THE AMINO ACID COMPOSITION OF HYPERTENSIN II AND ITS BIOCHEMICAL RELATIONSHIP TO HYPERTENSIN I

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Hypertensin has been demonstrated in the arterial bloods of many patients with hypertensive cardiovascular disease (1) as well as in dogs with experimental renal hypertension (2). Purification of this material led to the demonstration of the existence of two peptides with pressor activities (3). Both of these peptides, designated hypertensin I and hypertensin II, have been purified (4, 5). The amino acid composition of hypertensin I and its electrophoretic properties have been reported (6). An enzyme present in plasma and requiring chloride ion for activation was found to be necessary for the conversion of hypertensin I to hypertensin II (3). This enzyme has been partially purified, and is referred to as the hypertensin-converting enzyme (7). Recent work with isolated aortic strips suggested (8), and perfusion studies in the isolated rat kidney subsequently demonstrated (7) that hypertensin I itself has no vasoconstrictor properties. Hypertensin II, formed by the action of the converting enzyme on hypertensin I, was shown to be the substance responsible for vasoconstriction and the elevation of blood pressure produced by the renin-hypertensin system.

Carboxypeptidase is useful in the elucidation of polypeptide and protein structure by virtue of its ability to hydrolyze most carboxyl terminal peptide bonds with the release of amino acids (9). Study of the action of this enzyme on hypertensin I and hypertensin II has permitted an identification of the peptide bond hydrolyzed by the converting enzyme, and also a determination of the carboxyl terminal amino acid sequences of both peptides.

EXPERIMENTAL

Amino Acid Composition of Hypertensin II.—

A sample of hypertensin II (5) containing 5.46 mg. of solids (0.88 mg. of N with 12,000 Goldblatt units of pressor activity) was hydrolyzed in 2 ml. of 6 N HCl for 21.5 hours at 110°C. The hydrolysate, colorless and free of particles, was chromatographed on a 100 cm. dowex 50 × 8 ion exchange column by the procedure of Moore and Stein (10). Effluent

fractions were analyzed by the improved ninhydrin method of Moore and Stein (11). A duplicate hydrolysate was prepared from 3.28 mg. of solids containing 0.53 mg. of N with 7,250 Goldblatt units. This was chromatographed and analyzed in the same manner as the first sample. In order to obtain quantitative recoveries of the basic amino acids, a third sample, containing 5.46 mg. of solids (0.88 mg. of N) was hydrolyzed and chromatographed on a 15 cm. dowex 50 × 8 column as described by Moore and Stein (10). The effluent pattern of the first sample from the 100 cm. column is given in Fig. 1 which shows the pres-

