Inhibition of renin by conformationally restricted analogues of angiotensinogen

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[Cys⁵,Cys¹⁰]Angiotensinogen-(5–14)-peptide analogues and homologues were synthesized, in which a disulphide bond between residues 5 and 10 stabilized a 9→6 β -turn proposed for the substrate. These compounds were competitive inhibitors of human and pig renins with K_i values of the order of 10^{-6} – 10^{-5} M, indicating that the conformation proposed for the substrate is important for the interaction with the enzyme.

Renin (EC 3.4.99.19) is a highly specific enzyme that cleaves the decapeptide proangiotensin ('angiotensin I') from the *N*-terminal end of the protein substrate, angiotensinogen, contained in the α_2 -globulin fraction of plasma. This is the first step of the renin–angiotensin–aldosterone system, whose role in the pathogenesis of certain types of hypertension is well-recognized. The only peptide bonds that have been found to be hydrolysed by renin are the Leu–Leu bond in the equine (Skeggs *et al.*, 1957) substrate and the Leu–Val bond in the human substrate (Tewksbury *et al.*, 1981).

To date, most of the renin-specificity studies with synthetic peptides have been done with analogues and homologues of the *N*-terminal tetradecapeptide sequence of the equine substrate:

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser

Skeggs *et al.* (1968) showed that the tetradecapeptide is as good a substrate as the angiotensinogen protein and that the minimum substrate size needed for efficient renin activity is the (6–13)-octapeptide. The same minimum size applies to the competitive inhibitory activity of analogues of the *N*-terminal sequence of the renin substrate (Poulsen *et al.*, 1973). This requirement for a minimum size of the peptide substrate or inhibitor might be due to the interaction of renin's active centre with other side chains of the substrate besides those of the Leu–Leu or Leu–Val sequence. Another possibility is that the side chains are involved in maintaining a substrate conformation needed for optimal enzyme activity. We have investigated the latter hypothesis on the basis of the conformation proposed by Oliveira *et al.*

(1977) for the tetradecapeptide containing the *N*-terminal sequence of equine renin substrate. This model contains a 9→6 β -turn involving residues His⁶ (*i*) to His⁹ (*i* + 3), which is stabilized by interactions between the two β -antiparallel nucleating sequences Val³-Tyr⁴-Ile⁵ and Leu¹⁰-Leu¹¹-Val¹², and by electrostatic interactions between the *N*- and *C*-terminal ends of the molecule.

In order to find whether this proposed substrate conformation may be important for binding to renin, we have synthesized several analogues of the equine angiotensinogen-(5–14)-peptide sequence in which the proposed β -turn was stabilized by a covalent link between residues 5 and 10. For this purpose, we have replaced Ile⁵ and Leu¹⁰ by a cystine residue to obtain the [Cys⁵,Cys¹⁰]angiotensinogen-(5–14)-peptide, in which the β -carbons of residues 5 and 10 are correctly positioned for a 9→6 β -turn (Veber *et al.*, 1978). Since in this analogue a half-cystine residue replaces one of the leucine residues next to the bond split by renin, it should not be expected to be a substrate for that enzyme. However, if the other side chains in this conformationally restricted peptide maintain a favourable orientation for interaction with renin, it might be expected to be a competitive inhibitor, if our hypothesis is correct.

Besides the equine [Cys⁵,Cys¹⁰]angiotensinogen-(5–14)-peptide (compound 1, Table 1), homologues with an added lysine residue at the *N*-terminus (compound 2) or at the *C*-terminus (compound 3) were synthesized in view of the reported greater solubility of derivatives of the (5–14)-peptide sequence containing a *C*-terminal lysine residue (Cody *et al.*, 1980). An analogue of the human angiotensinogen-(4–14)-peptide sequence (compound 4), was also synthesized, as well as an homologue of compound 1 containing an extra histidine residue at the *endo*-9a position (compound

Abbreviations used: TNBS, 2,4,6-trinitrobenzenesulphonic acid; Boc, *t*-butoxycarbonyl.

