THE AMINO ACID SEQUENCE OF HYPERTENSIN II

By LEONARD T. SKEGGS, Jr., Ph.D., KENNETH E. LENTZ, Ph.D., JOSEPH R. KAHN, M.D., NORMAN P. SHUMWAY, M.D., and KENNETH R. WOODS, Ph.D.

(From the Department of Medicine and Surgery, Veterans Administration Hospital, and the Department of Pathology, Western Reserve University, Cleveland)

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The pressor substance hypertensin, which has been found in the blood of many human beings with hypertensive cardiovascular disease (1), as well as in animals with experimental renal hypertension (2), may actually exist in two forms (3). The first form, hypertensin I, is the initial product of the action of the renal enzyme renin upon its plasma substrate and is not a vasoconstrictor or a directly pressor substance. It may be rapidly changed to the second form, hypertensin II, by a chloride-activated converting enzyme contained in the plasma. Hypertensin II is an extremely powerful vasoconstrictor material and is therefore the effector substance of the renin-hypertensin system (4, 5).

Hypertensin I has been isolated from horse blood in a highly purified form (6). This material was treated with partially purified converting enzyme (4, 7) and after countercurrent distribution of the products, homogenous hypertensin II as well as a biologically inactive peptide residue was obtained. Quantitative amino acid analysis (8, 9) of these three substances together with the use of the enzyme carboxypeptidase (9) allowed the conversion to be expressed as a balanced biochemical equation.

$$\text{Hypertensin I} \xrightarrow{\text{converting enzyme}} \text{hypertensin II} + \text{dipeptide}$$

$$\text{(asp-arg-val-tyr-iso-hist-pro)-phe-hist-leu} \xrightarrow{\text{converting enzyme}} \text{(asp-arg-val-tyr-iso-hist-pro)-phe} + \text{hist-leu}$$

It is the purpose of this paper to report the amino acid sequence of hypertensin II as determined by the use of chymotrypsin, the fluorodinitrobenzene method, and stepwise phenylisothiocyanate degradation.

EXPERIMENTAL

Treatment of Hypertensin II with Chymotrypsin.—Chymotrypsin is known to hydrolyze peptide bonds which involve the carboxyl group of aromatic amino acids (10). Inasmuch as hypertensin II has only one such group it was

expected that this enzyme would divide the molecule into two fragments each of which might be amenable to further analysis.

A solution of hypertensin II containing 4.1 mg. of N and a minimum of 44,000 Goldblatt units (11) was treated with 0.5 mg. of a commercial preparation of crystalline chymotrypsin. The reaction mixture with a volume of 50 ml. was allowed to remain at room temperature and was maintained at pH 7.7 by occasional additions of 0.1 N NaOH. Small aliquots were withdrawn at suitable intervals and after inactivation of the enzyme by heat were diluted and assayed for pressor activity in the rat (12). After 37 minutes all but 50 units had been destroyed and the reaction was terminated by raising the temperature to 100°C. for 20 minutes. After cooling, the material was loaded in the first two tubes of a 200 tube Craig-Post countercurrent distribution apparatus (13). The machine was operated for 71 transfers using the solvent system *n*-butanol-water. Ninhydrin analyses (14) of the aqueous layer showed the presence of two components; peptide A and peptide B. The distribution coefficients of these two substances were 0.06 and 0.67 respectively. After pooling the solutions from the appropriate tubes were evaporated to convenient volumes and saved for further analysis. Descending paper chromatograms were obtained on both fractions by using a *n*-butanol–water–acetic acid (4:1:5) solvent system. Peptide A gave a single ninhydrin positive spot with an R_f of 0.38. Peptide B also gave a single ninhydrin spot with an R_f of 0.63. This spot stained with Durrum's reagent. The ultraviolet spectrum of peptide A was determined and found to correspond closely to that of tyrosine. Calculations based upon the molar extinction coefficient at 274.5 mμ showed the presence of 30.2 μM. The absorption of peptide B was small and not characteristic of tyrosine.

Aliquots of both peptides amounting to 8.0 μM, as determined by the ninhydrin method, were hydrolyzed under reflux for 24 hours in constant boiling HCl. These samples were subjected to quantitative chromatography upon 100 cm. dowex 50 × 8 columns according to the methods of Stein and Moore (15). The results obtained revealed that peptide A was composed of aspartic acid, arginine, valine, and tyrosine in approximately whole number molar ratios. Peptide B contained isoleucine, histidine, proline, and phenylalanine, also in nearly equimolar amounts.

It appeared from the foregoing experiments that chymotrypsin hydrolyzed a single peptide bond in the hypertensin II molecule yielding two peptides. These may be written as:

Peptide A		Peptide B
(asp-arg-val-tyr)	+	(iso-hist-pro)-phe

The position of phenylalanine at the C terminal of peptide B was known from previous experiments in which it was demonstrated that phenylalanine is released from the C terminal of hypertensin II by the action of carboxypeptidase (9). The presence of proline adjoining phenylalanine was suspected from the known inability of carboxypeptidase to remove terminal proline from peptide chains (16). The C terminal position of tyrosine in peptide A was also indicated from the known specificity of chymotrypsin.

