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High Resolution NMR Structure and Backbone Dynamics of Recombinant Bee Venom Melittin

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Abstract

In recent years there has been a resurgence of interest in melittin and its variants as their therapeutic potential has become increasingly evident. Melittin is a 26-residue peptide and a toxic component of honey bee venom. The versatility of melittin in interacting with various biological substrates, such as membranes, glycosaminoglycans and a variety of proteins has inspired a slew of studies to understand the structural basis of such interactions. However, these studies have largely focused on melittin solutions at high concentrations (> 1mM), even though melittin is generally effective at lower, (micromolar) concentrations. Here we present high-resolution NMR studies in the lower concentration regime using a novel method to produce isotope labeled (¹⁵N, ¹³C) recombinant melittin. We provide residue-specific structural characterization of melittin in dilute aqueous solution and in TFE-water mixtures, which mimic melittin structure-function and interactions in aqueous, and membrane-like environments respectively. We find that the *cis-trans* isomerization of Pro14 is key to changes in the secondary structure of melittin. Thus, this study provides residue-specific structural information on melittin in the free-state and in a model of the substrate-bound state. These results, taken together with published work from other labs, reveal the peptide's structural versatility which resembles that of intrinsically disordered proteins and peptides.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

Backbone assignments of [*L*-¹³C, ¹⁵N]-melittin in 0% TFE (Table S1), and 30% TFE (Table S2), SDS-PAGE gel showing the partitioning of the pTM-melittin fusion protein into insoluble and soluble fractions of the cell lysate (Figure S1), ESI mass spectra of labeled and unlabeled melittin samples (Figure S2), HSQC spectra of melittin samples with different relative amounts of *cis*-Pro-melittin and *trans*-Pro-melittin forms (Figure S3), effect of TFE concentration on HSQC spectra of melittin (Figure S4), output showing RCI and H-prob scores from the CSI analyses performed on melittin assignments (Tables S3-S6), helical propensities for different residues on melittin (Figure S5).

The authors declare no competing financial interest.

Keywords

Recombinant melittin; isotope labeling; NMR; HSQC; cis-trans isomerization

INTRODUCTION

Melittin, a 26-residue peptide, is the primary constituent of European bee venom (*Apis mellifera* L.).¹ This cationic peptide has hemolytic^{2, 3}, antibacterial^{2, 3}, antiviral⁴, and anticancer^{5, 6} properties. Melittin has been extensively used as a model peptide for studying protein folding^{7, 8} as well as protein-lipid associations in membranes.⁹⁻¹² Structural studies of melittin binding to calmodulin^{13, 14}, cell-surface glycosaminoglycans (GAGs)¹⁵ and the α crystallin molecular chaperone^{16, 17} have also been reported.

Several laboratories have investigated the structural basis of the membrane-active properties of melittin,^{2, 9} and there is a general consensus that melittin may exist in solution as a monomer or tetramer, depending on solution conditions.^{9, 18} Indeed the combined effects of concentration, pH, ionic strength, and temperature, dictate the conformation of melittin in solution. As a monomer, melittin may adopt either random coil or α -helical secondary structures.¹⁹⁻²¹ On the other hand, each melittin unit in a tetrameric system is said to be α -helical.²²⁻²⁴

Despite the numerous structural investigations noted here, there continues to be considerable interest in the structure determination of melittin under a variety of conditions - especially using nuclear magnetic resonance (NMR) spectroscopy. However, NMR studies to date have been hampered by the lack of uniformly isotope-labeled melittin.^{10, 25-27} Most previous studies have used selectively labeled melittin produced using solid-phase peptide synthesis,^{10, 25, 26} which is not cost effective. Uniform isotope-labeling by using recombinant protein expression is a relatively economical alternative to chemical methods, but this method can be complicated by the host-toxic effects of the peptide.²⁸⁻³¹ Ishida et al. expressed uniformly labeled [U - ^{13}C , ^{15}N]-melittin using recombinant techniques³¹, but the melittin so produced contains two extra residues that may affect the peptide's secondary and tertiary structure. In addition, the NMR assignments of the labeled sample in their report were not carried out.

It should be noted that most NMR experiments on melittin reported to date have used high concentrations^{19, 21, 25, 32} of the unlabeled or selectively labeled peptide (0.7 - 40 mM). For the sake of clarity, we note that the term "high concentration" in this report refers to the millimolar concentration regime, while "low concentration" corresponds to the sub-

millimolar regime. The higher concentration regime is consistent with the storage conditions of melittin in the bee venom sac, assuming that the amount of melittin contained per bee is about 250-500 micrograms.³³ In contrast, only a small amount of melittin is needed to achieve lysis and partial hemolysis of erythrocytes (up to 90% hemoglobin released), which can be accomplished with <0.001 mM melittin.³⁴ In fact, complete hemolysis appears to be possible at ~0.035 mM melittin.³⁴ Clearly therefore, it is important to characterize melittin structure in the low concentration (micromolar) regime - especially in view of the fact that the large majority of studies have been performed in the millimolar concentration range. Our studies reported here, performed in the low concentration, physiologically relevant regime, thus fill a well-needed gap in the structural characterization of melittin. We determined the NMR solution structure and backbone dynamics of melittin at neutral pH and low ionic strength, and at concentrations below 0.1 mM - conditions that are compatible with the melittin-induced hemolysis of erythrocytes. To obtain well-resolved spectra under these conditions, we used recombinant melittin samples uniformly labeled with ¹³C and ¹⁵N. Our protocol for expression and purification of the labeled sample is a first for melittin, and yields 0.1 mg of the peptide per liter of *E. coli*, which is purified to about 95% purity.