Table 1. *Conformationally restricted analogues and homologues of the equine angiotensinogen-(5-14)-peptide*

No.	Compound	Amino acid sequence
1	[Cys ⁵ ,Cys ¹⁰]Angiotensinogen-(5-14)-peptide	Cys-His-Pro-Phe-His-Cys-Leu-Val-Tyr-Ser
2	[Lys ⁴ ,Cys ⁵ ,Cys ¹⁰]Angiotensinogen-(4-14)-peptide	Lys-Cys-His-Pro-Phe-His-Cys-Leu-Val-Tyr-Ser
3	[Cys ⁵ ,Cys ¹⁰ ,Lys ¹⁴]Angiotensinogen-(5-14)-peptide	Cys-His-Pro-Phe-His-Cys-Leu-Val-Tyr-Lys
4	[Lys ⁴ ,Cys ⁵ ,Cys ¹⁰]Angiotensinogen-(4-14)-peptide (human)	Lys-Cys-His-Pro-Phe-His-Cys-Val-Ile-His-Ser
5	[Cys ⁵ ,endo-His ^{9a} ,Cys ¹⁰]Angiotensinogen-(5-14)-peptide	Cys-His-Pro-Phe-His-His-Cys-Leu-Val-Tyr-Ser

5). This latter compound was prepared in an attempt to achieve greater solubility, and to test the effect of having a 23-membered disulphide ring instead of the 20-membered one present in the other analogues.

Materials and methods

Materials

Pig renin (11 Goldblatt units/mg of protein) and human renin (0.05 Goldblatt units/mg of protein) were purified preparations kindly supplied by Dr. E. Haas, Mount Sinai Hospital, Cleveland, OH, U.S.A. The preparation of the human enzyme was shown to be free of other peptidases and suitable for studies of the enzyme kinetics (Haas *et al.*, 1966). This was confirmed and extended to the pig enzyme by incubations of 47 μ M-angiotensin I with pig renin (8.7×10^{-2} Goldblatt units/ml) or human renin (5×10^{-4} Goldblatt units/ml), in 0.1 M-phosphate buffer, pH 7.0. No change in the biological activity of proangiotensin was detected after 2 h at 36°C, as assayed by its contractile effect on the isolated guinea-pig ileum preparation.

Tetradecapeptide renin substrate was synthesized in this laboratory (Oliveira *et al.*, 1977), as well as oxytocin (490 i.u./mg). TNBS was prepared as described by Golumbic *et al.* (1946) and purified by the method of Fields (1972).

Methods

Enzyme assays. The reaction mixtures contained 12.5–46 μ M-tetradecapeptide substrate and pig renin (8.7×10^{-2} Goldblatt units/ml) or human renin (5×10^{-4} Goldblatt units/ml) in 0.1 M-phosphate buffer, pH 6.0 or 7.5. For the assay of inhibition, the peptide analogues were included in concentrations ranging from 70 to 100 μ M. The incubations were done at 37°C and the reaction was interrupted at the desired times by adding portions of the reaction mixture to a solution of 0.1 M-sodium tetraborate in 0.1 M-NaOH. The increase in free amino groups was measured by the TNBS method as described by Fields (1972).

Syntheses. The peptides were synthesized by the solid-phase method (Merrifield, 1963; Stewart & Young, 1969) in the scale of 1 mmol. The C-terminal Boc-amino acid was esterified to 1% cross-linked chloromethyl polymer, and chain elongation was

performed on an automatic peptide synthesizer (Merrifield *et al.*, 1966). The amino acids with reactive side chains were used in the form of the following derivatives: Boc-Ser(*O*-benzyl), Boc-His[*N*^{im}-tosyl (im = imidazole)]; Boc-Cys(*S-p*-methoxybenzyl); Boc-Tyr(*O*-2,6-dichlorobenzyl) and Boc-Lys(ϵ -2-chlorobenzyl). The coupling steps were carried out by shaking the resin in a solution of 2.5 equiv. of Boc-amino acid and dicyclohexylcarbodiimide dissolved in dichloromethane. The extent of coupling was monitored with the ninhydrin test (Kaiser *et al.*, 1970), and in all cases the reaction was completed after 2 h. The Boc groups were removed by treatment for 30 min with 25% (v/v) trifluoroacetic acid in chloroform containing 2% (v/v) anisole and 0.2% *o*-cresol. This was followed by neutralization with 10% (v/v) triethylamine in dichloromethane. After the coupling of the last residue, the peptide-resin was submitted to a last deprotection step with trifluoroacetic acid and washed with ethanol. The peptide was cleaved from the resin by treatment with anhydrous HF containing 5% (v/v) anisole, and then oxidized with 0.01 M-K₃Fe(CN)₆ and treated with an AG-3 anion-exchange resin as described by Live *et al.* (1977).

