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Synthesis, conformation, and antibody recognition of peptides built of the sequence of the flap of human renin.

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Abstract : Five peptides related to human renin flap region have been synthesized. Two of them are ring closed through properly designed disulfide bridges. Structure analysis involving IR, CD and NMR techniques and recognition by antibodies raised against human renin support our assumption that in the protein, the flap region adopts a β and γ turn leading to the carbonyl group of residue 83 (Tyr) interacting with the NH's of residues 85 (Thr) and 86 (Gly).

Understanding of protein function at the molecular level requires knowledge of the detailed three dimensional structure of the protein. Up to now, in spite of numerous structural studies including mainly X-ray diffraction and two dimensional NMR spectroscopy which require large amounts of material (i.e. mg), the precise conformation of many proteins is still unknown. Attempts to overcome these experimental difficulties have been developed and are based on structure prediction and conformational investigations on synthetic peptides mimicking fragments of proteins. For renins, aspartyl proteases which participate in the regulation of blood pressure, the situation is as follows :

-the primary structures of human and mouse species are known[1-2],

-several models based on X-ray diffraction data of pepsins have been proposed[3-4],

-the flexible region named the flap is thought to be of major importance for the understanding of the biological activity[5-6] of the renins.

Beside the conformational purpose, *i.e.* the elucidation of the conformation of the flap section, it was decided to synthesize peptides with the same sequence as the flap of human renin in order to verify the recent concept of using peptides, the sequences of which are small segments of proteins, as haptens. These haptens have been used for the detection of proteins predicted from their gene sequences, as synthetic vaccines and for mapping of natural epitopes of proteins. Many proposals have been made for designing these haptens, including the choice of putative flexible segments[7-8] or of rigid and fixed segments[9-10]. The first proposal is based on the assumption that if antibodies against many conformations are elicited, some clones may produce antibodies which fit with the peptide segment included in the native protein. In the second case, the conformation of the hapten is supposed to be very close to that presented by the corresponding sequence in the protein, so that most clones produce protein-fitting antibodies. In this case, it is very important to check the identity of the hapten conformation with that of the homologous protein segment. This can be achieved only if a crystallographic structure or at least a homologic three dimensional model is available.

Peptide design.

Figure 1. *A stereoview of the three dimensional structure of human renin model. The flap region is shown with a thicker line and with its side chain.*

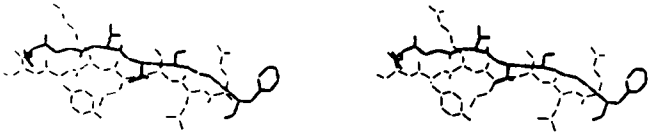

Figure 2. *A stereoview of the flap section (residues 78-91) showing that side chains of Phe 91 and Glu 78, of Ser 89 and Thr 80 are respectively facing each other on the same side of the flap mean plan.*

The localization of the flap in aspartyl proteases is shown in **Figure 1**, based on the human renin model of Blundell et al.[11-12]. This model has been based on the crystallographic structure of rhizopus pepsin from *Rhizopus arrhizus*. **Figure 2** shows the detail of the model of the flap for human renin : residue substitution is not the only deviation from the crystallographic structure as a residue deletion occurs when going from the starting pepsin to the renins (**Figure 3**).

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K W S I S Y G A G A S A S G N A V.....Endothiapepsin
T W S I S Y G D G S S A S G N V F.....Penicillopepsin
T W S I S Y G D G S S A S G I L A.....Rhizopuspepsin
D F T I H Y G S G R V  K G F L S.....Mouse Submaxillary renin
E L T L R Y S T G T V  S G F L S.....Human renin
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Figure 3. *Alignment of the flap region of several aspartyl proteases.*

