration and by recording 280 nm absorbance on an Esterline-Angus recorder. The reaction was started by addition of 7 μl of substrate solution (100 μm brought to pH 8.3 with NaOH) to 7 μl of enzyme solution which had been equilibrated at 40°C for 1 min. A blank was prepared by the substitution of 20 mM potassium phosphate, pH 8.2, for the enzyme solution. An enzyme dilution in either 0.25 M sucrose or bovine serum albumin (2 mg per ml) in the above buffer was selected in such a way that the degree of hydrolysis of substrate did not exceed 50%, the region in which the rate was linear. After a 5-min incubation period at 40°C, the reaction was stopped with 7 μl of 0.1 N HCl in 95% alcohol. Enzyme blanks, in which the order of addition was enzyme, alcohol, and substrate, were used to check the amino acid content of the enzyme solutions. The extent of reaction was determined by transferring a 6-μl aliquot of the mixture to a test tube to which were added 1 ml of water, 0.5 ml of sodium citrate (0.2 M, pH 4.7), and 1 ml of the Matheson-Tattrie ninhydrin reagent. After 7.5 min of boiling, and subsequent cooling, 2.5 ml of 60% alcohol were added and the resulting solutions read at 570 μm against the blank. The reaction was standardized by determining the ninhydrin values for suitable mixtures of amino acids and peptides. The difference in A_570 values between 0 and 50% hydrolysis of Ala-Gly is 0.660, the substrate blank values are 0.120 to 0.130, and the error of the method is less than 0.5%. Lecine aminopeptidase (22), prolidase (iminodipeptidase) (23), and tripeptidase (24) were determined by measurement of the ninhydrin color after incubation of the enzyme with the appropriate substrate for 5 min. In the case of the former two enzymes, preparations were previously incubated at 25°C for 30 min with 2 mM MnCl₂ giving a concentration of 1 mM Mn²⁺ in the reaction mixture (25).

The ninhydrin method could not be used for NHz-terminal glycol dipeptides, since there is too small a difference in ninhydrin color between the glycol peptides and their constituent amino acids for practical use. For assay of the hydrolysis of these dipeptides (e.g., Gly-Lys, the substrate for prolidase), the ultraviolet spectrometric method of Lindeberg-Lang and Holter (25) was used. A control of Ala-Gly hydrolysis estimated spectrophotometrically was run in the same experiment with each glycol peptide.

**Enzyme Units**—A unit of enzyme activity is defined as 1 μmole of substrate hydrolyzed per min at 40°C. The substrate concentrations were 50 μm except in the kinetic experiments. Unless otherwise specified, enzymatic activity is the rate of hydrolysis (40°C) of Ala-Gly (90 mM, pH 8.3). Enzyme concentrations are recorded as units per ml of solution. Specific activity is defined as enzyme units per mg of protein. Relative maximum velocity (V_max) is defined as the ratio of the maximum velocity with a given substrate to that with Ala-Gly.

**Enzyme Kinetics**—For determination of Michaelis constants and maximum velocities, peptide hydrolysis was followed by monitoring the loss of ultraviolet absorbance due to the peptide bond with a modification of the method of Schmitt and Siebert (26). Although the absorption peak for the peptide bond is at 187 μm (27), it was found that at 235 μm molar absorbance differences (ε₂₃₅, Gly-Pro = 56 μM⁻¹ cm⁻¹; ε₂₃₅, Gly-Gly = 16 μM⁻¹ cm⁻¹), for the millimolar range of substrate concentrations required, fell in a region measurable with accuracy in the Gilford spectrophotometer. The course of the reaction was recorded on a Honeywell strip chart recorder attached to the Gilford spectrophotometer. The reaction was carried out in silica microcells (2 X 10 mm) with a 155-μl volume of reaction mixture. The cell compartment was thermostated at 30°C, a temperature which was chosen for enzyme stability in the absence of albumin. Buffers used were phosphate or phosphate-borate which had low absorbances at 235 μm.

**Protein Determination**—The method of Nayar and Glick (28) was modified for determination of soluble proteins. Bovine serum albumin, which is an acidic protein, as is the dipeptidase, was used as the reference standard (29). The NaOH step was omitted and the citrate-sulfobromophthalein reagent (0.2 to 0.4 mg of sulfobromophthalein per ml of 0.5 M citric acid) was adjusted accordingly. By variation of the volumes of reagents and samples so that the weight ratio of reagent to protein was in the range 1.5 to 6, protein concentrations from 0.1 to 5 mg per ml were determined in three overlapping ranges.

