Evaluation of Conformational and Binding Characteristics of Various Natriuretic Peptides and Related Analogs†

Murielle Mimeault,‡ André De Léan,‡ Michel Lafleur,§ Danielle Bonenfant,§ and Alain Fournier*,‖

Department of Pharmacology, Faculty of Medicine, and Department of Chemistry, Faculty of Arts and Sciences, Université de Montréal, Québec, Canada, and Institut National de la Recherche Scientifique-Santé (INRS-Santé), Université du Québec, Pointe-Claire, Québec, Canada

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ABSTRACT: The conformational properties of atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and various analogs and homologs were studied by circular dichroism (CD) spectroscopy in solvent mixtures inducing secondary structures. The CD spectra obtained for rat ANF(99-126), porcine BNP32, and their related analogs indicated that these peptides exhibited mainly a random-coil conformation in pure water. However, the addition of increasing concentrations of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) gave rise for all peptides to a more ordered secondary structure. The analysis of the far-ultraviolet CD spectra suggested that the peptides exist under two conformational states, β-turn and β-sheet, in the presence of 20−60% HFIP/water solutions. Moreover, the characterizations of rANF(99-126) and the analog pBNP1, which combines the cyclic core of pBNP32 with the carboxy- and amino-terminal segments of rANF-(99-126), have been carried out by Fourier transform infrared spectroscopy (FTIR) in 40% HFIP/D_2O. The FTIR results indicated that these peptides exist predominantly under a β-turn and β-sheet mixed conformation. In addition, the amount of organized secondary structure obtained for human BNP32, bovine aldosterone secretion inhibitory factor, also known as ASIF(69-103) and β-rANF(92-126), in the presence of a 40% HFIP/phosphate buffer mixture, was similar to that of porcine BNP32, whereas rat BNP32 was found to be more structured. In the same solvent mixture, the CD spectra of Met(O)^{110}-human ANF(99-126) and chicken ANF(99-126) indicated that these peptides possess conformational features different to those of rANF(99-126) and hANF(99-126). Porcine CNP22, C-type natriuretic peptide, and the fragment C-ANF exhibited undefined secondary structure in the presence of 40% HFIP/phosphate buffer. These results suggest that the amino acid residues, not common to the various natriuretic peptides, would be involved in the stabilization of either β-turn and/or β-sheet conformations. Moreover, these secondary structures appear as particularly important for the recognition of the ANF-R_{1A} receptor subtype found in bovine adrenal cortex.

Natriuretic peptides are cyclic derivatives widely distributed in the central nervous system and peripheral tissues (Needleman et al., 1989; Gutkowska & Nemer, 1989; Saper et al., 1985; McKenzie et al., 1985; Inagaki et al., 1986). Among them, the atrial natriuretic factor (ANF)[1] and brain natriuretic peptide (BNP) were first isolated from human and rat atrial extracts and from porcine brain, respectively (Flynn et al., 1983; Kangawa & Matsuo, 1984; Sudoh et al., 1988). Subsequent studies allowed the identification of natriuretic peptides in various mammalian tissues (Sudoh et al., 1989, 1990; Nguyen et al., 1989; Kojima et al., 1989). Their physiological functions are related to diuresis, natriuresis, vasorelaxation, and inhibition of aldosterone and renin secretion (Needleman et al., 1989; Atlas, 1986; Cogan, 1990; Maack et al., 1984).

The natriuretic peptides are characterized by a high sequence homology in their 17-amino acid ring structure formed by a disulfide linkage between two cysteine residues, whereas the differences mainly exist in their amino- and carboxy-terminal portions (Table 1). Interestingly, the amino acid sequences of the various members of the BNP peptide group differ considerably among the species, and these peptides have been shown to possess different potencies in their receptor affinities and biological activities (Suga et al., 1992; Kambayashi et al., 1990). More recently, a 22-amino acid peptide was isolated from porcine brain, and it was designated as C-type natriuretic peptide (pCNP22) (Sudoh et al., 1990). This cyclic peptide which does not possess exocyclic C-terminal residues showed less potent diuretic and vasorelaxant activities than ANF and BNP (Koller et al., 1991; Stingo et al., 1992). Furthermore, it was observed

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* To whom correspondence and reprint requests should be addressed, at INRS-Santé, Université du Québec, 245 Hymus Blvd., Pointe-Claire (Québec), Canada H9R 1G6. Tel (514) 630-8800; Fax (514) 630-8850.

‡ Department of Pharmacology, Faculty of Medicine, Université de Montréal.

§ Department of Chemistry, Faculty of Arts and Sciences, Université de Montréal.

‖ INRS-Santé, Université de Québec.

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[1] The abbreviations for the amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [(1984) *Eur. J. Biochem. 138,* 9−37]. L-Isomers of amino acids were used. In addition: ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; C-ANF, [Cys^{116}]-atrial natriuretic factor-(102−116)-NH_2; ASIF, aldosterone secretion inhibitory factor; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)-phosphonium hexafluorophosphate; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; CD, circular dichroism; FTIR, Fourier transform infrared; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

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Table 1: Amino Acid Sequence of Natriuretic Peptides and Various Analogs

Peptides	Amino acid sequence
α-rat ANF(99-126)	S L R R S S C F G G R I D R I G A Q S G L G C N S F R - Y
α-human ANF(99-126)	S L R R S S C F G G R M D R I G A Q S G L G C N S F R - Y
β-rat ANF(92-126)	A A G P R S L R R S S C F G G R I D R I G A Q S G L G C N S F R - Y
chicken ANF(99-126)	M M R D S G C F G R R I D R I G S L S G M G C N G S R K N
C-ANF	R S S C F G G R I D R I G - - - - - - C[a]
porcine BNP32	S P K T M R D S G C F G R R L D R I G S L S G L G C N V L R R Y
porcine BNP26	D S G C F G R R L D R I G S L S G L G C N V L R R Y
human BNP32	S P K M V Q G S G C F G R K M D R I S S S S G L G C K V L R R H
rat BNP32	N S K M A H S S S C F G Q K I D R I G A V S R L G C D G L R L F
bovine ASIF	A L R G P K M M R D S G C F G R R L D R I G S L S G L G C N V L R R Y
porcine BNP1	S L R R S S C F G R R L D R I G S L S G L G C N S F R - Y
porcine BNP2	S P K T M R D S G C F G G R I D R I G A Q S G L G C N V L R R Y
porcine BNP3	S L R R S S C F G R R L D R I G S L S G L G C N V L R R Y
porcine BNP4	S P K T M R D S G C F G R R L D R I G S L S G L G C N S F R - Y
porcine CNP22	G L S K G C F G L K L D R I G S M S G L G C

[a] Presence of an amidated terminus.

that pCNP22 exhibited antinatriuretic activities in *in vitro* bioassays.

