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NMR studies on thermal stability of α-helix conformation of melittin in pure ethanol and ethanol–water mixture solvents

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Thermal stability of the α-helix conformation of melittin in pure ethanol and ethanol–water mixture solvents has been investigated by using NMR spectroscopy. With increase in water concentration of the mixture solvents (from 0 wt% to ~71.5 wt%) as well as temperature (from room temperature to 60 °C), the intramolecular hydrogen bonds formed in melittin are destabilized and the α-helix is partially uncoiled. Further, the hydrogen bonds are found to be more thermally stable in pure ethanol than in pure methanol, suggesting that their stability is enhanced with increase in the size of the alkyl groups of alcohol molecules. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: α-helix; ethanol; hydrogen bond; hydrophobic interaction; melittin; NMR; thermal stability

Introduction

It has been known that alcohols induce conformation change in proteins and peptides such as destruction of the native structure and/or formation of α-helices. Ubiquitin [1,2] and lysozyme [3], for example, transform to a molten globule-like conformation without tertiary structure by addition of alcohol to an aqueous solution, whereas cytochrome *c* [4] and β-lactoglobulin [5,6] to a peculiar conformation containing a large amount of helical structure. The alanine-based short peptides, on the other hand, are induced to form an α-helix, and its conformation is more stabilized in proportion to a concentration of alcohol [7].

Melittin, a main component of honeybee venom, is a short peptide composed of 26 amino acid residues: Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-Gln [8]. It takes a random coil conformation in an aqueous solution at low concentrations and acidic pH [9], while it undergoes a transition to α-helix conformation by adding alcohol. Hirota *et al*. demonstrated that although the helical conformation subsequent to the transition is independent of the kind of alcohol, the tendency of α-helix formation is enhanced with an increase in a size of alkyl groups of alcohol molecules by using CD spectroscopy [10]. They emphasized that hydrophobic interactions between alkyl groups of alcohol molecules and hydrophobic side chain groups of melittin play a significant role in forming α-helix conformation. Kinoshita *et al*. explained the size effect of alkyl groups and the mechanism of helix formation on the basis of the reference interaction site model theory [11]. These studies strongly suggest that thermal stability of helical conformation is also affected by the size of the alkyl groups, although experimental evidence has not been obtained.

Several NMR studies have focused on melittin in pure methanol and revealed the following conformational features: (i) melittin structure is composed of two regular α-helices (residues 2–11 and 13–26) and a kink section keeping some helical character (around residues 11–13) [12]; (ii) the intramolecular hydrogen bonds around the middle and the C-terminal have rather less stability [13]; and (iii) the α-helix conformation is mostly preserved up to 60 °C [14]. To elucidate the size effect of an alkyl group on thermal stability, investigation on melittin in alcohol of a larger-sized alkyl group is needed. In this paper, temperature dependence of the α-helix conformation in pure ethanol and ethanol–water mixture solvents is examined by ^{1}H NMR measurements, and moreover, thermal stability of the α-helix in those solvents and in a pure methanol is compared in the light of the intramolecular hydrogen bonds. To our knowledge, NMR study on melittin conformation in ethanol is the first attempt.

Materials and Methods

Melittin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used without further purification. Fully deuterated ethanol (C_2D_5OD with 99.97% one) and water (distilled H_2O and D_2O with 99.97% purity) were purchased from Nacalai Tesque (Kyoto, Japan) and partially deuterated ethanol (C_2D_5OH with 99.6% purity) from C/D/N Isotopes Inc. (Canada). For the NMR measurements, melittin was dissolved in pure ethanol and ethanol–water mixture solvents to a concentration of 1.0–3.3 mM. Because self-association of melittin molecules takes place by adding salts, pH measurements of a sample solution were not performed before the NMR measurements to avoid mixing with an internal KCl solution of a pH meter. After the accomplishment of all the NMR experiments, however, the pH values of all the sample solutions were 7.5–8.0.

All ^{1}H-NMR experiments were performed on a Varian Unity Inova 500 MHz NMR spectrometer. One-dimensional (1D) ^{1}H NMR spectra and two-dimensional (2D) ^{1}H-^{1}H NMR spectra of COSY and NOESY were acquired. The NMR experiments and data

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processes were carried out as previously described in the literature [14]. In Table 1, the ethanol–water mixture solvents and the sample concentrations used in the 2D NMR measurements are listed together with experiment temperatures.

Results and Discussion

Thermal Stability of α-helix Conformation in Pure Ethanol

The CD studies demonstrated that melittin forms α-helix conformation in an ethanol solvent at a room temperature [10]. In our study, we elucidate not only melittin conformation but also its thermal stability by NMR.

