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RENAL DIPEPTIDASE: LOCALIZATION AND INHIBITION

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SUMMARY

The principle locus of porcine renal dipeptidase activity has been found to be the microsomal fraction of kidney cortex. When the microsomal preparation was incubated with the enzymes, chymotrypsin, trypsin, lipase, and ribonuclease, the peptidase was released to no greater degree than other protein components of this fraction. Treatment of the microsomal fraction with deoxycholate and sodium dodecyl sulfate led to the identification of the dipeptidase within the resulting ribosomal fraction.

Relatively large-scale isolation methods applied to the preparation of renal dipeptidase resulted in an enzyme of purity comparable to that reported in previous studies. The molecular weight of the purified peptidase was estimated by gel filtration on Sephadex G-200 to be approximately 90 000. The enzyme was shown to be competitively inhibited by phosphate and phosphate esters, and the inhibition demonstrated to be instantaneous and completely reversible upon dilution. A comparison of K_i values for the adenine series indicated that the effectiveness of inhibition was in the order ATP > ADP > AMP > inorganic phosphate. It is suggested that the phosphate inhibition occurs as a result of the formation of a ternary (peptidase–Zn–inhibitor) complex at the active site of the enzyme. Inhibition of renal dipeptidase by 1,10-phenanthroline was measurably time dependent and could not be completely reversed by dilution or by the addition of excess zinc ions. However, removal of chelating agent followed by dialysis against zinc containing buffers led to reversal of inhibition. It seems likely that the inhibitory effect of 1,10-phenanthroline is caused by the removal of zinc from the active site of the peptidase to produce an inactive apoenzyme. The native enzyme can be reconstituted with additional zinc to restore full activity. When the rate of peptidase-catalyzed hydrolysis of dipeptides was measured in the presence of the free amino acid components, inhibition of hydrolysis was clearly demonstrated. The amino acids with bulkier side chains were the more effective inhibitors regardless of their positions as N-terminal or C-terminal components of the substrate. There was observed a lack of esterase activity against amino acid and peptidyl esters indicating that the free carboxyl group is required for renal dipeptidase activity.

INTRODUCTION

The physiological role played by peptidases in the function of the kidney is generally unknown. One attempt to discover a physiological role for kidney peptidases is to find where in the kidney peptidase activity is concentrated with the expectation that the distribution of materials in the kidney reflects distribution of function. The isolation of renal dipeptidase from particulate fractions of porcine kidney has been described in earlier investigations[1,2]. The present work is directed toward the identification and determination of dipeptidase concentration in renal substructures and subcellular fractions.

Biological control of enzymic catalysis is often exerted by inhibitors of the enzyme at the substrate level. Furthermore, studies of chelate inhibition of metalloenzymes have been employed to determine the nature of the metal–enzyme interactions[3]. This paper describes kinetic analyses of the effect of three different types of inhibitors upon the rate of dipeptide hydrolysis catalyzed by renal dipeptidase.

EXPERIMENTAL

Distribution of renal dipeptidase in kidney sub-structures

Fresh hog kidneys were dissected to obtain cleanly separated sub-structures. The medulla was separated from cortex material following a mid-sagittal cut dividing the kidney into equal halves. In order to isolate the medullary components of kidney tubules, the medulla was frozen and then sliced. These slices were allowed to thaw under a dissecting microscope while the tubules were removed with a needle probe. Each sub-structure was homogenized in an equal amount by weight of 0.066 M phosphate buffer at pH 7.0 and 0°. The homogenate was centrifuged at 22 500 × *g* for 30 min in the Sorvall RC2-B refrigerated centrifuge at 0°. The precipitate was discarded, and the supernatant centrifuged at 105 000 × *g* in the Spinco Model L Preparative Ultracentrifuge for 1 h. The supernatant was discarded, and the precipitate was washed repeatedly until the wash solution gave a negative test for protein with 10% trichloroacetic acid. The washed precipitate was suspended in the minimum volume of 0.002 M Tris–HCl buffer at pH 8.0. The protein suspension was cooled to 0° and *n*-butanol at 0° was added to make the final volume 20% with respect to alcohol. The mixture was stirred vigorously for 30 min at 0°. Following solubilization the protein solution was dialyzed against 0.002 M Tris–HCl buffer at pH 8 until the odor of butanol was no longer detected. The solubilized peptidase was then assayed employing the standard glycyldehydrophenylalanine assay.

