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KINETIC PROPERTIES OF PULMONARY ANGIOTENSIN-CONVERTING ENZYME. HYDROLYSIS OF HIPPURYLGLYCYLGLYCINE

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Summary

Some of the kinetic properties of angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) purified from hog lung have been determined using hippurylglycylglycine as substrate. The effects of pH and ionic environment on enzyme activity are complex and interdependent. At 0.1 M NaCl, the pH-activity curve shows an abrupt decrease in V/K_m as the pH rises from 6 to 6.5, implying that ionization of a group in the enzyme with a pK in this range aids in binding of the substrate. Chloride is required for enzyme activity; there are two phases in the effect of NaCl. At both pH 6 and 8, the first phase (up to 0.1 M NaCl) is activation. The second phase (above 0.1 M) at pH 6 is inhibition, while at pH 8 there is further activation which appears to be dependent upon ionic strength rather than a specific Cl^- effect. Activation by cobalt and inhibition by EDTA are somewhat more effective at pH 6 than at pH 8. The nonapeptide inhibitor <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro is nearly equipotent at both pH 6 and 8, but Arg-Pro-Pro is more inhibitory at pH 8 than at pH 6.

Introduction

Angiotensin-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) has been shown to occur in many tissues [1,2]. Relatively large amounts of the enzyme can be extracted from lung, and evidence indicates that the enzyme is situated on the luminal surface, including the caveolae, of pulmonary capillary endothelial cells [3,4]. In vitro, the enzyme "activates" angiotensin I by converting it to angiotensin II and inactivates bradykinin by hydrolyzing the Pro^7-

Abbreviations: Hip, hippuryl; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

Phe^8 bond [5—7]. Angiotensin II, a potent vasoconstricting substance, has been implicated in the etiology of various forms of experimental and human hypertension [8]. Bradykinin, also a potent vasoactive peptide, is capable of producing contraction of extravascular smooth muscle, vasodilation and increased capillary permeability, and has been implicated in the control of blood flow during response to injury and in shock [9,10]. Both angiotensin II and bradykinin stimulate the release of adrenal medullary catecholamines [11]. Because the lungs receive the entire cardiac output, and because angiotensin I and bradykinin are metabolized during a single passage through the pulmonary vascular bed, the pulmonary angiotensin-converting enzyme is in a unique position to play an important role in regulating the amounts of vasoactive peptides which are presented to the arterial circulation [12,13].

The substrate specificity of angiotensin-converting enzyme requires a single free carboxyl group at the C-terminal end of the peptide. Peptides with C-terminal esters or amides, and peptides with C-terminal acidic residues are not hydrolyzed [14]. There is evidence for an optimal chain length. Peptides of 13 and 14 residues are hydrolyzed at much slower rates than are 9-residue peptides with a common C-terminal sequence [15,16]. The degree of chloride dependence of the enzyme varies with the substrate. Bradykinin is hydrolyzed in the absence of chloride, although at a slower rate than in the presence of chloride [6]. Hip-Gly-Gly, on the other hand, is not hydrolyzed in the absence of chloride [17]. Several authors have measured the K_m of the enzyme with different substrates [6,18—21], but there has been no detailed study of the effects of the ionic environment on the kinetic constants of the enzyme, nor has the role of chloride in the reaction mechanism been defined.

Using the enzyme purified from hog lung [17], we have investigated the kinetic properties of the hydrolysis of Hip-Gly-Gly and the effects of the ionic environment on enzyme activity. Hip-Gly-Gly was chosen as substrate because its hydrolysis has an absolute requirement for chloride, and it does not have an ionizing group with a pK in the range pH 5—9.

Experimental

Hip-Gly-Gly was synthesized as described previously [17], HEPES was from Calbiochem, Gly-Gly was from Nutritional Biochemicals Corporation, Arg-Pro-Pro was from CycloChemical, and synthetic <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro was a gift from Dr. J.W. Ryan, Papanicolaou Cancer Research Institute. Angiotensin-converting enzyme was purified from hog lung [17].