Progress was realized in the determination of the structural requirements of the natriuretic peptides for an effective interaction with their receptors. Indeed, previous structure–activity relationship studies, carried out with various analogs and fragments of ANF(99-126), indicated that the disulfide bridge, the residues between Phe-106 and Ile-113, and the C-terminal segment of the native molecule are essential for the full expression of the biological responses while the N-terminal region is less important (Bovy, 1990; Chino et al., 1985; von Geldern et al., 1992; Hassman et al., 1988; Kitajima et al., 1989; Konishi et al., 1988). Heterogeneity of natriuretic peptide receptors has also been revealed by affinity cross-linking and photoaffinity labeling studies (Meloche et al., 1986; Leitman et al., 1986). Moreover, the existence of at least three distinct classes of ANF receptors, referred to as ANF-R_{1A}, ANF-R_2, and ANF-R_{1C}, has been confirmed by molecular cloning techniques (Chang et al., 1989; Chinkers et al., 1989). For instance, it has been shown that ANF and BNP mediate their biological actions through the ANF-R_{1A} receptor subtype by inducing an increase of cGMP production. On the other hand, the ANF-R_2 receptor would be implicated principally in the clearance of circulating peptides (Leitman & Murad, 1987; Maack et al., 1987) while the ANF-R_{1C} receptor subtype (Koller et al., 1991) would be the specific binding site for CNP-22.

Although a few structural investigations were carried out, still little is known about the biologically active conformations of the various natriuretic peptides. Conformational analyses of ANF and BNP, made by NMR spectroscopy, showed significant differences between the three-dimensional structures of rANF(99-126) and pBNP26. These differences include a specific folding-back of the C-terminal segment toward the cyclic core and a distinct orientation in space of the N-terminal portion (Kobayashi et al., 1988a; Inooka et al., 1990; Craik et al., 1991). On the other hand, certain NMR experiments revealed that ANF exhibited a disordered conformation in solvents like H_2O and dimethyl sulfoxide (DMSO) (Kobayashi et al., 1988a; Thériault et al., 1987; Ohkubo et al., 1986; Gampe et al., 1988). These results were explained by the flexibility of the natriuretic peptides in solution, thus resulting in the detection of averaged conformations. In contrast, the results of IR and CD spectroscopic studies, as well as other NMR investigations, of fragments and analogs of ANF(99-126), in the presence of various membrane-simulating agents such as anionic phospholipid vesicles or sodium dodecyl sulfate (SDS) micelles (Fesik et al., 1985; Olejniczak et al., 1988; Surewicz et al., 1987; Epand & Stahl, 1987) indicated ordered structures containing predominantly β-pleated sheet and β-turn arrangements. Then, it was suggested that the β-sheet conformation adopted by the natriuretic peptides would be an important structural feature for the binding and agonistic activities. On the other hand, the procedures of structural prediction based on Chou–Fasman calculations and molecular modeling studies using conformational constraints predicted that the ANF molecule would contain in the cyclic core an antiparallel β-sheet structure and a β-turn involving the residues 111–114 (Spear et al., 1989b; Faaruo et al., 1988). In contrast, another investigation instead suggested that the bioactive conformation of ANF would contain a β-reversed turn located near the carboxy-terminal portion. This structure would allow the folding-back of the C-terminal exocyclic residues toward the central core of the molecule (Brady et al., 1990).

The present study was undertaken in order to identify the differences between the conformational behaviors exhibited by various natriuretic peptides. Therefore, the conformations of rANF(99-126), pBNP32, pCNP22, and a variety of homologs and analogs were investigated by circular dichroism spectroscopy in structure-promoting solutions. Moreover, to confirm the components of the secondary structures of rANF(99-126) and its analog pBNP1 (Table 1), a conformational characterization was carried out by FTIR

spectroscopy in the presence of a 40% HFIP/D_2O mixture. In addition, the structure−binding relationships of the peptides were evaluated following assays performed with bovine adrenal cortex membrane preparations containing the ANF-R_{1A} receptor subtype.

MATERIALS AND METHODS

Peptide Synthesis and Cyclization. The parent-peptide molecules were purchased from Peninsula Laboratories while the chimeric peptide analogs were prepared by the solid-phase synthesis method (Merrifield, 1963) using a homemade automatized synthesizer and according to a procedure that we recently described (Forest & Fournier, 1990). Peptides were assembled on phenylacetamidomethyl resin, and BOP reagent was used for the coupling step. Side-chain protection of α-*tert*-butoxycarbonyl-amino acids was as follows: Arg-(Tos), Asp(OcHx), Lys(ClZ), Thr(Bzl), Ser(Bzl), and Cys-(Acm). Peptides were cleaved from the polymeric support and deprotected with liquid hydrofluoric acid (10 mL of HF/g) containing *m*-cresol (1 mL/g) at 0 °C for 60 min. After precipitation and washing with anhydrous diethyl ether, the crude peptides were extracted with pure trifluoroacetic acid followed by evaporation. After lyophilization, the crude linear peptides were cyclized by iodine oxidation as follows: the acetamidomethyl (Acm) group protected peptides were dissolved in a degassed 80% acetic acid solution (1 mg of peptide/mL) to which an equal volume of iodine solution (13 mM), dissolved in the same solvent, was added. The final solution was incubated for 2.5 h with occasional shaking. Then, zinc dust was added until a discolored solution was obtained. After filtration, the solution was evaporated to 50 mL, and 250 mL of 0.06% trifluoroacetic acid (TFA)/H_2O solution was added before lyophilization.

Peptide Purification and Characterization. The cyclized peptides were purified by preparative reversed-phase HPLC on a Waters Prep LC 3000 system equipped with a Waters Prep Pak Module and a Model 441 absorbance detector. The material was eluted with linear gradients of (A) H_2O containing 0.06% TFA and (B) 40% CH_3CN in TFA/H_2O (0.06%). The gradients used were 0−45% B in 25 min, 45−80% B in 60 min, and 80−100% B in 20 min. The flow rate was constant at 64 mL/min, and detection was at 230 nm. Fractions were analyzed by analytical reversed-phase HPLC on a 600 Multisolvent Delivery System with a Lambda-Max Model 481 LC spectrophotometer. Analyses were carried out with a Vydac C_{18} (10 μm) column (30 × 0.39 cm) and an eluant of (A) H_2O with 0.06% TFA and (B) CH_3CN in a linear gradient mode. The gradient used was 20−50% B in 15 min. The flow rate was maintained at 1.5 mL/min, and detection was at 230 nm. The fractions corresponding to the purified peptide were pooled and lyophilized. Final purification was achieved by reversed-phase HPLC using a C_{18} column and a gradient of acetonitrile in aqueous solution. The purified peptides were characterized using analytical HPLC, capillary electrophoresis, and amino acid analysis. Analytical HPLC was performed with a Super Pak Peps C_{18} (5 μm) column using the system described above. Capillary electrophoresis was carried out with an Applied Biosystems 270A instrument using the following conditions: 20 mM sodium citrate buffer, pH 2.5; capillary 45 cm × 50 μm; voltage: 15 kV; *T*: 30 °C; injection: 3 s in vacuum mode and detection at 200 nm. The amino acid hydrolysis of the cyclic peptides were performed with 6 N HCl containing 0.1% phenol for 24 h at 110 °C. The samples were analyzed after drying and derivatization with PITC, according to the method described by Waters chromatography. The purity of all the peptides was estimated to be >95%.