Distribution of renal dipeptidase in subcellular fractions of kidney cortex

Differential centrifugation of hog kidney cortex into subcellular components was performed according to the procedure of HOGEBOOM[4] as developed by ALLEN AND BEARD[5] for the treatment of renal tissue. In a typical fractionation fresh hog kidney cortex was cut into small pieces and then homogenized in 0.066 M phosphate buffer (pH 7.0) and 0.25 M sucrose for 5 min. The homogenizer was a smooth-wall glass vessel with a teflon pestle. Differential centrifugation was carried out in the Sorvall RC-2-B Refrigerated Centrifuge for speeds up to 22 500 × *g* and in the Spinco Model L ultracentrifuge at 105 000 × *g* according to the following schedule: nuclear

fraction, 270 × *g* for 10 min followed by two washes at 270 × *g* for 10 min; mitochondrial fraction, 3020 × *g* for 10 min followed by one wash at 2600 × *g* for 10 min; lysosomal fraction, 22 000 × *g* for 20 min; microsomal fraction, 105 000 × *g* for 1 h.

Differential centrifugation of the cortex homogenate was also carried out to obtain a composite preparation of nuclear, mitochondrial, and lysosomal fractions (22 500 × *g* for 30 min), a microsomal fraction (105 000 × *g* for 60 min), and a supernatant fraction.

Release of peptidase from microsomal fraction

Treatment with enzymes. The microsomal fraction of hog kidney cortex prepared as above after removal of heavier subcellular components was treated with various enzymes in an attempt to disrupt the microsomal fraction. The enzymes employed were pancreatic lipase, ribonuclease, trypsin, and chymotrypsin. The enzymes were separately mixed with microsomal fraction in 0.01 M Tris–succinate and 0.01 M magnesium acetate buffer at pH 7.6. The incubation mixtures were prepared on the basis of 50 μg of enzyme to 10 mg of microsomal protein, and they were allowed to incubate for 3 h at 30°. Following incubation each reaction mixture was centrifuged at 105 000 × *g* for 1 h. The pellet was subjected to solubilization with *n*-butanol prior to peptidase assay, and the supernatant was assayed directly. Appropriate controls were employed in which the microsomal fraction was treated with the same buffer under the same incubation conditions without addition of enzyme.

Treatment with detergents[6]. The microsomal preparation of kidney cortex was suspended in 0.01 M Tris–succinate and 0.01 M magnesium acetate buffer at pH 7.6. This suspension was adjusted to 0.5% deoxycholate and 0.2% sodium dodecyl sulfate. After standing for 45 min at 0°, NaCl was added to 1.0 M concentration, and the suspension was centrifuged at 105 000 × *g* for 90 min. The resulting pellet was solubilized by *n*-butanol prior to peptidase assay, and the supernatant was assayed directly. A control preparation was analyzed which had been treated under the same conditions without addition of detergents.

Purification of renal dipeptidase

Isolation of the purified enzyme from hog kidney cortex was carried out by procedures including solubilization with *n*-butanol, $(NH_4)_2SO_4$ precipitation, CM-cellulose chromatography and Sephadex gel filtration. Details regarding these techniques have been previously reported[1,2]. The gel filtration step was performed using a large (5.0 cm × 91.0 cm) column, and the procedure was standardized for comparison of the gel filtration properties of the peptidase with that of proteins of various molecular weights. Zinc analyses were carried out by atomic absorption spectroscopy using procedures described for the enzyme by RENÉ AND CAMPBELL[2]. The purified peptidase was examined for heterogeneity by analytical ultracentrifugation in the Spinco Model E ultracentrifuge at 59 780 rev./min in 0.02 M Tris–HCl buffer at pH 8.0. Polyacrylamide-gel electrophoresis experiments were performed using the disc electrophoresis method described earlier[2].

