Three-Dimensional Structure of the Mammalian Tachykinin Peptide Neurokinin A Bound to Lipid Micelles

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ABSTRACT The solution structure of NKA, a decapeptide of mammalian origin, has been characterized by CD spectropolarimetry and 2D proton nuclear magnetic resonance (2D ^{1}H-NMR) spectroscopy in both aqueous and membrane mimetic solvents. Unambiguous NMR assignments of protons have been made with the aid of correlation spectroscopy (DQF-COSY and TOCSY) experiments and nuclear Overhauser effect spectroscopy (NOESY and ROESY) experiments. The distance constraints obtained from the NMR data have been utilized to generate a family of structures, which have been refined using restrained energy minimization and dynamics. These data show that in water NKA prefers to be in an extended chain conformation whereas a helical conformation is induced in the central core and the C-terminal region (D4-M10) of the peptide in the presence of perdeuterated dodecylphosphocholine (DPC) micelles, a membrane model system. Though less defined the N-terminus also displays some degree of order and a possible turn structure. The conformation adopted by NKA in the presence of DPC micelles represents a structural motif typical of neurokinin-2 selective agonists and is similar to that reported for eledoisin in hydrophobic environment.

INTRODUCTION

Tachykinins are a family of biologically active peptides distributed in the central and peripheral nervous system. The earliest known members of the tachykinin family are those that are present in mammalian systems. Neurokinin A is a decapeptide found in mammalian neuronal tissue, with the sequence His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2. It was first isolated from the porcine spinal cord (Kimura et al., 1983). Other mammalian tachykinins include substance P, NKB and N-terminally extended forms of NKA, NPK, and NPγ. Substance P, NKA, NPγ, and NPK are derived from a single gene, preprotachykinin A (PPT-A), by alternative RNA splicing (Nakanishi, 1987; Nawa et al., 1984; Krause et al., 1987). Tachykinins elicit a wide and complex array of biological responses, such as the stimulation of extravascular smooth muscle, powerful vasodilation, hypertensive action, activation of immune system, regulation of pain transmission, and neurogenic inflammation. NKA and its N-terminally extended peptides have been identified as very potent bronchoconstrictors (Joos et al., 2000) and have been implicated as important mediators of inflammatory lung disorders, such as asthma (Sherwood et al., 1997). Hence there is considerable interest in these peptides as potential targets for drug design especially in the development of novel antiasthmatics. The tachykinin peptides are characterized by a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, where X represents either an aromatic (Phe, Tyr) or a branched aliphatic (Val, Ile) amino acid .The C-terminal region or the message domain is considered to be responsible for activating the receptor. The divergent N-terminal region or the address domain varies in amino acid sequence and length and is postulated to play a role in determining the receptor subtype specificity (Schwyzer, 1987).

The tachykinins mediate their effects through membrane bound receptors belonging to the superfamily of G-protein-coupled receptors, whose structure is believed to be similar to the heptahelical structure of rhodopsin (Palczewski, et al., 2000). Three pharmacologically distinct receptor subtypes have been identified and cloned for tachykinins designated as NK-1, NK-2, and NK-3 (Nakanishi, 1991; Masu et al., 1987; Hanley and Jackson, 1987), which all share a significant sequence similarity. All tachykinins bind to all the receptor subtypes, with substance P preferring NK-1, NKA preferring NK-2, and NKB preferring NK-3.The wide range of physiological activity of tachykinins has been attributed to the lack of specificity of tachykinins for a particular receptor type (Nakanishi, 1991). This lack of specificity can be accounted for by the conformational flexibility of these short, linear peptides. The characterization of the biologically active conformation, which controls receptor binding and subtype selectivity, is of significant interest.

Recently, several studies brought to light the biophysical properties of tachykinin neuropeptides. Various experimental structure studies have been carried out in aqueous and membrane mimetic solutions of substance P, its analogs and other tachykinins, NKA, NKB, NPK, Physalaemin, Kassinin, Eledoisin (Convert et al., 1988, 1991; Chassaing et al., 1986a,b; Szollosy et al., 1986; Woolley and Deber, 1987; Levian-Teitelbaum et al., 1989; Seelig and Macdonald,

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Abbreviations: NKA, neurokinin A; DPC, dodecylphosphocholine; CD, circular dichroism; DQF-COSY, double-quantum filtered correlation spectroscopy; NOESY, 2D nuclear Overhauser effect spectroscopy; ROESY, rotating frame Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; NK, neurokinin; NKB, neurokinin B; NPK, neuropeptide K; NPγ, neuropeptide gamma.

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1989; Lavielle et al., 1988, 1990; Sumner et al., 1990; Williams and Weaver, 1990; Seelig,1992; Ananthanarayanan and Orlicky,1992; Whitehead et al., 1998; Young et al., 1994; Horne et al., 1993; Wilson et al.,1994; Grace et al., 2001, 2003). In general, it has been found that the tachykinins display some elements of secondary structure in appropriate solution environment, though it has been suggested that they do undergo rapid conformational exchange (Sumner et al., 1990). There are no discernible trends in the conformation of the address segments of these peptides. However, the message domains are similar in each case. In general, the message domain of these peptides undergoes conformational averaging in aqueous environments (Woolley and Deber, 1987; Sumner et al., 1990). In hydrophobic environments, the message domain assumes helical conformations (Schwyzer, 1987; Woolley and Deber, 1987; Whitehead et al., 1998; Wu and Yang, 1983; Horne et al., 1993) or exists as a series of turns in dynamic equilibrium (Sumner et al., 1990).

