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INHIBITION OF HOMOGENEOUS ANGIOTENSIN-CONVERTING ENZYME OF RABBIT LUNG BY SYNTHETIC VENOM PEPTIDES OF *BOTHROPS JARARACA**

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SUMMARY

Seven synthetic peptides, identical to those found in the venom of *Bothrops jararaca*, inhibited the peptidolytic activity of homogeneous (275-fold purified) angiotensin-converting enzyme from rabbit lung on [Ile^5]-angiotensin I (angiotensin I), and on hippuryl-L-histidyl-L-leucine (Hip–His–Leu), with I_{50} values ranging from 0.06 to 76 μM. The nonapeptide, SQ 20 881 (Pyr–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro), and a decapeptide, SQ 20 858 (Pyr–Asn–Trp–Pro–His–Pro–Gln–Ile–Pro–Pro), were competitive with both substrates (angiotensin I and Hip–His–Leu), but the pentapeptide, SQ 20 475** (Pyr–Lys–Trp–Ala–Pro) was a "mixed" competitive and noncompetitive inhibitor. The K_i values (enzyme–inhibitor dissociation constants) calculated for SQ 20 881, SQ 20 858 and SQ 20 475 did not depend significantly on the substrate employed, but were altered by differences in pH. At pH 7.5 with angiotensin I as substrate, the K_i values were: SQ 20 475, 0.09 μM; SQ 20 881, 0.8 μM; and SQ 20 858, 4.3 μM. At pH 8.3, with Hip–His–Leu as substrate, the corresponding values were lower: 0.06, 0.10 and 0.74 μM. The most potent of the inhibitors *in vitro*, SQ 20 475, could be cleaved and inactivated when incubated with homogeneous angiotensin-converting enzyme in the absence of other substrates and of the activator, Cl^-; the other six peptides could not serve as substrates for the enzyme. The nonapeptide, SQ 20 881, appears to be the best of the synthetic venom peptides for use in studying the role of the renin (EC 3.4.4.15)–angiotensin system in physiological and pathological processes.

Abbreviations: angiotensin I, [Ile^5]-angiotensin I; Hip–His–Leu, hippuryl-L-histidyl-L-leucine.

* A preliminary report describing some of the material in this paper was presented at the International Symposium on the Renin-Angiotensin-Aldosterone-Sodium System in Hypertension, Mont Gabriel, Quebec, September 30 to October 4, 1971 (ref. 33).

** This pentapeptide was first synthesized by Stewart *et al.*[8] and named BPP 5a.

INTRODUCTION

Venom of a Brazilian pit viper, *Bothrops jararaca,* contains several small peptides that strongly inhibit angiotensin-converting enzyme, a dipeptide-releasing carboxypeptidase that "converts" the decapeptide, [Ile^5]-angiotensin I (angiotensin I), to the vasopressor octapeptide, angiotensin II, and also inactivates the vasodepressor nonapeptide, bradykinin. A crude mixture of these venom peptides, termed bradykinin-potentiating factor by Ferreira[1], was shown to inhibit destruction of bradykinin by plasma, to potentiate several pharmacological effects of bradykinin (see ref. 2), and to inhibit the activity of angiotensin-converting enzyme in lung extracts[3], perfused lung[4], or intact lung *in vivo*[5].

Ferreira, Greene and their associates[2,6-8] isolated from *Bothrops jararaca* venom nine bradykinin-potentiating peptides that ranged in size from pentapeptide to tridecapeptide; each of these purified peptides was also an inhibitor of angiotensin-converting enzyme[9]. Ferreira *et al.*[2,8] determined the amino acid sequence of the pentapeptide, and synthesized it by the solid-phase method. Ondetti *et al.*[10] isolated six similar peptides from *Bothrops jararaca* by following their activity as inhibitors of angiotensin-converting enzyme; they determined the amino acid sequences and synthesized each of the peptides, which contained from 9–13 amino acids. Five undecapeptides with similar activities were isolated by Kato and Suzuki[11-15] from the venom of a Japanese pit viper, *Agkistrodon halys blomhoffii*; they determined the amino acid sequences of three of these peptides and synthesized two.

Various synthetic venom peptides have been tested for their ability to inhibit activity of angiotensin-converting enzyme in cell-free preparations[16-24], in intact tissues *in vitro*[25], and in whole animals[20,24,26-32]. In a preliminary communication[33] we described the inhibition of a pure angiotensin-converting enzyme from rabbit lung by all seven of the *Bothrops jararaca* peptides that have so far been synthesized. The present paper describes the purification of angiotensin-converting enzyme from rabbit lung, its inhibition by the synthetic venom peptides, and kinetic studies on the interactions of these peptides with substrates and activators of the enzyme.