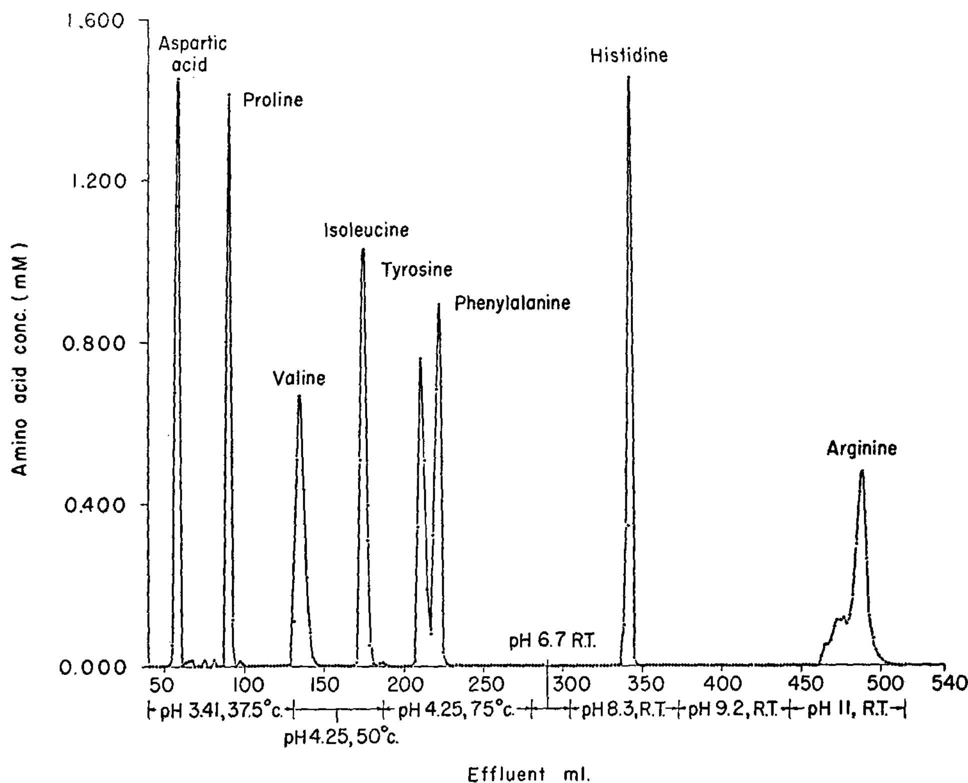

FIG. 1. Effluent patterns from a 100 cm. dowex-50 × 8 ion exchange column

ence of aspartic acid, proline, valine, isoleucine, tyrosine, phenylalanine, histidine, and arginine in hypertensin II.

The quantitative data for the two samples chromatographed on 100 cm. columns are presented in Table I, which for comparison also includes the molar amino acid composition of hypertensin I (6). The identity of isoleucine was confirmed by paper chromatography using a water-tertiary amyl alcohol solvent and Whatman No. 4 paper. When compared with reference leucine and isoleucine standards the unknown amino acid was indistinguishable from isoleucine.

The amino acid composition of hypertensin II differed from that of hyper-

tensin I in that it contains no leucine and only one mole of histidine. The minimum molecular weight would be 1305 for hypertensin I and 1036 for hypertensin II if it is assumed that both molecules are straight chain monomeric polypeptides.

The leucine and histidine present in hypertensin I, but not accounted for in hypertensin II, were sought in the filtrate which had resulted from the salting-out of hypertensin II following its formation from hypertensin I by the converting enzyme (5).

TABLE I

Amino Acid Analysis of Hypertensin II

	Nitrogen as per cent of total nitrogen			Amino acid residues per 100 gm. of sample			Molar proportions			
							Hypertensin II			Hypertensin I*
	1	2	Aver.	1	2	Aver.	1	2	Aver.	
	per cent	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	M	M	M	M
Aspartic acid	6.41	7.00	6.70	9.78	10.66	10.22	1.01	1.03	1.02	1.03
Proline	7.29	6.82	7.06	9.59	8.97	9.28	1.14	1.00	1.07	1.03
Valine	7.01	7.18	7.10	9.39	9.61	9.50	1.10	1.06	1.08	1.03
Isoleucine	6.14	6.42	6.28	9.23	9.63	9.43	0.97	0.95	0.96	0.95
Tyrosine	5.76	6.80	6.28	11.95	14.08	13.02	0.91	1.00	0.96	0.96
Phenylalanine	6.20	6.22	6.21	11.74	11.74	11.74	0.98	0.92	0.95	1.04
Histidine	16.82	19.30	18.06	9.97	11.41	10.69	0.88	0.95	0.92	1.87
Arginine‡	26.00	29.85	27.93	13.00	14.85	13.93	1.02	1.10	1.06	0.99
Leucine	—	—	—	—	—	—	—	—	—	1.09
Total	81.63	89.59	85.62	84.64	90.95	87.81				

* Data of Skeggs *et al.* (6).

‡ Calculated from the ratio of arginine to histidine found using the 15 cm. dowex 50 × 8 column for basic amino acids.

This filtrate had been saturated with sodium chloride and contained small amounts of unprecipitated hypertensin II and unconverted hypertensin I, as well as impurities introduced by the converting enzyme preparation. It was first extracted with secondary butyl alcohol at pH 1.4. The extract gave positive tests for ninhydrin-reactive material and for histidine when analyzed by the diazotized sulfanilic acid colorimetric method (12). It was distributed in a 200 tube Craig-Post countercurrent machine using 0.05 N HCl-5 per cent NaCl as the stationary phase and secondary butyl alcohol as the mobile phase. After 270 transfers, active pressor material was located in tubes 140 to 170, using the rat assay technique of Skeggs *et al.* (13). Tubes 50 to 90 were found to contain material which gave positive histidine and ninhydrin tests but did not increase the blood pressure of a rat when assayed for pressor activity. The buffer phase containing this latter fraction was collected from the machine and evaporated to dryness under reduced pressure. From the dry residue, material giving a positive histidine reaction equivalent to 8.6 mg. of histidine was extracted with secondary butyl alcohol. Appropriate aliquots of this butanol-soluble material were chromatographed on Whatman No. 4 paper using butanol-acetic acid-water solvent

(4:1:5). A single spot having an average R_f value of 0.47 was obtained upon development with ninhydrin. Leucine and histidine standards when chromatographed in the same manner, gave average R_f values of 0.63 and 0.15, respectively, demonstrating that the histidine-reactive material was not free histidine. After hydrolysis in constant boiling hydrochloric acid under reflux conditions for 21 hours the histidine-reactive material gave two spots corresponding precisely with histidine and leucine when chromatographed exactly as before. When the hydrolyzed material was mixed with unhydrolyzed material and chromatographed three spots were obtained. When mixed with leucine and histidine and chromatographed, it was indistinguishable from them.

