Conformations of Synthetic Tetradecapeptide Renin Substrate and of Angiotensin I in Aqueous Solution†

Maria C. F. Oliveira, Luiz Juliano, and Antonio C. M. Paiva*

ABSTRACT: The properties of aqueous solutions of synthetic renin substrate tetradecapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser) were examined through electrometric titrations, infrared and circular dichroism spectroscopy, and spectrofluorometry. Titration studies of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu) were also made, whose results indicated a flexible folded conformation similar to that previously proposed for the octapeptide angiotensin II, with a possible additional β turn at the C terminus. The experimental results of the tetradecapeptide study, associated with Chou and Fasman calculations and with an analysis of structure–activity relationships in renin substrates and competitive inhibitors, led to the proposal of a β turn involving the His-Pro-Phe-His sequence of the tetradecapeptide. This β turn would be stabilized by β-antiparallel interaction between residues 3–4 and 10–12 and by electrostatic attraction between the N-terminal ammonium and C-terminal carboxylate groups and would be destabilized below pH 5 by electrostatic repulsion between His^6 and His^9. The capacity to assume this conformation is related to structural requirements for renin substrates and competitive inhibitors.

Renin (EC 3.4.99.19) is an endopeptidase with very strict specificity, being able to hydrolyze only a Leu–Leu bond of peptides containing at least eight amino acid residues. Besides the natural protein substrate contained in the α_2-globulin fraction of blood plasma, the best known substrate for renin is a tetradecapeptide (Table I) obtained from tryptic hydrolysis of equine protein substrate (Skeggs et al., 1957, 1958). A kinetic study of the hydrolysis of several synthetic homologue peptides by purified hog renin (Skeggs et al., 1968) has shown that removal of Asp-Arg from the N terminus increases the K_m nearly 10-fold, without affecting V_{max}. Further shortenings from the N terminus result in small K_m increases and no changes in V_{max} until the nonapeptide His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser is reached. Removal of the histidyl residue from this peptide does not alter K_m significantly but decreases V_{max} about 20-fold. Removal of the C-terminal serine from the nonapeptide reduces k_{cat}/K_m to about one-half, and the heptapeptide His-Pro-Phe-His-Leu-Leu-Val is inactive as renin substrate.

This strict requirement for a minimum size of the peptide substrate suggests that conformational factors may play a role in renin specificity. Therefore, the study of the conformation of the tetradecapeptide substrate, besides being interesting in itself, may also contribute to the understanding of renin activity. Such a study, involving electrometric and spectroscopic properties of the synthetic tetradecapeptide, is presented in this paper. For the interpretation of the titration data, besides comparison with previously published results on angiotensin II (Table I), the pK_a values for angiotensin I were now determined, yielding some information also about this decapeptide's conformation in aqueous solution.

Experimental Procedure

Peptide Synthesis. Merrifield's solid phase method (Erickson and Merrifield, 1975) was employed. *tert*-Butyloxycarbonyl (Boc)[1] amino acids were purchased from Bachem, Inc., and the side chain protecting groups were: serine, *O*-benzyl; tyrosine, *O*-benzyl; histidine, N^{τ}-tosyl; arginine, N^{g}-nitro; aspartic acid, *O*-benzyl. Boc-Ser (Bzl) was attached to the Merrifield resin (1% cross-linked, 200–300 mesh, 0.75 mmol of Cl^-/g, from Bio-Rad Laboratories) by a standard method (Merrifield, 1963). Chain elongation was performed on an automatic peptide synthesizer (Merrifield et al., 1966), following standard techniques (Stewart and Young, 1969), with the following particularities. Dichloromethane was used as solvent for all reagents, except that deprotection was performed in 30% (v/v) trifluoroacetic acid in chloroform, and that Boc-Arg(NO_2) was dissolved in a 2:1 (v/v) mixture of dichloromethane and dimethylformamide. Couplings were done with 2.5 equiv of Boc amino acids and dicyclohexylcarbodiimide, and completeness of reaction was monitored with the ninhydrin reagent described by Kaiser et al. (1970). When the ninhydrin test remained positive after 6 h, the resin was washed with dichloromethane, and the coupling was repeated. In the few cases in which the ninhydrin test was not negative after 6 h recoupling, an acetylation step was performed with 10 equiv of acetic anhydride and triethylamine. After coupling of the last residue, the resin was treated with anhydrous hydrogen fluoride, containing 5% (v/v) anisole, for 60 min at 0 °C (Sakakibara and Shimonishi, 1965). The HF and anisole were removed by vacuum distillation and washing with ethyl acetate, and the peptide was extracted with glacial acetic acid and freeze-dried. The crude material was purified by 1400 transfers of countercurrent distribution in the system 1-butanol–acetic acid–water (4:1:5), with an automatic instrument made by H. O. Post Scientific Instrument Co. The tetradecapeptide, with a partition coefficient of 0.20, was located by a quantitative Pauly reaction; the fractions containing the material were pooled and concentrated in a rotary evaporator at 40 °C, followed by lyophilization. Further purification was done by chromatography on a 24 × 1.2 cm carboxymethylcellulose column equilibrated with 0.01 M ammonium acetate (pH 4.0) and eluted with a linear gradient between that solvent and 3.5 M acetic acid. The material was further purified by