**Metal Content**—A Varian-Techtron atomic absorption spectrophotometer was used for determination of soluble compounds. Bovine serum albumin, for which an acid protein, was used as the reference standard (29). The NaOH step was omitted and the citrate-sulfobromophthalein reagent (0.2 to 0.4 mg of sulfobromophthalein per ml of 0.5 M citric acid) was adjusted accordingly. By variation of the volumes of reagents and samples so that the weight ratio of reagent to protein was in the range 1.5 to 6, protein concentrations from 0.1 to 5 mg per ml were determined in three overlapping ranges.

1 The abbreviations used are: dansyl: 5-dimethylaminonaphthalene-1-sulfonfonyl; HEPES, N-2-hydroxyethylpipersaine-N’-2-ethanesulfonic acid.

2 S.-H. Hsiao, unpublished observations.
The effect of pH on dipeptidase activity. Ala-Gly, 0.10 M, was made up to the required pH with mixtures of 50 mM Na₂B₄O₇ with 0.1 M NaOH or 0.1 M KH₂PO₄. Seven microliters of an enzyme preparation (specific activity 164) diluted 5-fold with ovalbumin (1.5 mg per ml in 20 mM potassium phosphate buffer, pH 7.8) were incubated for 8 min at 40° with 7 ml of substrate. The pH values are given as measured at 25°. Relative activity refers to percentage of activity at the optimal pH.

**TABLE I**

**Purification of ascites tumor dipeptidase**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble fraction</td>
<td>10,500 mg</td>
<td>36,750 units</td>
<td>3.5 units/mg</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄ (50-75%)</td>
<td>3,200 mg</td>
<td>37,200 units</td>
<td>11.6 units/mg</td>
<td>101</td>
</tr>
<tr>
<td>Sephadex G-150</td>
<td>360 mg</td>
<td>33,000 units</td>
<td>91.7 units/mg</td>
<td>90</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fractions 90-91</td>
<td>1.9 mg</td>
<td>5,000 units</td>
<td>2,600 units/mg</td>
<td>14</td>
</tr>
<tr>
<td>Fractions 89-94</td>
<td>8.0 mg</td>
<td>14,300 units</td>
<td>1,800 units/mg</td>
<td>39</td>
</tr>
</tbody>
</table>

*The results in this table represent the best purification achieved. The figures in parentheses give the ranges obtained in all purifications.*

**Biographical Material**—The Ehrlich-Lettré hyperdiploid mouse ascites carcinoma was maintained by serial intraperitoneal transplantation of 0.2 to 0.3 ml of ascites into adult (24 to 3 month old) female mice of the ICR albino strain. After 7 to 8 days of growth, the peak of peptidase activity in the cells (34), tumor samples were aspirated by sterile syringe and collected in tubes in an ice bath. All subsequent operations were carried out at 2-5°. The ascites serum, which contains a dipeptidase inhibitor (34), was removed from the cells by centrifugation and subsequent washing with buffered 0.9% NaCl-KCl (sodium to potassium ratio, 10:1; 0.01 M NaHCO₃). The pelleted, washed ascites cells were then subjected to a 2 min hypotonic shock treatment with water, in order to lyse any contaminating red cells. After centrifugation and repetition of this treatment, the cell pellets were cream-colored.

**Homogenization of Ascites Cells**—The packed, washed cells, suspended in about one-sixth their volume of 20 mM potassium phosphate, pH 8.2, were transferred to an ice-cold Servall Omnitoolometer, fitted with an Aztec log expander and a Sola constant voltage transformer, was used for determination of the metal content of the enzyme preparations. Calibration of the instrument was made with appropriate dilutions of Fisher certified atomic absorption standard solutions. The buffer blanks contained less than 0.1 µg zinc, which was the noise level of the instrument.