Receptor Binding Assays. Competitive binding experiments to bovine adrenal cortex membrane preparations were performed essentially as previously described (Féthière & De Léan, 1991). Briefly, bovine adrenal cortex membranes corresponding to 25 μg of protein were incubated for 90 min at 22 °C in the presence of various concentrations of the unlabeled peptides, in 1 mL of 50 mM Tris·HCl buffer, pH 7.4, containing 0.1 mM EDTA, 0.5% bovine serum albumin, 5 mM $MnCl_2$ and 8−10 pM ^{125}I-rANF(99-126). After incubation, bound ^{125}I-rANF(99-126) was separated from free ligand by rapid filtration through GC/C glass fiber filters pretreated with 0.1% polyethylenimine, and the radioactivity retained on the filters was measured with a γ-counter. The competition binding curves were analyzed with the ALLFIT program, based on a four-parameter logistic equation, to obtain estimates of the IC_{50} and slope factor (De Léan et al., 1978). Moreover, the nonlinear least-squares curve-fitting program SCAFIT was used to determine equilibrium constants (K_d) for the different peptides (De Léan et al., 1982).

Circular Dichroism Spectroscopy. CD spectra were recorded on a Jobin-Yvon CD6 dichrograph. The instrument was calibred with (+)-10-camphorsulfonic acid and isoandrosterone. The peptide samples were contained between cylindral quartz windows with a path length of 0.1 cm. Spectra were recorded with a 2-nm bandwidth, a 0.5-nm step, and an integration time of 0.5 s. Each spectrum was the mean of 5−10 scans of two different samples, and it was corrected for solvent contribution. A smoothing routine was applied by using a digital low-pass filter. The rANF(99-126), pBNP32, and their chimeric analogs were studied in increasing concentrations (0−80% v/v) of HFIP in water. On the other hand, all peptides were also characterized in the presence of 40% (v/v) HFIP/phosphate buffer (10 mM, pH 7.0). The concentration of the peptides (15 μM) was estimated by UV light absorption measurements at 276 nm in 20 mM phosphate buffer, pH 6.5, containing 6 M guanidinium chloride. For the calculations, an extinction coefficient (ϵ_{276nm}) of 1500 M^{-1} cm^{-1} was used since the peptides contain only one tyrosine residue (Edelhoch, 1967). The molecular ellipticities ($[\theta]_\lambda$) are reported in deg·cm^2·$dmol^{-1}$.

Fourier Transform Infrared Spectroscopy. The samples for the FTIR measurements were prepared by dissolving known quantities of rANF(99-126) or the analog pBNP1 in deuterated water. Peptide samples were lyophilized and redissolved in D_2O. This step was repeated three times in order to ensure a complete hydrogen−deuterium exchange of the sample. Finally, peptide solutions at a final concentration of 3.5 mM were prepared using 40% (v/v) HFIP/D_2O as solvent. Sample aliquot of the peptide was placed in a demountable cell between two CaF_2 windows separated with a Teflon spacer of 5 μm. FTIR spectra were recorded at 20 °C on a BioRad FTS-25 spectrometer equipped with a mercury cadmium telluride detector. For each spectrum, covering the 400−4000 cm^{-1} region, 200 interferograms were coadded, apodized with a triangular function, and Fourier transformed to give a resolution of 2 cm^{-1}. The FTIR spectrum of the solvent was obtained under identical

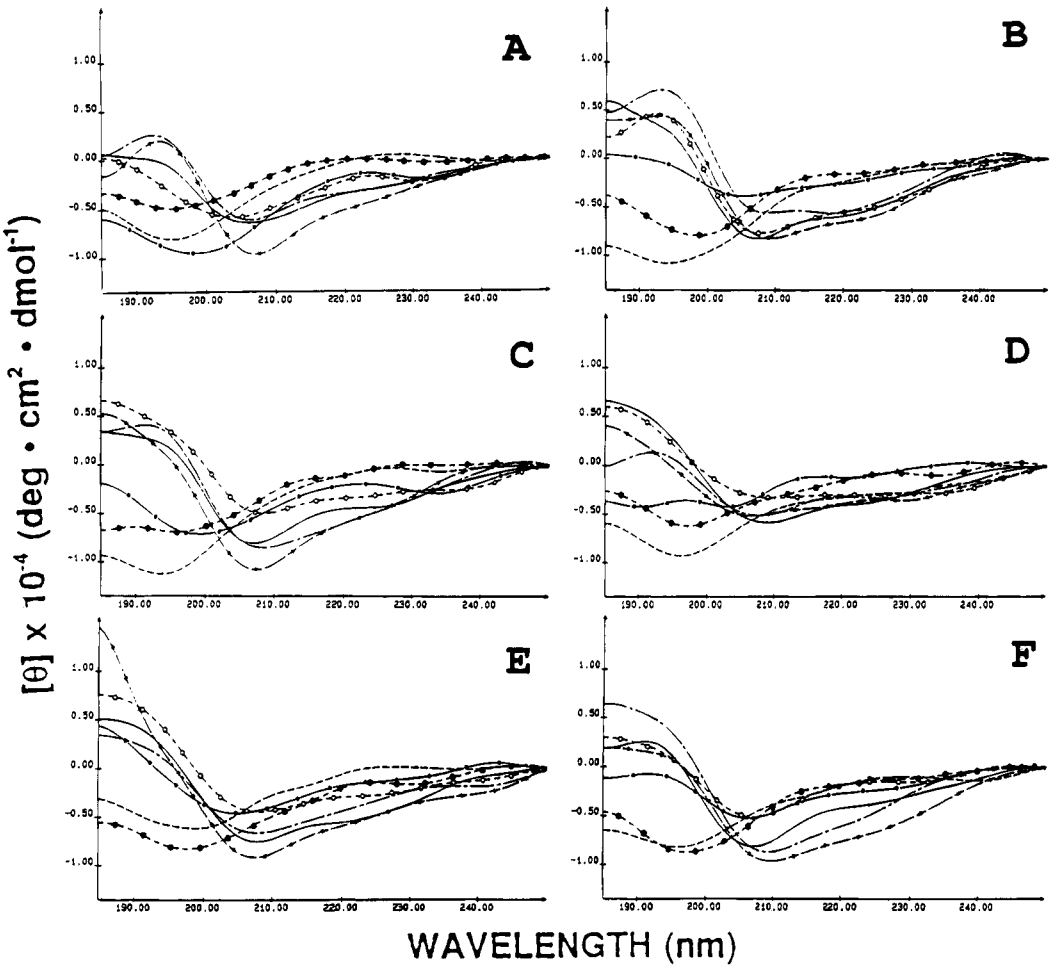

FIGURE 1: Circular dichroism spectra of rANF(99-126), pBNP32, and their related chimeric analogs in increasing concentrations of HFIP. CD spectra of peptides in 0% (- - -), 5% (- - ● - -), 10% (- - • - -), 20% (- -○ - -), 40% (—), 60% (— - —), and 80% (- - * - -) HFIP in water: (A) α-rANF(99-126), (B) pBNP32, (C) pBNP1, (D) pBNP2, (E) pBNP3, (F) pBNP4.

