Biophysical Chemistry 24 (1986) 25–31
Elsevier

BPC 01050

SELECTIVE ^{1}H-NMR RELAXATION INVESTIGATIONS OF MEMBRANE-BOUND DRUGS IN VITRO

2. ANGIOTENSIN II

Gianni VALENSIN [a], Maurizio DELFINI [b] and Elena GAGGELLI [a]

[a] *Department of Chemistry, University of Siena, Pian dei Mantellini 44, 53100 Siena, and* [b] *Istituto di Chimica e Tecnologia dei Radioelementi, CNR, Area delle Ricerche, 35100 Padova, Italy*

Received 31st May 1985
Revised manuscript received 14th January 1986
Accepted 18th February 1986

Key words: ^{1}H-NMR; Drug binding; Angiotensin II

Binding interactions between human angiotensin II and dipalmitoylphosphatidylcholine bilayer vesicles have been detected by measuring the selective proton spin-lattice relaxation rates of aromatic protons within the peptide. Involvement of the imidazole moiety of the His-6 residue has been demonstrated by the pH dependence of the NMR observables. A lower limit of the binding constant has been evaluated at 78.12 mol^{-1} dm^3 for the interaction involving nonionic intermolecular forces between aromatic residues and the lipid matrix.

1. Introduction

Human angiotensin II is a linear octapeptide whose primary structure is shown in fig. 1, where the pK values of the ionizable groups are also reported [1]. The broad range of biological activities presented by this hormone [2] has prompted extensive investigations especially dedicated to comprehension of the three-dimensional structure [3–7].

Several structure-function studies have been carried out in which various analog derivatives of the hormone have been prepared and their biological activities assayed. It transpired that the hormone is an extremely flexible molecule presenting several possible conformational states. Moreover, the biological activity of angiotensin II has been widely linked to the presence of a number of metal ions, especially Na^+ and Ca^{2+} [8–11], which prompted several reports about binding of metal ions to the hormone [12–16].

It is, however, generally believed that the biological function ought to be related to the interaction with a cellular receptor from which activation of some type of messenger system and, hence, biological response is brought about. From this point of view, the relevance of studying the interaction with lipid bilayer membranes is also validated by the following: (i) angiotensin II is able to mediate the transport of Mn^{2+} across phosphatidylcholine bilayers as detected by ^{1}H-NMR kinetic studies [17]; (ii) interference by some lipids is noticed in radioimmunoassays and receptor-binding assays for angiotensins [18,19].

In the present report the interaction of angiotensin II with dipalmitoylphosphatidylcholine (DPPC) bilayer membranes is delineated with particular emphasis on both pH and temperature effects. In fact, measuring the ^{1}H-NMR relaxation rates of angiotensin II in the presence of DPPC and titrating the His-6 ^{1}H resonances yield evaluation of the motional and environmental features of bound angiotensin II. Selective irradiation methods have been used throughout the experi-

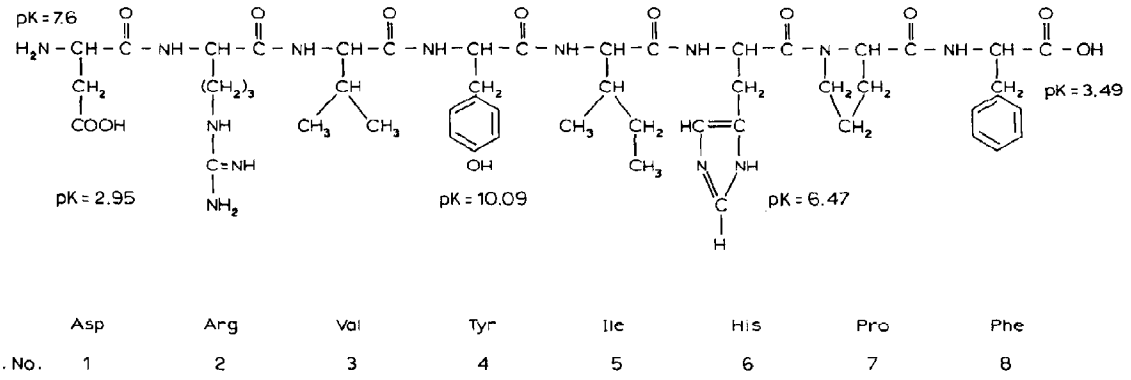

Fig. 1. Primary structure of angiotensin II and pK values of ionizable groups.

