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Helical Structure of Recombinant Melittin

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Abstract

Melittin is an extensively studied, 26-residue toxic peptide from honey bee venom. Because of its versatility in adopting a variety of secondary (helix or coil) and quaternary (monomer or tetramer) structures in various environments, melittin has been the focus of numerous investigations as a model peptide in protein folding studies as well as in studies involving binding to proteins, lipids, and polysaccharides. A significant body of evidence supports the view that melittin binds to these macromolecules in a predominantly helical conformation, but detailed structural knowledge of this conformation is lacking. In this report, we present nuclear magnetic resonance (NMR)-based structural insights into the helix formation of recombinant melittin in the presence of trifluoroethanol (TFE): a known secondary structure inducer in peptides. These studies were performed at neutral pH, with micromolar amounts of the peptide. Using nuclear Overhauser effect (NOE)-derived distance restraints from three-dimensional NMR spectra, we determined the atomic resolution solution NMR structure of recombinant melittin bearing a TFE-stabilized helix. To circumvent the complications with structure determination of small peptides with high conformational flexibility, we developed a workflow for enhancing proton NOEs by increasing the viscosity of the medium. In the TFE-containing medium, recombinant monomeric melittin forms a long, continuous helical structure, which consists of the N- and C-terminal α -helices and the noncanonical 3_{10} -helix in the middle. The noncanonical 3_{10} -helix is missing in the previously solved X-ray structure of tetrameric melittin and the NMR structure of melittin in methanol. Melittin's structure in TFE-containing medium provides insights into melittin's conformational transitions, which are relevant to the peptide's interactions with its biological targets.

GRAPHICAL ABSTRACT:

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Notes

The authors declare no competing financial interest.

INTRODUCTION

The toxic peptide melittin is the primary component of honey bee venom.¹ The hemolytic activity and antimicrobial properties of melittin have been extensively studied in the last few decades.^{2–4} Melittin has also been examined as a model peptide for studies on protein folding, in part due to its structural plasticity.^{5,6} Depending on solution conditions, melittin may form a monomer or a tetramer and may be helical or random coil.^{6–9}

Melittin is known to bind to membranes, polysaccharides, and proteins. The structure of melittin bound to these macromolecules has been found to be predominantly helical.^{10–13} Among proteins, melittin has been shown to bind to calmodulin,¹⁴ staphylokinase,¹⁵ centrin,^{12,16} and α -crystallin.^{17,18} The work of Farahbakhsh et al.¹⁸ suggests that melittin may assume helical or β -strand secondary structures while bound to α -crystallin, although the authors note that melittin does not reside in a hydrophobic cavity on α -crystallin as it does in membranes. In view of the observations of Sharma et al.¹⁷ that the substrate mimic, melittin, binds to a functionally important region on α -crystallin, the possible involvement of the helical form of the peptide in such binding assumes greater significance.

Previous studies have described the folding/unfolding of melittin in various media, notably trifluoroethanol (TFE)-water mixtures.^{5,19,20} TFE is known to stabilize secondary structures, such as α -helices and β -sheets, in proteins and peptides.^{21–26} It is thought that TFE induces α -helical structure through a variety of mechanisms, including promotion of intramolecular hydrogen bonding, perturbation of hydrophobic interactions, and molecular crowding.^{21–26} Studies on TFE-induced structural changes on melittin carried out by molecular dynamics (MD) simulations,²² circular dichroism (CD),¹⁹ fluorescence spectroscopy,⁵ and NMR^{20,27} spectroscopy suggest that TFE stabilizes the helical form of melittin. However, even with the substantial effort directed toward describing TFE–melittin associations, previous studies have not produced a Protein Data Bank (PDB) entry for melittin's three-dimensional (3D) structure solved in the presence of the TFE co-solvent. In addition to X-ray crystal structures of tetrameric melittin (PDB ID: 2MLT) and centrin-bound melittin (PDB ID: 3QRX), only two solution NMR, 3D structures of melittin are currently available in the PDB: the structure

of monomeric melittin (PDB ID: 2MW6) labeled with a ruthenium-containing organometallic fragment²⁸ and the solution NMR structure of D-Pro14-melittin (PDB ID: 1BH1).²⁹

As mentioned earlier, melittin's structure is generally dependent on solution conditions.^{6–9} In particular, structural changes on melittin have been observed by varying pH, ionic strength, temperature, and concentration.^{6,7} In deciding the solution conditions used for elucidating melittin's 3D structure, we took into consideration the fact that most structural studies on melittin have focused on “high” melittin concentrations, spanning the millimolar range ~1–40 mM,^{2,7} whereas even very “low”, micromolar concentrations of melittin were found to be toxic.^{3,30–33} Here, we have determined the atomic resolution NMR structure of melittin at micromolar concentrations, which are consistent with bioassays on melittin-induced hemolysis and inhibition of bacterial growth.^{3,30–33} Acquisition of high-quality 3D NMR spectra for micromolar amounts of melittin in this work is facilitated by the use of uniformly isotope-labeled (¹³C- and ¹⁵N-) recombinant melittin.²⁷

