CONFORMATIONAL STUDIES OF ANGIOTENSIN II AND ANALOGUES IN DIMETHYL SULFOXIDE BY ^{1}H NMR: LABILITY AND INTRAMOLECULAR INTERACTIONS OF THE TYROSINE HYDROXYL AND HISTIDINE IMIDAZOLE NH PROTONS

John MATSOUKAS[a], Glen BIGAM[b], Raghav YAMDAGNI[c] and Graham J. MOORE[d]

[a] *Department of Chemistry, University of Patras, Patras 26220, Greece*
[b] *Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada*
[c] *Department of Chemistry and*
[d] *Department of Medical Biochemistry,*
University of Calgary, Calgary, Alberta, Canada, T2N 4N1

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^{1}H NMR studies in dimethyl sulfoxide illustrate that the tyrosine hydroxyl proton and the histidine imidazole NH proton in [Sar^1]ANGII and analogues are labile at neutral pH but not at acid pH. This is attributable to intramolecular interactions of these groups with negatively charged groups. Methylation or elimination of the Tyr hydroxyl in [Sar^1]ANGII and analogues, invokes a small but consistent deshielding effect on the His C-2 and C-4 protons, suggesting an interaction between the Tyr hydroxyl and the His ring. Nuclear Overhauser Effect (NOE) enhancement experiments in [Sar^1]ANGII and the Sarmesin analogue [Des^1,Tyr(Me)^4]ANGII, support the Tyr/His interaction and confirm the presence of a *trans* His–Pro peptide bond.

Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe; ANGII), a potent agonist component of the renin angiotensin system, has been implicated in the pathogenesis of hypertension and congestive heart failure, and knowledge of its conformation is important for the design and synthesis of improved antagonists. Various models for the conformation of angiotensin II in solution have been proposed. These models include random coil[1], α-helix[2], β- and γ-turn[3,4], ion-dipole[5] and other more or less complex models[6–11]. Recent investigations have suggested the possible occurence of a charge transfer system involving the triad Tyr, His and Phe carboxylate[12,13]. Such an interaction would result in partial abstraction of the single ionizable proton from the imidazole by the α-carboxylate, which in turn would lead to the imidazole group seeking proton donation from the tyrosine hydroxyl group. The result is a tripartite network interaction in which the negative charge originating from the α-carboxylate becomes delocalized to the imidazole ring and then to the Tyr ring[14]. The interaction is analogous to the charge relay system believed to be present at the active site of the serine proteases[15].

In this study we have examined the ^{1}H NMR spectra of ANGII, ANGIII,[Sar^1]-

ANGII, [Sar^1, Tyr(Me)^4]ANGII, [Sar^1, Tyr(Me)^4, Ile^8]ANGII, [Des^1, Tyr(Me)^4, Ile^8]ANGII and [Des^1, Ile^8]ANGII in DMSO at neutral and acidic pH and at magnetic fields as low as 25 ppm, in order to investigate the nature of the Tyr hydroxyl and His NH protons and relate their lability with the possible existence of a charge relay system. We have also examined the ^{1}H NMR spectra of ANGII analogues with the hydroxyl group methylated or eliminated in order to investigate the spatial relationship between Tyr and His side chains. Nuclear Overhauser Effect (NOE) enhancement experiments carried out on [Sar^1]ANGII and the model ANGII analogue, [Des^1]Sarmesin, support the interaction of the Tyr and His rings and confirm the *trans* configuration for the His–Pro peptide bond. Saturation of the His C-2 and C-4 protons resulted in enhancement of the Tyr *ortho* and *meta* protons indicating proximity of the two aromatic rings. NOE enhancements for the two C_δ Pro protons illustrate the presence of a *trans* His–Pro bond.

Interresidue rotating frame Nuclear Overhauser Effect spectroscopy (ROESY) interactions observed in [Sar^1]ANGII between Tyr *ortho* and Phe ring protons, between Phe ring and Pro C_γ protons and between His C_α and Pro C_δ protons indisputably support a clustering of the Tyr, His and Phe aromatic rings.