Enzyme assays were performed by a modification of the automated method of Skeggs et al. [22] in which the rate of peptide bond hydrolysis is measured continuously by the ninhydrin reaction. The incubation mixture contained enzyme in 0.05 M sodium HEPES buffer with NaCl and other additions at the indicated concentration. When not otherwise specified, the NaCl concentration was 0.1 M. After a 10-min preincubation at 37°C, the substrate, Hip-Gly-Gly (final concentration 1 mM, except where otherwise specified), was added, and the mixture (total volume 5 ml) was sampled continuously for 8 min at 37°C. Enzyme velocities were calculated directly from the slopes of the recordings and were expressed as nmol of Gly-Gly formed per min in the 5-ml incubation

mixture. In all experiments, initial rates were measured. Sufficient enzyme was used to establish rates in the range 0.5—5.0 nmol/min. In no case was more than 5% of the substrate consumed. The rate curves were all linear over the 8-min observation period.

The purified enzyme shows some loss of activity over a period of time when diluted or after repeated freezing and thawing. The values given for enzyme activity have not been corrected for these changes. Rather, experiments in which comparisons are to be made have been done with the same enzyme preparation at the same time.

In situations where high ionic strength caused a slight decrease in ninhydrin reactivity, corrections were made by running calibration curves (Gly-Gly) in the appropriate medium. The pH values of the incubations were measured at room temperature at the end of the 8-min sampling period. The kinetic constants V (μmol/min per mg protein) and K_m (mM) were obtained by modifying the direct linear plot method of Eisenthal and Cornish-Bowden [23] to allow calculated rather than graphic determination of the kinetic parameters. All numbers for V and K_m are averages of duplicate values. Each value was determined from six experimental points with a 5—10-fold range of substrate concentration, overlapping the K_m value.

Results

During the purification of the enzyme reported previously, the enzyme assays were carried out in phosphate buffer. Since that time, we have found that the enzyme activity is influenced by the nature and concentration of the buffer. Of the buffers tested at pH 8 (HEPES, Bicine, phosphate, barbital), highest activity was found with HEPES; enzyme activity with the other buffers at pH 8 was 30—40% of that with HEPES. Fig. 1 compares the pH-activity curve obtained in 0.05 M sodium HEPES buffer with that previously found in 0.05 M sodium phosphate buffer [17]. The marked difference in height and shape of the two curves is explained by the data presented in Fig. 2. Phosphate is an inhibitor of the reaction, the inhibition being much more pronounced at pH 6 than at pH 8. These effects were identical when the sodium phosphate buffers (which had been prepared from NaH_2PO_4 and Na_2HPO_4) were replaced by potassium phosphate buffers, or when sodium phosphate buffers were prepared from H_3PO_4 and NaOH. An exception to our general findings was that one lot of Na_2HPO_4 at 0.05 M gave 90% inhibition at pH 8. The concentration of NaH_2PO_4 (as added NaH_2PO_4) over the pH range is shown in Fig. 1. It appears that it is $H_2PO_4^-$ which is inhibitory.

The unusual "double-optimum" pH curve in HEPES buffer shown in Fig. 1 led us to determine the kinetic constants V and V/K_m over the pH range. The results shown in Fig. 3 with the complex V curve explain the shape of the pH-activity curve in Fig. 1. That is, the maximum near pH 6 (Fig. 1) is a derivation of the falling V/K_m and rising V curves according to the relationship