† From the Department of Biophysics and Physiology of the Escola Paulista de Medicina, 04023 São Paulo, S. P., Brazil. *Received January 31, 1977.* This work was supported by grants from Financiadora de Estudos e Projetos (FINEP), the Brazilian National Council for Scientific and Technological Development (CNPq), and the São Paulo State Research Foundation (FAPESP).

[1] Abbreviation used: Boc, *tert*-butyloxycarbonyl.

TABLE I: Peptides of the Renin–Angiotensin System.

Name	Amino acid sequence
Tetradecapeptide	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser
Angiotensin I	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Angiotensin II	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe

chromatography on a 7.5 × 1.2 cm Dowex-50 column equilibrated with 0.1 M acetic acid and eluted stepwise with the equilibration solvent followed by 0.1 M ammonium acetate (pH 5.5) and by 2.0 M ammonium hydroxide.

The pure peptide was characterized by the following criteria. Only one Pauly-, ninhydrin-, and Sakaguchi-positive spot was obtained on paper electrophoresis (1000 V, 60 min) of 0.1 μmol of the peptide in three different buffers and on thin-layer chromatography (0.1-mm Eastman "Chromagram" silica gel plates). The relative migrations on electrophoresis were: 0.63, relative to histidine, at pH 2.8 (1 M acetic acid); 0.24, relative to arginine, at pH 4.9 (0.05 M pyridine acetate); 0.1, relative to picric acid, at pH 9.9 (0.05 M sodium carbonate/bicarbonate). The R_f values were: 0.14 in 1-butanol–acetic acid–water (4:1:1); 0.64 in 1-butanol–ethyl acetate–acetic acid–water (1:1:1:1); 0.63 in 1-butanol–pyridine–acetic acid–water (15:10:3:12). The amino acid analysis of an acid hydrolyzate (72 h, 6 N HCl at 110 °C), done on a Beckman Model 120C instrument, yielded the following molar ratios: Asp, 1.02; Arg, 1.01; Val, 1.88; Tyr, 2.10; Ile, 0.88; His, 2.05; Pro, 1.03; Phe, 0.95; Leu, 2.16; Ser, 0.96. $[\alpha]^{25}_D = -57.88°$ (*c* 0.28%, 1 M AcOH).

The activity as renin substrate was determined by incubating a 1.6×10^{-6} M solution of the tetradecapeptide, in pH 5.5 acetate buffer, with 0.6 Goldblatt units/mL of a partially purified renin preparation (Haas et al., 1953). After 2-h incubation, the reaction was stopped by boiling for 2 min and the angiotensin I activity in the solution was assayed in the isolated guinea pig ileum (Paiva et al., 1974), with a yield of 99.1% of the theoretical.

Angiotensin I was also synthesized by the solid phase method, starting from Boc-Leu polymer (2% cross-linking) and following the procedures described in detail elsewhere (Paiva et al., 1973).

Electrometric titrations were performed at 25.0 ± 0.1 °C, as previously described (Paiva et al., 1963; Juliano and Paiva, 1974). The pH meter (Radiometer Model PHM4C with G2028 glass and K100 calomel electrodes) was calibrated (Bates, 1973) with 0.025 M phosphate buffer, pH 6.855, and the sensitivity adjusted to within 0.01 pH unit in the range 4–9, with 0.01 M borate (pH 9.18) and 0.05 M phthalate (pH 4.01) buffers. At the end of each titration the calibration of the pH meter was checked to be within 0.01 pH unit of the initial value. Before titration, the peptide was repeatedly lyophilized from 0.1 N HCl to eliminate traces of ammonium acetate and to obtain the hydrochloride form. The solutions to be titrated were 5 mM, and the ionic strength was adjusted to 0.15 with KCl. Five-milliliter samples were titrated first from pH 2.5 to 11, with approximately 1 N KOH (recently standardized with potassium phthalate), and then titrated back to pH 2.5 with approximately 1 N HCl (standardized against the KOH). Each titration curve thus obtained contained more than 150 experimental points and was repeated six to eight times with concordant results. The pK_a values were calculated by a least-squares method with an iterative program on a Varian 620/L-100 computer.

TABLE II: pK_a Values for the Titratable Groups of Angiotensin I and the Tetradecapeptide, at 25 °C in 0.15 M KCl.