EXPERIMENTAL

ANGII, [Sar^1]ANGII, ANGIII, [Des^1,Ile^8]ANGII, [Arg^8]vasopressin (AVP) and oxytocin were purchased from Peninsula Laboratories and their purity was verified by HPLC. [Sar^1,Tyr(Me)^4]ANGII, [Sar^1,Tyr(Me)^4, Ile^8]ANGII, [Sar^1, Phe^4]ANGII and [Des^1,Tyr(Me)^4]ANGII, were synthesized by the solid phase technique using methods which have been described in detail previously[16,17]. Cleavage of the peptide from the Merrifield resin by HF and purification by reversed-phase HPLC, using TFA as a component of the solvent system[17], afforded the TFA salt of the peptide which was used for NMR studies in the acidic form. The neutral form was obtained by passing the HF product through a column of carboxymethyl cellulose (Whatman CM23) with a linear gradient of ammonium acetate, 0·01 mol l^{-1} and pH 5 to 0·5 mol l^{-1} and pH 8 at a flow rate of 45–50 ml/h[17]. The absorbance of the effluent was measured at 280 nm and fractions of the major product were lyophilized three times and the received peptide was used for NMR studies.

NMR experiments were carried out using a Bruker 400 MHz NMR spectrometer. The chemical shifts were reported relative to the undeuterated fraction of the methyl group of $(CD_3)_2SO$ at 2·50 ppm with respect to TMS. Data acquisition and data processing were controlled by an Aspect 3000 computer equipped with an array processor using 1987 DISNMR software. A total of 64 scans were accumulated to obtain a good signal-to-noise ratio. One-dimensional NOE enhancement measurements were carried out in the difference mode using multiple irradiation[18]. Each of the selected lines was irradiated 50 times for 100 ms (total irradiation time 5·0 s). Other irradiation times (0·2, 0·5, 1, and 3 s) were also employed in some experiments to monitor the NOE buildup. A total of 1 000 scans for each line was required, and total relaxation time was 2 s. Under the experimental conditions which we have used for the NOE experiments (low power, different preirradiation times τ, saturation of control areas) spin diffusion and partial saturation were minimized for the interactions under discussion. NOE enhancements were determined as the point increase in signal per proton after saturation of a functionally distinct proton.

Unsymmetrized ROESY spectra for [Sar^1] ANGII in DMSO, were recorded in the phase-sensitive mode. Cross-peaks for the Tyr *ortho*-Phe ring protons, together with cross-peaks for Pro C_γ–Phe ring protons and Pro C_δ–His C_α protons illustrate the proximity of Tyr with both His and Phe and suggest that the three aromatic rings in ANGII are in close proximity. Full details of the ROESY experiments and of all ROESY intra- and interresidue interactions for [Sar^1] ANGII in DMSO appear elsewhere[26].

RESULTS

Table I shows the chemical shifts for the labile aromatic ring protons of angiotensin analogues in $(CD_3)_2SO$ at 400 MHz in the neutral and protonated forms. These analogues have been synthesized by known methods[16,17]. The Tyr hydroxyl proton in ANGII and analogues, in their neutral form, could not be clearly detected in the ^{1}H NMR spectra. In contrast, the same proton in free tyrosine and AVP, was clearly observed at δ 9·2 ppm as a broad signal. The protonated form of [Sar^1]ANGII, and analogues, also gave a broad signal at δ 9·2 ppm attributable to the Tyr hydroxyl proton (D_2O exchangable). The protonated form of the molecule is obtained by lyophilization from strong acids such as trifluoroacetic acid (TFA) or hydrochloric acid (HCl) but not from acetic acid. It can be inferred that the imidazolium trifluoroacetate or hydrochloride salt is stable in DMSO, whereas the acetate salt of imidazole dissociates. Protonation of the imidazole ring disrupts the charge transfer system, resulting in a "random coil" conformation with all side chains freely rotating. Such random conformation is manifested in the NMR spectra of these analogues by distinct signals with measurable coupling constants (Fig. 1). A low field signal at δ 14·2 ppm, observed in the NMR spectra of [Des^1,Ile^8]ANGII, [Sar^1,Tyr(Me)^4, Ile^8]ANGII (Fig. 1) and [Des^1,Tyr(Me)^4, Ile^8]ANGII in their protonated forms, is due to the NH proton of the protonated His ring. This is accompanied by correspondingly low field shifts of the C-2 and C-4 His protons in these three analogues.