In view of reports in the literature that in membrane interactions, functional melittin assumes an α -helical conformation,^{2, 20, 35} we measured the standard NMR spectra of the peptide in aqueous solutions with and without the co-solvent 2,2,2-trifluoroethanol (TFE), which has been used to stabilize the α -helical form of melittin.⁸ Interestingly, even at low concentrations, the spectra of the ¹³C and ¹⁵N labeled melittin showed two distinct populations attributed to the *cis-trans* isomerization of the Leu13-Pro14 peptide bond. Therefore, for the first time, we provide a complete list of backbone assignments for melittin including ¹³C and ¹⁵N, which distinguish between *cis*-Pro and *trans*-Pro conformations of the peptide. Analysis of chemical shifts and steady state heteronuclear nuclear Overhauser effect (NOE) patterns indicated that melittin is unstructured without TFE, but assumes stable helical conformation above a certain TFE concentration threshold. We also find that in the physiologically relevant low concentration regime, under our solution conditions, melittin is monomeric. We envision that our findings on the secondary structure and backbone dynamics of melittin presented here will enhance the understanding of interactions of the peptide with biological partners such as proteins and membrane lipids.

EXPERIMENTAL SECTION

Cloning and Expression

The cDNA of melittin (GIGAVLKVLTTGLPALISWIKRKRQQ) codon optimized for *E. coli* was cloned into a pTM vector that conferred kanamycin resistance.³⁶ The 5' end of the synthetic gene was engineered with a *Hind*III recognition sequence followed by a methionine codon, while the 3' end had a stop codon and a *Bam*HI recognition sequence. The vector was digested with *Bam*HI HF and *Hind*III (both enzymes from New England Biolabs, NEB) following the manufacturer's instructions. The linearized vector was purified then ligated to the melittin cDNA insert using T4 ligase (NEB). Presence of the melittin cDNA insert in the expression plasmid, pTM-melittin, was confirmed by DNA sequencing at the Molecular Core Facility in Life Sciences at the University at Albany.

With the pTM-melittin expression system (Figure 1A), a fusion protein is produced with an N-terminal 9x-histidine tag (9xHis) followed by a modified Tryptophan Leader (TrpLE) peptide, a methionine residue, and then the melittin peptide.

For peptide expression we selected the *E. coli* strain C41(DE3), instead of the commonly used BL21(DE3). The C41(DE3) strain has been previously used as an alternative to BL21(DE3) for expressing proteins that are toxic to the host.³⁷ Freshly transformed C41(DE3) cells (Lucigen) were pre-grown in 25 mL of Luria Broth (LB) with 1% glucose and 35 mg/L kanamycin overnight (16 h) at 37°C. Each seed culture was inoculated into 1 liter of LB with 1% glucose and 35 mg/L kanamycin and incubated at 37°C until the optical density (OD₆₀₀) reached 0.8. This typically took 2.5 to 3 h. The cells from 2 L of LB were harvested by centrifugation and suspended in 1 L of minimal medium (5 mM Na₂HPO₄, 2.5 mM KH₂PO₄, 1 mM NaCl, 2mM MgSO₄, 0.1 mM CaCl₂, and 1 mg/L thiamine-HCl with pH at 7.0). For [*U*-¹³C, ¹⁵N] labeling, (99%) ¹⁵N-ammonium chloride (1 g/L) was used as the sole nitrogen source, and 2 g (99%) ¹³C-glucose as the carbon source (Cambridge Isotope Laboratories). Overexpression was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated at 37°C and cells were harvested after 4 hours of induction.

Purification of the pTM-Melittin Fusion Protein and CNBr Cleavage

The harvested cells were re-suspended in lysis buffer (10 mM sodium phosphate, pH 7.0) and sonicated. The lysate was treated with benzonase (1 unit of benzonase per milliliter of lysate) for 1 hour at room temperature. The lysate was combined with denaturing buffer (100 mM NaH₂PO₄/Na₂HPO₄ and 8M guanidinium hydrochloride (Gu-HCl) at pH 8.7) to a final Gu-HCl concentration of 6M and a pH of 8.5. The lysate was centrifuged and the supernatant fraction was incubated with nickel-nitriloacetic acid (Ni-NTA) resin (Qiagen) overnight.

Immobilized Metal Affinity Chromatography (IMAC) was performed using the following protocol. The Ni-NTA resin with bound fusion protein was loaded onto a column and washed with the following buffers in sequence: 6M urea at pH 6.3 with 1M NaCl and 50 mM NaH₂PO₄/Na₂HPO₄, 2M NaCl adjusted at pH 6.0 with 50 mM NaH₂PO₄/Na₂HPO₄, and 6 M urea at pH 5.5 with 0.5M NaCl. The fusion protein was eluted using 6M urea solution at pH 3.6 with 0.5M NaCl. The eluted fractions enriched with the fusion protein were desalted by extensive dialysis against water. The desalted fusion protein was freeze-dried and stored at -80°C until it could be processed further.

The pTM-melittin fusion protein was dissolved in 70% formic acid (5 mg protein per mL of 70% formic acid) with 100-fold molar excess of cyanogen bromide. The cleavage was performed in the dark at room temperature for 2 hours. The reaction was quenched by drying the mixture under a stream of nitrogen to evaporate the formic acid and cyanogen bromide. Water was added to dissolve the cleavage products and the mixture was freeze-dried. SDS-PAGE was used to assess the efficiency of the cleavage reaction (Figure 1B).