Renal dipeptidase assays

Spectrophotometric assay. A spectrophotometric peptidase assay was employed in which the rate of enzyme-catalyzed hydrolysis of the unsaturated dipeptide, glycyl-

dehydrophenylalanine, was measured by observing the fall in absorbance at 275 nm of a solution of $5.00 \cdot 10^{-5}$ M glycyldehydrophenylalanine as described previously[2].

Inhibition by phosphate and phosphate esters. The inhibition of peptidase by inorganic phosphate and by phosphate esters was measured by employing the spectrophotometric assay in 0.025 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer (TAPS) at pH 7.60. The inhibitor constants for inorganic phosphate and phosphate esters were determined by a study of the rate of hydrolysis as a function of substrate concentration over the range $8.00 \cdot 10^{-5}$ to $1.5 \cdot 10^{-3}$ M glycyldehydrophenylalanine. The enzyme concentration employed was 2.68 μg/ml in the assay mixtures. The time dependence of phosphate inhibition was determined by measuring the residual peptidase activity in 10^{-3} M phosphate at various times from initial mixing to 120 min. The effect of dilution upon phosphate inhibition was observed by diluting the inhibited peptidase (10^{-3} M phosphate) 25-fold and 50-fold with 0.002 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer prior to assay.

Inhibition by 1,10-phenanthroline. The effect of 1,10-phenanthroline upon peptidase activity was determined by using the spectrophotometric assay in 0.002 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer at pH 7.6. Time and temperature dependence of 1,10-phenanthroline inhibition were measured by incubating the enzyme with 10^{-4} M and 10^{-3} M solutions of inhibitor followed by assay of aliquots of the reaction mixtures at various times. Reversal of 1,10-phenanthroline inhibition by dilution was studied by dilution of a peptidase preparation which had been incubated in 10^{-3} M 1,10-phenanthroline at 25° for 90 min. A 50-fold dilution of the inhibited enzyme was carried out by the addition of 0.002 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer at pH 7.6 with and without the presence of 10^{-3} M $ZnCl_2$. Reversal of 1,10-phenanthroline inhibition by dialysis was performed by dialyzing a peptidase preparation which had been incubated in 10^{-3} M 1,10-phenanthroline at 25° for 90 min. The inhibited enzyme was dialyzed in succeeding stages as follows: (1) against 0.002 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer at pH 7.6 for 8 h with three changes of buffer; (2) against 0.002 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer containing 10^{-3} M $ZnCl_2$ for 8 h; (3) against 0.002 M tris(hydroxymethyl)methylaminopropane sulfonic acid buffer (pH 7.6) which did not contain added zinc for 2.5 days. The effect of substrate concentration upon 1,10-phenanthroline inhibition was determined by measuring the rate of hydrolysis produced by the inhibited enzyme (10^{-4} M and 10^{-5} M 1,10-phenanthroline for 70 min) over the concentration range $8.00 \cdot 10^{-5}$ to $1.5 \cdot 10^{-3}$ M glycyldehydrophenylalanine. The final concentration of peptidase in the assay mixtures was 6.7 μg/ml.

Titrimetric assay. The rate of peptidase-catalyzed hydrolysis of saturated dipeptides, amino acid esters, and dipeptide esters was followed by potentiometric titration using a method reported by BRYCE AND RABIN[9]. If $\bar{h}$ is the average number of ionizable protons bound/mole of ligand and $\Delta\bar{h}$ is the number of moles of protons required to keep the pH constant for complete hydrolysis of one mole of substrate, then for the typical peptide, L-alanylglycine (AG)

$$\Delta\bar{h} = \bar{h}_A + \bar{h}_G - \bar{h}_{AG} - 1$$

The $\bar{h}$ values were calculated from the pK's of the ionizable groups on the appropriate ligands and the pH of the assay system. The pK values of ligands which

were not available in the literature were determined by potentiometric titration according to the method previously reported by CAMPBELL *et al.*[8]. The μmoles of protons added to maintain constant pH was measured as a function of time and compared with the $\Delta\bar{h}$ for complete hydrolysis to determine the μmoles peptide hydrolyzed/min. In the case of ester substrates standardized NaOH was employed as titrant. Enzyme concentration was measured by the method of LOWRY *et al.*[7] to obtain values for μmoles peptide hydrolyzed/min per mg enzyme.