MATERIALS AND METHODS

Materials

Seven peptides corresponding in structure to those of known sequence from the venom of *Bothrops jararaca*[2,10] were synthesized by Dr M. A. Ondetti and his associates at the Squibb Institute for Medical Research; hippuryl-L-histidyl-L-leucine (Hip–His–Leu) was prepared by Dr F. Sipos and Mr D. Gaston of the Squibb Institute. The [Ile^5]-angiotensin I (angiotensin I) employed in our studies was purchased from Schwarz/Mann (lot No. 6905); results obtained from quantitative amino acid analysis, enzymic degradation, and chromatography of this peptide were consistent with a triacetate salt of high purity. When compared on a molar basis (as determined from quantitative amino acid analyses of both peptides), the angiotensin I was found to be equipotent with [Asn^1, Val^5]-angiotensin II (Hypertensin, Ciba) for elevation of blood pressure in the anesthetized dog.

Acetone powder of rabbit lung was obtained from Pel-Freez Biologicals, Inc., Whatman DE-52 microgranular DEAE-cellulose from H. Reeve Angel and Co., Ltd

BioGel P-300 polyacrylamide resin from Bio-Rad Laboratories, and *o*-phthaldialdehyde from Sigma Chemical Company. Calcium phosphate gel was prepared according to Keilin and Hartree[34]; it was concentrated by sedimentation and decantation to yield a suspension containing 50 mg (dry) per ml. Other chemicals employed were of reagent grade, and were obtained from various commercial sources.

Preparation of homogeneous angiotensin-converting enzyme

50 g of acetone powder of rabbit lung was extracted by blending for three 1-min intervals with 1 l of 50 mM potassium phosphate buffer (pH 8.3); the resulting homogenate was centrifuged 40 min at 37 000 × *g*, the sedimented residue was extracted by the same procedure, and the two supernatant solutions were combined. This and succeeding steps were performed at 5 °C. The acetone-powder extract was acidified to pH 5 by multiple dialyses against 5-l volumes of 5 mM sodium acetate buffer (pH 4.9), with a change of buffer every 12 h; precipitated protein was removed by centrifugation at 10 000 × *g* for 20 min. The acidified extract was stirred 1 h with 0.8 mg of calcium phosphate gel per mg of protein; the gel, with unwanted protein adsorbed, was removed by centrifugation at 10 000 × *g* for 10 min, and discarded. Fresh gel, added at three times the original amount, was stirred with the extract for 1 h to adsorb the enzyme, and was collected by centrifugation. The enzyme was eluted by stirring the gel for 30 min with 500 ml of 10 mM potassium phosphate buffer (pH 8.3) and centrifuging as above. The eluate was placed on a 2.5 cm × 18 cm column of DEAE-cellulose, which was then washed with 500 ml of 10 mM phosphate buffer (pH 8.3) and developed with a 500-ml linear gradient of 0 to 100 mM KCl in the same buffer. The DEAE-cellulose fractions (10 ml) that contained enzyme activity, usually from 15 to 75 mM KCl, were concentrated to 5 or 10 ml, using a BioMed UF-1 ultrafiltration concentrator, and further purified by reverse-flow gel-permeation chromatography on a 2.5 cm × 80 cm column of BioGel P-300 that had previously been equilibrated with 50 mM phosphate buffer. The peak of enzyme activity was eluted from the P-300 column at a volume of about 200 ml (Fraction 40), with a constant specific activity of 22 units per mg over most of the peak. A much smaller amount of a lower-molecular-weight oligomeric form of the enzyme was also obtained at this step. Enzyme from the major peak was homogeneous by the criterion of disc electrophoresis[35] at pH 8.3; only a single, slowly moving protein band could be detected.

Table I summarizes the purification of angiotensin-converting enzyme from

TABLE I

PREPARATION OF HOMOGENEOUS ANGIOTENSIN-CONVERTING ENZYME OF RABBIT LUNG

Step	*Protein (mg)*	*Activity (units)**	*Specific activity (units/mg protein)*	*Recovery (%)*	*Purification (-fold)*
Acetone powder extract	9700	750	0.08	100	1.0
Acidification	6100	710	0.12	95	1.5
Calcium phosphate gel	300	360	1.2	48	15
DEAE-cellulose	34	260	7.6	35	95
BioGel P-300	8	178	22	24	275

* μmoles of Hip–His–Leu hydrolyzed per min.

acetone powder of rabbit lung. Protein concentrations were determined in the first three steps by the biuret method[36], and afterward by the spectrophotometric method[37]. Angiotensin-converting enzyme activity in the various fractions was routinely determined spectrophotometrically[18] with Hip–His–Leu as substrate, but the ratio of activities on Hip–His–Leu and angiotensin I, assayed as described below, was found to be constant at about 20 in all fractions throughout the purification. Homogeneous angiotensin-converting enzyme, thus obtained, is stable in dilute solution for at least 6 months at 5 °C; the enzyme in acetone powder extracts has remained active under similar conditions for 2 years.