Purification and characterization of peptides. The crude peptides were submitted to 100–200 transfers of countercurrent distribution in butanol/acetic acid/water (20:1:19, by vol.) followed by gel-filtration chromatography on a 1.8 cm $\times$ 110 cm column of Bio-Gel P-4 resin eluted with 3.5 M-acetic acid. Further purification, if necessary, was done by ion-exchange chromatography on a 2 cm $\times$ 21 cm CM-cellulose column with a linear gradient of ammonium acetate from 0.01 M (pH 5) to 0.5 M (pH 6.5). Each peptide was purified until the following criteria for purity were met. (a) The amino acid analysis of the acid hydrolysates, done on a Beckman model 120C amino acid analyser, yielded a molar ratio within 5% of the theoretical value for all the amino acid residues. Peptide samples were hydrolysed *in vacuo* for 72 h at 110°C in 6 M-HCl with 5% (w/v) phenol, both with and without previous treatment with performic acid for conversion of cysteine to cysteic acid (Moore, 1963). (b) Only one spot was detected with Pauly, ninhydrin and chlorine reagents after t.l.c. on silica gel (Eastman Chromagram plates; 0.1 mm thick) with

the following solvent systems: butanol/acetic acid/water (4:1:1, by vol.); butanol/ethyl acetate/acetic acid/water (1:1:1:1, by vol.); butanol/acetic acid/pyridine/water (15:3:10:12, by vol.). (c) Only one component, with the expected mobility, could be detected after paper electrophoresis at 1000 V for 60 min in three different buffers: 1 M-acetic acid (pH 2.8), 0.1 M-pyridine/acetate (pH 4.9) and 0.025 M-sodium carbonate/bicarbonate (pH 9.9).

Estimation of the peptides' molecular size was done by gel permeation on a 2.5 cm $\times$ 97 cm column of Bio-Gel P-4 eluted with 3.5 M-acetic acid at the rate of 15 ml/h. The column's void volume was 92.5 ml, as determined by the elution of Blue Dextran.

Results

The disulphide bond formation by oxidation of the deprotected [Cys⁵,Cys¹⁰] analogues gave relatively high yields of the monomeric cyclic disulphides, no significant amounts of dimers or higher polymers being detected by gel-filtration chromatography. As Fig. 1 shows, the five compounds were eluted somewhat later than predicted by their molecular weight. However, since the column was calibrated

Fig. 1. Behaviour of angiotensinogen analogues on gel-filtration chromatography

The ratio of elution volume (V_e) to void volume (V_0), from a Bio-Gel P-4 column, as a function of log (molecular weight). $\circ$, Cyclic disulphide peptides: numbers 1–5 refer to the compounds listed in Table 1; 6, oxytocin. $\bullet$, Linear peptides: 7 Tyr-Ile-His-Pro-Phe-NH₂; 8, Val-Tyr-Ile-His-Pro-Phe; 9, [Pro³,Pro⁵]angiotensin; 10, bradykinin; 11, [Ile⁸]proangiotensin; 12, angiotensinogen-(1–11)-peptide.

with linear peptides, the apparently smaller molecular size may be attributed to the cyclic nature of the disulphide compounds. A similar behaviour was

Fig. 2. Effect of conformationally restricted angiotensinogen analogues on renin kinetics

Lineweaver-Burk plots for the action of human renin at pH 7.5 (a) and pig renin at pH 6.0 (b) on angiotensinogen-(5–14)-peptide in the absence ($\square$) and in the presence of the following analogues, numbered as listed in Table 1: $\bullet$, compound 1 (99 μ M); $\circ$, compound 2 (77 μ M); $\blacktriangle$, compound 3 (73 μ M); $\triangle$, compound 4 (72 μ M); $\blacksquare$, compound 5 (70 μ M).

Table 2. Inhibitory constants (K_i) for the effect of conformationally restricted substrate analogues on the action of human and pig renin on the tetradecapeptide substrate

Compound*	K_i (μM)		
	Renin ...	Human	Pig
		pH 6.0 pH 7.5	pH 6.0
1		53 —†	17.1
2		66 33	8.6
3		48 8.6	6.9
4		54 30	9.8

* See Table 1.