Table II shows the chemical shifts for the aromatic protons of reference peptides (e.g. [Sar^1,D-Pro^7]ANGII, random coil[13], [Arg^8]vasopressin, Tyr/Phe ring interaction[19,27]) and angiotensin analogues in which the Tyr hydroxyl is methylated or eliminated. The His C-2 and C-4 protons in ANGII, ANGIII and [Sar^1]ANGII are observed as sharp signals indicating very fast averaging (on the NMR time scale) for the His residue. Upon methylation of the Tyr hydroxyl, the His C-2 and C-3 proton signals in [Sar^1,Tyr(Me)^4]ANGII and [Des^1,Tyr(Me)^4]ANGIII are broadened. Broad His C-2 and C-4 proton signals in proteins and enzymes have been suggested to indicate different environments for the His residue[20,21]. A similar situation, i.e. slower averaging, may apply to His ring in the two methylated ANGII analogues, where the Tyr/His interaction cannot be present. These changes may be related to the loss of pressor activity in these two analogues.

Figure 2 shows the NOE difference spectrum for [Des^1]sarmesin after saturation of the His C_α proton at δ 4·64 ppm. An AB quartet at δ 3·85 ppm is attributed to

TABLE I

Chemical shifts (relative to TMS) for the aromatic protons of angiotensin analogues in $(CD_3)_2SO$ at 400 MHz, in the neutral and protonated forms

Peptide		His			Tyr[a]			Phe
		C-2	C-4	N[b]	*m*	*o*	OH[b]	
ANGII	(pH 1)	9·10	7·40	—	7·08	6·70	9·2	7·27 s
	(pH 7)	7·47	6·84	—	6·99	6·60	—	7·18—7·12 m
ANGIII	(pH 1)	8·00	7·21	—	7·04	6·64	9·2	7·27 s
	(pH 7)	7·47	6·87	—	6·99	6·60	—	7·18—7·12 m
[Sar[1]]ANGII	(pH 1)	8·50	7·25	—	7·02	6·62	9·2	7·27 s
	(pH 7)	7·48	6·87	—	6·98	6·59	—	7·18—7·11 m
[Sar[1],Tyr(Me)[4]]ANGII	(pH 1)	8·40	7·22	—	7·17	6·79	—	7·27 s
	(pH 7)	7·50	6·90	—	7·15	6·70	—	7·12—7·06 m
[Sar[1],Tyr(Me)[4],Ile[8]]ANGII	(pH 1)	9·00	7·38	14·2	7·16	6·78	—	—
	(pH 7)	7·60	6·88	—	7·10	6·70	—	—
[Tyr(Me)[3],Ile[7]]ANGII	(pH 1)	9·00	7·38	14·2	7·16	6·78	—	—
	(pH 7)	7·50	6·88	—	7·10	6·70	—	—
[Ile[7]]ANGIII	(pH 1)	8·70	7·28	14·2	7·02	6·62	9·2	—
	(pH 7)	7·54	6·92	—	7·00	6·60	—	—
AVP([Arg[8]]vasopressin)	(pH 7)	—	—	—	6·91	6·62	9·2	7·32—7·22 m
Tyrosine	(pH 7)	—	—	—	7·07	6·70	9·2	

[a] *Meta* and *ortho* refer to the Tyr hydroxyl group; [b] labile protons of the His and Tyr rings.