MATERIALS

Glycyldehydrophenylalanine was synthesized by methods previously described[8]. Lysozyme, ovalbumin and alkaline phosphatase were purchased in the crystalline form from Worthington Biochemical Corporation. Hemoglobin and γ-globulin were purchased from the Sigma Chemical Company. The saturated dipeptides and amino acids were all purchased from Mann Research Laboratories and were reported to be chromatographically pure in two solvent systems.

RESULTS

Localization of renal dipeptidase

The data in Table I demonstrate that renal dipeptidase is found in the cortex portion of the hog kidney. No evidence of activity as measured by the spectrophoto-

TABLE I

DISTRIBUTION OF RENAL DIPEPTIDASE IN KIDNEY SUBSTRUCTURES

Substructure	*Tissue wt. (g)*	*Protein (mg/ml)*	*Total protein (mg)*	*Enzyme units**	*Units/g tissue*
Cortex	0.50	0.278	2.59	0.052	0.103
Medulla					
Whole medulla	1.98	0.315	3.15	0	0
Medulla papilla	0.50	0.238	2.23	0	0
Medulla tubules	0.50	0.400	4.00	0	0

* μmoles glycyldehydrophenylalanine hydrolyzed per min.

metric assay over a period of 15 min was obtained in fractions obtained from medulla or tubular components of the medulla. The distribution of particulate renal dipeptidase is shown in Table II. When subcellular fractionation was carried out to separate the kidney cortex into a composite fraction (nuclear, mitochondrial, and lysosomal), the microsomal fraction and the soluble fraction, the peptidase was distributed in the composite fraction, 19.5%; the microsomal fraction 64.2%; and the soluble fraction, 16.3%. The results of attempts to release the peptidase activity from the microsomal fraction are presented in Table III. Treatment of the microsomal fraction with the detergents, deoxycholate and sodium dodecyl sulfate, resulted in the release of approximately 1% of the peptidase activity into the supernatant. This release of

TABLE II

SUBCELLULAR DISTRIBUTION OF RENAL DIPEPTIDASE

Fraction	*Total protein (mg)*	*Total units* × 100*	*Specific activity***	*Distribution (%)*
Nuclear	86.0	104	1.21	29
Mitochondrial	93.0	67	0.72	19
Lysosomal	137.1	48	0.35	13
Microsomal	83.1	138	1.66	39

* μmoles glycyldehydrophenylalanine hydrolyzed per min.
** Units/mg × 100.

activity was less than that produced by the control treatment which employed the same buffers without the presence of detergents.

Purification of renal dipeptidase

Examination of schlieren photographs of the sedimentation analysis of purified renal dipeptidase revealed the presence of only one symmetrical peak after 60 min at 59 780 rev./min, and polyacrylamide disc electrophoresis of the purified enzyme at pH 8.0 resulted in the appearance of only one protein band. These results are the same as those reported for earlier preparations using the same techniques[1,2]. The specific activity of the enzyme employed in the present report was assayed at 585 μmoles of glycyldehydrophenylalanine hydrolyzed/min per mg enzyme × 100. Analysis of zinc content of the purified enzyme gave values of 1.34 and 1.35 μg of zinc per mg of protein which confirms earlier reports[1,2] that 47 200 g of renal dipeptidase contain 1 g atom of zinc. Results obtained from the gel filtration of proteins of various molecular weights on the Sephadex G-200 column were treated according to the method of ANDREWS[10]. A plot of elution volume *vs.* $\log_{10}$ molecular weight is presented in Fig. 1. The molecular weight of renal dipeptidase estimated from these data is approximately 90 000.