intrumental conditions and subtracted from the spectrum of the peptide using solvent bands around 1380 cm^{-1} as an internal standard. Despite the fact that the spectrometer was continuously purged with dry air, weak spectral contributions of water vapor had to be subtracted in certain cases. Moreover, as described by Hollosi et al. (1994), a correction was carried out in order to minimize the effects due to the absorptions of the trifluoroacetate counterions. Spectral Fourier self-deconvolution method was carried out for band narrowing in the region between 1500 and 1800 cm^{-1} using Spectra Calc software (Galactic Industries Corp.). Finally, the secondary structure composition of the peptides was estimated from the amide I′ region by band curve-fitting analysis.

RESULTS

CD Spectroscopic Characterization of the Secondary Structures of rANF(99-126), pBNP32, and Their Chimeric Analogs in Various HFIP/Water Mixtures. The far-UV CD spectra of rANF(99-126), pBNP32, and their chimeric analogs were measured in the absence and presence of the structure-promoting solvent HFIP. A low peptide concentration (15 μM) was utilized in order to allow the evaluation of the secondary structure of the peptide molecule in its monomeric state. As shown in Figure 1, the CD spectra of all these peptides measured in pure water showed a negative maximum near 200 nm, thus indicating a random-coil structure. However, with the addition of increasing concentrations of HFIP (up to 60%), the spectral profiles characterized by maxima at approximately 190, 208, and 223 nm suggested that the peptides adopt a more ordered conformation. In these solvent systems, the CD data (Table 2) are compatible with the presence of type I/III β-turn and β-sheet structures or the α-helix arrangement. In contrast, the CD spectra of these peptides when dissolved in 80% HFIP/water showed a decrease of the negative maxima, suggesting that they exist under an associated form following intermolecular aggregation.

FTIR Spectroscopic Analysis of the Secondary Structures of rANF(99-126) and the Analog pBNP1. To obtain further information about the secondary structures adopted by these peptides, FTIR measurements were recorded in a 40% (v/v) HFIP/D_2O mixture. Deuterium oxide (D_2O) was used in order to minimize the strong overlapping absorption of H_2O in the amide I region of the peptide spectrum. The FTIR absorption spectra in the 1800−1500 cm^{-1} region, obtained for rANF(99-126) and pBNP1, were characterized by the presence of two bands in the amide I′ region (Figure 2, panels A and B, respectively). The deconvolved spectrum of pBNP1 clearly showed two components at about 1672 and 1650 cm^{-1} and a broad shoulder in the region 1635−1637 cm^{-1}. In addition, the spectrum revealed bands at about 1610, 1588, and 1515 cm^{-1} which can be attributed to infrared absorption of amino acid side chains (Surewicz et

Table 2: CD Spectral Parameters of Various Natriuretic Peptides and Their Binding Affinities to ANF-R_{1A} Receptor Subtype in Bovine Adrenal Cortex Preparations

	molecular ellipticity		ANF-R_{1A} affinity
peptide	λ (nm)	$[\theta]_\lambda$[a] (deg·cm^2·dmol^{-1})	K_d (M)[b]
α-rANF(99-126)	207	−5751 ± 465	(2.5 ± 0.5) × 10^{-11}
	223	−2356 ± 613	
β-rANF(92-126)	208	−8391 ± 221	(1.7 ± 0.3) × 10^{-11}
	223	−7521 ± 233	
α-hANF(99-126)	207	−6017 ± 464	(3.1 ± 0.3) × 10^{-11}
	223	−3104 ± 297	
Met(O)^{110}-hANF	208	−7430 ± 440	(4.0 ± 0.1) × 10^{-10}
	223	−3391 ± 189	
chANF(99-126)	204	−6655 ± 166	(7.1 ± 1.0) × 10^{-10}
	223	−3722 ± 211	
C-ANF	200	−4766 ± 404	> 10^{-6}
pBNP32	208	−8378 ± 273	(3.6 ± 0.3) × 10^{-11}
	223	−4998 ± 410	
pBNP26	207	−5656 ± 594	(6.0 ± 1.0) × 10^{-11}
	223	−2934 ± 221	
bASIF(69-103)	208	−7388 ± 339	(1.4 ± 0.6) × 10^{-11}
	223	−5306 ± 182	
hBNP32	206	−9639 ± 372	(4.3 ± 0.1) × 10^{-11}
	223	−6062 ± 460	
rBNP32	208	−10305 ± 973	(4.6 ± 0.4) × 10^{-10}
	223	−8992 ± 122	
pBNP1	208	−8621 ± 438	(7.9 ± 0.2) × 10^{-12}
	223	−4328 ± 954	
pBNP2	208	−4205 ± 413	(4.0 ± 0.4) × 10^{-10}
	223	−1496 ± 586	
pBNP3	208	−8594 ± 260	(5.0 ± 0.7) × 10^{-12}
	223	−4758 ± 751	
pBNP4	208	−6255 ± 317	(1.3 ± 0.1) × 10^{-10}
	223	−2768 ± 25	
pCNP22	201	−7037 ± 273	> 10^{-6}
	218	−5154 ± 498	

a Mean residue molecular ellipticity ± standard error of 5−10 CD measurements determined in a 40% HFIP phosphate buffer mixture. b K_d; mean ± standard error of 2−6 receptor binding assays using ^{125}I-rANF(99-126) as radioligand.

al., 1987). Using these results as input, the non-deconvolved spectrum was then curve-fitted with 6 bands. A similar data treatment has been applied to the spectrum of rANF(99-126). The deconvolved spectrum showed two components at about 1670 and 1647 cm^{-1}; this latter contribution was broad, suggesting unresolved components. Based on the similarity of pBNP1 and rANF(99-126), the number of bands used in the curve-fitting analysis included two components for that broad feature. Thus, the curve-fitting results indicated an additional component at about 1635−1637 cm^{-1}.