In general, the NMR-based solution structure determination of small peptides is challenging due to their high conformational flexibility and fast tumbling in solution.^{34–36} For a small peptide, the interconversion among various conformers is usually fast on the NMR time scale, and this may result in NMR observables, such as chemical shifts and nuclear Overhauser effect (NOE) cross peaks, which only provide information on the “average” structure of multiple conformers.^{34–36} Another common problem is weak NOEs due to unfavorable values of the rotational correlation time, τ_c .^{34,37,38} Moreover, using low (micromolar) peptide concentrations exacerbates the problem of weak NOEs. Altogether, these difficulties hamper NMR solution structure calculations, which require an abundance of NOE-derived distance restraints. Therefore, in the present study, we employed a systematic approach to enhance the nuclear Overhauser effect of melittin by adjusting the viscosity of the solvent. The restraints derived from the NOEs were used to solve the 3D solution NMR structure of recombinant melittin in its TFE-stabilized helical form (PDB ID: 6DST), which serves as the only solution NMR structure in the PDB for monomeric melittin without organometallic labels and without modification of the Pro14 residue.

METHODS

Preparation of NMR Samples.

Deuterated glycerol (D₈, 99%), ¹⁵N-ammonium chloride (¹⁵N, 99%), and ¹³C-glucose (U-¹³C₆, 99%) were purchased from Cambridge Isotope Laboratories. Deuterated 2,2,2-trifluoroethanol (D₃, 99.5%) was purchased from Sigma-Aldrich. Unless otherwise stated, all NMR samples were prepared with deuterated glycerol and deuterated TFE.

Recombinant [U-¹³C, ¹⁵N]-melittin was expressed and purified using a previously published protocol.²⁷ Briefly, *Escherichia coli* (E. coli) strain C41(DE3) was transformed with a plasmid encoding a fusion protein engineered with a poly-histidine tag at its N-terminus followed by methionine and the melittin sequence (GIGAVLKVLTTGLPALISWIKRKRQQ). Colonies expressing the melittin-containing fusion protein were cultured and grown in M9 medium containing ¹⁵NH₄Cl and [U-¹³C]-

glucose to produce [U- ^{13}C , ^{15}N]-labeled fusion protein. Overexpression was induced using 1 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG). Following expression, cells were pelleted and lysed. The fusion protein was purified by nickel affinity chromatography under denaturing conditions. The fusion protein was cleaved using cyanogen bromide to release the recombinant melittin fragment, and the melittin peptide was purified by reversed phase high-performance liquid chromatography. The masses of purified recombinant melittin determined by electrospray ionization mass spectrometry (ESI-MS) were 2847 Da without uniform labeling and 3000 Da with uniform ^{13}C - and ^{15}N -labelings. Recombinant melittin of at least 95% purity was lyophilized and stored at -80°C until it was used in NMR experiments.

Screening of Optimal Conditions for Acquiring NOESY Spectra.

NMR spectra were acquired using Bruker Ascend 600 MHz or Avance II 700 MHz NMR spectrometers equipped with ultrasensitive cryoprobes. Standard triple resonance experiments (HNCA, HNCOC, HNCACB), 3D ^1H - ^{15}N nuclear Overhauser effect spectroscopy (NOESY), 3D ^1H - ^{13}C NOESY, 2D ^1H - ^{15}N heteronuclear single quantum coherence (HSQC), 2D ^1H - ^{13}C HSQC, 2D ^1H - ^1H NOESY, and 2D ^1H - ^1H rotating frame Overhauser effect spectroscopy (ROESY) were acquired with the WATERGATE pulse sequence for water suppression.³⁹ The NOESY mixing time was 200 ms for 3D ^1H - ^{13}C NOESY, 3D ^1H - ^{15}N NOESY, 2D ^1H - ^1H NOESY, and 2D ^1H - ^1H ROESY. NOESY experiments for generating distance restraints were recorded at 285 K. Data were processed with TOPSPIN 3.1 and analyzed using CARRA.⁴⁰

