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Fluorescence properties of angiotensin II analogues in receptor-simulating environments: relationship between tyrosinate fluorescence lifetime and biological activity

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Nanosecond time-resolved decays from excited-state tyrosinate fluorescence of angiotensin II analogues were measured from the emission at 350 nm. Fluorescence lifetimes were determined in several different solvents using *N*-acetyltyrosinamide as the reference standard. Long-lifetime tyrosinate fluorescence (LTF) of angiotensin II (ANG II) was observed in propylene glycol, trifluoroethanol and isopropanol but not in DMSO or water. The addition of SDS at a concentration sufficient to induce micelle formation in water resulted in LTF for ANG II. LTF for ANG II was longer in propylene glycol (21 ns) than in isopropanol (16 ns) whereas the % conformer(s) producing LTF was higher in isopropanol (79%) than in propylene glycol (19%). For a series of ten angiotensin analogues, LTF values determined in propylene glycol and isopropanol were a reflection of the contractile activities of these analogues in the rat uterus assay. In propylene glycol, with the notable exception of ANG III, biologically active analogues had longer LTFs (13–21 ns) than inactive analogues (0–11 ns). In isopropanol, strong agonists had longer LTFs (13–16 ns) than weak agonists/inactive analogues (0–11 ns). Structure-fluorescence relationships suggest that the primary TyrOH acceptor in ANG II is the His^6 imidazole group, and that the C-terminal carboxylate has an essential auxiliary role in generating long-lived tyrosinate fluorescence. The present findings appear to support the proposition that the receptor conformation of ANG II contains a tripartite interaction of Tyr, His and carboxylate groups which is analogous to that found at the active site of serine proteinases, and that the tyrosinate nucleophile may activate angiotensin receptors. Solvents of intermediate polarity such as propylene glycol and isopropanol appear to induce conformations for small peptides such as angiotensin which resemble those present at membrane receptors.

Introduction

The octapeptide angiotensin II has an important role in the maintenance of blood pressure and in the pathophysiology of hypertension [1]. Knowledge of its conformation is required for the rational design of angiotensin receptor antagonists for therapeutic application. During the last three decades a great deal of effort has been expended on determining the conformation of angiotensin II in solution, and this has resulted in numerous conformational models (for reviews, see Refs. 2 and 3). The tyrosine hydroxyl group of ANG II has been shown to have an important role in receptor activation, since its removal [4] or methylation [5] produces antagonists. Moreover, chemical reactivity studies on ANG II in aqueous solution have suggested that the tyrosine hydroxyl group has unusual nucleophilic properties, particularly in relation to pH dependence, and may participate in an intramolecular hydrogen bond [6]. Structure-activity studies have shown that the Tyr hydroxyl, the His imidazole and the C-terminal carboxylate are required for biological activity [2], and it has been suggested that ANG II may contain a charge relay system analogous to that found at the active site of serine proteinases [6,7].

In the present study, a more direct approach to investigating intramolecular hydrogen bonding of the tyrosine hydroxyl group in ANG II was applied, by

Abbreviations: PrOH, propane-2-ol; NAYA, *N*-acetyltyrosinamide; $Pr(OH)_2$, propane-1,2-diol; SDS, sodium dodecylsulphate; TFE, 2,2,2-trifluoroethanol; DMSO, dimethylsulphoxide; ANG II, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe. Abbreviations for amino acids and analogues are as recommended by the IUB. LTF, long-lifetime tyrosinate fluorescence (τ_3).

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taking advantage of the characteristic fluorescent properties of the excited-state tyrosinate species [8]. Thus, the fluorescent properties of ANG II and a number of analogues were investigated in several solvents of differing dielectric constants. The use of solvents with decreased polarity was prompted by recent evidence from site-specific receptor mutation studies which have suggested that small ligands such as ANG II bind to a site in one of the transmembrane domains of the receptor protein [9], and may therefore have biologically active conformations which are determined by the bulk dielectric constant of the membrane environment or are 'lipid-induced' [10].