Assays of angiotensin-converting enzyme activity

The activity of angiotensin-converting enzyme was assayed both by spectrophotometric determination of the rate of formation of hippuric acid from Hip–His–Leu[18], and by fluorometric determination[38] of the rate of formation of histidylleucine from either angiotensin I or Hip–His–Leu. For either assay method, incubations were carried out at 37 °C in 13 mm × 100 mm tubes containing a final volume of 0.25 ml. Depending on the substrate employed (angiotensin I or Hip–His–Leu), one of the following two sets of optimal incubation conditions was employed: 100 mM potassium phosphate buffer (pH 8.3), 300 mM NaCl, and 5 mM Hip–His–Leu; or 100 mM potassium phosphate buffer (pH 7.5), 30 mM NaCl, and 0.3 mM angiotensin I. Enzymic reactions in both assay methods were initiated by addition of enzyme. For spectrophotometric assays, reactions were terminated by addition of 0.25 ml of 1 M HCl, and the assay was continued as previously described[18]. For fluorometric assays, the enzymic reactions were terminated by addition of 1.45 ml of 0.3 M NaOH; 0.1 ml of 0.2% *o*-phthaldialdehyde in methanol was then added, followed after 10 min by 0.2 ml of 3 M HCl (final volume equals 2.0 ml). The fluorometric assay was otherwise performed as described by Piquilloud *et al.*[38].

A unit of angiotensin-converting enzyme activity is defined, for the reaction with either substrate, as the amount catalyzing the hydrolysis of 1 μmole of substrate in 1 min under the assay conditions that are optimal for reaction with the substrate employed. When such optimal conditions are employed for each substrate, Hip–His–Leu is hydrolyzed 20 times more rapidly than angiotensin I by the same quantity of angiotensin-converting enzyme; thus, 1 Hip–His–Leu unit is equivalent to 0.05 angiotensin I unit of angiotensin-converting enzyme activity. For 30-min fixed-time assays, the rate of formation of histidylleucine from angiotensin I was linear with amounts of enzyme as high as 0.1 angiotensin I munit (0.1 μg of homogeneous enzyme); and the rate of formation of histidylleucine or hippuric acid from Hip–His–Leu was linear with 5-fold greater amounts of enzyme, up to 10 Hip–His–Leu munits (0.5 μg of homogeneous enzyme). For assays of duration shorter than 30 min, as employed for kinetic studies, correspondingly larger amounts of enzyme could be used.

Inhibition studies

Activity of angiotensin-converting enzyme in the presence of inhibitors was determined as described above, without pre-incubation of the inhibitors with the enzyme. For the determination of I_{50} values (concentration of inhibitor producing a 50% inhibition of angiotensin-converting enzyme), assay tubes containing a wide

range of concentrations of inhibitor were incubated for 30 min. For kinetic studies, in which concentrations of substrate, as well as of inhibitor, were varied, each activity measurement was based on incubations of 2, 5, 10 and 15 min duration, to ensure the measurement of initial velocities. Kinetic results (K_i values and type of inhibition) were confirmed by four graphical methods ($1/v$ *vs* $1/[S]$; $[S]/v$ *vs* $[S]$; v *vs* $v/[S]$; and $1/v$ *vs* $[I]$) described by Webb[39], although only one type of plot is shown in the figures.

TABLE II

STRUCTURE AND NOMENCLATURE OF PEPTIDES FROM THE VENOM OF *Bothrops Jararaca*

Equivalent designations			*Structures*
V-3-A*	BPP 5a†	SQ 20 475‡	Pyr–Lys–Trp–Ala–Pro
V-6-1	BPP 9a	SQ 20 881	Pyr–Trp–Pro–Arg–Pro–Gln–Ile –Pro–Pro
V-6-2	BPP 10b	SQ 20 861	Pyr–Asn–Trp–Pro–Arg–Pro–Gln–Ile –Pro–Pro
V-2	BPP 11d	SQ 20 661	Pyr–Trp–Pro–Arg–Pro–Thr–Pro–Gln–Ile –Pro–Pro
V-7	BPP 10c	SQ 20 858	Pyr–Asn–Trp–Pro–His–Pro–Gln–Ile –Pro–Pro
V-9	BPP 13a	SQ 20 718	Pyr–Gly–Gly–Trp–Pro–Arg–Pro–Gly–Pro–Glu–Ile –Pro–Pro
V-8	BPP 10a	SQ 20 859	Pyr–Ser –Trp–Pro–Gly–Pro–Asn–Ile –Pro–Pro

* These codes refer to the fractions in which the seven peptides were isolated by the investigators who determined their structures: V-3-A by Ferreira *et al.*[2], the other peptides by Ondetti *et al.*[10].

† This nomenclature was suggested by Stewart *et al.*[8]. The three peptides of known structure from the venom of *Agkistrodon halys blomhoffii*[11–15] would be BPP 11a, 11b and 11c; it is not clear whether BPP 10c should, instead, be named [His^5]-BPP 10b.

‡ These numbers designate peptides synthesized at the Squibb Institute; they are used to distinguish these peptides from the isolated peptides, or those synthesized by other groups.

