TITRATION OF THE PHENOXYL GROUPS OF ANGIOTENSIN PEPTIDES WITH A SENSITIVE SPECTROPHOTOMETRIC METHOD

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SUMMARY

The possibility that the biological activities of angiotensin might depend on the presence or absence of secondary bonding involving the phenolic hydroxyl of the tyrosyl residue, was examined. Val^5-angiotensin II, Asp(NH$_2$)^1-Val^5-angiotensin II, Asp(NH$_2$)^1-homotyrosyl^{4,5}-Val^5-angiotensin II and Asp(NH$_2$)^1-Val^5-Pro^7-angiotensin II were titrated spectrophotometrically using the increase in absorption at 2425 Å as a measure of the appearance of the phenoxide form. The peptides were titrated normally, with pK_0 values 10.29, 10.30, 10.52 and 10.35, respectively, compared to the value 10.15 found for free tyrosine. Participation of the phenolic hydroxyl in secondary bonding was thus excluded for the four peptides.

INTRODUCTION

Studies on the relation of angiotensin structure to biological activity[1-5] have shown that many of the groups on the peptide side chains are required for a wholly active molecule. It is conceivable that not all of these groups would be directly concerned in the interaction with the cell "receptors", but that some of them might be important for establishing secondary bonds that would hold the molecule in an active spacial configuration. Although the presence of secondary bonds has not yet been demonstrated for peptides the size of the angiotensins, the theoretical possibility exists, and the observations that oxytocin[6] and angiotensin[5] octapeptides are inactivated by incubation with urea suggest that hydrogen bonding may play a role in the maintenance of an active configuration in these peptides. In angiotensin, such bonds could be formed by the phenolic hydroxyl pertaining to the tyrosyl residue. The tyrosyl side chains have been shown to contribute to the stability of the secondary structure of several proteins through either hydrogen, ion-dipole or hydrophobic bonding[7], and the phenolic hydroxyls engaged in such bonding are more difficultly titrated with base, having pK values higher than normal[8]. In the case of angiotensin, an ion–dipole interaction between the phenolic hydroxyl and the C-terminal carboxyl groups is suggested by examination of molecular models, and by the fact that both groups are essential for activity[1,3].

Abbreviations: Angiotensin, Val^5-angiotensin II; AA, Asp(NH$_2$)^1-Val^5-angiotensin II; PAA, Asp(NH$_2$)^1-Val^5-Pro^7-angiotensin II; HTAA, Asp(NH$_2$)^1-homotyrosyl^{4,5}-Val^5-angiotensin II.

TABLE I

AMINO ACID SEQUENCE OF ANGIOTENSIN PEPTIDES

Peptide	*Abbreviation*	*Amino acid sequence*
Val^5-angiotensin II	Angiotensin	Asp·Arg·Val·Tyr·Val·His·Pro·Phe
$Asp(NH_2)^1$,Val^5-angiotensin II	AA	$Asp(NH_2)$·Arg·Val·Tyr·Val·His·Pro·Phe
$Asp(NH_2)^1$-Val^5-Pro^7-angiotensin II	PAA	$Asp(NH_2)$·Arg·Val·Tyr·Val·His·Pro
$Asp(NH_2)^1$-homotyrosyl^{4,5}-Val^5-angiotensin II	HTAA	$Asp(NH_2)$·Arg·Val·Tyr·Tyr·Val·His·Pro·Phe

In the present paper we report an investigation of the possible role played by tyrosyl secondary bonds in determining the biological effects of four synthetic angiotensin peptides (Table I) through the spectrophotometric titration of the phenoxyl groups in these peptides. In view of the small amount of peptides available to us, we developed a more sensitive spectrophotometric titration than the one introduced by CRAMMER AND NEUBERGER[9], which has been commonly employed for the titration of tyrosyl side-chains in peptides and proteins. In that method, the dissociation of the phenoxyl group is measured by the increase in absorption at some wavelength near 2935 Å, where the phenoxide ion has an absorption maximum (molar extinction coefficient, $\varepsilon = 2.33 \cdot 10^3$) and the undissociated phenoxyl group absorbs very little. However, a greater difference in absorption is encountered near 2400 Å, since the phenoxide ion spectrum goes through a maximum at 2400 Å ($\varepsilon = 1.105 \cdot 10^4$) and the phenoxyl group goes through a minimum at 2450 Å ($\varepsilon = 1.7 \cdot 10^2$) (see ref. 8). In the experiments reported here, advantage was taken of this fact to follow the titration of the phenoxyl groups in angiotensin peptides with the technique of differential spectrophotometry[10].