Experimental procedures

Materials

ANG II, ANG III and [Sar^{1}Ile^8]ANG II were obtained from Sigma Chem. Co. or Peninsula Labs. and were found to contain a single peptide by reversed-phase HPLC. All other analogues of angiotensin were synthesized, purified, analyzed and bioassayed as described previously [11]. Purification by reversed-phase HPLC gave peptides of > 99% purity for which the absorbance at 230 nm and 254 nm was characteristic of tyrosine-containing peptides devoid of tryptophan-like impurities. 1,2-Propanediol ($Pr(OH)_2$) was dried by refluxing over calcium oxide for 8 h, collected by distillation and stored over a molecular sieve. Water content was estimated by ^{1}H-NMR or by the Karl Fisher method. Isopropanol (PrOH) was of HPLC grade (Caledon Laboratories Ltd.) and expected to contain less than 1% H_2O. Dimethylsulphoxide (DMSO) and trifluoroethanol (TFE) were used without further treatment. Aqueous solvents were prepared from distilled water which had been passed through Fisher ion-exchange cartridges. *N*-Acetyltyrosinamide (NAYA) was obtained from Sigma.

Methods

Fluorescence experiments were performed at 21°C and samples used for fluorescence were typically between 0.25 and 1.0 mg/ml. All samples were warmed in a 50°C water bath to aid in dissolving the peptide, and any insoluble material was removed by filtration. Cuvettes were cleaned between samples with sulphochromic acid and were soaked in the solvent of the experiment. Solvents involving sodium dodecylsulphate (obtained from BDH Biochemicals, specially pure) were performed three ways. SDS(mix); 0.5 ml of 10% SDS was gently mixed with 2 mg/ml of ANG II in 10 mM Tris (pH 7.5). SDS(add); SDS was added in solid form to buffered ANG II solution in increments of 10 mM up to 100 mM. SDS(mix + NaCl); the procedure was the same as SDS (mix) except that 0.1 M NaCl was present and the mixture was vortexed for 5 min.

Fluorescence measurements were conducted as a single blind study in which the fluorescence spectroscopist had no prior knowledge of the biological activities of the peptides. Nanosecond time-resolved fluorescence decays were measured at 21°C using Photochemical Research Associates (PRA) fluorescence lifetime instrumentation (System 3000). This instrument utilizes the time correlated single photon counting technique [12]. A PRA 510 flash lamp was utilized as the light source and was operated at 18.6 kHz, with 5.8 kV applied across a 4 mm electrode gap under −44 kPa of H_2. The excitation and emission wavelengths were selected using Jobin Yvon monochromators with slits giving an 8 nm bandpass. The lamp decay profile was obtained by measuring the scattering of light by a suspension of 2.02 μm polyvinyltoluene latex spheres in glycerol/water (1 : 1, v/v) with the excitation and emission monochrometers set at the emission wavelength of the sample. In all experiments data were collected until $2.5 \cdot 10^5$ photon counts were obtained. Background counts were obtained for each solvent and were subtracted from the sample data; the background obtained during the time of the sample collection was less than 7% of the counts at the tail end of the sample decay. The observed decay data were deconvoluted beginning from five channels before the channel maximum to the channel which contained 0.05% of the photon counts present in the channel of maximum counts. The deconvolution method used was that of iterative non-linear least squares [13] which has been demonstrated to be the best method for this type of data [14]. Acceptance of a least-squares fit at 95% confidence was evaluated by the reduced chi-squared test, and the quality of fit was evaluated from the residuals, the autocorrelation function of the residuals, and the Durbin-Watson parameter [15].

The experimentally obtained fluorescence decay, $f(\lambda, t)$, is described as a sum of exponentials:

$$f(\lambda, t) = \Sigma\alpha_i(\lambda) \exp[-t/\tau_i(\lambda)] \quad (1)$$

where $\alpha_i(\lambda)$ and $\tau_i(\lambda)$ are the preexponential weighting factor and fluorescence lifetime of the ith component for a given emission wavelength, respectively. The fraction of the fluorescence intensity that arises from each component is related by;

$$I\%(\lambda) = \frac{\alpha_i(\lambda)\tau_i(\lambda)}{\sum\alpha_i(\lambda)\tau_i(\lambda)} \times 100 \quad (2)$$

Results

Fig. 1 shows the normalized fluorescence decay curves (data fitted to Eqn. 1) for ANG II in a number of different solvents. Observation of the emission at 305 nm due to tyrosine [16] showed typical tyrosine fluores-