$$v_0 = \frac{V[S]}{K_m + [S]},$$

while the maximum near pH 8 reflects the V and the plateau in the V/K_m curve

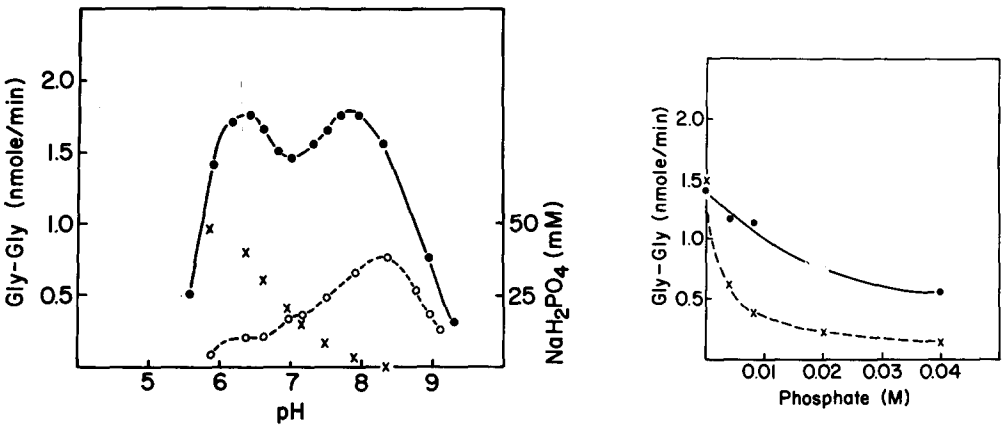

Fig. 1. Converting-enzyme activity as a function of pH: comparison of HEPES (●———●) and phosphate (○- - - - - -○) buffers. Phosphate buffers (0.05 M) were prepared from NaH_2PO_4 and Na_2HPO_4. The concentration of added NaH_2PO_4 is shown (X). Assays were performed as described in the text with 0.125 μg enzyme per tube.

Fig. 2. Inhibition of converting-enzyme activity by sodium phosphate at pH 6 (X- - - - - -X) and pH 8 (●———●). Enzyme assays were in HEPES buffers as described in the text. Phosphate was added as NaH_2PO_4 and Na_2HPO_4 in the following molar ratios: pH 6, 9 : 1; pH 8, 0.4 : 9.6. Assays were performed as described in the text with 0.125 μg enzyme per tube.

between pH 7.5 and 8. The inflection point between pH 6 and 6.5 in the V/K_m curve of Fig. 3 indicates that a group(s) in the free enzyme with a pK in this region (histidine?) must be protonated for optimal substrate binding to occur. The steepness of the V/K_m change indicates that more than one proton is involved.

Because the value for V/K_m is constant in the range pH 6.5 to 8, the V curve in this range may be interpreted in terms of dissociation of groups in an uncompetitive manner in the enzyme-substrate complex, but not in the free enzyme. That is, only the rate constant for conversion of enzyme-substrate

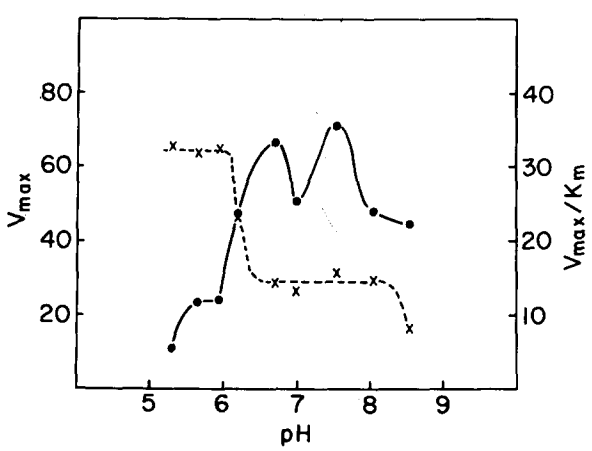

Fig. 3. Converting-enzyme activity as a function of pH. Enzyme activity expressed as V (●———●) or V/K_m (X- - - - - -X). Kinetic data were obtained as described in the text.

complex to products is being affected in this region of the pH curve. The unusual shape of the V curve with the minimum at pH 7 was confirmed in a separate set of experiments with a different batch of purified enzyme.

The NaCl-pH interrelationship is illustrated in Fig. 4, in which pH-activity curves are shown at different NaCl concentrations. In other experiments of this type, KCl was substituted for NaCl, and similar results were obtained. Because of the marked differences in response of the enzyme to pH and chloride concentration at pH 6 and pH 8 (Figs. 3 and 4), we have examined the effect of various conditions on enzyme activity at pH 6 and 8. Most of the subsequent work reported here was done at these two pH values in an attempt to distinguish between the "binding" and "hydrolytic" reactions.