ments, since the selective proton spin-lattice relaxation rate (R^s) is linearly affected by changes in the motional correlation time and, hence, by receptor-binding interactions, as has been shown elsewhere [20,21]. In fact R^s, and not R^{ns}, is substantially contributed by the zero-quantum relaxation transition probability between two of the nuclear spin energy levels of a given proton pair. As a consequence, slowing down of molecular motions brings about a progressive increase in R^s, whereas, in the case of R^{ns}, a maximum is reached at $\omega_0\tau_c \simeq 1$ (ω_0, Larmor frequency; τ_c motional correlation time), followed by a progressive decrease.

2. Materials and methods

Human angiotensin II was purchased from Sigma Chemical Co. The hormone was dissolved in 99.95% ^2H_2O and the concentration was determined spectrophotometrically by using a molar absorption of 1.4×10^3 at 275 nm. The pH was adjusted with NaO^2H or ^2HCl.

Synthetic DPPC was obtained from Sigma and used without further purification.

Sonicated DPPC vesicles were prepared from a 50 mg/ml suspension in 10 mM Tris-chloride and 1 mM EDTA to reduce the effect of metal ion impurities. The suspension was deoxygenated by bubbling nitrogen and sonicated for 10 min at relatively low power, while maintaining the sonicator chamber at 50°C throughout the sonification. An MSE 150 W ultrasonic disintegrator was used for sonifications.

The sonicated suspension was centrifuged for 30 min at 45 000 rpm at 45°C; the zone containing the clear supernatant was removed and kept at 50°C. The stability of DPPC during the NMR experiments was checked by observing the ^{1}H-NMR spectral features of the lipid.

NMR measurements were performed on a Varian XL-200 NMR spectrometer in the pulsed FT mode. The temperature was controlled to ± 1°C and was measured from the chemical shift of ethylene glycol. The non selective spin-lattice relaxation rates (R^{ns}) were measured using the inversion recovery pulse sequences $(\pi - \tau - \pi/2 - t)_n$; the selective spin-lattice relaxation rates (R^s) were measured with inversion recovery pulse sequences where the π pulse was given by the proton decoupler at the selected frequency for relatively long times (typically 19–21 ms).

The R^{ns} and R^s values were evaluated by a three-parameter exponential regression analysis of the recovery curve of longitudinal magnetization.

3. Results and discussion

Typical non-selective and selective proton spin-lattice relaxation rate measurements of the low-field protons of angiotensin II in ^2H_2O are reported in figs. 2 and 3, respectively, while the relaxation data are summarized in table 1. From the R^{ns}/R^s ratio much lower than 1.5 two differ-