NMR studies have been reported on NKA in water and methanol (Chassaing et al., 1986c), in 28% 1,1,1-trifluoroethanol (TFE) /water mixture (Horne et al., 1993) and in 50 mM sodium dodecyl sulfate (SDS) micelles (Whitehead et al., 1998). There have also been reports of NMR studies on analogs and fragments of NKA in membrane mimetic solvents (Gao and Wong, 1999; Zhang and Wong, 1993). NKA was found to be in a random coil conformation in water, but displayed definite secondary structure in methanol and in TFE/water mixture. A series of dynamic turns in equilibrium involving the segment T3–M10 was suggested as the structure for NKA in TFE/water mixture (Horne et al., 1993). Structure of NKA in SDS micelles was reported to adopt a helical structure from F6–L9 with an extended N-terminus (Whitehead et al., 1998).

It has been postulated that the binding of neuropeptides to their cell surface receptors may be catalyzed by nonspecific interactions with membrane lipids and the binding of the peptide to the receptor occurs in at least two sequential steps: the binding of the peptide to the membrane, followed by the binding of the peptide to the receptor in the membrane (Schwyzer et al., 1986). Although the neuropeptides in aqueous solution exist as randomly distributed conformers, the biologically active forms of these neuropeptides are likely to be ordered and stabilized within the lipid bilayers of the cell membrane before binding with their receptors (Schwyzer, 1987; Woolley and Deber, 1987; Schwyzer et al., 1986). Thus conformational features of NKA in lipid medium, which control receptor binding and which govern its activity, are of much interest.

NMR is particularly suited for the study of micelle-bound peptides. Almost two decades ago, it was demonstrated that the high-resolution proton NMR spectra could be acquired on peptides bound to micelles of perdeuterated lipid (Brown, 1979). Micellar systems have been used frequently in high-resolution NMR studies of peptide/membrane interaction as membrane mimics. Since the pioneering work on melittin and glucagon in micelles (Brown, 1979; Lauterwein et al., 1979), there have been a large number of NMR studies of peptide structure and their interactions in micellar systems and the conditions for determining the conformations of peptides bound to micelles using NMR have been established by several research groups (Braun et al., 1983; McDonnell and Opella, 1993; Opella, 1997; Kallick et al., 1995; Maurer et al., 1991). DPC is one of the well-characterized model membrane systems in current use for the study of peptides and proteins that bind to the lipids. It forms a stable micelle, which freely rotates in solution, making it an excellent tool to mimic the anisotropic environment of a lipid membrane while providing motional properties desirable for solution NMR. It has electrostatic and hydrophobic components, which approximate a cell membrane. It has been shown that membrane mimetic systems (Brown, 1979; Lauterwein et al., 1979; Braun et al., 1983; Rizo et al., 1993; Maurer and Rüterjans, 1994; Pellegrini et al., 1996; Cowsik et al., 1997; Grace et al., 2001 , 2003) are quite capable of inducing structures upon several small neuropeptides holding biological relevance, and here the study has been extended to NKA.

In the current study, CD and NMR spectroscopic techniques have been used to investigate the secondary structure of NKA in different solvents. The 3D structure of NKA bound to micelles of DPC has been reported for the first time (the structure has been deposited in Protein Data Bank and the PDB ID code is 1N6T). Also, the conformational properties of NKA in water and perdeuterated DPC micelles have been described and compared. Several homonuclear 2D NMR techniques (Wüthrich, 1986), such as TOCSY, DQF-COSY, ROESY, and NOESY have been utilized in deriving the complete proton resonance assignments for NKA, in water and in the lipid medium. The NOESY crosspeak volumes have further been used to determine the interproton distances in 3D space. An ensemble of model conformations has been generated for NKA in the lipid medium using the program DYANA (dynamic algorithm for NMR applications, Güntert et al., 1997).

EXPERIMENTAL PROCEDURES

MATERIALS AND METHODS

NKA was obtained from Sigma Chemical Company (St.Louis, MO). Perdeuterated DPC (d_{38}) was obtained from Cambridge Isotope Laboratories (Andover, MA). NMR reagents were obtained from Aldrich Chemical Company (Milwaukee, WI).

CD spectropolarimetry

The CD spectra have been recorded on a Jasco J-720 spectropolarimeter (Jasco, Tokyo, Japan). The instrument was calibrated using *d*-10-camphorsulfonic acid (Chen and Yang, 1977). The spectra were collected between 190 and 250 nm at room temperature using a quartz cell having a path length of 1 mm. The peptide concentration was around 117 μM. The spectra are the average over four scans, each recorded with a bandwidth of

1 nm, 0.25-nm step size and a 0.2-s time constant. After baseline correction, the observed ellipticity was converted to a mean residue ellipticity ([θ] deg cm^2 dmol^{-1}), using the relationship [θ] = θ/lcN, where θ is the observed ellipticity, l is the path length in mm, c is the molar concentration, and N is the number of residues in the peptide. The spectra reported have not been smoothed. To mimic different biomembrane compartments, different solvents were used .The aqueous environment was mimicked by a 10-mM sodium phosphate buffer at pH 7.4, the charged surface by the anionic detergent SDS, and the hydrophobic interior by TFE. The CD spectra for all the peptides were recorded in water, sodium phosphate buffer, in increasing concentrations of TFE, anionic detergent SDS, and in Zwitterionic lipid DPC. The spectra recorded in the presence of DPC, SDS micelles, and TFE were corrected by subtracting the spectra of the corresponding DPC, SDS, and TFE solutions.