In a typical 3D NMR experiment, 50 μM [U- ^{13}C , ^{15}N]-melittin was dissolved in the NMR buffer, 10 mM potassium phosphate buffer at pH 7.0 with 10% v/v D_2O . Immediately after dissolving melittin, 30% v/v-deuterated TFE was added to the sample, followed by either 0 or 10% w/v-deuterated glycerol. Freshly reconstituted recombinant melittin was used for all 3D NMR experiments, so that only the *trans*-Pro14 isomer of melittin was observed in the NMR spectra. In a previous report, we determined that the *cis-trans* isomerization of the proline residue in recombinant melittin occurs slowly in the NMR buffer without TFE at 298 K, and the population of the *cis* isomer was virtually absent in a 1 day old solution of recombinant melittin, but gradually increased to ~20–27% of the total melittin population after 2 weeks of storage in the NMR buffer.²⁷ For 2D ^1H - ^1H NOESY and ^1H - ^1H ROESY experiments, freshly reconstituted synthetic melittin (Genscript, Piscataway NJ) was used. Melittin concentration was determined at 280 nm using a molar extinction coefficient of $5500\text{ M}^{-1}\text{ cm}^{-1}$. Homonuclear NMR experiments were also carried out on “blank” samples at 285 K which contained 30% v/v TFE in the NMR buffer and a combination of 30% v/v TFE and 10% w/v glycerol in NMR buffer to determine spectral artifacts due to incompletely deuterated TFE and glycerol.

To evaluate the effect of decreasing temperature and increasing viscosity on the transverse relaxation rate (R_2), one-dimensional jump-return Hahn echo experiments were performed, as described previously.⁴¹ The pulse sequence included gradient purge pulses for the efficient water suppression. The signal intensities of the amide $^1\text{H}^{\text{N}}$ envelope from spectra of

melittin recorded at two echo delays, $\tau_1 = 1.1$ ms and $\tau_2 = 6.1$ ms, were measured. The ratio of signal intensities in the two spectra, I_2/I_1 , is related to R_2 in the following equation⁴²

$$R_2 = \left\{ -1/[2(\tau_2 - \tau_1)] \right\} \ln(I_2 - I_1) \quad (1)$$

The reciprocal of R_2 is equal to T_2 , the transverse relaxation time constant.⁴²

Chemical shift perturbations (CSPs) were calculated using a previously described method.²⁷ CSPs are the weighted average chemical-shift deviations for each backbone $^1\text{H}/^{15}\text{N}$ pair in the ^1H - ^{15}N HSQC spectra of melittin in the low-viscosity condition (melittin at 298 K without glycerol) and the high-viscosity condition (melittin at 285 K with 10% w/v glycerol). Chemical shift index (CSI) analysis⁴³ version 3.0 was used to calculate the helical probability scores (abbreviated H-prob) of melittin residues in the high-viscosity and low-viscosity conditions. Assignments of backbone ^{15}N , ^{13}C , and ^1H were uploaded to the CSI 3.0 web server at <http://csi3.wishartlab.com>. Backbone assignments of melittin in the low-viscosity condition were taken from our previous report,²⁷ whereas those for melittin in the high-viscosity condition were taken from Biological Magnetic Resonance Bank (BMRB) entry 30481.

Resonance Assignment of Recombinant Melittin in a TFE/Water/Glycerol Environment.

The extent of assignment is 96% for the peptide backbone (N, H^{N} , C^{α} , H^{α} , and C^{β}) and 95% for the amino acid side chains (Biological Magnetic Resonance Bank, BMRB entry 30481). Sequence-specific resonance assignments for the ^{15}N , ^{13}C , and ^1H nuclei of the polypeptide backbone of $[\text{U}-^{13}\text{C}, ^{15}\text{N}]$ -melittin were obtained through analysis of 3D NMR spectra recorded at 298 K, as detailed in a previous report.²⁷ Side-chain assignments were completed by recording HCCH-Total Correlation Spectroscopy, TOCSY spectra at 298 K and in the presence of 10% w/v glycerol.⁴² All assignments made at 298 K were transferred to the corresponding NOESY spectra recorded at 285 K (Figure S3). ^1H - ^{13}C HSQC spectra of the $[\text{U}-^{13}\text{C}, ^{15}\text{N}]$ -melittin sample with 10% w/v glycerol recorded at various temperatures (298, 295, 292, 290, and 285 K) were used as guides in transferring side-chain assignments at 298 K to spectra recorded at 285 K. Notably, for the P14 residue, the $^{13}\text{C}^{\beta}$ chemical shift is ~ 31 ppm, corresponding to the *trans* conformation of the L13-P14 peptide bond.⁴⁴

Structure Calculations.

CYANA⁴⁵ version 3.98 was used to calculate the 3D structure of melittin in a TFE/water/glycerol environment. The input consisted of a chemical-shift list obtained from the resonance assignment, a 3D ^1H - ^{13}C NOESY (optimized for the aliphatic ^{13}C region) and a 3D ^1H - ^{15}N NOESY.