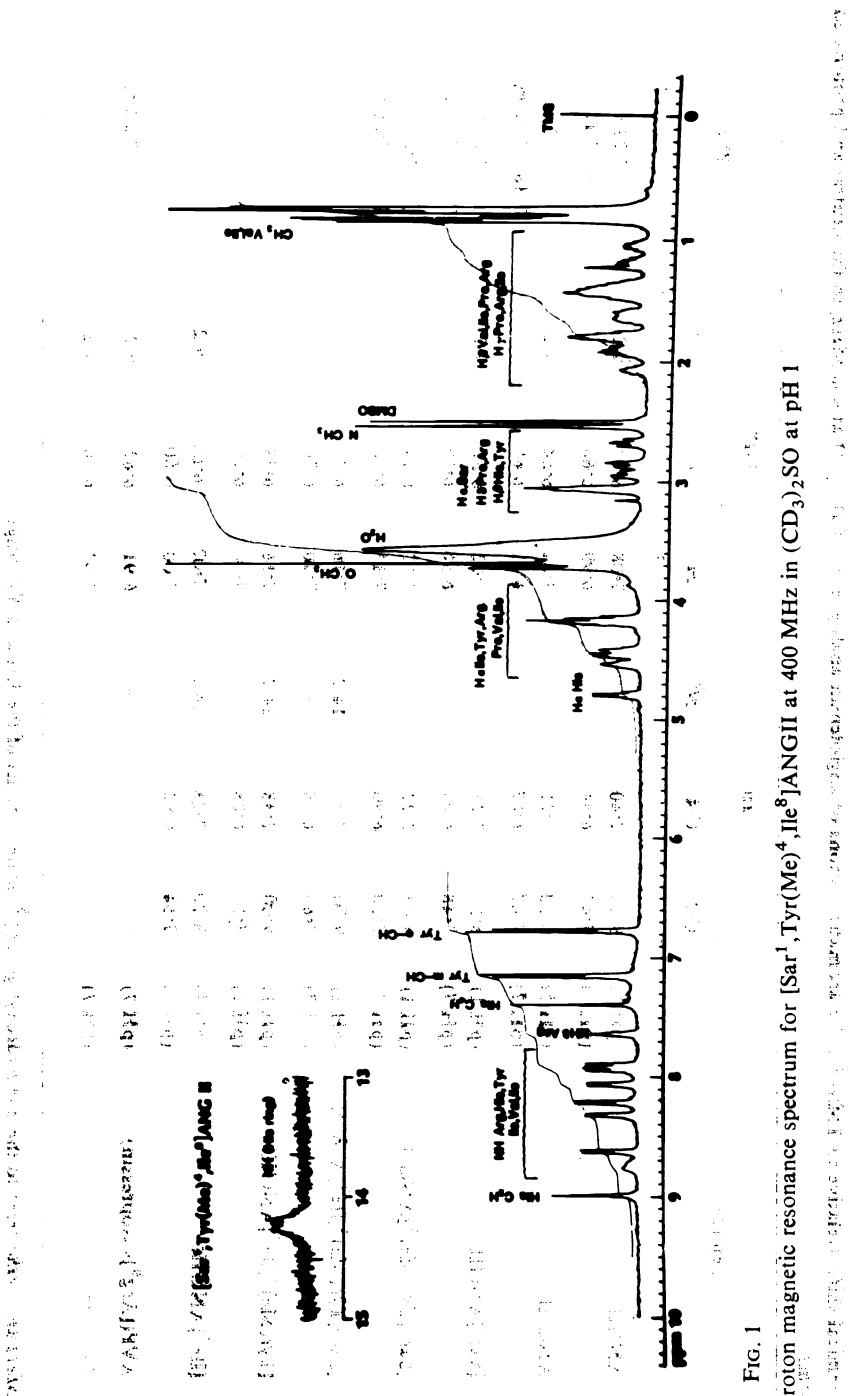

FIG. 1
Proton magnetic resonance spectrum for [Sar^1,Tyr(Me)^4,Ile^8]ANGII at 400 MHz in $(CD_3)_2SO$ at pH 1

the two vicinal His C_β protons. Two strong resonances at δ 3·18 ppm and 3·50 ppm are due to enhancement of the two Pro C_δ protons. Both Pro C_δ protons are almost equally affected, revealing close proximity and equidistance of both of these protons from the His C_α proton. The His C_α/Pro C_δ interaction indicates a *trans* configuration for the His–Pro peptide bond. Enhancement of the Tyr *meta* (δ 7·10 ppm) and *ortho* (δ 6·72 ppm), illustrate also proximity of the His residue with the Tyr ring.