These results show that the hypertensin-converting enzyme acts as a peptidase, splitting histidine and leucine from hypertensin I as an intact peptide.

The N-terminal amino acid of the dipeptide was determined by Sanger's fluorodinitrobenzene technique (14) as modified by Levy (15).

The dipeptide, after reaction with fluorodinitrobenzene, followed by hydrolysis with constant boiling hydrochloric acid, was chromatographed on Whatman No. 4 paper. Bisdinitrophenylhistidine and dinitrophenylleucine, as well as free histidine and leucine were used as reference standards. One yellow spot corresponding to bisdinitrophenylhistidine was obtained in the butanol–acetic acid–water solvent. On spraying the chromatogram with ninhydrin a single blue spot corresponding to free leucine was found. Neither free histidine nor dinitrophenylleucine was found.

The fluorodinitrobenzene technique thus established the amino acid sequence of the dipeptide as histidylleucine.

Action of Carboxypeptidase on Hypertensin I.—

A 0.2 ml. suspension of carboxypeptidase (Nutritional Biochemical Co., Cleveland) was twice washed with cold water and then resuspended in 10 ml. of 0.1 per cent sodium bicarbonate solution. Molar sodium hydroxide was added until the suspension just dissolved, whereupon the pH was quickly adjusted to 8.0 by the addition of dilute hydrochloric acid. Aliquots of the enzyme solution prepared in this manner were used in each of the experiments.

In the first experiment 5 ml. of a solution of hypertensin I at pH 8.0 containing 5.25 mg. of solid (0.84 mg. of N, with 8,100 Goldblatt units of activity) was incubated with 0.10 ml. of the carboxypeptidase preparation at 38°C. for 165 minutes. At various intervals during incubation suitable aliquots of the reaction mixture were withdrawn and the enzymatic activity was quickly destroyed by boiling. Each aliquot was analyzed for ninhydrin reactive material and also assayed for pressor activity in the rat.

The results, illustrated in Fig. 2, indicate that soon after initiation of the reaction there was an increase in ninhydrin-reactive material, presumably due to splitting of the peptide, which continued for 160 minutes. It is noteworthy that pressor activity diminished very rapidly, but returned to a considerable extent after 30 minutes, only to decrease again until at the end of the experiment there was virtually no activity remaining. This observation was confirmed upon repetition of the experiment.

The appearance of amino acids in the samples withdrawn as the reaction

proceeded was studied by paper chromatography. After 165 minutes leucine, histidine, and phenylalanine were the only free amino acids found. The presence of a single peptide was also revealed when the paper chromatograms were stained by Durrum's procedure (16). This peptide was presumably the product of hypertensin I after degradation to the limit of carboxypeptidase action.

Leucine was the first amino acid to be liberated in major quantities and appeared during the first 10 minutes of the incubation. Histidine next appeared,

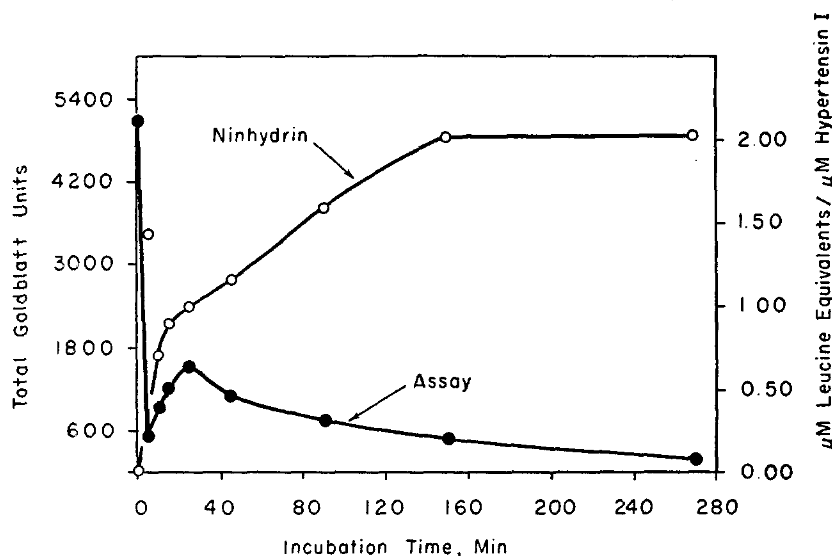

FIG. 2. Incubation of hypertensin I with carboxypeptidase

being maximally liberated in 25 minutes, and phenylalanine appeared last, increasing in amount until 145 minutes. This qualitative interpretation of the sequence in which the amino acids were liberated was based on a visual estimate of the relative intensities of the ninhydrin spots resulting from chromatographs at each time interval. A quantitative procedure was next devised to test the validity of these interpretations.