Nuclear magnetic resonance experiments

The NMR samples were prepared by dissolving 5 mg of NKA in ~0.5 ml of water (90% H_2O, 10% D_2O, pH 5.0). The experiments in lipid environment were performed with an identical peptide sample to which 39 mg of perdeuterated DPC was added, yielding in solution a lipid concentration of 260 mM, which is well above the critical micelle concentration (1 mM) for DPC. The lipid/peptide ratio of the NMR sample was 30:1. All NMR spectra in DPC and water were recorded on a Bruker DRX 500 (Bruker, Zurich, Switzerland) spectrometer operating at 500 MHz proton resonance frequency. The data was processed by the XWINNMR program on a Silicon Graphics Indigo workstation (SGI, Mountain View, CA).

All the 2D spectra were acquired in the phase-sensitive mode. The homonuclear ROESY, NOESY, and TOCSY spectra were recorded with 64 scans, a relaxation delay of 1.5 s, a spectral width of 5020 Hz in both dimensions, and with 512 increments in t_1 and 2 K data points in t_2. After zero filling and Sine apodization in t_1 and t_2 dimensions, the final size of the data matrix was 1 K × 1 K.The NOESY spectra were recorded with mixing times of 50, 100, 150, 200, and 250 ms. Different mixing times were used to evaluate the linear build-up of NOE and to find the mixing time appropriate to the two-spin approximation. NOESY spectrum recorded with a mixing time of 150 ms was chosen for obtaining the distance constraints.

Structure determination

For the determination of internuclear distances, the NOESY peak volumes on the 150-ms NOESY spectra were classified as strong, medium, and weak, corresponding to the upper-bound interproton distance restraints of 2.7, 3.5, and 5.0 Å, respectively. The NOEs used for the structure calculation have been taken from the NOESY spectrum (150 ms), which lies within the initial build-up of the NOE curve. Appropriate pseudo atom corrections were applied to nonstereospecifically assigned methylene and methyl protons. A total of 102 NOE constraints (55 intra, 26 i to $i+1$, 12 i to $i+2$, and 9 i to $i+3$) were originally applied. A total of 50 structures were initially generated using DYANA (Güntert et al., 1997). Dihedral angles (ϕ), which were derived from the measured $^3J_{NH}$ values, were also used as constraints for the ϕ values.

RESULTS

CD studies

Fig. 1 shows the CD spectra of NKA in 10 mM phosphate buffer (pH 7.4), in 90% TFE, SDS micelles (16 mM), and DPC (12 mM), the SDS and DPC concentrations being well above their CMC (8 mM and 1 mM, respectively). The titrations were performed with various concentrations of TFE (10–90%), SDS (4–32 mM), and DPC. The spectrum in aqueous solution shows the peptide as being primarily

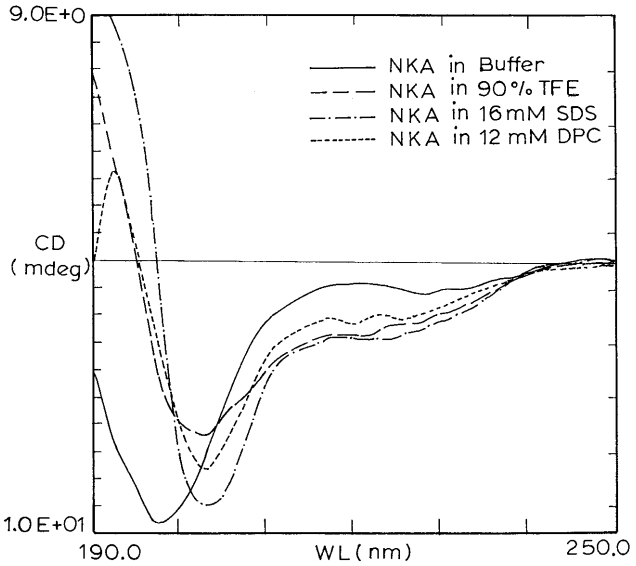

FIGURE 1 CD Spectra of NKA in buffer (*solid line*), SDS micelles (*dash/dot line*), 90% TFE (*dashed line*), and DPC (*dotted line*).

unstructured and having a weak maximum around 220 nm and a strong minimum around 198 nm (Woody, 1992). The addition of the structure-inducing SDS and DPC micelles induced a shift toward helical conformation (at concentration as low as respective CMC), as all spectra had a minimum at 222 nm (helical $\pi\pi^*$ transition) and a second minimum between 203 and 208 nm (overlapping helical and random coil $\pi\pi^*$ transition at 208 nm and 200 nm respectively) (Holzworth and Doty, 1965; Alder et al., 1973; Chang et al., 1978). CD data were also analyzed using two parameters, R_1 and R_2, which are independent of inaccuracies in the determined peptide concentration, as well as those caused by small shifts in wavelength (Bruch et al., 1991). Here R_1 is the ratio of the intensity of the maximum between 190 and 195 nm and the intensity of minimum between 200 and 210 nm and R_2 is the ratio of the intensity of minimum near 222 nm and the intensity of minimum between 200 and 210 nm. For a random structure, R_1 is positive and R_2 is close to zero. On the other hand, in a highly helical state, R_1 will be close to -2 and R_2 will approach 1 (Rizo et al., 1993; Bruch et al., 1991). These parameters calculated for NKA in buffer, TFE, DPC, and SDS show an induction of the helical structure on addition of TFE ($R_1 = -1.09, R_2 = 0.43$), SDS ($R_1 = -1.29, R_2 = 0.31$), and DPC ($R_1 = -1.19, R_2 = 0.30$). Thus the CD results indicate that NKA associates with SDS and DPC micelles undergoing a conformational transition from a prevalently random coil state (in water) to α-helical state.

