Rapid Papers

Formation of Angiotensin III by Angiotensin-Converting Enzyme

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Angiotensin III is formed from des-Asp¹-angiotensin I by angiotensin-converting enzyme. The K_m (11 μ M) of the reaction is one-third of that for the conversion of angiotensin I into angiotensin II. As suggested by the K_m values, bradykinin, peptide BPP_{9a} and angiotensins II and III are better inhibitors of the formation of angiotensin II than of the formation of angiotensin III.

A number of investigators have postulated that angiotensin III (des-Asp¹-angiotensin II) may be a primary secretagogue of aldosterone (Chiu & Peach, 1974; Peach & Chiu, 1974; Campbell *et al.*, 1974; Spielman *et al.*, 1975). Although angiotensin III is less active than angiotensin II on vascular smooth muscle, it has a faster onset of action and is slightly more potent than angiotensin II in stimulating the release of aldosterone from dispersed cells of the adrenal cortex (Chiu & Peach, 1973). Angiotensin III also differs from angiotensin II in terms of its response to antagonists. For example, [Sar¹,Ala⁸]-angiotensin II is a good antagonist of the effects of angiotensin II on the adrenal cortex but is a poor antagonist of angiotensin III (Bravo *et al.*, 1975). A related lower homologue, des-Sar¹-[Ile⁸]angiotensin II, is a good antagonist of the effects of both angiotensins II and III on aldosterone secretion.

At present, it is not known whether angiotensin III occurs *in vivo* in concentrations sufficient to affect aldosterone secretion. Similarly, little is known of how angiotensin III might be formed *in vivo*. Plasma, erythrocytes and homogenates of some tissues contain enzymes capable of releasing the *N*-terminal aspartic acid residue of angiotensin II (for review see Nagatsu *et al.*, 1970; Cheung & Cushman, 1971; Ryan, 1974); however, the enzymes have not been purified to homogeneity, and little is known of their specificity or selectivity of action.

In early studies on the immediate metabolic fate of [[¹⁴C]Leu¹⁰]angiotensin I during passage through the lungs, a radioactive product was found that possessed properties like those expected of des-Asp¹-angiotensin I (Ryan *et al.*, 1970). On molecular-sieve chromatography, the product was

eluted like angiotensin I, but migrated on electrophoresis like a compound having a net positive charge greater than that of angiotensin I. In view of the position of the radioactivity within the parent compound and of the distribution of charged residues in angiotensin I (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH), it seems likely that the product was des-Asp¹-angiotensin I.

Considering the abundance of angiotensin-converting enzyme in plasma, lung and other tissues and the accessibility of the lung enzymes to circulating substrates (Skeggs *et al.*, 1956; Roth *et al.*, 1969; Cushman & Cheung, 1971; Ryan *et al.*, 1972, 1975), we have begun to examine the possibility that angiotensin III may be formed from des-Asp¹-angiotensin I. Angiotensin-converting enzyme acts as a dipeptidyl carboxypeptidase, but its selectivity of action appears to depend in part on *N*-terminal residues distant from the site of hydrolysis (Dorer *et al.*, 1974a, 1975). None of the *N*-terminal higher homologues of the nonapeptide bradykinin (e.g. Lys-bradykinin or Met-Lys-bradykinin) is hydrolysed as rapidly as is bradykinin itself. Similarly, the tetradecapeptide renin substrate is hydrolysed at less than one-tenth the rate observed for hydrolysis of its *C*-terminal nonapeptide (Dorer *et al.*, 1975). In the present study we have found that des-Asp¹-angiotensin I is a better substrate for angiotensin-converting enzyme than is angiotensin I.