RESULTS

Inhibition of homogeneous angiotensin-converting enzyme by synthetic Bothrops jararaca venom peptides

The seven inhibitors of known amino acid sequence from *Bothrops jararaca* venom are shown in Table II, along with designations used by various investigators for natural or synthetic peptides having these structures. A single, generally accepted system of nomenclature for these peptides and their synthetic analogs may not be possible unless a common mechanism is demonstrated for their action as inhibitors of angiotensin-converting enzyme and as bradykinin-potentiating compounds. In this paper, the synthetic peptides will be referred to by the numbers shown in the third column of Table II.

Two I_{50} values were determined for each synthetic *Bothrops jararaca* venom peptide (Fig. 1), one for inhibition of the homogeneous enzyme acting on Hip–His–Leu (pH 8.3, 300 mM NaCl), the other for inhibition with angiotensin I as substrate (pH 7.5, 30 mM NaCl). As shown in Table III, all seven peptides were less potent under the latter set of conditions, with the most potent peptides showing the greatest differences. The I_{50} values obtained using homogeneous enzyme with Hip–His–Leu as substrate (Table III) are virtually identical to I_{50} values obtained with acetone powder extract as the source of enzyme.

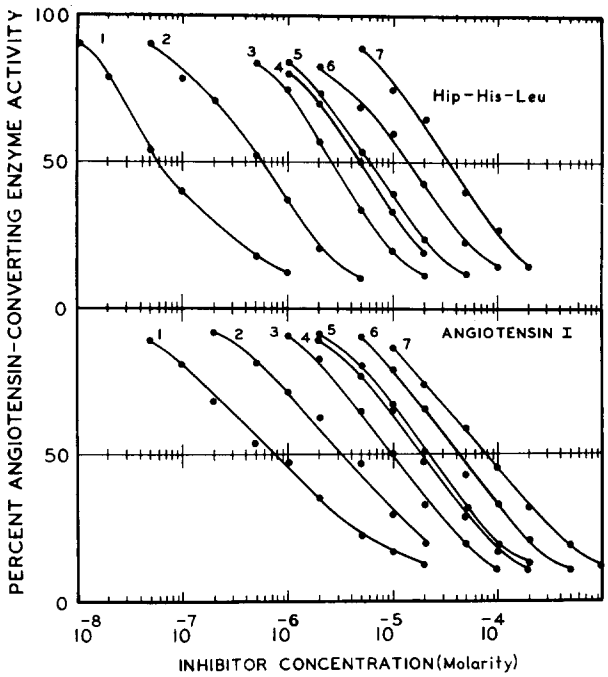

Fig. 1. Inhibition of homogeneous angiotensin-converting enzyme of rabbit lung by synthetic *Bothrops jararaca* venom peptides. The following synthetic venom peptides were tested over a wide range of concentrations for inhibition of homogeneous angiotensin-converting enzyme acting on angiotensin I or hippuryl-L-histidyl-L-leucine: 1, SQ 20 475, Pyr–Lys–Trp–Ala–Pro; 2, SQ 20 881, Pyr–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro; 3, SQ 20 861, Pyr–Asn–Trp–Pro–Arg–Pro–Gln–Ile–Pro–Pro; 4, SQ 20 661, Pyr–Trp–Pro–Arg–Pro–Thr–Pro–Gln–Ile–Pro–Pro; 5, SQ 20 858, Pyr–Asn–Trp–Pro–His–Pro–Gln–Ile–Pro–Pro; 6, SQ 20 718, Pyr–Gly–Gly–Trp–Pro–Arg–Pro–Gly–Pro–Glu–Ile–Pro–Pro; 7, SQ 20 859, Pyr–Ser–Trp–Pro–Gly–Pro–Asn–Ile–Pro–Pro. Activity of angiotensin-converting enzyme on angiotensin I was assayed by fluorometric determination of the amount of histidylleucine produced in 30 min in a 0.25-ml incubation mixture containing 0.3 mM angiotensin I, 30 mM NaCl, 100 mM potassium phosphate buffer (pH 7.5) and 0.1 μg of enzyme protein per assay; hydrolysis of Hip–His–Leu by angiotensin-converting enzyme was assayed by fluorometric determination of the amount of histidylleucine and by spectrophotometric determination of the amount of hippuric acid formed in 30 min in a 0.25-ml incubation mixture consisting of 5 mM Hip–His–Leu, 300 mM NaCl, 100 mM potassium phosphate buffer (pH 8.3) and 0.25 or 0.5 μg, respectively, of homogeneous enzyme.

Effect of substrate concentration on inhibition

Kinetic studies were performed with three of the most potent synthetic peptides (Figs 2–4), to determine the effect of substrate (Hip–His–Leu or angiotensin I) concentration on the inhibition obtained. The two larger venom peptides, SQ 20 881 and SQ 20 858, were competitive with both substrates (Figs 3 and 4). The pentapeptide, SQ 20 475, gave "mixed inhibition" (Fig. 2), intermediate between competitive and non-competitive; the deviation from competitive inhibition was much greater, however, with Hip–His–Leu as substrate.