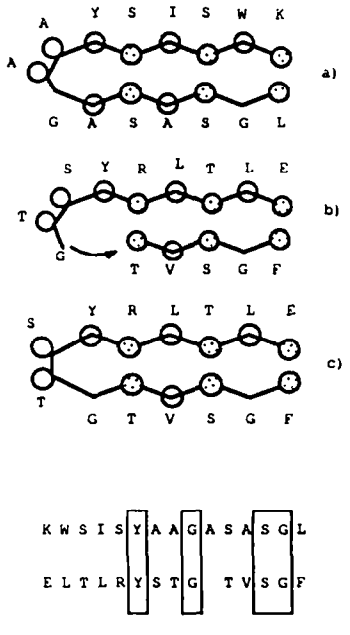

Figure 4. *Alignment of human renin and endothia pepsin sequences. (a) schematic representation of the α carbon of endothia pepsin flap determined by X-ray crystallographic techniques, (b) alignment of the human sequence on the α carbon of endothia pepsin and creation of a deletion, (c) the proposed model of the human flap α carbon for this study. The circles represent the side chain of amino-acid.*

This deletion can be accomodated either by pulling the chain oriented toward the C-terminus of the protein, or by pulling the chain oriented towards the turn section (**Figure 4**). This latter possibility appears very unlikely as strong perturbations can be expected in the pre- or post-flap sections. A more plausible operation causes a modification of the type of reversal of the peptide chain, going from a β to a γ turn or *vice-versa* maintaining the overall dimensions of the flap (**Figure 4**). However, this implies modifications of the orientations of the side-chains of one of the hairpin strands. Thus, the localization and the type of the reversal is one of the key for the determination of the flap structure.

Examination of the relative positions of residues engaged in a hairpin structure, particularly the relative orientations of the CαH bonds of residues of both strands indicate that the identification of the reversal could be easily achieved through ^{1}H NMR spectrometry. Indeed, CαH---CαH NOEs will be informative of the type and the localization of the reverse turn structure. For example CαH---CαH NOEs between two residues in position j and j+4 or j and j+5 are expected for γ and β turns respectively.

Hence, five peptides will be considered in this study as shown in **Figure 5**. The first one (peptide 1) is based on the sequence which is thought to correspond to the flap of human renin, is a 17-peptide. The four others were selected on the basis of preliminary results obtained on peptide 1 which suggested the presence, in DMSO, of a β turn involving the Tyr-Ser-Thr-Gly sequence. Therefore the sequences of these other peptides are centered around this β turn, two of them are linear (peptides 2 and 3) and were made for classical immunological studies while the two others are the homologous peptides cyclized by appropriate disulfide bridges (peptides 4 and 5) designed especially to improve the stability of the hairpin conformation. In the cyclic peptides, facing homologous residues, Thr80 and Ser 89 for the 10-peptide 4, Glu78 and Phe91 for the 14-peptide 5, are replaced by cysteine; in the linear peptides 2 and 3, these residues are substituted by alanine. In the β-hairpin structures, the alternation of facing hydrophilic (outside the protein) and hydrophobic side chains (inside the protein) is a striking feature.

T E L T L R Y S T G T V S G F L S : Peptide 1 : [77-93]-human renin

A L R Y S T G T V A : Peptide 2 : (Ala80, Ala89)[80-89]-human renin

A L T L R Y S T G T V S G A : Peptide 3 : (Ala78, Ala91)]78-91]-human renin

C L R Y S T G T V C : Peptide 4 : (Cys80,Cys89)[80-89]-human renin

C L T L R Y S T G T V S G C : Peptide 5 : (Cys78, Cys91)[78-91]-human renin

***Figure 5.** Sequences of the synthesized peptides.*

Instrumentation :

Infrared spectra were recorded on a 983 model Perkin Elmer spectrograph. NMR samples were prepared in DMSO-d6 at 12-20 mM peptide concentration. ^{1}H NMR spectra were recorded at 305°K on a Bruker WM 360 WB spectrometer (working at 360 MHz for ^{1}H) operating in the FT mode with quadrature detection. COSY[13-14], relayed COSY[15] and NOESY[16-17] were used for proton resonance assignments.

Purity of the peptides was assessed by amino acid analysis on a automated pre-column derivatization with *ortho*-phtalaldehyde with a Waters system. Capacity factor was calculated in the following conditions : a C18 column reversed phase Lichrosorb Merck (4 x 250 mm, 5 mm); monitoring at 214 and 254 nm with an isocratic eluent system (acetonitrile/water) 0.1 % of trifluoroacetic acid; flow rate : 1.5 mL/min.

