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Trifluoroethanol-induced conformational changes in α- and β-neoendorphins monitored using hydrogen/deuterium exchange mass spectrometry and circular dichroism spectroscopy

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This article is dedicated to my Guru Michael Gross on the occasion of his 70th birthday. Happy Birthday, Michael, and many 2 happy returns of this blissful day.

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Alpha-neoendorphin
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Conformation of peptides
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Circular dichroism spectroscopy
Hydrogen/deuterium scrambling

ABSTRACT

Trifluoroethanol (TFE)-induced conformational changes in two endogenous opioid peptides α-neoendorphin (α-NE; YGGFLRKYPK) and β-neoendorphin (β-NE; YGGFLRKYP) were studied using hydrogen/deuterium exchange (HX), coupled with electrospray ionization mass spectrometry (ESI-MS). HX data revealed that both peptides predominantly exist in an unfolded conformation in water and assume a compact conformation with increasing concentrations of TFE. Through tandem mass spectrometry it was identified that, because the LRKY segment in α-NE and N-terminal YG and RKY regions in β-NE contain the unexchanged hydrogens, they participate in secondary structure formation. The circular dichroism (CD) spectra of the peptides acquired in water and in mixed solvents that are comprised of varied amounts of TFE in water showed the presence of both β-turn and helical type secondary structures in these peptides. With increasing concentrations of the helical-inducing solvent, TFE, the α-helical content increases and that of β-turn decreases.

1. Introduction

Alpha-neoendorphin (α-NE; YGGFLRKYPK) and beta-neoendorphin (β-NE; YGGFLRKYP) are the endogenous peptides that belong to the dynorphin family within the super family of endogenous opioid peptides [1]. They are derived from the same precursor protein that also produces dynorphin A and dynorphin B [2]. α-NE and β-NE are also referred to as "BIG" leu-enkephalins and were first isolated from porcine hypothalamus [3]. Their amino acid sequences are identical, except for an additional lysine at the C-terminus of α-NE. Both these peptides have affinity for kappa opioid receptors, but their physiological significance is not yet completely understood [4].

In general, endogenous peptides encounter both aqueous and lipid-rich environments *en route* to their membrane embedded protein receptors. During the approach to the binding site, the medium in which the peptide moves through gradually becomes more nonpolar. Change in the environmental conditions is known to induce conformational changes in peptides, which often accompany changes in their functions. In order to gain understanding of how a peptide performs its biological functions, it is essential to identify its precise conformational states that exist in the receptor membrane environment [5]. In vivo studies have frequently used trifluoroethanol (TFE) [6,7], methanol [8,9], and dimethyl sulfoxide [10] to mimic the membrane environments. Especially, TFE is the preferred solvent because it simulates the hydrophobic environment and better stabilizes the secondary structures in peptides owing to its hydrophobicity and hydrogen bond-forming properties [11,12]. There is a clear understanding in the literature of why TFE favorably induces secondary structures in proteins/peptides. Its dielectric constant approximates that of the interior of proteins and is one-third of that of water. This property strengthens intramolecular interactions between charged groups. The differences in solvent acidity and basicity between TFE and water are responsible for increased stability of hydrogen bonds. TFE forms

Abbreviations: ACTH, adrenocorticotropic hormone; CD, circular dichroism; CID, collision-induced dissociation; ESI, electrospray ionization; FT-IR, Fourier-transform-infrared; HX, hydrogen/deuterium exchange; MS/MS, tandem mass spectrometry; NE, neoendorphin; NMR, nuclear magnetic resonance; TFE, trifluoroethanol.

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weaker bonds with carboxyl and amide groups of peptides, thus stabilizing intramolecular hydrogen bonds in peptides. Also, the ability of TFE to form clusters in aqueous solution could lead to the development of secondary structures.

Circular dichroism [13,14], X-ray crystallography [13,14], nuclear magnetic resonance (NMR), [14,15], Fourier-transform-infrared (FT-IR) [14], Raman, ultraviolet (UV)–visible absorption and fluorescence spectroscopy [14] are some of the techniques that are frequently used in protein/peptide conformational studies. In recent years, ESI-MS, in combination with hydrogen/deuterium exchange (HX), has emerged as a viable option for conformational studies [16–22], primarily owing to speed, sensitivity and specificity of the analysis. Also, the mass spectrometry analysis performed in the gas phase is relevant to the exchange chemistry carried out and subsequently quenched in the solution-phase [23]. Currently, HX has become a standard method to probe the conformations and folding/unfolding dynamics of peptides and proteins [18–22,24–27]. The underlying basis of the HX protocol is that various hydrogens in the peptide/protein structure, depending upon their accessibility to the deuterated solvent, have distinct exchange rates [20]. The hydrogens that are involved in the secondary structure formation are not fully exposed to the solvent; therefore they either exchange at slow rate or do not exchange at all. These hydrogens upon their release from secondary structural elements, (e.g., hydrogen bonding), undergo faster exchange. Intramolecular hydrogen bonds are found in secondary structures such as α-helices and β-sheets. Information on the extent of isotopic exchange at individual amino acid residue level can be gleaned using tandem mass spectrometry (MS/MS), which for last several decades has been an indispensable tool in sequencing of peptides. This information is obtained by comparing the *m/z* of sequence-specific fragment ions produced by collision-induced dissociation (CID) of the peptide before and after HX. This methodology has been successfully used to probe the higher-order structures of proteins and peptides [24–27]. We have also applied these methodologies (HX-ESI-MS and HX-ESI-MS/MS) to investigate solvent-induced conformational changes in adrenocorticotropic hormone (ACTH), β-endorphin, methionine and leucine enkephalins, dynorphin (1–13) and lipid vesicles-bound angiotensins [28–32].