Although the degree of inhibition of an enzyme by a competitive inhibitor is dependent on the particular substrate employed, and on its concentration, the K_i value (enzyme–inhibitor dissociation constant) should be the same, whichever substrate is employed for the kinetic studies. As shown in Table III, each of the three

TABLE III

INHIBITION OF HOMOGENEOUS ANGIOTENSIN-CONVERTING ENZYME BY SYNTHETIC VENOM PEPTIDES

Two sets of I_{50} values and two sets of K_i values were determined, one set for inhibition of the enzyme acting on each of the substrates (angiotensin I and Hip–His–Leu). Optimal assay conditions (see text) employed with each substrate are identified only by the pH value: 8.3 for Hip–His–Leu and 7.5 for angiotensin I. One set of K_i values was determined for inhibition of enzyme acting on Hip–His–Leu under the same conditions (pH 7.5) usually employed for assays with angiotensin I.

Peptide	I_{50} *(μM)*		K_i *(μM)*			*Type of inhibition*
	Hip–His–Leu pH 8.3	*Angiotensin I pH 7.5*	*Hip–His–Leu pH 8.3*	*Hip–His–Leu pH 7.5*	*Angiotensin I pH 7.5*	
SQ 20 475	0.06	0.7	0.06	0.08	0.09	mixed
SQ 20 881	0.56	3.4	0.10	0.60	0.84	competitive
SQ 20 861	2.5	10				
SQ 20 661	5.0	18				
SQ 20 858	5.8	21	0.74	3.3	4.3	competitive
SQ 20 718	14	43				
SQ 20 859	34	76				

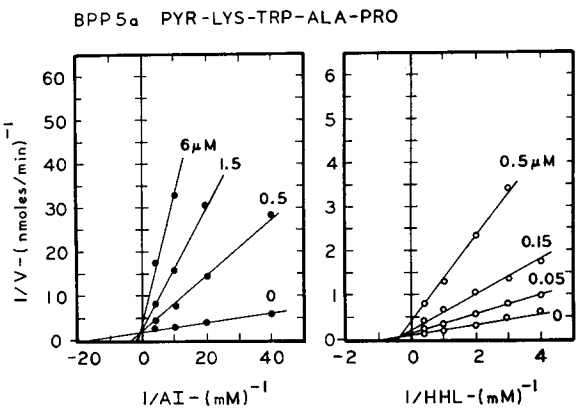

Fig. 2. Double-reciprocal plots of the effect of substrates, angiotensin I (AI) and hippuryl-L-histidyl-L-leucine (HHL), on the inhibition of homogeneous angiotensin-converting enzyme by the pentapeptide BPP 5a (SQ 20 475). Activity of the enzyme on either substrate was assayed fluorometrically under the conditions described in the legend for Fig. 1, with 0.25 μg of homogeneous enzyme employed per assay. For each rate determination, incubations were carried out for 0-, 2-, 5-, 10- and 15-min intervals to ensure measurement of initial velocities. The structure of the pentapeptide and the concentrations employed are shown in the figure.

K_i values calculated for each of the three venom peptide inhibitors tested was the same, within experimental error, whether angiotensin I or Hip–His–Leu was employed as the substrate for the enzyme, as long as the enzyme assays were performed under identical incubation conditions (pH 7.5, 30 mM NaCl) instead of the usual two sets of conditions optimal for the two substrates.

Effect of pH on binding of inhibitors and substrates to the enzyme

Although, as with most enzymes, the substrate employed for kinetic studies with angiotensin-converting enzyme does not affect the magnitude of the K_i value

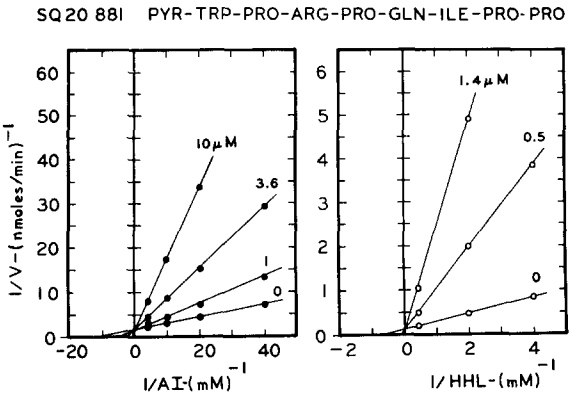

Fig. 3. Double-reciprocal plots of the effect of substrates, angiotensin I (AI) and hippuryl-L histidyl-L-leucine (HHL), on the inhibition of homogeneous angiotensin-converting enzyme by the nonapeptide, SQ 20 881. Assays were performed as described in the legends for Figs 1 and 2. The structure of the nonapeptide and the concentrations employed are shown in the figure.