The bands observed at 1670−1672 and 1635−1637 cm^{-1} can be assigned to β-turn/antiparallel β-sheet and H-bonded β-sheet/β-turn secondary structures, respectively (Haris et al., 1986; Surewicz et al., 1987; Dong et al., 1990; Prestrelski et al., 1991; Fabian et al., 1992; Mantsch et al., 1993; Hollosi et al., 1994). These structures were dominant in both peptides. From the curve-fitting analysis assuming identical absorptivity coefficients for all the different structures, the amount of β-turn/antiparallel β-sheet and H-bonded β-sheet/β-turn for rANF(99-126) was estimated to be about 32% and 49%, respectively (Figure 2C). The analog pBNP1 contained about 59% β-turn/antiparallel β-sheet and 17% H-bonded β-sheet/β-turn (Figure 2D).

Structural Analysis by CD Spectroscopy and Binding Study of Model Peptides Belonging to the ANF Family. The secondary structure adopted by the natriuretic peptides was determined in an amphiphilic environment in order to pinpoint the conformational features which might be important for the recognition of the peptide ligand by the ANF-R_{1A} receptor subtype. Therefore, a CD characterization of rANF(99-126), pBNP32, pCNP22, and the fragment C-ANF was carried out in the presence of a 40% HFIP/phosphate buffer mixture. The CD spectrum obtained for rANF(99-126) revealed only two strong absorptions at approximately 190 and 207 nm while that of pBNP32 showed two maxima near 190 and 208 nm and a broad shoulder at around 223 nm (Figure 3). In contrast, in the same solvent conditions, the CD spectral characteristics of pCNP22 and the short fragment C-ANF indicated no well-defined secondary structure. Thus, these CD patterns suggest, under these experimental conditions, precise structures for rANF(99-126) and pBNP32. In parallel, binding assays indicated that the more structured peptides, rANF(99-126) and pBNP32, possessed a high affinity for the ANF-R_{1A} receptor subtype while the least organized peptides, pCNP22 and C-ANF, did not exhibit a significant inhibition of the binding of ^{125}I-rANF(99-126) to ANF-R_{1A} receptors present in bovine adrenal cortex preparations (Table 2).

Relationships between the CD Data and ANF-R_{1A} Receptor Affinities for Various Analogs and Homologs of rANF-(99-126) and pBNP32. The CD spectra of the peptides were compared to those obtained with rANF(99-126) and pBNP32 (Figure 4). In parallel, the receptor binding affinities of these peptides were evaluated using bovine adrenal cortex membranes, a preparation containing only the ANF-R_{1A} receptor subtype (Table 2). The CD spectra of the peptides were analyzed, taking into account the structural elements identified by the FTIR analysis of rANF(99-126) and pBNP1. CD data indicated that the chimeric analogs pBNP1 and pBNP3, which possess the cyclic core of pBNP32 and the N-terminal segment of rANF(99-126), show a β-turn/β-sheet content comparable to that of pBNP32. Under the same experimental conditions, pBNP4 and pBNP2 were found much less structured. Similarly, the analogs pBNP3 and pBNP1 exhibited higher affinities than pBNP4 and pBNP2 for ANF-R_{1A} receptors (Table 2). The CD data and ANF-R_{1A} receptor affinity obtained for hANF(99-126) were very similar to those of rANF(99-126), indicating identical conformational and binding behaviors for those two peptides (Figure 4A and Table 2). In contrast, as compared to hANF(99-126), we observed, for the oxidized form Met(O)^{110}-hANF(99-126), a slightly higher content of organized structure but a weaker affinity for the ANF-R_{1A} receptor subtype (Table 2). Likewise, as compared to rANF(99-126), chicken ANF(99-126) exhibited a more ordered secondary structure but an affinity about 30 times lower. Nevertheless, the primary structure of chicken ANF(99-126) shows more homology with that of pBNP32. Thus, when using pBNP32 as the reference, it is observed that the structural stability and affinity of chANF(99-126) are lower. Similarly, pBNP26 and hBNP32 possess a secondary structure content lower than that of pBNP32. However, these peptides exhibit affinities comparable to that of pBNP32. The CD spectra of bASIF(69−103) and β-rANF(92-126) showed profiles close to that of pBNP32, suggesting similar folded structures for these peptides (Figure 4B). Moreover, these homologous compounds exhibited affinities close to that of pBNP32 on the ANF-R_{1A} receptor (Table 2). Finally, the CD spectrum of rat BNP32 in the structure-inducing solution showed, as indicated by the intensity of the negative molecular ellipticities near 208 and 223 nm (Figure 4B), that this peptide

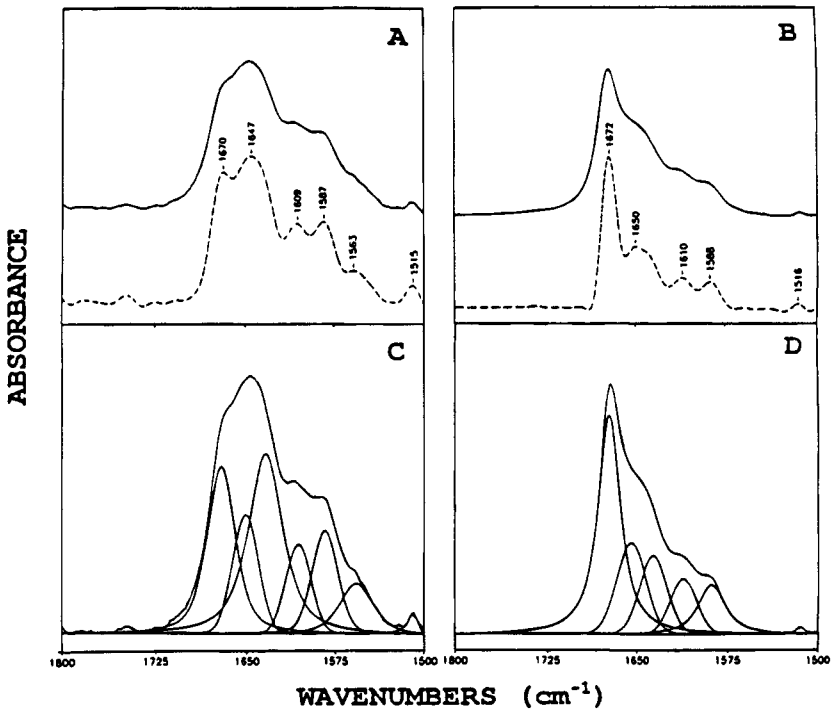

FIGURE 2: Fourier transform infrared spectra of the amide I′ and II′ regions of rANF(99-126) and analog pBNP1 in a 40% HFIP/D_2O mixture. FTIR spectra of (A) rANF(99-126) and (B) pBNP1 before (—) and after (- - -) band narrowing by deconvolution. Best fitted individual component bands for (C) rANF(99-126) and (D) pBNP1.