Materials and Methods

All of the following experiments used pig lung angiotensin-converting enzyme (fraction G as described by Dorer *et al.*, 1972). Angiotensin I was obtained from Corning Glass Works, Medfield, MA,

U.S.A., through the courtesy of Mr. Roger N. Piasio. [Sar¹,Ala⁸]Angiotensin II was obtained from Norwich Pharmacal Co., Norwich, NY, U.S.A., through the courtesy of Dr. Alan W. Castellion. Angiotensin III was purchased from Peninsula Laboratories, San Carlos, CA, U.S.A. Bradykinin, angiotensin II-(3-6)-hexapeptide and L-histidyl-L-leucine were purchased from Schwarz/Mann, Orangeburg, NY, U.S.A. Bradykinin-potentiating peptides (BPP_{9a} and BPP_{5a}; for structures see Tominaga *et al.*, 1975), angiotensin II, des-Asp¹-angiotensin I and lower homologues (angiotensin I-(3-10)-octapeptide, -(4-10)-heptapeptide and -(5-10)-hexapeptide; structures shown in Table 1) were synthesized by the solid-phase technique (Stewart & Young, 1969). The polypeptides were purified by chromatography on Sephadex G-25 and/or by countercurrent distribution.

Rates of hydrolysis of angiotensin I or des-Asp¹-angiotensin I by pig lung angiotensin-converting enzyme were measured in terms of the formation of the dipeptide His-Leu by the automated fluorescence technique described by Boucher *et al.* (1972). The dipeptide His-Leu forms a fluorophor on reaction with *o*-phthalaldehyde. Under the conditions of the assay, the products of reaction of *o*-phthalaldehyde with free histidine and leucine do not interfere. Except for studies for estimating K_m and V_{max} , each substrate was used in a final concentration of 25 or 50 μ M. Incubations were carried out at 37°C in 50mM-sodium phosphate buffer, pH 7.5. Except where indicated, the incubation mixtures contained 100mM-NaCl. Reactions were begun by adding angiotensin-converting enzyme at a final concentration of 0.286 ng of protein/ml. Corrected for purity (approx. 35%) and assuming a molecular weight of 129000 (Das & Soffer, 1975), the enzyme was at a final concentration of 770 pM. After it was established that the reactions proceeded linearly over a period of 60 min, all subsequent incubations were for 60 min. Reactions were terminated by heating the mixtures in a boiling-water bath for 5 min. In studies on the effects of inhibitors, the enzyme was preincubated at 37°C with a given inhibitor for 2 min.

Results and Discussion

At pH 7.5, in the presence of 100mM-NaCl, the K_m for the hydrolysis of des-Asp¹-angiotensin I is one-third of that for angiotensin I (Table 1). However, the K_m values for lower C-terminal homologues are greatly elevated, a finding that suggests that, if these lower homologues are formed *in vivo*, they are unlikely to interfere with the action of converting enzyme on angiotensin I. Values for K_m and V_{max} were estimated from Lineweaver-Burk plots, and it can be argued that the threefold difference between the K_m of des-Asp¹-angiotensin I and that of angiotensin I may be near to experimental error. Since the hydrolysis of both substrates was measured in terms of the formation of His-Leu, we were unable to determine whether des-Asp¹-angiotensin I inhibits the hydrolysis of angiotensin I. Possibly the question can be resolved definitively by using tracer amounts of [³H]Leu¹⁰angiotensin I.

In view of the different pharmacological effects of angiotensin II and III, whether converting enzyme has a greater affinity for des-Asp¹-angiotensin I than for angiotensin I could well be a question of physiological significance. Should des-Asp¹-angiotensin I and angiotensin I occur in equimolar quantities, the formation of an aldosterone secretagogue would be favoured over the formation of a pressor agent (cf. Bravo *et al.*, 1975). A higher affinity of the enzyme for des-Asp¹-angiotensin I is favoured by data shown in Table 2. Angiotensins II and III, bradykinin and peptide BPP_{9a} are better inhibitors of the hydrolysis of angiotensin I than of the hydrolysis of des-Asp¹-angiotensin I.

