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NMR studies on the monomer–tetramer transition of melittin in an aqueous solution at high and low temperatures

Yoshinori Miura

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Abstract Melittin, a peptide of 26 amino acid residues, has been used as a model peptide for protein folding and unfolding, and extensive research has been done into its structure and conformational stability. Circular dichroism (CD) studies have demonstrated that melittin in an aqueous solution undergoes a transition from a helical tetramer to a random coil monomer not only by heating but also by cooling from room temperature (i.e., heat- and cold-denaturation, respectively). The heat-denaturation has been also examined by nuclear magnetic resonance (NMR) experiments, however, no NMR data have been presented on the cold-denaturation. In this paper, using proton (^{1}H) NMR spectroscopy, we show that melittin undergoes conformational transitions from the monomer to the tetramer to the monomer by elevating temperature from 2 to 70 °C. Only melittin including a *trans* proline peptide bond participates in the transitions, whereas melittin including a *cis* proline one does not. The tetramer has maximum conformation stability at around 20 °C, and cooperativity of the heat-denaturation is extremely low.

Keywords Melittin · Self-association · Monomer–tetramer transition · NMR spectroscopy · *cis* and *trans* proline peptide bonds · Cold- and heat-denaturation

Introduction

Melittin, which is a major ingredient of honeybee venom and has a hemolytic activity, is a small polypeptide composed of 26 amino acid residues: GIGAVLKVLT TGLPALISWIKRKRQQ (Dempsey 1990). Its conformations have been elucidated under various solution conditions by gel filtration, ultracentrifugation, circular dichroism (CD) and nuclear magnetic resonance (NMR) studies. Melittin forms a monomer helix in a methanol solution or on binding to lipids in membranes (Bazzo et al. 1988; Inagaki et al. 1989; Miura 2011). While in an aqueous solution it takes either a random coil monomer or a helical tetramer depending on pH, peptide concentration and salt concentration. The monomer conformation is realized at acidic pH and low peptide concentration (e.g., at pH 3 and 3 mM), whereas the tetramer one at alkaline pH or high peptide concentration or high salt concentration (e.g., at pH 9 and 6 mM) (Brown et al. 1980; Iwadate et al. 1998; Lauterwein et al. 1980). The monomer and the tetramer coexist in a solution at alkaline pH and low peptide concentration (e.g., at pH 9 and 2 mM). The tetramer is stabilized mainly by inter-molecular hydrophobic interaction among hydrophobic side chain groups (Lauterwein et al. 1980). The helix structure in the tetramer is maintained by intra-molecular hydrogen bonds, while in the random coil state the hydrogen bonds are broken, and instead inter-molecular hydrogen bonds are formed between melittin and surrounding water molecules. Furthermore, it has been known that the peptide bond of L13–P14 in melittin is either in a *trans* or in a *cis* configuration due to isomerization, and melittin including the *trans* proline peptide bond has a stronger tendency to form the tetramer (Lauterwein et al. 1980). Monomeric melittin having the *trans* and *cis* proline, if it is necessary to make a clear distinction, will be referred to as the *trans*- and *cis*-monomer, respectively.

By variation in pH or salt concentration or temperature of an aqueous solution, a conformation transition between the tetramer and the monomer is induced (Bello et al. 1982; Faucon et al. 1979; Goto and Hagihara 1992; Hagihara et al.

Y. Miura (✉)
Center for Advanced Instrumental Analysis, Kyushu University, Kasuga 816-8580, Japan
e-mail: yoshi@mm.kyushu-u.ac.jp

1992, 1994; Iwadate et al. 1998; Miura 2011; Quay and Condie 1983; Ramalingam et al. 1991, 1992; Wilcox and Eisenberg 1992). Interestingly, melittin undergoes the transition not only by heating but also by cooling from room temperature like a globular protein such as myoglobin or staphylococcal nuclease (Privalov 1990). These transitions correspond to the heat- and cold-denaturation of the tetramer, respectively, which were revealed by CD studies on temperature dependence of the helix content in tetramer melittin. On the basis of the CD studies, thermal stability of the tetramer was examined, and further the problem of protein folding was addressed (Faucon et al. 1979; Ramalingam et al. 1991, 1992; Wilcox and Eisenberg 1992). Differential scanning calorimetry (DSC) measurements have been also performed to explore structural stability of tetramer melittin, and a similarity with a molten globule conformation of cytochrome *c* has been suggested (Hagihara et al. 1994).

NMR studies on the heat-denaturation of the tetramer has been performed by many researchers, however, there have been no NMR data on the cold-denaturation. In this paper we show the NMR data on transitions from the monomer to the tetramer to the monomer in melittin induced by temperature variation from 2 to 70 °C at various peptide concentrations. These transitions correspond to the renaturation from the cold-denatured state and the heat-denaturation, respectively. Since an NMR spectrum reflects conformation of melittin, NMR signals from the monomer and the tetramer are distinguishable. Thus, information on population change of these conformations can be obtained as a function of temperature. We compare the present results with the previous ones gained by other researchers, and discuss a process of the conformational transitions.

