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Supplementary Material Available

Sequence analysis of complete RNase T_1 and pancreatic RNase digestion products of wheat germ $tRNA_1^{Gly}$ (11 pages). Ordering information is available on any current masthead page.

References

Allende, J. E. (1969), in Techniques in Protein Biosynthesis, Vol. 2, Campbell, P. N., and Sargent, J. R., Ed., New York, N.Y., Academic Press, p 55.

Dudock, B. S., and Katz, G. (1969), *J. Biol. Chem. 244,* 3069.

Dudock, B. S., Katz, G., Taylor, E. K., and Holley, R. W. (1969), *Proc. Natl. Acad. Sci. U.S.A. 62,* 941.

Gillam, I., Millward, S., Blew, D., Von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6,* 3043.

Katz, G., and Dudock, B. S. (1969), *J. Biol. Chem. 244,* 3062.

Marcu, K., and Dudock, B. (1974), *Nucleic Acids Res. 1,* 1385.

Marcu, K., Mignery, R., Reszelback, R., Roe, B., Sirover, M., and Dudock, B. (1973), *Biochem. Biophys. Res. Commun. 55,* 477.

Marcu, K. B., and Dudock, B. S. (1976), *Nature (London) 261,* 159.

Marcus, A. (1972), in Protein Biosynthesis in Nonbacterial Systems, Last, J. A., and Laskin, A. L., Ed., New York, N.Y., Marcel Dekker, p 127.

Molinaro, M., Sheiner, L. B., Neelson, F. A., and Cantoni, G. L. (1968), *J. Biol. Chem. 243,* 1277.

Nishimura, S. (1974), in Biochemistry of Nucleic Acids, Burton, K., Ed., Baltimore, Md., University Park Press, p 289.

Pearson, R. L., Weiss, J. F., and Kelmers, A. D. (1971), *Biochim. Biophys. Acta 228,* 770.

Piper, P. W., and Clark, B. F. C. (1974), *FEBS Lett. 47,* 56.

Robillard, G., Hilbers, C., Reid, B., Gangloff, J., Dirheimer, B., and Shulman, R. G. (1976), *Biochemistry 15,* 1883.

Roe, B., Marcu, K., and Dudock, B. (1973), *Biochim. Biophys. Acta 319,* 25.

Singhal, R. P., and Cohn, W. E. (1973), *Biochemistry 12,* 1532.

Stahl, S., Paddock, G., and Abelson, J. (1973), *Biochem. Biophys. Res. Commun. 54,* 567.

Yoshida, M. (1973), *Biochem. Biophys. Res. Commun. 50,* 779.

Circular Dichroism Studies of Angiotensin II and Analogues: Effects of Primary Sequence, Solvent, and pH on the Side-Chain Conformation†

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ABSTRACT: Conformational aspects of the pressor hormone angiotensin II and 11 of its structural analogues were studied by circular dichroism. Each position of the peptide was singly substituted with an aliphatic residue and alterations of the CD spectra of the resulting analogues in the peptide and aromatic spectral regions (320–250 nm, 250–190 nm) were examined. The spectra of these peptides in 2,2,2-trifluoroethanol solution permit estimation of the relative importance of the various side chains in maintaining the backbone conformation of the hormone. The evolution of the CD spectra in both spectral regions of the peptides in aqueous solution during a titration from pH 1 to pH 12 makes it possible to elucidate further the role of ionizable groups and their interaction with aromatic amino acids such as tyrosine. The results obtained indicate that substitutions in aspartic acid 1, proline 7, and phenylalanine 8 of angiotensin II entail changes in the backbone conformation. On the other hand, the side chains of valine 3, isoleucine 5, and the biologically essential histidine 6 serve mainly to correctly align the phenolic ring of tyrosine in position 4.

In previous papers from our laboratory (Fermandjian et al., 1971a,b, 1976; Greff et al., 1976) we have pointed out the usefulness of circular dichroism measurements for obtaining an overall picture of the conformation of the peptide hormone angiotensin II, its truncated peptides and several of its analogues. Moreover, a recent study showed that CD can give even more detailed information about the relative importance and the function of the side chains in the peptide (Lintner et al., 1975).

Angiotensin II (Asp-Arg-Val-Tyr-(Val or Ile)-His-Pro-Phe) is an extremely potent pressor peptide and has been studied

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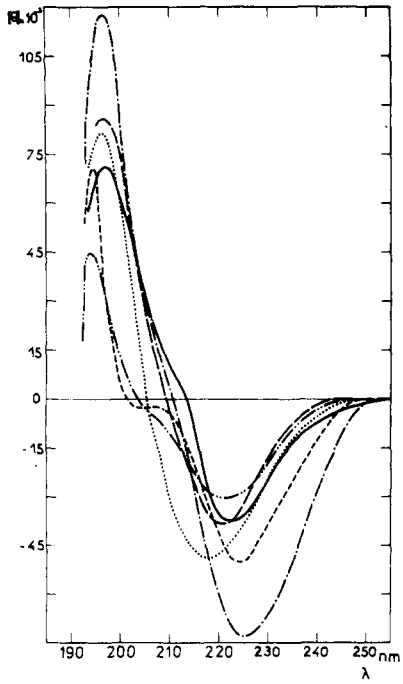

FIGURE 1: Circular dichroism spectra in the peptide region of the structural analogues of angiotensin II, in TFE. (—) [Asp[1]] A II and [Asn[1]] A II; (— —) [Ala[3]] A II; (—·—) [Pro[3]] A II; (—··—) [Me_2Gly[1]] A II; (- - -) [Ala[4]] A II; (· · ·) [Ala[5]] A II.