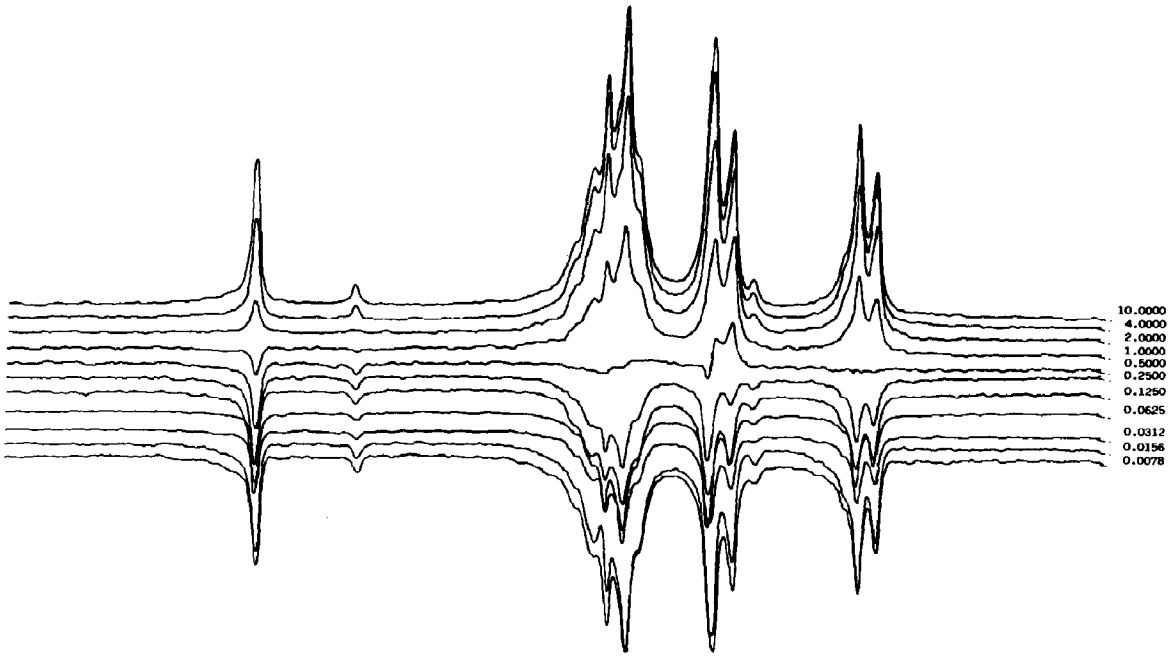

Fig. 2. Non selective partially relaxed proton spectra of the aromatic protons of angiotensin II (14 mg/ml in ^2H_2O at pH = 6.0 and $T = 298$ K).

ent inferences are possible: (i) extreme narrowing conditions do not apply and/or (ii) relaxation mechanisms other than ^1H-^1H dipole-dipole coupling are effective. The latter point is unlikely to determine the observed R^{ns}/R^s ratio, as it has already been discussed for an angiotensin pentapeptide [22]. As a consequence, the motional correlation time could be evaluated [20] ($\tau_c = 0.72$ ns at 298 K), in agreement with that calculated for the angiotensin pentapeptide [22].

The binding interaction was investigated by adding DPPC vesicles (8.33 mg/ml) to the peptide solution. The data obtained at room temperature are reported in table 2. The presence of DPPC bilayers membranes induces enhancements of the R^s value of all the aromatic protons. In contrast,

Table 1

Relaxation parameters for angiotensin II

Conditions: angiotensin-II, 14 mg/ml in ^2H_2O at pH 7.2; $T = 298$ K. ± values denote 95% confidence limits of the regression analysis.

Resonance	R^{ns} (s^{-1})	R^s (s^{-1})	R^{ns}/R^s
His H-2	0.41 ± 0.01	0.405 ± 0.01	1.01 ± 0.05
Phe	1.11 ± 0.02	1.05 ± 0.02	1.06 ± 0.04
Tyr_{meta}	1.74 ± 0.06	1.55 ± 0.04	1.12 ± 0.07
His H-4	0.52 ± 0.02	0.50 ± 0.01	1.04 ± 0.06
Tyr_{ortho}	1.15 ± 0.06	1.09 ± 0.02	1.06 ± 0.07

Table 2

Selective relaxation rate enhancement of aromatic protons of angiotensin II

Conditions: angiotensin II, 14 mg/ml in ^2H_2O in the presence of DPPC vesicles (8.33 mg/ml) at pH 6.8 and $T = 298$ K. ± values denote approx. 95% confidence limits of the regression analysis.

Resonance	R^s_{blank} (s^{-1})	R^s_{obs} (s^{-1})
His H-2	0.505 ± 0.01	2.62 ± 0.09
Phe	1.05 ± 0.02	3.61 ± 0.15
Tyr_{meta}	1.55 ± 0.04	4.15 ± 0.19
His H-4	0.50 ± 0.01	2.70 ± 0.10
Tyr_{ortho}	1.09 ± 0.02	3.34 ± 0.14

the R^{ns} values are almost unaffected, yielding a decrease in the R^{ns}/R^{s} ratio down to 0.55.

The decrease in the R^{ns}/R^{s} ratio and the enhancement in R^{s} are both consistent with slowing down of the reorientational molecular tumbling rate that, in turn, results from binding interactions with the DPPC bilayers. 'True' binding rather than viscosity effects can be suitably suggested on the basis of the pH and concentration dependences of the observed effect (see below). It can therefore be stated that the vasoactive peptide hormone angiotensin II interacts with phospholipids in vitro. Such an interaction, previously suggested on the basis of partitioning of radioactively labelled angiotensin II into chloroform [19], may be relevant for the physiological and pathologic behavior of the peptide.