Peptide synthesis :

Table 1 lists the analytical data of the peptides prepared for this study. The peptides were synthesized by the solid phase technique using a p-methylbenzhydrylamine resin . The side chain protecting groups were the following : O-2,6 dichlorobenzyl (tyrosine); O-benzyl (glutamic acid, serine, threonine); N-tosyl (arginine); S-p-methoxybenzyl (cysteine). The temporary protection of the Nα function was by tert-butyloxycarbonyl, which was removed in 30 min with a trifluoroacetic acid/dichloromethane/ethanedithiol solution : 30/70/5. The coupling reactions were carried out with a twofold excess of protected amino acid and benzotriazolyl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP)[18-19]. The protected peptidyl-resin was treated with anhydrous hydrogen fluoride containing 10% anisole for one hour at 0°C. After removal of the hydrogen fluoride, the residue was washed with diethylether, extracted with water or 1M acetic acid and the aqueous solution was lyophilized. Peptides 1, 2, 3 and 4 were purified by reverse phase high performance liquid chromatography while peptide 5 was first cyclized and then purified as the others.

Cyclization of peptides 4 and 5 :

The disulfide bond formation was completed in one hour by air oxidation of an aqueous solution (pH 8) at a concentration of 0.3 mg of peptide per milliliter. The oxidized product was then recovered by lyophilization and purified once more. The air cyclization process was followed by Ellman's test[20] and by HPLC analysis with dual wavelength monitoring at 214 and 254 nm, the absorbance ratio at these wavelengths between the cyclized peptide and the linear peptide was different (ε 214nm/ ε 254nm = 20 and 40 respectively). Peptide 4 was purified by HPLC before cyclization. The oxidation of 5 was performed on the crude peptide under the same conditions as 4, and it was purified after cyclization.

Table 1. Physical characteristics of the peptides.

Sequence	HPLC capacity factor : k'	amino acid analysis
peptide 1 TELTLRYSTGTVSGFLS	5.1(27%CH_3CN)	Thr 4.05(4); Glu 0.98(1); Leu 3.03(3); Arg 1.01(1); Tyr 0.97(1); Ser 3.08(3); Gly 2.02(2); Val 1.02(1); Phe 0.95(1).
peptide 2 ALRYSTGTVA	3.95(25 % CH_3CN)	Leu 0.99(1); Arg 1.02(1); Tyr 0.95(1); Ser 1.05(1); Thr 1.99(2); Gly 1.03(1); Val 1.01(1); Ala 1.98(2).
peptide 3 ALTLRYSTGTVSGA	4.3(32%CH_3CN)	Leu 1.98(2); Thr 3.04(3); Arg 1.01(1); Tyr 0.97(1); Ser 2.08(2); Gly 2.02(2); Val 1.02(1); Ala 1.96(2).
peptide 4 CLRYSTGTVC	3.3(17%CH_3CN)	Leu 1.01(1); Arg 0.98(1); Tyr 0.95(1); Ser 1.08(1); Thr 1.95(2); Gly 0.97(1); Val 0.95(1).
peptide 5 CLTLRYSTGTVSGC	9.1(18%CH_3CN)	Leu 1.98(2); Thr 3.05(3); Arg 0.97(1); Tyr 0.98(1); Ser 2.05(2); Gly 2.02(2); Val 1.01(1).

Peptide purity :

The peptides homogeneity was controlled by HPLC in isocratic and gradient elution modes at 214 and 254 nm; the capacity factors (k') are given in **Table 1** as well as their amino acid analysis.

Antigenicity of peptides :

Peptides were tested by ELISA for their ability to be bound by nine human renin antisera (HRas).

Wells of plastic microtiter plates (Falcon 3915 Pro-bind, Becton Dickinson Labware CA, USA) were coated with 50 µL of peptides (5 µg/mL) in Tris-HCl buffer 0.1 M pH 9.6.

Plates were first incubated for 30 min at 37°C and overnight at room temperature. After washing three times with Tween-containing phosphate-buffered saline (PBS-Tween 0.1% pH 7.4), 50 µL of serial dilutions of antisera in PBS-BSA 1% pH 7.4 were added in peptides-coated duplicate wells and incubated for 2 hours at 37°C. Plates were washed three times again with PBS-Tween 0.1%, and wells were then treated with 50 µL of alkaline phosphatase-labelled sheep anti-rabbit IgG diluted 1:500 (Sigma, MO, USA). Incubation was carried out for 1 hour at 37°C.