Figure 1 shows three portions of the ^{1}H-^{1}H NOESY spectra of melittin in pure ethanol (C_2D_5OH) at 25 °C; (A) the main chain amide proton (NH) region, (B) the fingerprint region and (C) the region of cross peaks between an α proton (αH) and a side chain. As shown in Figure 1(A), the cross peaks between the NH protons of the ith and $i+1$th residues ($d_{NN}(i, i+1)$ NOE peaks) are sequentially connected. However, connectivities are interrupted at some residues in addition to Pro-14 for which there is no NH proton. NH protons of Gly-1, Ile-2 and Gly-3 are not detected owing to fast exchange with hydroxyl protons (OHs) of the surrounding ethanol molecules. Those of Lys-7, Leu-13 and Lys-23 cannot be identified owing to an overlap with another NOE peak, whereas in the fingerprint region (Figure 1(B)), a number of inter-residue and intra-residue cross peaks between the αH and NH protons emerge. In particular, the cross peaks between the αH of the ith residue and the NH of the $i+3$th/$i+4$th one ($d_{\alpha N}(i, i+3)$ and $d_{\alpha N}(i, i+4)$ NOE peaks) are enclosed with broken squares. The observation of these peaks provides a clear evidence for the presence of an α-helix conformation in melittin [15]. Appearance of the cross peaks between the αH of the ith residue and the βH of the $i+3$th one ($d_{\alpha\beta}(i, i+3)$ NOE peaks) also indicates that helical conformation is formed in the region between the ith and $i+3$th residues (Figure 1(C)).

Table 1. Summary of mixture solvents, concentrations and sample temperatures in 2D ^{1}H NMR

Solvent	Concentration (mM)	2D NMR	Temperature (°C)
100 wt% C_2D_5OH	1.0	COSY	0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60
		NOESY	20, 25
100 wt% C_2D_5OD	1.3	NOESY	15, 20, 25, 30, 35, 40, 45, 50, 55, 60
74.5 wt% C_2D_5OH/ 25.5 wt% H_2O	2.8	COSY	25, 30, 35, 40, 45, 50, 55, 60
48.4 wt% C_2D_5OH/ 51.6 wt% H_2O	3.3	COSY	10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60
		NOESY	25, 30
48.6 wt% C_2D_5OD/ 51.4 wt% D_2O	2.8	NOESY	25, 30, 35, 40, 45, 50, 55, 60
28.9 wt% C_2D_5OH/ 71.1 wt% H_2O	3.1	COSY	20, 25, 30, 35, 40, 45, 50
		NOESY	25
28.0 wt% C_2D_5OD/ 72.0 wt% D_2O	3.2	NOESY	30, 35, 40, 45, 50, 55, 60

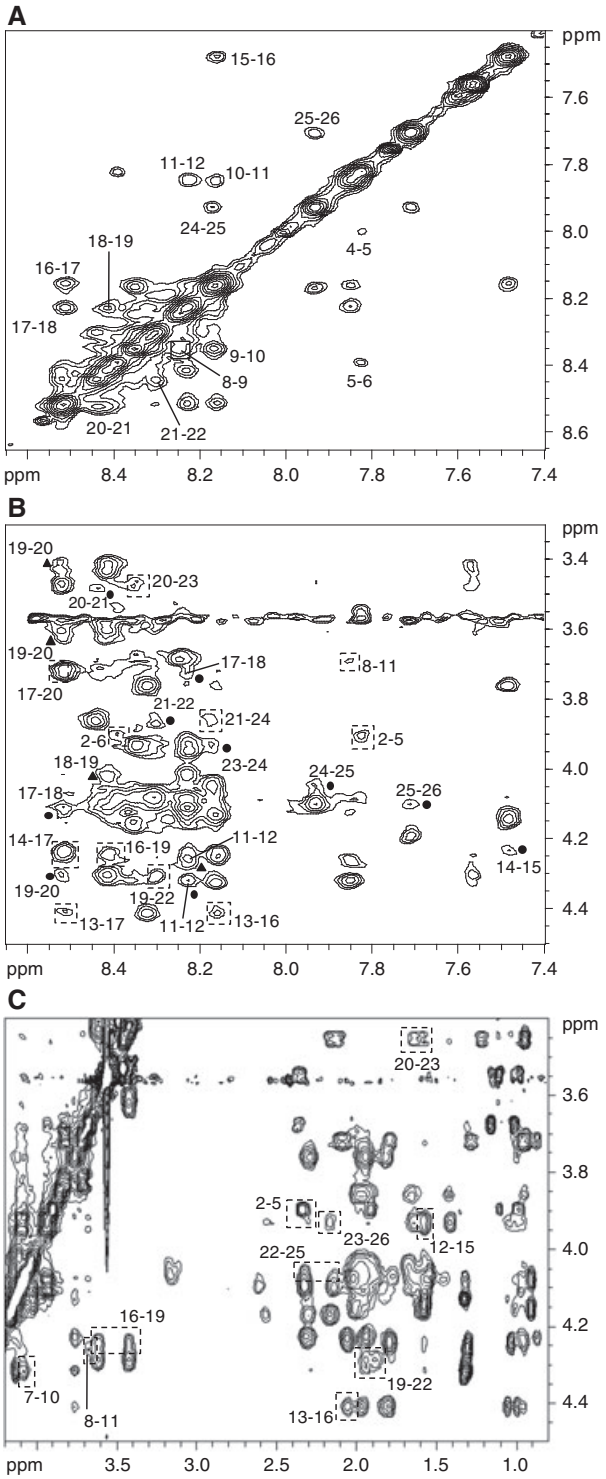

Figure 1. Three regions of the ^{1}H-^{1}H NOESY spectrum of melittin in pure ethanol (C_2D_5OH) at 25 °C. (A) The NH proton region. The $d_{NN}(i, i+1)$ NOE peaks are labeled with residue numbers; (B) The fingerprint region. The $d_{\alpha N}(i, i+1)$ and $d_{\beta N}(i, i+1)$ NOE peaks are marked with circles and triangles, respectively. The peaks enclosed with the broken squares are assigned to the $d_{\alpha N}(i, i+3)$ and $d_{\alpha N}(i, i+4)$ NOE ones; (C) The cross peak region between an αH and a side chain proton. The $d_{\alpha\beta}(i, i+3)$ NOE peaks are enclosed by broken squares.