The NOE peaks were assigned automatically and converted into distance constraints using the standard CYANA protocol with seven cycles of NOE assignment and simulated annealing in torsion angle space. The CISPROCHECK routine of CYANA 3.98 classified the P14 conformation as *trans*, based on the average value and standard deviations for the difference between the chemical shifts of C^{β} and C^{γ} .⁴⁶ Backbone Φ and Ψ dihedral angle

constraints were determined using TALOS ⁴⁷ version 3.80. These constraints were used as input data in each cycle of the structure calculation. To determine the optimal geometry of W19, a 2D ¹H-¹H NOESY spectrum was recorded in D₂O solvent. The lack of inter-residue NOEs involving aromatic protons was used to exclude structures showing W19 aromatic protons in close proximity ($\ll 5$ Å) with nonlabile protons on neighboring residues. The ensemble of 20 conformers with the lowest residual CYANA target function values obtained in the final cycle of the structure calculation was chosen to represent the solution structure of recombinant melittin. Model 10 is the most similar to the other models in the final bundle of 20 conformers of melittin, therefore this model was considered to be the “representative” conformer of melittin. PyMOL version 1.7.4.5 (Schrödinger, LLC) and Yet Another Scientific Artificial Reality Application (YASARA)⁴⁸ version 18.2.7 were used to prepare Figures showing the final ensemble of conformers.

Analysis of 3D Structures of Melittin.

The following 3D structures of melittin were compared: the crystal structure (PDB ID: 2MLT) of tetrameric melittin (wild-type, with a sequence GIGAVLKVLTTGLPALISWIKRKRQQ-NH₂), the solution NMR structure of wild-type melittin bearing an organometallic fragment and solved in methanol (PDB ID: 2MW6), the solution NMR structure of recombinant melittin in TFE/water/glycerol determined in this report (PDB ID: 6DST), and the crystal structure of melittin bound to centrin (PDB ID: 3QRX). 6DST has 20 conformers in the final ensemble of NMR structures, whereas 2MW6 has 10 conformers. As mentioned earlier, the representative structure for 6DST is model 10. In the 2MW6 ensemble, the representative structure is also model 10, which is most similar to other models in the 2MW6 ensemble.

MOLMOL⁴⁹ was used to determine the bend angles in the 3D structures of melittin. The bend angle is formed by lines drawn through helix axes of residues 5–9 and 14–20. Swiss Protein Data Bank (PDB) Viewer⁵⁰ version 4.1.0 was used to generate the molecular surface and electrostatic potential map of melittin.

The Dictionary of Protein Secondary Structure (DSSP)^{51,52} was used to determine secondary structural elements and backbone hydrogen bond patterns. DSSP assigns secondary structural elements (unstructured, α -helix, 3_{10} -helix, π -helix, hydrogen-bonded turn, bend, extended strand, and β -bridge) based on hydrogen bonding and geometric criteria, however, in case of overlaps of helical elements, priority is given to α -helix over 3_{10} -helix.⁵¹ In the work of Cao and Bowie,⁵³ helical segments assigned as “ α -helix” by DSSP but containing both CO_{*i*}-NH_{*i*+4} and CO_{*i*}-NH_{*i*+3} hydrogen bonds were classified as neither canonical α -helices nor canonical 3_{10} -helices. In this article, we adopted the classification scheme of Cao and Bowie⁵³ but added specific hydrogen bonding criteria: we therefore use the term “ α -helix” to refer to regions assigned as α -helix by DSSP and containing CO_{*i*}-NH_{*i*+4} hydrogen bonds with bond energies < -1.0 kcal mol⁻¹ and “noncanonical 3_{10} helix” for regions assigned as α -helix by the program but containing a combination of CO_{*i*}-NH_{*i*+4} and CO_{*i*}-NH_{*i*+3} hydrogen bonds with bond energies < -1.0 kcal mol⁻¹.

The melittin structures in PDB entry 2MW6 contain an organometallic label on W19 $[(C_5H_5)Ru]^+$. The atomic coordinates of the organometallic label were omitted in calculations of the bend angle, electrostatic potential, and molecular surface of melittin's solution NMR structure in methanol. The recombinant melittin structures in PDB entry 6DST have a C-terminal carboxylate group (COO^-). The COO^- group was replaced with a primary amide group ($CONH_2$) to match the sequence of wild-type melittin, and the resulting atomic coordinates were used in calculations of the bend angle, electrostatic potential, and molecular surface of melittin's solution NMR structure in TFE/water/glycerol. The atomic coordinates of melittin from 2MLT and 3QRX lack hydrogens. Hydrogen atoms were added to the coordinates using the MolProbity web server^{54,55} at <http://molprobity.biochem.duke.edu>.

Accession Numbers.

Analysis of the stereo chemical quality of the NMR structure was accomplished using the PDB validation server (<http://deposit.pdb.org/validate>). The atomic coordinates of the bundle of 20 conformers used to represent the melittin structure have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (<http://www.rcsb.org/pdb>) with the PDB ID: 6DST.