In the present study, ESI-MS, has been used to probe the conformational changes in α- and β-neoendorphins in water and in helical-inducing solvent, TFE. These results are supplemented by the CD spectroscopy experiments.

2. Methods and materials

α-Neoendorphin and β-neoendorphin were purchased from American Peptide Company (Sunnyvale, CA, USA). Trifluoroethanol and deuterated solvents (TFE-d_3, 99 atom %D, acetic acid-d_1, deuterium oxide 99.9 atom %D) were obtained from Aldrich (Milwaukee, WI, USA).

2.1. Hydrogen/deuterium exchange-ESI mass spectrometry

Stock solutions (800 μM) of α-NE and β-NE were prepared by dissolving appropriate amounts separately in deionized water and in various mixed solvents that contained 20–80% TFE in water. HX was initiated by diluting an aliquot of the stock solution with deuterated solvent to a final peptide concentration of 40 pmol/μL and a deuterium content of 95%. The exchange was studied at various time points that ranged from 30 s to 24 h. The pH of all HX solutions was adjusted to 7.0 by adding acetic acid or acetic acid-d_1 to the solution. The exchange reaction was quenched immediately after the incubation period with an ice-cold solution of 20% acetic acid-d_1/D_2O (to lower the pH to about 2.5 and the temperature to 0 °C). The resulting solutions were then immediately infused into the ESI source of the LCQ^{deca} quadrupole ion trap mass spectrometer (Thermo-Finnigan) at a flow rate of 0.4 μL/min. The full scan mass spectra were acquired by scanning the instrument in the *m/z* range of 200–2000. The zoom scan of the $[M+H]^+$ ion region was obtained by scanning the ion trap in the appropriate narrow mass range. The collision energy was set at 40% of the maximum for CID experiments. The ESI source was operated at 100 °C. Each MS and MS/MS spectrum is the average of several scans, and each HX experiment was repeated thrice. Isolating the source from the surroundings, flushing nitrogen continuously at a flow rate of 15 mL/min, and equilibrating the syringe and transfer lines with a deuterated solvent helped to minimize the extent of back-exchange. Our previous work has shown that under these conditions the extent of back-exchange is less than 6% [30]. During the short analysis time (10–30 s), the forward exchange of backbone amide hydrogens and of involved in secondary structure formation is unlikely under these ion source conditions owing to their very slow exchange rate. Rest of the sidechain hydrogens have already undergone fast exchange.

2.2. Calculations of deuterium incorporation in the peptide molecules

The number of exchanged hydrogens (H_x) in the peptide at several time points can be directly calculated from the MS data by observing the *m/z* ratio of the most abundant isotopic peak of the *n*th charge state and using the following expression [20];

$$H_x = M_D - M = n[(m/z)_D - D^+] - M \quad (1)$$

where *M* is the molecular mass of the non-deuterated species and M_D is the measured molecular mass of the partially deuterated species. The information about the number of exchanged hydrogens from the MS/MS spectra is derived from the *m/z* values of b and y ions by using the following expressions [33].

$$b_j^{n+}: \quad H_x = n[M_D(b_j^{n+}) - M(b_j^{n+}) \quad (2)$$

$$y_j^{n+}: \quad H_x = n[M_D(y_j^{n+}) - M(y_j^{n+}) - 1] - 1 \quad (3)$$

2.3. Circular dichroism spectra

Circular dichroism spectra were acquired on AVIV 62DS circular dichroism spectropolarimeter. Samples were prepared by dissolving α-NE and β-NE separately in water (0.2 mg/mL; in 0.01 M Tris–HCl, pH 7.0), and in mixed solvents containing 20, 50, and 80% TFE in water. The data points were collected in delta epsilon units with the start and final values of the wavelength set at 190 and 260 nm, respectively, and wavelength step size at 1.0 nm.

3. Results

3.1. Hydrogen/deuterium exchange experiments

The full-scan spectra of both peptides were acquired in the *m/z* range of 200–2000 (spectra not shown); each spectrum is characterized to contain singly, doubly, and triply protonated peptide molecules, with doubly charged ion ($[M+2H]^{2+}$) being the most abundant. For simplicity in calculations, the $[M+H]^+$ ion was chosen for the zoom scan spectra; Fig. 1A and B show these spectra for α-NE in water and after 24 h of HX in D_2O, respectively. The *m/z* of the $[M+H]^+$ ion of the non-deuterated α-NE increases from 1228.7 to 1249.7 (the $[M+D]^+$ ion) upon deuteration indicating that a total of 20 (out of 21) hydrogens have exchanged in the aqueous medium. Similar data for β-NE showed exchange of 17 (out of 18) hydrogens