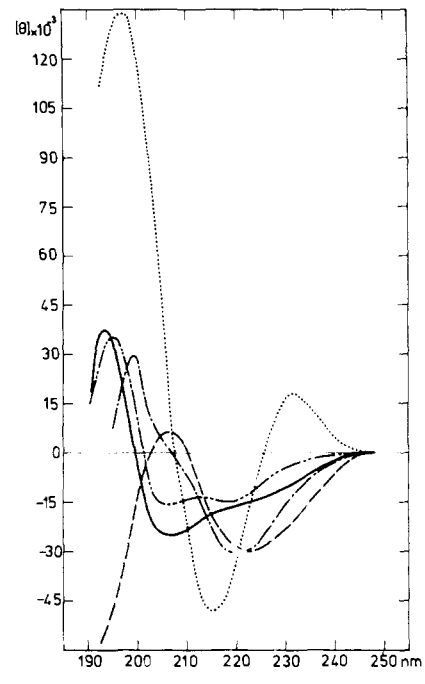

FIGURE 2: Circular dichroism spectra in the peptide region of the structural analogues of angiotensin II, in TFE. (· · ·) [Ala[6]] A II; (—) [D-His[6]] A II; (— —) [Ile[8]] A II; (— - - —) [Gly[7]] A II; (— - —) [Pro[3]-Ile[8]] A II.

extensively in regard to its physiology, pharmacology, conformation, and interaction with membrane receptors (Devynck et al., 1974; Khosla et al., 1973; Regoli et al., 1974; Juliano and Paiva, 1974; Marshall et al., 1974). The initial aim in these studies was to define the relationship of primary sequence to biological activity of the peptide. However, primary sequence changes often result in a modified peptide conformation so, before drawing conclusions from biological data on side group–receptor interactions, it is necessary to know any conformational modifications brought about by sequence changes. Although it has recently been claimed (Marshall et al., 1974) that no single model proposed so far for the conformation of angiotensin II is consistent with all physical data (NMR ^1H, ^{13}C, ^{19}F, deuterium, and tritium exchange, CD) available, it is generally agreed that: in solutions with concentrations below 10^{-2} M, angiotensin II is monodispersed (Greff et al., 1976); the high flexibility of the molecule allows no single conformation to dominate, at least not in aqueous solution; some type of folding or bending seems inherent in the peptide's conformation (Fermandjian et al., 1976); solvents like 2,2,2-trifluoroethanol (TFE) introduce a certain conformational selection (Fermandjian et al., 1971b; Greff et al., 1976; Nabedryk-Viala et al., 1975).

This paper reports CD data on a number of angiotensin II derived peptides. The CD spectra in two spectral regions (320 to 250 nm, 250 to 190 nm) of analogues mainly singly substituted are compared in the solvents water, as a function of pH in aqueous solution, and in TFE. They are interpreted in conformational terms, after spectral features such as aromatic contributions are taken into account.

Materials and Methods

The peptides (Asn[1]) A II and (D-His[6]) A II are a gift from Dr. Riniker (Ciba-Geigy, Basel); (Ala[5]) A II and (Gly[7]) A II are a gift from Dr. Park (University of Sherbrook, Quebec). Angiotensin II, (Me_2Gly[1])[1] A II, (Ala[3]) A II, (Pro[3]) A II, (Ala[4]) A II, (Ala[6]) A II, (Ile[8]) A II, and (Pro[3]-Ile[8]) A II have been synthesized and purified as previously described (Khosla et al., 1973).

The dried, weighed peptides were dissolved in water or TFE to obtain concentrations of 1 to 2 mg/ml. The aqueous solutions were acidified to pH 1 with concentrated HCl and final pH adjustments were effected by addition of small volumes of concentrated NaOH. The peptide concentration remained unaffected by this method because, after reacidification, the ultraviolet absorption at 275 nm was equal to the original value. Solvents used were 2,2,2-trifluoroethanol UVASOL (Merck) dried over washed and activated molecular sieve and doubly deionized water.

Spectra were recorded on the Jobin Yvon Dichrograph III model using fused quartz cells with path lengths of 0.01 and 0.02 cm for the peptide region and 0.5 and 1.0 cm for the aromatic region. Pathlengths had been calibrated with a solution of epiandrosterone in dioxane ($\Delta\epsilon_{304nm} = 3.31$).

Circular dichroism results are expressed in units of molar ellipticity, $[\theta] = 3300\ \Delta\epsilon$.

Results and Discussion

Spectra of Angiotensin II and Analogues in TFE Solution. It has been shown previously that solutions of angiotensin II in TFE give CD spectra of the type found for the β-sheet conformation. These spectra have been interpreted in terms of a "cross-β" or bent conformation (Devynck et al., 1973). As the parameters for the appearance of this type of spectrum have been defined with respect to solvent and to peptide length (Greff, 1973; Greff et al., 1976), it is interesting to compare the CD spectra of angiotensin II with those of its analogues recorded under the same conditions. In the series of analogues presented in this work, each position within the sequence of

[1] Abbreviations used: Me_2Gly, *N,N*-dimethylglycine; NMR, nuclear magnetic resonance; CD, circular dichroism; UV, ultraviolet; TFE, 2,2,2-trifluoroethanol.