NMR studies

Some preliminary 1D and 2D spectra of NKA were recorded in aqueous solution at various temperatures. Temperature-dependent 1D and 2D spectra were recorded and

were assigned completely. On the basis of observation of a relatively small number of inter-residue crosspeaks in the ROESY spectra, it was concluded that NKA adopts essentially a random coil conformation in water. Aliquots of d_{38}-DPC were then added to an aqueous solution of NKA, and another series of 1D and 2D proton NMR spectra were recorded at 500 MHz. The structural stabilization was apparent in the NMR spectra on addition of 12 mg or more of DPC. All subsequent experiments were performed under these solution conditions (DPC concentration 260 mM; lipid/peptide ratio 30:1).

Spectral assignment

Assignment of the proton spectra of NKA in the presence of membrane mimetic solvent (DPC) was accomplished using the technique of sequence-specific resonance assignments developed by Wüthrich (1986). The amide region of the NOESY (150 ms mixing time) spectrum of NKA is shown in Fig. 2 *b*. The assignment of the various resonances in the sequence of the peptide is indicated on the spectrum in Fig. 2 *a*. Complete proton resonance assignments for NKA in the presence of membrane mimetic solvent (DPC) and in water, thus obtained, are given in Tables I and II in Supplementary Material.

Analysis of chemical shift values

In this analysis the simple method of Wishart and co-workers (Wishart et al., 1992) is adopted for predicting the secondary structure of proteins based on changes in their α-H proton chemical shifts. A local $\alpha-$helical structure is identified by three consecutive negative secondary shifts (the resonances shifted to high field relative to the corresponding random coil values). The results for the analysis of the α-H proton chemical shifts for NKA in DPC (Fig. 3), show that for the residues 3–10 the difference in the chemical shifts is continually negative, which suggests that in this part of the peptide a helical secondary structure is favored in the presence of DPC. This is not observed in the case of water, which further confirms the absence of any structure for NKA in an aqueous medium.

It is very difficult to determine the exact on/off rates for the binding of NKA with DPC micelles from the NMR data. However, the percentage of bound conformers compared to the free conformer may be obtained by determination of the amount of α-helix observed. A semiquantitative estimation of the helical content of NKA when going from water to DPC micelles may be obtained from the average upfield shifts of the α_H protons. In this procedure the upfield shifts of the α_H protons of the region assumed to be helical are added together and averaged. The averaged up-field shifts are divided by 0.35 (0.35 ppm is assumed to correspond to 100% α-helix) yielding percentage of α-helix (Rizo et al., 1993). The semiquantitative estimate of helical content for residues 3–10 for NKA in DPC micelles thus calculated was 40%.