The chemical-shift list has been deposited in the BMRB (<http://www.bmrb.wisc.edu> accession number: 30481). ROSETTA⁵⁶ version 3.80 and CS-ROSETTA Toolbox version 3.3 were used to predict the atomic model of recombinant melittin from the deposited chemical shifts. The results of the structure prediction are available at <https://csrosetta.bmrb.wisc.edu/csrosetta/entry/b11ca2f7faf9>.

RESULTS

Increasing Viscosity Improves the Quality of Melittin's NOESY Spectra.

The proton NOEs are a function of the product of the proton Larmor frequency ω_0 and the rotational correlation time, τ_c .³⁷ Near the condition $\omega_0\tau_c = 1.12$, the proton NOEs reach a null point.³⁷ This is typically the case for molecules with molecular weights in the range ~ 0.5 to ~ 2 kD, and melittin (~ 2.8 kD) may fall under this condition.³⁸ When $\omega_0\tau_c > 1.12$, the null effect is diminished.³⁸ Therefore, a sufficient increase in τ_c by increasing the solvent viscosity could result in observable NOE signals.

We first performed the 3D 1H - ^{15}N NOESY experiment on 50 μM recombinant $[U-^{13}C, ^{15}N]$ melittin in TFE/water (without glycerol) at 298 K with a mixing time of 200 ms. However, only a few, weak NOEs were observed (Figure 1A). We hypothesized that by increasing the solvent viscosity of the melittin sample, the NOESY spectra of melittin could provide more useful cross peaks for 3D structure determination. We tested this hypothesis by adding deuterated glycerol (10% w/v) to the recombinant melittin sample and lowering the temperature to 285 K. This resulted in the increased number of NOE cross peaks (Figure 1B) observed in the 3D 1H - ^{15}N NOESY spectrum. Melittin's transverse relaxation time, T_2 ,⁴¹ in TFE/water and TFE/water/glycerol mixtures, was monitored as a function of temperature. Figure 1C shows T_2 determined within the temperature range 298–285 K.

Within this range of conditions, we found that the minimum T_2 was achieved when the glycerol concentration was 10% w/v and temperature was 285 K. This condition was thus used for recording 3D ^1H - ^{15}N and ^1H - ^{13}C NOESY spectra to determine distance restraints.

We used NMR spectroscopy to confirm that melittin retains its helical secondary structure under the two solution conditions tested: the “high-viscosity” condition (melittin in TFE/glycerol/water at 285 K) and the “low-viscosity” condition (melittin in TFE/water at 298 K). Two ^1H - ^{15}N HSQC spectra of recombinant melittin (Figure 2A) recorded under these conditions show well-dispersed amide proton and nitrogen chemical shifts and are consistent with a structured form of melittin²⁷ that we determined to correspond to the helical *trans*-Pro14 isomer of melittin. Figure S2 shows the chemical-shift perturbations (CSPs) in the overlaid ^1H - ^{15}N HSQC spectra that compare the positions of $^1\text{H}/^{15}\text{N}$ peaks in the low-viscosity and high-viscosity conditions. All CSP values were low, mostly below 0.1 ppm (Figure S2), suggesting that the melittin helical secondary structure was preserved. Chemical shift index (CSI) analysis of backbones ^{15}N , ^{13}C , and ^1H identified α -helical segments on melittin in both solution conditions (Figure 2B,C). Notably, the melittin secondary structure in the high-viscosity condition has a slightly higher overall helical propensity. The preservation of the helical secondary structure of melittin in the high- and low- viscosity conditions enabled a straightforward transfer of melittin assignments from spectra recorded at 298 K to those acquired at 285 K and in the presence of 10% w/v glycerol (Figure S3).

Recombinant Melittin in a TFE/Water/Glycerol Environment Assumes an Extended Helical Structure.

CYANA⁴⁵ was used to calculate the solution NMR structure of recombinant melittin in TFE/water/glycerol (Table 1). The bundle of 20 conformers has an extended helical structure (Figure 3A,B). In all structures, one continuous, bent helix is defined by residues ~4–23. This ensemble of NOE-derived structures, which we collectively refer to as the “NMR structure in TFE/water/glycerol”, is different from the compact structure of melittin (Figure 3C,D) predicted by CS-ROSETTA; the predicted structure is solely based on the melittin chemical shifts and does not take into account structural changes introduced by adding the helix-favoring cosolvent, TFE. In contrast, the NMR structure was solved using interproton distance restraints determined from NOESY data. In the NMR structure, the presence of a helical region spanning residues ~4–23 is consistent with our observation of d_{NN} NOEs (Figure S4) for residues 3–13, then 15–23. The interruption of d_{NN} connectivities is due to the fact that residue 14 is a proline, which does not have an amide proton. Residues 24–26 are highly flexible, as suggested by the loss of d_{NN} connectivities in this region.