**Other Determinations**—Phosphatase was analyzed by the procedure of Kolb, Weidner, and Toennies (30) scaled down for 5-µl aliquots. Chloride was measured by the method of Schales and Schales (31) modified for use with 10 to 20-µl samples. Mouse serum albumin was estimated by a micromodification by
mixer and homogenized at full speed (14,000 rpm) for 5 min. After addition of sufficient 2 M sucrose in 20 mM potassium phosphate (pH 7.6) to give 0.25 M sucrose, final homogenization was carried out for 2 min.

Preparation of Soluble Fraction—The homogenate was centrifuged at 17,500 rpm (20,000 × g) in a Spinco model L centrifuge for 20 min in order to sediment the remaining whole cells, nuclei, and mitochondria. The supernatant fluid was then centrifuged at 17,500 rpm (20,000 × g) for 2 hours. After removal of the top lipid layer, the clear soluble fraction (pH 6.9 to 7.0) was pipetted off, leaving behind the microsomal pellet and the fluffy layer. The dipeptidase is localized entirely in the soluble fraction of the ascites cell (36).

RESULTS
Dipeptidase Purification
Buffer and pH Optimum—As seen from Fig. 1, the pH optimum of the enzyme is pH 8.3. Since the highest enzyme activities were obtained with preparations in phosphate (see “Effect of Added Metal Ions on Dipeptidase Activity”), this buffer (20 mM, pH 8.2) was used throughout the purification.

Ammonium Sulfate Fractionation—Table I summarizes the purification of the enzyme. Approximately 400 ml of ascites-soluble fraction from 350 mice were fractionated between 50 and 75% saturated ammonium sulfate. The resultant precipitate was dissolved in the minimal volume of buffer and dialyzed against the same buffer overnight. Yields of enzyme in the

### Table II

 Stability of dipeptidase preparations

<table>
<thead>
<tr>
<th>Treatment and buffer</th>
<th>Specific activity</th>
<th>Temperature</th>
<th>Time</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Storage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium phosphate, 20 mM</td>
<td>3.5-9.0</td>
<td>-30°</td>
<td>18 mos</td>
<td>Excellent</td>
</tr>
<tr>
<td>Sucrose, 250 mM, pH 8.1</td>
<td>1000-2600</td>
<td>-30°</td>
<td>10 min</td>
<td>Slow loss</td>
</tr>
<tr>
<td>400</td>
<td>20°, 22°</td>
<td>7 hrs</td>
<td>Excellent</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>20°, 22°</td>
<td>24 hrs</td>
<td>10-20% loss</td>
<td></td>
</tr>
<tr>
<td><strong>Dilution</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium phosphate, 20 mM</td>
<td>3.5-600</td>
<td>5°</td>
<td>18 hrs</td>
<td>Excellent</td>
</tr>
<tr>
<td>plus sucrose, 250 mM</td>
<td>50-2600</td>
<td>40°</td>
<td>10 min</td>
<td>Poor</td>
</tr>
<tr>
<td>plus tRNA, 0.2 mg/ml</td>
<td>50-2000</td>
<td>40°</td>
<td>10 min</td>
<td>Poor</td>
</tr>
<tr>
<td>plus bovine serum albumin, 2 mg/ml</td>
<td>50-2600</td>
<td>40°</td>
<td>10 min</td>
<td>Excellent</td>
</tr>
</tbody>
</table>

An artifact of several Sephadex G-150 runs has been the occurrence of a rapid marked shrinkage of the column during the period of sample addition. This phenomenon has rendered the use of either upward flow elution or of flow adaptors impossible, but does not appear to impair the resolution unduly. It is probably due to the osmotic effect described by Edmond et al. (37).
log molecular weight of standard proteins and dipeptidase. Three milligrams of blue dextran, 40 mg of rabbit γ-globulin, 10 mg of ovalbumin were dissolved in 0.5 ml of 0.25 M sucrose. The concentrations plotted refer to the molarities in the previously incubated mixture. After 8 min of prior incubation at 22°C, 7 ml of an Ala-Gly solution (0.1 M) were added, and the mixture was incubated for 5 min at 40°C. The values for initial activity were obtained when 1 ml of water was substituted for the phenanthroline solutions. The values obtained with α-phenanthroline are plotted O--O; □--□, m-phenanthroline; Δ--Δ represent data from an enzyme preparation at the (NH₄)₂SO₄ step (specific activity 12) treated with α-phenanthroline. In this case, the enzyme preparation was diluted 52-fold with 20 mM potassium phosphate buffer, pH 8.2, containing 0.25 M sucrose.