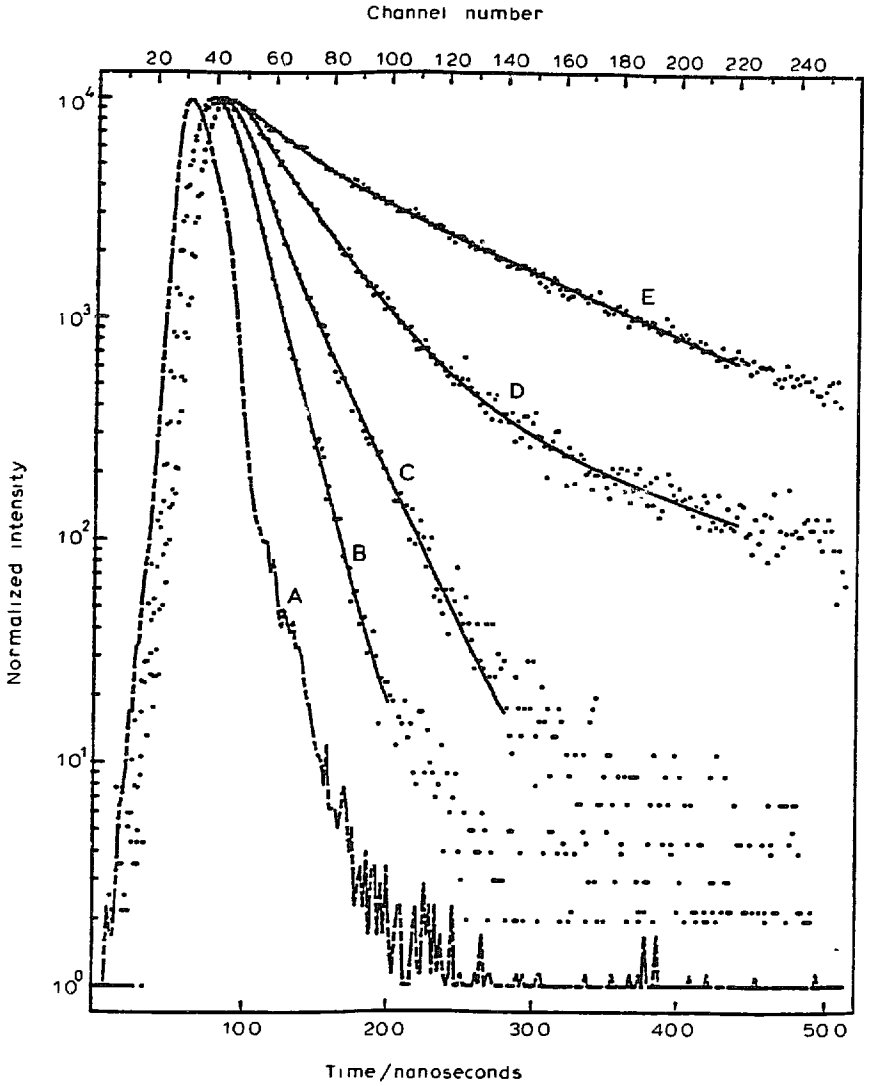

Fig. 1. Normalized fluorescence decay durves for angiotensin II in different solvents. Curve A: Lamp profile, B: dimethyl sulphoxide, C: 15 mM Tris-HCl buffer (pH 7.5), D: 1,2-propanediol, E: isopropanol. All samples are 0.75 mg/ml. Each decay curve was collected with $2 \cdot 10^5$ photon counts at 0.2 ns/channel.

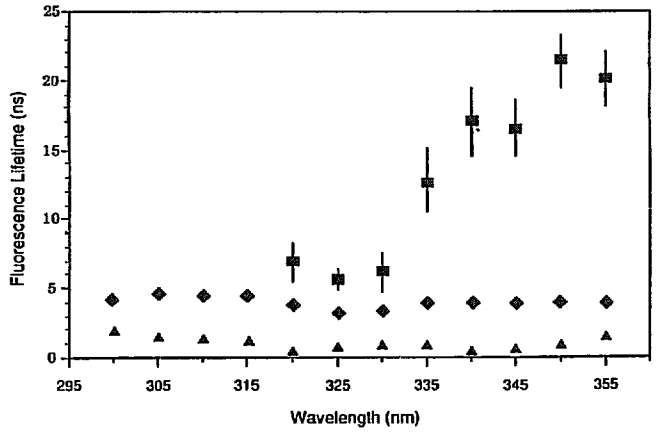

Fig. 2. Fluorescence lifetimes for angiotensin II in 1,2-propanediol. Symbols represent the first (▲), second (◆), and third (■) lifetime components of the exponential decay (Eqn. 1). Standard deviations fall within the contours of the plotting symbol unless otherwise indicated.