DISCUSSION

One important feature in assessing the conformation which is assumed by ANGII and analogues in solution is the positioning of the aromatic rings in relation to each other, and the configuration of the His[6]–Pro[7] peptide bond[7,11,22]. NMR and chemical reactivity studies in dimethyl sulfoxide and water have suggested proximity of the histidine and phenylalanine rings[23], and a charge relay system mechanism involving the Tyr hydroxyl, His ring and the Phe-carboxylate[12,13]. In this respect, the absence of the Tyr OH and His ring NH protons in the ^{1}H NMR spectra of ANGII and analogues studied in their neutral form (Tables I and II) suggests a fast exchange between different environments which is in accord with the proposed interaction

TABLE II

Chemical shifts (relative to TMS) for the aromatic protons of angiotensin analogues and reference peptides in $(CD_3)_2SO$ at 400 MHz

Peptide	His		Tyr[a]		Phe
	C-2	C-4	*meta*	*ortho*	
ANGII	7·47	6·86	6·99	6·60	7·18–7·12
ANGIII	7·47	6·85	6·99	6·60	7·18–7·12
[Sar[1]]ANGII	7·48	6·87	6·98	6·59	7·18–7·11
[Sar[1],Tyr(Me)[4]]ANGII	7·50	6·90	7·15[b]	6·70[b]	7·12–7·06
[Des[1],Tyr(Me)[4]]ANGII	7·50	6·90	7·15[b]	6·70[b]	7·12–7·06
[Sar[1],Phe[4]]ANGII	7·52	6·92	—	—	7·21–7·13
[Sar[1],D-Pro[7]]ANGII	7·60	6·84	7·06	6·64	7·24
Amino acid[c]	7·68[d]	7·04[d]	7·07	6·70	
			7·20[b]	6·86[b]	7·30
CF_3CO-Gly-Gly-X-Ala-OCH_3[e]	8·35	7·35	7·02	6·64	7·26
Oxytocin[f]	—	—	7·12	6·68	—
[Arg[8]]vasopressin[f]	—	—	6·91	6·62	7·32–7·22

[a] *Meta* and *ortho* refer to the Tyr hydroxyl group; [b] Tyr(Me); [c] at 200 MHz; [d] for imidazole, His is insoluble in DMSO; [e] linear reference peptides[25] in which X = His (protonated), Tyr or Phe; [f] after D_2O exchange.

between Tyr(OH)-His-Phe(COO^-). Interestingly, the ^1H NMR spectrum of [Arg^8]-vasopressin, taken in its neutral form, clearly shows the Tyr hydroxyl proton at δ 9·2 ppm. The absence of intramolecular proton transfer in vasopressin should result in the Tyr hydroxyl proton being less labile (and therefore NMR visible).