Those NOE peaks, along with $d_{\alpha N}(i, i+1)$ and $d_{\beta N}(i, i+1)$ NOE ones, are summarized in Figure 2. In the upper row, the amino acid sequence of melittin is described. The bars connect the

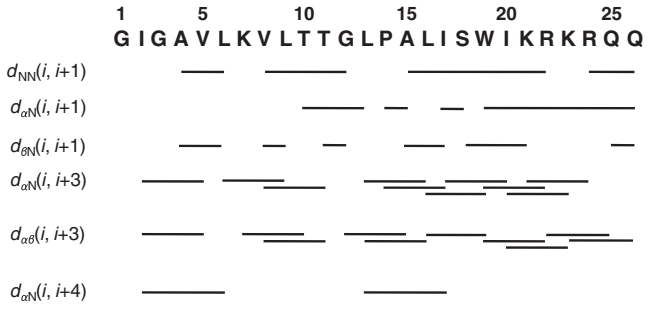

Figure 2. Summary of the NOE peaks observed for melittin in pure ethanol at 25 °C. A series of the capital letters in the upper row represents the amino acid sequence of melittin. The bars connect the *i*th and $i+1$th residues for the $d_{NN}(i, i+1)$, $d_{\alpha N}(i, i+1)$ and $d_{\beta N}(i, i+1)$ NOE peaks, the *i*th and $i+3$th residues for the $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOE ones and the *i*th and $i+4$th residues for the $d_{\alpha N}(i, i+4)$ NOE ones.

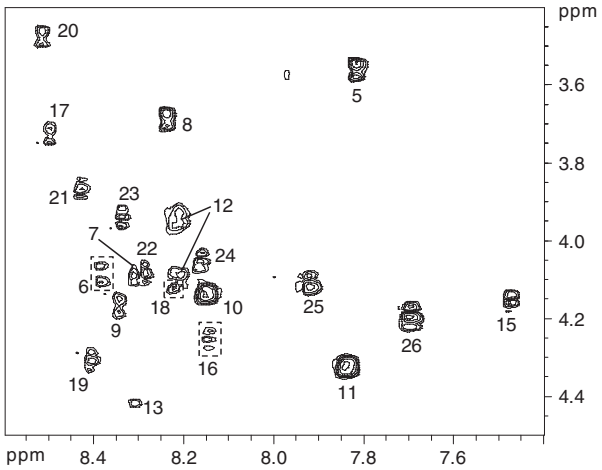

Figure 3. The NH-αH cross peak region of ^{1}H-^{1}H COSY spectra of melittin in pure ethanol (C_2D_5OH) at 25 °C. The cross peaks are labeled with residue numbers.

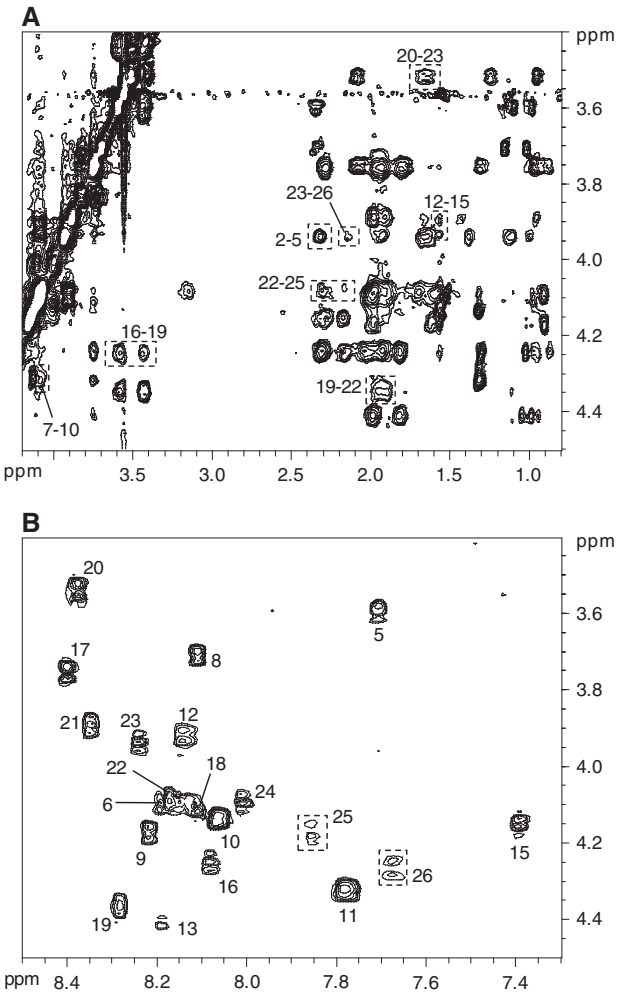

Figure 4. The cross peak region between αH and side chain proton of ^{1}H-^{1}H NOESY (A) and the NH-αH cross peak region of ^{1}H-^{1}H COSY (B) spectra of melittin in pure ethanol (C_2D_5OH) at 50 °C. The $d_{\alpha\beta}(i, i+3)$ NOE peaks are enclosed by broken squares and labeled with residue numbers. The NH-αH COSY cross peaks are labeled with residue numbers. The weak NH-αH peaks are enclosed with the broken squares.

two residues between which NOE peaks are detected. The connectivities of the NOE peaks indicate that the helical conformation is formed between the Ile-2 and Gln-26 residues.

