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INHIBITION OF ANGIOTENSIN CONVERTING ENZYME WITH N^{α}-PHOSPHORYL-L-ALANYL-L-PROLINE AND N^{α}-PHOSPHORYL-L-VALYL-L-TRYPTOPHAN

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SUMMARY

The inhibition constants (K_i) and modes of inhibition have been determined for $N\alpha$-phosphoryl-L-alanyl-L-proline and $N\alpha$-phosphoryl-L-valyl-L-tryptophan against rabbit pulmonary angiotensin converting enzyme (dipeptidyl carboxpeptidase, E.C. 3.4.15.1). Phosphorylalanylproline and phosphorylvalyltryptophan are noncompeptitive inhibitors of the hydrolysis of hippuryl-L-histidyl-L-leucine in 100 mM phosphate buffer at pH 8.3 with K_i's of 9 nM and 51 nM respectively. In 50 mM Tris-hydrochloride buffer at pH 7.5, however, phosphorylalanylproline is a competitive inhibitor of the hydrolysis of hippuryl-L-histidyl-L-leucine with a K_i of 1.4 nM, and thus is one of the most potent inhibitors known for this enzyme.

INTRODUCTION

Angiotensin converting enzyme is a dipeptidyl carboxpeptidase (E.C. 3.4.15.1) which *in vivo* catalyzes the hydrolysis of the COOH-terminal dipeptide L-histidyl-L-leucine from the nonpressor decapeptide antiotensin I to produce the pressor octapeptide angiotensin II (1). Based on the similarity of this zinc dependent exoprotease to the well characterized zinc enzyme carboxypeptidase A, Cushman, et al., (2) proposed a schematic model of the active site of the enzyme which accounts for most of its known catalytic properties (see Figure 1). This model enabled them to rationally design a number of powerful inhibitors of the enzyme, culminating in D-3-mercapto-2-methylpropanoyl-L-proline which is a competitive inhibitor with a K_i of 1.7 nM and which is an orally active antihypertensize agent (3).

The structure-activity relations established for competitive dipeptide inhibitors (4), and the powerful competitive inhibition of the zinc endoprotease thermolysin by the N^{α}-phosphoryldipeptide phosphoramidon (5) suggested that N^{α}-phos-

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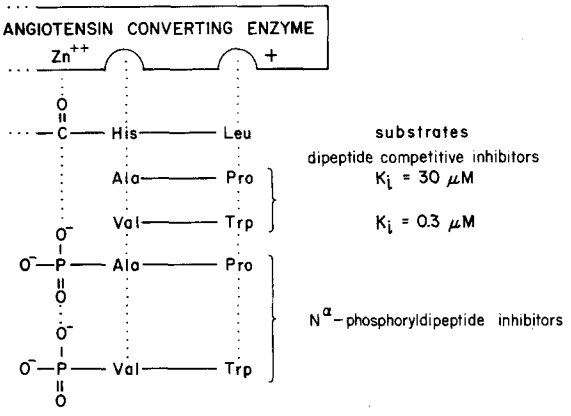

Figure 1: A schematic model of the active site of angiotensin converting enzyme adapted from Cushman, et al (2). The vertical dots indicate interactions known to be important for binding and/or hydrolysis of inhibitors and substrates by the enzyme. Using the model, and the known coordination of one phosphate oxygen anion of phosphoramidon by zinc in the x-ray structure of the phosphoramidon-thermolysin complex (6), a competitive mode of binding for phosphoryl-dipeptides is indicated. The K_i's of the dipeptide competitive inhibitors are from Cheung, et al (4).