Purification of Melittin Using Reversed Phase High Performance Liquid Chromatography (RP-HPLC) and Ultrafiltration

The lyophilized cleavage products were dissolved in HPLC Buffer A (0.065% trifluoroacetic acid (TFA) in 95% water/ 5% acetonitrile (ACN)) then injected into a Pursuit C18 column (Varian), operated at room temperature at a flow rate of 1 mL/min, and a solvent gradient with increasing %B buffer (95% ACN / 5% water / 0.05% TFA). The retention time of melittin was determined using a synthetic melittin standard (Genscript) with 95% purity as specified by the manufacturer. HPLC eluates containing recombinant melittin were freeze-dried.

Eluted fractions containing both 9xHis-TrpLE fragments and recombinant melittin were reconstituted in water and the mixture was subjected to ultrafiltration using a 10-kD molecular weight cut-off filter (Amicon). Melittin was collected in the filtrate.

Characterization of Recombinant Melittin by Electrospray Ionization Mass Spectrometry (ESI-MS)

Recombinant melittin was characterized using the Agilent 6530B LC-MS equipped with an electrospray ionization source. The MS was operated in the positive ion mode and scans were acquired in the 90 –1500 m/z range, at a gas temperature of 325°C, drying gas flow at 10.0 L/min, nebulizer pressure at 40 psi, and Vcap voltage at 4000V. Data were acquired and processed using the Agilent Masshunter Workstation Data Acquisition and Qualitative Analysis software respectively. Chromatographic separation (Figure 1C) was carried out on an Agilent Zorbax C-18 column with 2.1 mm inner diameter and 50 mm column length using buffers A (0.1% formic acid in 95% water /5% acetonitrile), and B (0.1% formic acid in 95% acetonitrile /5% water), at a flow rate of 0.2 mL/min, and a gradient of 5% - 95% B buffer. Formic acid was used as the acidic modifier of the mobile phase instead of TFA, because TFA is known to suppress the ESI-MS signal response.³⁸ The ESI mass-to-charge spectra were deconvoluted using Agilent Qualitative Analysis software (Figure S2). In addition, molecular weights of the unlabeled, ¹⁵N-labeled, and ¹³C,¹⁵N-labeled recombinant melittin were calculated using the ESIProt software.³⁹

Determination of % Purity and % Isotope Enrichment

Only recombinant, isotope-labeled melittin samples with >90% purity and >90% incorporation of isotope labels were used in the NMR experiments. The procedures for determining % purity of samples are as follows. Percent purity was determined by using two complementary techniques: 1. peak integration analysis on the LC traces from our ESI-LC-MS experiments (traces are in the form of total ion chromatograms, TIC, with counts plotted against retention time). In this analysis we used unlabeled, synthetic melittin (Genscript) with 95% purity as a calibration standard. 2. we performed SDS-PAGE on the recombinant melittin sample and the synthetic melittin standard, and calculated % purity from band intensities. The percentages of incorporated isotopes (¹³C and ¹⁵N) in [¹⁵N]-labeled and [¹³C,¹⁵N]-labeled samples were calculated as follows: Molecular weights of labeled, recombinant melittin (C₁₃₁H₂₂₈N₃₈O₃₂) samples were determined by deconvoluting ESI mass spectra ("M_x"). The theoretical molecular weight of completely labeled melittin ("M_{CL}") was calculated assuming atomic masses of 13 Da for all carbon atoms and 15 Da

for all nitrogen atoms in the [U - ^{13}C , ^{15}N]-labeled samples. For [U - ^{15}N]-labeled samples, M_{CL} was calculated using an atomic mass 15 Da for all nitrogen atoms. The percent isotope enrichment (% IE) was calculated using the following equation:

$$\% \text{ IE} = 100 - \left[100 * (M_{\text{CL}} - M_{\text{X}}) / (N) \right] \quad (1)$$

In the above equation, N is the theoretical number of ^{13}C and ^{15}N labels in a completely labeled sample. N is equal to 38 for [U - ^{15}N]-labeled samples, and 169 for [U - ^{13}C , ^{15}N]-labeled samples.

NMR Experiments

Melittin samples for NMR measurements were prepared by dissolving [U - ^{13}C , ^{15}N]-labeled melittin in 10 mM potassium phosphate, pH 7.0 with 10% (v/v) D_2O to a concentration of 0.05 mM - 0.09 mM. To prepare the sample containing 30% TFE, deuterated trifluoroethanol (Sigma) was combined with the melittin stock solution.

NMR spectra were acquired at 298 K using a 700 MHz Bruker Avance II NMR spectrometer equipped with a TXI cryoprobe. The experiments were performed with Watergate⁴⁰ water suppression. The ^1H , ^{13}C and ^{15}N backbone resonances were assigned using 2D ^{15}N -edited heteronuclear single quantum coherence (HSQC) spectra, 3D HNCA, 3D HNCOCa, and 3D HNCACB experiments.⁴¹ Spectra were processed using Topspin 2.1 (Bruker, Inc.) and assigned manually using CARA.⁴² The side chain proton resonances were assigned by using 3D ^1H - ^{15}N -HSQC TOCSY.⁴¹ The 3D ^1H - ^{15}N -NOESY-HSQC spectra of both 0% and 30% TFE samples at varying mixing times showed no cross peaks associated with $^{\text{NH}}(\text{i})$ - $^{\text{NH}}(\text{i}+1)$ connectivities, presumably due to unfavorable rotational correlation time.⁴¹ Chemical shift perturbations (CSPs) induced by increasing TFE concentration from 0 to 30% TFE were calculated as described previously.⁴³ CSPs were determined as weighted average chemical shift deviations for each $^1\text{H}/^{15}\text{N}$ pair from two ^{15}N -HSQC spectra for melittin: without TFE (Figure 2B), and with 30% TFE (Figure 2C).