A deeper insight into the binding features can be gained from the temperature dependence of the enhancement in R^{s}. Such a dependence is shown in fig. 4 for two aromatic protons, however the other aromatic protons also display the same characteristics. It is evident that, at temperatures above 320 K, the R^{s} enhancement has almost completely disappeared such that the aromatic residues of angiotensin II can be thought of as no longer being involved in binding the bilayer.

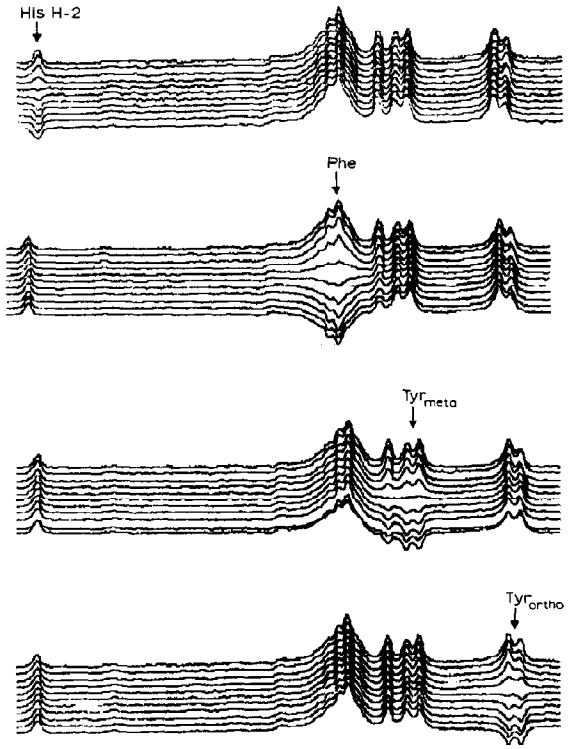

Fig. 3. Selective partially relaxed proton spectra of the aromatic protons of angiotensin II (14 mg/ml in ^2H_2O at pH = 5.0 and T = 298 K). Arrows indicate the frequencies of selective irradiation.

For the peptide-lipid interaction, formation of complexes through ionic bonds and/or formation of adducts through non ionic intermolecular forces are the mechanisms likely to be involved. The temperature dependence in fig. 4 points to a rapid and complete reversal of the binding interaction with increasing temperature and, as a consequence, nonionic interactions between lipid and aromatic moieties within the peptide can be consistently claimed. However, since metal ions were shown to inhibit binding of angiotensin II to lipids [19], two different interacting sites must be conceived within the peptide molecule. The main interaction is likely to occur through ionic forces involving the lipid head groups and charged groups within the peptide, most probably localized in the Asp-1 terminal residue [17]. The second interaction occurs through non-ionic forces involving at least one of the aromatic residues. The observed temperature dependence shows that, above the gel-to-liquid crystal phase transition temperature (T_c = 313 K), binding through non-ionic forces does not play any role in the angiotensin II-bilayer interaction. The temperature effect on the binding features of the aromatic moieties is in agreement with the observed increase in the rate of angiotensin II-mediated transport of Mn(II) across lecithin bilayers with rising temperature [17]. In fact, disruption of non-ionic intermolecular forces between lipid and peptide is expected to favor binding of metal ions, which has been recently shown to imply coordination to the His-6 imidazole moiety [16]. In conclusion, the effect of DPPC vesicles on the relaxation features of aromatic protons of angiotensin II can be suitably explained in terms of binding occurring in different ways, probably corresponding to different conformations of angiotensin II, depending upon the temperature.