phoryl-L-alanyl-L-proline and N^{α}-phosphoryl-L-valyl-L-tryptophan would be competitive inhibitors of converting enzyme (see Figure 1). However, under the assay conditions employed by the Squibb group (7,8) for the substrate hippuryl-L-histidyl-L-leucine (phosphate buffer at pH 8.3, assay A in Materials and Methods), the phosphoryldipeptides were found to be noncompetitive (or mixed) inhibitors with K_i phosphoryl-L-alanyl-L-proline < K_i phosphoryl-L-valyl-L-tryptophan, the reverse of that predicted in Figure 1. In Tris-hydrochloride buffer at pH 7.5 (assay B in Materials and Methods), N^{α}-phosphoryl-L-alanyl-L-proline was a competitive (or possibly mixed) inhibitor, but N^{α}-phosphoryl-L-valyl-L-tryptophan was noncompetitive against hippuryl-L-histidyl-L-leucine as substrate. With angiotensin I as substrate, both inhibitors were competitive.

Materials and Methods

Pure Angiotensin converting enzyme (9) from rabbit lung was a gift from Dr. Richard L. Soffer, Department of Medicine and Biochemistry, New York Hospital, Cornell University, New York 10021. [Asn^1, Val^5]angiotensin I was synthesized as previously described (10). Nα-Phosphoryl-L-alanyl-L-proline and Nα-phosphoryl-L-valyl-L-tryptophan were synthesized by a general method (5) and were greater than 90% homogeneous by thin layer chromatography on cellulose plates and by paper electrophoresis. Synthetic details will be reported in a subsequent publication. Concentrated stock solutions were prepared daily

in distilled water (final pH 9-11) since phosphoramidates are known to be unstable at neutral pH and below (11). 3-D-Mercapto-2-methylpropanoyl-L proline was the generous gift of Dr. Z.P. Horovitz of the Squibb Institute for Medical Research.

Four different assay conditions at 37° were employed. Hippuryl-L-histidyl-L-leucine was employed as substrate in 100 mM potassium phosphate buffer at pH 8.3, 300 mM in sodium chloride (9), (assay A), lit.K_m = 2.2(4), 2.3(9), or in 50 mM Tris-hydrochloride adjusted to pH 7.5 with sodium hydroxide, 300 mM in sodium chloride (assay B). Angiotensin I was employed as substrate in 100 mM potassium phosphate buffer, pH 7.5, 30 mM in sodium chloride (7) (assay C), lit.K_m = 0.05(7), or in 50 mM Tris-hydrochloride adjusted to pH 7.5 with sodium hydroxide, 30 mM in sodium chloride (9) (assay D), lit. K_m = 0.07 (9). Free hippuryl-L-histidyl-L-leucine was detected by the fluorescence assay (7). To establish the linearity of initial rate measurements, L-histidyl-L-leucine released as a function of time was determined for each assay condition without inhibitor present and a single time point selected for initial rate determination in the presence of inhibitors. This time-point was normally ten minutes for hippuryl-L-histidyl-L-leucine and 20 minutes for angiotensin I. K_m was determined from Lineweaver-Burke plots. Ki was determined from Dixon plots (12). For determination of all Ki's, enzyme concentration was much less than K_i to avoid mutual depletion of enzyme and inhibitor (13).

RESULTS

Figure 2 shows both Lineweaver-Burke and Dixon plots for inhibition of converting enzyme hydrolysis of hippuryl-L-histidyl-L-leucine in phosphate buffer at pH 8.3 (assay A) and Tris buffer at pH 7.5 (assay B) by N^{α}-phosphoryl-L-alanyl-L-proline. Table I gives the K_i's, and the modes of inhibition determined for the two inhibitors against the two substrates under each assay condition. 2-D-Mercapto-3-methylpropanoyl-L-proline was found to be a purely competitive inhibitor of converting enzyme against hippuryl-L-histidyl-L-leucine with a K_i of 1.4 nM (1.7 nM (2)) in assay A. Potassium phosphate was found to inhibit the hydrolysis of hippuryl-L-histidyl-L-leucine. It's K_i could not be determined since several modes of inhibition seemed to be occuring, but appeared to be in the range of 100 mM. Tris-hydrochloride was also found to inhibit the enzyme (Assay B) probably due in part to its chloride content at high Tris-hydrochloride concentrations (8). Its K_i appeared to be in the range of several hundred millimolar. Substrate inhibition was observed with hippuryl-L-histidyl-L-leucine (8) in both phosphate (assay A) and Tris-hydrochloride buffer (assay B) and prevented determinations of 1/V at high S. Loss of linearity prevented determination of 1/V at low S with the fixed time point assay. Determination of K_m (and K_i) was limited to a narrow range of S, and