cence for all the samples studied. Fig. 2 shows that the long-lifetime fluorescence component in Fig. 1 emitted only at wavelengths greater than 330 nm, and is assignable to excited-state tyrosinate fluorescence centered around 345 nm [8,17]. Changing the concentration of the sample did not affect the parameters obtained, therefore dimerization or multiple aggregates can be ruled out as possible structures responsible for the LTF component (τ_3). The triexponential fits given in Tables II–IV were in all cases better than biexponential fits of the same data. Increasing the number of counts to 10^6 did not change the number of exponentials in the fit to the fluorescence decay, nor did it change the values of the fit, although it did improve the standard deviations for the parameters measured. The number of photon counts collected was kept constant for all analogues examined and provided a method for measuring the ability of a given peptide to produce the long-lifetime

TABLE I

Nanosecond time-resolved fluorescence data from N-acetyltyrosinamide

Excitation and emission wavelengths at 275 and 350 nm, respectively.

	α_1 [a]	α_2	τ_1 [a]	τ_2	I_1 [b]	I_2	χ^2
Buffer pH = 7.0	0.54±0.01	0.11±0.01	1.19±0.04	3.01±0.10	65.5	34.5	0.908
DMSO	0.17±0.12	0.49±0.15	0.95±0.60	1.83±0.12	15.5	84.5	1.135
Propanediol	0.04±0.06	0.23±0.003	0.59±0.82	4.10±0.03	2.5	97.5	0.900
Isopropanol	0.28±0.19	0.08±0.19	3.70±0.55	5.20±0.86	71.5	28.5	0.905
Trifluoroethanol	0.65±0.01	0.05±0.02	1.27±0.03	2.94±0.33	85.6	14.4	1.150
80% Glycerol	0.12±0.02	0.26±0.01	0.94±0.22	3.36±0.04	10.9	89.1	1.123
5% SDS	0.46±0.02	0.41±0.03	0.92±0.07	1.98±0.04	34.5	65.5	0.822

[a] Preexponentials and fluorescence lifetimes as in Eqn. 1.
[b] Percentage of the intensity of each decay component (Eqn. 2).

TABLE II

Nanosecond time-resolved fluorescence data from angiotensin II

Parameters as in Table I. 1,2-Propanediol ($Pr(OH)_2$); isopropanol (PrOH). $\epsilon^{25°C}$, dielectric constant. Buffer; 10 mM Tris (pH = 7.5). SDS solutions were made up in this buffer. SDS(mix); 0.5 ml of 10% SDS in H_2O was mixed with 2 mg/ml Ang II solution by slowly inverting the test tube. SDS(add); SDS was added in solid form at 10 mM steps to buffered Ang II solution up to 100 mM. SDS(mix + NaCl); same as SDS(mix) but added salt to 0.1 M then vortexed 5 min.

	$\epsilon^{25°C}$	α_1	α_2	α_3	τ_1	τ_2	τ_3	I_1	I_2	I_3	χ^2
Buffer	79	0.45±0.01	0.16±0.01		1.01±0.05	3.18±0.06		46.5	53.5		1.328
DMSO	45	0.66±0.03	0.10±0.04		1.09±0.06	2.30±0.22		75.5	24.4		1.334
Propanediol	32	0.09±0.02	0.19±0.01	0.01±0.001	0.77±0.24	4.05±0.12	20.77±2.25	6.8	75.5	18.7	0.966
Isopropanol	18		0.06±0.001	0.06±0.002		3.88±0.18	15.49±0.25		21.0	79.0	1.201
Trifluoroethanol	30	0.17±0.01	0.19±0.01	0.01±0.001	0.98±0.10	3.38±0.11	13.03±0.54	16.7	64.7	18.6	0.999
$Pr(OH)_2$: H_2O (9:1)	37	0.25±0.04	0.18±0.004	0.01±0.002	0.57±0.10	3.94±0.09	12.97±1.11	14.2	72.9	12.9	1.059
PrOH : H_2O (9:1)	25	0.10±0.01	0.24±0.11	0.01±0.001	1.05±0.20	3.39±0.09	11.82±1.30	10.7	83.0	6.3	1.102
PrOH : DMSO (9:1)	20	0.52±0.03	0.21±0.01	0.02±0.002	0.59±0.05	3.40±0.09	12.79±0.62	24.8	57.9	17.3	1.211
Glycerol : H_2O (4:1)	54	0.35±0.01	0.25±0.02		1.83±0.08	4.12±0.07		38.0	62.0		1.333
SDS(add)	–	0.25±0.01	0.92±0.11	0.01±0.001	0.92±0.11	3.38±0.08	13.73±1.32	17.1	76.3	13.7	1.344
SDS(mix)	–	0.25±0.02	0.34±0.01	0.02±0.006	0.70±0.11	3.08±0.09	7.15±0.57	13.0	76.8	10.2	0.897
SDS(mix + NaCl)	–	0.30±0.03	0.28±0.01	0,01±0.003	0.73±0.10	3.25±0.08	8.93±0.70	17.9	74.6	7.5	0.919

TABLE III

Nanosecond time-resolved fluorescence data from angiotensin II analogues in 1,2-propanediol

Parameters as in Table I. Analogues are residue changes to angiotensin II (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8).