For both samples (0% TFE and 30% TFE), the ^1H - ^{15}N steady-state heteronuclear values were obtained by recording spectra in an interleaved manner, with and without applying ^1H saturation ("sat" and "unsat" respectively). Protons were irradiated with a 120° high power pulse every 5 ms over the course of a 1s relaxation delay. Steady-state NOEs were calculated as the ratio of peak amplitudes, $I_{\text{sat}}/I_{\text{unsat}}$, with standard deviations estimated using the method⁴⁴ of Farrow et al.:

$$\sigma_{\text{NOE}} / \text{NOE} = \left[(\sigma_{\text{sat}}/I_{\text{sat}})^2 + (\sigma_{\text{unsat}}/I_{\text{unsat}})^2 \right]^{1/2} \quad (2)$$

where σ_{sat} and σ_{unsat} are the root-mean-square noise levels.

Chemical Shift Index (CSI) Analysis

Melittin secondary structures were predicted using chemical shift index (CSI) analysis.⁴⁵ Assignments for H^N, H^A, C^A, C^B, N^H were uploaded to the CSI 3.0 web server at <http://csi3.wishartlab.com>. Secondary structure was defined in terms of probabilities for a particular segment to fold into α -helix, random coil, or β -sheet.

RESULTS

Expression and Purification of Recombinant Melittin

The design of the expression system and the results of the expression and purification of recombinant melittin are shown in Figure 1A and 1B-D respectively.

We expressed melittin as a fusion protein with an insoluble TrpLE fragment on the N-terminal side of melittin. TrpLE is a 11 kDa protein that promotes the formation of inclusion bodies in the cell.³⁶ Based on earlier reports,²⁹⁻³¹ we reasoned that confinement of the pTM-melittin fusion protein within inclusion bodies would attenuate melittin's toxicity and circumvent protein degradation and poor host-cell growth issues. Interestingly however, only about 55% of the total fusion protein was found in the insoluble fraction of the bacterial lysate (Figure S1), which suggests that the highly charged melittin fragment probably helps to partially solubilize the fusion protein, thereby reducing the influence of TrpLE on solubility. We note that a similar approach involving an insoluble fusion partner³⁰ has been used in expressing melittin with the F4 polypeptide as the fusion partner, instead of TrpLE.

Another key feature of the pTM-melittin construct was the modification of the TrpLE sequence by replacing the two cysteines and six methionines with alanines and leucines, respectively,³⁶ in order to facilitate the His-tag based affinity purification and cyanogen bromide cleavage of the fusion protein. Thus, the entire fusion protein contains only two methionines, one at the start codon and the other between TrpLE and the melittin sequence. This design allowed us to obtain wild type melittin directly after CNBr cleavage of the fusion protein. Figure 1B shows the SDS-PAGE analysis corresponding to the several steps that track the expression and purification of recombinant melittin as the overexpressed pTM-melittin fusion protein (16 kDa) in C41(DE3) cells. In Figure 1B, we also show that the pTM-melittin fusion protein was extracted from the cell lysate by IMAC. We used high-salt wash buffers (containing 500 mM NaCl up to 2M NaCl) in IMAC to break up complexes that may have been formed between DNA/RNA and the fusion protein. This step ensures the efficient binding of the fusion protein to the IMAC column. The fusion protein was about 60% pure after IMAC and before the CNBr cleavage step (determined by quantifying band intensities from SDS-PAGE). We observed that the presence of contaminants (mainly DNA and RNA, as detected by the absorption at 260 nm) greatly impeded the cleavage reaction, presumably by limiting access to the cleavage site.

In Figure 1B-1C, we show that the cleavage of the fusion protein produces two fragments corresponding to melittin and the N-terminal region containing the 9xHis-tag and TrpLE (abbreviated as 9xHis-TrpLE fragment). The deconvoluted mass spectrum of unlabeled recombinant melittin (Figure 1D) is consistent with that of the synthetic melittin standard. The mass-to-charge ratios for different charge states of our recombinant, unlabeled sample

(Figure S2A) are consistent with previously reported data.³⁸ With 70% formic acid as solvent, we did not detect formylated melittin in LC-MS. We did not use neat (i.e. 88%) formic acid because of its tendency to formylate about 10% of melittin. The [U - ^{15}N] and [U - ^{13}C , ^{15}N]-labeled melittin obtained after cleaving the respective labeled-fusion peptides have deconvoluted molecular weights of 2883.7 ± 1 Da and 2999.6 ± 4 Da, respectively. These masses are consistent with the percent isotope enrichment values of 93.2% and 90.1% for [U - ^{15}N]- and [U - ^{13}C , ^{15}N]-labeled melittin, respectively (Figure S2 B-C).

Figure 1C shows the TIC from LC-MS analysis of the cleavage reaction mixture and the purified recombinant melittin. Under these run conditions, we were unable to obtain a clear separation between melittin and 9xHis-TrpLE (black trace) because the melittin peak significantly overlapped with the 9xHis-TrpLE peak, although other contaminants such as uncleaved fusion protein and truncated fragments of the fusion protein were easily separated from melittin. Thus, a final ultrafiltration step was carried out to separate melittin from 9xHis-TrpLE. Melittin obtained after ultrafiltration (red trace) had a purity of 91-95% (see Experimental Section). The final yield of pure melittin was found to be 0.1 mg/liter of *E. coli*.

We note that our work on the expression of uniformly labeled recombinant melittin is distinct from those of other labs.²⁸⁻³⁰ In most previous reports the isotope labels were not incorporated in the recombinant melittin peptide.²⁸⁻³⁰ The only report on the production of isotope-labeled melittin³¹ was by Ishida et al., but their melittin preparation after cleavage of the fusion protein contained an N-terminal Gly-Thr dipeptide artifact, as also noted by the authors.