TABLE III

ENZYMIC RELEASE OF RENAL DIPEPTIDASE FROM MICROSOMAL FRACTION

Enzyme	*Fraction*	*Total protein (mg)*	*% Protein solubilized*	*Peptidase units*	*% Peptidase released*
Lipase	Soluble	13.2	31	0.53	28
	Pellet	29.2		1.37	
Trypsin	Soluble	6.9	19	0.27	18
	Pellet	29.4		1.23	
Ribonuclease	Soluble	6.6	29	0.26	27
	Pellet	16.2		0.70	
Chymotrypsin	Soluble	6.6	21	0.27	22
	Pellet	24.4		0.98	
Control	Soluble	8.8	21	0.35	20
	Pellet	32.6		1.39	

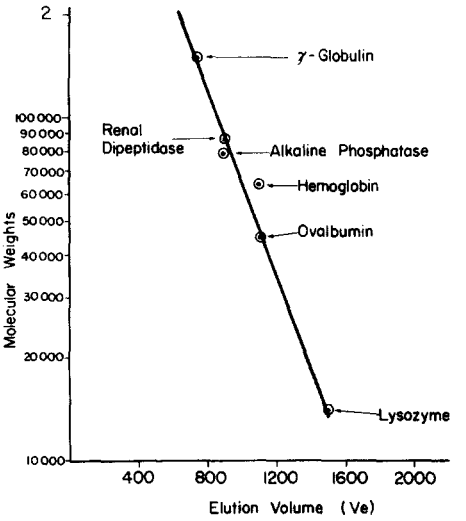

Fig. 1. Estimation of the molecular weight of renal dipeptidase by gel filtration. The gel employed was Sephadex G-200. The column dimensions were 5.0 cm × 91.0 cm, and the flow rate was 10 ml/h at 8°. Molecular weights of standard proteins employed are: lysozyme 13 900; ovalbumin 45 000; hemoglobin 64 000; alkaline phosphatase 80 000; γ-globulin 150 000.

Inhibition by phosphate and phosphate esters

The effect of substrate concentration upon phosphate inhibition of peptidase-catalyzed hydrolysis is shown in the LINEWEAVER–BURK[11] plots presented in Fig. 2. The reliability of the K_i values obtained from the spectrophotometric assay has previously been established to be within 10% (refs. 2, 12). The inhibition constants of phosphate and nucleoside phosphate esters are listed in Table IV. An independent

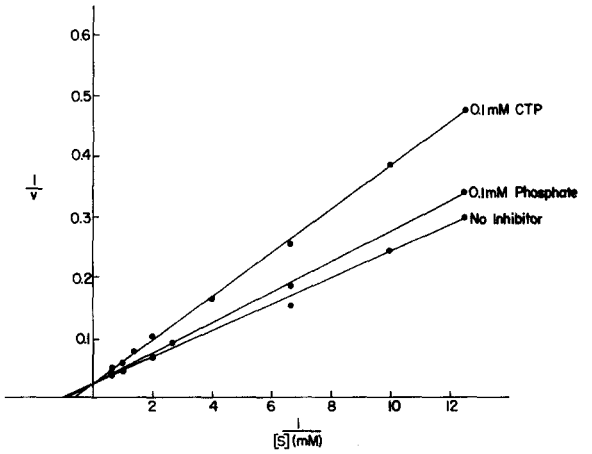

Fig. 2. Lineweaver–Burk plots of inhibition of renal dipeptidase by phosphate and cytosine triphosphate (CTP). Reactions were followed by measuring the fall in absorption at 275 nm of appropriate concentrations of glycyldehydrophenylalanine in the presence of 2.68 μg/ml enzyme in the reaction mixture. All reactions were observed at pH 7.60 in 0.025 M tris(hydroxymethyl)-methylaminopropane sulfonic acid buffer at 35°. Velocity, v, is given in μmoles of glycyldehydrophenylalanine hydrolyzed/min per mg enzyme × 100.