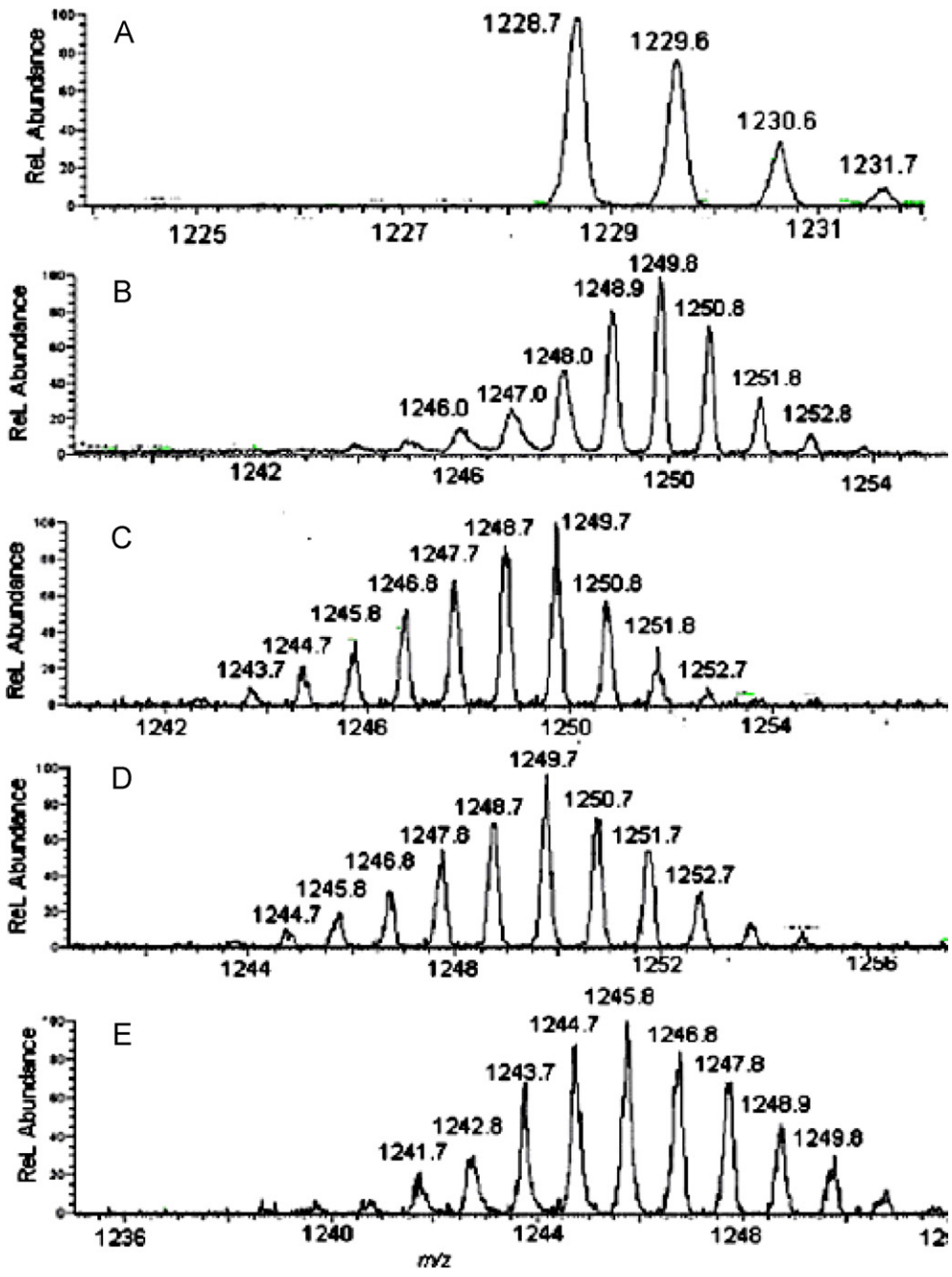

Fig. 1. Zoom scan spectra of the $[M+H]^+$ ion region of α-NE in (A) water and after 24 h HX in (B) D_2O and in mixed solvent system of (C) 20% (D) 50% and (E) 80% TFE-d_3 in D_2O.

(see Fig. 2A and B). Thus, only one of the labile hydrogens remains shielded from the solvent in both peptides.

To determine TFE-induced conformational changes in α-NE and β-NE, HX was performed at various concentrations (20, 50, and 80% TFE-d_3 in D_2O) of TFE. It was found that for α-NE, 1, 3 and 5 labile hydrogens remain unexchanged in the 20, 50 and 80% TFE-d_3–D_2O mixed solvents (see Fig. 1C–E), respectively, and for β-NE, those numbers are 2, 4 and 5 in the respective TFE-d_3–D_2O mixed solvents (see Fig. 2C–E).

Further evidence of the secondary structure formation in TFE comes from the time-resolved HX experiments that were performed in D_2O and in 50% TFE-d_3–50% D_2O and 80% TFE-d_3–20% D_2O mixed solvent systems (data not shown). In water, all of the labile hydrogens were exchanged within 30 s. No further deuterium incorporation was observed even when HX was continued for longer periods of 24 h and 48 h.

To probe the site specific deuterium incorporation at a single amino acid level, the CID-MS/MS spectra of the doubly charged peptide ions were acquired in water, D_2O, and 50% TFE-d_3–50% D_2O mixed solvent before and after 1 h HX. In the literature, it is a common practice to choose 50–60% TFE in water as the solvent composition for determining the secondary structure of peptides, because this composition offers a unique blend of hydrophilic and hydrophobic environment, which mimics extensively the *in vivo* membrane environment. α-NE and β-NE both exhibit identical fragmentation patterns to yield mostly b- and y-type sequence-specific ions; a few a-type ions are also observed. The type of sequence-specific ions observed for α-NE in water, D_2O and 50% TFE-d_3–50% D_2O mixed solvent, their *m/z* values and the numbers