	α_1	α_2	α_3	τ_1	τ_2	τ_3	I_1	I_2	I_3	χ^2
ANG II	0.09±0.02	0.19±0.01	0.01 ±0.001	0.77±0.24	4.05±0.12	20.77±2.25	6.8	75.5	18.7	0.966
[Sar^1,Ile^8]ANG II	0.13±0.02	0.10±0.02		3.22+0.24	5.45+0.25		44.2	55.8		1.030
[Sar^1,$PheNH_2^8$]ANG II	0.07±0.01	0.22±0.01	0.007±0.008	1.30+0.30	4.30±0.20	9.16±2.06	8.0	85.8	6.2	0.981
ANG III	0.06±0.01	0.26±0.01	0.016±0.017	0.78±0.37	4.05±0.19	7.41±1.60	3.8	86.3	9.8	1.184
[Sar^1,His(3-Me)^6]ANG II		0.06±0.001	0.015±0.001		3.82±0.11	13.06±0.47		54.2	45.8	0.971
[Sar^1,His(1-Me)^6]ANG II	0.15±0.02	0.16±0.004	0.016±0.006	0.55±0.12	4.73±0.17	10.62±1.19	8.3	74.5	17.1	0.988
[Sar^1,D-Pro^7]ANG II	0.10±0.01	0.19±0.003	0.008±0.007	0.88±0.16	4.33±0.18	10.24±2.13	9.1	82.9	8.0	0.961
[Sar^1,Cha^8]ANG II [a]	0.05±0.01	0.18±0.01	0.009±0.002	1.20±0.35	4.56±0.15	14.95±1.74	5.4	81.2	13.3	1.092
[Des^1,Cha^8]ANG II [a]	0.06±0.01	0.17±0.01	0.006±0.002	1.55±0.31	4.77±0.18	16.24±2.55	8.9	81.3	9.8	1.072
[Sar^1,Ala^6]ANG II	0.14±0.15	0.16±0.02	0.053±0.020	0.33±0.35	3.70±0.27	6.65±0.52	4.8	59.8	35.4	1.186
[Sar^1,Phe^6]ANG II	0.08±0.00	0.17±0.01	0.006±0.001	1.19±0.17	4.66±0.13	18.80±2.85	9.2	79.5	11.3	0.936

[a] Cha, L-cyclohexylalanine; Des, amino acid omitted.

TABLE IV

Nanosecond time-resolved fluorescence data from angiotensin II analogues in isopropanol

Parameters as in Table I. Analogues are residue changes to angiotensin II (Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]).

	α_1	α_2	α_3	τ_1	τ_2	τ_3	I_1	I_2	I_3	χ^2
ANG II		0.06±0.001	0.06±0.002		3.88±0.18	15.49±0 25		21.0	79.0	1.201
[Sar[1],Ile[8]]ANG II	0.23±0.01	0.19±0.01	0.03±0.01	0.75±0.10	3.21±0.23	6.49±0.69	17.4	62.7	19.9	0.967
[Sar[1],PheNH$_2^8$]ANG II	0.07±0.01	0.24±0.01	0.00±0.001	0.94±0.23	3.70+0.08	11.63±2.43	6.9	89.7	3.4	1.031
ANG III	0.68±0.05	0.23±0.004	0.02±0.004	0.45±0.04	3.60±0.11	10.18±0.74	23.2	62.8	14.0	1.279
[Sar[1],His(3-Me)[6]]ANG II	0.09±0.04	0.19±0.04	0.01±0.003	1.86±0.51	3.86±0.32	13.14±1.97	16.1	74.5	9.4	1.006
[Sar[1],His(1-Me)[6]]ANG II	0.29±0.15	0.21±0.003	0.02±0.003	0.31±0.15	3.50±0.08	9.43±0.65	9.2	74.7	16.1	0.971
[Sar[1],D-Pro[7]]ANG II		0.24±0.003	0.01±0.004		3.67±0.05	8.50±0.72		88.1	11.9	1.176
[Sar[1],Cha[8]]ANG II	0.12±0.00	0.21±0.005		1.32±0.10	3.92±0.03		16 6	83.4		1.191
[Des[1],Cha[8]]ANG II	0.11±0.02	0.18±0.02		2.38±0.22	4.18±0.09		26.6	73.4		1.001
[Sar[1],Ala[6]]ANG II	0.16±0.01	0.74±0.01		2.02±0.09	4.65±0.01		31.9	68.1		1.296
[Sar[1],Phe[6]]ANG II	0.09±0.01	0.21±0.004	0.01±0.007	0.82±0.18	3.80±0.15	9.27±1.55	7.5	81.2	11.38	0.984