Properties

Molecular Weight—An estimate of the molecular weight of the dipeptidase was made by gel filtration in Sephadex G-150 of a small aliquot of the ammonium sulfate-precipitated enzyme, in conjunction with several standard proteins and blue dextran (38) (Fig. 4). The resolution was excellent and it was notable that the dipeptidase peak was eluted just prior to, and overlapping that of, dansyl-bovine serum albumin. With the assumption that the dipeptidase is a globular protein, a molecular weight of 85,000 ± 5,000 was calculated from the mean of four experiments. When more highly purified enzyme (after DEAE-cellulose chromatography which removes RNA) was used, the elution profile was similar and the molecular weight obtained was 87,000.

Effect of Metal Chelators—Fig. 5 shows the inhibitory effect of α- and m-phenanthroline on dipeptidase activity. One microliter of a solution of α- or m-phenanthroline of the required concentration was added to 6 μl of a dipeptidase solution (specific activity 2500) which had been diluted 15 times with a 2 mg per ml bovine serum albumin solution in 20 mM potassium phosphate, pH 8.2. The concentrations plotted refer to the molarities in the previously incubated mixture. After 8 min of prior incubation at 22°C, 7 ml of an Ala-Gly solution (0.1 M) were added, and the mixture was incubated for 5 min at 40°C. The values with initial activity were obtained when 1 ml of water was substituted for the phenanthroline solutions. The values obtained with α-phenanthroline are plotted O--O; □--□, m-phenanthroline; Δ--Δ represent data from an enzyme preparation at the (NH₄)₂SO₄ step (specific activity 12) treated with α-phenanthroline. In this case, the enzyme preparation was diluted 52-fold with 20 mM potassium phosphate buffer, pH 8.2, containing 0.25 M sucrose.

The zinc values are discussed below.

Stability—The stability of the dipeptidase is described in Table II. Except for the requirement of added sucrose for stability to freezing, the enzyme is completely stable until the Sephadex step, when dilution in albumin is compulsory for stability during the assay at 40°C. After DEAE-cellulose chromatography, a further degree of instability is evidenced by intolerance to dialysis, to freezing, and to further chromatographic steps, such as rechromatography on DEAE-cellulose or Sephadex.

No increase in stability was achieved by addition of MgCl₂ or tRNA (which was removed in the DEAE-cellulose step), or replacement of phosphate buffer by triethanolamine.
FIG. 6. Difference spectra of o-phenanthroline as compared with metal o-phenanthroline and dipeptidase-o-phenanthroline solutions. The absorbance of a ZnCl₂ (0.1 mM)-o-phenanthroline (Zn-OP) (0.1 mM) solution was read with a blank of o-phenanthroline (0.1 mM), ΔΔ. Similarly, the absorbance of a MgCl₂ (10 mM)-o-phenanthroline (Mg-OP) (0.2 mM) solution was read with a blank of o-phenanthroline (0.2 mM). The absorbance of a solution of dipeptidase (specific activity 2200)-o-phenanthroline (Dipeptidase-OP) (0.37 mM) mixture was read against water subtracted from the absorbance of the dipeptidase solution read against water with a blank of o-phenanthroline (0.2 mM), ΔΔΔ. The absorbance of a MgCl₂ (0.1 mM) solution was read with a blank of o-phenanthroline (0.37 mM) and the absorbance of the dipeptidase-o-phenanthroline (Dipeptidase-OP) (0.37 mM) mixture was read with a blank of o-phenanthroline (0.37 mM) and the absorbance of the dipeptidase solution read against water subtracted from the former values, O-Δ. The buffer for this enzyme preparation contained no magnesium.

15-min prior incubation (23°) with o-phenanthroline gave full inhibition. EDTA gave an inhibition curve similar to that of o-phenanthroline, but the concentration for 50% inhibition was 0.13 μM, in contrast to the 0.13 mM concentration of o-phenanthroline. This factor of 10⁴ difference between the two chelators was not caused by differences in ionic strength, inasmuch as the enzyme activity was not affected by 0.15 M NaCl or KCl. Histidine (50 mM) caused an 88% inhibition of the hydrolysis of Ala-Gly (12.5 mM) by the dipeptidase and (ma) dithiothreitol caused an 85% inhibition of the hydrolysis of Ala-Gly by the dipeptidase, whereas mercaptoethanol (5 mM) inhibited only 38%. The rough correlation of degree of inhibition by the thiols with chelating ability (40), rather than reducing power, leads to the conclusion that inhibition is due to metal chelation.