Additional extensive washing with PBS-Tween 0.1% was followed by incubation with 150 µL of alkaline-phosphatase substrate (2 tablets/10 ml of Sigma 104-105) dissolved in 0.1 M Glycine-NaOH buffer pH 10.4 containing $MgCl_2$ and $ZnCl_2$ 1M/L. The enzymatic reaction was allowed to proceed for 2 hours at 37°C and stopped by addition of 50 µL of Na_2CO_3 1.5 M.

Absorbance was read at 405 nm in a Titerteck multiskan ELISA reader (Flow laboratories).

Titer expression : Optical density was multiplied by the maximal dilution giving an absorbance three times as high as the negative control (consisting of pooled normal rabbit sera diluted 1:100).

Conformational investigations :

Attempts to identify the conformation of the five peptides were made in two different solvents DMSO, and TFE. Clearly, from infrared spectroscopy (**Figure 6**),as the spectra show strong differences in the Amide I band region : two Amide I bands in DMSO and a single Amide I band in TFE, it can be concluded that the conformations in both these solvents are different. Now, what are these conformational differences?

In DMSO -NMR studies :

Figure 7 shows the ^1H NMR spectra (NH regions) of peptides 3 and 5. That the NH resonances cover a wide range of chemical shifts is a good indication that we are dealing with an ordered structure. As already mentionned above, the finding of a Hα Arg82---Hα Thr87 NOE, which corresponds to position j and j+5, suggests the presence of a β turn for peptide 1. This conclusion is confirmed by the temperature coefficients of the NH's (**Table 2** and **Figure 8**) and by the NOE data (see Figure 9) obtained on the cyclic compounds. As for peptides 2 and 3, although no interstrand NOEs could be detected, the strong similarity of the NH's temperature coefficients (Figure 8) suggests that they adopt basically the same conformation with, however, a higher mobility of the two strands. The proposed model for 4 and 5 is presented in **Figure 9** which also shows the detailed structure of the turn. Indeed, the low temperature coefficients of the Thr85 NH (see **Table 2**) and OH (-3.4. 10^{-3} for peptide 4) compared to the others OH's temperature coefficients suggest that all these protons are engaged in hydrogen bonds. All these results, together with examination

...olecular models allowed us to propose a turn which strongly resembles that already reported by Gierasch et al.[21] for cyclo(Gly-Pro-Gly)$_2$ which is stabilized by a Ser84 CO---Thr85 OH hydrogen bond.

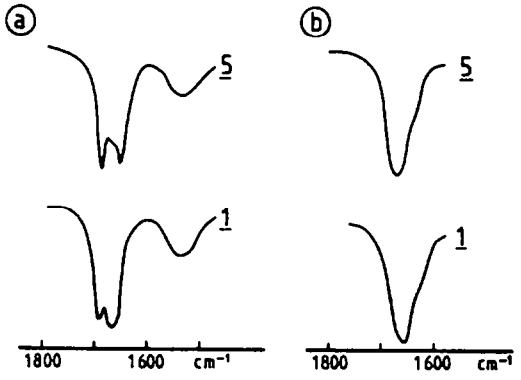

Figure 6. Infrared spectra of peptides 1 and 5 in DMSO (a) and in TFE (b).