Group	Tetradecapeptide: Ascending titration	Tetradecapeptide: Descending titration	Angiotensin I
β-Carboxyl	2.76	2.85	2.92
α-Carboxyl	3.96	3.80	3.37
Imidazole	5.54	5.50	6.14
	6.67	6.50	6.94
Amino	8.69	7.30	7.62
Phenol[a]	10.21	10.15	10.20

[a] Obtained from spectrophotometric titrations.

Spectrophotometric titrations were performed on a Beckman Acta V double monochromator instrument, using the absorption at 242.5 nm (Paiva and Paiva, 1962), at 25.0 °C, 0.15 ionic strength, in the pH range 9.5–12.

Spectroscopic Measurements. All solutions used for spectroscopic measurements were filtered through a GSWPO 1300 Millipore filter and were free of visible suspensions. Infrared spectra of D_2O solutions were obtained with a Zeiss-Jena Model UR20 instrument in a cell with CaF_2 windows and 1-mm optic path. Solid films were obtained by vacuum desiccation of thin layers of D_2O solutions over CaF_2 surfaces.

Fluorescence measurements were done on an Aminco-Bowman Model SPF1 spectrofluorimeter, calibrated with a quinine sulfate solution in 0.1 N H_2SO_4. Excitation was at 280 nm, and a 10^{-5} M tyrosine solution (pH 6.00) was used as reference. The quantum yield was estimated by the method of Parker and Rees (1960), using the value of 0.14 for the quantum efficiency of tyrosine (Chen et al., 1969).

Circular dichroic spectra were obtained on a Cary 61 spectropolarimeter using fused quartz cells of 1-mm and 10-mm optical paths.

Results

Titrations. The electrometric titrations of angiotensin I with base (ascending titration) and with acid (descending titration) were reproducible and superposable, and the pK_a values calculated for the titratable groups are shown in Table II. The 95% fiducial limits were estimated to be ±0.05 for the carboxyls, ±0.03 for the imidazole and amino groups, and ±0.07 for the phenolic groups. Spectrophotometric titrations of the latter groups were in very good agreement with the electrometric measurements, but the error was smaller (95% fiducial limits: ±0.04).

The ascending titration of the tetradecapeptide proceeded normally until pH 3.8, when the solution became increasingly opalescent as the pH was raised. A fine suspension was apparent from pH 5.6 to pH 9.5, but above pH 10 the solution was again clear. Upon back titration with HCl (descending titration), insoluble material was present between pH 9 and pH 4. In the pH region where precipitate was present, a longer time was necessary for stabilization of the pH reading after each addition of acid or base, but it was possible to attain a stable value (within 0.001 pH unit) in 10 min. Under these conditions, reproducible titration curves were obtained. One example is shown in Figure 1, where the ascending and descending titrations are displaced on the ordinate axis in order to avoid overlap of the curves. A pronounced and reproducible hysteresis was observed in the region between pH 6 and pH 9.

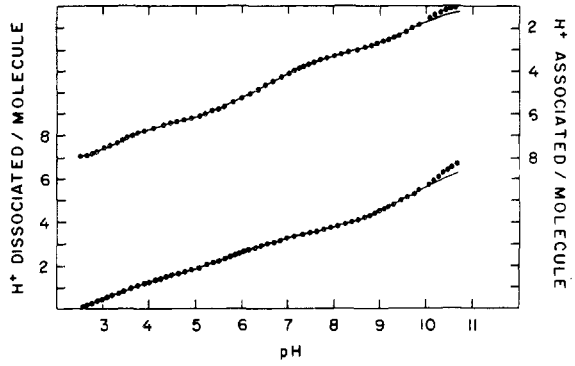

FIGURE 1: Titration curve of the tetradecapeptide in 0.15 M KCl, at 25 °C. The points represent experimental data from one titration, and the curves were drawn using the pK_a values of Table II. The ordinate scale for the ascending titration (lower curve) is on the left side, and that for the descending titration (upper curve) is on the right side of the graph.

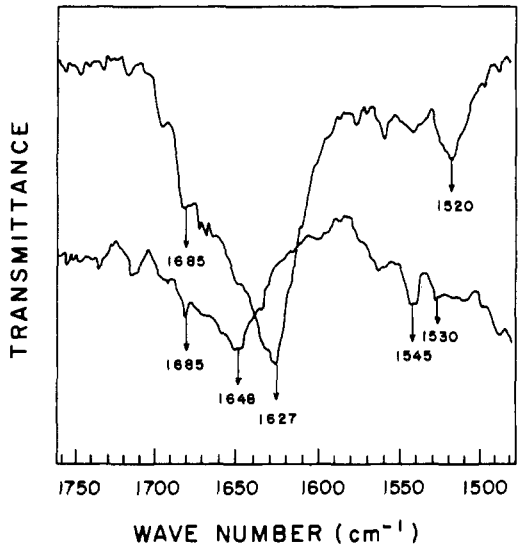

FIGURE 2: Infrared spectra of the tetradecapeptide in solid film (upper tracing) and in D_2O solution (lower tracing), pD = 4.04.