The technique of differential spectrophotometry (41) was used in order to detect whether a metal ion in the enzyme was chelated by o-phenanthroline. Fig. 6 shows the difference absorption curves of Zn⁺⁺ o-phenanthroline so compared with o-phenanthroline, Mg⁺⁺-o-phenanthroline as compared with o-phenanthroline, and dipeptidase (specific activity 2500, 0.86 mM) o-phenanthroline corrected for absorbance of enzyme and o-phenanthroline. The fact that the dipeptidase-o-phenanthroline curve resembled the metal-o-phenanthroline curves, with a major peak in absorbance in the 290 μM region and minor peaks at 327 and 343 μM, strongly suggests that the dipeptidase is a metalloenzyme. A similar experiment in which m-phenanthroline was substituted for o-phenanthroline resulted in the absence of a difference spectrum. Identification of the metal, however, could not be made with any certainty by comparison of the enzyme o-phenanthroline curve with those studied of the complexes of o-phenanthroline with Zn⁺⁺, Mg⁺⁺, Co⁺⁺, and Mn⁺⁺. The correspondence of the minor peaks in the longer wave length regions with those of the zinc-o-
was found at lower metal ion concentrations. The sensitivity of the dipeptidase to metal ions was studied with highly purified enzyme preparations. As seen from Table III, the majority of metal ions commonly found in biological materials are inhibitory to the hydrolysis of Ala-Gly (HzO), Gly-Gly (Co*), and Pro-Gly (Mn*). However, no significant activation was observed, the fact that a net value of zero magnesium was obtained, the ratio of moles of zinc concentration with highly purified enzyme (Fig. 3). On the other hand, there was a decrease in the ratio of moles of water in the case of Ala Gly, O-O ; mM CoCl₂ in the case of Gly-Gly, O-O ; mM MnCl₂ in the case of Pro-Gly, O-O. The control values were those obtained at zero time.

The effect of added metal ions on dipeptidase activity—As seen from Table III, the majority of metal ions commonly found in biological materials are inhibitory to the hydrolysis of Ala-Gly at concentrations less than millimolar. No significant activation was found at lower metal ion concentrations. The sensitivity of the dipeptidase to metal ions was studied in experiments in which compounds which act as better buffers at pH 8.2 were substituted for phosphate. Tris, N-ethylmorpholine, HEPES, sodium 5, 5'-diethyl barbiturate and triethanolamine (all at 0.02 M) were inhibitory to varying extents. Less inhibition was observed with highly purified buffers. This result suggested that the inhibition was due to trace metal contamination in the buffers.

When the effect of millimolar concentrations of selected divalent metal ions was studied with highly purified enzyme preparations and various substrates, a complex pattern of inhibition and activation emerged. As seen from Table IV, although the hydrolysis of Ala-Gly was inhibited by Co**, Mn**, and Cd**, the hydrolyses of Gly-Thr, Gly-Ser, and especially Gly-Gly were activated by Co**, and those of Pro-Gly and Hyp-Gly by Mn**. Since Gly Gile dipeptidase is known to be activated by Co** (42) and prolipase (iminodipeptidase) by Mn** (43), it seemed that at least three dipeptidases might be present in the enzyme preparations. Nevertheless, these activities could not be separated by Sephadex G-150 gel filtration (before or after the DEAE-cellulose step) or by DEAE-cellulose chromatography with a very slow phosphate gradient. Activities toward the three substrates, when normalized for their differences in hydrolysis velocity, were completely superimposable around the peaks. Moreover, the ratios of activities throughout purification were identical. The optimum pH for hydrolysis of Gly-Gly, Co**-activated, was 8.2, similar to that of Ala-Gly. In addition, as seen in Fig. 7, when an enzyme preparation (specific activity 600) was held at 30° for 24 hours, the rates of loss in activity toward Ala-Gly (H₂O), Gly-Gly (Co**), and Pro-Gly (Mn**) were approximately the same for 24 hours. Therefore, no evidence has been obtained thus far for separate enzymes.