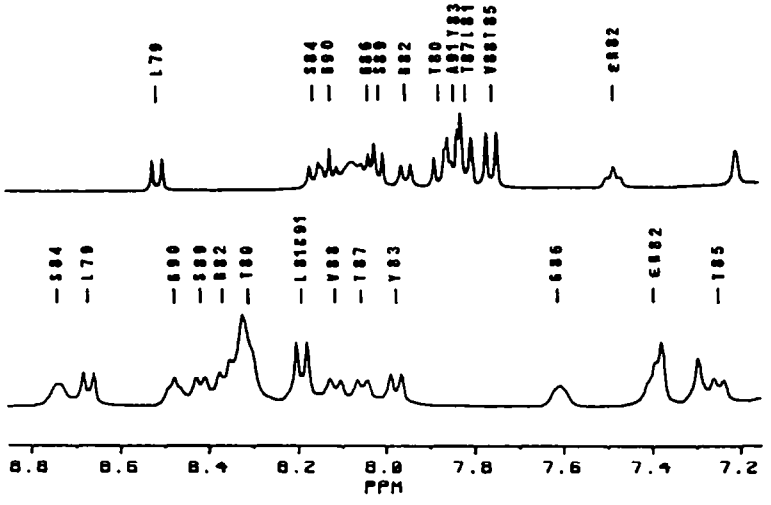

Figure 7. ^1H NMR NH region of peptides 3 (top) and 5 (bottom) in DMSO.

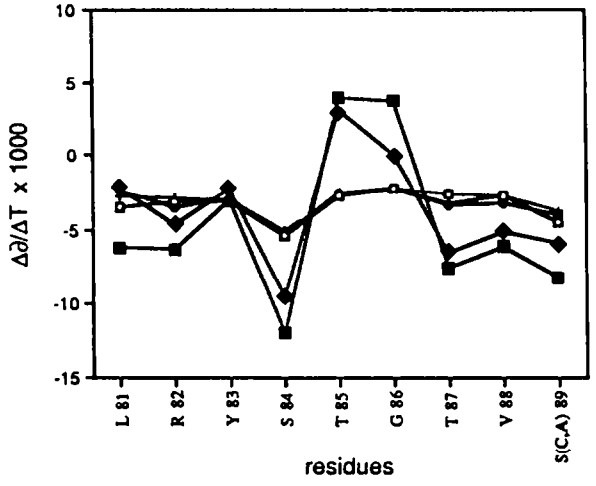

Figure 8. Temperatures coefficients of common residues NH group. (+) peptide 1, (o) peptide 2, (□) peptide 3, (◆) peptide 4, (■) peptide 5.

In TFE - CD studies :

As mentionned above, from infrared data it was suggested that in TFE the conformation of the peptides differs from that identified in DMSO. The CD spectra of the five peptides are shown in **Figure 10**. From the IR (position of Amide I band) and CD (intensities and positions of the extrema) spectra it can be stated that peptide 1 adopts in TFE an α helical structure. Similarly peptides 2 and 3 have CD spectra characterized by an α helical contribution which depends on the length of the peptide : the shorter it is, the lower the α contribution. This interpretation has, however, to be taken with care as it is known that some cyclic peptides which posses two β turns show CD[22] spectra which are

Table 2. Temperature coefficients of peptide NH resonances ($\Delta\delta/\Delta T \times 10^3$).

Residue	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5
Thr 77					
Glu or Cys or Ala 78	-3.3				
Leu 79	-3.3		-2.3		-2.0
Thr or Cys or Ala 80	-4.6		-3.2		-9.2
Leu 81	-2.7	-2.4	-3.5	-2.3	-6.2
Arg 82	-2.9	-3.5	-3.1	-4.6	-6.3
Tyr 83	-3.0	-2.9	-3.1	-2.3	-3.0
Ser 84	-5.0	-5.3	-5.4	-9.5	-11.8
Thr 85	-2.7	-2.5	-2.7	+3.0	+3.9
Gly 86	-2.3	-2.2	-2.3	0	+3.7
Thr 87	-3.2	-3.4	-2.6	-6.5	-7.5
Val 88	-2.7	-3.2	-2.7	-5.2	-6.1
Ser or Cys or Ala 89	-3.8	-4.0	-4.5	-5.9	-8.2
Gly 90	-3.2		-4.1		-7.6
Phe or Cys or Ala 91	-3.4				
Leu 92	-4.6				
Ser 93	-3.4				