TABLE I: Ellipticities [θ] at 275 nm.

TFE	Peptide	H_2O, pH 5
+270	Angiotensin II	+250
+280	[Asn^1] A II	+150
−200	[Me_2Gly^1] A II	+40
+400	[Ala^3] A II	+80
+900	[Pro^3] A II	−30
	[Ala^4] A II	
−1000	[Ala^5] A II	+320
+1700	[Ala^6] A II	−100
+100	[D-His^6] A II	+340
−100	[Gly^7] A II	+350
+180	[Ile^8] A II	+280
+1590	[Pro^3-Ile^8] A II	−70

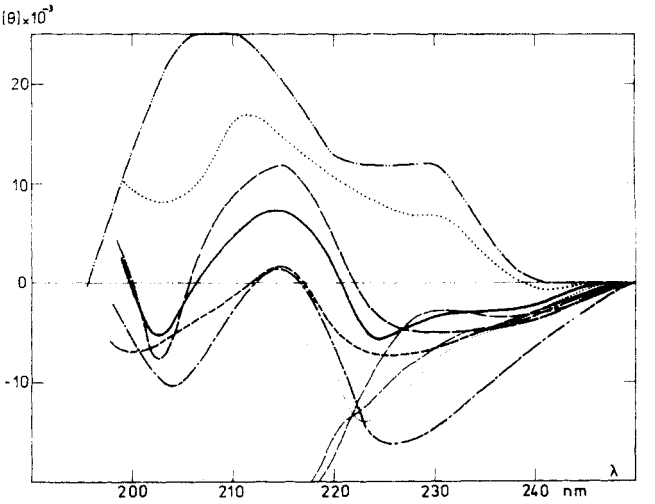

FIGURE 3: Circular dichroism spectra in the peptide region of the structural analogues of angiotensin II, in water at pH 5. (—) Angiotensin II; (— · —) [Pro^3] A II; (— —) [Ala^3] A II; (- - -) [Ala^4] A II; (• • •) [Ala^5] A II; (· · ·) [Ala^6] A II; (— · · —) [Gly^7] A II; (— —) [Ile^8] A II; (— · —) [Pro^3-Ile^8] A II.

angiotensin II (with the exception of amino acid No. 2, arginine) is consecutively substituted with a simple aliphatic residue such as glycine, alanine or, in two cases, isoleucine. A few substitutions of a more drastic nature complete the series.

Figures 1 and 2 show the CD spectra in the peptide region of angiotensin II and its analogues in TFE solution. We find that the 12 peptides studied can be grouped into two classes, the first of which gives rise to CD spectra that resemble, within reasonable limits, the spectrum of angiotensin II. The second group of peptides, substituted in the C-terminal sequence His-Pro-Phe, deviate greatly in their CD spectra from that of the native hormone. The comparison of the spectra of these peptides in the aromatic region, where we observe the signals of tyrosine and phenylalanine (^1L_b-transitions), permits further interpretation. Table I gives the [θ] values at 275 nm, the wavelength of the maximum of the tyrosine signal, which is very sensitive to local and global structural variations. These values help explain the differences seen in the peptide region of the spectra, as the contributions of the two aromatic amino acids in these octapeptides are of importance. Taking this into account, it seems plausible to assign similar backbone conformations to the peptides angiotensin II, [Asn^1] A II, [Ala^3] A II, and [Ala^5] A II (Figure 1).

The value of $[\theta]_{275nm}$ of −1000 found for [Ala^5] A II in TFE solution is surprising, as its sign and magnitude are different from the value found for angiotensin II and [Ala^3] A II under the same conditions. The side chain in position 5 reflects a stronger influence than position 3 on the tyrosine ring, and this difference in the CD signal at 275 nm may also explain the apparent band shift at 220 nm (Figure 1) in these analogues as being caused by a slightly modified aromatic ^1L_a contribution. The CD spectra of [Ala^4] A II and [Me_2Gly^1] A II reflect, in comparison with angiotensin II, the reduced aromatic contributions as do the increased intensities of the CD signals of [Pro^3] A II, which have been explained previously as being due to a steric influence on the tyrosine ring ($[\theta]_{275nm}$) and a higher rigidity of the entire molecule on account of the second proline residue ($[\theta]_{225nm}$, $[\theta]_{197nm}$) (Greff, 1973; Fermandjian et al., 1973; Greff et al., 1976). The lack of the tyrosine chromophore in [Ala^4] A II becomes evident from the increased (with respect to angiotensin II) [θ] value at 225 nm and the decreased one at 200 nm. For [Me_2Gly^1] A II we find a negative signal for tyrosine in the aromatic region and a diminished signal at 195 nm in the peptide region of the spectrum. The substitution of aspartic acid by *N,N*-dimethylglycine apparently affects the β-turn conformation (Greff et al., 1976; Fermandjian et al., 1976) to a small degree in the N-terminal tetrapeptide part, such as to perturb the tyrosine contribution and the overall stability of the peptide. Although the spectra of the peptides presented in Figure 2 deviate more substantially from the CD spectrum of angiotensin II, they, too, can be interpreted in conformational terms by taking several factors into account.