Another unusual feature of this enzyme, which we showed in an earlier publication [17], is the shape of the chloride-activity curve at pH 8. The activation appears to be biphasic, that is, a plateau is reached in the region of 0.1 M NaCl. Then, as the NaCl concentration is increased to 1.0 M, further activation occurs. The chloride-activity relationship at pH 6 is also biphasic; the second phase in this case is inhibition rather than activation. These chloride-activity curves at pH 6 and 8 are shown on a semi-logarithmic plot in Fig. 5.

Resolution of these curves into V and V/K_m parameters revealed the following. At pH 6, both V and V/K_m increase as chloride concentration is increased from 0.001 M to 0.1 M. Above 0.1 M NaCl, both V and V/K_m reverse direction. These data (continuous change in both V and V/K_m) imply that it is the total enzyme (both free and substrate-bound) that is being affected by NaCl, causing

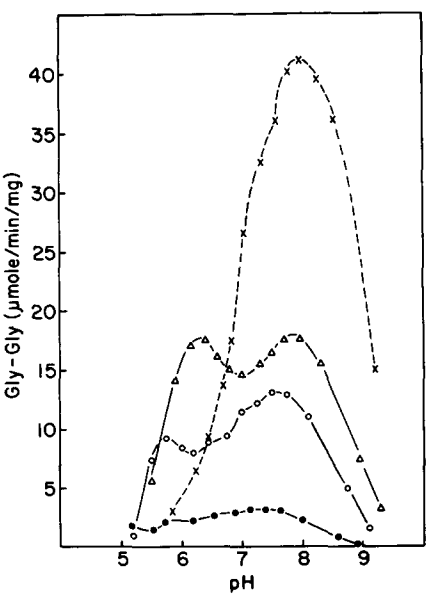

Fig. 4. Converting enzyme activity as a function of pH. NaCl concentrations: 0.001 M (●———●), 0.01 M (○———○), 0.1 M (△———△), 1.0 M (X------X). Velocities are expressed as specific activity to permit comparison of experiments performed with different amounts of enzyme.

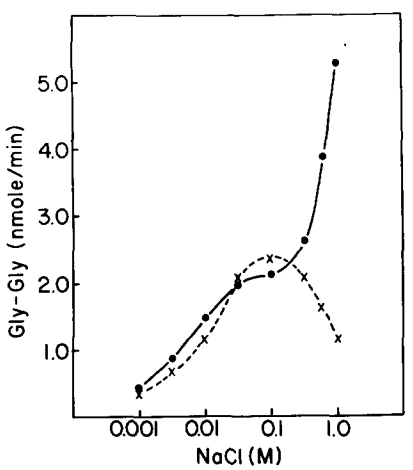

Fig. 5. Converting enzyme activity as a function of NaCl concentration at pH 6 (X------X) and pH 8 (●———●). Assays were performed as described in the text with 0.25 μg enzyme per tube.

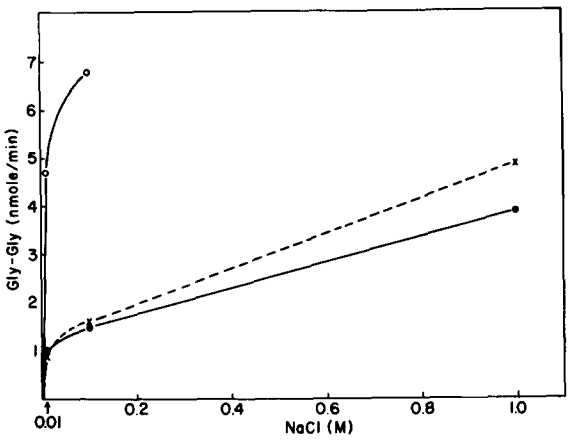

Fig. 6. Converting enzyme activity as a function of NaCl concentration at pH 8 in the absence of Na_2SO_4 (●———●), in the presence of 0.1 M Na_2SO_4 (X------X), and in the presence of 0.6 M Na_2SO_4 (○———○). Assays were performed as described in the text with 0.125 μg enzyme per tube.

in the first phase activation, and in the second phase inhibition. At pH 8, the first phase represents activation of the total enzyme as in the case of pH 6 above. The second phase at pH 8 shows further activation, again involving the total enzyme. At least qualitatively, substrate binding does not appear to affect the changes in the enzyme induced by chloride.