An interaction of the phenoxyl proton with the His side-chain should manifest

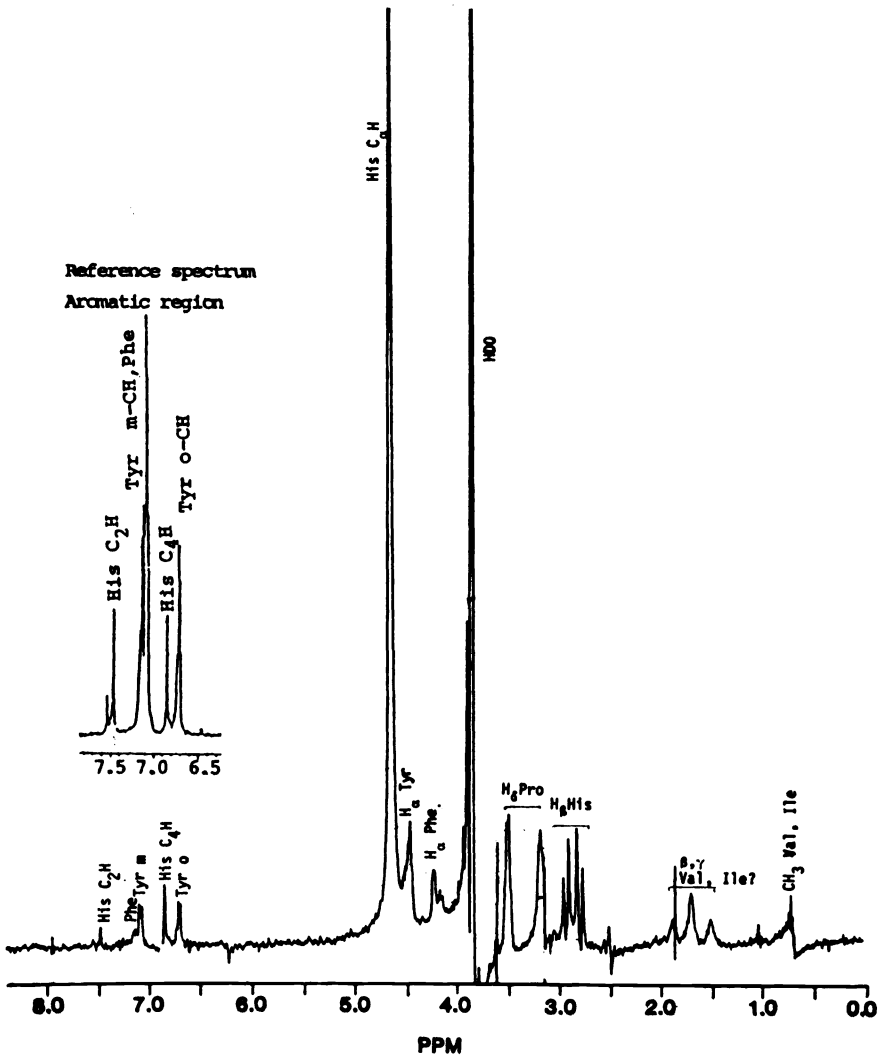

FIG. 2

NOE difference spectrum for [Des^1,$Tyr(Me)^4$]ANGII (acetate form), in $(CD_3)_2SO + D_2O$ (2 drops) obtained upon saturation of the C_α proton line for His

itself in the NMR spectrum of ANGII, although this effect would probably only be detectable if the chemical shifts for the His ring protons of ANGII were compared directly with the same protons in analogues lacking the phenoxyl proton. For this reason we have examined the ^{1}H NMR spectra for the superagonist [Sar^1]ANGII, the natural agonist ANGII, the heptapeptide ANGIII, and compared them with the NMR spectra for the antagonists [Sar^1,Tyr(Me)^4]ANGII, and [Sar^1,Phe^4]-ANGII as well as the inactive analogue [Des^1,Tyr(Me)^4]ANGII (which lack the phenoxyl proton[24]). The chemical shifts (Table II) demonstrate a small shielding influence on the His C-2 and C-4 protons in ANGII, ANGIII and [Sar^1]ANGII which is absent in [Sar^1,Tyr(Me)^4]ANGII, [Des^1,Tyr(Me)^4]ANGII and [Sar^1, Phe^4]ANGII. Thus, the His C-2 and C-4 proton shifts are deshielded for [Sar^1, Tyr(Me)^4]ANGII (δ 7·50 and 6·90 ppm), [Des^1,Tyr(Me)^4]ANGII (δ 7·50 and 6·90 ppm) and [Sar^1,Phe^4]ANGII (δ 7·52 and 6·92 ppm) when compared with the same shifts for ANGII (δ 7·47 and 6·86 ppm) and [Sar^1]ANGII (δ 7·48 and 6·87 ppm). Apparently a shielding influence of the His ring by the phenoxyl group in ANGII is dependent on the presence of the single ionizable phenoxyl proton. Previously[23] we have suggested that a shielding influence on the His ring of ANGII might originate from an interaction with the Phe ring. However it appears that part of this shielding influence on the His ring originates from the phenolic hydroxyl group. This is consistent with the original proposal[12] based largely on chemical reactivity studies, that the Tyr hydroxyl in ANGII is involved in an intramolecular interaction, probably by hydrogen bonding to the His ring.