The pK_a values estimated from the ascending and descending titrations are listed in Table II. From the six independent titrations that were performed, the 95% fiducial limits were estimated to be within ±0.15 for the α- and β-carboxyls, and 0.07 for the imidazole and amino groups. The electrometric and spectrophotometric titrations of the phenolic groups closely agreed, but a smaller error was associated with the spectrophotometric pK_a determinations (95% fiducial limits: ±0.05). Only one phenolic pK_a value could be determined for the titration of the two tyrosyl side chains of the tetradecapeptide.

Infrared Spectra. In the solid state, the intense symmetrical amide I band at 1627 cm^{-1}, with a component at 1685 cm^{-1} (Figure 2), is characteristic of antiparallel β structure, although the weak amide II band at 1520 cm^{-1} is indicative of unordered conformation (Krimm, 1962).

In D_2O solution (Figure 2), at pD 4.04, the amide I component at 1685 cm^{-1} was present, indicating antiparallel β structure (Krimm, 1962), but the main peak was broadened, with a less defined maximum at 1648 cm^{-1}. This maximum could be due to a composition of amide I bands from unordered (1658 cm^{-1}) and antiparallel β (1630 cm^{-1}) structures. The amide II bands at 1530 and 1545 cm^{-1} also indicate that antiparallel β structure is present (Krimm, 1962).

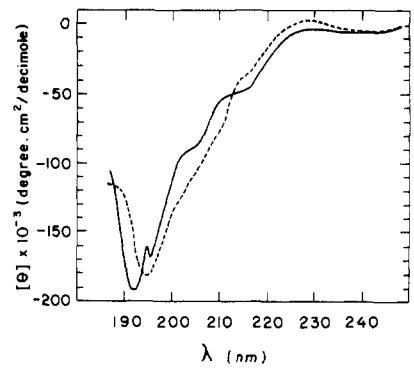

FIGURE 3: Circular dichroism spectra of the tetracecapeptide at pH 2.53 (- - -) and at pH 5.86 (—).

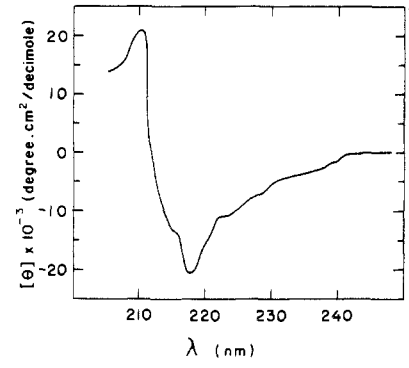

FIGURE 4: Difference circular dichroism spectrum obtained by subtracting the spectrum at pH 2.53 from that at pH 5.86, and corrected by subtracting the difference spectrum due to histidine deprotonation.

Circular Dichroism. The dichroic spectra of the tetradecapeptide at pH 2.53 and pH 5.86 showed, in the aromatic region, the normal positive bands due to tyrosine (Adler et al., 1973). In the region 180–250 nm, the spectra (Figure 3) did not conform with those described for any of the well-defined conformations. However, some differences between the two spectra are apparent, and in order to better analyze them a difference spectrum was obtained. From this, the calculated difference spectrum due to histidine protonation was subtracted, using the data of Grebow and Hooker (1975), and admitting Gaussian bands (Beychok, 1966). The resulting corrected difference spectrum (Figure 4) presented a minimum at 218 nm, characteristic of β structure (Adler et al., 1973), indicating that this type of structure appeared, or was accentuated, when the pH was raised from 2.53 to 5.86.

Fluorescence. The tetradecapeptide presented an emission spectrum identical with that of tyrosine, with a maximum at 305 nm (pH 6.0). A value of 0.039 was found for the quantum yield of tyrosine in the tetradecapeptide, at pH 6.0. The emission did not vary significantly in the pH range 4–6, in which the average fluorescence intensity relative to that at pH 6.0, measured at 0.1 pH unit intervals, was 0.97 with standard deviation 0.04. A small decrease in emission was observed when the pH was lowered below 4, but in the pH range 2.5–3.6 the relative intensity did not vary significantly from the average value of 0.83 (standard deviation: 0.02).

The quenching of the fluorescence at pH 6.0 by potassium iodide (in concentrations varying from 0.05 to 0.25 M, and 0.3 ionic strength obtained by adding KCl) followed a linear Stern–Volmer plot (Moon et al., 1965), indicating collision as the only quenching mechanism.