Materials and methods

Materials

Melittin was purchased from Sigma Chemical Co. (St. Louis, Mo, USA) and used without further purification. For acquisition of NMR spectra, melittin was dissolved in deuterated water (D_2O) with 99.97 % purity obtained from Nacalai Tesque, Inc. (Japan). Sample solutions with peptide concentrations of 0.65, 3.27, 6.56 and 10.11 mM were prepared. All the solutions were salt-free and their pH values were 7.5–8.0. Turbidity of the solutions was not observed. 3-trimethylsilyl-propionate (TSP) was used as an internal standard for chemical shifts in NMR spectra.

NMR experiments

All ^{1}H NMR experiments were performed on a Varian Unity Inova 500 MHz NMR spectrometer. One-dimensional (1D) ^{1}H NMR spectra were acquired between 2 and 70 °C in step of 2 or 4 degrees. In this temperature range, dependence of the spectra on temperature was reversible. Prior to data acquisition, the sample solutions in the NMR probe were kept for 30 min at the desired temperature to ensure proper equilibration. The spectra were normally recorded with 6,000 data points and a spectral width of 6,000 Hz. Accumulation of free induction decay was carried out 64 or 200 times with a pulse flip angle of 45 degrees, an accumulation time of 1.998 s and a relaxation delay time of 3.5 s. For an improvement in signal-to-noise ratio of the 1D ^{1}H NMR spectra, the resonance of the residual water (HDO in the D_2O solvent) was suppressed by a pre-saturation method during the relaxation delay time.

The data were processed using a commercial NMR data analysis soft-ware Nuts on a PC. The resolution of the 1D ^{1}H NMR spectra was enhanced by applying an exponential window function and zero filling; the digital resolution was about 0.4 Hz/point. Assignments of NMR signals were performed on the basis of 1D ^{1}H NMR spectra of monomer and tetramer melittin acquired by other researches (Brown et al. 1980; Lauterwein et al. 1980; Iwadate et al. 1998).

Results

Self-association of melittin at room temperature

A ^{1}H NMR spectrum of melittin has a large number of sharp resonance lines when melittin forms a monomeric random coil conformation, whereas their lines become broad when it self-associates (Brown et al. 1980; Dempsey 1990; Iwadate et al. 1998; Lauterwein et al. 1980). Figure 1 shows peptide concentration dependence of ^{1}H NMR spectra of melittin in a D_2O solvent at 26 °C. The NMR signals of the W19 indole ring protons and labile ones emerge in the high frequency region, however, labile proton signals fully disappear owing to complete replacement of the labile protons by OD deuterons of surrounding solvent molecules. The signals of the W19 indole ring protons at C-2, C-4, C-5, C-6 and C-7 are labeled as 2, 4, 5, 6 and 7, respectively. In the low frequency region, on the other hand, the signals of α protons and non-labile side chain ones except the ring ones are observed. In the spectrum of 0.65 mM melittin solution a large number of sharp signals are observed, while they gradually broaden in proportion to peptide concentration. This change indicates self-association of monomeric melittin and its resultant reduction of monomer molecules present in the solutions. Increase in the peptide concentration enhances probability of contacts between monomeric melittin molecules. Several signals reflecting self-associated melittin are marked with the closed squares in Fig. 1.

As evidenced by emergence of the I2 $C\delta H_3$ signal at about 0.45 ppm in the spectrum of 10.11 mM melittin