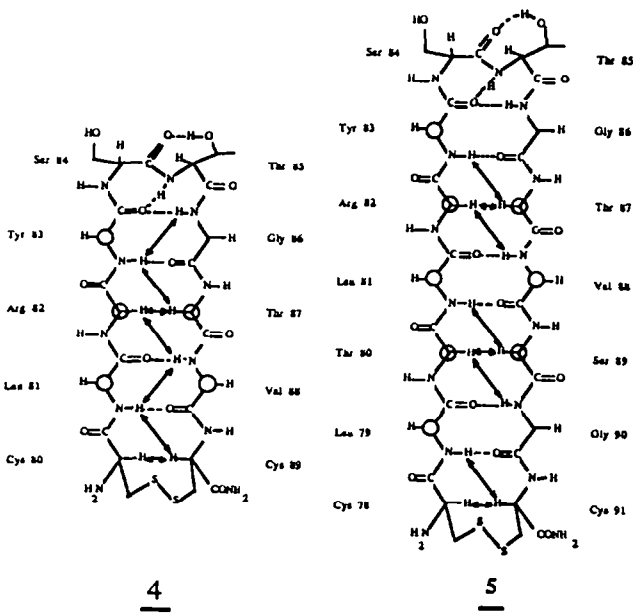

Figure 9. *The proposed model of human renin flap (-----) hydrogen bond, (⟷) observed NOEs.*

similar to that of an α helical polypeptide. Nevertheless, the far UV (190-200 nm) CD spectrum (strong positive extremum at 193 nm), together with the modifications of the spectrum when going from a 10 to a 17-peptide rather favor the presence of α helices. As for compounds **4** and **5**, beside a small positive contribution around 240-250 nm attributed to the disulfide bridge, their CD spectra could suggest an unordered structure for peptide **4** and a low helical contribution for peptide **5**. The latter case illustrates the above remark concerning CD spectra of cyclic and α helical peptides as the presence of a disulfide bridge precludes the formation of an α helix. The CD spectrum of **5**, which also resembles that reported by Azzena and Luisi[23] for a peptide which adopts a hairpin structure, is then in accordance with the conformation identified in DMSO by ^1H NMR studies. Therefore, owing to the similarity of their IR spectra (Amide I band centered around 1674 cm^{-1}) we conclude that peptides **5** and also **4** adopt in TFE a hairpin structure but with rather poor interstrand hydrogen bonds as suggested by the high wavenumber position of Amide I band.

Addition of water to the TFE solutions does not induce any conformational modifications except for peptides **1** and **3** for which a decrease of the α helical content is observed leading to CD spectra which closely resemble that of peptide **5** under identical conditions (**Figure 11**). This observation leads to the conclusion that addition of water favors the formation of the hairpin structure. This latter point is of great interest with regard to the synthesis of peptides **4** and **5** and particularly the kinetics of formation of the S-S bond. In water, i.e. the same solvent as that used in the cyclization procedure, the linear peptide adopts a hairpin conformation with the thiol groups close to one another favoring the rapid formation of the disulfide bridge as described in the experimental section.

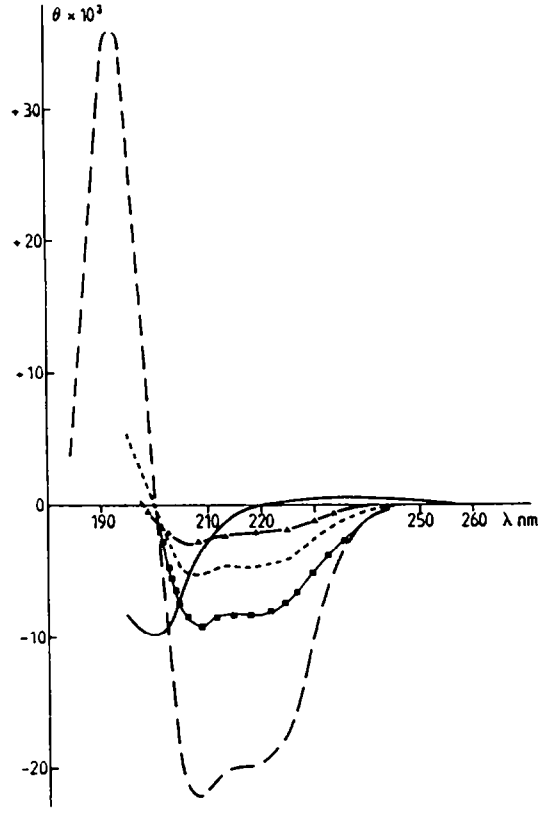

*Figure 10. CD spectra of the five peptides in TFE (c = 0.1 mg/mL; cell path 1 mm). (— —) peptide **1**, (▲) peptide **2**, (■) peptide **3**, (———) peptide **4**, (– – – –)peptide **5**.*