The Amino Acid Sequence of Peptide B.—The N terminal stepwise degradation of 4.5 μM of peptide B was then performed using the phenylisothiocyanate method as outlined in detail by Fraenkel-Conrat, Harris, and Levy (17).

The phenylthiohydantoin derivatives of isoleucine, histidine, and proline were each removed in turn from the peptide and identified by direct paper chromatography. No difficulty was experienced except in the case of the third step when it proved necessary to heat the prolyl phenylalanine peptide derivative to 50°C. in the presence of 3N HCl in order to remove the phenylthiohydantoin derivative of proline.

Additional confirmation of the progress of the degradation was obtained by hydrolysis and paper chromatography of the peptide fraction remaining after the removal of each N terminal amino acid derivative. The results of the chromatograms thus secured were consistent with the aforementioned N terminal sequence. The final peptide fraction remaining after these degradation steps was found to contain free phenylalanine.

As a result of these studies it was then possible to assign the following partial amino acid sequence:—

(asp-arg-val-tyr)-iso-hist-pro-phe

The N Terminal Sequence of Hypertensin I and Hypertensin II.—Application of the fluorodinitrobenzene technique of Sanger (18), as modified by Fraenkel-Conrat *et al.* (17) to a 1.0 μM sample of hypertensin II revealed the N terminal amino acid to be aspartic acid. Similar results were obtained with a small sample of hypertensin I.

Owing to the ready availability of hypertensin I this compound was used for further determinations. A 20 μM sample of hypertensin I was subjected to one cycle of phenylisothiocyanate degradation. The phenylthiohydantoin derivative of aspartic acid thus obtained was identified as the free amino acid by paper chromatography following hydrolysis. An aliquot of the remaining peptide fraction was then treated with fluorodinitrobenzene and after hydrolysis and chromatography the dinitrophenyl derivative of arginine was identified. The peptide fraction minus aspartic acid was again treated with phenylisothiocyanate. In this case the amino acid derivative released could not be separated from the peptide fraction. This is consistent with the behavior of the phenylthiohydantoin derivative of arginine. The peptide fraction, now minus aspartic acid and arginine was then treated for the third time with the phenylisothiocyanate method yielding a phenylthiohydantoin amino acid which was identified after hydrolysis as valine. This finding was confirmed by application of the fluorodinitrobenzene technique.

The fourth step in the phenylisothiocyanate degradation of this material was unsuccessful and yielded equivocal results. Additional direct evidence as to the position of tyrosine in the molecule was obtained in a partially sucessful acid degradation experiment.

A sample of carboxypeptidase treated hypertensin I containing all of the amino acids of hypertensin II except the C terminal phenylalanine was heated at 100°C. for 3 hours in the presence of 3 N HCl. Among the reaction products the tripeptide arginyl-valyl-tyrosine was isolated by dowex 50 × 2 chromatography and its sequence determined by the phenylisothiocyanate method.

It was therefore possible to establish the amino acid sequence of hypertensin II as follows:—

asp-arg-val-tyr-iso-hist-pro-phe

DISCUSSION

The purification of a pressor peptide derived from ox serum has recently been described by Peart (19). This material was not identified as being either hypertensin I or hypertensin II, nor have the experimental procedures been described in enough detail to allow an identification. In a still more recent communication Elliott and Peart (20) report an amino acid sequence for this material which corresponds to that of horse hypertensin I with the exception that valine is substituted for isoleucine.

It is of paramount interest to discover a therapeutic method of lowering the blood pressure of human beings afflicted with hypertensive cardiovascular disease. Owing to the knowledge now available concerning the structure of hypertensin I, hypertensin II, and the converting enzyme it becomes possible for the first time to approach this problem upon rational grounds. Three separate avenues of attack appear to be feasible. It may now be possible to discover and protect by structural analogs the bond which the enzyme renin dissociates when hypertensin I is formed from renin substrate. It is possible that the C terminal leucine of hypertensin I is involved in this bond since the converting enzyme does not release histidylleucine from the renin substrate. Highly purified renin is now available for this study, owing to the work of Haas, Lamfrom, and Goldblatt (21).

It may also be possible to prevent the formation of hypertensin II from hypertensin I by the converting enzyme, for example by providing a structural analog of the phenylalanyl-histidylleucine bond or by an immunological approach using the converting enzyme in a manner similar to that which is partially successful in the case of renin.

Finally, it might be possible to prevent the vasoconstrictive action of hypertensin II upon smooth muscle. Although the nature of this mechanism is completely unknown the action might be blocked by analogs based upon the structure of hypertensin II.

SUMMARY

The amino acid sequence of horse hypertensin II has been determined by the use of chymotrypsin, the fluorodinitrobenzene method, and stepwise phenylisothiocyanate degradation. The results indicate that the amino acids of hypertensin II are arranged in the following order: asp-arg-val-tyr-iso-hist-pro-phe.

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