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Studies on angiotensin II and analogs: Impact of substitution in position 8 on conformation and activity

(structure–function relationship/peptide hormone/side chain steric effect/spectroscopy)

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ABSTRACT **Affinity, residual agonist activity, and inhibitor properties of a series of angiotensin II analogs modified at the COOH-terminal position (X^8-substituted peptides) have been probed for structure/conformation–biological activity relationships. The results emphasize (*i*) the large impact that subtle conformational variations caused by structural alterations in the position 8 side chain have on biological properties, (*ii*) the implication of the COOH-terminal carboxyl group in both affinity and intrinsic activity, and (*iii*) the influence that the bulkiness of the side chain in position 8 of antagonists has on the local conformation at the COOH terminus and thus on the inhibitory properties. In the hormone, the phenylalanine-8 ring is required for its steric influence and aromaticity to ensure a fully active conformation at the COOH terminus. Especially, correct orientation of the position 8 carboxyl group relative to the phenyl group of the phenylalanine residue may be necessary for agonistic activation of the angiotensin receptor complex. Replacement of the aromatic ring on the COOH-terminal residue by a nonaromatic group leads to incorrect orientation of the carboxyl group and causes the appearance of antagonist properties. Although the steric effects of the side chain can be modulated by specific interaction of its chemical groups (if any) with the peptide backbone, we found a good correlation between the size of the side chain—e.g., the steric parameter V_γ (the van der Waals volume consisting of the C_α, C_β, and C_γ atoms), the conformational properties in the backbone (^3J HC_α–NH), and the binding capacities in all compounds tested.**

Numerous analogs of peptide hormones have been synthesized and tested for structure–activity relationships (1–3). Although such studies have led to progress in elucidating hormonal function, the fundamental question remains unanswered; how do amino acid side chains interact in a peptide chain to confer structural and dynamical properties to the molecules that ensure specificity and high binding affinity at the receptor site, as well as stimulation of target cells.

Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, AII) physiological receptors are found on plasma membranes of several types of cells including smooth muscle cells. The interaction of smooth muscle cells with the hormone or its agonist analogs stimulates a cascade of reactions leading to muscle contraction. This particular action of AII on vascular smooth muscle is in general considered to result in the direct pressor response of the peptide. It is not surprising, therefore, to find good correlation between the *in vivo* pressor response in an animal and contraction of isolated smooth muscle preparations in various *in vitro* assays. Another element of interest concerns inhibitors because both agonists and antagonists bind to the receptor but only agonists stimulate a biological response. The mechanism to account for this differential effect is currently unknown although studies on this field have been a focus of research since the discovery of angiotensin (4, 5). The most potent antagonists have been obtained by replacing the COOH-terminal aromatic residue (phenylalanine) by an aliphatic residue (e.g., [Sar^1, Ala^8]AII and [Sar^1, Ile^8]AII. In these peptides, sarcosine in position 1 appears to increase the half-life partly by reducing the degradation caused by amino peptidase action and partly by increasing the affinity for the receptors. These peptides are currently being used as experimental tools to understand the role of the renin/angiotensin system in hypertension (1, 2). However, their therapeutic use is limited by two factors: (*i*) a short biological half-life because of their weak resistance to enzymatic degradation and (*ii*) a small (1–2%) but significant residual agonistic action.

It has been shown previously that the binding of AII and related peptides to receptors requires well-defined conformational and dynamical properties of the tyrosine-4 and histidine-6 side chains (6–11). It has also been shown that high agonist activity requires the presence of both the phenyl ring and the free carboxyl group at the COOH terminus (12) and that the juxtaposition of these groups is very important (13, 14). In this paper, we evaluate the contributions of the side chain in position 8 and of the COOH-terminal carboxyl group to the conformation and the biological activity of AII and its antagonist homologues.

METHODS

Peptides were synthesized by the solid-phase procedure using protocols previously described (3). CD spectra were recorded with a computer-assisted Jobin Yvon Dichrograph model III using optical cells having a path length of 0.01–0.1 cm and equipped with quartz windows. Results are expressed in molar ellipticity, $[\theta] = 3300\ (\Delta\varepsilon\ \text{deg}\cdot\text{cm}^2\cdot\text{dmol}^{-1})$. NMR spectra were obtained at 500 MHz on a Bruker WM model 500. NMR samples were prepared as follows: peptides were lyophilized from aqueous solutions adjusted to pH 1 with 1 M HCl to obtain a constant cationic species and dissolved at low concentration (3 mM) in [$^2H_6$]dimethyl sulfoxide. Nuclear Overhauser effect studies were carried out at 295 K, using D1 and D2 delays of 2 and 3 sec, respectively, with 45L decoupling power in the HG mode.