The important feature of the spectrum of [Ala^6] A II is the positive band at 232 nm and the tyrosine signal at 275 nm ([θ] = 1700) which is six times higher than in angiotensin II. The intensity of the 275-nm band indicates that the band at 232 nm might only be the strong aromatic contribution of tyrosine in this analogue. This assumption is confirmed by the evolution of the spectra of [Ala^6] A II in going from TFE to water solutions (Figure 3) (Lintner, 1975), where the band at 232 nm disappears quickly, paralleling a drop in band intensity at 275 nm, and allowing the usual "β-type" spectrum to appear at solvent mixtures of 50% TFE–50% water. The spectra of the [Ala^6] A II analogue confirm again the important role of the histidine side chain (or the absence thereof) in position 6 in regard to the orientation of the tyrosine residue, as has been suggested by Lintner et al. (1975).

The analogue [D-His^6] A II gives a surprising spectrum for in the peptide region it strongly resembles an α-helix type spectrum. It would be hasty, though, to attribute α-helical conformation to this analogue. It has to be kept in mind that (a) histidine is a chromophore with its proper CD signal which, in the D-form, will be inverted; (b) the spectrum of [D-His^6] A II, which in the aromatic region is quite different from the one of angiotensin II, hints at changed contributions of tyrosine and phenylalanine in the peptide region; (c) the sequences of D-X-L-Pro in peptides tend to give rise to CD spectra of α-helix type by themselves (Laiken et al., 1967; Woody, 1974).

The situation is different for [Gly^7] A II, where we find a similar α-helical type spectrum in the peptide region. When the conformationally restricted Φ and Ψ angles of proline are eliminated, the octapeptide is quite likely to form an α-helix in a solvent like TFE.

The important role of the C-terminal amino acid phenylalanine for the conformation as well as for the biological activity has been amply demonstrated (Khosla et al., 1974). The kind of conformational change that takes place in going from [Phe^8] A II to [Ile^8] A II cannot be determined precisely from the CD spectra, but some arguments are in favor of only minor

conformational modifications at the C-terminal end of the peptide, e.g.: the CD signal of tyrosine at 275 nm in [Ile^8] A II is quite similar to the one in angiotensin II, and its increase in intensity in [Pro^3-Ile^8] A II analogue parallels that in [Pro^3] A II; the n–π^* bands at 222 nm correspond well to the ones found in angiotensin II, the difference in the spectra being mainly at 190–205 nm. Loss of positive optical activity in [Ile^8] A II in this region is certainly, to some extent, due to the lack of contribution of the phenylalanine side chain, although it could also reflect a loosening conformational stability of the molecule. This becomes clear from the spectrum of [Pro^3-Ile^8] A II where the recurrence of a positive signal at 200 nm must come from the additional rigidity in the N-terminal part of the molecule, stemming from the second proline residue.

The comparative survey of the 12 analogues studied showed that substitutions in positions 1 through 6 do not greatly affect the overall conformation of the peptides as it is found in TFE solution. This does not mean that substitutions do not entail local conformational changes, principally in the vicinal side chains. However, replacing proline by glycine in position 7 permits the formation of an α-helix, whereas substitution of phenylalanine by isoleucine seems mainly to destabilize the C-terminal end of the peptide.

Spectra of Angiotensin II and Analogues in Aqueous Solution—Titration from pH 1 to pH 12. Aromatic Region. As has been pointed out previously, the CD signal of the tyrosine side chain at 275 nm in angiotensin II is very sensitive to changes in local environment. It is therefore useful to monitor changes of this CD signal by varying parameters such as primary sequence, solvent, and pH in aqueous solution or any combination of these. Table I lists the ellipticities [θ] at 275 nm measured for 12 angiotensin II analogues in aqueous solution at pH 5. It is clear from these figures that the signal itself cannot be proof for or against a given conformation of the entire peptide since such similar analogues as [Ala^3] A II, [Pro^3] A II, and [Ala^5] A II (see preceding paragraphs) have distinctly different CD spectra in the near-UV region. Differences in local environment of the 4th residue, due to direct influences of one amino acid on the angles Φ, Ψ, and χ_1 of the following or preceding amino acid (Pullmann and Pullmann, 1974) must be invoked as the causes. More information, however, can be gained by comparing the changes these tyrosine signals undergo as a common parameter, such as the pH, is varied. Figure 4 shows that the signal at 275 nm undergoes variations with changes in protonation state of three of the four titrable groups in angiotensin II. In all cases but three ([Me$_2$Gly^1] A II, [β-Asp^1] A II, and pentapeptide 4–8 A II, Figure 4a and b) the deprotonation of the carboxyl group(s) leads to an increase ($\Delta[\theta]$ positive) in the aromatic tyrosine signal, varying between 20 and 100 units of ellipticity at 275 nm. Interestingly, the three exceptions mentioned have in common a changed or disrupted N-terminal sequence. The most striking evolution of the spectra in this region occurs during the titration of the histidine side chain (pK = 6.25–6.50). As the causes for the high negative values of $\Delta[\theta]_{275}$, either major or local conformational effects could be considered. The series of analogues studied allows an initial estimate of the process involved. Within the values of $\Delta[\theta]_{275} = -500 \pm 100$ are found angiotensin II and the analogues [Asn^1] A II, [Ala^3] A II, [Pro^3] A II, and [Ile^8] A II; the titration effect appears less pronounced in analogues [Me$_2$Gly^1] A II, [Ala^5] A II, and [Pro^3-Ile^8] A II, whereas [D-His^6] A II, [Gly^7] A II, [Ala^4] A II, and [Ala^6] A II show little or no significant change in the spectra. Clearly the respective orientations of the histidine and tyrosine side chains affect each other in a way depending principally on the state of protonation of the imidazole group. Evidently peptides whose arrangement of the first six amino acids is close in its backbone conformation to the one found in angiotensin II (cf. spectra in TFE) also show similar titration behavior.