Backbone Resonance Assignments of *cis*-Pro-melittin and *trans*-Pro-melittin

The 2D ^1H - ^1H NOESY spectrum of melittin at pH 7.0, in D_2O (Figure 2A) shows NOEs in the spectral region corresponding to proline resonances. The proton NOE cross peaks suggest the presence of *cis* and *trans* conformers of the peptide under physiological conditions. Most melittin molecules are found in the *trans*-Pro14 configuration, indicated by the presence of a strong NOE⁴⁶ between H^α of Leu13 and $\text{H}^{\delta 2}$ of Pro14. A much weaker NOE cross peak is observed between the H^α protons of Leu13 and Pro14, indicating the presence of a small population of Pro14 in the *cis* conformation.

The ^{15}N -HSQC, spectra for [U - ^{13}C , ^{15}N] melittin in the NMR buffer was acquired under two conditions: without TFE (Figure 2B), and with 30% TFE (Figure 2C). TFE, a helix-stabilizing agent, has been previously used to induce helix formation in melittin and other peptides.^{8, 47, 48} Low concentrations (0.05 mM to 0.09 mM) were used to mimic conditions in which melittin lyses erythrocytes in a dilute suspension.³⁴

Within this low concentration and low ionic strength regime, melittin is a monomer.^{19, 32} Gly1 amino protons were not visible in both ^{15}N -HSQC spectra (Figure 2B, C) presumably due to fast exchange with the solvent. In spectra taken without TFE (Figure 2B), weak cross peaks corresponding to alternate conformations of only Leu13, Ala15, and Leu16 from the *cis* form were observed, while more cross peaks attributed to this conformer appeared after adding TFE (Figure 2C, Figure S3). These additional peaks in Figure 2C corresponded to

residues other than 1-6, 14, 22-23. We found that the NH peak for Ile2 disappeared upon addition of TFE probably because the peak was broadened beyond detection due to exchange with water. Figure 2D shows that adding up to 30% TFE to melittin resulted in large (>0.1 ppm) chemical shift perturbations for both *trans* and *cis* conformers of melittin. Adding TFE clearly leads to an increase in the chemical shift dispersion along the ^1H dimension indicating a transition to a more structured form. Peak amplitude analysis of the NH cross-peaks in Figure 2C and Figure S4 indicated that the *cis* form comprised ~20-27 % of the population.

The ^{15}N -HSQC spectrum of melittin in aqueous solution at pH 7.0 without TFE (Figure 2B) showed a limited chemical shift dispersion along the ^1H dimension indicative of an unstructured form. This spectrum is generally consistent with the ^{15}N -HSQC shown in the work of Ishida et al.³¹ although their spectrum showed more broadened signals, and was acquired in the higher, 6.5 mM, concentration regime of melittin. Their NMR sample was prepared in water with 10% D_2O and contained 0.5 mM 2,2-dimethyl-2-silapentanesulfonic acid (DSS) and 0.03% sodium azide. The authors suggested that the signal broadening in their spectrum could have been due to the tetramerization of melittin. The similarities between the general pattern of NH cross peaks in our ^{15}N -HSQC spectrum and theirs suggest that their spectrum may also correspond to the unstructured form of melittin. Thus, these results are not consistent with the previously reported α -helical structure of melittin in a tetrameric system.²²⁻²⁴ We conclude that, if melittin were to assume an α -helical structure in a tetrameric unit *while in aqueous solution*, there would be a much larger chemical shift dispersion along the ^1H dimension in the ^{15}N -HSQC spectrum - which would more closely resemble our ^{15}N -HSQC spectrum measured with 30% TFE.

Secondary Structure Prediction

From the NMR assignments obtained with 0% and 30% TFE, the secondary structures (Figure 3A-B) were predicted by means of the Chemical Shift Index (CSI) analysis. This method uses NMR backbone chemical shifts in identifying probable secondary and super-secondary structures in protein segments. The complete lists of secondary structure assignments are shown in the Supporting Material (Tables S3 - S6). In this report, secondary structure assignments are reported in terms of CSI consensus values (Figure 3B) as well as helical probability scores, abbreviated as “H-prob” (Figure S5). The CSI consensus values can only be equal to -1, 0, or +1, pertaining to α -helix, random coil, and β -sheet structures.⁴⁵ On the other hand, H-prob scores may range from 0 to 1, corresponding to 0 to 100% probabilities of forming an α -helix.⁴⁵ CSI consensus values serve as overall indicators of the secondary structure assignments, while H-prob scores provide a more conservative means of assessing a segment’s propensity for the α -helical structure.

The results of the secondary structure prediction clearly suggest that melittin at low (i.e. micromolar) concentrations in phosphate buffer is unstructured, and that TFE induces helicity in melittin. It should be noted that although the consensus CSI values (Figure 3B) for ‘unstructured’ melittin were zero and suggest a random coil conformation, the C-terminal region for both *cis* and *trans* conformers had H-prob scores (Figure S5).

In 30% TFE, two helical regions are formed in *trans*-Pro-melittin, while only one helix at the N-terminal end is formed for *cis*-Pro-melittin. In *trans*-Pro-melittin, the segments that consisted of residues 2-9 and 13-22 have consensus CSI values of -1 pertaining to α -helices. In *cis*-Pro-melittin, only one helix at the N-terminal end is formed by residues 2-11. Although the C-terminal region in *cis*-Pro-melittin in 30% TFE has a consensus CSI score of zero, a closer examination shows that the H-prob scores for residues 15-18 are higher than those of the *cis* form at 0% TFE (Figure S5). After residue 18, however, the H-prob fluctuates drastically. Thus, it appears that in the presence of TFE, the C-terminal region of the *cis* conformer shows partial helical character.