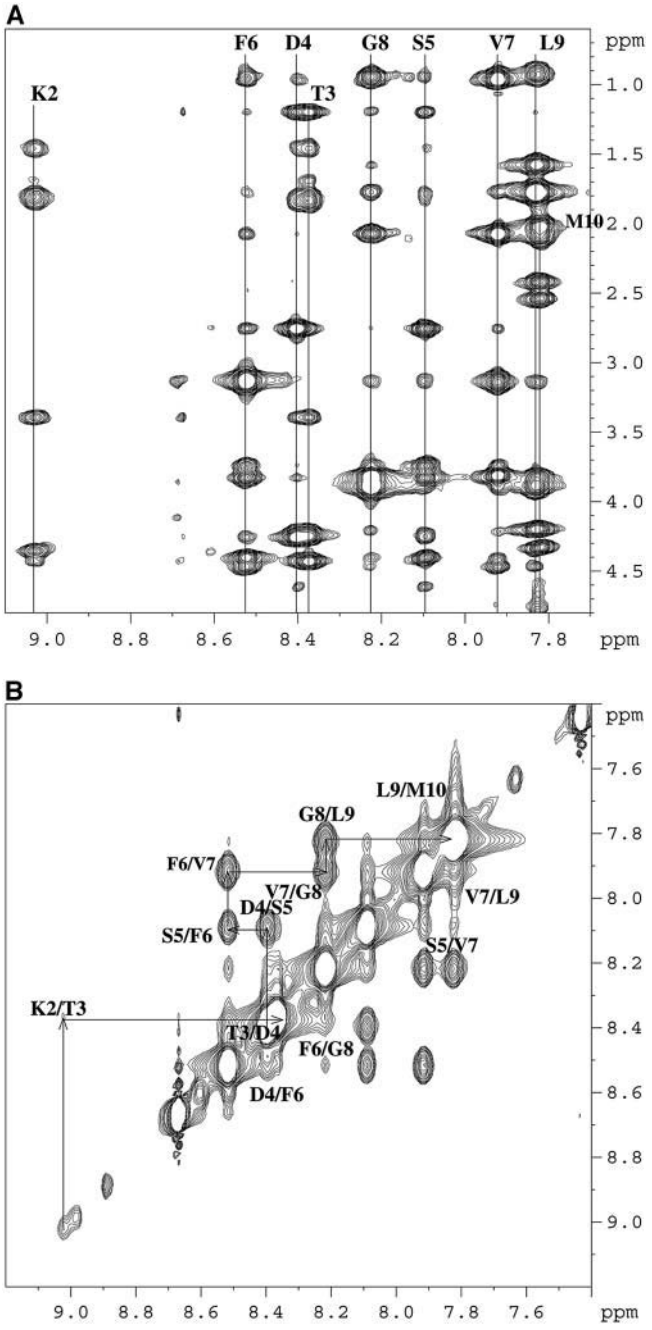

FIGURE 2 (*a*) The NH-α,β,γ region of the 500 MHz NOESY spectrum of NKA in the presence of membrane mimetic solvent (DPC), recorded with a mixing time of 150 ms. (*b*) The NH-NH region of the 500 MHz phase-sensitive NOESY spectrum of NKA in the presence of membrane mimetic solvent (DPC), recorded with a mixing time of 150 ms.

Analysis of NOE connectives

Wüthrich and co-workers (Wüthrich, 1986, 1984) have reported that the observation of a group of specific sequential and medium-range NOEs can be used to determine the existence of secondary structural features such as α-helix or β-sheet. NOEs that are important to characterize the

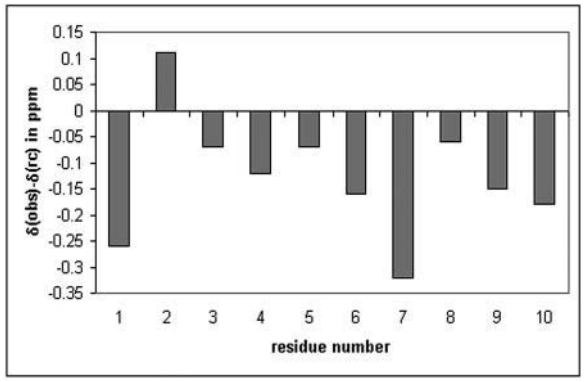

FIGURE 3 Analysis of the α-H proton chemical shifts for NKA in the presence of DPC micelles. Residues 3–10 adopt a helical structure.

secondary structure of NKA in the presence of DPC micelles are summarized in Fig. 4. For NKA (D4-M10), the intraresidue crosspeaks are more intense, indicating the presence of helical structure. Similarly, variations in relative intensities of d_{NN} and $d_{\alpha N}$ sequential NOEs support the proposal of a helical structure for NKA (D4-M10) as the d_{NN} contacts are stronger than the $d_{\alpha N}$ contacts (Wüthrich et al., 1984). On the other hand, the ROESY spectrum of NKA in water shows only intraresidue and i to $i + 1$ ROEs among the amide protons and α-protons, confirming the random coil nature of NKA in water.

A dense grouping of NOEs, the five $d_{\alpha N}$ (i, $i + 3$), two $d_{\alpha\beta}$ (i, $i + 3$), nine sequential d_{NN} NOEs, and five d_{NN} (i, $i + 2$) NOEs coupled with six $d_{\alpha N}$ (i, $i + 2$) NOEs (Fig. 4), supports the presence of helical structure in this region of NKA (involving residues 4–10). The observation of six $d_{\alpha N}$ (i, $i + 2$) connectivity (K2-D4, T3-S5, S5-V7, F6-G8, V7-L9, G8-M10) suggests the presence of 3_{10}-helical structure. NOE connectivities [$d_{\alpha N}$ (i, $i + 4$)] suggestive of

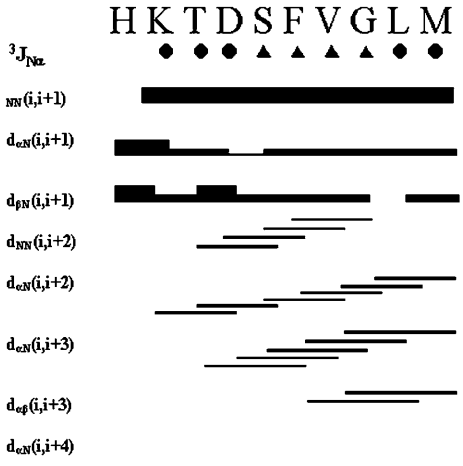

FIGURE 4 The NOEs that are important to characterize the secondary structure of NKA in the presence of DPC micelles. Filled triangles refer to a coupling constant of 4–5 Hz and filled circles to 6 Hz and above.

regular $\alpha-$helical structure were not observed. Overlap of some of the resonances under water interfered with the NOE analyses. However, the number of $\alpha H_i-\beta H_{i+3}$, NOEs observed indicate that NKA is substantially folded from residues 6–10. Observation of these various types of NOEs simultaneously suggests that some degree of conformational averaging is present around a predominantly helical core. In the N-terminus of the helical segment there are crosspeaks characteristic of a β-turn or a 3_{10}-helix. Therefore, the possibility of turn conformations must be considered for the region near the N-terminus as seen by the presence of $d_{\alpha N}$ (i, $i + 2$) NOE between K2 and D4, T3 and S5. In these structures, distances $d_{\alpha N}$ (i, $i + 2$) and $d_{\alpha\beta}$ (i, $i + 2$) are very short and the NOE crosspeaks are usually observed (Wüthrich et al., 1984; Dyson et al., 1988).