component, i.e. adopt a stable conformation showing tyrosinate fluorescence. Two parameters were used to evaluate results: (1) the fluorescence lifetimes (LTF), and (2) the percentage of the intensity arising from a given decay component (% LTF).

Table I shows that there was no LTF component (τ_3) due to tyrosinate fluorescence from NAYA in any of the solvents investigated, whereas fluorescence components due to tyrosine (τ_1 and τ_2) were present as expected [8]. For ANG II, LTF was absent in water and DMSO, but was present in all solvents of lower dielectric constant (Table II). The longest LTF for ANG II (20.8 ns) was observed in propylene glycol, although the % LTF conformer was highest (79%) in isopropanol. In trifluoroethanol, which has a similar dielectric constant to propylene glycol, the LTF for ANG II was shorter than in propylene glycol but had the same % LTF as in propylene glycol. In 90% aqueous propylene glycol or isopropanol, the LTF and % LTF for ANG II were markedly lower than in the dry solvents, and in 80% aqueous glycerol LTF was absent. The addition of 10% DMSO to isopropanol also reduced LTF and% LTF (Table II). ANG II produced LTF in water in the presence of SDS; both LTF and % LTF were greater when the SDS micelles were formed in the presence of ANG II than when preformed SDS micelles were added to a solution containing ANG II (Table II).

An investigation of the structural dependence of ANG II LTF was carried out with a number of ANG II analogues in propylene glycol (Table III) and isopropanol (Table IV). Data on tyrosinate fluorescence (LTF and % LTF) in these two solvents, together with the biological activities of the analogues investigated, are shown in Table V. With the exception of the hepta-

TABLE V

Fluorescence properties and biological activities of angiotensin analogues

Peptide	Propane-1,2-diol		Isopropanol		Agonist activity [a] (%)
	LTF (τ_3) (ns)	%LTF (I_3)	LTF (τ_3) (ns)	%LTF (I_3)	
ANG II	20.8	19	15.5	79	100
[Sar[1],His(3-Me)[6]]ANG II	13.1	46	13.1	10	27
[Sar[1],Phe[6]]ANG II	18.8	11	9.3	11	7
[Sar[1],Cha[8]]ANG II	14.9	13	0	–	4 [c]
[Des[1],Cha[8]]ANG II	16.2	10	0	–	5 [c]
[Sar[1],PheNH$_2^8$]ANG II	9.2	6	11.6	3	0.2
[Sar[1],Ala[6]]ANG II	6.6	35	0	–	< 0.1
[Sar[1],His(1-Me)[6]]ANG II	0.6	17	9.4	16	< 0.1
[Sar[1],D-Pro[7]]ANG II	0.2	8	8.5	12	< 0.1
[Sar[1],Ile[8]]ANG II	0	–	6.5	20	< 0.1 [d]
ANG III	7.4	10	0.2	14	10

[a] Rat isolated uterus bioassay.

[b] Cha, L-cyclohexylalanine; Des, amino acid omitted.

[c] Potent receptor antagonist with residual agonist activity.

[d] Potent receptor antagonist.

peptide ANG III, LTFs were higher in propylene glycol for biologically active analogues (13–21 ns) than for inactive analogues (0–11 ns). In isopropanol long lifetime fluorescence was present for the two strong agonists, ANG II (16 ns) and [Sar^1,His(3-Me)^6]ANG II (13 ns), and the remaining nine analogues had shorter LTFs (0–11 ns).