Stability of the α-helix is mainly ascribed to intramolecular hydrogen bonds between a main chain carbonyl oxygen atom of the *i*th residue and an NH proton of the $i+4$th one [13]. Therefore, the NH protons play a key role for investigating the stability of the hydrogen bonds. When the hydrogen bonds are stably formed, strong and clear NMR signals of NH protons emerge because of slow exchange between the NHs and solvent OH protons. In contrast, when the hydrogen bonds lose their stability or are broken, the signals become broad or disappear because of fast exchange with solvent. Figure 3 shows the fingerprint region of the ^{1}H-^{1}H COSY spectra of melittin in pure ethanol (C_2D_5OH) at 25 °C. In this spectral region, only the cross peaks between NH and αH protons in intra-residues (NH-αH peaks) emerge. Clear observation of the peaks assigned to Val-5–Gln-26 suggests that the NH protons of these residues participate in stable hydrogen bonds.

A helical conformation is found to be still retained even at 50 °C because several $d_{\alpha\beta}(i, i+3)$ NOE peaks can be observed (Figure 4(A)). The helices are formed at least in the region of Ile-2–Val-5, Lys-7–Thr-10 and Gly-12–Gln-26. Further, appearance of a number of NH-αH COSY peaks implies that the hydrogen bonds are stably formed even at the temperature (Figure 4(B)). The intensity of the COSY peaks of Gln-25 and Gln-26 somewhat decreases, suggesting that destabilization of the hydrogen bonds takes place to a certain extent around the C-terminal.

To support the evidence for presence of helical conformation, the $^3J_{NH\text{-}\alpha CH}$ coupling constants measured from the 1D ^{1}H-NMR spectrum at 50 °C are given in Table 2. The typical value of the $^3J_{NH\text{-}\alpha CH}$ for the α-helix is about 4.0 Hz [15]. The $^3J_{NH\text{-}\alpha CH}$ values of Ala-15–Ala-24 are in the range of 3.2–4.8 Hz, which indicates that the α-helix conformation is realized in the residue region, whereas, the coupling constants of Thr-10, Thr-11 and Gln-25 are more than 6 Hz, which implies that in these regions some deviation from the helical conformation is to be expected. This seems to be inconsistent with the results from NOESY (Figure 4(A)).

To compare qualitatively conformations of melittin at low and high temperatures, the appearance/disappearance of $d_{\alpha\beta}(i, i+3)$ NOE peaks and NH-αH COSY peaks are examined. The use of $d_{\alpha\beta}(i, i+3)$ NOE peaks is suitable for examining conformation because the intensity of their peaks, unlike the $d_{\alpha N}(i, i+3)$ or $d_{\alpha N}(i,$

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Table 2. $^{3}J_{NH\text{-}\alpha CH}$ coupling constants at 50 °C measured from 1D ^{1}H-NMR spectrum with ~0.4 Hz/point digital resolution

Residue[a]	$^{3}J_{NH\text{-}\alpha CH}$ (Hz)[b]
Leu-9	4.3
Thr-10	6.3
Thr-11	7.0
Gly-12	—
Leu-13	—
Pro-14	No[c]
Ala-15	4.8
Leu-16	—
Ile-17	3.8
Ser-18	—
Trp-19	3.7
Ile-20	3.9
Lys-21	3.2
Arg-22	—
Lys-23	4.8
Arg-24	4.4
Gln-25	6.2
Gln-26	—

[a]The coupling constants of residues 1–8 could not be measured owing to an overlap with another NMR signal or a fast exchange with solvent.
[b]The bars indicate that the coupling constants cannot be measured owing to an overlap with another NMR signal.
[c]There is no NH proton for the proline residue.

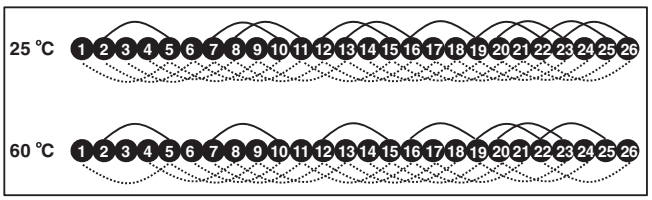

Figure 5. The connectivity of the $d_{\alpha\beta}(i, i+3)$ NOE and NH-αH COSY peaks observed for melittin in pure ethanol at 25 and 60 °C. The amino acid residues of a melittin molecule are schematically shown by closed circles. The numbers in the circles represents residue numbers. The solid lines connect the pairs of residues giving $d_{\alpha\beta}(i, i+3)$ NOE peaks. The dotted lines schematically represent the hydrogen bonds presumed from observation of the NH-αH COSY peaks.