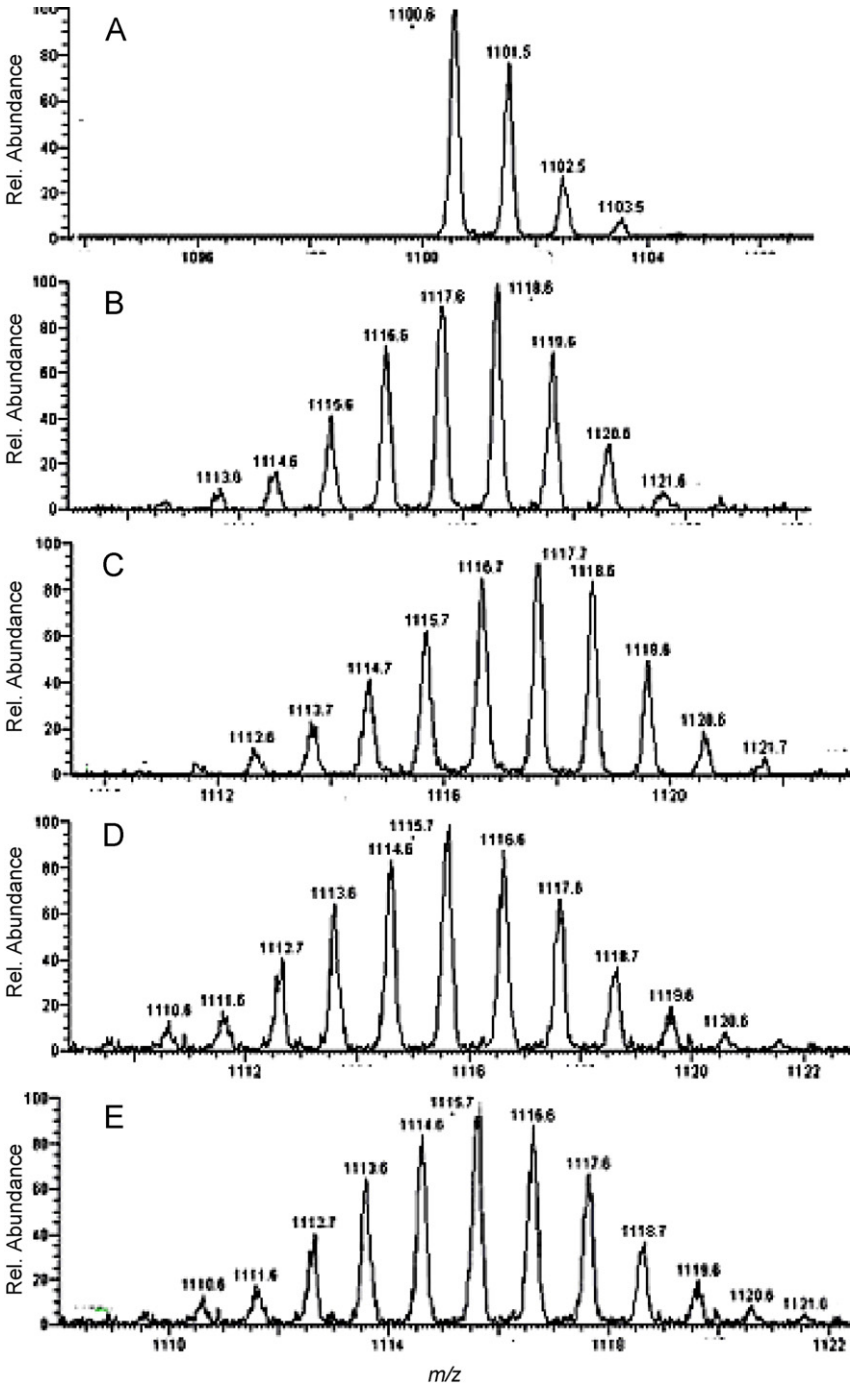

Fig. 2. Zoom scan spectra of the $[M+H]^+$ ion region of β-NE in (A) water and after 24 h HX in (B) D_2O and in mixed solvent system of (C) 20% (D) 50% and (E) 80% TFE-d_3 in D_2O.

of exchanged hydrogens calculated using Eqs. (2) and (3) are listed in Table 1. In water, the N-terminal sequence ions were detected at *m/z* 221.3(b_2), 278.4(b_3), 822.3(b_7), 951.6(b_8), and 193.3(a_2) and the C-terminal y-type ions at *m/z* 244.2(y_2), 533.7(y_9^{2+}), 804.5(y_6), and 951.6(y_7) (see Fig. 3). The y_9^{2+} sequence ion is the most abundant ion in the spectrum. The CID of deuterated α-NE also yielded the same pattern of sequence-specific ions (i.e., the b- and y-type ions dominated the spectrum).

The CID-MS/MS data for β-NE in water, D_2O, and 50% TFE-d_3–50% D_2O mixed solvent are reproduced in Fig. 4 and Table 2. In water, the N-terminal sequence-specific ions were detected at *m/z* 193.0(a_2), 221.0(b_2), 397.1(a_4), 822.4(b_7) and 985.4(b_8) and the C-terminal y-type sequence-specific ions at *m/z* 279.1(y_2), 563.2(y_4), 676.3(y_5), and 880.5(y_7). The most abundant ion is of *m/z* 493.4, which is identified as b_8^{2+} ion.

3.2. Circular dichroism experiments

Although HX-ESI-MS can reveal the existence of the secondary structure and pinpoint the amino acid residue participating in the secondary structure formation, the type of secondary structure is difficult to predict from the MS data. This task can be accomplished through circular dichroism spectroscopy. Representative CD spectra of α-NE and β-NE in water and in various mixed solvent systems that contain 20, 50, and 80% TFE in water are shown in Fig. 6A and B, respectively. The exact contribution of secondary structural

Table 1
The number of hydrogens exchanged in the sequence-specific ions of α-NE.