Figure 4 shows the structural elements and the NOEs that define the bend angle of melittin. Based on a database of secondary structure assignments, DSSP,^{51,52} melittin structure can be classified as consisting of N-(4–6) and C-(14–23) terminal α -helices interrupted by a noncanonical 3_{10} -helix (residues 7–13). Sequential d_{NN} connectivities in the non-canonical 3_{10} -helix are present from T10–G12. More importantly, a strong, medium-range NOE is observed between the amide protons of T10 and G12. Other medium-range NOEs involving T11 are also observed. Figure 4B shows NOEs involving H^{N} and H^{β} of T11 with H^{α} of V8 and H^{α} of T11 with $\text{H}^{\delta 2}$, $\text{H}^{\delta 3}$, and $\text{H}^{\gamma 3}$ of P14.

Figure 5 shows NMR evidence that P14 is part of an α -helix and is in a *trans* conformation. This is observed in all 20 structures of the final ensemble. Strong sequential $d_{\text{N}\delta}$ and $d_{\text{H}\beta}$ connectivities between L13 and P14 were observed in the NOESY spectra (Figure 5A,B), and these are characteristic of *trans* X-Pro peptide bonds.³⁷ The fact that P14 is part of an α -helical structure (Figure 5C) is consistent with our observation of $d_{\text{a}\text{N}}(i, i+3)$ and $d_{\text{a}\text{N}}(i, i+4)$ connectivities with I17 and S18, respectively.³⁷

Structural Comparison between NMR and Crystal Structures of Melittin.

The solution NMR structure of recombinant melittin in TFE/water/glycerol (PDB ID: 6DST) was compared with the crystal structure of tetrameric melittin (PDB ID: 2MLT, chains A and B),^{57–59} the crystal structure of a melittin segment (residues 2–21) in complex with centrin (PDB ID: 3QRX), and the solution NMR structure of melittin containing a tryptophan residue labeled with an organometallic fragment and solved in methanol (PDB ID: 2MW6).²⁸ The overall bend angles in the melittin structures are comparable. The ensemble of 20 solution NMR structures in TFE/water/glycerol (6DST) has an average bend angle of $131 \pm 8^\circ$. For 2MW6, the ensemble of 10 conformers has an average bend angle of $119 \pm 7^\circ$. The bend angle in the crystal structure of tetrameric melittin (2MLT) is $122 \pm 2^\circ$ (averaged over melittin chains A and B in the PDB entry), and the bend angle in centrin-bound melittin (3QRX) is 163° .

Backbone hydrogen bonding patterns (Figure 6A,B and Table S1) of the four PDB entries were compared. At least 70% of conformers of the NMR structure in TFE/water/glycerol (6DST) shows hydrogen bonds of the type $\text{CO}_i\text{---HN}_{i+3}$ and $\text{CO}_i\text{---HN}_{i+4}$, corresponding to 3_{10} -helices and α -helices. α -helices have 3.6 residues per turn, a $5.4 \text{ \AA}$ helical pitch, contiguous $\text{CO}_i\text{---HN}_{i+4}$ hydrogen bonds, and a 100° angle formed by consecutive residues about the helical axis, whereas 3_{10} -helices have 3 residues per turn, a $5.8\text{--}6 \text{ \AA}$ helical pitch, contiguous $\text{CO}_i\text{---HN}_{i+3}$ hydrogen bonds, and a 120° angle formed by consecutive residues.^{60,61} The observation of $\text{CO}_i\text{---HN}_{i+4}$ hydrogen bonds for the pairs Val8–Gly12, Thr11–Ala15, and Gly12–Leu16 as well as $\text{CO}_i\text{---HN}_{i+3}$ hydrogen bonds between Lys7 and Thr10 and Thr10 and Leu13 (Figure 6B) indicates that the segment spanning residues 7–13 is neither a canonical α -helix nor a canonical 3_{10} -helix, therefore we refer to this segment as a noncanonical 3_{10} -helix.^{61,62} The segments flanking this region have hydrogen bonds consistent with only α -helices. In contrast to the structure in 6DST, at least 70% of the conformers for the solution structure in methanol (2MW6) contains only $\text{CO}_i\text{---HN}_{i+4}$ hydrogen bonds. This structure has two α -helical segments (residues 2–9 and 14–25) containing $\text{CO}_i\text{---HN}_{i+4}$ hydrogen bonds, which are separated by a flexible central region (residues 10–13) that lacks hydrogen bonds.

The crystal structure of tetrameric melittin (2MLT chains A and B) has mostly hydrogen bonds typical of α -helices,⁵⁸ with one exception: a $\text{CO}_i\text{---HN}_{i+5}$ bond between Val8 and Leu13 (in chain A) that is consistent with a π -turn.⁶³ The continuous α -helix in 2MLT chain B spans residues 2–25, whereas in chain A the two α -helices (residues 2–10 and 12–25, respectively) are separated by the π -turn. The centrin-bound melittin structure (3QRX) has two α -helical segments (3–7 and 13–19) interrupted by a π -helix (residues 8–12). The π -helix has two $\text{CO}_i\text{---HN}_{i+5}$ hydrogen bonds between Lys7 and Gly12 and Val8 and Leu13.