Discussion

The pK_a values found for the β-carboxyl and amino groups of angiotensin I were within 0.03 unit of those described (Juliano and Paiva, 1974) for the octapeptide angiotensin II. The α-carboxyl pK_a was 0.12 unit lower in the decapeptide, which might be due to the differences in the C-terminal side chains. The pK_a values for the amino and β-carboxyl groups in angiotensin II have been interpreted as indicative of proximity between the N- and C-terminal ends of that peptide (Juliano and Paiva, 1974). The occurrence of the same pK_a values in angiotensin I may indicate a similar folding of this decapeptide. This is also suggested by the pK_a of the tyrosyl side chain. Juliano and Paiva (1974) have shown that the phenoxyl pK_a of [Pro^3,Pro^5]angiotensin II (where the tyrosyl side chain is thought to be freely accessible to solvent) is 9.92, while that of angiotensin II is 10.09. This higher pK_a was attributed to a somewhat restricted freedom of the tyrosyl side chain (Juliano et al., 1974). The still higher pK_a of angiotensin I (Table II), in spite of the removal of the C-terminal carboxylate and addition of the imidazolium side chain, indicates that the tyrosyl residue may be more restricted in the decapeptide.

The pK_a of one the imidazoles in angiotensin I is 6.94 (Table II), which is not significantly different from the 6.90 found in Ac-Gly-Gly-His-Gly (Bryce et al., 1966) and may be ascribed to His^9. The other imidazole pK_a is the same as that in [Asn^1]-angiotensin II-α-amide (Juliano and Paiva, 1974), and may be assigned to the histidyl residue in position 6. Blocking of the β-carboxyl group reduced the His^6 pK_a of angiotensin II by 0.17 unit, and further blocking of the α-carboxyl group decreased that pK_a an additional 0.16 unit (Juliano and Paiva, 1974). One would then expect that blocking of the C-terminal carboxyl group should lower the His^6 pK_a of angiotensin II by about 0.15 unit. Since the pK_a of His^6 in angiotensin I is 0.33 unit lower than that in angiotensin II, the positive charge of His^9 appears to be influencing the ionization of the imidazole side chain in position 6. This is further indication that His^6 is closer to the C terminus of angiotensin I than would be expected in an entirely random conformation.

We conclude from the results of angiotensin I titrations that this peptide in aqueous solutions tends to assume folded conformations similarly to angiotensin II, with added interactions in the C-terminal portion due to the two-residue extension of the peptide chain. These interactions may involve a β turn comprising the sequence His-Pro-Phe-His, similarly to what is proposed below for the tetradecapeptide renin substrate.

Analysis of the tetradecapeptide titration results is more difficult because the occurrence of precipitation brings greater uncertainty to the interpretation of pK_a differences. We believe, for instance, that the lower pK_a values observed in the descending titration, as compared with the ascending one (with the exception of the β-carboxyl groups) may be due to the lower accessibility of the titratable groups in the precipitate. However, some features of the titration behavior are marked enough to be considered significant. Such is the case of the large hysteresis (Table II), mostly due to the high amino group pK_a observed in the ascending titration. This might indicate masking of the protonated amino group in the insoluble species, and it is interesting to note that the titration of this group coincided with disappearance of the precipitate. However, during the descending titration, the aggregation appeared to coincide with the protonation of tyrosine. The amino group pK_a was normal, as compared with that of [Pro^3,Pro^5]angiotensin II (Juliano and Paiva, 1974), in spite of the fact that its titration took place in a pH region where precipitate was present.

The appearance and disappearance of precipitate in the ascending and descending titrations around pH 4-5 seem to be related to deprotonation and protonation of one of the imidazole groups, which was found to have the abnormally low pK_a of 5.5. The other imidazole group, which is titrated in the region of precipitation, had a pK_a close to the value of 6.54 found in the case of [Pro^3,Pro^5]angiotensin II (Juliano and Paiva, 1974). The data do not permit assignment of this normal pK_a to either of the two histidines in the tetradecapeptide. It is plausible that the conformation of this peptide tends to bring the two histidines close to each other, which would lead to a greater acidity of the first imidazole to be titrated. A similar situation was proposed for ribonuclease, in which the low pK_a of His^{119} (5.8, in 0.2 M NaCl at 25 °C) was attributed to its proximity to the positive charges of His^{12} and Lys^4 (Roberts et al., 1969).

The two phenoxyl groups of the tetradecapeptide could not be distinguished by titration, but the single pK_a value (Table II) is higher than expected from totally exposed tyrosyl side chains (Juliano and Paiva, 1974). The fluorescence behavior indicates absence of tyrosyl-tyrosyl or tyrosyl-phenylalanyl interactions. The quantum yield of 0.039 is significantly lower than that of 0.064 found for angiotensin II (Paiva et al., 1975), where the tyrosyl side chain is partially restricted (Juliano et al., 1974) and appears to be in a hydrophobic environment (Lintner et al., 1975). According to their quantum yield, the tetradecapeptide's tyrosines may be classified as Cowgill's (1968) type II, whose fluorescence is quenched by hydrated peptide carbonyl groups. The change in fluorescence around pH 4 may be the influence of titration of the C-terminal carboxyl group on Tyr^{13}, since -COOH is known to quench tyrosine fluorescence (Cowgill, 1963).