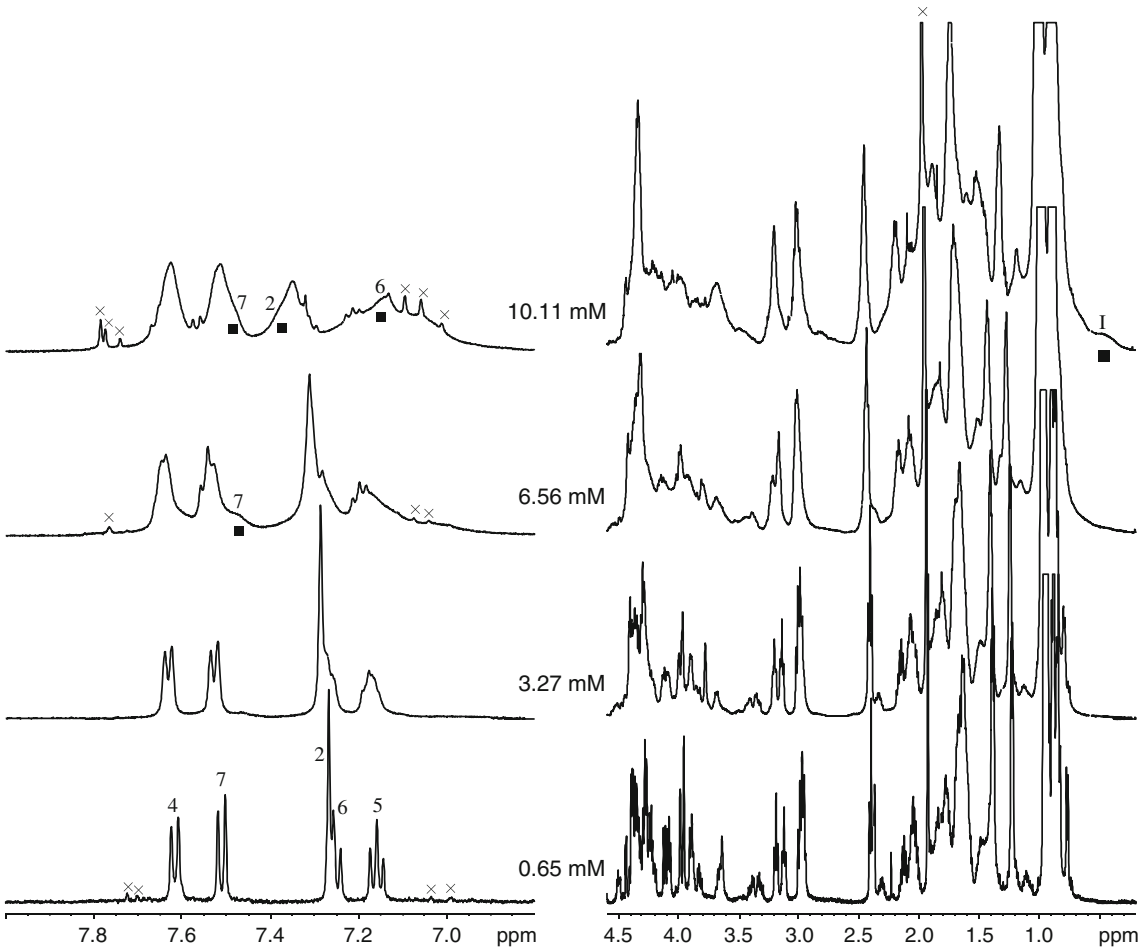

Fig. 1 The high and low frequency regions of 1D ^{1}H NMR spectra of melittin in a D_2O solvent at 26 °C and at peptide concentrations of 0.65, 3.27, 6.56 and 10.11 mM. The increasing peptide concentrations correspond to the spectra from *bottom* to *top*. The signals of the W19 indole ring protons at C-2, C-4, C-5, C-6 and C-7 are labeled as 2, 4, 5, 6 and 7, respectively, and the I2 CδH$_3$ signal as I. The signals marked with the *closed squares* correspond to those of the I2 CδH$_3$ and indole ring protons reflecting self-associated melittin. The signals marked with the *crosses* are those from impurities

solution, self-associated melittin corresponds to the tetramer (Brown et al. 1980). This signal emerges at about 0.9 ppm when melittin takes the random coil monomer, however, it shifts to the lower frequency side due to formation of the tetramer and its resultant proximity of the I2 residue in one monomeric subunit to the indole ring of the W19 residue in another (Brown et al. 1980; Iwadate et al. 1998). In 3.27 and 6.56 mM melittin solutions, observation of the signal from the tetramer is difficult because of a small population of tetramer molecules. While a possibility of formation of dimer or aggregation is extremely low, because dimer conformation is very unstable (Schubert

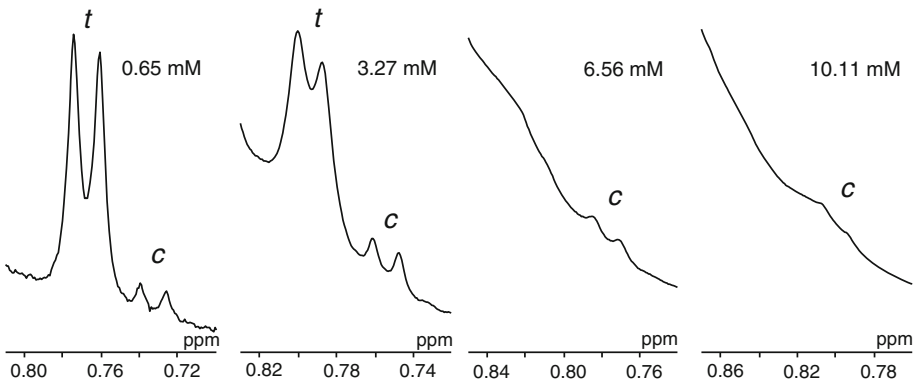

Fig. 2 The I17 CγH$_3$ doublet signal region of ^{1}H NMR spectra of melittin in a D_2O solvent at 26 °C and at peptide concentrations of 0.65, 3.27, 6.56 and 10.11 mM. The increasing peptide concentrations correspond to the spectra from *left* to *right*. The I17 CγH$_3$ doublet signals reflecting the *cis*- and *trans*-monomers are labeled as *c* and *t*, respectively

et al. 1985) and the melittin solutions for the NMR measurements were not turbid.