It is also possible to acquire information about the ϕ angles along the peptide chain by measuring $^3J_{N\alpha}$ coupling constants. Helical structures result in coupling constants of 4–5 Hz whereas extended structures have coupling constants in the range of 8–9 Hz (Wüthrich, 1986; Pardi et al., 1984). Wüthrich has suggested that a series of three or more $^3J_{N\alpha}$ coupling constants <6 Hz is diagnostic of $\alpha-$helical structure (Wüthrich, 1986). Most of the measurable coupling constants for NKA are in the range of 4–6 Hz, with the exception of T3, D4 (6.24 Hz) and L9, M10 (7 Hz). The low $^3J_{N\alpha}$ values (<6 Hz) suggest that there is a large population of helical structures in the stretch between residues 5 and 10. L9 and M10 also show a large $^3J_{N\alpha}$ (>6 Hz), which may be due to fraying of helix at the terminus.

From the above NMR results, it is concluded that residues 4–10 clearly meet the criteria for the existence of helical structure: the presence of sequential $d_{NN}(i, i + 1)$ crosspeaks, medium-range $d_{\alpha N}(i, i + 3)$, and $d_{\alpha\beta}(i, i + 3)$ crosspeaks and a series of $^3J_{N\alpha}$ coupling constants of 6 Hz or less. However, the entire peptide is not helical. Due to the lack of medium-range crosspeaks and the large $^3J_{N\alpha}$ coupling constants, we conclude that the first three residues are in turn conformation. No evidence for helix stabilization through salt bridge formation was observed.

Generation of 3D structure

Given the indication of a helical structure along the central core of NKA in DPC, it was of interest to use the observed NOEs to obtain information on the 3D structure of the peptide. This was done using the torsion angle dynamics algorithm for NMR applications, DYANA (Güntert et al., 1997). Initially, 50 structures were generated by DYANA. The 20 conformations with the lowest target function value (i.e., least violations of experimental restraints and van der Waals distances) were chosen for further refinement using restrained energy minimization. The resulting structures are shown in Fig. 5, after superimposing the backbone atoms. Pair wise RMSD calculated for backbone atoms for residues 1–10 for all 20 refined structures ranged from 0.14–1.06

FIGURE 5 Stereo views showing the superimposition of the backbone atoms of NKA for 20 structures generated by DYANA.

Å, with a mean value of 0.57 Å and a standard deviation of 0.18 Å.

In a stable secondary structure, both Φ and Ψ dihedral angles should have well-defined values. The Ramachandran plots of all 20 refined structures (included in Supplementary Material) indicate that the backbone dihedral angles consistently lie in the α-region and are solely within the allowed ranges. A helical-type backbone arrangement is indicated for the central region of NKA, in particular the stretch from D4 through M10 (Fig. 5), with some dynamic fraying of the helix termini. The measurement of $C{=}O_i$, NH_{i+3} versus $C{=}O_i$, NH_{i+4} distances along the stretch was made and the i, $i + 3$ distances correlated with the NKA having a preference for 3_{10} helix over regular helix. For the N-terminus of NKA the αH_i–αH_{i+3} distances were measured to be within 7 Å in the ensemble of conformations obtained. This distance is the threshold for defining a $\beta-$turn. However, it may not be appropriate to interpret this data in terms of a single turn conformation.

NMR spectroscopy is the method of choice for determining the 3D solution structure of peptides. However, a number of factors such as precision in the estimate of NOE values, use of short interproton distances, approximation of the rotational reorientation of the peptide in solution with a single-correlation time model, and the internal mobility of the peptide chain complicate the structure determination.

Due to the relatively large size of the DPC micelles, the correlation times in a micellar environment are expected to be much longer than the correlation times observed in an aqueous environment (Rizo et al., 1993). An additional complicating factor that must be recognized is the possibility of different regions of NKA experiencing different degrees of association with the micelles due to variations in hydrophobic and electrostatic effects. These factors make accurate quantification of the observed NOEs a major concern. The net result of the longer correlation times is that the observed NOEs may be weighted in favor of conformations that are induced by interactions with the micelles. This could result in an apparently higher percentage of secondary structure than is actually present.

DISCUSSION

CD and NMR studies reported here indicate that in aqueous solution NKA prefers to be in an extended chain conformation whereas in the presence of membrane mimetic solutions helical conformation is induced. The NMR studies reported here suggest that, in hydrophobic environment, part of the address domain and the whole of the message domain are folded whereas in water NKA has extended conformation. The likely situation is that NKA exists in equilibrium between two states in which there is a turn over residues 1–3 followed by a stretch of helical structures for residues 4–10, another where the helical region extends over residues from 4 through 10. In DPC, the structural equilibrium is biased toward a 3_{10}-helix from residues 4–10, though small populations of regular α-helix cannot be excluded in the solution ensemble since 3_{10}-helices are intermediates in the folding/unfolding pathways of regular helices. Also short linear peptides like NKA may be too short to sustain a well-defined regular helix in solution. In support of this hypothesis we may note that the NMR data for other tachykinins like eledoisin (Grace et al., 2003), kassinin (Grace et al., 2001), substance P in SDS (Young et al., 1994), DPC (Cowsik et al., 1997; Keire and Fletcher, 1996), and physalaemin in methanol (Chassaing et al., 1986a; Sumner et al., 1990) were also explained in terms of equilibrium between α-helix and 3_{10}-helix.