EXPERIMENTAL

L-Tyrosine was a product of Nutritional Biochemicals Corporation; angiotensin, AA, PAA and HTAA were synthetic products prepared at Ciba Limited, Basel[11].

The spectral measurements were made on a Beckman model DU quartz spectrophotometer with silica cells of 1-cm optical path. The titration curves were obtained by using a solution at pH 7.00–7.50 as a reference (absorbance taken as zero) against which the absorbances of solutions of the same concentration, but at other pH's, were read. The pH of each solution was adjusted by adding 0.1 *N* or 0.5 *N* NaOH and enough CO_2-free water or 0.1 *M* NaCl to obtain the desired concentration of tyrosine or peptide, and an ionic strength of 0.05. Usually, $2 \cdot 10^{-5}$ *M* solutions of HTAA and $4 \cdot 10^{-5}$ *M* solutions of tyrosine, angiotensin, AA and PAA were used to obtain the titration curves. The pH readings were made on a Beckman model G pH meter equipped with type E high pH glass electrodes and calibrated with standard buffers manufactured by the Cambridge Instrument Company. All measurements were made at room temperature (20° ± 3). The apparent pK' values were obtained graphically from the equation: $pH = pK' + \log \alpha/(1-\alpha)$, where α is the ratio between the differential absorbance at a given pH and the maximum differential absorbance. The latter value was obtained from the titration curve, at a point where 100% ionization was assumed. The pK' values were corrected to zero ionic strength with the aid of the simplified Debye equation: $pK_0 = pK' + 0.5\sqrt{\mu}$

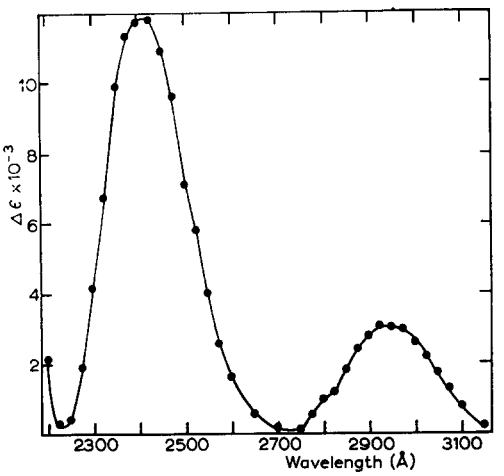

Fig. 1. Difference spectrum of tyrosine at pH 13 *versus* pH 7.0.

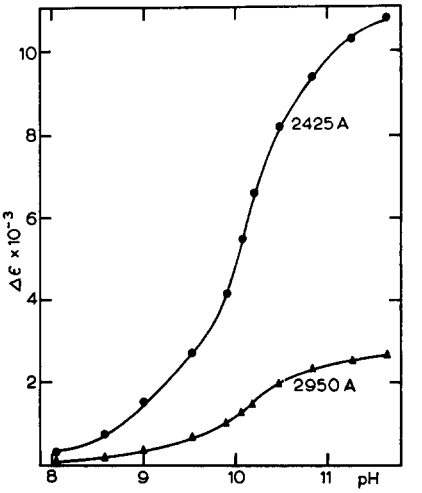

Fig. 2. Tyrosine titration curves obtained at 2425 Å and at 2950 Å.

RESULTS AND DISCUSSION

The titration of tyrosine

The difference spectrum obtained for tyrosine at pH 13 *versus* pH 7.0 is shown in Fig. 1. The absorption difference observed at 2425 Å ($\Delta\varepsilon_{max} = 11.8 \cdot 10^3$) was considerably greater than at 2950 Å ($\Delta\varepsilon_{max} = 3 \cdot 10^3$). Titration curves for tyrosine, obtained at 2425 and 2950 Å, are shown on Fig. 2, where it can be seen that the use of the differential absorbance maximum at 2425 Å instead of that at 2950 Å, results in a fourfold increase of sensitivity. Yet, titrations performed at either wavelength yielded the same pK_0 value, 10.15, which agrees with previously reported determinations[9,12–14]. The titration was reversible and a pK_0 of 10.0 was found when a tyrosine solution in 0.03 *N* NaOH was titrated with hydrochloric acid. These results indicate that the titration of the phenoxyl group of tyrosine may be advantageously followed by measurements of the absorbance at 2425 Å, provided the differential technique is used to correct for the significant absorbance of the undissociated phenoxyl group at that wavelength. This correction is even more necessary in the titration of peptides or more complex molecules because of the presence of other groups that absorb light in that region of the spectrum, the peptide bond itself having some absorption at 2425 Å due to the long-wave tail of its absorption band, which has a maximum at 1900 Å (see ref. 15). As for the usual side chains present in peptides, cysteyl residues show significant increase of absorption at 2425 Å on alkalinization owing to the appearance, in the difference spectrum, of an absorption band with maximum at 2300–2380 Å caused by dissociation of the thiol group[16]. For this reason, titration of phenoxyl groups in cystein-containing peptides may not be followed by the absorbance at 2425 Å.