The influence of chloride concentration on the hydrolysis of des-Asp¹-angiotensin I by converting enzyme differed from that found when angiotensin I was used as substrate. Hydrolysis of des-Asp¹-angiotensin I proceeded at its maximum rate (783 nmol/min per mg of enzyme protein) when chloride was in a final concentration of 10mM, the optimal chloride concentration for hydrolysis of bradykinin (Dorer *et al.*, 1974b). The rate was decreased by 33% when chloride was at 1 or 50mM and by 51% when chloride was at 100mM, the optimal chloride

Table 1. Kinetics of the reaction of angiotensin I and its C-terminal lower homologues with angiotensin-converting enzyme. Values for V_{max} are corrected for apparent purity of the enzyme and are expressed as μ mol/min per mg of enzyme protein. Details of the incubation conditions are described in the text.

	K_m (μ M)	V_{max}	V_{max}/K_m
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu*	33	1.98	0.060
Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	11	0.99	0.090
Val-Tyr-Ile-His-Pro-Phe-His-Leu	170	7.94	0.047
Tyr-Ile-His-Pro-Phe-His-Leu	100	8.83	0.088
Ile-His-Pro-Phe-His-Leu	1000	37.71	0.038

*Angiotensin I.

Table 2. Inhibition of the reaction of angiotensin-converting enzyme with angiotensin I or des-Asp¹-angiotensin I

Reaction mixtures contained substrate at 25 μ M in 50mm-sodium phosphate buffer, pH7.5, plus 100mm-NaCl. Converting enzyme was in a final concentration of 770pM.

Inhibitor	Concn. of inhibitor	Inhibition (%)	
		Angiotensin I 0 (731 nmol/min per mg)	Des-Asp ¹ -angiotensin I 0 (417 nmol/min per mg)
None	0	0	0
Bradykinin	5 μ M	90.9	50
Bradykinin	25 μ M	95.0	80.8
Angiotensin II	25 μ M	13.6	0
Angiotensin II	50 μ M	34.1	7.7
Angiotensin III	25 μ M	70.4	11.5
Angiotensin III	50 μ M	79.5	26.1
Angiotensin II-(3-8)-hexapeptide	25 μ M	0	0
Angiotensin II-(3-8)-hexapeptide	50 μ M	0	0
[Sar ¹ ,Ala ⁸]Angiotensin II	25 μ M	54.2	13
[Sar ¹ ,Ala ⁸]Angiotensin II	50 μ M	75.0	20
[Sar ¹ ,Ala ⁸]Angiotensin II	100 μ M	83.3	*
Peptide BPP _{9a}	5 nM	36.5	13.3
Peptide BPP _{9a}	50 nM	76.5	33.5
Peptide BPP _{5a}	5 nM	9.2	*
Peptide BPP _{5a}	50 nM	70	*

*Not tested.

concentration for hydrolysis of angiotensin I (Dorer *et al.*, 1972). Like bradykinin, des-Asp¹-angiotensin I is hydrolysed in the absence of chloride (25% of its maximum rate). Under these conditions hydrolysis of angiotensin I is almost negligible (approx. 5% of its maximum rate).

Results of the present study raise several points of possible importance. If des-Asp¹-angiotensin I occurs *in vivo*, it can be converted readily into angiotensin III. Judged by the relatively low K_m of the reaction of des-Asp¹-angiotensin I with converting enzyme, the formation of angiotensin III might be favoured over the formation of angiotensin II. In view of the potency of angiotensin III as an aldosterone secretagogue, it may be of interest that the formation of angiotensin III is accelerated when chloride concentrations fall below the physiological range (approx. 100mM). Dorer *et al.* (1972) have shown that the effects of chloride on the conversion of angiotensin I into angiotensin II are biphasic, with the highest rate occurring at 100mM-chloride. The possibility is suggested that the formation of angiotensin II is suppressed when chloride concentrations fall on either side of the physiological range.

An unexpected finding was that [Sar¹,Ala⁸]angiotensin II, an antagonist of pharmacological effects of angiotensin II (Pals *et al.*, 1971), is a good inhibitor of the conversion of angiotensin I into angiotensin II. As shown in Table 2, when [Sar¹,Ala⁸]angiotensin II and angiotensin I are used in equimolar quantities the rate of formation of angiotensin II is halved.