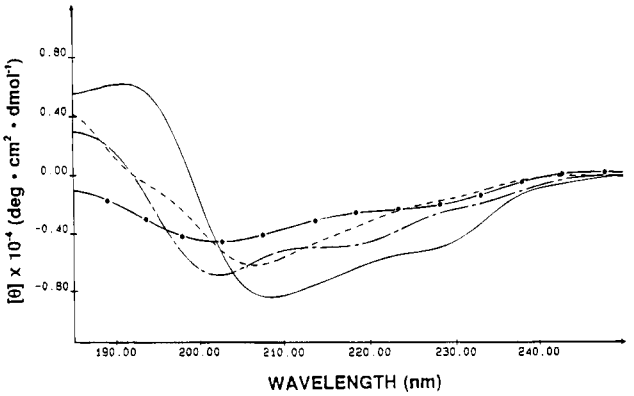

FIGURE 3: Circular dichroism spectra of natriuretic peptides in 40% HFIP in phosphate buffer. CD spectra of rANF(99-126) (- - -), pBNP32 (—), pCNP22 (— - —), and C-ANF (- ● - -).

exhibits a higher amount of β-turn/β-sheet structures than porcine BNP32. However, rBNP32 possessed a weaker affinity ($K_d = 4.6 \times 10^{-10}$ M) for the ANF-R_{1A} receptors as compared to pBNP32 ($K_d = 3.6 \times 10^{-11}$ M) (Table 2).

When comparing the whole series of peptides, the affinities for the ANF-R_{1A} receptor were, in decreasing order, as follows: pBNP3 ≥ pBNP1 ≈ bASIF(69-103) ≈ β-rANF-(92-126) ≈ α-rANF(99-126) ≈ α-hANF(99-126) ≈ pBNP32 ≥ hBNP32 > pBNP26 > pBNP4 > pBNP2 ≈ $Met(O)^{110}$-hANF(99-126) ≈ rBNP32 ≥ chANF(99-126) ≫ C-ANF and pCNP22. Thus, binding affinity of the various natriuretic peptides requires a significant content of the secondary structures, as estimated by the relative intensity of the molecular ellipticity at 207−208 and 222−223 nm. However, as observed with $Met(O)^{110}$-hANF(99-126) and rBNP32, other parameters are obviously involved in the binding process since these highly organized peptides showed an important loss of affinity for the ANF-R_{1A} receptor subtype.

DISCUSSION

In the present study, we reported the structural characterization by CD spectroscopy of various natriuretic peptides in a variety of solvent conditions. The CD data indicated that rANF(99-126), pBNP32, and their related chimeric analogs exist predominantly, in pure water, in a random-coil structure (Figure 1). These results are in agreement with the data obtained by different NMR and IR spectroscopic techniques which showed no well-defined secondary structure for various ANF fragments and pBNP26 in the presence of polar solvents such as H_2O, D_2O, and deuterated dimethyl sulfoxide (Kobayashi et al., 1988a; Inooka et al., 1990; Craik et al., 1991; Thériault et al., 1987; Gampe et al., 1988; Surewicz et al., 1987). However, the addition of increasing concentrations of HFIP (20−60%) in water resulted in a conformational stabilization of the peptides. Interestingly, the CD spectral characteristics of these peptides, in the presence of 40% HFIP in water, resemble those related to an α-helix structure (Table 2). However, the presence of maxima near 190, 208, and 223 nm is not by itself a sufficient evidence for helical secondary structure. Indeed, several recent CD studies revealed that the type C spectrum characterizing various peptides containing a type I/III β-turn looks like that obtained for peptides having an α-helix arrangement (Lang et al., 1992; Tatham et al., 1990; Woody, 1985; Gierasch et al., 1981; Mimeault et al., 1993b). Furthermore, the analysis of CD spectra obtained for rANF-(103-126) in the presence of SDS micelles or anionic phospholipid vesicles revealed one negative maximum near 207 nm which was associated to a high proportion of β-structure in the molecule (Epand & Stahl, 1987). In addition, previous NMR and IR studies combined with molecular modeling analyses strongly suggested a significant amount of antiparallel β-sheet and β-turn secondary structures in the ANF molecule but no α-helix (Fesik et al., 1985;

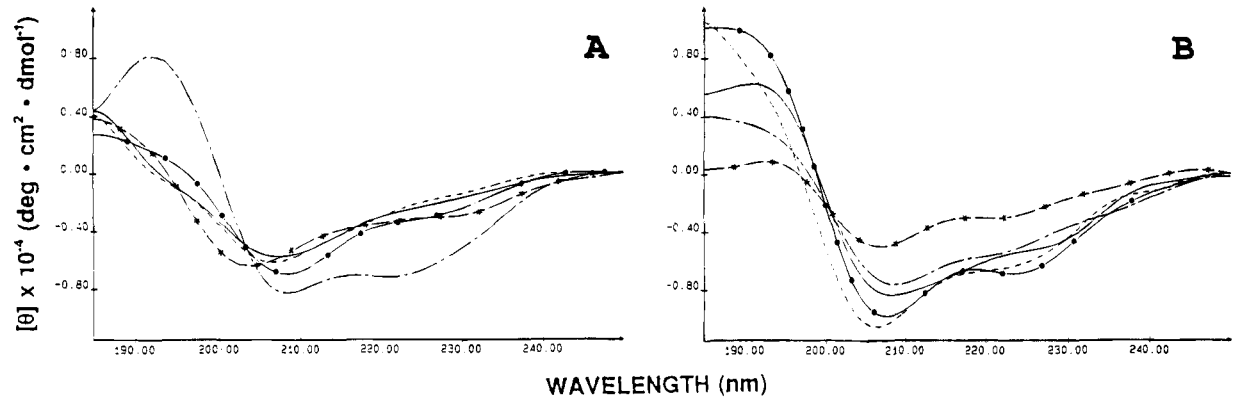

FIGURE 4: Circular dichroism spectra of various homologs of rANF(99-126) and pBNP32. (A) CD spectra of α-rANF(99-126) (- - -), α-hANF(99-126) (—), Met(O)^{110}-hANF(99-126) (— ● —), ch-ANF(99-126) (- - * - -), and β-rANF(92-126) (— - —) in 40% HFIP in phosphate buffer. (B) CD spectra of pBNP32 (—), pBNP26 (- - * - -), hBNP32 (- - -), rBNP32 (— ● —), and bASIF(69-103) (— - —) in 40% HFIP in phosphate buffer.