**Specificity Studies and Kinetics**—The enzyme is a true dipeptidase (3, 4) in that it hydrolyzes only L-α-dipeptides with a free amino and carboxyl group. Compounds which are not hydrolyzed are Gly-α-Leu, β-Ala-Gly, L-phenylalanineamide, L-Leu-
Gly-Ala. The \( l/(S) \) values are given in terms of molar concentrations and the \( \frac{1}{v} \) values in pmoles per ml per min. An enzyme preparation of specific activity 758 was diluted 8-fold for the Ala-Gly assays and 4-fold for Gly-Ala. Details of methods used are given above Table V.

Leu, and L-Ala-Gly-Gly. The dehydropeptides, Gly-dehydro-L-Ala (0.24 mM) and Gly-dehydro-L-Phe (50 \( \mu \)M), are split 0.03% and 0.02% as fast as Ala-Gly (50 \( \mu \)M), respectively.

The availability of a spectrophotometric assay permitted the determination of the kinetic parameters of the enzyme for a variety of aliphatic dipeptides. The values obtained are listed in Table V in the order of decreasing \( V'_{\text{max}} \). Significant enzyme inhibition was observed with many substrates in the 3 to 50 \( \mu \)M range, the concentration at which inhibition occurred being lower, the lower the \( K_m \). For instance (Fig. 8), the hydrolysis of Ala-Gly (\( K_m \) 2.5 \( \mu \)M) is inhibited by substrate concentrations over 25 \( \mu \)M, whereas no inhibition is observed at 50 \( \mu \)M Gly-Ala (\( K_m \) 22 \( \mu \)M).

A molecular activity (micromoles of Ala-Ile hydrolyzed at 40° per min per \( \mu \) mole of enzyme) of \( 2 \times 10^6 \) can be calculated for the best substrate found thus far, Ala-Ile. This value is based on the \( V'_{\text{max}} \), the specific activity of 2,600 with Ala-Gly as substrate and a molecular weight of 85,000. Since the assumptions used include that of a pure enzyme, the molecular activity would be even higher if the dipeptidase was contaminated with extraneous protein.

**Discussion**

The choice of Ehrlich-Letterre mouse ascites carcinoma cells as the enzyme source was dictated by the reproducibility of a constant single cell type (avoidance of isoenzymes), the high specific activity (3.5), and the restriction of the dipeptidase to the soluble cell fraction (36). In material commonly used for dipeptidase purification, hog kidney (10, 13) and E. coli (44), the enzymes are distributed between the particulate and soluble fractions.

Estimation of the purity of the ascites dipeptidase is difficult because of the liability of the most highly purified preparations. By the criterion of congruity of enzyme and protein peaks (after the last step, DEAE-cellulose chromatography), the dipeptidase is obviously not homogeneous over the peak. A stable preparation of a specific activity of 1,900 was estimated to be 80% pure, with the assumption that the strongest band after acrylamide gel electrophoresis was the enzyme. If this assumption was valid, enzyme of specific activity 2,600 should be homogeneous, but preparations originally of this specific activity showed multiple bands, possibly due to deamination. A preparation of this specific activity has a molecular activity of \( 2 \times 10^4 \) with the best substrate, Ala-Ile. This value is very high compared to other purified proteolytic enzymes; for instance, the 1,200 times purified prolidase (11) had a turnover number of \( 3.5 \times 10^9 \) moles of Gly-Pro hydrolyzed per min per 100 \( \mu \)g of enzyme of molecular weight 150,000. Therefore it was felt that studies of the properties of the ascites dipeptidase of specific activity values of 2,000 to 2,600 were worthwhile. For kinetic studies, a more stable enzyme of specific activity 1,360 was used. Since the relative rates at 50 \( \mu \)M substrate determined spectrophotometrically compared favorably with those determined titrimetrically with enzyme of 2,600 specific activity, the use of the former enzyme preparations seemed acceptable.