The presence of 1 M NaCl does not lead to an irreversible change in the enzyme. This was shown by dialyzing the enzyme for 15 h against 1000 vol. of 1 M NaCl. After 100-fold dilution with distilled water to reduce the chloride concentration, the enzyme was assayed at pH 8 in the presence of 0.1 and 1.0 M NaCl. All values for enzyme activity were the same as before dialysis.

The change in enzyme activity in the second phase of the chloride-activity curves (inhibition at pH 6 and activation at pH 8) appears to be due to an increase in ionic strength rather than to a specific effect of chloride. Evidence for this was obtained by replacing part of the NaCl with Na_2SO_4. The experi-

TABLE I

KINETIC CONSTANTS AT HIGH IONIC STRENGTH

The kinetic parameters V, K_m and V/K_m were determined as described in the text at the indicated pH and ionic strength. V and K_m are expressed as μmol/min per mg protein and mM respectively.

	V	K_m	V/K_m	Ionic strength (calculated)
pH 6: 0.1 M NaCl	30	0.9	33	0.1
1.0 M NaCl	20	1.3	15	1.0
0.1 M NaCl + 0.6 M Na_2SO_4	18	1.8	10	1.9
pH 8: 0.1 M NaCl	65	3.0	22	0.1
1.0 M NaCl	179	2.9	62	1.0
0.1 M NaCl + 0.6 M Na_2SO_4	310	2.6	119	1.9

TABLE II

EFFECT OF $CoSO_4$ ON CONVERTING ENZYME ACTIVITY

Converting enzyme activity was determined as described in the text at the indicated pH.

$CoSO_4$ (mM)	Hip-Gly-Gly hydrolysis (μmol/min per mg protein)	
	pH 6	pH 8
0.0	14	13
0.01	16	14
0.1	19	17
0.5	25	19
1.0	30	18

ments done at pH 8 (Fig. 6) show that addition of Na_2SO_4 at a final concentration of 0.6 M to incubations containing 0.01 or 0.1 M NaCl causes an increase in enzyme activity equal to or greater than that obtained with 1.0 M NaCl. The same experiments at pH 6 showed that inhibition similar to that found at 1.0 M NaCl can be obtained with 0.6 M Na_2SO_4 in the presence of 0.1 M NaCl. In the absence of NaCl, there is no hydrolysis of Hip-Gly-Gly at any Na_2SO_4 concentration (in the range 0.001—0.6 M) at either pH 6 or 8. Results similar to those shown in Fig. 6 were obtained with Li_2SO_4. The kinetic constants at high NaCl and Na_2SO_4 concentrations are compared in Table I. Using the calculated ionic strength values, the velocities at 0.1 M NaCl/0.6 M Na_2SO_4 appear to be extensions of the NaCl curves to concentrations above 1.0 M. Concentrations above 1.0 M NaCl or 0.6 M Na_2SO_4 could not be used because of interference with the AutoAnalyzer assay system.

The stimulation of converting enzyme by divalent metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}) at concentrations from 0.01 to 1.0 mM has been investigated at both pH 6 and 8. Only cobalt stimulated the enzyme activity at either pH (Table II). Extending the time of preincubation to 2 h had no effect. At both pH 6 and 8, the effect of $CoSO_4$ is to increase both V and K_m, indicating an effect of cobalt on breakdown of the enzyme-substrate complex.

EDTA is an inhibitor of the enzyme, being slightly more effective at pH 6 than at pH 8. At pH 6, complete inhibition of the enzyme activity was obtained at an EDTA concentration of 2 μM, whereas at pH 8, 6% of the activity remained even at an EDTA concentration of 10 μM. After dialysis of a concentrated sample of converting enzyme against 1 mM EDTA at pH 7.5, followed by dilution of the enzyme 1000-fold in glass-distilled water, 85% of the enzyme activity was present when assayed in 0.1 M NaCl at pH 8. This finding implies that EDTA must be present at the time of assay in order to be an effective inhibitor; dialysis against EDTA, in itself, is not sufficient to cause inhibition. It is possible, of course, that the reagents used in the assay contain traces of metal ions which cause reactivation of the enzyme after the EDTA is removed.