TABLE IV

INHIBITION OF RENAL DIPEPTIDASE BY PHOSPHATE AND PHOSPHATE ESTERS

Inhibitor	$K_i \times 10^4$ *(M)*	*Inhibition* (%)*
Phosphate	28	7
AMP	15	24
ADP	12	32
ATP	9	35
UTP	7	36
GTP	5	45
CTP	4	46

* Substrate concentration $5 \cdot 10^{-4}$ M and inhibitor concentration $5 \cdot 10^{-4}$ M.

determination of the K_i for phosphate inhibition obtained by varying the concentration of the inhibitor according to the method of DIXON[13] gave a value of $31 \cdot 10^{-4}$ in agreement with the value presented in Table IV. In time dependence studies it was determined that 10^{-3} M phosphate at pH 7.6 inhibited the enzyme-catalyzed hydrolysis of $5 \cdot 10^{-5}$ M glycyldehydrophenylalanine 44% upon initial addition of inhibitor and that this degree of inhibition did not change after incubation of the

TABLE V

INHIBITION OF RENAL DIPEPTIDASE BY 1,10-PHENANTHROLINE

Time dependence of inhibition

Incubation time (min)	*% Residual activity*	
	10^{-4} M 1,10-phenanthroline	*10^{-3} M 1,10-phenanthroline*
5	72.8	41.5
15	66.2	30.5
30	57.4	14.9
60	50.3	0.0
120	50.3	0.0

Effect of dilution upon inhibition

Treatment of peptidase	*% Activity recovered*
Incubated with 10^{-3} M 1,10-phenanthroline (90 min)	0.0
50-fold dilution with buffer	7.5
50-fold dilution with 10^{-3} M $ZnCl_2$ in buffer	52.7

Effect of dialysis upon inhibition

Treatment of peptidase	*% Activity recovered*
Step 1. Incubation with 10^{-3} M 1,10-phenanthroline (90 min)	0.0
Step 2. Dialysis against buffer at pH 7.60	27.5
Step 3. Dialysis against 10^{-3} M $ZnCl_2$ in buffer	81.6
Step 4. Dialysis against buffer at pH 7.60	97.5

enzyme with inhibitor over a period of 120 min. When peptidase which had been inhibited by incubation in 10^{-3} M phosphate was diluted 25-fold, 96% of the original activity was recovered; and when 50-fold dilution was carried out 100% of the peptidase activity was recovered. No inhibition was observed when the enzyme was treated with sulfate, arsenate, adenine, guanine, cytosine or thymine. In these studies the reaction mixtures contained $5.00 \cdot 10^{-4}$ M substrate and 10^{-4} M inhibitor at pH 7.60.

Inhibition by 1,10-phenanthroline

The time dependence of the inhibition of renal dipeptidase by 1,10-phenanthroline is demonstrated by the data presented in Table V. The results of attempts to reverse 1,10-phenanthroline inhibition by dilution and by dialysis are also reported in Table V. Lineweaver–Burk plots presented in Fig. 3 show the effect of increasing

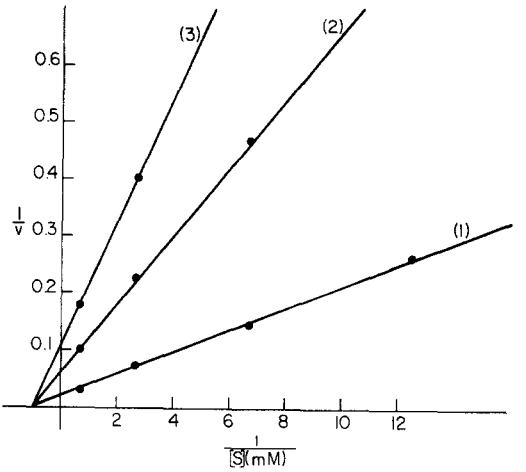

Fig. 3. Lineweaver–Burk plots of inhibition of renal dipeptidase by 1,10-phenanthroline. The peptidase at a concentration of 6.7 μg/ml was incubated in: (1) buffer with no 1,10-phenanthroline present, (2) 10^{-5} M 1,10-phenanthroline and (3) 10^{-4} M 1,10-phenanthroline at pH 7.6 in 0.025 M tris(hydroxymethylaminopropane sulfonic acid buffer prior to assay as described in Fig. 2.

substrate concentration upon 1,10-phenanthroline inhibition of enzyme-catalyzed hydrolysis. In these experiments the peptidase was incubated with inhibitor for 70 min prior to addition of substrate to insure that complete reaction of enzyme with inhibitor had taken place.