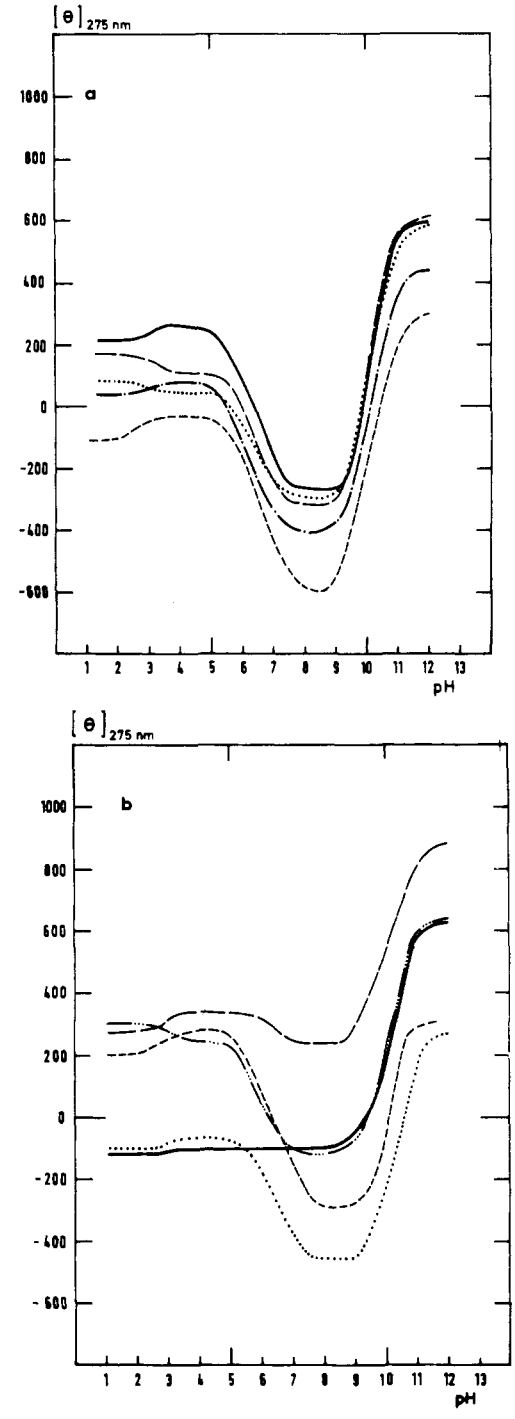

FIGURE 4: Ellipticities [θ] at 275 nm, plotted as a function of pH. (a) (—) Angiotensin II; (— —) [β-Asp^1] A II; (· · ·) [Me$_2$Gly^1] A II; (— · —) [Ala^3] A II; (- - -) [Pro^3] A II. (b) (—) [Ala^6] A II; (— —) [D-His^6] A II; (- - -) [Ile^8] A II; (· · ·) [Pro^3-Ile^8] A II; (— · · · —) [4–8] A II.

On the other hand, the conformational differences with respect to angiotensin II inherent in the peptides [D-His^6] A II, [Gly^7] A II and, to a lesser extent, in [Me$_2$Gly^1] A II and [Ala^5] A II, as evidenced by their spectra in TFE solution, are well reflected in their strongly diminished values of $\Delta[\theta]_{275nm}$.

TABLE II: Variations of Ellipticities ($\Delta[\theta]$) between pH 1 and pH 4.5 and pH 4.5 and pH 7.5, Respectively, Measured at 275, 225, and 205 nm.

Peptide	$\Delta[\theta]_{275}$		$\Delta[\theta]_{225}$		$\Delta[\theta]_{205}$	
	pH 1–4.5	pH 4.5–7.5	pH 1–4.5	pH 4.5–7.5	pH 1–4.5	pH 4.5–7.5
Angiotensin II	+40	−500	−7 500	0	+11 000	−25 000
[Asn^1] A II	+50	−430	−9 000	0	+7 000	−22 000
[Me_2Gly^1] A II	−35	−350	−10 000	0	+7 500	−14 000
[Ala^3] A II	+40	−480	−8 000	0	+10 000	−29 000
[Pro^3] A II	+90	−570	−12 000	0	+5 000	−23 000
[Ala^4] A II	+20 (θ_{261})	0	−5 000	0	+13 000	0
[Ala^5] A II	+20	−250	−6 500	−3000	+9 500	−15 000
[Ala^6] A II	+20	0	−4 000	0	+10 000	0
[D-His^6] A II	+60	−100	−7 500	−30000	0	0
[Gly^7] A II	+100	−175	−6 500	0	+22 000	−7 000
[Ile^8] A II	+70	−550	−5 000	−2500	−13 000	−14 000
[Pro^3-Ile^8] A II	+30	−380	−5 000	−3000	−13 000	−9 000

The titration of the N-terminal group ($NH_3^+ \rightarrow NH_2$) does not result in visible effects on the near-UV CD spectra of the analogues studied. Furthermore, no conformational information can be deduced from the important spectral changes that occur with the ionization of the tyrosine side chain. In all cases studied a strong band appears around 295 nm, caused by the shift of the ^1L_b transition from 275 to 293 nm. The observed values of $[\theta]_{275}$ reflect this phenomenon, showing little difference from one analogue to the other.