The *cis* and *trans* state of proline in melittin

The L13–P14 peptide bond of melittin takes either the *cis* or *trans* states owing to isomerization. As evidence of the isomers, the I17 CγH$_3$ doublet signals from the *cis*- and *trans*-monomer are clearly observed in the spectra of 0.65 and 3.27 mM melittin solutions (Fig. 2). In the spectra of 6.56 and 10.11 mM melittin solutions, the signal from the *trans*-monomer is obscured by another signal and self-association.

Figure 3 shows temperature dependence of ^{1}H NMR spectra of 0.65 mM melittin solution. As temperature is raised, all NMR signals become narrower and increase in intensity due to motional narrowing. The chemical shifts of most signals remain almost unchanged against temperature change. The I17 CγH$_3$ doublet signals from the *cis*- and *trans*-monomers are clearly observed, and the peak height ratio between the doublet signals is nearly the same at all temperatures. These behaviors indicate that melittin

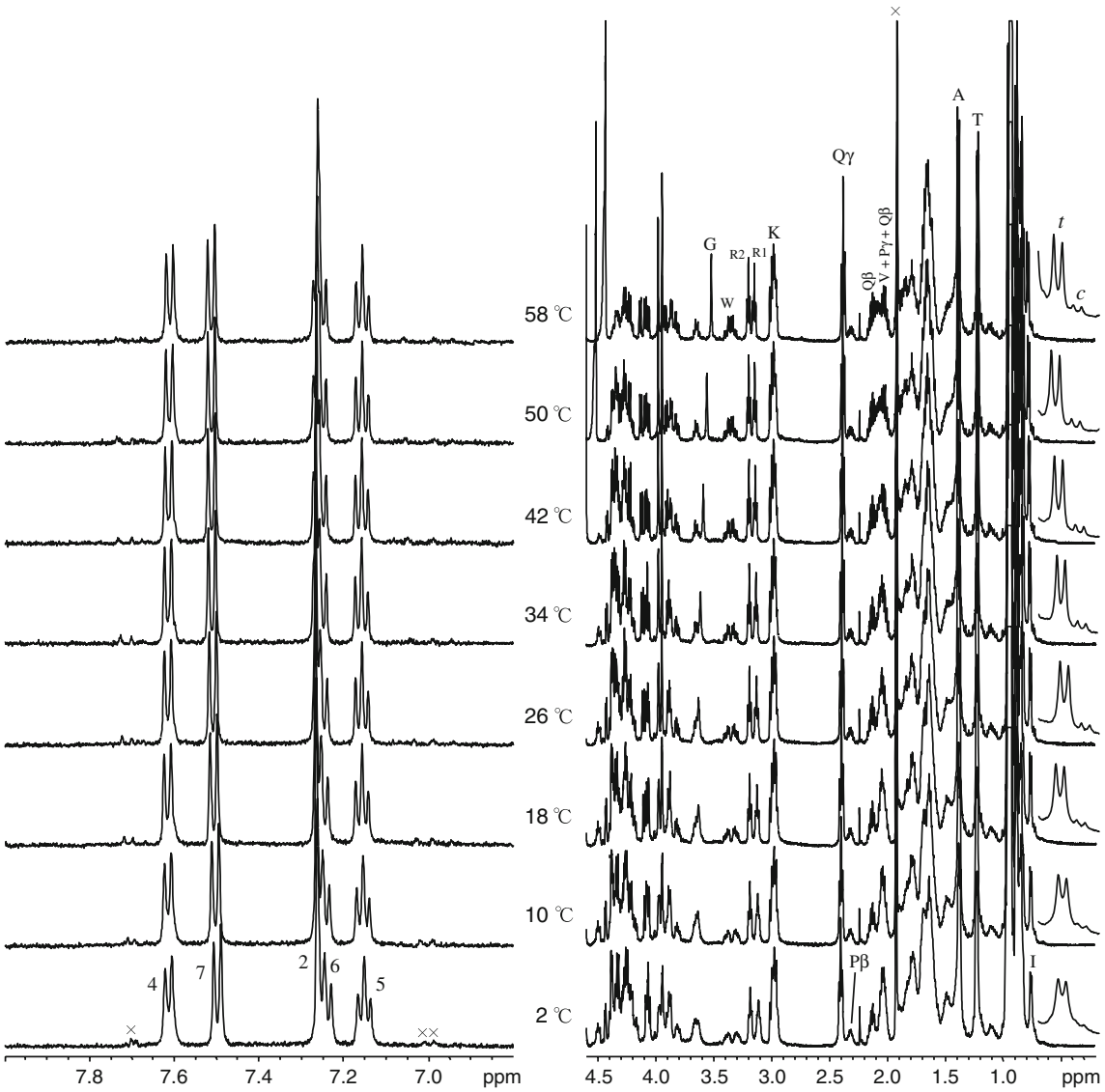

Fig. 3 The high and low frequency regions of 1D ^{1}H NMR spectra of melittin in a D_2O solvent at 0.65 mM peptide concentration as a function of temperature. The signals of the W19 