Discussion

In order for fluorescence emission from tyrosinate to occur there must be proton transfer from the phenolic hydroxyl to an appropriate acceptor group. Based on the pK_a values of tyrosine in the ground state [10.4] and excited-state (≤ 5.4) [17,18], protolysis in the excited-state would be more efficient at neutral pH [19,20]. Since excited-state proton transfer is facilitated by the preexistence of ground state H-bonding, tyrosinate fluorescence lifetimes provide information on the stability of ground state H-bonding interactions. Excited-state proton transfer to buffer ions (e.g. acetate, phosphate, imidazole) can occur when the buffer is in high enough concentration [21,22]. However, at the low buffer concentrations used in biochemical experiments, a more likely acceptor in a peptide or protein would be a conformationally predisposed amino acid side-chain or peptide terminus. Candidate amino acid residues involved as acceptors might include aspartate and glutamate as well as the C-terminal carboxyl group; it has also been pointed out that hystidyl and lysyl side chains are also possibilities [23].

The existence of a long lived fluorescence component emitting at 350 nm for the single tyrosine-containing protein S-100b has been reported previously [24,25]. This characteristic emission makes it possible to differentiate between the ability of different peptides to form stable conformations (on the nanosecond time scale) which allow excited-state proton transfer to occur. Since the fluorescence lifetime is very sensitive to the microenvironment of the fluorophore, subtle changes in the lifetime can provide evidence for conformational variability of the peptides. However, it should be pointed out that when the long-lived component has a small amplitude (Tables II–V), differences between say 20 and 15 ns may not be significant. Using specific analogues of ANG II it is possible to identify proton acceptors for the excited-state protolysis reaction, and probe the ability of a peptide to fold into the conformation which gives rise to the long-lifetime. It has been previously suggested [26] that the fluorescence from tyrosinate could be enhanced by both restricted motion and reduced solvent accessibility. The quantum yield for tyrosinate also depends on both the fluorophore environment and the degree of ionization [27]. This infers that the strength of the hydrogen bond as well as the encompassing environment could play a role in determining the duration of the long-lifetime component.

In nonpolar solvents, intramolecular electrostatic interactions and conformational stability of angiotensin would be increasingly favoured as the dielectric constant of the solvent is decreased. In polar solvents, the angiotensin molecule is in an environment which promotes internal motion and side-chain mobility, which would diminish the likelihood of stable intramolecular hydrogen bond formation of the TyrOH on the space-time scale. Although conformational averaging could explain the absence of tyrosinate fluorescence in water and DMSO (Table II), other factors such as fluorescence quenching have to be considered because previous findings suggest that H-bonding of the TyrOH occurs to some extent in both water [3] and DMSO [28]. Since DMSO is an aprotic solvent which can function as a weak hydrogen bond acceptor, it is possible that the ANG II hydroxyl group might prefer to hydrogen bond to this solvent. However, the fluorescence properties of NAYA in the different solvents used in these experiments, and in particular the absence of LTF (Table I), establish that tyrosinate formation in ANG II is associated with intramolecular proton transfer and not with proton transfer to the solvent. The addition of 10% water or DMSO to isopropanol or propylene glycol decreases both the LTF and % LTF (I_3) for ANG II (Table II), suggesting that it is not the dielectric constant alone which determines the stability of intramolecular hydrogen bonding, and that a quenching effect (originating from dipole–dipole interactions between solvent and solute molecules) is present in water and DMSO. This conclusion is substantiated by recent proton NMR (2D-ROESY) studies which support the existence of a charge relay conformation for ANG II in DMSO [28].

LTF is a measure of the stability of the excited-state tyrosinate species, whereas % LTF reflects the percent of those conformer(s) present which give rise to LTF. These two variables must be interdependent to some degree. The % LTF for ANG II (Table II) was considerably higher ($I_3 = 79\%$) in isopropanol than in propylene glycol ($I_3 = 19\%$) suggesting that the less polar isopropanol provides an environment which stabilizes the tyrosinate-containing conformer(s). However, the LTF for ANG II was longer in propylene glycol (20.8 ns) than in isopropanol (15.5 ns) illustrating greater excited-state tyrosinate stability in the former solvent. When the results for ANG II in propylene glycol and trifluoroethanol, which have similar dielectric constants, are compared (Table II), it is apparent that whereas the dielectric constant of the solvent may determine the % LTF (19% in each solvent), it is not the only factor influencing the LTF. Thus, the tyrosinate lifetime in propylene glycol is 20.8 ns compared to 13.0 ns in trifluoroethanol, suggesting that the structure and dy-

namics of the solvent cage formed around the tyrosine hydroxyl group and the acceptor group may be significant determinants of excited-state tyrosinate stability. In addition, the photophysical behaviour of ANG II in different solvents may depend not only on bulk dielectric constant, but also on different dipolar relaxation effects in these solvents.