Fragment ions	m/z in H_2O	$(m/z)_D$ in 50% TFE-d	$(m/z)_D$ in D_2O	$H_x(D)$ in 50% TFE-d	$H_x(D)$ in D_2O
$[M+H]^+$	1228.7	1247.8	1249.8	18.1	20.1
a_2	193.3	197	197	3.7	3.7
b_2	221.3	225.1	225.1	3.8	3.8
y_2	244.2	250.1	250.1	3.9	3.9
b_3	278.4	283.1	283.1	4.7	4.7
y_9^{2+}	533.7	542.7	543.7	15.0	17.0
y_6	804.5	818.5	820.4	12.0	13.9
b_7	822.3	835.5	836.4	13.2	14.1
y_7	951.6	966.6	968.6	13.0	15.0
b_8	985.4	999.6	1001.6	14.2	16.2

elements was calculated by using the in-built software that was supplied with the instrument. The results of these calculations are presented in Table 3.

4. Discussion

α-NE (MW 1227.62 Da) contains 21 exchangeable hydrogens; eight are the backbone amide hydrogens, 10 side chain hydrogens (eight in Arg and Lys amino groups and two in Tyr hydroxyl groups), and three at two termini of the peptide. Similarly, β-NE (MW 1099.58 Da) contains a total of 18 exchangeable hydrogens, three less than α-NE owing to one missing lysine in its sequence. Comparison of the zoom-scan spectra shows that nearly all labile hydrogens (to be precise 20 out of 21) have been replaced with deuterium in both peptides. The evidence from CD spectroscopy (see Table 3) of the existence of secondary structural elements for

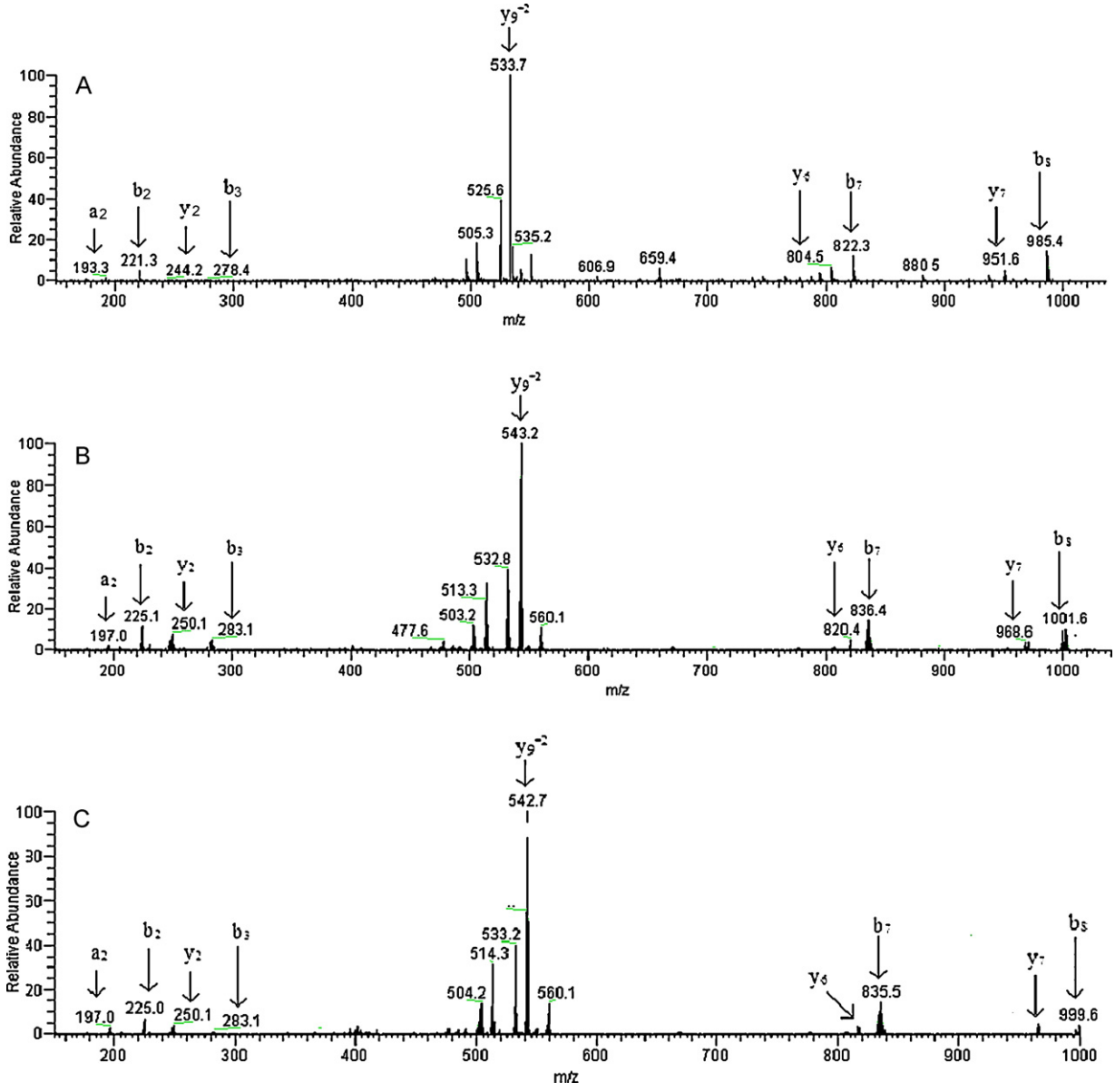

Fig. 3. CID-MS/MS mass spectra of α-NE in (A) water and after 24 h HX in (B) D_2O and in mixed solvent system of (C) 50% TFE-d_3 in D_2O.