Backbone Dynamics

To identify the boundaries of the folded protein structure, we performed steady-state ^{15}N - ^1H NOE experiments on melittin samples in 0% and 30% TFE (Figure 4A-B). Segments of the protein that do not participate in the folded structure have negative heteronuclear NOE values due to their high degree of local flexibility and motions in the sub-nanosecond to nanosecond time-scale.⁴⁹ Terminal residues were omitted from the analysis because the very high flexibility demonstrated by these regions resulted in unreliable estimates of heteronuclear NOE intensities.⁵⁰

In the data without TFE (Figure 4A), only a slight difference in the heteronuclear NOE values is observed between the *cis* and *trans* forms. Most heteronuclear NOEs were negative, indicative of highly flexible regions that show faster motions relative to the overall tumbling of the peptide. A small C-terminal segment formed by residues 13-20 had low, positive heteronuclear NOE values (ranging 0.05 - 0.45) indicative of more restricted motion compared to that of the rest of the peptide. For both *cis* and *trans* conformers, heteronuclear NOEs for the C-terminal segment are too low to be associated with a fully formed helix. We conclude that at 0% TFE, an extended, flexible conformation is maintained. This is in agreement with our secondary structure predictions generated by the CSI.

At 30% TFE (Figure 4B), there is an overall reduction in flexibility manifested by the higher positive heteronuclear NOEs. In general, the *trans* conformer was less flexible than the *cis*, and the difference is more pronounced for residues 15-17, and 19-21. The *trans* conformer has large positive heteronuclear NOEs (0.57 - 0.94 for residues 4-24) throughout the chain, and this is consistent with the formation of stable helices. Similarly, the large positive heteronuclear NOEs in the N-terminal region (0.64-0.87 for residues 4-13) of the *cis* conformer point to the presence of an N-terminal helix. The C-terminal region (0.10-0.67 for residues 15-24) for the *cis* conformer is still unstructured and shows a degree of flexibility similar to that of the *cis* and *trans* forms of melittin in 0% TFE. The lack of a stable C-terminal helix in the *cis* form may result from the restriction imposed by the Pro14 residue on helix formation. The Protein Data Bank (PDB)-generated structures of both melittin conformers (Figure 5) show that Pro14 is situated at or near a helix break – thus its isomerization state may determine the probability of forming a C-terminal helix.⁵¹

DISCUSSION

Melittin has long been recognized as a model peptide representing anti-microbial peptides^{2, 3} and amphiphilic membrane-binding peptides.^{2, 9} Its specific properties such as hemolysis,³⁴ as well as binding to calcium-binding proteins,¹³ have also been well examined. Such wide-ranging properties in a naturally occurring, 26 amino acid peptide inspired a slew of structural studies of melittin beginning in the 1970s. Most of these studies were carried out in the millimolar concentration regime, and focused on the monomer-tetramer equilibrium, as well as the two helical segments observed in the monomer.

Much less attention has been given to the structure of melittin in the micromolar concentration regime, even though the peptide is functional at that concentration. It is known that melittin is unstructured at low concentrations in aqueous solutions,¹⁹ and more structured when bound to lipids,²⁰ cell-surface GAGs,¹⁵ and proteins.⁵² Moreover, solution conditions, such as pH and ionic strength, also affect the structure of melittin. This plasticity of structure is very reminiscent of intrinsically disordered proteins (IDPs).⁵³ Perhaps, because of this structural plasticity, melittin, just like IDPs, binds to a variety of molecules. A cursory analysis for the prediction of disordered regions, for example, using the Protein Disorder Prediction System (PrDOS) server at prdos.hgc.jp, shows the four N-terminal residues (GIGA) and five C-terminal residues (RKRQQ) as disordered regions - i.e., about a third of the total residues. While disordered termini in peptides and proteins are not unusual, their presence, taken together with other observations, clearly highlight the structural origin of the versatility of melittin. Our steady-state heteronuclear NOE data for melittin in 0% TFE (Figure 4A) show that these terminal regions are also highly flexible – more so than the other regions of the peptide. Therefore, under these solution conditions (0% TFE, 10 mM potassium phosphate at pH 7.0), a correlation may be made between flexibility and the predicted degree of disorder in melittin.

We observed – as did others earlier – that under certain conditions, melittin can assume an α -helical structure. The presence of two helices in melittin (N-terminal and C-terminal) has been reported in previous X-ray studies,^{22, 24, 47} as well as in NMR studies of solutions of melittin in methanol,²¹ and aqueous solutions of micelle-bound melittin.²⁰ To the best of our knowledge, this is the first report showing that melittin in the low concentration regime forms just one stable N-terminal helix, while the C-terminal region remains unstructured.

As stated above, an α -helical form is often the structure of melittin in the bound state - hence its significance. Here we used TFE to induce helix formation in melittin. TFE is often used in aqueous solution to enhance the structural propensity of peptides, through various proposed mechanisms including lowering of the dielectric constant of aqueous solutions of the peptide, enhancing intrapeptide hydrogen bonding, and preferential solvation of some groups on the peptide.⁵⁴ We have observed in our experiments that the helical structure of melittin is more favored in TFE-enriched solutions. However, we cannot claim that this trend is always followed in conditions with larger ratios of TFE/water and higher melittin concentrations. In a study by Othon et al.,⁸ the titration of a more concentrated, aqueous solution of tetrameric, α -helical melittin with TFE resulted in only a slight increase in helicity, from 60 to 80% (monitored by circular dichroism) in the concentration range 0-10%

TFE. Their study also showed that the percent helicity remained consistent in the range 10-75% TFE, then declined at TFE concentrations approaching 100% TFE. These authors suggested that such a change in helicity demonstrates the “unfolding of the melittin tetramer”, with the tetrameric form favored at low TFE concentrations (<10%), and the monomeric form favored at higher TFE concentrations. With the availability of the ^{13}C , ^{15}N -labeled melittin reported here, the unfolding process may also be studied at the residue level.