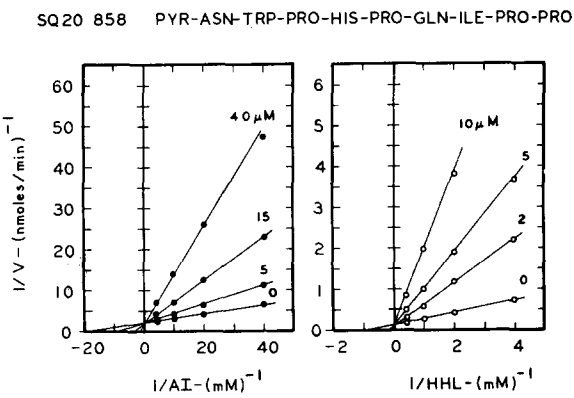

Fig. 4. Double-reciprocal plots of the effect of substrates, angiotensin I (AI) and hippuryl-L-histidyl-L-leucine (HHL), on the inhibition of homogeneous angiotensin-converting enzyme by a decapeptide, SQ 20 858. Assays were performed as described in the legends for Figs 1 and 2. The structure of the decapeptide, and the concentrations employed, are shown in the figure.

calculated for an inhibitor, other assay conditions, such as pH, do have such an effect. The K_i values calculated for SQ 20 881 and SQ 20 858 with Hip–His–Leu as substrate were 6-fold and 4.5-fold higher, respectively, at pH 7.5 than at pH 8.3 (Table III). No such difference was observed in the two K_i values obtained for SQ 20 475, although the I_{50} values obtained for this peptide did differ greatly at the two pH values. However, both the "mixed inhibition" obtained with SQ 20 475, and its ability to serve as a substrate for angiotensin-converting enzyme (see below) make the results of kinetic studies, such as K_i values, obtained with this peptide difficult to interpret.

Unlike such venom peptide inhibitors as SQ 20 881 and SQ 20 858, substrates for angiotensin-converting enzyme that we have employed showed no change in their affinity for the enzyme between pH 7.5 and pH 8.3, even though the rate of hydro-

lysis of both substrates varied with pH over this range. Enzyme–substrate dissociation constants (K_m values), determined in triplicate at both pH values with both substrates, were as follows: angiotensin I (pH 7.5), 0.05 ± 0.01 μM; angiotensin I (pH 8.3), 0.05 ± 0.006 μM; Hip–His–Leu (pH 7.5), 1.3 ± 0.1 μM; Hip–His–Leu (pH 8.3), 1.2 ± 0.1 μM. Thus, even though the venom peptides are competitive inhibitors of angiotensin-converting enzyme, results at different pH values suggest that their mode of binding to the enzyme is at least slightly different from that of substrates.

Effect of activator concentration on inhibition

The nonapeptide, SQ 20 881, was tested for inhibition of homogeneous angiotensin-converting enzyme, with angiotensin I as substrate, over a wide range of concentrations of the activator, Cl^-. The results of two such kinetic studies indicated that the inhibition produced by SQ 20 881 was "uncompetitive" with respect to Cl^-. The data, which were more variable than those shown in Figs 2–4, could have been plotted in such a manner as to have indicated noncompetitive inhibition, but in no way could they be used to indicate competition between the activator ion and the inhibitor.

Effect of pre-incubation of inhibitors with the enzyme

Pre-incubation of synthetic *Bothrops jararaca* venom peptides with homogeneous angiotensin-converting enzyme did not, in any case, increase the amount of inhibition obtained. The six nonapeptide to tridecapeptide inhibitors could be pre-incubated with the enzyme for at least 2 h without any effect on their inhibitory potency. The pentapeptide, SQ 20 475, however, lost all of its inhibitory potency within 10–15 min after pre-incubation with the enzyme. Thin-layer chromatography of products resulting from pre-incubation of SQ 20 475 with the enzyme indicated the presence of the dipeptide, Ala–Pro, and of another product, assumed to be Pyr–Lys–Trp. Thus, the pentapeptide inhibitor can also serve as a substrate for angiotensin-converting enzyme, when incubated with the enzyme alone. Unlike angiotensin I or Hip–His–Leu, however, SQ 20 475 is cleaved more slowly in the presence of Cl^-; in our normal assay mixtures, containing Cl^- as well as a high concentration of another substrate, such as angiotensin I or Hip–His–Leu, SQ 20 475 does not appear to be hydrolyzed appreciably within 30 min.

DISCUSSION

Ferreira *et al.*[2] were the first to isolate from the venom of *Bothrops jararaca* peptides that potentiated activities of bradykinin and inhibited angiotensin-converting enzyme; however, they determined the sequence of only one of these peptides, a pentapeptide that they named BPP 5a (SQ 20 475 in this paper). Sequences of the other six peptides discussed in this paper were determined by Ondetti and coworkers[10], who independently isolated them from *Bothrops* venom. All seven peptides that we have used were synthesized by the latter workers, and are referred to in this paper by their synthetic code numbers (see Table II).