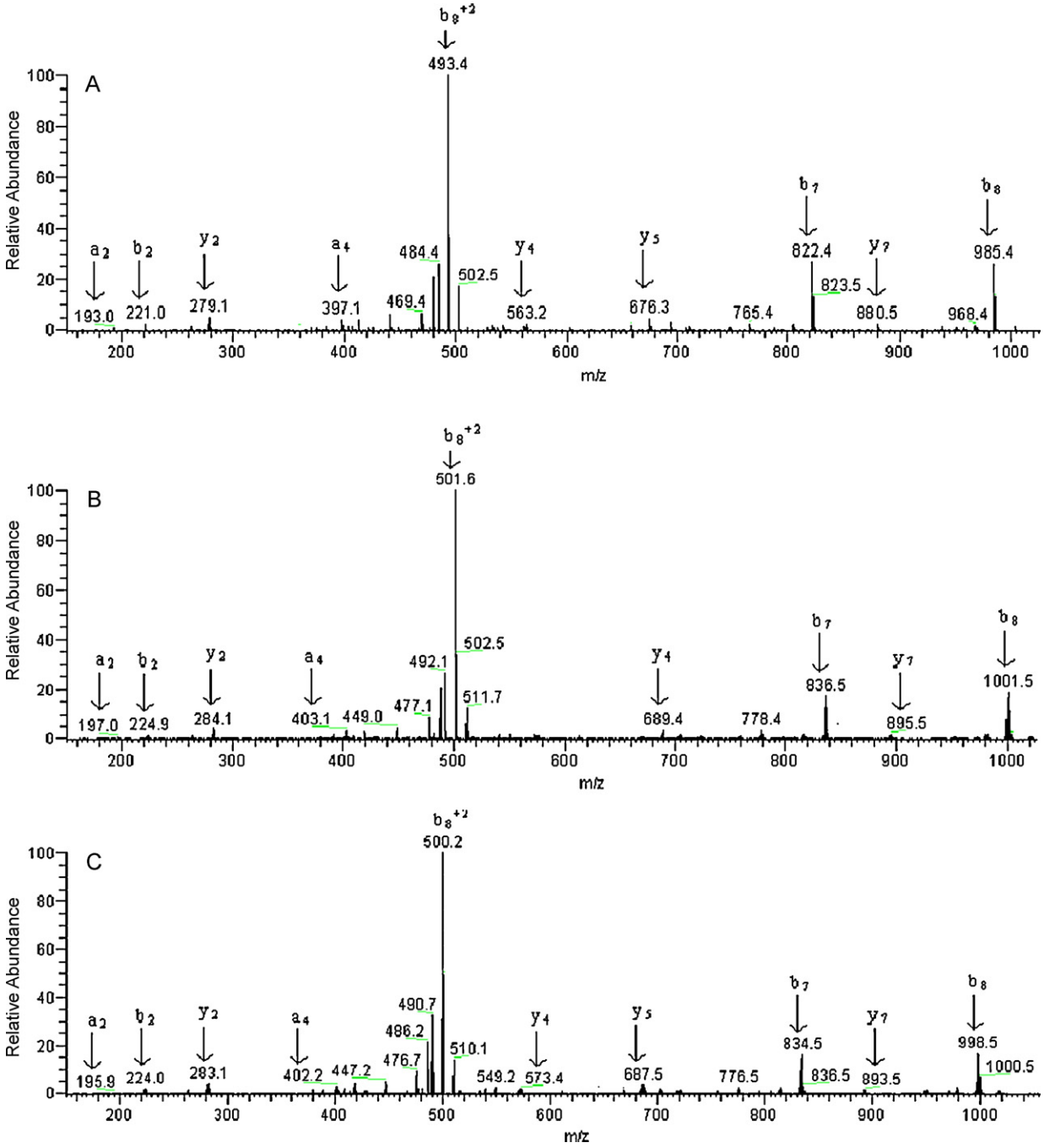

Fig. 4. CID-MS/MS mass spectra of β-NE in (A) water and after 24 h HX in (B) D_2O and in mixed solvent system of (C) 50% TFE-d_3 in D_2O.

Table 2
The number of hydrogens exchanged in the sequence-specific ions of β-NE.

Fragment ions	m/z in H_2O	$(m/z)_D$ in 50%TFE-d	$(m/z)_D$ in D_2O	$H_x(D)$ in 50% TFE-d	$H_x(D)$ in D_2O
$[M+H]^+$	1100.6	1115.7	1118.6	14.1	17.0
a_2	193	195.9	197	2.9	4.0
b_2	221	224	224.9	3.0	3.9
y_2	279.1	283.1	283.1	2.0	2.0
a_4	397.1	402.2	403.1	5.1	6.0
b_8^{2+}	493.4	500.2	501.6	13.4	16.4
y_4	563.2	573.4	575.4	8.2	10.2
y_5	676.3	687.5	689.4	9.2	11.1
b_7	822.4	834.5	836.5	12.1	14.1
y_7	880.5	893.5	895.5	11.0	13.0
b_8	985.4	998.5	1001.5	13.1	16.1

Table 3
Secondary structural elements of α-NE and β-NE calculated from CD spectra.

	Water (%)	20% TFE/water	50% TFE/water	80% TFE/water
β-NE				
Helix	7.20	11.10	15.10	23.40
β-Turn	29.80	32.30	21.30	19.90
Random coil	64.20	59.80	47.20	45.20
α-NE				
Helix	9.20	12.10	14.40	26.10
β-Turn	31.30	34.30	23.40	21.30
Random coil	67.70	59.80	48.20	46.10

both peptides in water suggests that this hydrogen is involved in secondary structure development. The possibility of it being the back-exchanged hydrogen cannot be ruled out. These observations suggest predominantly random coil open conformation for both these peptides in the aqueous environment with some elements of secondary structures.