The infrared spectra of the tetradecapeptide (Figure 2) indicate the presence of some antiparallel β structure, not only in the solid films, but also in D_2O solution at a pD (4.04) in which both imidazole groups are protonated. An increase in β structure content was further observed when the pH was raised from 2.53 to 5.86 (Figure 4), causing most of the more acidic imidazole (pK_a 5.50) to be titrated. As mentioned above, this titration coincides with the precipitation that was observed in the more concentrated solutions used for the electrometric titrations.

Models for structure in the tetradecapeptide may involve either intra- or intermolecular interactions. The use of Chou and Fasman's (1974) method indicates two tripeptide sequences with high probability of β-sheet nucleation, involving residues 3-5 (Val-Tyr-Ile) and 10-12 (Leu-Leu-Val). It is possible to juxtapose these two regions of two tetradecapeptide molecules either in a parallel or in an antiparallel fashion, yielding an association model. This would account for the observed precipitation, if one admits that the association is destabilized by electrostatic repulsion between adjacent protonated imidazole groups and stabilized by attraction between C-terminal carboxylate and N-terminal ammonium groups (in the antiparallel model). However, in both the parallel and the antiparallel association models, there is juxtaposition of the sequence His-Pro-Phe-His from two adjacent molecules, and three of these four residues are β breakers.

That tetrapeptide sequence has a probability of occurrence in a β turn ($\langle P_t \rangle = 4.7 \times 10^{-5}$) which is well above the average value of 2.4×10^{-5} and close to the "cut-off" point (5×10^{-5}) proposed by Chou and Fasman (1974). The stability of this β turn would be increased by hydrogen bonds between the two β-structure nucleating sequences (residues 3-5 and 10-12), as illustrated in Figure 5. This model would also be stabilized

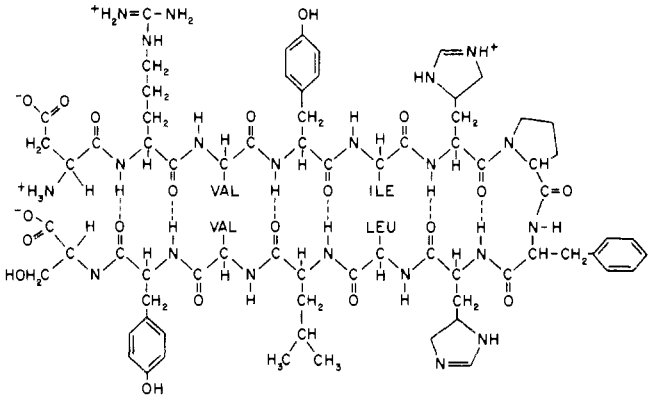

FIGURE 5: Schematic representation of β-turn model for the tetradecapeptide.

by electrostatic interaction of the C-terminal carboxylate group with the protonated amino group, which might account for the low α-carboxyl pK_a and high amino pK_a observed in the titration (Table II). The involvement of the peptide groups in hydrogen bonding might confer only a marginal aqueous solubility to the molecule at pH values below 5 and above 10. This would explain the precipitations upon protonation of the phenolic groups, or deprotonation of one of the imidazoles. The proximity of the two histidine side chains, in this model, might cause an electrostatic interaction leading to the abnormally low pK_a of one of the imidazoles that was observed experimentally.

The possibility that the β-turn conformation depicted in Figure 5 occurs in the tetradecapeptide may help to understand the requirements for renin substrates and competitive inhibitors. The large loss of renin affinity observed when Asp-Arg is removed from the tetradecapeptide's N terminus (Skeggs et al., 1968) may indicate that at the pH optimum of 4.5 (Favre et al., 1973), where both histidine side chains are protonated, the $-COO^-\ldots{}^+H_3N-$ interaction is important for stabilizing the β-turn structure, which would be required for binding to renin's active site. We also propose that the β turn is important for catalytic activity since removal of His from His-Pro-Phe-His-Leu-Leu-Val-Tyr reduces V_{max} 20-fold (Skeggs, 1968) and also would destabilize the β turn maintained by the His-Pro-Phe-His sequence. The dependence of enzyme activity on a β turn in the substrate, proposed here, is analogous to Chou and Fasman's (1975) proposal that a β turn involving glucagon's residues 2 to 5 is needed for its biological activity.

It is also interesting to note that the most effective competitive renin inhibitors hitherto described (Poulsen et al., 1973; Parikh and Cuatrecasas, 1973; McKown et al., 1974; Burton et al., 1975) characteristically contain the β-turn-forming His-Pro-Phe-His sequence. Some of the most potent inhibitors (Burton et al., 1975) do not have β-association nucleating sequences, further favoring the β-turn over the β-association model.