indole ring protons at C-2, C-4, C-5, C-6 and C-7 are labeled as 2, 4, 5, 6 and 7, respectively. The assignments for several signals in the low frequency region are as follows: G, G1 CαH$_2$; W, W19 CβH$_2$; R1, R22 CδH$_3$; R2, R24 CδH$_3$; K, K7, 21 and 23 CεH$_3$; V, V5 and 8 CβH; Pβ and Pγ, P14 CβH and CγH$_2$; Qβ and Qγ, Q25 and 26 CβH$_2$ and CγH$_2$; T, T10 and 11 CγH$_3$; A, A4 and 15 CβH$_3$; I, I17 CγH$_3$. The signal labeled as V + Pγ + Qβ is superposition of the signals corresponding to V, Pγ and Qβ. The spectral region of the I17 CγH$_3$ doublet signals is expanded, and their signals from the *cis*- and *trans*-monomers are labeled as *c* and *t*, respectively. The sharp signal at about 4.5 ppm observed at 42, 50 and 58 °C corresponds to the pre-saturated resonance line of HDO proton in the solvent. The signals marked with the *crosses* are those reflecting impurities

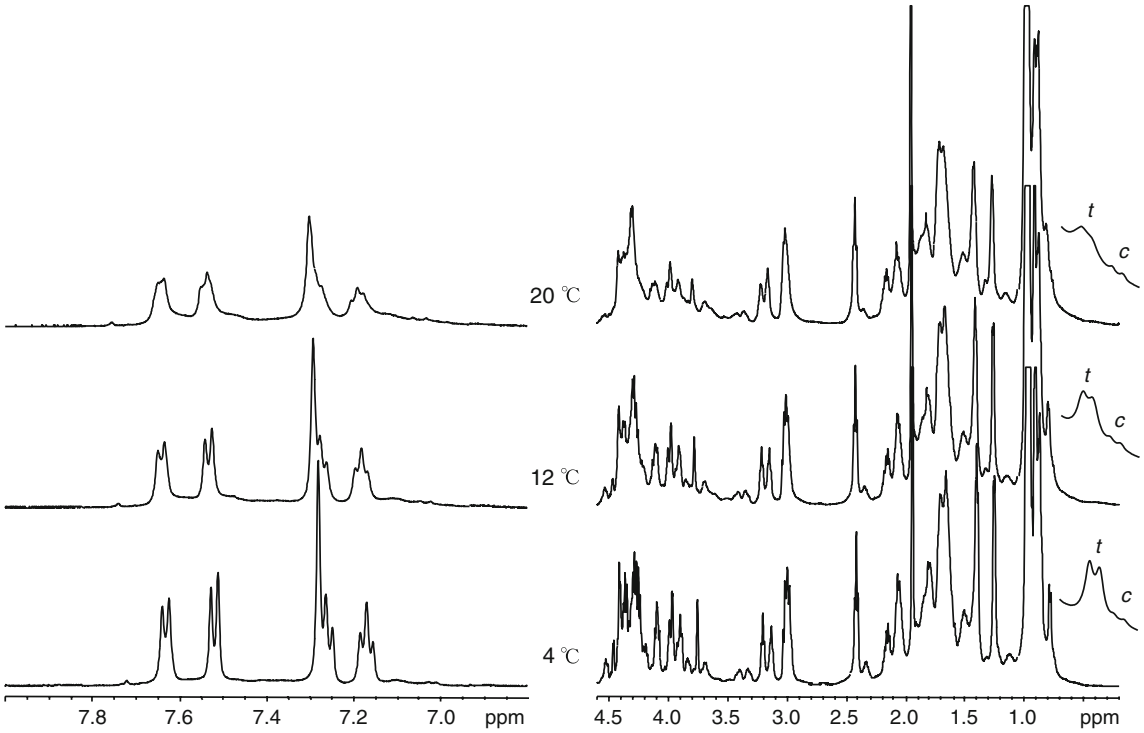

Fig. 4 1D ^{1}H NMR spectra of melittin in a D_2O solvent at peptide concentration of 6.56 mM and at 4, 12 and 20 °C. The spectral region of the I17 CγH$_3$ doublet signals is expanded, and their signals from the *cis*- and *trans*-monomers are labeled as *c* and *t*, respectively