Olejniczak et al., 1988; Surewicz et al., 1987; Spear et al., 1989b; Faaruo et al., 1988; Brady et al., 1990). Other NMR studies carried out with pBNP26 indicated also that the conformation of this peptide could contain at least two β-turn-like structures involving the segments Leu^9—Ile^{12} and Leu^{18}—Asn^{21} which would permit the formation of a β-pleated sheet in the proximity of the C-terminal stretch (Inooka et al., 1990; Craik et al., 1991). Therefore, the most probable conformation adopted by the natriuretic peptides would correspond to mixed I/III β-turn and β-sheet secondary structures. Accordingly, FTIR spectral measurements of rANF(99-126) and its analog pBNP1, in 40% HFIP/D_2O, suggested that these peptides exist predominantly under these two conformational states. As a matter of fact, our FTIR spectra of rANF(99-126) and pBNP1 showed major amide I′ bands in the regions 1670—1672 and 1635—1637 cm^{-1} (Figure 2). These bands, on the basis of infrared data reported in the literature for other peptides or proteins, can be assigned to β-turn and/or antiparallel β-sheet (Prestrelski et al., 1991; Fabian et al., 1992; Mantsch et al., 1993; Haris et al., 1986; Dong et al., 1990). Thus, the FTIR spectroscopic data suggest that rANF(99-126) and its analog pBNP1 exhibit predominantly in the HFIP solution a mixture of β-turn/antiparallel β-pleated sheet. These results are in agreement with a previous FTIR spectroscopic study which showed that rANF (103-126) adopts a β-turn/β-sheet conformation in presence of anionic phospholipid vesicles (Surewicz et al., 1987). However, a quantitative analysis of the FTIR spectra, carried out by the curve-fitting method, revealed a significant difference between the amount of organized secondary structures for these peptides (Figure 2C,D). In particular, the analog pBNP1 possessed a more intense β-turn/antiparallel β-sheet band (1672 cm^{-1}) than rANF(99-126). This suggested that this peptide exhibits a specific behavior in an amphiphilic environment.

When using high concentrations of HFIP in water (80%; Figure 1), the CD data indicated that another type of conformation, or a destabilization of the organized structure, prevails for these peptides. Moreover, as observed with rANF(99-126) and pBNP32, a large decrease in intensity of the negative maxima at 208 and 223 nm was observed in pure HFIP (data not shown). As suggested previously after the CD analysis of calcitonin gene-related peptide and vasoactive intestinal peptide (Mimeault et al., 1993b; Fournier et al., 1988), this effect probably results from aggregation of the peptides. Therefore, the conformation observed for rANF(99-126), pBNP32, and their chimeric analogs, in the presence of high cosolvent concentrations, would correspond to a β-structure, resulting from the phenomenon of peptide aggregation. This secondary structure would be at the origin of the known tendency of ANF molecules to associate intermolecularly. This is in agreement with previous results of IR and CD studies which indicated the presence of β-structure in ANF fragments upon high peptide concentrations (Surewicz et al., 1987; Epand & Stahl, 1987). Thus, these observations suggest that rANF(99-126), pBNP32, and their chimeric analogs adopt well-defined spatial arrangements only in an amphiphilic solvent such as a mixture of HFIP/water.

In addition, the results showed significant conformational differences between the natriuretic peptides including rANF-(99-126), pBNP32, pCNP22, and C-ANF. More particularly, the amount of ordered secondary structure exhibited by pBNP32, in the presence of 40% HFIP/phosphate buffer, was higher than that of rANF(99-126). Interestingly, both peptides showed high affinities for the ANF-R_{1A} receptors found in bovine adrenal cortex membrane preparations. Thus, these differences between the β-sheet/β-turn-forming propensity of rANF(99-126), as compared to that of pBNP32, suggest that minor conformational variations of the ligand can be tolerated by the ANF-R_{1A} receptor. These structural characteristics between rANF(99-126) and pBNP32 may also explain the disparity of affinities and agonistic activities observed with these peptides in various biological systems (Suga et al., 1992; Kambayashi et al., 1990; Féthière & De Léan, 1991; Mimeault et al., 1993a).

On the other hand, as indicated by the profiles of their CD spectra (Figure 3 and Table 2), pCNP22 and the ring-contracted fragment C-ANF, which have been shown as ligands for the ANF-R_{1C} and ANF-R_2 receptors, respectively (Sudoh et al., 1990; Suga et al., 1992; Koller et al., 1991; Stingo et al., 1992; Mimeault et al., 1993a; Féthière et al., 1989), appeared as only slightly structured peptides in the 40% HFIP/phosphate buffer solution. The results of the binding assays indicated that these less ordered peptides do not bind to the ANF-R_{1A} receptor subtype, even at a concentration as high as 1 μM (Table 2). This suggests that the amino acid substitutions found in the ring structure and N-terminal portion of pCNP22, as compared to rANF(99-126) and pBNP32, caused structural modifications harmful

for the interaction of the ligand with ANF-R_{1A} binding site. Of course, the absence of the exocyclic C-terminal segment in pCNP22 and C-ANF might also be a structural element unfavorable to the formation or stabilization of the conformation recognized by ANF-R_{1A} receptors. Therefore, these results suggest that the conformational features observed for the above-mentioned natriuretic peptides in the presence of a structure-promoting solvent may be related to their selectivities for the different ANF receptor subtypes.

Relationships were observed between the stability of the secondary structure of the natriuretic peptides and their ANF-R_{1A} receptor affinities (Table 2). In particular, the CD spectroscopic analysis of the chimeric analog pBNP2, which contains the ring structure of rANF(99-126) and the N- and C-terminal segments of pBNP32, revealed that this peptide possesses much less organized structure than the parent compounds. This observation was related to the results of the binding assays which showed that the conformation adopted by this peptide does not allow an optimal interaction with the receptor (Mimeault et al., 1993a). Thus, this suggests the possibility of intramolecular interactions between the cyclic core of rANF(99-126) and the N- and C-terminal segments of pBNP32. This particular arrangement would disfavor the binding to the ANF-R_{1A} receptor subtype. Moreover, the chimeric analogs pBNP1 and pBNP3 containing the core of pBNP32 and the N-terminal portion of rANF-(99-126) showed CD spectral characteristics comparable to those of pBNP32. Nevertheless, these peptides possessed affinities significantly superior to that of pBNP32, indicating that the presence of the N-terminal segment of rANF(99-126) helped for the recognition of the ANF-R_{1A} receptor without affecting the peptide conformation (Mimeault et al., 1993a). In contrast, as shown by the data obtained with the analog pBNP4, as compared to those of pBNP32 (Table 1), the substitution of the carboxy-terminal portion of pBNP32 with that of rANF(99-126) seemed to result in a significant loss of secondary structure and affinity for the ANF-R_{1A} receptors. This effect can be explained by the results of a previous NMR study suggesting the possibility of a hydrogen bond between the residues Ser-17 and Arg-24 in pBNP26 (Inooka et al., 1990). Indeed, the replacement of the C-terminal portion of pBNP32 with the segment of rANF-(99-126) would disrupt this intramolecular interaction and destabilize the conformation of the peptide which is essential for the ANF-R_{1A} receptor recognition (Mimeault et al., 1993a). Taken together, these results suggest that the structural properties of rANF(99-126) and pBNP32 molecules are not only related to the intrinsic contribution of the amino acid residues but also to their intramolecular interactions which probably give rise to subtle modifications in the three-dimensional arrangements. In addition, the structural and pharmacological properties exhibited by the human and rat ANF(99-126) were very similar, suggesting that the substitution of Met^{110} by Ile did not induce a conformational change in the ANF molecule (Figure 4A and Table 2). However, the CD data obtained for $Met(O)^{110}$-hANF(99-126) indicated a higher content of stabilized secondary structure than for the parent compound hANF-(99-126), while a significant decrease of the ANF-R_{1A} receptor affinity was observed with this analog. These results are in agreement with previous NMR experiments which showed significant differences between the three-dimensional structure of $Met(O)^{110}$-hANF(99-126) and hANF(99-126) (Kobayashi et al., 1988b). Moreover, the oxidized form $Met-(O)^{110}$-hANF(99-126) has been reported to possess lower receptor binding affinities and agonistic activities than hANF-(99-126) in many paradigms (Chino et al., 1985; Hirata et al., 1985). Thus, this suggests that the presence of an oxygen atom on the Met residue in position 110 of the peptide sequence would create a local conformational restriction in the ANF molecule which would not allow an optimal interaction of the ligand with the receptor. Accordingly, structure−activity studies based on modeling techniques indicated that conformational restrictions of the ANF molecule, obtained by bicyclization or deletion of certain amino acids in the cyclic core, stabilize an antiparallel β-structure but result in a reduction of the biological activity and affinity for the ANF-R_{1A} receptors (Spear et al., 1989a,b; Schiller et al., 1986). Another explanation is that the side chain of Met-110 could be directly involved in the interaction with the receptor (Chino et al., 1985; Kitajima et al., 1989). Hence, the oxidation of the sulfur atom of Met-110 in hANF(99-126) could result in a steric hindrance disfavoring the ANF receptor recognition.