The most potent inhibitor of angiotensin converting enzyme in our experience is the nonapeptide <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, which occurs in the venom of *Bothrops jararaca* [24]. It is very effective at both pH values tested, giving I_{50} values of $1.2 \cdot 10^{-8}$ M at pH 6 and $1.5 \cdot 10^{-8}$ M at pH 8. Cheung and Cushman [19] reported that the K_i value for the nonapeptide was

six-fold higher at pH 7.5 than at pH 8.3 when Hip-His-Leu was used as substrate. Another inhibitor of converting enzyme is the N-terminal tripeptide fragment of bradykinin, Arg-Pro-Pro [25]. We have found that this tripeptide is a more effective inhibitor at pH 8 than at pH 6. At pH 8 the I_{50} value obtained for Arg-Pro-Pro is 2.5 μM, whereas at pH 6 only 40% inhibition can be obtained at an inhibitor concentration of 20 μM.

Discussion

Other than K_m values for different substrates, there is little information in the literature concerning the kinetic properties of angiotensin converting enzyme. The experimental data reported here show a very complex and interdependent role of pH and ionic environment in the regulation of the activity of the enzyme in vitro.

The resolution of the observed initial velocity into the two components V/K_m (proportional to velocity at low substrate concentration) and V (velocity at saturating concentration) allows us to look at the "binding" and "hydrolytic" reactions individually. The fact that the substrate, Hip-Gly-Gly, does not have a pK within the pH range studied becomes important here, because changes in V/K_m with pH can be interpreted in terms of ionization of the enzyme. Thus the sharp decrease in V/K_m which takes place between pH 6 and 6.5 leads to speculation that a histidine group(s) in the enzyme must be protonated for optimal binding of substrate to occur. When the data of Fig. 3 are plotted in the form pK_m vs. pH according to Dixon [26], the concave side of the curve faces upward, indicating the ionization of a group in the enzyme-substrate complex with a pK in the region of 6.5. That is, at pH values below 6.5, there is a net increase of one positive charge when the substrate binds to the enzyme; and at pH values above 6.5, there is no change in net charge on formation of the enzyme-substrate complex.

In general, the effect of chloride appears to be on the total enzyme, that is, both free and substrate-bound. This is true at both pH 6 and 8. Bakhle [14] has raised the question of a common role of chloride in dipeptidyl peptidase mechanisms, since chloride is also an activator of dipeptidyl aminopeptidase [27]. However, Gorter and Gruber [28] have observed that the V for cathepsin C (dipeptidyl aminopeptidase) is independent of chloride concentration, a finding which contrasts with our results for the dipeptidyl carboxypeptidase enzyme. In the case of another chloride-activated enzyme, dopamine-β-hydroxylase, a shift to higher pH optimum in the presence of chloride was interpreted in terms of binding of chloride to a basic group adjacent to the active site. No evidence was found for chloride-induced conformational change in the enzyme [29]. Although the biphasic nature of the chloride-activation curve was noted previously [17], the importance of ionic strength in the second phase of chloride activation at pH 8 was not appreciated at that time. The ionic strength effect at high concentration of NaCl or Na_2SO_4 could be due to major conformational changes in the enzyme which affect both free and substrate-bound enzyme. Oshima et al. [30] have studied the ultraviolet spectra of the converting enzyme purified from bovine kidney cortex. Their results indicate different conformations for the enzyme in the presence and absence of NaCl; addition of

NaCl to a concentration of 0.2 M leads to increased exposure of tryptophan and tyrosine residues.

In vitro, the K_m for angiotensin-converting enzyme with bradykinin as substrate is lower by at least a factor of ten than the K_m with angiotensin I [6]. Under these conditions, bradykinin would be "favored" at physiological substrate concentrations which are well below K_m for both peptides. Because of the sensitivity of enzyme activity toward pH and ionic composition, it is possible that these factors, within the micro-environment of the endothelial cell surface and caveolae, could exert a controlling influence on the reaction rates or specificity of the enzyme as it exists in the lungs.

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