The global Ca RMSD values for the segment spanning residues 2–21 on melittin were determined while comparing the structure of melittin in TFE/water/glycerol (6DST) to those in other PDB entries. These values are 2.6 Å (between 6DST and 2MW6), 2.5 Å (between 6DST and 3QRX), 2.9 Å (between 6DST and 2MLT chain A), and 3.0 Å (between 6DST and 2MLT chain B). Molecular surface and electrostatic potential maps (Figure S5) show that the NMR structure in TFE/water/glycerol is more elongated than the structures from 2MLT due to the presence of the noncanonical 3_{10} -helix. As shown in the overlaid backbone traces of the melittin structures in Figure 6C, the NMR structure in TFE/water/glycerol deviates from the other previously solved structures because the segment spanning residues 7–13, which contains the noncanonical 3_{10} -helix in the NMR structure in TFE/water/glycerol, is more tightly wound than the counterpart in other models. Thus, the NMR structure in TFE/water/glycerol represents an extended, overwound conformation of melittin not previously seen in other melittin PDB entries.

DISCUSSION

Melittin has been extensively studied by NMR spectroscopy, although elucidation of its solution structure has been hampered by the inherent conformational flexibility of the peptide in solution^{34–36} and the lack of uniformly isotope-labeled material, which only became recently available.²⁷ The motivation for these studies also arises from the long-term goal of examining the structural details of melittin binding to the prototypical small heat-shock protein (sHSP) α -crystallin, which is also an established molecular chaperone in the ocular lens.⁶⁴ A prior report in the literature suggests that the substrate mimic, melittin, binds to the region in α -crystallin that is directly involved in its chaperone function.^{18,65}

For the present study, we used recombinant melittin with uniform ^{13}C - and ^{15}N -labeling to collect NMR spectra with minimal resonance overlap and at micromolar concentrations that appear to be physiologically relevant. We also devised a plan to restrict the conformational flexibility of melittin by increasing the viscosity of the medium. In this study, viscosity was increased through the combined effects of adding co-solvents TFE and glycerol and decreasing the temperature of the medium. This approach allowed us to obtain NOE-derived distance restraints for solution NMR structure calculations. Using this strategy, we determined the 3D solution structure of melittin in TFE/water/glycerol (PDB ID: 6DST). The ensemble of calculated NMR structures exhibited atomic precision (0.7 Å). Other methods, such as the exact NOE (eNOE) approach, may be used to further refine the structural ensemble.^{66,67}

In acquiring melittin's NOESY spectra, we minimized the presence of co-solvent artifacts by using deuterated TFE and deuterated glycerol. Nevertheless, examination of the 1H NMR spectra of "blank" samples containing only 30% v/v TFE and 10% w/v glycerol in NMR buffer at 285 K showed some weak proton peaks arising from incomplete deuterium labeling. We examined the presence of these artifacts in the 3D NOESY data. The peak for H^{α} of Ile17 on several planes of the 3D 1H - ^{13}C NOESY overlapped with a glycerol spectral artifact at 3.39 ppm (1H dimension) and 65.16 ppm (^{13}C dimension). Therefore, most NOEs involving H^{α} of Ile17 were excluded from the structure calculation. This did not hamper our structure calculations, however, because the automated routine of CYANA for NOE

assignment and structure annealing is tolerant to omission of up to 50% of cross peaks in heteronuclear-resolved NOESY spectra.⁶⁸ Fortunately, other glycerol artifacts did not overlap with melittin NOEs. For TFE, we observed weak intermolecular NOEs between some melittin residues with TFE at ~0.60 ppm. Most methyl groups of hydrophobic residues are found at the same chemical shift, however, which meant that we could not unambiguously assign TFE–melittin intermolecular NOEs for most of the melittin residues. We found some cross peaks at ~0.60 ppm that point to TFE–melittin interactions involving residues T10, T11, G12, A15, R22, R24, Q25, and Q26 (Figure S6). Notably, the residues T10–G12 belong to the central region of the peptide, whereas residues R24–R26 belong to the unstructured C-terminus.