remains a random coil conformation at all temperatures, and the proportion of *cis*-monomer molecules to *trans*-monomer ones is largely unaffected by temperature. By calculating the ratio of the I17 CγH$_3$ signal peak height for the *cis*-monomer to that for the *trans*-monomer, about 85 % of monomeric melittin molecules are evaluated to be in the *trans*-monomer state. The *cis*- and *trans*-monomers are in equilibrium throughout the temperature variation, and the conformation exchange between the monomers are slow on the NMR timescale; since the I17 CγH$_3$ signals are about 0.04 ppm apart and do not exhibit noticeable exchange-broadening, this implies a conformational interconversion of quite a bit less 20 s^{-1}.

Figure 4 shows ^{1}H NMR spectra of 6.56 mM melittin solution at 4, 12 and 20 °C. With increasing temperature, most of the resonance lines gradually broaden and decrease in intensity due to self-association of the monomer into the tetramer. In accordance with the conformation change, the I17 CγH$_3$ doublet signal from the *trans*-monomer also broadens and decreases, whereas, the doublet from the *cis*-monomer can be clearly observed even at 20 °C. This gives clear evidence that only the *trans*-monomer is involved in formation of the tetramer, which is in agreement with the result obtained by Lauterwein et al. (1980).

Melittin in a methanol solution or bound to micelles takes a monomer helix with the *trans* proline, whereas a monomer helix with the *cis* proline has not yet been discovered (Bazzo et al. 1988; Inagaki et al. 1989). This implies that the *trans* proline peptide bond is essential for realization of the helix conformation of melittin. The monomer subunits of the tetramer form a helical conformation, and thus only the *trans*-monomer participates in formation of the tetramer.

Monomer-tetramer transition in melittin

Temperature dependence of ^{1}H NMR spectra of 10.11 mM melittin solution is shown in Fig. 5a, b. In the high frequency region (Fig. 5a), the strong singlet, doublet and triplet signals and the weak broad ones, which correspond to the W19 ring protons, are observed at 2 °C. The former strong signals and the latter broad ones reflect the monomer and the tetramer, respectively, and are labeled as the closed triangles and squares, respectively. Upon heating from 2 °C, intensity of the signals from the monomer reduce, however, this reduction is almost stopped above about 26 °C. In contrast to this behavior, the signals from the tetramer increase in intensity up to 18–22 °C, and then gradually decrease. On the other hand, the new signals marked with the open triangles emerge at 18–22 °C, and then increase in intensity as temperature is raised. They eventually become the singlet, doublet or triplet signals akin to the line shapes reflecting the monomer. These rather complicated behaviors demonstrate that melittin