RESULTS AND DISCUSSION

We used AII competitive inhibitors of the type [Sar^1, X^8]AII or [Asp^1, X^8]AII as well as three COOH-terminal amide

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Abbreviation: AII, angiotensin II.

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Table 1. Structural, spectroscopic, and biological parameters of AII antagonists

Compound	Type of side chain in position 8	V_γ	^3J HC_α–NH,* Hz	$\Delta[\theta]_{275\ nm}$†	PD 2	PA 2	% pressor activity‡
[Sar^1, Gly^8]AII	Hydrogen	23.62	6.0	310		8.29	0
[Sar^1, Ala^8]AII	Methyl	39.615	7.2	335		8.38	0.1
[Sar^1, Ser^8]AII	Hydroxymethyl	46.196	7.7			8.08	0.7
[Sar^1, Leu^8]AII	γ-Branched	51.048	7.9	395		8.64	0.2
[Asp^1, Cha^8]AII§	γ-Branched	51.048	7.9			(see text)	20
[Asp^1, Phe^8]AII	γ-Branched	51.12	7.7	490	8.86¶	100	
[Sar^1, Phe^8]AII	γ-Branched	51.12	7.8				150
[Asp^1, Tyr^8]AII	γ-Branched	51.12	8.0		7.94¶		83
[Sar^1, Thr^8]AII	β-Branched	57.629	8.6			8.79	0.6
[Sar^1, Ile^8]AII	β-Branched	62.481	8.5	460		9.17	1.0
[Asp^1, Phe^8 amide]AII‖			8.2				0–3
[Sar^1, Gly^8 amide]AII			6.0				0
[Sar^1, Ile^8 amide]AII			8.7				0

V_γ, volume of the portion of the amino acid side chain consisting of the C_α, C_β, and C_γ atoms, as defined in ref. 11; PD 2, negative logarithm of the concentration of peptide producing 50% of maximal response (used as a measure of affinity for rabbit aorta strips); PA 2, negative logarithm of the concentration of antagonist that reduces the effect of a double dose of agonist to that of a single dose (used as a measure of affinity for rabbit aorta strips).

*Estimated to an accuracy of 0.1 Hz.

†pH 4 vs. pH 8.

‡From nephrectomized rats.

§Cha, cyclohexylalanine.

¶These values are underestimated as compared with those obtained when sarcosine is at the NH_2 terminus (see ref. 1 and refs. cited therein).

‖This compound has no inhibitory activity (see ref. 1).

analogs and the partially active [Tyr^8]AII (Table 1). CD and NMR measurements were made under various experimental conditions. Since conclusions require rigorous comparison of data for one peptide with those for another, all samples were studied as exactly the same ionic species or in the same solvent environment.

The CD spectra of four analogs having side chains of differing bulkiness in position 8 and dissolved in trifluoroethanol are shown in Fig. 1. In both the peptide region (250–190 nm, Fig. 1*A*), and the aromatic region (310–250 nm, Fig. 1*B*), we observed band patterns that support the hypothesis that conformation is influenced by the position 8 residue. The similarity between the changes observed in the aromatic spectral region and in the peptide spectral region, where aromatic residues also contribute (^1L_a band) suggests that it is mostly the spatial orientation or the surrounding of the phenolic chromophore in the tyrosine-4 side chain and not the backbone that is affected by the substitutions in position 8. The ellipticity in both spectral regions was found to decrease (in either the positive or the negative direction) in the order Ile → Leu → Ala → Gly.

To further evaluate the degree of perturbation of the tyrosine-4 side chain, we recorded CD curves as a function of pH at 275 nm, where the ^1L_b band of the phenolic group is observed. It has previously been shown for the case of analogs modified at position 5 that the amplitude of titration (Δ [θ]) at 275 nm between pH 4 and pH 8 (the pK of histidine ≈ 6) is a measure of the time-averaged distance between the side chains of the tyrosine-4 and and the histidine-6 residues. The change in ellipticity thus obtained also decreases from peptide to peptide in the order Ile (β branched) → Leu (γ branched) → Ala (unbranched) → Gly (no side chain) (Table 1).