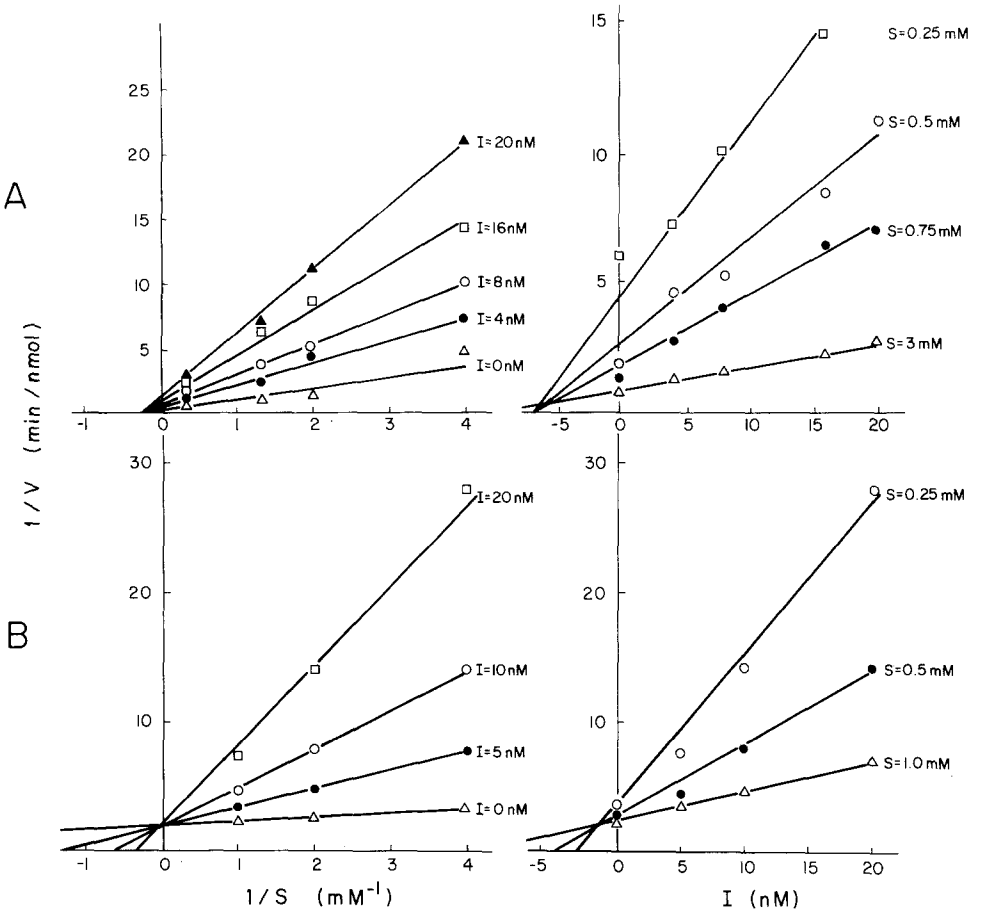

Figure 2: Lineweaver-Burke (1/V versus 1/S) and Dixon (1/V versus I) plots for inhibition of angiotensin converting enzyme catalyzed hydrolysis of hippuryl-L-histidyl-L-leucine in phosphate buffer at pH 8.3 (A) and Tris-hydrochloride buffer at pH 7.5 (B). Details of the assay conditions are given in Materials and Methods.

thus hippuryl-L-histidyl-L-leucine is probably not the most convenient substrate for accurately determining K_i's for converting enzyme.