Although the initial isolation of peptides from *Bothrops jararaca* venom was achieved on the basis of the bradykinin-potentiating activity of these compounds[2], most of these isolated peptides were also tested by Ferreira *et al.*[9] for inhibition of a

particulate preparation of angiotensin-converting enzyme from dog lung, using a biological assay method for the enzyme. In addition to the pentapeptide V-3-A (BPP 5a), which is identical to synthetic peptide, SQ 20 475, several of the other peptides isolated and tested by Ferreira and coworkers[9] probably have the same structures as peptides that we have employed that were isolated and synthesized by Ondetti *et al.*[10]: SQ 20 881 appears to be identical with Fraction IV-1-D isolated by Ferreira *et al.*[9], SQ 20 859 with Fraction IV-1-A, and SQ 20 718 with Fraction III-1-A. Fraction IV-1-B of Ferreira and coworkers apparently contains at least two peptides (*cf.* refs 2 and 9) that are probably identical to SQ 20 858 and SQ 20 661; none of the peptides isolated by Ferreira and coworkers, however, was comparable in amino acid composition to SQ 20 861.

The results of Ferreira and coworkers on inhibition of angiotensin-converting enzyme by the isolated peptide fractions are consistent with our own results with the synthetic peptides, but two apparent discrepancies deserve comment. First, they obtained greater inhibition than we did with all of the peptides except the pentapeptide. This difference is easily explained by the low concentrations of substrate that they employed, since the inhibitors are competitive with substrates for the enzyme. Inhibition obtained using the pentapeptide (BPP 5a or SQ 20 475) is the second discrepancy; it was much less potent in their studies than in ours. The pentapeptide, however, can be degraded by enzymes (including the angiotensin-converting enzyme) present in some crude enzyme fractions; it is possible that under the conditions employed by Ferreira and coworkers, the pentapeptide was significantly degraded during incubation with their particulate enzyme preparation. As previously mentioned, even though the pentapeptide can serve as a substrate for angiotensin-converting enzyme, neither homogeneous enzyme nor acetone powder extract destroys the pentapeptide under our normal assay conditions. We have, however, obtained much higher I_{50} values for the pentapeptide (unpublished results) when it was tested for inhibition of angiotensin-converting enzyme of human seminal plasma[22], a preparation that had not been treated with acetone.

A few of the same synthetic peptides that were synthesized by Ondetti *et al.*[10] and that were described in this paper have also been tested by other investigators. Yang *et al.*[19] found SQ 20 475 to be less potent than SQ 20 881 for inhibition of angiotensin-converting enzyme (kininase II) of hog plasma, assayed by spectrophotometric methods; they also obtained evidence for the destruction of SQ 20 475 by their enzyme preparation. Bakhle[21,24] tested SQ 20 475, SQ 20 881 and SQ 20 859 for inhibition of a particulate fraction of angiotensin-converting enzyme from dog lung, assayed by biological methods; he also found SQ 20 881 to be superior to SQ 20 475, but did not test for possible destruction of SQ 20 475 by his enzyme preparation.

The synthetic venom peptides of *Bothrops jararaca*, with the possible exception of SQ 20 475, are competitive inhibitors of angiotensin-converting enzyme with respect to substrate; Sander *et al.*[17] found the same type of inhibition with peptide C from *Agkistrodon halys blomhoffii*. The simplest explanation for such competitive inhibition is that the inhibitors bind to the active site of the enzyme in a manner similar to that of substrates for the enzyme. Studies with analogs of the *Bothrops jararaca* peptides (unpublished observations in collaboration with Ondetti and coworkers) tend to confirm this concept, and emphasize the importance of the last three amino acid residues

at the carboxyl end of both substrates and inhibitors for binding to the active site of the enzyme. Most of the venom peptides have a proline residue in the second position from the C-terminal end of their structure; such a penultimate proline prevents peptides from serving as substrates for angiotensin-converting enzyme[18,40]. The pentapeptide inhibitor, SQ 20 475, lacking a penultimate proline residue, can be degraded by angiotensin-converting enzyme under certain conditions; such degradation may contribute to the short duration of inhibitory activity of the pentapeptide *in vitro* and *in vivo*, and probably accounts for the unusual results obtained with this peptide in kinetic studies.