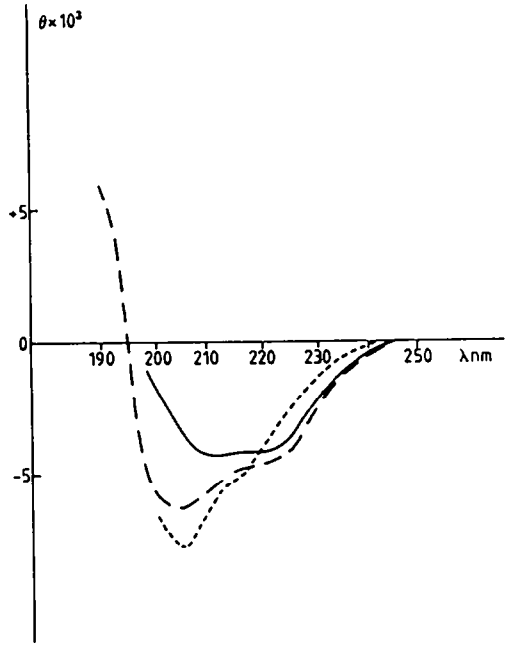

*Figure 11. CD spectra of peptides **1**, **3** and **5** in TFE/H_2O (c = 0.1 mg/mL; cell path 1 mm) (— —) peptide **1**, (———) peptide **3** (– – – –)peptide **5**.*

Peptides antigenicity : recognition by human renin antibodies

The ability for the peptides to be bound by a collection of nine polyclonal human renin antisera was tested by ELISA and the results are summarized in **Figure 12**. Peptide **1** was not bound by any antiserum, so it doesn't appear in the results. Clearly antisera R14, R15, R24, R25 exhibit significant binding towards both cyclized peptides. It can be seen that recognition of cyclized haptens is always higher than that of the linear ones. In the same antisera, recognition of 10-peptides is significantly higher than that of the 14-peptides. Antisera R11, R21, R22, R26 and Donc are clearly oriented towards other parts of the protein.

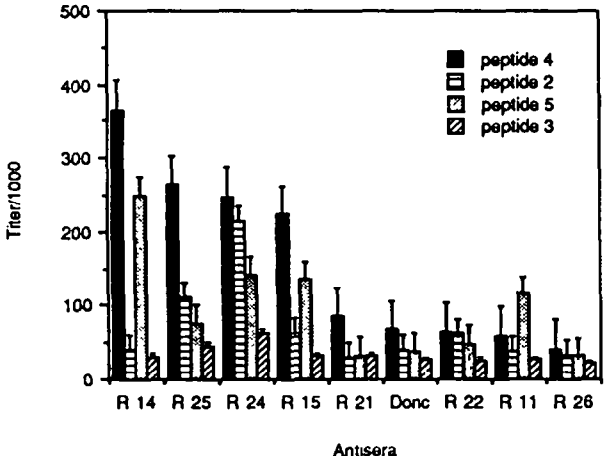

Figure 12. Recognition of the peptides by polyclonal human renin antibodies.

The main comments related to these results can be outlined as follow :

-Cyclic peptides are better recognized than the linear ones.

-Shorter peptides are better recognized than the longer.

-The longest, linear, unbalanced stranded **1** is not recognized at all.

These results support the hypothesis that the native conformation of the human renin flap closely resembles that which exists in the cyclized peptides. The disulfide bond thus imposes a secondary structure on the molecule that correspond to the renin model in this region. The fact that the peptides are recognized by all the renin antisera indicates that the flap overlaps an immunopotent region of human renin. This epitope can be assumed to be the common part of all these haptens, that is the β turn. However, as the peptides have to adopt a fixed conformation to bind, an additionnal entropy is to be paid to bind the linear haptens ; the longer the peptide, the higher the energy required for immobilization.