† Compound 1 was not assayed at pH 7.5 because of low solubility.

described for oxytocin (Zahn & Folsche, 1968), which we have also observed (Fig. 1).

None of the five compounds studied was detectably hydrolysed by either pig renin at pH 6.0 or human renin at pH 6.0 or 7.5. The renins from both sources were studied at pH 6.0, which is within the range of the pH optimum for their activity on the synthetic tetradecapeptide substrate (Galen *et al.*, 1978; Favre *et al.*, 1973). Human renin was also studied at pH 7.5, which is above its pH optimum, because of the interest in testing possible inhibitors at the physiological pH.

With the exception of compound 5, which was inactive, the disulphide analogues behaved as competitive inhibitors of the action of renin on the tetradecapeptide substrate. This is illustrated by the Lineweaver–Burk plots of Fig. 2.

Compound 1 could not be assayed against human renin at pH 7.5 because of its low solubility at that pH, but it was soluble enough at pH 6.0 to behave as a competitive inhibitor of both human and pig renin at that pH. Table 2 shows the inhibitory constants (K_i) determined with the equine angiotensinogen-(1–14)-peptide as substrate. These constants should be compared with the K_m values for the action of the enzymes on that substrate. For pig renin at pH 6.0 we found a K_m of 11.3 μM , which is of the same order as the value 17.8 μM found by Galen *et al.* (1978) under the same conditions. For human renin at pH 6.0, we found a K_m of 115 μM , which is comparable with the value of 143 μM determined by Poulsen *et al.* (1973) for the same substrate at the same pH. At pH 7.5, we obtained a K_m value of 25 μM , which is the same value as that found by Poulsen *et al.* (1976) under the same conditions.

Discussion

Oliveira *et al.* (1977) presented evidence for a reverse turn in the solution conformation of the tetradecapeptide renin substrate and suggested that

this structure might be important for the interaction with the enzyme. In the compounds studied here, the 9→6 β -turn is stabilized by the disulphide bond between the cysteine residues occupying positions 5 and 10 of the sequence, as has been shown to occur in the similar case of the conformationally restricted somatostatin analogues described by Veber *et al.* (1978).

The K_i for compound 1 at pH 6.0 was smaller than the K_m for the tetradecapeptide in the case of human renin, and of the same order in the case of pig renin. This contrasts with the finding of Skeggs *et al.* (1968), that the K_m for the action of pig renin on the (5–14)-peptide (at pH 7.5), was 30.7 μM , compared with 3.68 μM for the hydrolysis of the tetradecapeptide. The fact that this 10-fold difference does not occur in the conformationally restricted analogues indicates that the proposed conformation may be important for the interaction of renin with its substrate.

The addition of an *N*-terminal lysine residue (compound 2) did not change the affinity for the human enzyme significantly, but improved binding to pig renin. On the other hand, the introduction of lysine as the *C*-terminal residue in compound 3 yielded the best inhibitor of the series for both human and pig renin.

The presence of the sequence of the human substrate in compound 4 was expected to make it a more selective inhibitor for human renin. However, we found that this compound had about the same inhibitory effects on human and pig renins as its analogue with the pig substrate sequence (compound 2). This indicates that the sites responsible for the species difference in renin specificity lie beyond the (5–13)-peptide sequence of the substrate.

Renin inhibitors may have important applications in the study of the physio-pathological roles of the renal pressor system and in the treatment of renin-dependent forms of arterial hypertension. The most specific renin inhibitors hitherto described have been peptide analogues of substrate fragments. Among these, the most potent are analogues and derivatives of the (6–13)-octapeptide (Haber & Burton, 1979), which, however, are generally not active *in vivo* because of solubility problems. Recently, the decapeptide:

has been shown to have a K_i of 2 μM *in vitro* and to be efficient for use *in vivo* (Cody *et al.*, 1980).

Compound 3, being soluble at pH 7.5, and having inhibitory constants of the same order of magnitude as those of the most potent inhibitors previously described, may also be useful in future studies *in vivo*. We believe that conformationally restricted analogues of renin substrate hold promise as useful inhibitors of that enzyme.

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