We found that replacement of aspartate in position 1 by

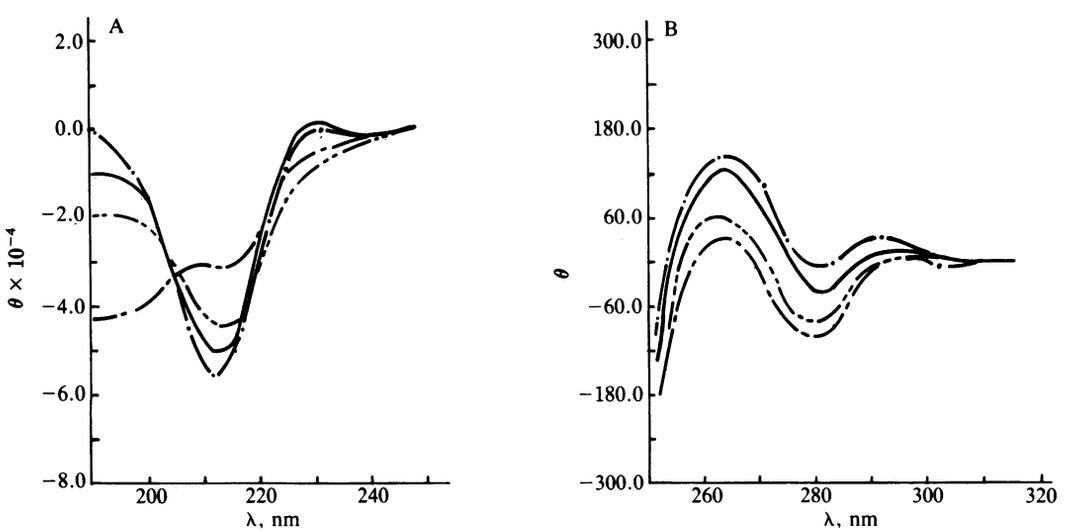

FIG. 1. CD spectra of AII inhibitors in trifluoroethanol. (*A*) Peptide region. (*B*) Aromatic region. —·—, [Sar^1, Ile^8]AII; —, [Sar^1, Leu^8]AII; — - - —, [Sar^1, Ala^8]AII; — · —, [Sar^1, Gly^8]AII.

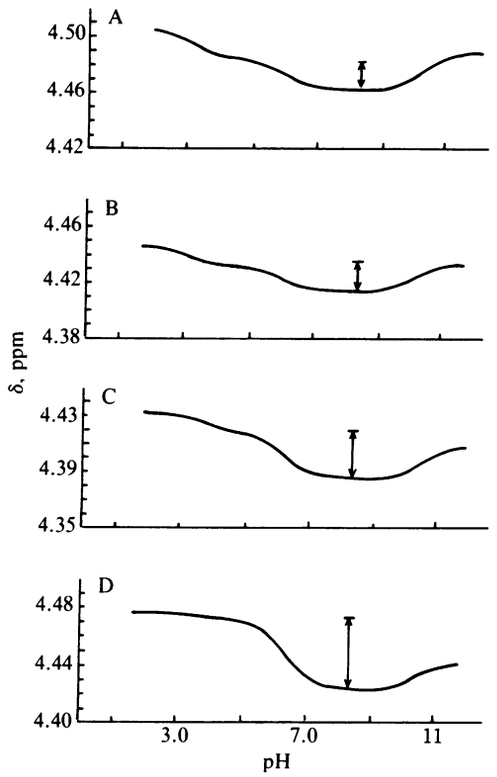

FIG. 2. Titration curve of the chemical shift of the α proton of proline-7 in angiotensin position 8-substituted analogs. (*A*) [Ile^8]AII. (*B*) [Leu^8]AII. (*C*) [Ala^8]AII. (*D*) [Gly^8]AII.

sarcosine did not significantly modify the backbone conformation of the remainder of the molecule. The same is true when the phenylalanine in position 8 is replaced by any other residue. Thus, we focused our interest on the coupling constants ^3J HC_α–NH tied to the main chain geometry in position 8. These are reported in Table 1 together with the type of side chains tested and the steric parameters (Van der Waals volume, V_γ) (15).

Among the noticeable elements for position 8, there is the parallelism between the backbone coupling constant and the bulkiness of the side chain. First, there is again a continuous decrease in the order β-branched → γ-branched → unbranched → no side chain. Second, we observed that amidation of the COOH-terminal carboxyl group can change the local conformation (^3J HC_α–NH) of the position 8 residue. The extent of modification appears, however, to be controlled by the side chain in that position because the effect is greater for phenylalanine-8 and null for glycine-8 (compare the coupling constant of [Asp^1, Phe^8]AII with that of [Asp^1, Phe^8 amide]AII). Third, we can discern even more subtle effects from titration curves of proton chemical shift versus pH. Among these, the most representative is the sensitivity of the α proton of proline-7 to histidine titration, which also reflects an effect of side chain size from residue 8 (Fig. 2). In this connection, but not shown, the β and β' protons of histidine-6 display small but significant nuances in their pH behavior during titration of the carboxylic and phenolic groups.