Peptide Region. The notions acquired from these titration curves are borne out and can be expanded by studying the CD spectra in the peptide region during titration. Although the observed far-UV CD spectrum of angiotensin II is composed of several electronic transitions (such as $n-\pi^*$ and $\pi-\pi^*$ of the peptide bonds, reflecting the conformational equilibrium existing in aqueous solution (Fermandjian et al., 1971a), the ^1L_a and the ^1B transitions of phenylalanine and tyrosine, and even the still quite unknown contribution of the $\pi-\pi^*$ transition in histidine), their respective importance is unequal and can be estimated to a large extent by studies of model peptides (Lintner, 1975), by correlation of near-UV and far-UV CD spectra and by comparison of selected analogues.

The spectra of angiotensin II and its analogues in aqueous solution at pH 5 (Figure 3) do not lend themselves to simple interpretation in terms of α-helix, β-sheet, or random coil, but are an indication of their sensitivity toward conformational changes. Again, angiotensin II, [Ala^3] A II, [Pro^3] A II, and [Ala^4] A II have spectra of reasonable likeness, with the modifications already explained, while [Ala^5] A II, [Ala^6] A II, [Gly^7] A II, and [Ile^8] A II appear distinctly different.

Evidently, the substitutions in positions 5([Ala^5] A II) and 6 ([Ala^6] A II) influence greatly the ^1L_b and ^1L_a transitions, respectively, of the tyrosine ring and their contribution to the CD spectrum. Theory predicts that the ^1L_b transition is strongly under the influence of the angle χ_1 in the tyrosine side chain, while the ^1L_a transition is modified by changes in the angle Ψ of the same residue (Woody, 1974; Snow and Hooker, 1975). The substitution of isoleucine by alanine 5 is likely to disturb Ψ_{Tyr} (Pullmann and Pullmann, 1974), and thus the ^1L_a transition at 225 nm (Figure 3), whereas the replacement of histidine 6 by alanine 6 leads, among other things, to changes in χ_1, reorienting the ring (Lintner et al., 1975), thereby influencing ^1L_b at 275 nm (Table I).

Again, more information can be gained by following the evolution of these spectra as a function of pH. The results of the titration of the 12 peptides in this spectral region are summarized in Figures 5 and 6 and in Table II.

The wavelengths selected for the titration curves and the figures in Table II are 275 nm for the tyrosine signal in the aromatic region (261 nm for the phenylalanine signal in [Ala^4] A II which contains no tyrosine), 225 nm for the $n-\pi^*$ transition of the peptide bond, and 205 nm as the wavelength farthest in UV that could be followed with a reasonable degree of precision (the optical density of the solutions during titration made measurements at lower wavelenghts impossible). At 205 nm we can expect to see the influence of the $\pi-\pi^*$ transition and the ^1B transitions of the phenylalanine and tyrosine residues. The titration curves for angiotensin II (Figures 4–6) at different wavelengths reflect different phenomena as the various ionizable groups in the molecule change their state of protonation. In this respect it is reasonable to assume that pH-induced changes in the CD spectra visible at all wavelengths are due to conformational changes, whereas localized perturbations of side chains should only affect the corresponding transitions.

The deprotonation of the carboxyl group(s) in angiotensin II is evident at all wavelengths by spectral changes (Figures 4–6). The peptide apparently undergoes a conformational transition from the quite randomized form at very acid pH to a state of one or more better defined conformations; especially the appearance of the negative band at 225 nm, corresponding to the $n-\pi^*$ transition, and the positive values of $\Delta[\theta]$ at 200–205 nm underline this assumption. It is to be noted, however, that studies with model peptides containing an aromatic residue (Tyr, Phe) in the C-terminal position showed a two- to threefold increase of the ^1B contribution to the spectrum at about 200 nm (Lintner, 1975), during the ionization of the carboxyl group. In all cases these signals have been observed to be positive; consequently the decrease of negative ellipticity around 200 nm (Figure 5) in going from pH 1 to pH 4 stems partially from the increased aromatic contribution of the C-terminal phenylalanine.