$i+4$) NOE ones, are not affected by exchange with solvent. The connectivity of the $d_{\alpha\beta}(i, i+3)$ NOE peaks are summarized in Figure 5, together with the connectivity of the hydrogen bonds presumed from the NH-αH peaks observed in the COSY spectra. The amino acid residues of melittin are schematically depicted as closed circles and the numbers in the circles stands for residue numbers. The solid lines connect the pairs of residues giving $d_{\alpha\beta}(i, i+3)$ NOE peaks. The dotted lines schematically represent the hydrogen bonds formed between the ith and $i+4$th residues. In the region linked by solid and/or dotted lines, the α-helices are formed. The connectivity indicated by these lines shows that the helical conformation is formed between the Gly-1 and Gln-26 residues, that is, a full α-helix, in the temperature range from 25 to 60 °C.

Thermal stability of α-helix conformation in ethanol–water mixture solvents

As demonstrated by CD studies, conformation change in melittin is induced by adding water to an ethanol solution. [10]. Figure 6

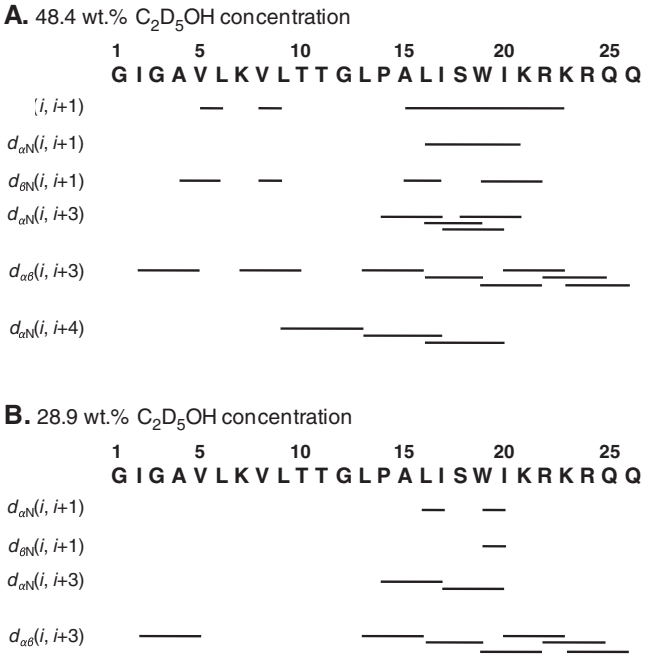

Figure 6. Summary of the NOE peaks observed for melittin in (A) a 48.4 wt% C_2D_5OH/51.6 wt% H_2O mixture solvent and in (B) a 28.9 wt% C_2D_5OH/71.1 wt% H_2O mixture solvent at 25 °C. A series of the capital letters in the upper row represents the amino acid sequence of melittin. The bars connect the ith and $i+1$th residues for the $d_{NN}(i, i+1)$, $d_{\alpha N}(i, i+1)$ and $d_{\beta N}(i, i+1)$ NOE peaks, the ith and $i+3$th residues for the $d_{\alpha N}(i, i+3)$ and $d_{\alpha\beta}(i, i+3)$ NOE ones and the ith and $i+4$th residues for the $d_{\alpha N}(i, i+4)$ NOE ones. The $d_{NN}(i, i+1)$ and $d_{\alpha N}(i, i+4)$ NOE peaks were not observed at 28.9 wt% C_2D_5OH concentration.

shows the summary of the NOE peaks observed for melittin in the ethanol–water mixture solvents at 48.4 and 28.9 wt% ethanol concentrations at 25 °C. The connectivities of the peaks indicate that the helical conformation is formed between the Ile-2 and Gln-26 residues at 48.4 wt% ethanol concentration, whereas at 28.9 wt% ethanol concentration, the helix at the Leu-6–Gly-12 region is uncoiled.

On the other hand, the fingerprint region of the ^{1}H-^{1}H COSY spectra of melittin in the mixture solvents is shown in Figure 7. Disappearance of the NH-αH peaks is observed with increase in water concentration and temperature. This is ascribed to a larger exchange rate between the NHs and the solvent OHs at higher water concentration and temperature, which indicates destabilization of the hydrogen bonds. The complete disappearance of all the peaks in the spectrum at 45 °C and 28.9 wt% C_2D_5OH concentration (Figure 7(D)) suggests that all the hydrogen bonds are almost broken.

Figure 8 shows temperature dependence of the $d_{\alpha\beta}(i, i+3)$ NOE peak region of the ^{1}H-^{1}H NOESY spectra of melittin in C_2D_5OD/D_2O mixture solvents at 48.6 and 28.0 wt% C_2D_5OD concentrations. The number of the $d_{\alpha\beta}(i, i+3)$ NOE peaks decreases with increasing temperature and/or water concentration, which reflects uncoiling of the α-helix.