In addition, we find nuclear Overhauser effects (not reported in Table 1) between the α proton of proline-7 and the position 8 peptide proton that vary from peptide to peptide in a range between 10 and 30 (±2)%, probably reflecting changes in the ψ value of proline-7.

We have thus collected spectroscopic data in different media [H_2O (function of pH), dimethyl sulfoxide, trifluoroethanol] that show the same trends. These can be correlated with the calculated steric parameter V_γ (Table 1 and Fig. 3*A*) and also with the biological properties of the inhibitors (Fig. 3*B*). One may thus assume that the size of residue 8 has a major effect on binding by inducing specific conformational and dynamical properties in the main chain at the COOH terminus. The analog [Sar^1, Ser^8]AII shows, however, weaker affinity than expected on steric grounds (V_γ). This may be due to orientational restriction in the terminal carboxyl group because of interaction with the β-OH group of the serine side chain. Such interactions, which are usual in serine (16, 17), are supported by values of $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ (5.0 and 4.0 Hz, respectively), which indicate a preferred orientation of the side chain toward the COOH-terminal carboxyl group.

There is considerable evidence suggesting that the carboxyl group of AII is involved in the binding process, since suppression, sequential positional shift, amidation, or change of configuration destroy or greatly reduce the biological activity (18, 19). The present conformational study indicates that in AII the free carboxyl group is subjected to specific steric restriction by the aromatic side chain of phenylalanine. For instance, amidation of the carboxyl group in the hormone simultaneously alters the side chain organization ($J_{\alpha\beta}$ and $J_{\alpha\beta'}$ = 5.5 and 8.4 Hz in AII versus 5.0 and 8.7 Hz in AII amide) and the backbone conformation (^3J CH_α–NH = +0.5 Hz), while effects are very weak or null when position 8 is occupied by isoleucine or glycine (Table 1). However, direct information on the carboxyl spatial orientation is not available and one assumes that variations of ^3J HC_α–NH reflect connected changes of ϕ and ψ since these two angles are interdependent (19–23). This holds

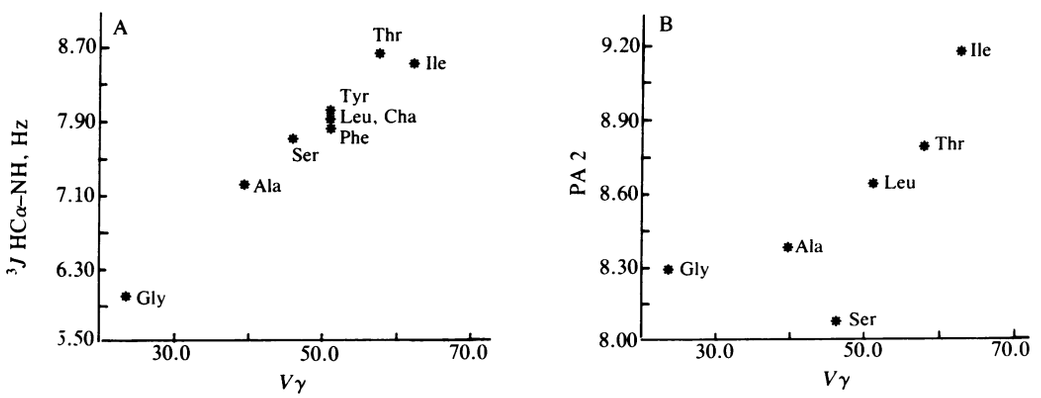

FIG. 3. Effect of residue in position 8 of AII and analogs. (*A*) ^3J HC_α–NH coupling constant vs. V_γ steric parameter. (*B*) V_γ steric parameter vs. negative logarithm of the concentration of peptide producing 50% of maximal response (PA 2) of analogs using aorta strip preparations.