The finding that the addition to ANG II in water of SDS above critical micelle concentration induces tyrosinate fluorescence suggests that intramolecular hydrogen bond formation of the tyrosine hydroxyl in ANG II could occur in the presence of a cell membrane but not in its absence. Both the stability of the tyrosinate species (LTF) and the percent of conformer(s) providing for the tyrosinate species (% LTF) were significantly increased when the SDS micelles were formed in the presence of ANG II compared to when preformed SDS micelles were added to a solution containing ANG II (Table II). The former represents a situation where ANG II becomes trapped within the hydrophobic interior of the micelles, whereas the latter represents binding of ANG II to the negatively charged exterior surface of the micelles. Differences in the tyrosinate fluorescence presumably reflect the nature of the interactions of ANG II with the micelle interior versus exterior. The data for 'trapped' ANG II also suggest that the tyrosinate species is stabilized even in extremely hydrophobic ($\epsilon = 2$) environments.

Observation of tyrosinate fluorescence in angiotensin analogues in non-polar solvents is of interest in relation to the biological activities of these analogues because tyrosinate has been proposed to be the active group in ANG II which stimulates or 'triggers' the angiotensin receptor to initiate the intracellular cascade of molecular events leading to the response [6,7]. Table V reveals that in propylene glycol, biologically active analogues had longer LTFs (> 13 ns) than inactive analogues (< 11 ns). There is a suggestion here of a threshold tyrosinate stability in propylene glycol which reflects the expression of biological activity in the membrane environment of the receptor. However, the LTF was particularly low for the biologically active ANG III ($\tau_3 = 7.4$ ns); this may relate to the finding that ANG III takes up a significantly different conformation from ANG II in certain phospholipid environments [29], and is perhaps a good illustration that solvent environments can only roughly approximate the receptor environment.

Isopropanol also induced tyrosinate fluorescence properties which related to the biological activities of the analogues studied (Table V). The preponderance of the tyrosinate-producing conformer for ANG II, which was by far the dominant conformer (79%) in this solvent, could illustrate that isopropanol more closely mimics the receptor environment which ANG II encounters at its receptor. As with propylene glycol, the LTFs in isopropanol correlated with the biological activities of analogues, although for isopropanol only the two most biologically active analogues, ANG II (16 ns) and [Sar^1,His(3-Me)^6]ANG II (13 ns), had substantial LTFs, and the remaining nine analogues had shorter LTFs (0–11 ns). Apparently isopropanol discriminates between strong agonists and weak agonists/inactive analogues. The two strong agonists, ANG II and [Sar^1,His(3-Me)^6]ANG II, gave values for LTF and % LTF which were in line with their relative biological activities (Table V). It is not known how closely isopropanol ($\epsilon = 18$) or propylene glycol ($\epsilon = 32$) simulate the receptor environment; presumably the central lipid phase ($\epsilon = 2$) of the membrane bilayer must have its effective polarity increased to some degree by the presence of the transmembrane domains of the receptor protein. Complementary charged groups at the receptor binding site which provide Coulombic ligand binding forces will also influence the conformation of the approaching ligand.

Potential strong hydrogen bond acceptors in ANG II and [Sar^1] analogues capable of producing stable tyrosinate species are the imidazole and C-terminal carboxylate, although backbone carbonyl groups, if accessible to the TyrOH, could act as acceptors (and may account for some of the shorter LTFs observed). Recent ^{1}H-NMR (2D-ROESY) experiments [28] have illustrated that, even in relatively polar solvents such as dimethylsulphoxide, ANG II exists in a tightly folded conformation in which the three aromatic rings cluster together, so that the likelihood of the TyrOH gaining access to groups in the peptide backbone is minimized. The absence of 'potent' LTF for [Sar^1,Ala^6]ANG II and for [Sar,PheNH$_2^8$]ANG II (Table V) implicates both the imidazole and C-terminal carboxylate of ANG II in acceptor function. Thus the present findings support the proposition that the receptor conformation of ANG II contains a tripartite interaction of Tyr hydroxyl, His imidazole and C-terminal carboxylate groups, which is analogous to that found at the active site of serine proteinases [6,7]. However, it should be emphasized that there are substantial functional differences between Tyr and Ser charge relay systems, and that the hormone-receptor interaction is not likely to involve cleavage of the peptide backbone of either ligand or receptor. However, formation of a transient bond between the TyrO$^-$ of ANG II and the receptor is a possibility, and could represent an important step in the receptor 'triggering' mechanism.

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