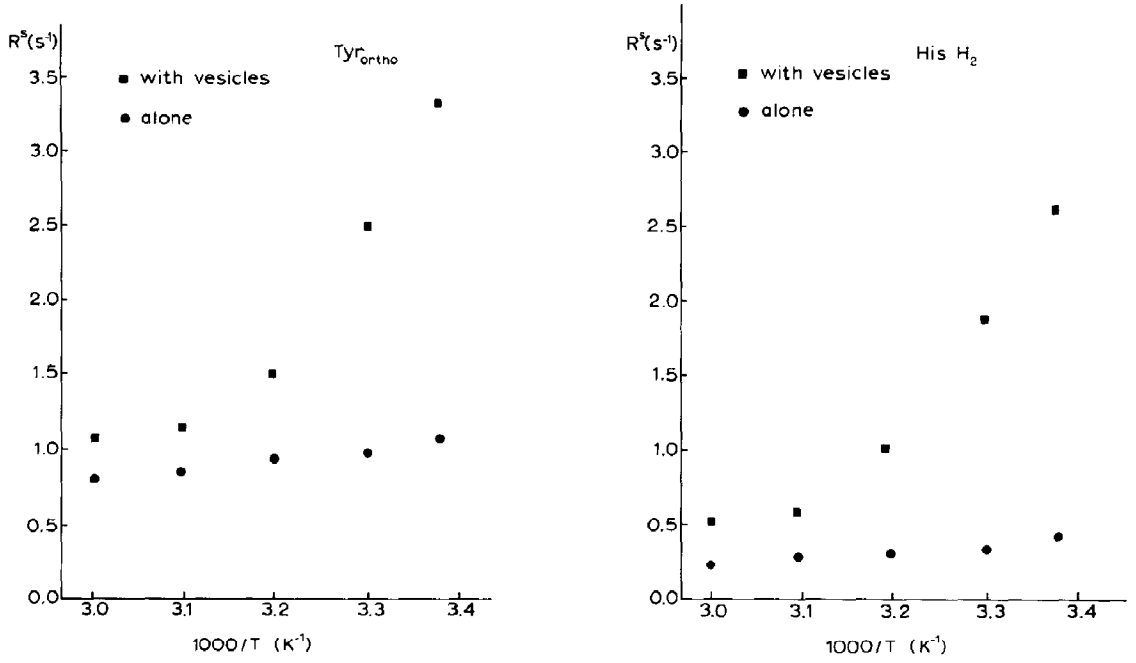

Fig. 4. Temperature dependence of the selective proton relaxation rates of the Tyr_{ortho} and His H-2 protons of angiotensin II (14 mg/ml in ^2H_2O at pH = 7.0) in the absence and presence of DPPC vesicles (8.33 mg/ml).

The pH dependence of the observed $\Delta R^s = R^s_{lipid} - R^s_{blank}$ is reported in fig. 5. The enhancement in R^s is strongly pH dependent such that at pH $\leq$ 5 the addition of lipid vesicles has only a slight effect on the relaxation rate of any aromatic proton. Since changes in pH are not likely to give rise to dramatic changes in viscosity, the pH dependence of the enhancement in R^s is indirect proof that the relaxation effect derives from true binding interactions. The same conclusion is reached on consideration of the dependence of ΔR^s on the concentration of angiotensin II, which, in the same way, does not affect the viscosity. Since a certain flattening can be noticed at pH > 7 in fig. 5 and since the only ionizable group having a pK in that pH range is the imidazole side chain of the His-6 residue, it can be suggested that deprotonation of the imidazole ring somehow favors binding of the peptide to the lipid matrix. However, interpretation of such an effect is not straightforward, since direct involvement of His-6 binding is not likely to occur. In the light of the key role played by protonation equilibria at the imidazole ring on

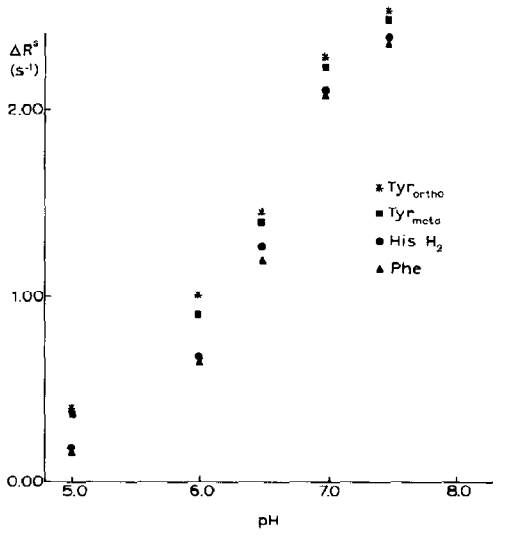

Fig. 5. pH dependence of the selective relaxation rate enhancement for the aromatic protons of angiotensin II (14 mg/ml in ^2H_2O at T = 298 K) in the presence of DPPC vesicles (8.33 mg/ml).

the conformational features of angiotensin II [7], the most probable interpretation is that a folded structure of angiotensin II, stabilized by the deprotonated imidazole of His-6, is responsible for binding to the lipid matrix at the level of the aromatic moieties. It has in fact been shown that the imidazole moiety is directly involved in a pH-dependent, extensive intramolecular structuring within the peptide molecule [5], resulting in either a C_7–C_5 structure or a distorted β-turn.