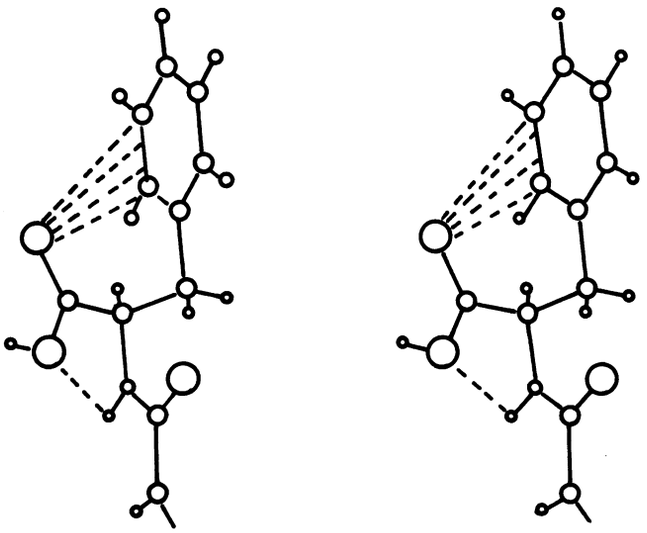

FIG. 4. Stereo view of the COOH terminal end of AII showing the specific effects of the phenylalanine side chain on the backbone conformation, including the carboxyl-group orientation.

particularly true if a strongly stabilized secondary structure cannot be invoked, which is the case in linear octapeptides. Thus, in AII three concomitant local effects could ensure the proper orientation of the carboxyl group. These are (*i*) the steric hindrance on the backbone produced primarily by the C_β, C_γ part (V_γ) of the phenylalanine side chain, (*ii*) local interactions in the backbone—e.g., that of an oxygen belonging to carboxyl group with the amide proton of the phenylalanine residue (interdependence of ϕ and ψ), and (*iii*) the interaction of a carboxyl oxygen with the electrons of the aromatic ring (Fig. 4). The interaction of a phenyl residue with the oxygen of a carbonyl group has been demonstrated from examination of 170 phenylalanine residues in 28 crystal protein structures and also by *ab initio* calculations (24). In small peptides in which aromatic residues are at the COOH terminus, the aromatic ring is often oriented toward the carboxyl group (25). The question arises now whether a specific arrangement of the phenyl residue and the carboxyl group is strictly required for eliciting a biological response? Which of these moieties is more critical for binding or for intrinsic activity? Are other functional groups implicated?

Binding is good provided the position 8 carboxyl group is free and improves as the size of the side chain on the COOH-terminal residue increases to an optimal size. Aromaticity is not required for binding but binding is significantly improved, when compared with that of AII, when a β-branched side chain is located at the COOH terminus—e.g., replacement of phenylalanine-8 by isoleucine.

Agonist activity is integrally maintained even when binding is reduced—e.g., replacement of phenylalanine-8 by tyrosine. Although both tyrosine and phenylalanine contain aromatic rings and the two have the same V_γ steric parameter, they display different $J_{\alpha\beta}$ and $J_{\alpha\beta'}$ values (5.4 and 7.4 vs. 5.5 and 8.4 Hz, respectively) for their side chains as well as slightly different ^3J HC_α–NH values for their main chain (Table 1). Although weak, this latter difference is sufficient to account for the reduction of the binding capacity shown by the analog considering the high sensitivity of this property to conformation at the COOH terminus.

Further information is gained about the origin of stimulation activity when phenylalanine-8 is replaced by cyclohexylalanine (Cha), a residue that mimics phenylalanine with the exception of ring planarity and aromaticity (26). Its spectroscopic and steric parameters are exactly the same as those of the γ-branched leucine in the good antagonist [Sar^1, Leu^8]AII but still closely resemble those of phenylalanine in the parent AII (Table 1). As expected, the resulting [Cha^8]-AII analog behaves as a good agonist (20% of pressor response of AII *in vivo*) (14). These examples give additional evidence for a nondirect effect of the aromatic ring in the stimulation process. They also prove how specific the influence of the aromatic side chain on the local conformation can be (Fig. 4) and, in turn, how critically important the local conformation is for biological activity.

However, the present CD and NMR data have shown that modifications in position 8 may affect the orientation of the histidine and tyrosine side chains, both of which are also implicated in the binding process (7–11). Such long-distance effects are usually weak in peptides and require careful spectroscopic measurements. They are not predictable in chemical structure–activity relationship studies. Yet, they cannot be neglected if we wish to have an exact picture of the role played by each residue in biological activity. In AII, quantitative estimation of individual roles is difficult because of the marked interdependence between various side chains and groups in the molecule, but is, however, an important point to investigate in the future.

In conclusion, the combination of steric parameters calculated from spectroscopic data and biological data provides a realistic view of the influence residue 8 exerts on the conformational and biological properties of AII and its antagonists. These results may be helpful in drawing guidelines for a more rational design of AII antagonists.

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