The amidated C-terminus of NKA in DPC comprises 3_{10}-helix or turn-like elements with some possible fraying of helix terminus. The ''address'' segment of NKA, although undergoing greater conformational averaging than the message domain, also retains substantial conformational order in DPC. This order may be interpreted as a loosely defined turn or an unstable continuation of 3_{10}-helix along the message domain. However, the stability of turns is not known since there are few reports on turns occurring in membrane mimetic solvents (Sonnichsen et al., 1992). However, the identification of folded conformation in N-terminus under hydrophobic conditions has some significance, as it may represent an essential feature of NK-2 binding. It is significant to note that N-terminus of NK-2 selective agonists, neuropeptide K, and NKA were also found to be folded in TFE (Horne et al., 1993).

Several structure-activity studies have been reported on NKA and its analogs. These studies have led to the identification of the amino acid residues in NKA that confer selec-

tivity and specificity for NK-2 receptors and the minimum structure required for activation of NK-2 receptors. The binding of tachykinin peptides and their fragments to NK-2 receptor sites in hamster urinary bladder membranes were examined and compared with the binding to NK-1 receptor sites in rat submandibular glands and it was found that NKA and its C-terminal fragments exhibited highest affinity and selectivity for bladder NK-2 receptor binding sites, whereas the N-terminal fragments of NKA lacked NK-2 receptor affinity and selectivity (Buck and Shatzer, 1988). The presence of aspartate residue in NK-2 agonists at the position analogous to residue 5 of substance P was found critical for the NK-2 receptor binding, suggesting that an ionic interaction may contribute to the binding energy (Cascieri et al., 1992; Buck and Shatzer, 1988). This hypothesis is further strengthened by a study involving probing of the binding domain of the NK-2 receptor with fluorescent ligands labeled with environment-sensitive probes (Turcatti et al., 1995). This study indicated that the N-terminal regions of all NK-2 agonists bound to the receptor were accessible to the solvent, which points to the extracellular regions of the receptor as the major binding determinant for the N-terminal address region of NK-2 agonists (Turcatti et al., 1995). Structure-activity studies on NKA performed using uterus from estrogen-primed rat indicated that although the N-terminal residue His was not essential for receptor activity, substitution of Lys-2 and Thr-3 decreases the potency of NKA, and that the amino acids at positions 4, 6, 7, 9, and 10 are very important for contractile activity of NKA (Fisher and Pennefather, 1998). The modification of residues Lys-2, Asp-4, and Ser-5 of NKA as reported in binding assays (Fisher and Pennefather, 1998; Cascieri et al., 1992; Buck and Shatzer, 1988; Warner et al., 2002) modulates its affinity and selectivity for the receptors. The residue at position 5 of NKA was shown to be critical in determining the selectivity between NK-2 and NK-3 receptors (Comis and Burcher, 1999). Studies on interaction of NKA with NK-2 receptor using site-directed mutagenesis and affinity labeling indicated that C-terminus of NKA binds within the transmembrane bundle of NK-2 receptor and the hydrophilic N-terminus of NKA interacts with the N-terminus and extracellular loop regions of the receptor (Bhogal et al., 1994; Huang et al., 1995; Labrou et al., 2001; Lecat et al., 2002). Further, structural comparison of NK-2 receptor agonists and antagonists by simulated annealing techniques revealed that there was a close superimposition of the three key residues, Phe, Leu, and Met, which are hypothesized to be the binding site for the NK-2 receptor (Giolitti and Maggi, 1994). Studies have also indicated the importance of C-terminal amidation for the agonist activity of natural tachykinins at NK-2 receptor (Patacchini et al., 1993). The C-terminal free acid derivatives of NKA and NKA (4–10) were much weaker agonists of NK-2 receptors than NKA or NKA (4–10), suggesting that the C-terminal amide group may not be directly involved in stimulation of the tachykinin receptors, but could induce agonist activity through a conformation effect (Patacchini et al., 1993).

Conventional binding assays have shown that substance P is the preferred ligand for NK-1 receptor and NKA, NKB binds more weakly than substance P. The current model of binding of substance P and other agonists at the NK-1 receptor suggests that the three C-terminal residues (G-L-M-NH_2) interact with a transmembrane region of the receptor and a change in the conformation of the three C-terminal residues will affect receptor binding. In a previous study, our group (Cowsik et al., 1997) determined that the structure adopted by substance P (the primary ligand for NK-1 receptor) is comprised of a helical mid-region with an extended C-terminus in the presence of DPC micelles. Seelig and co-workers (Seelig and Macdonald, 1989; Seelig et al., 1996) have suggested that the hydrophobic (F7, F8, L10) and a hydrophilic (Q6, G9) side chains in the C-terminus will position substance P at the receptor binding site in such a fashion as to lead to optimal binding. NMR studies have shown that a helical structure is the predominant structure adopted by NKA and NKB in the presence of membrane model system but in comparison to substance P, the helix length is extended in both NKA and NKB to include Gly-8 and Leu-9 residues (Whitehead et al., 1998, and our present investigation). Such a change in helix length alters the positions of the hydrophobic and hydrophilic side chains for the C-terminus, decreasing the ability of NKA to bind as effectively to the NK-1 receptor (Fig. 6). Furthermore,