Involvement of the imidazole moiety in the secondary binding interaction between aromatic moieties within the peptide and the phospholipid bilayer was also confirmed by titrating the histidine H-2 proton chemical shift in the absence and presence of DPPC vesicles. Computer fitting of the titration curves (shown in fig 6) demonstrated a shift in pK of about 0.4 units due to the presence of vesicles such that a relevant change in chemical environments for histidine protons could be pointed out.

It has been previously shown [24] that plotting the reciprocal of the selective relaxation rate enhancement vs. the substrate concentration yields evaluation of the association constant for small molecules bound to macromolecular sites, provided fast exchange occurs between free and bound environments. In the limit of one peptide molecule bound to one receptor site, a linear plot is expected that extrapolates to $1/\Delta R^s = 0$ when the concentration of the substrate is equal to $-1/K_{ass}$. The concentration-dependent, selective relaxation rate enhancements are plotted in fig. 7 where the linear plot apparently yields, upon extrapolation, a value of 78.1 mol^{-1} dm^3 for the association constant. However, it must be recognized that, since experimental difficulties necessitated scan-

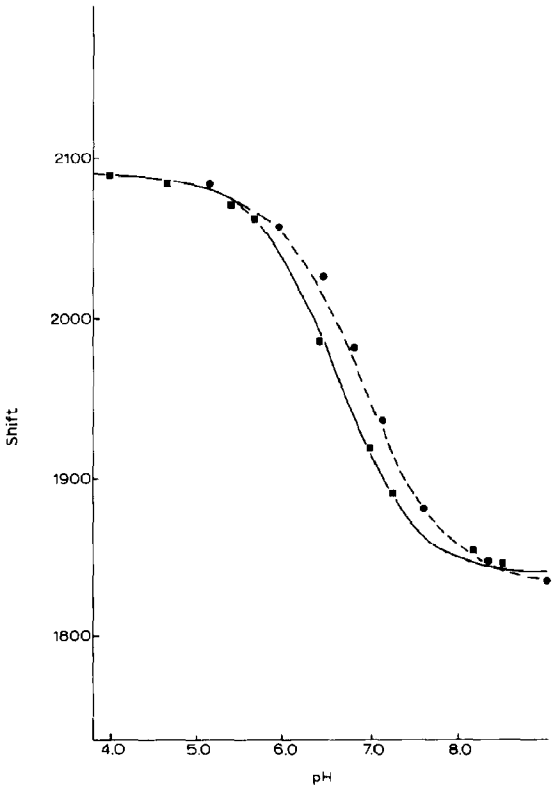

Fig. 6. Chemical shift (arbitrary units) vs. pH titration curves of the His H-2 proton of angiotensin II (14 mg/ml in ^2H_2O at $T = 298$ K) in the absence (———) and (- - - - -) of DPPC vesicles (8.33 mg/ml).

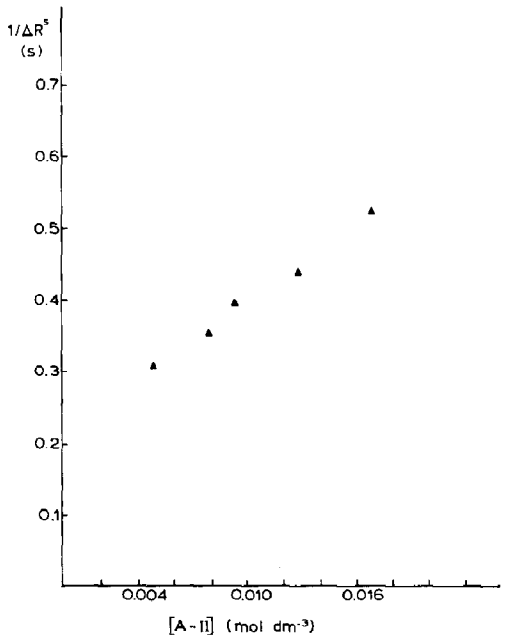

Fig. 7. $1/R^s$ vs. angiotensin II plot for the His H-2 protons in the presence of DPPC vesicles (8.33 mg/ml); pH = 6.8, $T = 298$ K.

ning of only a limited range of concentrations, deviations from linearity could well have escaped detection; the evaluated binding constant should therefore be considered as a lower limiting value.