Furthermore, we thought it of interest to compare the chemical shifts of the aromatic protons of ANGII with those of vasopressin and oxytocin, two hormones whose conformations have been studied in greater depth. Previous studies have indicated that the aromatic rings of the neighbouring Tyr and Phe residues in vasopressin are involved in a stacking interaction in D_2O (ref.[19]). However, the Phe side-chain of vasopressin in DMSO (Table II) shows evidence of restricted rotation without notable shielding, whereas the chemical shifts for the Tyr residue illustrate that the *meta* protons, but not the *ortho* protons, are subjected to a strong shielding influence (compare chemical shifts for Tyr in vasopressin with free amino acid, reference peptide and ANGII). Possibly there is only partial overlap of the Tyr and Phe rings in vasopressin in DMSO, although this does not provide an adequate explanation for the absence of shielding for any of the Phe protons. These data are in fact in accord with a perpendicular plate interaction[23,27] for the two rings in vasopressin. We have suggested previously that shielding and restricted rotation for the Phe side-chain in ANGII might be due to an interaction with the His side-chain[23]. It is possible that the strong shielding influence on the Phe ring in ANGII is due to the presence of a partically delocalized negative charge on the His ring[14], as predicted by the charge relay system conformation for ANGII (ref.[13]), or due to shielding by the proximal C-terminal carboxylate.

In order to investigate the proposed occurrence of an ion dipole bond[13] between the Phe carboxylate and the His ring, we examined the ^{1}H NMR spectrum of the analogue [Sar^1,D-Pro^7]ANGII which, according to molecular modelling experiments, would be unable to form this bond (and thereby the charge relay system) because of steric constraints. With the exception of the His C-4 proton, the aromatic protons of [Sar^1,D-Pro^7]ANGII show no evidence of shielding influences when the chemical shifts are compared to those of a simple reference peptide and the free amino acids (Table II). In addition, the Phe side-chain of [Sar^1,D-Pro^7]ANGII, unlike ANGII and [Sar^1]ANGII, shows up as a singlet demonstrating free rotation. The chemical shifts of the aromatic protons of this biologically inactive analogue (Table II) demonstrate that the ordered compact tertiary structure of the ANGII molecule becomes completely relaxed when the Pro^7 residue is switched to the D configuration. These findings suggest that a proline residue in the L configuration is mandatory for maintaining a restricted biologically active conformation for ANGII. This is in accord with chemical reactivity studies[12] which have suggested that the nucleophilic groups in [Sar^1,D-Pro^7]ANGII, unlike ANGII, freely interact with the solvent.

The close proximity of the Tyr and His rings was also demonstrated by Nuclear Over-hauser Effect (NOE) enhancement experiments in [Sar^1]ANGII and the model compound [Des^1]sarmesin ([Des^1,Tyr(Me)^4]ANGII). [Des^1]sarmesin was selected because its sharp Tyr(OCH_3) singlet at δ 3·60 ppm, provides a distinct probe for studying the spatial relationship between the Tyr and His rings. Saturation of the Tyr(OMe) resonance resulted in weak enhancement of the His C-2 and C-4 proton resonances at δ 7·47 ppm and δ 6·85 ppm indicating proximity of the Tyr and His rings. Similarly, saturation of the His C_α proton resulted in enhancement of the Tyr *meta* and *ortho* protons (Fig. 2). Enhancement of the two Pro C_δ protons confirms the *trans* configuration of the His–Pro peptide bond.

Recent 2D NMR studies of ANGII and [Sar^1]ANGII in DMSO by Nuclear Overhauser Effect spectroscopy in the Rotating Frame have confirmed a clustering of the three aromatic rings (Tyr, His, Phe) and the *trans* configuration of the His–Pro bond[26].

In conclusion, we suggest that the present findings are consistent with the proposed charge relay system mechanism and ring clustering for ANGII. The data in Table I

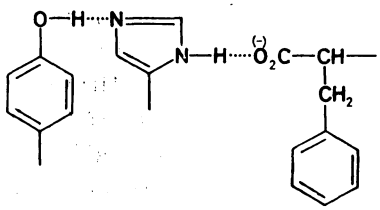

FIG. 3
Charge relay system in ANGII envolving the triad Tyr,His,Phe

indicate that the Tyr hydroxyl and the His imidazole NH protons are labile in ANGII, in agreement with the presence of a charge relay system (Fig. 3). The data in Table II indicate that a shielding influence on the His ring orginates not only from an interaction involving the Phe ring[23], but is also dependent on an interaction of the Tyr phenoxyl proton with the this ring.

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