However, as used clinically, [Sar¹,Ala⁸]angiotensin II has been reported to reach concentrations in venous blood of up to 1 μ g/ml (Pettinger *et al.*, 1975). Thus, *in vivo*, the molar concentration of [Sar¹,Ala⁸]angiotensin II may exceed that of angiotensin I by more than 10000-fold. Under these conditions it is possible that conversion of angiotensin I into angiotensin II does not occur. It must be emphasized, however, that the present studies examined reactions of angiotensin-converting enzyme in a simple system *in vitro*, and the results may not be applicable in full to reactions occurring *in vivo*.

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References

- Boucher, R., Saidi, M. & Genest, J. (1972) in *Hypertension '72* (Genest, J. & Koiv, E., eds.), pp. 512–523, Springer-Verlag, Berlin, Heidelberg and New York
- Bravo, E. L., Khosla, M. C. & Bumpus, F. M. (1975) *J. Clin. Endocrinol. Metab.* **40**, 530–533
- Campbell, W. B., Brooks, S. N. & Pettinger, W. A. (1974) *Science* **184**, 994–996
- Cheung, H. S. & Cushman, D. W. (1971) *Biochim. Biophys. Acta* **242**, 190–193
- Chiu, A. T. & Peach, M. J. (1973) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 765
- Chiu, A. T. & Peach, M. J. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 341–344

- Cushman, D. W. & Cheung, H. S. (1971) *Biochim. Biophys. Acta* **250**, 261–265
- Das, M. & Soffer, R. L. (1975) *J. Biol. Chem.* **250**, 6762–6768
- Dorer, F. E., Kahn, J. R., Lentz, K. E., Levine, M. & Skeggs, L. T. (1972) *Circ. Res.* **31**, 356–366
- Dorer, F. E., Ryan, J. W. & Stewart, J. M. (1974a) *Biochem. J.* **141**, 915–917
- Dorer, F. E., Kahn, J. R., Lentz, K. E., Levine, M. & Skeggs, L. T. (1974b) *Circ. Res.* **34**, 824–827
- Dorer, F. E., Kahn, J. R., Lentz, K. E., Levine, M. & Skeggs, L. T. (1975) *Biochem. Pharmacol.* **24**, 1137–1139
- Nagatsu, I., Nagatsu, T., Yamamoto, T., Glenner, G. G. & Mehl, J. W. (1970) *Biochim. Biophys. Acta* **198**, 255–270
- Pals, D. T., Masucci, F. D., Denning, G. S., Jr., Sipos, F. & Fessler, D. C. (1971) *Circ. Res.* **29**, 673–681
- Peach, M. J. & Chiu, A. T. (1974) *Circ. Res.* **34–35**, Suppl. 1, I-7–I-13
- Pettinger, W. A., Keeton, K. & Tanaka, K. (1975) *Clin. Pharmacol. Ther.* **17**, 146–158
- Roth, M., Weitzman, A. F. & Piquilloud, Y. (1969) *Experientia* **25**, 1247
- Ryan, J. W. (1974) in *Handbook of Experimental Pharmacology* (Page, I. H. & Bumpus, F. M., eds.), vol. 37, pp. 81–110, Springer-Verlag, New York
- Ryan, J. W., Stewart, J. M., Leary, W. P. & Ledingham, J. G. (1970) *Biochem. J.* **120**, 221–223
- Ryan, J. W., Smith, U. & Niemeyer, R. S. (1972) *Science* **176**, 64–66
- Ryan, J. W., Ryan, U. S., Schultz, D. R., Whitaker, C., Chung, A. & Dorer, F. E. (1975) *Biochem. J.* **146**, 497–499
- Skeggs, L. T., Kahn, J. R. & Shumway, N. P. (1956) *J. Exp. Med.* **103**, 295–299
- Spielman, W. S., Davis, J. O. & Freeman, R. H. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 312 (Abstr. no. 526)
- Stewart, J. M. & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, W. H. Freeman, San Francisco
- Tominaga, M., Stewart, J. M., Paiva, T. B. & Paiva, A. C. M. (1975) *J. Med. Chem.* **18**, 130–133