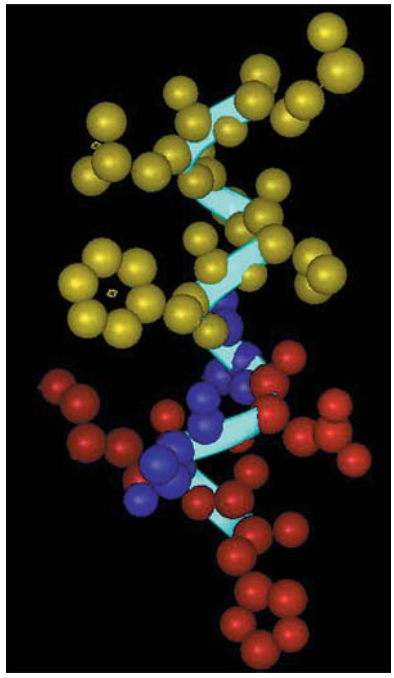

FIGURE 6 A graphic representation of the lipid-bound NKA conformation. The peptide backbone is shown as a ribbon tube (*blue*). Ionic residues are colored red, polar residues are colored purple, and the hydrophobic residues are colored yellow. The helical segment is clearly visible.

stabilization of helix through an increase in helix length results in a reduction of flexibility of the message domain, a situation determined to be unfavorable for NK-1 receptor binding (Seelig et al., 1996). It is interesting to note that increasing helical content in the conformation of C-terminus of NKA and prediction of helical content by Seelig et al. (1996) are consistent with the poor binding property of NKA to the NK-1 receptor. Further, the decreased ability of NKA in its binding to NK-1 can also be attributed to the absence of proline residue in the position analogous to that of residue 4 of substance P, as indicated by binding studies to be essential for high-affinity binding to NK-1 (Cascieri et al., 1992).

The results from our structural studies agree well with the findings of structure-activity studies and receptor-ligand interaction studies. It can be seen from Fig. 6 that the C-terminus presents a hydrophobic upper half comprised of Phe-6, Val-7, Leu-9, and Met-10 residues and the N-terminus presents a hydrophilic lower half comprised of the His-1, Lys-2, Thr-3, Asp-4, and Ser-5 residues. We propose that the hydrophobic C-terminus of NKA is being inserted into the transmembrane region of the receptor, with Phe-6, Leu-9, and Met-10 forming the anchoring points and contributing a major portion of the binding energy. The hydrophilic and solvent accessible N-terminus of NKA probably helps in maintaining the peptide conformation and positioning the C-terminus within the transmembrane region. The N-terminus interacts with the extracellular regions of the receptor and plays an important role in NK-2 receptor binding with His-1, Lys-2, and Asp-4 as anchoring points.

In conclusion, the results obtained in this investigation correlate well with the other findings of receptor-ligand interaction studies and are consistent with the proposed biologically active conformation of NK-2 receptor agonist. Moreover, the NMR results presented here agree well with the theoretical secondary structure predictions for turns and helices in NKA using program ALB by Wilson and coworkers (Wilson et al., 1994). Our studies indicate that the helical central core of NKA is better defined in DPC micelles than in SDS and TFE. The presence of helical core is more pronounced in DPC micelles than in TFE, wherein a series of dynamic turns in equilibrium involving the Thr3-Met10 segment was suggested (Horne et al., 1993). An increase in helical content is observed in the presence of lipid micelles with the helix extending from residue 4 to residue 10, in comparison to SDS wherein the helix extends from 6 to 10 (Whitehead et al., 1998). The presence of a loosely defined turn in the N-terminus preceding the helical core in the C-terminus of NKA is consistent with that observed in TFE. The overall conformational features adopted by NKA in DPC micelles correlate well with that reported for NKA in TFE and SDS micelles (Horne et al., 1993; Whitehead et al., 1998) and with that of other NK-2 agonists like eledoisin in DPC micelles (Grace et al., 2003). In a surface analysis of the averaged structures for the NK-2 selective agonists NKA and eledoisin, it is seen that the hydrophobicity profiles of both

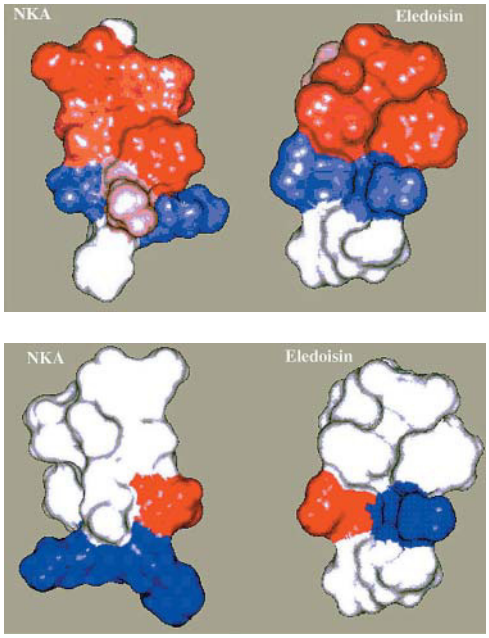

FIGURE 7 Comparison of (*top*) hydrophobicity and (*bottom*) charge state topography for NK-2 selective agonists NKA and eledoisin. The two structures have the same relative orientation to each other. For side-chain charges, red denotes a negative residue, blue denotes a positive residue, and gray denotes a neutral residue. For hydrophobicity, increasing red denotes increasing hydrophobicity, while blue denotes increasing hydrophilicity.

NKA and eledoisin are very similar (Fig. 7). On the basis of this correlation, it is interesting to note that conformation adopted by NKA in the presence of DPC micelles provides a biologically relevant structure and presents the structural motif typical of neurokinin-2 selective agonists.

SUPPLEMENTARY MATERIAL

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

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