The finding of greater number of labile hydrogens remaining unexchanged in water–TFE mixed solvents points to formation of secondary structures in these solvents. The time-resolved HX experiments further support the existence of secondary structures for neoendorphins in the presence of TFE. In water, all of the labile hydrogens were exchanged within 30 s. This type of fast HX behavior is a common phenomenon for several other peptides [28–35]. The HX rates were relatively slower in 50% and 80% solvent systems. The faster exchange rates of the two target peptides in water can be attributed to the highly dynamic nature of hydrogen bonds and to the presence of a random conformation in water. The slower exchange rates for peptides dissolved in mixed solvents are consistent with more compact structures.

The identity of the amino acid residues involved in the secondary structure formation of α-NE was obtained from the CID-MS/MS spectra (see Fig. 5A). This involves identifying the location of three unexchanged hydrogens in α-NE. Comparison of the *m/z* values

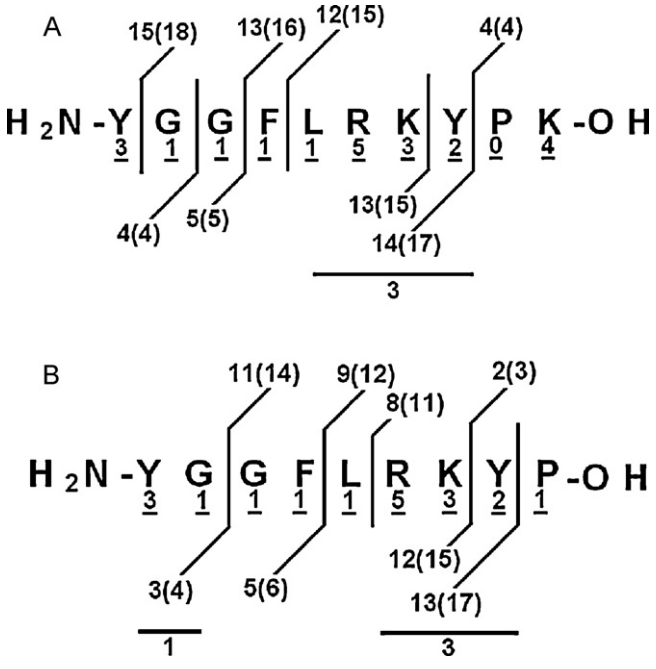

Fig. 5. The number of hydrogens exchanged on N-terminal b-type and C-terminal y-type fragment ions of (A) α-NE and (B) β-NE, calculated from ESI-MS/MS spectra in mixed solvent system of 50% TFE-d_3 in D_2O. The number of labile hydrogens present in individual amino acids is underlined below each residue and those presents in a particular sequence-specific ion are listed in the parenthesis; the number of exchanged hydrogens in those sequence-specific ions is shown outside the parenthesis, and the unexchanged hydrogens are given under the bar.

of the sequence-specific ions observed in water and in the 50% TFE-d_3–50% D_2O mixed solvent (see Table 1) indicates none of the three unexchanged hydrogens is present in the N-terminal YGG and C-terminal PK regions of α-NE as complete exchange of all labile hydrogens was observed in a_2, b_2, b_3, and y_2, sequence-specific ions. The shift in the *m/z* of b_7 and b_8 ions to 835.5 and 999.6 reveals that the two of the three unexchanged hydrogens are present in the YGGFLRK segment, and the third one belongs to Tyr-8. Comparison of the *m/z* values also reveals that the Phe amide hydrogen is free to exchange because the LRKYPK (y_6) and FLRKYPK (y_7) both contain all three unexchanged hydrogens. Thus all three unexchanged hydrogens have been identified: two are located in LRK and one at Y-8. The analysis of the y_9^{2+} ion (GGFLRKYPK) also supports this conclusion. This segment also contains three unexchanged hydrogens, and they are part of the FLRKY portion of this segment (YGG and PK portions were concluded above to have all hydrogens exchanged).

β-NE contains four protected hydrogens, their identity was determined from the MS/MS spectra (Fig. 4 and Table 2). Similar comparison of the *m/z* values of the sequence-specific ions observed in water and 50% TFE-d_3–50% D_2O indicates that one of the four protected hydrogens in β-NE resides in the N-terminal YG pair of residues and three in the RKY fragment (see Fig. 5B). All these four protected hydrogens are believed to participate in secondary structure formation.

However, caution must be exercised in using the CID data owing to the possibility of H/D scrambling [36–46], which is strongly influenced on the peptide structure, nature of the charge carrier and instrumental conditions. The extent of scrambling is a debatable matter. Some researchers have reported complete scrambling in both b- and y-ions [36,41], whereas others have reported low degree of scrambling with b-type ions being free from scrambling [37,42–46]. Hamuro et al. have developed a peptide probe (RPYIL) and a simple test to detect the occurrence of H/D scrambling [41]. The peptide was completely deuterated and then back-exchanged to replace all but the amide deuteriums on Y, I, and L residues. Complete scrambling was observed in this peptide upon CID as was evident by the extent of deuterium incorporation in b_2 and other b-type ions, which is proportional to the number of labile hydrogen sites in those ions. These authors also reported that scrambling is absent at 30% or higher collision energy. Another research group has recently reported that scrambling does not occur when higher charge state precursor is subjected to CID [46]. In the present study, the precursor ion was of 2+ charge state and 40% collision energy was used to fragment it. Therefore, scrambling may not be occurring to a significant extent, because in the event of scrambling, the deuterium will be equally distributed among all of the exchangeable hydrogen positions [41]. As a corollary, all of the protected hydrogens should also be equally distributed all along the peptide, which is not the case; they are mainly localized in the LRKY region in both α-NE and β-NE. If at all scrambling is occurring, it is not significant and will not have adverse impact on the structure derived.