To examine qualitatively conformation change under these ethanol concentration conditions, the connectivity of the $d_{\alpha\beta}(i, i+3)$ NOE peaks are summarized in Figure 9 along with the connectivity of the hydrogen bonds inferred from the observation of NH-αH COSY peaks (Figure 7). The figure symbols in Figure 9 are the same as those in Figure 5. In a ~48.5 wt% ethanol solution, melittin forms a full α-helix conformation at 25 °C, whereas the

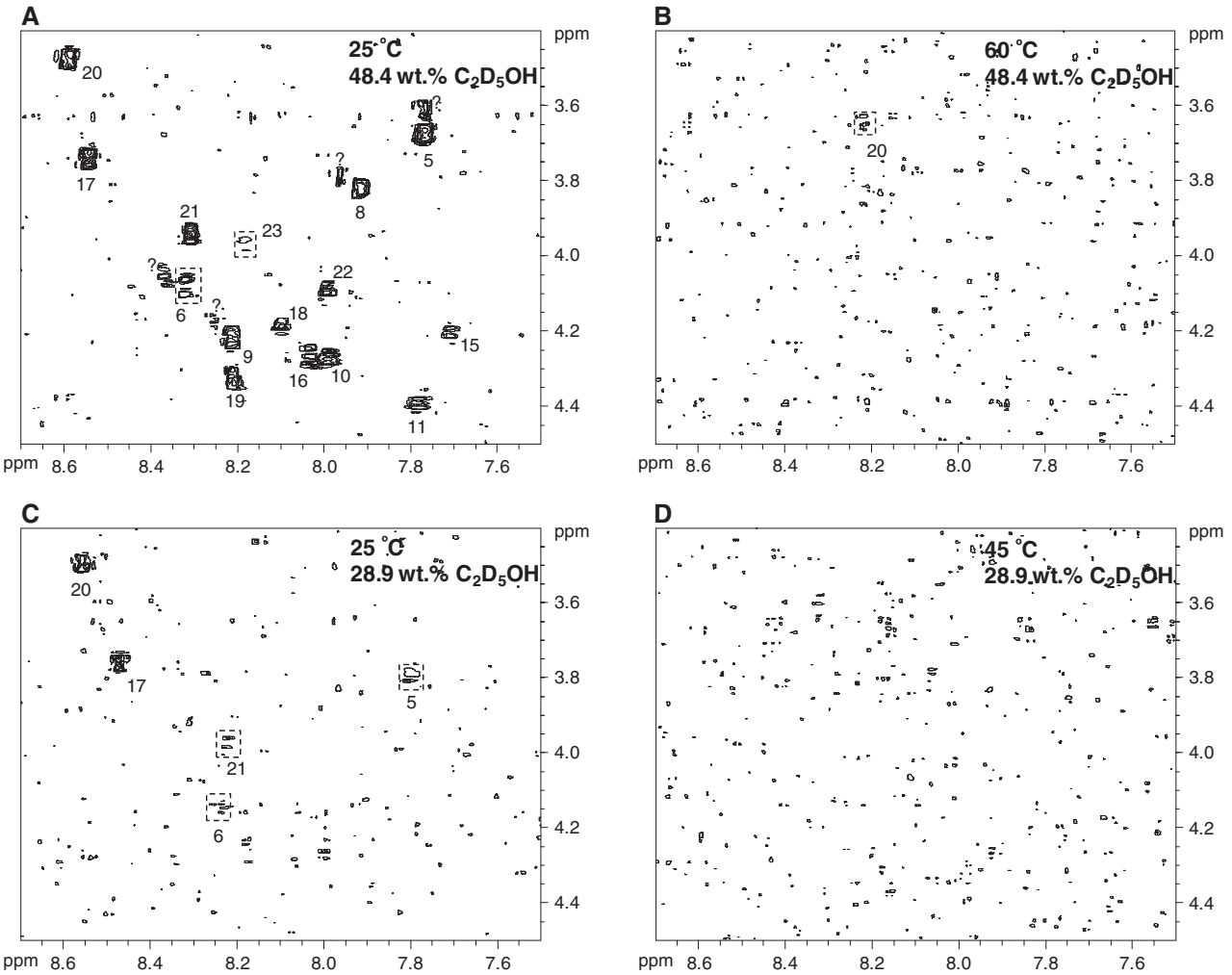

Figure 7. The NH-αH cross peak region of ^{1}H-^{1}H COSY spectra of melittin in (A, B) a 48.4 wt% C_2D_5OH/51.6 wt% H_2O mixture solvent at 25 and 60 °C and in (C, D) a 28.9 wt% C_2D_5OH/71.1 wt% H_2O mixture solvent at 25 and 45 °C. The NH-αH cross peaks are labeled with residue numbers. The weak NH-αH peaks are enclosed with the broken squares to be distinguished from noise peaks. Assignments for some peaks labeled with question marks are unclear.

α-helices are formed only at the regions of the Ile-2–Val-5, Lys-7–Thr-10 and Leu-16–Gln-25 residues at 60 °C (Figure 9(A)). The conformation taken at 60 °C corresponds to an intermediate one between a full α-helix and a fully random coil, that is, a partial α-helix. In a ~28.5 wt% ethanol solution, on the other hand, melittin forms a partial α-helix between 30 and 60 °C (Figure 9(B)). The α-helices of the Ile-2–Val-5 and Leu-16–Arg-22 residues are preserved even at 60 °C, suggesting that their thermal stability is extremely high.

Comparison of thermal stability of the intramolecular hydrogen bonds

The results obtained in the previous two sections are qualitatively similar to those obtained recently for melittin in pure methanol and methanol–water mixture solvents [14]. To elucidate the effect of an alkyl group of alcohol, conformation stability in ethanol and methanol solutions is compared in the light of the intramolecular hydrogen bonds.