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References

Adler, A. J., Greenfield, N. J., and Fasman, G. D. (1973), *Methods Enzymol. 27D,* 675.

Bates, R. G. (1973), Determination of pH: Theory and Practice, New York, N.Y., Wiley, p 59.

Beychok, S. (1966), *J. Biol. Chem. 241,* 5150.

Bryce, G. F., Roeske, R. W., and Gurd, F. R. N. (1966), *J. Biol. Chem. 241,* 1072.

Burton, J., Poulsen, K., and Haber, E. (1975), *Biochemistry 14,* 3892.

Chen, R. F., Edelhoch, H., and Stimer, R. F. (1969), in Physical Principles and Techniques of Protein Chemistry, Part A, Leach, S. J., Ed., New York, N.Y., Academic Press, p 171.

Chou, P. Y., and Fasman, G. D. (1974), *Biochemistry 13,* 222.

Chou, P. Y., and Fasman, G. D. (1975), *Biochemistry 14,* 2536.

Cowgill, R. W. (1963), *Arch. Biochem. Biophys. 100,* 36.

Cowgill, R. W. (1968), *Arch. Biochem. Biophys. 168,* 417.

Erickson, B. W., and Merrifield, R. B. (1975), in The Proteins, Neurath, H., and Hill, R. L., Ed., New York, N.Y., Academic Press, Vol. 2, p 255.

Favre, L., Roussel-Deruyck, R., and Valloton, M. B. (1973), *Biochim. Biophys. Acta 302,* 102.

Grebow, P. E., and Hooker, T. M. (1975), *Biopolymers 14,* 1863.

Haas, E., Lamfrom, H., and Goldblatt, H. (1953), *Arch. Biochem. Biophys. 42,* 368.

Juliano, L., Boschcov, P., and Paiva, A. C. M. (1974), *Biochemistry 13,* 4263.

Juliano, L., and Paiva, A. C. M. (1974), *Biochemistry 13,* 2445.

Kaiser, E., Colescott, R. L., Bossinger, C. D., and Cook, P. I. (1970), *Anal. Biochem. 34,* 595.

Krimm, S. (1962), *J. Mol. Biol. 4,* 528.

Lintner, K., Fermandjian, S., Fromageot, P., Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1975), *FEBS Lett. 56,* 366.

McKown, M. M., Workman, R. J., and Gregerman, R. I. (1974), *J. Biol. Chem. 340,* 2770.

Merrifield, R. B. (1963), *J. Am. Chem. Soc. 85,* 2149.

Merrifield, R. B., Stewart, J. M., and Jernberg, N. (1966), *Anal. Chem. 38,* 1905.

Moon, A. Y., Poland, D. C., and Scheraga, H. A. (1965), *J. Phys. Chem. 69,* 2960.

Paiva, A. C. M., Nouailhetas, V. L. A., Miyamoto, M. E., Mendes, G. B., and Paiva, T. B. (1973), *J. Med. Chem. 16,* 6.

Paiva, A. C. M., Oliveira, M. C. F., Juliano, L., Ventura, M. M., and Aragão, J. B. (1975), in Peptides 1974, Wolman, Y., Ed., Wiley, New York, N.Y., p 339.

Paiva, A. C. M., and Paiva, T. B. (1962), *Biochim. Biophys. Acta 56,* 339.

Paiva, T. B., Juliano, L., Nouailhetas, V. L. A., and Paiva, A. C. M. (1974), *Eur. J. Pharmacol. 25,* 191.

Paiva, T. B., Paiva, A. C. M., and Scheraga, H. A. (1963), *Biochemistry 2,* 1327.

Parikh, I., and Cuatrecasas, P. (1973), *Biochem. Biophys. Res. Commun. 54,* 1356.

Parker, C. A., and Rees, W. T. (1960), *Analyst 85,* 587.

Poulsen, K., Burton, J., and Haber, E. (1973), *Biochemistry 12,* 3877.

Roberts, G. C. K., Meadows, D. H., and Jardetzky, O. (1969), *Biochemistry 8,* 2053.

Sakakibara, S., and Shimonishi, Y. (1965), *Bull. Chem. Soc. Jpn. 38,* 1412.

Skeggs, L. T., Jr., Kahn, J. R., Lentz, K. E., and Shumway, N. P. (1957), *J. Exp. Med. 106,* 439.
Skeggs, L. T., Jr., Lentz, K. E., Kahn, J. R., and Hochstrasser, H. (1968), *J. Exp. Med. 128,* 13.
Skeggs, L. T., Jr., Lentz, K. E., Kahn, J. R., and Shumway, N. P. (1958), *J. Exp. Med. 180,* 283.
Stewart, J. M., and Young, J. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman.