By summarizing all the experimental findings the following conclusions can be advanced:

(i) Angiotensin II interacts with phospholipid bilayer vesicles in vitro;

(ii) The interaction is likely to occur at least at two binding sites, involving one or more aromatic moieties of angiotensin II;

(iii) The binding interaction involving the aromatic moiety occurs through nonionic intermolecular forces (e.g. hydrogen bonds or hydrophobic interactions), is easily reversed on increasing the temperature and is characterized by an association constant $K_{ass} \geq 78.1$ mol^{-1} dm^3;

(iv) The detected binding interaction is strongly pH dependent with an apparent pK which could be interpreted in terms of involvement of the imidazole moiety within the His-6 residue in stabilizing a folded conformation of the peptide that binds to the lipid matrix. The observed pK is also consistent with involvement of the N-terminal amino group, but chemical shift titration of the imidazole H-2 in the absence and presence of the lipid seems to confirm that a major role is played by the His-6 residue.

References

1 M.J. Peach, in: Chemistry and biology of peptides, ed. J. Meienhofer (Ann Arbor Science Publishers, Ann Arbor, 1972) p. 471.

2 I.N. Page and F.M. Bumpus, Handbook of experimental pharmacology: angiotensin II (Springer-Verlag, Berlin, 1979) vol. 37.

3 P.W. Schiller, Int. J. Peptide Protein Res. 16 (1980) 259.

4 R.E. Lenkinski and R.L. Stephens, J. Inorg. Biochem. 15 (1981) 95.

5 R.E. Lenkinski, R.L. Stephens and N.R. Krishna, Biochemistry 20 (1981) 3122.

6 F. Piriou, K. Lintner, S. Fermandjian, P. Fromageot, M.C. Khosla, R.R. Smeby and F.M. Bumpus, Proc. Nat. Acad. Sci. U.S.A. 77 (1980) 82.

7 S. Fermandjian, S. Piriou, C. Sakarellos, K. Lintner, M.C. Khosla, R.R. Smeby and F.M. Bumpus, Biopolymers 20 (1981) 1971.

8 G. Schaechtelin, R. Walter, H. Salomon, J. Jelinek, P. Karen and J.H. Cort, Mol. Pharmacol. 10 (1974) 57.

9 G. Schaechtelin, D. Surovec and R. Walter, Experientia 31 (1975) 346.

10 E. Blank, J. Sraer, J.D. Sraer, L. Baud and R. Ardaillou, Biochem. Pharmacol. 27 (1978) 517.

11 S. Gunther, M.A. Gimbrone and R.W. Alexander, Circ. Res. 47 (1980) 278.

12 R.E. Lenkinski, J.D. Glickson and R. Walter, Bioinorg. Chem. 8 (1978) 363.

13 R.G. Canada, Biochem. Biophys. Res. Commun. 99 (1981) 813.

14 R.E. Lenkinski, R.L. Stephens and N.R. Krishna, Biochim. Biophys. Acta 667 (1981) 157.

15 R.E. Lenkinski and R.L. Stephens, J. Inorg. Biochem. 18 (1983) 175.

16 R. Basosi, E. Gaggelli and G. Valensin, J. Inorg. Biochem. 20 (1984) 263.

17 H. Degani and R.E. Lenkinski, Biochemistry 19 (1980) 3430.

18 C.E. Odya, T.L. Goodfriend, J.M. Stewart and C. Pena, J. Immunol. Methods 19 (1978) 243.

19 M.E. Elliott and T.L. Goodfriend, Biochim. Biophys. Acta 586 (1979) 357.

20 G. Valensin, T. Kushnir and G. Navon, J. Magn. Reson. 46 (1982) 23.

21 G. Valensin, A. Casini, A. Lepri and E. Gaggelli, Biophys. Chem. 17 (1983) 297.

22 N. Niccolai, M.P. Miles and W.A. Gibbons, Biochem. Biophys. Res. Commun. 91 (1979) 157.

23 E. Giralt, R. Viladrich and E. Pedroso, Org. Magn. Reson. 21 (1983) 208.

24 G. Valensin, A. Lepri and E. Gaggelli, Biophys. Chem. 22 (1985) 83.