The NH-αH COSY peaks can be observed up to high temperatures in proportion to thermal stability of the hydrogen bonds. Figure 10 shows the maximum temperature for detectable NH-αH peaks as a function of the amino acid residue number. The maximum temperatures are represented as closed symbols: the peaks can be observed only below the temperature marked with the symbols. On the other hand, the open symbols denote that the peaks can be observed even at 60 °C. As indicated in Figure 10 (A), most of the NH-αH peaks can be detected even at 60 °C in pure ethanol, whereas many peaks disappear at low temperatures with increasing water concentrations. The number of detectable peaks is appreciably reduced at 28.9 wt% ethanol concentration even at room temperature. The maximum temperatures are different depending on residues, which reflect that disappearance of the peaks is hardly affected by the concentration of ions of H^+, $C_2D_5O^-$ and OH^- in the solutions. If the concentration of these ions affects the appearance/disappearance of the peaks, then all the peaks disappear simultaneously at a certain temperature. It is clear that the thermal stability of hydrogen bonds decreases with an increase in water concentration. In particular, the hydrogen bonds formed by NHs of Ile-17 and Ile-20 have very high stability.

In methanol, on the other hand, the hydrogen bonds formed around the middle and both terminals have rather lower stability, whereas those at the region of Ile-17–Lys-23 residues have high

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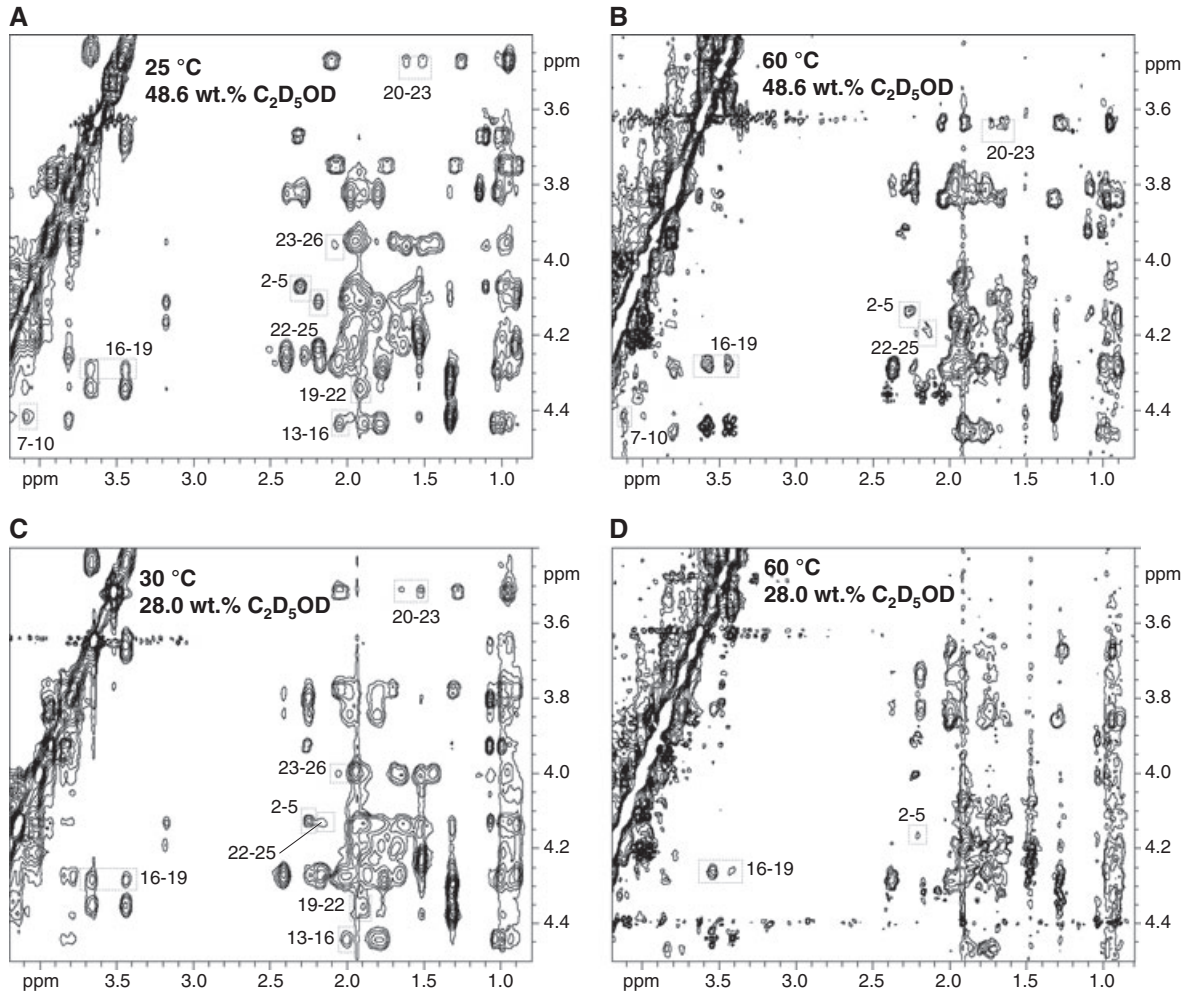

Figure 8. The ^{1}H-^{1}H NOESY spectra of melittin in (A, B) a 48.6 wt% C_2D_5OD/51.4 wt% D_2O mixture solvent at 25 and 60 °C and in (C, D) a 28.0 wt% C_2D_5OD/72.0 wt% D_2O mixture solvent at 30 and 60 °C. The peaks enclosed with the broken squares are the $d_{\alpha\beta}(i, i+3)$ NOE peaks and are labeled with residue numbers.