Study of the Secondary Structure of the Luteinizing Hormone Releasing Factor Using Intramolecular Charge Transfer Complexes†

Bernard Donzel,* Jean Rivier, and Murray Goodman

ABSTRACT: The syntheses and conformational properties of three analogues of the luteinizing hormone releasing factor (LRF)—the C^{δ}-nicotinamidium derivative of [8-norvalyl]-LRF, the C^{δ}-nicotinamidium derivatives of [8-norvalyl,6-D-alanyl]- and [8-norvalyl,6-L-alanyl]-LRF—are reported. This series of analogues was designed for the purpose of analyzing the probability of folding of the LRF peptide skeleton as a function of the steric nature of the residue in position 6 by measuring the charge-transfer interaction between the nicotinamidium ring on residue 8 and the side chain of tryptophan in position 3. Nuclear magnetic resonance studies in water show evidence for a pronounced conformational flexibility of the peptide backbones and side chains, similar to the case of LRF itself. The ultraviolet–visible electronic spectra of all three compounds contain a shoulder in the long wavelength region which is identified as the *intramolecular* charge transfer spectrum of the nicotinamidium–indolyl complex. The relative population of the molecules in the complexed state was evaluated in each case from the intensity of the charge transfer band at 380 nm, using the molar extinction coefficient of the *intermolecular* complex between acetyltryptophanamide and *N*-methylnicotinamide chloride. It is highest in the [6-D-alanyl] analogue (ca. 15%) and lowest in the [6-L-alanyl] analogue (ca. 9%). This trend is in qualitative agreement with the theoretical predictions of the stability of β turn of type II, provided that the amino acid substitution was made at the third residue of the turn.

Studies on structure–activity relationships of the luteinizing hormone releasing factor (LRF)[1] (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2) have shown that most residues present in the peptide molecule are necessary for full biological potency (Vale and Rivier, 1975). Substantial efforts have been made to delineate both the amino acid residues responsible for binding to the receptor and also those for triggering the biological response, but no simple relationships have been found. From these studies, however, some positions along the peptide chain were found to be critical for biological activity. Among them are:

(a) Histidine in Position 2. Deletion or substitution of this residue by D-amino acids with aromatic side chains produces analogues with antagonist properties. Their affinity constant to the receptor sites, however, was found to be lower than in the case of the hormone (LRF) itself (Vale et al., 1976).

(b) Glycine in Position 6. Replacement of this residue by any L-amino acid drastically decreases the biological potency, whereas replacement by a D-amino acid residue dramatically increases the activity. The nature of the side chain in this case was found to have a profound influence, as reflected in the biological potencies of LRF (100%), [D-Ala^6]-LRF (450%), [D-Phe^6]-LRF (750%), [D-Tyr^6]-LRF (1500%), and [D-Trp^6]-LRF (3600%) (Monahan et al., 1973; Rivier et al., 1975; Coy et al., 1975).

(c) Glycinamide in Position 10. Substitution of this residue by a variety of alkylamide groups (Fujino et al., 1972b) provokes a substantial increase of the LH-releasing activity, with a maximum obtained for the ethylamide derivative (300%).

Simultaneous modifications at positions 6 and 10, as well as at positions 2 and 6, produce effects which vary in a geometric fashion while modifications at positions 2 and 10 apparently are not interrelated.

These modifications have been used in our laboratories in an attempt to determine whether such drastic variations in the biological activities could be correlated with conformational changes. This paper deals with the conformational effects caused by the replacement of glycine in position 6 with L-alanine or D-alanine. A preliminary report has been previously presented (Donzel et al., 1975).

Recently, several conformational studies of LRF have been reported. From ^{1}H and ^{13}C NMR studies on LRF and component fragments, Wessels et al. (1973) concluded that the hormone, when dissolved in polar solvents such as water or dimethyl sulfoxide, adopts a random structure. No intramolecular hydrogen bonding and no stacking of the aromatic side chains could be detected. Such an interaction between tyrosine-5 and tryptophan-3 was previously proposed by Chang and co-workers (1972) based upon model considerations. Furthermore, ^{13}C spin–lattice relaxation studies (Deslauriers et

† From the Department of Chemistry, University of California, San Diego, La Jolla, California 92093, and The Salk Institute for Biological Studies, San Diego, California 92112. *Received December 9, 1976.* This work was supported by a grant to Dr. Murray Goodman from the National Institutes of Health (USPHS AM 15410) and by a grant to Dr. Roger Guillemin from The Salk Institute for Biological Studies (NICHD Grant No. HD 09690-02).

[1] Abbreviations used: LRF, luteinizing hormone releasing factor; LH, luteinizing hormone; NMR, nuclear magnetic resonance; UV, ultraviolet; vis, visible; CD, circular dichroism; CT, charge transfer; DEAE, diethylaminoethyl; CM, carboxymethyl; Boc, *tert*-butoxycarbonyl; HMDS, hexamethyldisiloxane; CPK, Corey–Pauling–Koltun.