Comparison of the relative substrate specificity of the ascites dipeptidase with Ala-Gly-hydrolyzing enzymes from different sources can be made only on the basis of the high substrate concentration (17 or 50 \( \mu \)M) at which the hydrolyses were

![Graph](image-url)
measured. Although the relative data for a given enzyme may be misleading, because of our finding that aspartic peptidase is inhibited at these substrate levels, the comparison (Table VI) may shed some light on the plurality of dipeptidases. In addition to some of the dipeptides listed in Table V, Table VI includes dipeptides with aromatic residues. It should be emphasized that the aspartic enzyme has the highest specific activity toward Ala–Gly of the four enzymes and, on a relative substrate specificity basis, most closely resembles the enzyme purified from yeast autolysates by Cordonnier (19). Although the hog kidney particulate dipeptidase of Campbell et al. (13) has been crystallized, the specific activity of 69 is comparatively low. This enzyme and that from the same source purified in 1953 by Robinson, Birnbaum, and Greenstein (10) differ from the aspartic enzyme in their relatively rapid hydrolysis of dehydropeptides and cleavage of peptides with COOH-terminal d-amino acids, and therefore clearly represent a separate class of enzymes.

The question of multiplicity of enzymes within a given dipeptidase preparation has repeatedly arisen because Co²⁺ and Mn²⁺ specifically activate hydrolyses of some substrates and inhibit those of others. As seen from Table VI, the hydrolysis of Ala–Gly by both the aspartic and yeast enzymes is inhibited by Co²⁺, whereas the cleavages of Gly–Gly and Gly–Ser are strongly activated by this metal ion. As in the case of the aspartic enzyme, Cordonnier (19) was unable to separate these activities in the yeast preparations. Similarly, Capobianco and Veccia (14), who purified a dipeptidase 6000-fold from hog kidney, believe that they have a single enzyme, although Co²⁺ enhances the activity toward Gly–Gly and inhibits the hydrolysis of Gly–Leu and Leu–Gly. A precedent for the dependence of enzymatic specificity upon specific metals exists in the case of carboxypeptidase (45) in which, for example, replacement of Zn²⁺ by Co²⁺ abolishes peptidase activity while enhancing esterase activity. Since there is no evidence of separation of Ala–Gly, Gly–Gly, and Pro–Gly activities in the aspartic preparations either by chromatography or by inactivation, it is tentatively concluded that all three activities are functions of a single enzyme molecule. Purification of this enzyme to homogeneity would clarify this question.

Since carboxypeptidase (46) and leucine aminopeptidase (47, 48) are both zinc metalloenzymes and hydrolyze peptide bonds with adjacent free carboxyl and amino groups, respectively, it is not surprising that the aspartic dipeptidase is a metalloenzyme. Positive identification of the metal ion in the enzyme cannot be made at this time. Despite the correlation of zinc and enzyme concentrations around the early part of a chromatographic peak and the value of 0.9 ± 0.1 g atom of zinc per mole of enzyme of specific activity 2600, until the enzyme is proven homogeneous, it may shed some light on the plurality of dipeptidases. In addition to some of the dipeptides listed in Table V, Table VI includes dipeptides with aromatic residues. It should be emphasized that the aspartic enzyme has the highest specific activity toward Ala–Gly of the four enzymes and, on a relative substrate specificity basis, most closely resembles the enzyme purified from yeast autolysates by Cordonnier (19). Although the hog kidney particulate dipeptidase of Campbell et al. (13) has been crystallized, the specific activity of 69 is comparatively low. This enzyme and that from the same source purified in 1953 by Robinson, Birnbaum, and Greenstein (10) differ from the aspartic enzyme in their relatively rapid hydrolysis of dehydropeptides and cleavage of peptides with COOH-terminal d-amino acids, and therefore clearly represent a separate class of enzymes.

Tentative generalizations can be drawn from the kinetic data with different dipeptides (Table V). The substrates that are hydrolyzed most rapidly are those with bulky COOH-terminal R groups, especially those branching at the β carbon, isoleucine and valine. Most noteworthy are the low or zero values for the peptides with COOH-terminal polar groups and Gly–Gly. The enzyme appears to prefer small NH₂-terminal R groups. In general, NH₂-terminal alanyl substrates have lower Kₘ values than the corresponding NH₂-terminal glycyl peptides. Results with such peptides as Leu–Ala and Val–Gly indicate that even greater bulk in the NH₂-terminal amino acid causes a further lowering of the Kₘ. Although both Ala–Gly and Gly–Ala have the same V_max, the markedly higher Kₘ for Gly–Ala indicates that this substrate interacts less well with the active site of the enzyme.

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