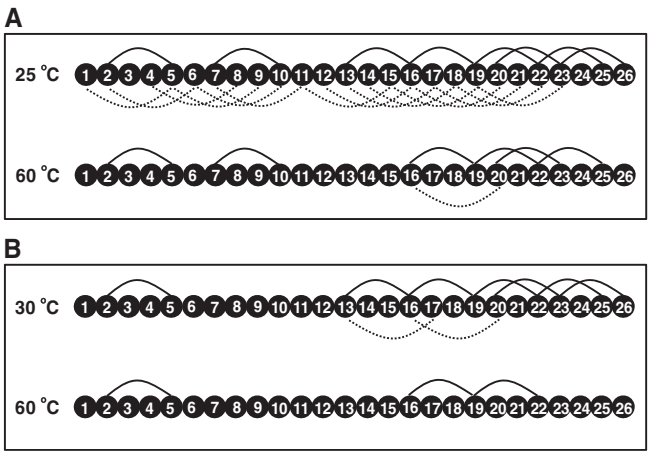

Figure 9. The connectivity of the $d_{\alpha\beta}(i, i+3)$ NOE and NH-αH COSY peaks observed for melittin in (A) a ~48.5 wt% ethanol solution at 25 and 60 °C and in (B) a ~28.5 wt% ethanol one at 30 and 60 °C. The amino acid residues of a melittin molecule are schematically shown by closed circles. Numbers in the circles are residue numbers. The solid lines connect the pairs of residues giving $d_{\alpha\beta}(i, i+3)$ NOE peaks. The dotted lines schematically represent the hydrogen bonds presumed from an observation of the NH-αH COSY peaks.

stability (Figure 10(B)). Compared with the data on the pure ethanol solution, this result provides evidence that the hydrogen bonds are more thermally stable in pure ethanol than in pure methanol.

Contact between the alkyl groups of alcohol molecules and hydrophobic side chain groups of melittin is crucial for avoiding formation of intermolecular hydrogen bonds with hydroxyl group of alcohol and for promoting formation of the intramolecular hydrogen bonds. This contact is induced and stabilized by hydrophobic interaction. The reference interaction site model theory suggests that the hydrophobic interaction between peptides and alcohols strengthens with increase in the size of alkyl groups of an alcohol molecule [11]. Thus, the interaction of melittin with ethanol is stronger than with methanol, which contributes significantly to more stable intramolecular hydrogen bonds in ethanol.

Conclusions

Temperature dependence of helical conformation of melittin in pure ethanol and ethanol–water mixture solvents has been explored by NMR measurements. In pure ethanol, melittin maintains

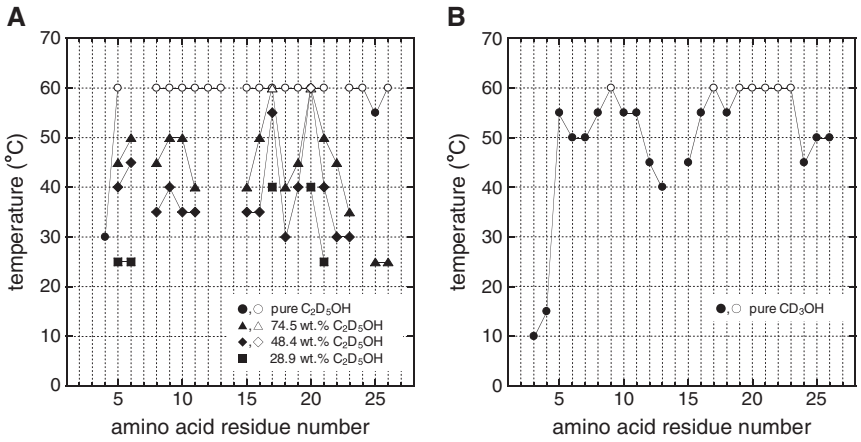

Figure 10. The maximum temperature for detectable NH-αH COSY peaks of melittin in (A) the ethanol–water mixture solutions and in (B) pure methanol, as a function of the amino acid residue number. The maximum temperatures are represented as the closed symbols, and the open symbols denote that the peaks can be observed even at 60 °C. Data points for the mixture and pure methanol solutions are represented as follows: the circle for pure ethanol and methanol, the triangle for 74.5 wt% ethanol concentration, the diamond for 48.4 wt% ethanol one and the square for 28.9 wt% ethanol one. The data points adjoining each other are connected with lines.

a full α-helix up to 60 °C at least. At ~48.5 wt% ethanol concentration, it undergoes a transition from a full α-helix to a partial one upon heating, and at ~28.5 wt% ethanol concentration, it takes a partial α-helix between 30 and 60 °C. We demonstrated that the intramolecular hydrogen bonds become more stable in proportion to an ethanol concentration in ethanol–water mixture solvents, and they are more thermally stabilized in pure ethanol than in pure methanol. The latter finding shows that thermal stability of hydrogen bonds is enhanced with increase in the size of the alkyl groups in alcohol molecules. To acquire experimental support for this conclusion, further investigations are required on the α-helix conformation in propanol (C_3H_7OH) and butanol (C_4H_9OH) solutions.

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