While the values of $\Delta[\theta]_{225}$ fall mostly around −8000 with the extremes of [Ala^6] A II (−4000) and [Pro^3] A II (−12 000), the values of $\Delta[\theta]_{205}$ are more dispersed and varied. Although the given figures have to be interpreted with caution due to the complexity of the CD signal at this wavelength, the analogues [D-His^6] A II, [Gly^7] A II, and [Ile^8] A II, [Pro^3-Ile^8] A II are clearly distinguished from the others. As we have found for the aromatic region, the titration between pH 5 and pH 7, changing the protonation state of the histidine side chain, yields more telling results. In fact, for the analogues substituted in positions 1 through 6 the values of $\Delta[\theta]$ found at 275 nm correspond semiquantitatively to the ones at 205 nm:

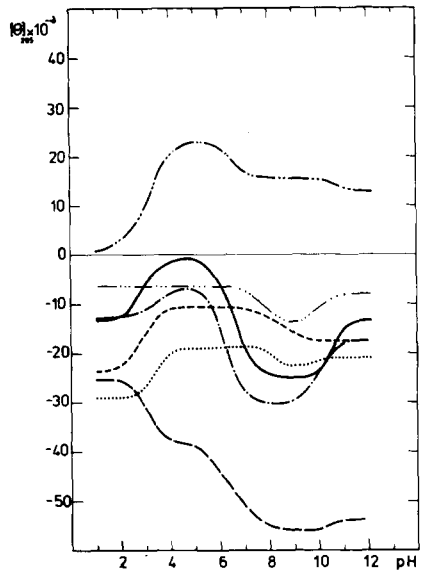

FIGURE 5: Ellipticities [θ] at 205 nm plotted as a function of pH. (—) Angiotensin II; (— · —) [Pro^3] A II; (- - -) [Ala^4] A II; (· · ·) [Ala^6] A II; (— · · · —) [D-His^6] A II; (— · · —) [Gly^7] A II; (— —) [Ile^8] A II.

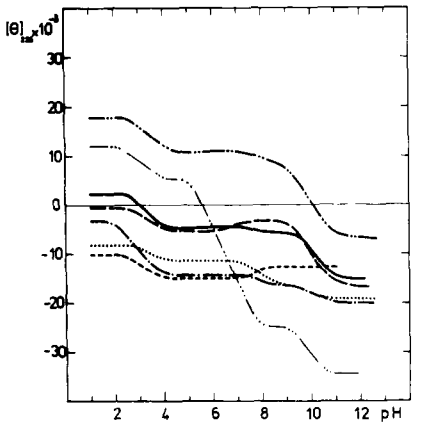

FIGURE 6: Ellipticities [θ] at 225 nm, plotted as a function of pH. (—) Angiotensin II; (— · —) [Pro^3] A II; (- - -) [Ala^4] A II; (· · ·) [Ala^6] A II; (— · · · —) [D-His^6] A II; (— · · —) [Gly^7] A II; (— —) [Ile^8] A II.

$$\frac{\Delta[\theta]_{275}}{\Delta[\theta]_{205}} = 21 \pm 4\ (\times 10^{-3})$$

On the other hand, the spectra of the analogues, [Ala^4] A II and [Ala^6] A II, do not show any variation at these wavelengths between pH 5 and 7. This confirms the assumption that the $\Delta[\theta]$ values at 205 nm in the other analogues are due to some form of coupling of the ^1L_b and ^1B transitions of tyrosine. Its apparent absence in [D-His^6] A II again reveals this analogue's peculiarity, as is also evident from its $\Delta[\theta]_{225}$ value of −30 000.

These same analogues substituted in positions 1 through 6, on the other hand, do not show any variation of the spectra at 225 nm, demonstrating once more that the mutual influence of the tyrosine and histidine side chains is confined to these two aromatic rings, whereas no conformational change of importance takes place in the backbone of the peptide during the titration of the imidazole group (cf. titration curves of [Ala^4] A II).

Furthermore, we notice in analogues [Ile^8] A II and [Pro^3-Ile^8] A II, despite the usual titration behavior at 275 nm, a factor $(\Delta[\theta]_{275})/(\Delta[\theta]_{205})$ of 40 and 36, respectively. The values at 225 nm, however, show that these two latter molecules do undergo a certain conformational change (Figure 6) which will certainly influence the ellipticity at 205 nm too.

The N-terminal amino group of aspartic acid is titrated at higher pH values. Spectral variations with this reaction are only slight, at 275 nm and at 205 nm probably overshadowed by the large titration effect of histidine, but somewhat more visible at 225 nm and at 205 nm in the analogues [Ala^4] A II and [Ala^6] A II. Although no major change in conformation can be deduced from these small variations, it seems that certain modifications at the N-terminal end can introduce slight changes in the conformation of the molecule. This may also be true with the deprotonation of the NH_3^+ group. As to the effects of the ionization of the tyrosine ring at about pH 10.0, the large spectral shifts of the aromatic transitions (275 → 293 nm, 225 → 240 nm, 200 → 205 nm) implicate more than half of the spectral range and preclude herewith any conformational interpretation of the changes observed. It is to be noted, however, that in all cases studied (model peptides included [Lintner, 1975]), the shift of the ^1L_a band at 225 to 240 nm leads to negative values of $\Delta[\theta]_{225}$ and positive values of $\Delta[\theta]_{240}$, thus proving that the ^1L_a band contributes *positive* ellipticity to the spectrum of angiotensin II.