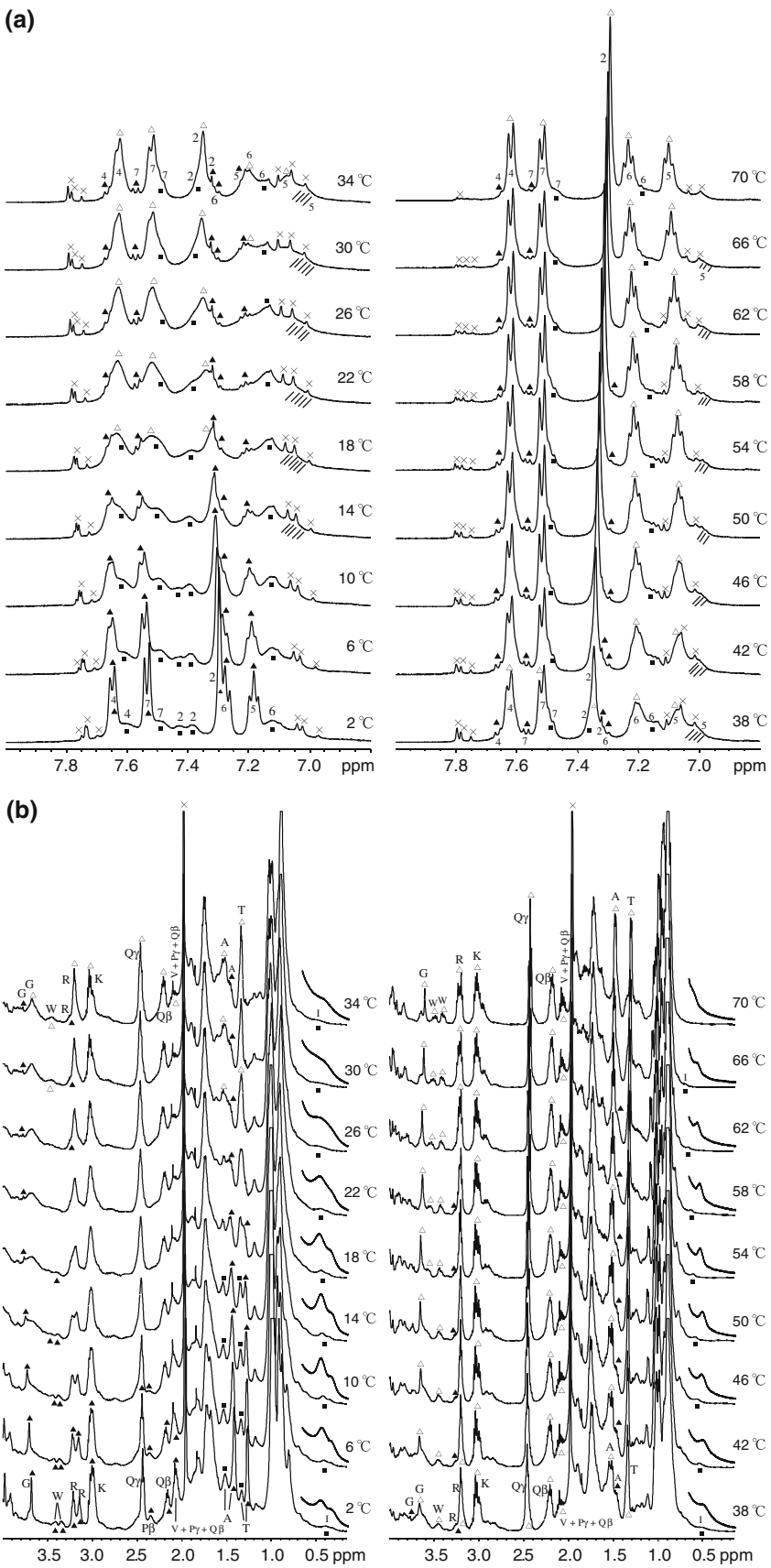

Fig. 5 1D ^{1}H NMR spectra of melittin in a D_2O solvent at 10.11 mM peptide concentration as a function of temperature. The signals marked with the *closed triangles* reflect the monomer which does not participate in self-association. The signals from the tetramer and those from the monomer separated from the tetramer are marked with the *closed squares* and the *open triangles*, respectively. The signals marked with the *crosses* are those reflecting impurities. **a** The high frequency region. The signals corresponding to the W19 indole ring protons at C-2, C-4, C-5, C-6 and C-7 are labeled as 2, 4, 5, 6 and 7, respectively. In particular, the signal of the C-5 proton reflecting the tetramer is indicated as *hatching*. **b** The low frequency region. The assignments for several signals are as follows: G, G1 CαH$_2$; W, W19 CβH$_2$; R, R22 and 24 CδH$_3$; K, K7, 21 and 23 CεH$_3$; V, V5 and 8 CβH; Pβ and Pγ, P14 CβH and CγH$_2$; Qβ and Qγ, Q25 and 26 CβH$_2$ and CγH$_2$; T, T10 and 11 CγH$_3$; A, A4 and 15 CβH$_3$; I, I2 CδH$_3$. The signal labeled as V + Pγ + Qβ is superposition of the signals corresponding to V, Pγ and Qβ. The spectral region of the I2 CδH$_3$ signal is expanded

undergoes the monomer-to-tetramer transition from 2 to about 22 °C, and the tetramer-to-monomer transition above about 22 °C. Thus, the signals marked with the closed triangles reflect the monomer which does not participate in self-association, while those marked with the open ones the monomer which is dissociated subsequently to formation of the tetramer, i.e., the *trans*-monomer. The same explanation is true for variation of the NMR signals observed in the low frequency region (Fig. 5b). Incidentally, the I2 CδH_3 signal from the tetramer turns to the singlet at around 34 °C owing to a fast rotational motion of the intra-residue side chain bond, which indicates enhancement of conformational fluctuation of the tetramer.

As shown in Fig. 5a, b, the signal positions reflecting the *trans*-monomer are quite different from those reflecting the monomer not involved in tetramer formation. This implies that the *trans*-monomers dissociated from the tetramers are still undergoing fast exchange with the tetramer at higher temperatures. Thus, the signals from the *trans*-monomer have unique chemical shifts that are strongly affected by the exchange.