Discussion

N^{α}-phosphoryl-L-alanyl-L-proline, with a competitive K_i of 1.4 nM in Tris-hydrochloride buffer at pH 7.5, is among the most potent inhibitors known for angiotensin converting enzyme catalyzed hydrolysis of hippuryl-L-histidyl-L-leucine. This inhibition is somewhat less potent (K_i = 9 nM) in phosphate buffer and appears to be noncompetitive under conditions where 3-D-mercapto-2-

Table I: Inhibition constants (K_i) for N^{α}-phosphoryl-L-alanyl-L-proline (PAP) and N^{α}-phosphoryl-L-valyl-L-tryptophan (PVT) against angiotensin converting enzyme.

Substrate			Inhibitor	
Peptide	Buffer	K_m (mM)	K_i PAP (nM)	K_i PVT (nM)
Hippuryl-His-Leu	pH 8.3 phosphate (A)	1.7	9, N[a]	51, N[a]
	pH 7.5 Tris (B)	0.5	1.4, C	12, N
Angiotensin I	pH 7.5 phosphate (C)	0.08	26, C	28, C
	pH 7.5 Tris (D)	0.03	6, C	7, C[a]

The assay conditions A, B, C, and D are given in Materials and Methods. K_i's were determined from Dixon plots only and did not differ significantly (within a factor of 2) from K_i's determined from Lineweaver-Burke plots. The previously reported K_i of 5 nM for N^{α}-phosphoryl-Ala-Pro and 95 nM for N^{α}-phosphoryl-Val-Trp- were from Lineweaver-Burke plots alone (14). N = noncompetitive, C = competitive.

[a]These modes of inhibition were assigned as competitive or noncompetitive, but could also be mixed.

methylpropanoyl-L-proline (SQ 14,225) is purely competitive. Therefore, the mode of binding of these inhibitors to the enzyme must be different in phosphate buffer. N^{α}-Phosphoryl-L-alanyl-L-proline is, however, a competitive inhibitor of the hydrolysis of angiotensin I in both phosphate and Tris-hydrochloride buffer, as predicted using the simple model presented in Figure 1. Based on the K_i's of the free dipeptides in Figure 1, N^{α}-phosphoryl-L-valyl-L-tryptophan should have a K_i about 100-fold lower than that of N^{α}-phosphoryl-L-alanyl-L-proline. Its K_i was found to be higher than that of N^{α}-phosphoryl-L-alanyl-L-proline against hippuryl-L-histidyl-L-leucine and the same as that of N^{α}-phosphoryl-L-alanyl-L-proline against angiotensin I. Therefore, the structure activity relations established for the relative potencies and modes of inhibition of free depeptide inhibitors (4) cannot be extended to the corresponding N^{α}-phosphoryl-dipeptides. This suggests the possibility that some other N^{α}-phosphoryl-dipeptide may be a more potent inhibitor than N^{α}-phosphoryl-L-alanyl-L-proline.

The K_i's of these N^{α}-phosphoryl-dipeptides against converting enzyme are in the range of those reported for the most potent N^{α}-phosphoryl-dipeptide inhibitors of thermolysin (5) and are up to ~700-fold better inhibitors of converting enzyme than the best N^{α}-monophenylphosphoryldipeptide inhibitor reported by B. Holmquist and B.L. Vallee (15). These results suggest the importance of two ionizable (or one ionizable, and one hydrogen bond donating) phosphate oxygen atoms, as found by Kam et al (5), for phosphoramidate inhibitors of thermolysin.

N^{α}-Phosphoryl-L-alanyl-L-proline blocks the pressor effect of angiotensin I in the rat pressor assay and reduces mean arterial pressure in rats made hypertensive by ligating the aorta between the two renal arteries (14).

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