The titration of angiotensin peptides

Before titrating the phenoxyl groups of angiotensin peptides, we have ascertained that no other groups present in their molecules caused alteration in absorbance at

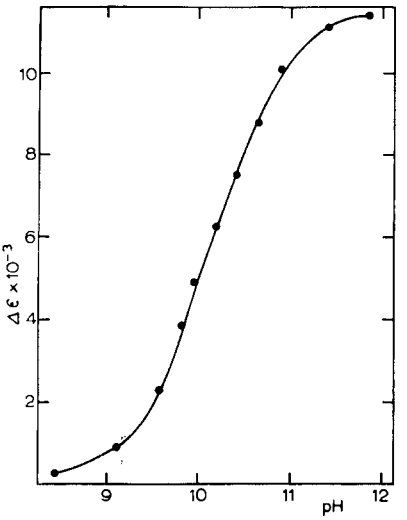

Fig. 3. Titration curve obtained for angiotensin at 2425 Å.

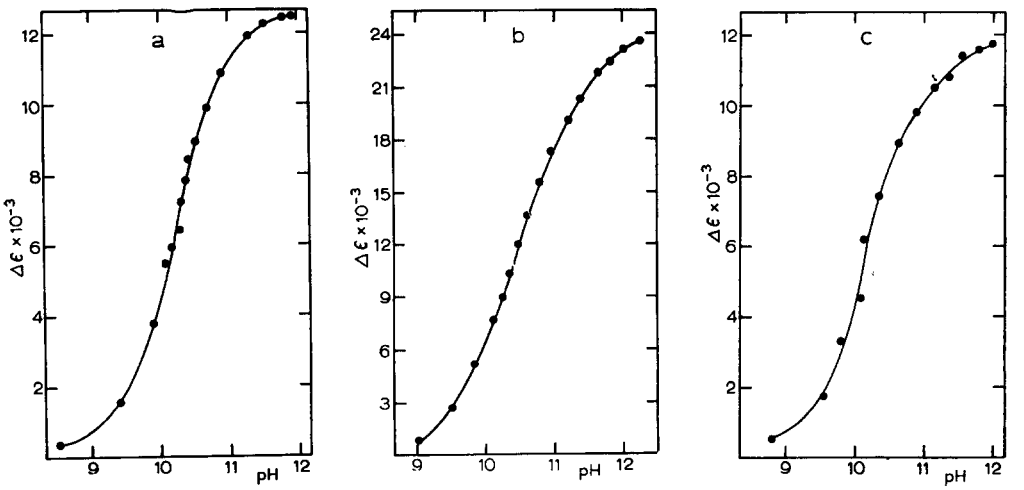

Fig. 4. Titration curves obtained at 2425 Å for AA(a), HTAA (b) and PAA (c).

2425 Å that might interfere with the titration: no significant difference in absorbancy was detected when 10^{-4} *M* solutions of either leucylglycylglycine or a mixture of aspartic acid, arginine, valine, isoleucine, histidine, proline and phenylalanine had their pH's raised from 7 to 12.

The phenoxyl group of angiotensin was titrated normally (Fig. 3) and its pK_o was 10.29. The titration was reversible and no change of absorption with time was observed. This indicates that the tyrosyl side chain of angiotensin is not engaged in secondary bonding, and gives rise to the hypothesis that in some of the angiotensin analogues that were found inactive the lack of activity might be due to the presence of secondary bonds that would hold the molecule in an inappropriate spacial arrangement. Thus, in analogues with an altered distance between the phenoxyl and the

carboxyl groups, the ion–dipole interaction, that does not occur in angiotensin, might be favored. For this reason, the titrations of AA, PAA and HTAA were performed. Fig. 4 shows that the three analogues had normal titration curves, and the pK_0 found for AA, PAA and HTAA were 10.30, 10.35 and 10.52, respectively. These values are within the normal range for tyrosyl residues in peptide combination[8].

Our results show that the biological behaviour of angiotensin and the three analogues studied cannot be explained by the possible role of the tyrosyl side-chain in maintaining an active or inactive spacial configuration. Other types of secondary bonding in the angiotensin molecule, however, may not be excluded and their presence should be investigated.

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