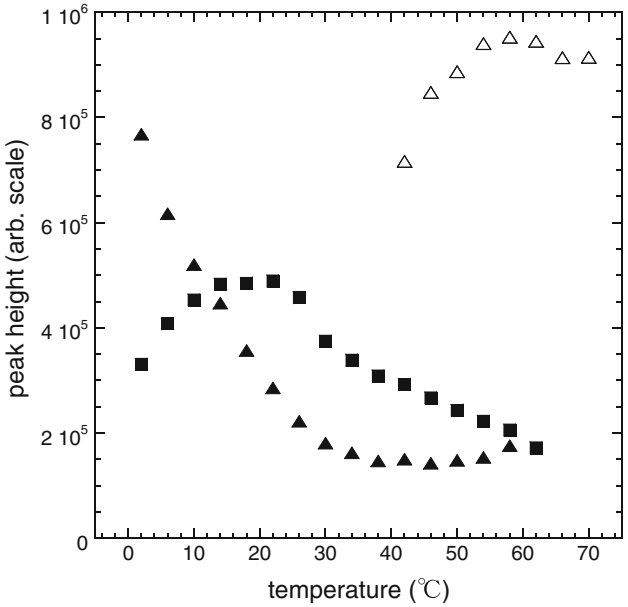

Fig. 6 The peak height of the signal corresponding to the W19 C(6)H indole ring proton from the monomer and the tetramer as a function of temperature. The *closed triangles* correspond to the peak height of the signal reflecting the monomer which is not involved in formation of the tetramer, the *closed squares* reflecting the tetramer and the *open triangles* reflecting the monomer separated from the tetramer

Discussion

To understand fully the results of the NMR experiments for 10.11 mM melittin solution, the peak height, as a typical example, of the W19 C(6)H indole ring proton signal from the monomer and the tetramer are plotted as a function of temperature in Fig. 6. The closed triangles correspond to the peak height of the signal reflecting the monomer which is not involved in formation of the tetramer, the closed squares reflecting the tetramer and the open triangles the *trans*-monomer separated from the tetramer. The changes in the peak heights qualitatively correspond to the population change of tetramer, *cis*- and *trans*-monomer molecules. From 2 to about 20 °C, due to the *trans*-monomer to tetramer transition, the population of the monomer and that of the tetramer decreases and increases, respectively, while, from about 26 to 70 °C the population of the *trans*-monomer increases and that of the tetramer decreases due to the tetramer to *trans*-monomer transition. Therefore, a series of conformational and oligomeric state equilibria proceeds with increasing temperature from low to intermediate to high temperatures; i.e., from *cis*-monomer ↔ t*rans*-monomer to *trans*-monomer ↔ tetramer to tetramer ↔ *trans*-monomer ↔ *cis*-monomer.

The population of the tetramer achieves the maximum at 18–22 °C, which indicates that the tetramer conformation is the most stable at around 20 °C. This indication is consistent with the result based on the CD studies (Wilcox and Eisenberg 1992; Ramalingam et al. 1992). On the other hand, since the *cis*-monomer does not participate in the transitions throughout temperature variation, the value of the peak height in the flat region between 30 and 60 °C are proportional to the *cis*-monomer population. By calculating the ratio of the peak height of the monomer signal at 2 °C to that of *cis*-monomer signal between 30 and 60 °C, about 80 % of monomeric melittin molecules are evaluated to be in the *trans*-monomer state at 2 °C.

Formation of the tetramer is induced by increase in salt concentration or pH as a result of shielding of repulsive interaction between positive charge on the side chain groups of the Lys and Arg residues and its resultant increase in inter-molecular hydrophobic interactions. NMR, CD and DSC studies have shown that thermal stability of the tetramer enhances with increase in salt concentration and/or pH, and the tetramer conformation is maintained at high salt concentrations even at 70–80 °C (Brown et al. 1980; Hagihara et al. 1994; Iwadate et al. 1998; Wilcox and Eisenberg 1992). Since the melittin solutions used for the present NMR experiments are weak alkaline pH and salt-free, packing of the hydrophobic contacts in the tetramer is weaker. As indicated in Fig. 6, the transition range for the high temperature side covers more than forty degrees; the transition begins at about 26 °C, however, does not finish even at 60 °C. This low conformational stability and transition cooperativity are ascribed mainly to weakness of hydrophobic packing.

Hydrophobic interaction and hydration have been recognized to be important keys for understanding the mechanism of the cold-denaturation of the tetramer as well

as a globular protein such as myoglobin. Recently, the studies on hydration dynamics in melittin have demonstrated that hydration is intimately related to structural stability of the tetramer and the monomer–tetramer transition (Othon et al. 2009; Qiu et al. 2005). Despite the exhaustive research, a satisfactory explanation for the cold-denaturation has not yet emerged (Chandler 2005; Dias et al. 2008; Kinoshita 2009; Marques et al. 2003; Murphy et al. 1990; Privalov and Gill 1988; Privalov 1990, 1992; Soda 1993; ten Wolde and Chandler 2002; Yoshidome and Kinoshita 2009). It is necessary to examine much more about the monomer-tetramer transition to deepen understanding on the stability and the cold- and heat-denaturation of melittin. To address this problem rigorously, two and three dimensional NMR studies and ^{15}N-labeled melittin will be helpful. Further, elucidation of the problem is significant to understand the biological function of melittin, because the tetramer-to-monomer transition is a part of the process of binding to lipids in membranes and forming a monomer helix.

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