In addition to helix stabilization, TFE is also useful in modeling membrane and protein microenvironments,⁵⁴ although the basis for this is not understood in a comprehensive manner. However, the structures generated in TFE-enriched aqueous media may serve as representative model structures of melittin in the membrane-bound or protein-bound states.

The role of Pro14 in melittin is of particular interest: Its isomerization gives rise to two populations of melittin, which differ significantly in the secondary structure of the C-terminal region. An earlier report⁵⁵ on monomeric D-Pro14 melittin showed that in methanol the two helices (N- and C-terminal) were laterally displaced from their corresponding positions in wild-type melittin, and that there was a small rotation of the C-terminal helix relative to the N-terminal, around the long axis of the molecule. The importance of Pro14 was also noted by Rex et al.⁵⁶ based on their studies of the P14A variant of melittin, and they concluded that Pro14 not only has a direct structural influence, but also indirectly controls the electrostatic properties of the peptide. It should be stressed that we observed the residue-specific structural change due to the isomerization of Pro14 primarily because we successfully expressed uniformly ^{15}N - and ^{13}C -labeled melittin.

Our recombinant melittin samples exhibited a mixture of both *cis* and *trans* forms of the Leu13-Pro14 bond, which is consistent with studies on smaller Pro-containing peptides.⁵⁷ A previous NMR study on melittin in methanol²¹ did not report the presence of a *cis* form, but this may be due to a number of factors. First, in studies with small model peptides, the *trans*-Pro conformer is reported to be slightly more stable than the *cis*-Pro conformer.^{58, 59} Second, the *trans-cis* conversion has a high energy barrier⁵⁸ because of the partial double-bond character of the X-Pro bond, and this may have resulted in a transition too slow to be observable within the time frame of the previously conducted experiments.²¹ Third, methanol is less polar than water, and may have inhibited the conversion of *trans*-Pro melittin into the *cis* form, since the *cis* form is favored in polar solvents.⁵⁸ In our present study, we observed that the population of the *cis* form increased after a prolonged period (2 weeks) of storing the melittin sample in aqueous solution at room temperature (Figure S3). When the incubation time was decreased, only the *trans* form was observed in the ^{15}N -HSQC spectra. Our results may be consistent with those of Lauterwein et al.¹⁹ In their study of melittin in aqueous solution, they observed two populations of monomeric melittin, and suggested that the low-abundance form may have arisen from the *cis-trans* isomerization of the Leu13-Pro14 bond.¹⁹ Arguably, more comprehensive NMR structural studies using varying solvents and temperatures with our uniformly isotope-labeled melittin need to be performed to characterize the kinetics and thermodynamics of the X-Pro isomerization process.

Previous structural studies on melittin were conducted with selectively-labeled melittin, in which the ^{13}C and/or ^{15}N labels resided only in a few residues.^{26, 60} These studies, while very informative, could only reveal localized structural changes. A recent study on uniformly ^{13}C - and ^{15}N - labeled melittin, reported an ^{15}N -HSQC spectrum, but the melittin sample contained additional Gly and Thr residues at the N-terminus, and the authors have not yet assigned their HSQC spectrum.³¹ Thus, our study reported here is the first to present NMR structural dynamics in the low concentration regime (<1 mM) of melittin, that addresses the structure of melittin in aqueous solutions, and in its putative bound state in TFE-containing solutions.

SUMMARY AND CONCLUSIONS

Arguably, melittin has been extensively studied, and the bio-applications of this versatile peptide continue to grow.^{61, 62} Structural analysis of melittin under physiological conditions is necessary to understand its biological activities including hemolysis, disruption of lipid micelles, and association with multifunctional proteins such as calmodulin and the alpha-crystallin molecular chaperone. The NMR measurements reported here were performed on melittin samples at neutral pH, low ionic strength, and low concentration, while most previous NMR studies focused on characterizing melittin in the high concentration regime. Thus our data provide this missing link. Using a variety of triple resonance experiments, we obtained complete ^1H , ^{13}C , and ^{15}N backbone assignments for melittin in its unstructured and helical forms. The assignments were used to predict the secondary structures for different regions of melittin. To provide insights into the flexibility and backbone dynamics of melittin we carried out steady-state heteronuclear NOE experiments. Our findings suggest that melittin at neutral pH, low ionic strength, and low concentrations assumes an extended, flexible conformation, which is in agreement with previous NMR studies. Upon association with TFE, melittin assumes a helical structure, consistent with previously reported X-ray and NMR data.^{22, 24, 47}

We have characterized the transition of monomeric melittin from coil to helix with the consideration of both isomerization states of the Leu13-Pro14 peptide bond, and have demonstrated that the *cis* and *trans* conformers show markedly different secondary structures and backbone dynamics. Thus, while our study marks a step forward in the distinction between the biological roles of these two conformers, the kinetics of this transition needs to be explored in detail. Furthermore, with the availability of the [U - ^{13}C , ^{15}N]-melittin, we are now in a position to conduct further high-resolution NMR studies on the interactions of melittin with other substrates such as micelles and molecular chaperones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
CSP	chemical shift perturbation
HSQC	heteronuclear single-quantum coherence
NOESY	nuclear Overhauser effect spectroscopy
TOCSY	total correlation spectroscopy
Gu-HCl	guanidinium hydrochloride
TFE	trifluoroethanol
PrDOS	Protein Disorder Prediction System
PDB	Protein Data Bank
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
ESI	electrospray ionization
LC	liquid chromatography
MS	mass spectrometry
CSI	chemical shift index
RCI	random coil index
H-prob	propensity for α -helix
C-prob	propensity for random coil
B-prob	propensity for β -sheet

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Figure 1.