Inhibition by amino acids

The dissociation constants (pK_{NH_2}) of the dipeptides, glycyl-L-phenylalanine and L-phenylalanylglycine, were experimentally determined to be 8.15 and 7.55, respectively. The dissociation constants of the free amino acids and remaining dipeptides were obtained from data reported in ref. 14. Inhibition of renal dipeptidase by amino acid products is demonstrated by the data reported in Table VI. The reliability of the velocity determination estimated by repeated measurements of

TABLE VI

INHIBITION OF RENAL DIPEPTIDASE BY AMINO ACID PRODUCTS

Substrate (0.25 M)	*Inhibitor (0.050 M)*	*Velocity**	*Inhibition (%)*
L-Alanylglycine	—	119	
Glycyl-L-alanine	—	75.9	
	L-Alanine	48.1	36.4
	Glycine	60.7	20.0
Glycyl-L-leucine	—	105	
	L-Leucine	54.6	47.8
	Glycine	86.5	17.4
L-Leucylglycine		87.4	
	L-Leucine	48.7	50.0
	Glycine	66.2	23.3
Glycyl-L-phenylalanine	—	75.4	
	L-Phenylalanine	37.8	50.0
	Glycine	62.2	11.1
L-Phenylalanylglycine	—	27.1	
	L-Phenylalanine	15.4	43.4
	Glycine	21.6	20.0
Glycylglycine	—	61.5	
	Glycine	33.9	45.4

* μmoles substrate hydrolyzed/min per mg peptidase.

peptidase-catalyzed hydrolysis of glycylglycine was 10.7%. When the esters, glycine ethyl ester and glycylglycine ethyl ester, were employed as substrates in the titrimetric assay no change in pH over a period of 60 min was observed. These results indicate that renal dipeptidase does not exhibit esterase activity against amino acid esters or dipeptide esters, nor does it catalyze the hydrolysis of the peptide bond if the C-terminal group is blocked by the ester group.

DISCUSSION

The data presented in Table I indicate that renal dipeptidase activity is located in the kidney cortex. Within the limits of the sensitivity of the spectrophotometric assay the medulla appears to be devoid of dipeptidase activity. Since the medulla components analyzed would include collecting ducts, straight portions of proximal and distal tubules, and the ascending and descending limbs of the loops of Henle, it seems unlikely that appreciable amounts of peptidase are located within these substructures. The cortex, however, contains the renal corpuscles, the convoluted parts of the proximal and distal tubules, and the arched portions of the collecting ducts[15]. It is interesting that alkaline phosphatase, carbonic anhydrase, glutamic dehydrogenase and malic dehydrogenase are all zinc metalloenzymes that have been reported to be located in highest activity in the proximal and/or distal convolutions of mammalian kidney cortex[16,17].

It is observed in Table II that the distribution of particulate enzyme is rather diffuse, with the highest concentration of enzyme found in the microsomal fraction. The diffuse pattern obtained could have resulted from contamination of heavier fractions by adsorption of the microsomal enzyme during centrifugation. Evidence

for such contamination is provided by the composite fractionation procedure in which the microsomal content of peptidase increased from 39 to 64%.

The results of attempts to release the peptidase from the microsomal fraction presented in Table III indicate that the enzyme is firmly bound to this fraction. The fact that the percent total protein released in each case was essentially equal to the percent peptidase activity released suggests that the peptidase is bound to the microsomal fraction to the same degree as the structural proteins of this fraction. Treatment with detergents to obtain a ribosomal preparation also led to the identification of the major portion of the peptidase within the ribosomal fraction. Ribosomal peptidases have been reported previously in bacterial systems[6,18] and in preparations obtained from hog kidney[19,20]. The presence of peptidase in microsomal and ribosomal fractions in bacterial and mammalian preparations suggests that these enzymes may play some part in the biological role of these subcellular components.