Conclusion :

From the above investigations, it can be concluded that the flap of human renin possesses the same conformation as that of the cyclized peptides. What is this conformation? The flap is a β hairpin with a loop region of four residues leading to the carbonyl group of Tyr 83 interacting with the NH's of Thr 85 and Gly 86. In the peptides examined, the β hairpin loop structure is strongly stabilized by an appropriate disulfide bridge as revealed by the human renin antisera recognition. Such peptides appear to be excellent models for studying the structural features of β hairpin structures; they also can be used for the epitope mapping of proteins in view of obtaining specific antibodies.

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References :

[1]Imai, T.; Miyazuki, H.; Hirose, S.; Hori, H.; Hayashi, T.; Kageyama, R.; Ohkubo, H.; Nakanishi, S.; Murakami, K. *Proc. Natl. Acad. Sci. USA* **1983** *80*, 7405-7409.

[2]Misono, K. S.; Chang, J. J.; Inagami T. *Proc. Natl. Acad. Sci. USA* **1982** *79*, 4858-4862

[3]Carlson, W.; Karplus, M.; Haber, E. *Hypertension* **1985** *7*, 13-26.

[4]Akahane, K.; Umeyama, H.; Nakagawa, S.; Moriguchi, I.; Hirose, S.; Iizuka, K.; Murakami, K. *Hypertension* **1985** *7*, 3-12.

[5]James, M. N. G.; Sielecki, A. R. *Biochemistry* **1985** *24*, 3701-3713.

[6]James, M. N. G.; Sielecki, A. R. *Nature (London)* **1986** *319*, 33-38.

[7]Talner, J. A.; Getzoff, E. D.; Alexander, H.; Houghten, R. A.; Olson, A. J.; Lerner, R. A.; Hendrickson, W. A. *Nature (London)* **1984** *312*, 127-134

[8]Westhof, E.; Altschuh, D.; Moras, D.; Bloomer, A. C.; Mondragon, A.; Klug, A.; Van Regenmortel, M. H. V. *Nature (London)* **1984** *311*, 123-126

[9]Dyson, H. J.; Cross, K. J.; Ostresh, J.; Houghten, R. A.; Wilson, I.A.; Wright, P. E.; Lerner, R. A. *Synthetic peptides as antigens* **1986** (Wiley, Chichester, Ciba Foundation Symposium 119) 58-75

[10]Audibert, F.; Jolivet, M.; Chedid, L.; Alouf, J. E.; Boquet, P.; Rivaille, P.; Siffret, O. *Nature (London)* **1981** *289*, 593-595

[11]Blundell, T.; Sibanda, B. L.; Pearl, L. *Nature (London)* **1983** *304*, 273-275.

[12]Sibanda, B. L.; Blundell, T.; Hobart, P. M.; Fogliano, M.; Bindra, J. S.; Dominy, B. W.; Chirgwin, J. M. *Febs Letters* **1984** *174*, 102-111.

[13]Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976** *64*, 2229-2246

[14]Nagayama, K.; Kumar, A.; Wuthrich, K.; Ernst, R. R. *J. Magn. Res.* **1980** *40*, 321-334.

[15]Wagner, G. *J. Magn. Res.* **1983** *55*, 151-156.

[16]Bodenhausen, G.; Kogler, H.; Ernst, R. R. *J. Magn. Res.* **1984** *58*, 370-388.

[17]Otting, G.; Widmer, H.; Wagner, G.; Wuthrich, K. *J. Magn. Res.* **1986** *66*, 187-193.

[18]Dormoy, J. R.; Castro, B. *Tetrahedron Lett.* **1979** *35*, 3321-3322.

[19]Le Nguyen, D.; Rivier, J. *Int. J. Peptide Protein Res.* **1986** *27*, 285-292.

[20]Ellman, G. L. *Arch. Biochem. Biophys.* **1959** *82*, 70-77

[21]Gierasch, L. M.; Deber, C. M.; Madison, V.; Nice, C. H.; Blout, E. R. *Biochemistry* **1981** *20*, 4730-4738.

[22]Urry, D. W.; Ohnishi, M. in "Spectroscopic approaches to Biomolecular Conformation, D. W. Urry, Ed. American Medical Association, Chicago, Illinois **1970**

[23]Azzena, U.; Luisi, P. L. *Biopolymers* **1986** *25*, 555-570