Expression and purification of recombinant melittin. **A**) Scheme for the pTM-melittin plasmid for expressing a fusion protein containing an N-terminal 9x-Histidine tag followed by the modified Tryptophan Leader Peptide (TrpLE) sequence. The C-terminal region contains the melittin sequence which is preceded by a methionine residue. Cyanogen bromide (CNBr) cleaves between this Met residue and Gly1 of melittin. **B**) SDS-PAGE (15% acrylamide gel) was used to monitor expression and purification of melittin. Positions of the fusion protein, 9xHis-TrpLE and recombinant ¹³C, ¹⁵N-melittin are indicated. Lane 1) Molecular weight markers. Lanes 2-3) Total cell lysate obtained before (2) and after (3) IPTG induction, respectively. Lane 4) Fusion protein (~16 kDa) purified by affinity chromatography. Lane 5) Products of the cleavage mixture (9xHis-TrpLE at ~13 kDa and melittin below the 10 kD marker), with some fusion protein left unreacted. Lane 6) HPLC-purified recombinant ¹³C, ¹⁵N-labeled melittin. **C**) Total ion current (TIC) chromatogram from LC-MS showing overlaid traces for the cleavage mixture (black) and purified (>90% purity) recombinant melittin (red) obtained after RP-HPLC and ultrafiltration. A C18 column was used to resolve components of the cleavage mixture. The mobile phase gradient (grey trace) used was 5% - 65% Buffer B from t = 1 to t = 25 min, followed by 65% - 95% Buffer B from t = 25 to t = 30 min. **D**) Deconvoluted ESI mass spectrum of recombinant unlabeled melittin (black trace). Inset (red trace): mass spectrum of the synthetic melittin standard.

Figure 2. NMR characterization of melittin. **A)** 2D ^1H - ^1H NOESY spectrum of melittin in 10 mM potassium phosphate at pH 7.0 and D_2O as solvent. Note that the application of the Watergate⁴⁰ pulse sequence to suppress residual water signal led to the bleaching of one of the NOEs on the other side of the diagonal that corresponded to H^α Leu13 and H^α Pro14. **B)** [U - ^{13}C , ^{15}N]-melittin in 10 mM potassium phosphate buffer at pH 7.0, without TFE. Spin systems for all residues except for G1 and P14 are assigned. Peaks enclosed in Box (a) correspond to the amide side chains of Q25 and Q26. The peak at ~ 10 ppm (^{15}N dimension) in Box (b) is assigned to the side chain indole of W19. For residues A15, L16, and L13, two peaks were found; the smaller peaks (labeled A15_c, L16_c, and L13_c) are from melittin in the *cis* conformation. **C)** [U - ^{13}C , ^{15}N]-melittin in 10 mM potassium phosphate buffer at pH 7.0 with 30% TFE. Spin systems for all residues except for G1, I2, and P14 are assigned. Peaks

enclosed in Box (a) correspond to the amide side chains of Q25 and Q26 from both *cis*-P14 and *trans*-P14 melittin. The peaks in Box (b) are assigned to the side chain indole of W19 in both the *cis*-P14 (smaller peak) and *trans*-P14 (larger peak) conformations. The subscript “c” indicates the assignments from the *cis* conformation. **D**) Chemical shift perturbations plotted against residue number. The perturbations were induced by increasing TFE concentration from 0% to 30% TFE in the [U - ^{13}C , ^{15}N]-melittin sample with 10 mM potassium phosphate at pH 7.0. Most perturbations are greater than 0.1 ppm (threshold indicated by a broken line).

Figure 3.

Secondary structure of melittin in 0% TFE and 30% TFE. A) Scheme of the secondary structures adopted by melittin in 0% TFE and 30% TFE. Melittin is initially unstructured, but after addition of TFE two helices form for the *trans* conformer while only one stable helix remains in the *cis* conformer. These structures were predicted from backbone assignments using CSI 3.0.⁴⁵ B) Consensus CSI values were plotted against residue number. Scores of -1, 0, and +1 were assigned to α -helix, random coil, and β -sheet secondary structures, respectively.

Figure 4. Steady-state heteronuclear NOE for the amide backbone of melittin in 10 mM potassium phosphate buffer at pH 7.0 (A) without TFE and (B) with 30% TFE. Values for Pro14 are not shown. A) Negative heteronuclear NOE values for C-terminal (21-22) and N-terminal (4-12) residues are indicative of highly flexible regions that move faster than the overall melittin tumbling. Residues 13-20 had low positive heteronuclear NOEs suggesting the formation of a partial helix. Heteronuclear NOE values for residues at the termini (2-3, 23-26) were outside the acceptable range due to very high flexibility, and were thus omitted.

B) Upon addition of 30% TFE, melittin adopted a less flexible structure, and *trans* and *cis* forms showed distinct backbone dynamics. In general, more positive heteronuclear NOEs were obtained for the *trans* conformation. The standard deviation of the heteronuclear NOEs was noticeably higher for peaks present in *cis* but not in *trans*, which is due to the weaker heteronuclear NOE signals associated with the lower population of the *cis* form

Figure 5. Structural representations of *trans*-Pro14-melittin (A-B) and *cis*-Pro14-melittin (C-D) generated using Swiss PDB Viewer 4.10. Proline residues are highlighted in magenta. The structure of *trans*-Pro14-melittin was solved (PDB code 2MLT). The structure for *cis*-Pro14-melittin was generated from *trans*-Pro14-melittin by isomerizing the Leu13-Pro14 peptide bond, followed by energy minimization. Panels B and D show the enlarged profile of the hinge regions.