In the quantitative experiment separate 2.0 ml. samples containing 2.10 mg. of hypertensin I were incubated at 38°C. for 5, 15, and 90 minutes with 0.05 ml. of the carboxypeptidase preparation. The reaction was stopped by lowering the pH to 1.5 with hydrochloric acid. The sample was evaporated to dryness. The residues were taken up in 2.0 ml. of 0.01 N HCl and transferred to 0.9 × 15 cm. dowex 50 × 8 columns. The following series of buffers was used in eluting the samples: (*a*) 0.1 M sodium citrate, pH 4.00, to elute leucine; (*b*) 0.1 M sodium citrate, pH 4.25, to elute phenylalanine; (*c*) an intermediate buffer, 0.1 M sodium citrate, pH 6.7 (15–20 ml.); and (*d*) 0.2 M $NaHCO_3$, pH 8.3 to elute histidine. These buffers, except for the pH 4.00 buffer, are those described by Moore and Stein (10).

The results of these experiments, given in Table II, demonstrate that the first amino acid removed from hypertensin I by carboxypeptidase is leucine, the second histidine, and the third phenylalanine. Of particular interest was the finding that leucine is the carboxyl terminal amino acid of hypertensin I and that it is linked through its amino group to the carboxyl of histidine. This is the same sequence previously established for the peptide histidylleucine, obtained by the action of the hypertensin converting enzyme on hypertensin I.

Action of Carboxypeptidase on Hypertensin II.—

1.0 ml. of hypertensin II solution containing 1.37 mg. of solid (0.22 mg. of N with 3,000 Goldblatt units) was chilled in ice and mixed with 0.1 ml. of cold molar Na_2HPO_4 buffer

TABLE II

Release of Amino Acids from Hypertensin I by Carboxypeptidase

Products	Time of incubation at 38°C.	Amount of amino acid released per μM* of hypertensin I
	min.	μM
Leucine	5	0.81
	15	0.91
	90	0.67
Histidine	5	0.12
	15	0.37
	90	0.82
Phenylalanine	5	0.00
	15	0.22
	90	0.73

* Based on the tentative minimum molecular weight of 1305.

containing 0.05 ml. of carboxypeptidase solution. The mixture was incubated at 38°C. and samples were withdrawn at 0, 15, 45, 105, and 165 minutes. Each sample was analyzed for ninhydrin-reactive material and assayed for pressor activity in the rat.

The results are given in Fig. 3. Identity of the ninhydrin-reactive material was established by paper chromatography of aliquots from the reaction mixture after incubation for 165 minutes. With a descending secondary butyl alcohol–4 per cent ammonia 3 to 1 system, only one ninhydrin-reactive spot, corresponding to phenylalanine, was visible. Chromatography of this material alone and with reference phenylalanine in butanol–acetic acid–water gave a single spot in either case. No traces of free phenylalanine could be found when a zero time sample of the reaction mixture was similarly chromatographed. A second incubation of hypertensin II with carboxypeptidase for 240 minutes did not result in the appearance of even traces of amino acids other than

phenylalanine. In this latter experiment 0.90 μM of phenylalanine was liberated per μM of hypertensin II.

It is apparent that the loss in pressor activity of hypertensin II coincides with the liberation of its carboxyl terminal amino acid, phenylalanine. The failure of carboxypeptidase to hydrolyze either type of hypertensin beyond the release of phenylalanine, suggests that the amino acid present in the pep-