Conclusions

By the comparison of the CD spectra of angiotensin II with those of a selected series of analogues taken under conditions such as to allow for a general view of the conformation (TFE) and to observe effects due to ionizable groups in the molecule (as a function of pH) it is shown that the amino acids do not all have the same importance for secondary structure of the hormone and that the spatial organization of aromatic side chains is well defined. Analysis, position by position, reveals a number of important details. Aspartic acid (and asparagine) seems to have a small but distinct influence on the angiotensin II conformation as a whole, as substitutions of the sort [Me_2Gly^1] A II modify the CD spectra. The side chain of valine in position 3 is of minor importance, although it partially secures the correct orientation of the tyrosine side chain, as can be seen by the only small variations in CD spectra encountered in analogues substituted in this place. The β-branched hydrophobic side chain of isoleucine in position 5, however, seems to play a major role in orienting the neighboring tyrosyl ring, such that its replacement with alanine causes noticeable changes in spectral behavior. Elimination of the tyrosine side chain ring does not perturb conformation to a large extent, as the CD spectra of [Ala^4] A II underline. The determining factor at this location in the hormone seems thus to be the exact orientation of the aromatic ring which is sensitive to substitutions (Ala^5, Ala^6) and not to others (Ala^3).

Histidine, too, does not seem to participate in the stabilization of the angiotensin II conformation; rather the side chain itself, subjected to the well-established orienting effects of the proline residue (Pullmann and Pullmann, 1974), exerts directional effects through space on the tyrosine ring. It has long been recognized that proline in position 7 must be of conformational importance in angiotensin II, a fact that is confirmed by our spectra and pH titration of the analogue [Gly^7] A II which exhibits quite different behavior from the native hormone (Figures 2, 4b, and 5).

Finally, the analogues substituted in the C-terminal position present a special interest, as a number of them are shown to be good competitive inhibitors of angiotensin II (Khosla et al.,

1974). Their spectral behavior is in some respects quite different from most of the other analogues; yet it appears that an important part of the molecule's conformation is conserved intact, while mainly the C-terminal end is affected by the substitution in going from [Phe^8] A II to [Ile^8] A II. For instance, the tyrosine signal at 275 nm behaves in TFE, and during titration just as it does in angiotensin II (Figure 4a,b) indicating the sequence Tyr-Ile-His to be aligned much the same as in the native hormone. The effect of introducing a proline residue in position 3, either in the analogue ([Pro^3-Ile^8]) A II or in the hormone ([Pro^3] A II), gives the same results (Figure 4a,b).

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References

Devynck, M. A., Pernollet, M. C., and Meyer, P. (1974), *Nature (London) 249,* 67.

Devynck, M. A., Pernollet, M. C., Meyer, P., Fermandjian, S., and Fromageot, P. (1973), *Nature (London), New Biol. 245,* 55–58.

Fermandjian, S., Greff, D., and Fromageot, P. (1973), in Dynamic Aspects of Conformation Changes in Biological Molecules, Sadron, C., Ed., Dordrecht, Holland, D. Reidel Publishing Co., p 493.

Fermandjian, S., Lintner, K., Haar, W., Fromageot, P., Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1976), in Peptides 1976, Proceedings of the 14th European Peptide Symposium, Loffet, A., Ed., Bruxelles, Belgium, Editions de l'Université de (in press).

Fermandjian, S., Morgat, J. L., and Fromageot, P. (1971a), *Eur. J. Biochem. 24,* 252.

Fermandjian, S., Morgat, J. L., Fromageot, P., Lutz, M., and Leicknam, J. P. (1971b), in Proceedings of the Second International Symposium, Liege: Structure Activity Relationships of Protein and Polypeptide Hormones, Margoulies, M., and Greenwood, F. C., Ed., Netherlands, Excerpta Medica, p 460.

Greff, D. (1973), Ph.D. Thesis, Université de Paris.

Greff, D., Fermandjian, S., Fromageot, P., Khosla, M. C., Smeby, R. R., and Bumpus, F. M. (1976), *Eur. J. Biochem. 61,* 297.

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

Khosla, M. C., Hall, M. M., Smeby, R. R., and Bumpus F. M. (1974), *J. Med. Chem. 17,* 1156.

Khosla, M. D., Smeby, R. R., and Bumpus, F. M. (1973), *Handb. Exp. Pharmacol. 37,* 126.

Laiken, S., Printz, M., and Craig, L. C. (1967), *J. Biol. Chem. 244,* 209.

Lintner, K. (1975), Dissertation, Technische Universität Wien.

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

Marshall, G. R., Bosshard, H. E., Vine, W. H., Glickson, J. D., and Needleman, P. (1974), in Recent Advances in Renal Physiology and Pharmacology, Wesson, L. C., and Fanelli, G. M., Ed., Baltimore, Md., University Park Press, p 215.

Nabedryk-Viala, E., Thiéry, C., Calvet, P., and Fermandjian, S. (1975), *FEBS Lett. 58,* 273.

Printz, M. P., Williams, H. P., and Craig, L. C. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 378.

Pullmann, B., and Pullmann, A. (1974), *Adv. Protein Chem. 28,* 346.

Regoli, D., Park, W. K., and Rioux, F. (1974), *Pharmacol. Rev. 26,* 69.

Snow, J. L., and Hooker, T. M., Jr. (1975), *J. Am. Chem. Soc. 97,* 3506, and references therein.

Woody, R. (1974), in The Rehovot Symposium on Poly(aminoacids), Polypeptides and Proteins and their Biological Implications, Blout, G. R., Bovey, F. A., Goodman, M., and Lotan, N., Ed., New York, N.Y., Wiley.