In contrast, β-rANF(92-126) showed an amount of organized structure and an affinity for ANF-R_{1A} receptor higher than those of rANF(99-126). Consequently, the N-terminal amino acid extension of β-rANF(92-126) seems to improve the stabilization of the secondary structure and favor the recognition by the ANF-R_{1A} binding sites. Interestingly, the results of several structure−activity studies have indicated that the N-terminal segment of rANF(99-126) does not play a determinant role in the binding to the receptor (Hirata et al., 1985; Schiller et al., 1986; Bovy, 1990). Therefore, the addition of amino acid residues in the N-terminal segment of rANF(99-126) could promote the stabilization of the secondary structures found in other regions of the molecule and improve the receptor recognition.

The CD data obtained for chicken ANF(99-126) showed that this peptide is more structured than rat ANF(99-126) but less than pBNP32, a peptide with which it shows more homology. On the other hand, chANF(99-126) possessed a weaker affinity for ANF-R_{1A} receptors than rANF(99-126) and pBNP32. These results are in agreement with a previous study indicating that chANF displays weaker biological activities than hANF(99-126) (Miyata et al., 1988). Therefore, the amino acid differences found in these peptides are apparently involved in the formation or stabilization of the structural features important for the binding to ANF-R_{1A} receptor. In particular, the chANF(99-126) differs from BNP32 by only two amino acids (Ile-110 and Met-118) in the ring structure. Furthermore, one of these (Ile-110) is also identified in rANF(99-126). Thus, the C-terminal residues Gly-122, Ser-123, Lys-125, and Asn-126, and the residue Met-118 found in chANF(99-126), did not seem to induce an appropriate structure for an effective binding of the ligand to the ANF-R_{1A} receptor subtype. Additionally, the receptor affinity and molecular ellipticities obtained for bovine BNP, known as bASIF(63−109), were close to that of pBNP32 (Figure 4B and Table 2). This suggested that the additional N-terminal amino acid residues of bASIF(63−109) did not affect the stability of the BNP conformation (Féthière & De Léan, 1991). In contrast, the significant loss of organized structure and affinity observed with pBNP26, as compared to pBNP32, suggested that the N-terminal residues 27−32 of pBNP32 are important for the mainte-

nance of the affinity for the ANF-R_{1A} receptor subtype. However, as aforementioned for the β-rANF(92-126), the residues 27−32 of pBNP32 could participate in the formation of a particular folding of the N-terminal tail or contribute to the stabilization of neighboring segments in the BNP molecule.

Structural differences were observed between the human and porcine BNP32, but these peptides exhibited similar binding affinities to ANF-R_{1A} receptor. The disparity in the primary structures of hBNP32 and pBNP32 (Table 1) did not appear to modify the capacity of the ANF-R_{1A} receptors present in bovine adrenal cortex membrane preparations to recognize the BNP32 ligand. Nevertheless, the structural differences between these two peptides could explain the results of previous pharmacological characterizations which described variations in affinities and agonistic potencies between pBNP32 and hBNP32, in tissue preparations containing the ANF-R_{1A} receptor subtype (Suga et al., 1992; Kambayashi et al., 1990). Finally, the CD data indicated a higher amount of ordered secondary structure for rat BNP32 than for its porcine form while the affinity of rBNP32 was significantly lower than that of pBNP32. Although the rBNP32 molecule contains a highly stabilized secondary structure, it appears that its sequence which shows only a 47% homology with that of pBNP32 would not fulfil the structural requirements for a potent interaction with the ANF-R_{1A} receptor. Accordingly, as shown in Table 1, the amino acids which differ between rBNP32 and pBNP32, including the Glu^{13}-Lys^{14} dipeptide and the C-terminal residues, have been reported to be important for the recognition of the ANF receptor (Bovy, 1990; von Geldern et al., 1992; Hassman et al., 1988). Furthermore, rat BNP32 is the only peptide of this series containing an arginine residue at position 23. Therefore, it can also be hypothesized that this additional particular feature may be unfavorable for the binding of rBNP32 to the ANF-R_{1A} receptors.

In conclusion, the CD and FTIR spectroscopic data provided information about the conformations prevailing in a variety of natriuretic peptides. As a matter of fact, the results are in agreement with the presence of a mixture of antiparallel β-sheet/β-turn secondary structures, when the peptides are in an amphiphilic environment. In addition, the results suggested that structural features of the molecules, including the presence of a significant content of β-turn and β-sheet secondary structures, would be important for the binding to the ANF-R_{1A} receptors found in bovine adrenal cortex. The CD and FTIR data did not allow the precise identification of the residues participating in the secondary structures. Nevertheless, it appears that the variable amino acids found within the cyclic portion of the natriuretic peptides would be involved in the particular folding observed in the peptides belonging to the ANF and BNP family. Furthermore, the presence of specific exocyclic residues in the C- and N-terminal regions of the peptides seems to be required for the stabilization of the structural elements that are important for the ANF-R_{1A} receptor recognition.

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