Although they are competitive inhibitors, the most potent of the venom peptides can bind to angiotensin-converting enzyme as much as 500 times more tightly than angiotensin I and up to 10 000 times more tightly than Hip–His–Leu. Assuming a molecular weight of about 150 000 for the angiotensin-converting enzyme, one can calculate from the data given in Table II that peptides such as SQ 20 475 or SQ 20 881 occupy half of the available active sites of the enzyme when present in solution at a ratio of only 10–20 molecules of inhibitor per molecule of enzyme. Thus, although the three C-terminal amino acid residues participate in the binding of the inhibitors and are responsible for the competitive nature of the inhibition, one must assume that groups in another region of the peptide structures are involved in the binding of these inhibitors to the enzyme, in order to produce overall affinities for the enzyme that are so much greater than those of substrates for the enzyme. Such residues may be responsible for the change in the enzyme-binding affinities (K_i values) of SQ 20 881 and SQ 20 858 that occur between pH values of 7.5 and 8.3 (Table III). The differences between the I_{50} values obtained with angiotensin I as substrate at pH 7.5 and those obtained with Hip–His–Leu as substrate at pH 8.3 are probably due both to differences in the affinities of the inhibitors at the two pH values and to differences in the interactions of these competitive inhibitors with the two substrates.

Some of the results described in this paper should be considered in relation to two recent observations regarding the kininase activity of angiotensin-converting enzyme. Although Yang *et al.*[19] have demonstrated that angiotensin-converting enzyme is capable of degrading bradykinin, and is identical to their previously described kininase II, others have shown that the bradykininase activity of partially purified angiotensin-converting enzyme preparations from lung was not stimulated by Cl^- (refs. 17, 24), a fact that might suggest that the kininase activity was due to a different enzyme. In our own studies with homogeneous angiotensin-converting enzyme, however, we found that the concentration of Cl^- required for optimal activity depended on the substrate employed (Hip–His–Leu or angiotensin I), and that with a substrate such as the pentapeptide inhibitor, SQ 20 475, activity was even inhibited by Cl^-. Cl^- appears to serve as an "allosteric" modifier, subtly changing the shape of the active site of the enzyme; such a change could make the active site more suitable for binding of some substrates (angiotensin I or Hip–His–Leu), less suitable for others (SQ 20 475), and cause no change in its affinity for still others, such as bradykinin. Freer and Stewart[26] showed that the mercaptoethanol-resistant kininase activity of perfused lung was inhibited by lower concentrations of the pentapeptide, BPP 5a (SQ 20 475) than that required to inhibit conversion of angiotensin I; this result was presented as evidence that different enzymes were involved in the two

reactions. However, the amount of such a competitive (or mixed) inhibitor required for inhibition of the enzyme would be expected to depend very much on the affinity for the enzyme of the particular substrate employed (angiotensin I or bradykinin), as well as on the concentration of the substrate.

The *Bothrops jararaca* venom peptides may be quite specific, as well as potent, inhibitors of the dipeptide-releasing carboxypeptidase, variously referred to as angiotensin-converting enzyme or as kininase II[19]. The pentapeptide, SQ 20 475, did not inhibit leucine aminopeptidase (EC 3.4.1.1), carboxypeptidase A (EC 3.4.2.1), trypsin (EC 3.4.4.4), or chymotrypsin (EC 3.4.4.5)[18,20]. Stewart and coworkers[8], however, studying fragments obtained after perfusion of lung with labelled bradykinin, have postulated the existence in lung of an enzyme that cleaves the Ser[6]–Pro[7] bond of bradykinin, and which is inhibited by the pentapeptide. It is the opinion of the present authors that the labelled fragments that led to the postulation of such an enzyme could also have been formed by action of the angiotensin-converting enzyme (kininase II) of lung in concert with the action of other lung peptidases. It is tempting to speculate, in spite of some evidence to the contrary, that the bradykinin-potentiating activity of the venom peptides might be due entirely to their inhibition of angiotensin-converting enzyme (kininase II); this enzyme is present in all tissues in which potentiation of the biological effects of bradykinin has been demonstrated[22,33]. Certainly, if there exists a kininase other than the angiotensin-converting enzyme that is inhibited by the venom peptides, the most direct proof would be the isolation of the enzyme in a fraction of sufficient purity to allow direct determination of the chemical nature of the reaction that it catalyzes.

Peptidic inhibitors of angiotensin-converting enzyme from the venom of *Bothrops jararaca* have already proved to be useful tools for confirming the presence of the enzyme in tissues or extracts[22,25], and for demonstrating its role in various physiological processes[27–32]. The most useful peptide for such purposes appears to be SQ 20 881. The potentially more potent peptide, SQ 20 475, has a much shorter duration of activity *in vivo*[7,8,20,27–29,32], perhaps due to enzymic destruction, such as that occurring *in vitro*. Both BPP 5a (SQ 20 475)[32] and SQ 20 881[29] have been shown to lower the blood pressure of rats made hypertensive by clamping one renal artery, while leaving the contralateral kidney intact. The involvement of the renin (EC 3.4.4.15)–angiotensin system in this model of renal hypertension is borne out by studies with a phospholipid inhibitor of renin[41], with antibodies to angiotensin II[42], and with specific angiotensin–receptor antagonists[43,44]. Thus, in addition to their great utility as experimental tools, it is hoped that synthetic *Bothrops jararaca* venom peptides such as SQ 20 881 might be useful as diagnostic, or even as therapeutic, agents for diseases, such as some forms of renal hypertension, in which the renin–angiotensin system plays a role.

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