In the CD spectrum, characteristic features of the random coil structure are a strong negative band at near 200 nm and a weak maximum at 220 nm, both are clearly visible in Fig. 6. With increasing content of TFE, the random coil structure is replaced by α-helix and β-turn secondary structural elements as evident from the decrease in intensity of these two peaks. From Table 3, [14] it is apparent that both peptides exist predominantly in random coil conformation in water and exhibit some elements of helix and β-turn in TFE. The helix-inducing property of TFE is evident from these results. The helical content of both these peptides gradually increases (from 7.2 to 23.4%) with increase in the TFE concentration from 0 to 80%. On the other hand, the content of β-turn is highest at 20% TFE concentration and decreases at higher concentrations

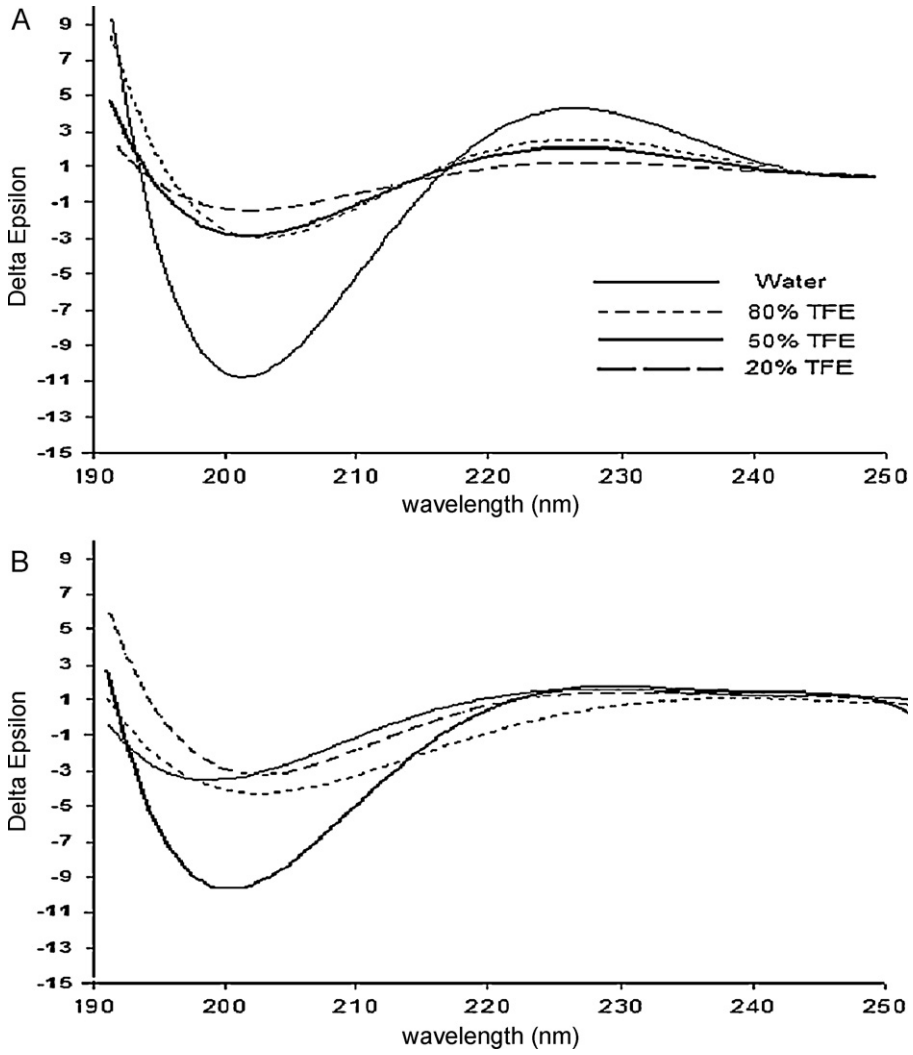

Fig. 6. Circular dichroism spectra of α-NE (A) and β-NE (B) in various mixed solvent systems of 20–80% TFE in water.

of TFE. These results corroborate the conclusions drawn from the HX-ESI-MS data, which have shown that both peptides exist in open conformation in water and adopt a compact conformation at higher concentrations of TFE.

5. Conclusions

In this study, we have demonstrated the usefulness of HX-ESI-MS to investigate the conformational changes in small peptides. This study has established that α-NE and β-NE both exist predominantly in random coil conformations in water and assume secondary structures in the presence of TFE. The first five amino acid residues in α-NE and β-NE are identical with the sequence of leucine enkephalin, which was shown by us [30] and others to exist as a 5 → 2 β-turn structure in mixed media containing TFE and water [8,47]. Therefore, it is suggested that the N-terminal YGGFL exists as a 5 → 2 β-turn structure and the C-terminal half in helical form. Circular dichroism experiments showed that α-helical and β-turn elements both are present in the secondary structure. It was also clear from the CD data that, consistent with the helical-inducing ability of TFE, the helical content of α-NE and β-NE increases and β-turn decreases with the increase of TFE concentration in the surrounding solvent environment. Thus, both these peptides undergo a progressive conformational change in mixed TFE/water solvents.

Acknowledgements

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