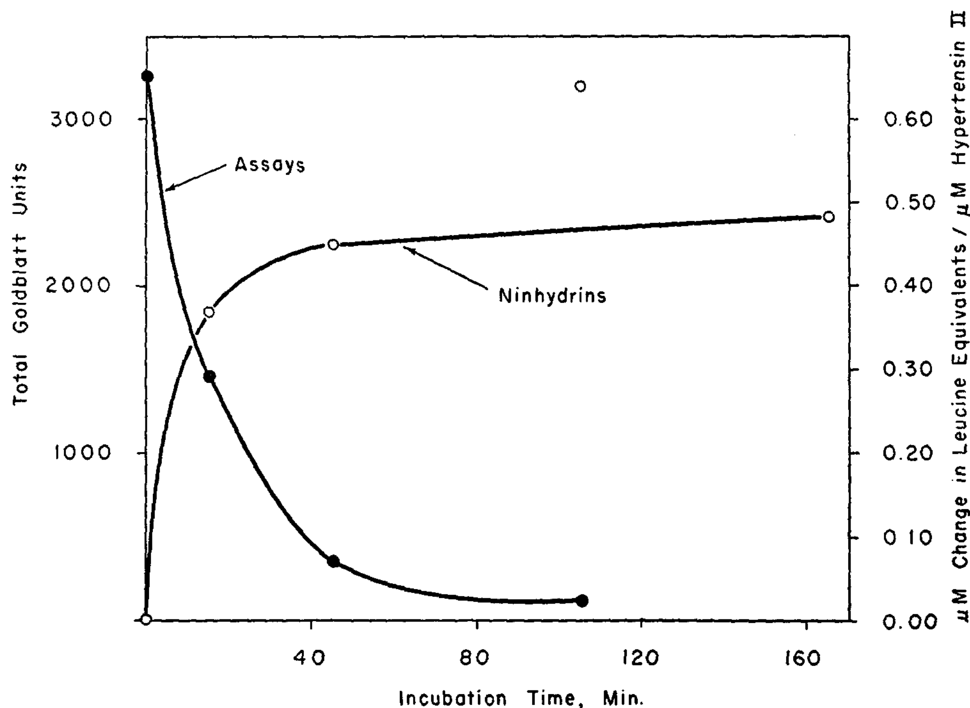

FIG. 3. Incubation of hypertensin II with carboxypeptidase

tide bond beyond phenylalanine is proline, an amino acid which resists the attack of carboxypeptidase (10).

DISCUSSION

The preparations of hypertensin I and II used in these studies had each previously been purified and isolated as a single component by countercurrent distribution. Hypertensin II was found to consist of eight amino acids in approximately molar ratios. These eight amino acids, plus the peptide histidylleucine, account for the ten amino acids previously found in hypertensin I, thus providing strong additional evidence for the purity of the hypertensin preparations.

The action of carboxypeptidase on hypertensin I produced an unusual effect

on the biological activity as assayed in the intact rat. The loss in activity coincident with the first appearance of free amino acids was followed by the return of activity as the amount of liberated amino acids increased. Finally the activity fell to 3 per cent of the initial level. By correlating the sequence of appearance of the various free amino acids with the changes in biological activity and the demonstration that the vasoconstrictor effect of hypertensin II is completely destroyed by the removal of phenylalanine a tentative explanation of the results may be given. The zero time sample of hypertensin I shows high pressor activity because it is readily converted to hypertensin II by the converting enzyme present in the circulating plasma of the rat used for assay. The first abrupt drop in physiological activity corresponds closely to the appearance of free leucine during the first 5 to 15 minutes; at this stage, the converting enzyme may not be capable of removing histidine to form hypertensin II. The subsequent increase in activity closely parallels the appearance of free histidine and therefore of free hypertensin II in the medium. The final drop in activity, which corresponds to the liberation of phenylalanine, would represent the inactivation of hypertensin II.

SUMMARY

Preparations of hypertensin II, obtained from the treatment of hypertensin I by the action of the hypertensin converting enzyme of plasma and purified by countercurrent distribution, were quantitatively analyzed for their amino acid content. Chromatography on ion exchange columns showed the presence of equimolar amounts of aspartic acid, proline, valine, isoleucine, tyrosine, phenylalanine, histidine, and arginine.

Hypertensin I was found to contain one mole of leucine and one mole of histidine in addition to the amino acids of hypertensin II. These two amino acids were isolated from the conversion products of hypertensin I and identified as the peptide histidylleucine.

Carboxypeptidase digestion of hypertensin I showed the carboxyl terminal sequence of amino acids to be residue–phenylalanyl–histidylleucine. Similar studies of hypertensin II demonstrated residue-phenylalanine. It was concluded that the conversion of hypertensin I by the plasma hypertensin converting enzyme involved hydrolysis of the phenylalanyl-histidine bond to form hypertensin II and histidylleucine. The further removal by carboxypeptidase of phenylalanine from hypertensin II destroyed all of the vasoconstrictor activity.

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