The molecular weight of renal dipeptidase estimated to be approximately 90 000 from the gel filtration studies is consistant with recent preliminary observations in which the sedimentation equilibrium of renal dipeptidase in dilute solution has been studied by the methods of YPHANTIS[21].

The time-independent inhibition of renal dipeptidase by phosphate and phosphate esters suggests the rapid formation of an equilibrium complex between inhibitor and enzyme. Furthermore, the instantaneous reversibility of such an inhibitor–enzyme complex is indicated by the complete recovery of initial peptidase activity upon 50-fold dilution of inhibited enzyme with buffer. The form of the Lineweaver–Burk plots presented in Fig. 2 is characteristic of classical competitive inhibition in which the inhibitor competes with substrate for the active site of the peptidase. Failure of arsenate or sulfate to inhibit the enzyme provides some indication of specificity for the observed polyvalent anion inhibition. A comparison of the K_i values for phosphate inhibitors presented in Table IV shows that ATP is the most effective inhibitor of the adenine series which is in the order ATP > ADP > AMP > inorganic phosphate. The stabilities of the metal chelate compounds of this series[22] parallel their inhibitory effectiveness suggesting that inhibition is due to the formation of a ternary complex (peptidase–Zn–inhibitor) within the active site. Since the primary locus of peptidase activity in renal cortex seems to be in the microsomal fraction *(vide supra)*, inhibition of peptidase by nucleotides may exist as a possible control mechanism at the biological site of peptidase action in this tissue.

It is clear from the results shown in Table V that the inhibition of renal dipeptidase by 1,10-phenanthroline is time dependent and that it cannot be completely reversed by dilution or addition of excess zinc ions. These results are the opposite of those obtained for phosphate inhibition of peptidase in which the formation of a mixed peptidase–Zn–phosphate complex is proposed. The dialysis experiments in Table V indicate that essentially complete reactivation can be achieved after removal of chelating agent and addition of zinc ions. The 27.5% residual activity that is observed following removal of 1,10-phenanthroline (Step 2) suggests that all of the zinc has not been removed. Upon treatment with 10^{-2} M $ZnCl_2$, 81.6% is recovered, indicating that inhibition with excess zinc can occur. Essentially complete recovery of activity is obtained when the excess zinc is removed in the final dialysis.

Thus it seems likely that inhibition of renal dipeptidase by 1,10-phenanthroline occurs as a time-dependent removal of the metal ion from the protein to form an

apoenzyme. Evidence in favor of this mechanism of inhibition is also shown in Fig. 3 where the Lineweaver–Burk plots indicate what appears to be noncompetitive inhibition. However, plots of this type would also result from removal of zinc from native enzyme to produce a nonactive apoenzyme fraction in the reaction mixture:

$$\text{Peptidase–zinc} + \text{nOP} \rightarrow \text{peptidase} + \text{zinc–OPn}$$

The time dependency and inability to reverse the inhibition upon dilution support this interpretation rather than that of classical noncompetitive type inhibition.

According to the rate measurements reported in Table VI, the substrate most susceptible to peptidase catalyzed hydrolysis was L-alanylglycine. The effect of amino acids upon the rates of cleavage of dipeptides tested clearly demonstrates the inhibitory action of amino acids upon renal dipeptidase. Also it is evident that the amino acids with larger side chains are the more effective inhibitors regardless of their position as N-terminal or C-terminal components of the substrate. Attempts to treat kinetic data according to standard graphical procedures did not yield results amenable to mechanistic interpretation. Nevertheless the inhibition observed suggests that product inhibition of renal dipeptidase represents a possible means of physiological control for peptidase catalysis.

Previous studies have demonstrated a requirement for an unblocked amino group in renal dipeptidase substrates[1]. In the present investigation the observed lack of activity against amino acid and peptidyl esters indicates that the free carboxyl group is required for enzymatic catalysis. It is possible that the negatively charged carboxyl end of the dipeptide substrate binds to the same site as does the anionic competitive inhibitor.

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