When we compared the NMR structure in TFE/water/glycerol (Figure 3A,B) to the CS-ROSETTA-predicted structure in the same solvent system (Figure 3C,D), we found that the latter structure was more compact and had a much smaller bend angle ($26 \pm 10^\circ$). The CS-ROSETTA protocol selects protein/peptide fragments from a structural database based on backbone chemical shifts and amino acid sequence similarity and uses such fragments to build 3D atomic models.⁵⁶ Thus, CS-ROSETTA makes a correlation between the chemical shifts of proteins/peptides and the 3D structure. However, we suggest that this correlation may not be applicable to recombinant melittin's structure in the TFE/water/glycerol medium used in this report, especially considering that the TFE co-solvent's effect on chemical shift may not be identical to that of other, more 'conventional' NMR solvents for protein samples, such as water. Our previous report showed that backbone (^1HN and ^{15}N) chemical shifts of melittin in an aqueous solution without TFE deviated significantly from those of melittin in 30% v/v TFE.²⁷ Moreover, a quick search of structures with the keyword "trifluoroethanol" in the PDB generated ~135 solution NMR structures solved in the presence of TFE, out of ~12 000 solution NMR structures deposited in the database. Therefore, statistically speaking, the chemical shifts employed by CS-ROSETTA are likely to correspond to other solution conditions and are not representative of protein structures determined in the presence of TFE. This may explain the striking differences between the solution NMR structure of melittin in TFE/water/glycerol and that of CS-ROSETTA (Figure 3). Similarly, the secondary structural elements predicted by CSI⁴³ version 3.0 (Figure 2C) deviate from those assigned by DSSP^{51,52} to the ensemble of 3D NMR structures in TFE/water/glycerol, possibly due to the limited accuracy (~80%) of CSI analysis. Misrepresentation of the 3D structure of melittin by both CSI and CS-ROSETTA in this report reflects some drawbacks of predicting the structure of small, flexible peptides solely based on NMR chemical shifts, especially in unconventional solvent systems. Arguably, strategies for improving the collection of NOEs, including the methods described in this report, may be used to overcome challenges of peptide structure determination.

Of particular interest is melittin's bent conformation represented by the ensemble of solution NMR structures in PDB ID: 6DST (Figure 3A,B). The bend in the middle of the peptide is thought to arise from P14.⁵⁸ Proline is known to introduce kinks in helices, in part because it cannot form hydrogen bonds to proton acceptors, such as backbone carbonyl oxygen atoms, thereby interrupting the hydrogen bonding pattern expected for an α -helix.^{61,69} The presence of proline in the central region of melittin's bent helix is reminiscent of the published 3D structures of transmembrane helices, many of which also feature a proline

residue in the center of the membrane-spanning regions.^{70,71} Proline-containing structural motifs in transmembrane helices are thought to serve as conformational switches, which, for example, aid in signal transduction across membranes.^{72,73}

The NMR structure in TFE/water/glycerol (6DST) was compared to structural models found in other PDB entries for melittin: the crystal structure of tetrameric melittin (2MLT), the crystal structure of centrin-bound melittin (3QRX), and the solution NMR structure of melittin in methanol (2MW6). The hydrogen bonding patterns in these structures reflect the different conformations adopted by melittin in a variety of solvent/buffer conditions. When comparing the four PDB entries, we observed shifted hydrogen bond partners (Figure 6A,B). It was previously proposed that the shifting of hydrogen bond donor–acceptor pairs may contribute to flexibility of transmembrane helical regions in membrane proteins.⁵³ Therefore, the diversity of hydrogen bond patterns in melittin structures may have important implications on melittin's ability to associate with and permeabilize membranes.^{2,7}

Although all PDB models of melittin structure (available to date) show helical regions, the NMR structure in TFE/water/glycerol (6DST) is distinct in that it features both α - and 3_{10} -helices. It must be noted that helices with combined α -helical and 3_{10} -helical character are also observed in 3D structures of other proteins,^{53,62,74} and it has been suggested that helix kinks may originate from adjoined segments of 3_{10} - and α -helical structures, in which the C-terminal side of a 3_{10} -helix is contiguous with the N-terminal side of an α -helix.⁶² Transitions between α -helical and 3_{10} -helical conformations are of interest in studying protein folding pathways, as previous studies have suggested that the 3_{10} -helix is an intermediate in the folding of α -helices.^{75,76} α -helix to 3_{10} -helix transitions were also observed in molecular dynamics simulations of other model peptides in the nanosecond time scale.^{60,77,78} Interestingly, Fourier transform infrared spectroscopy studies on melittin–centrin interactions¹² suggest that upon binding to centrin the 3_{10} -helical character on melittin increases, although the crystal structure of melittin-bound centrin (3QRX) features only α -helices flanking a π -helix, based on our analysis of secondary structure using DSSP.^{51,52} Our findings on melittin's structure in TFE/water/glycerol therefore provide insight into melittin's conformational transitions, which may be relevant to the peptide's interactions with its biological targets.

CONCLUSIONS

The 3D solution NMR structure in this report was solved with atomic precision (0.7 Å) in a TFE/water/glycerol environment. This marks the first report of an atomic resolution NMR structure of recombinant melittin in the presence of TFE. We suggest that the use of this structure in docking experiments and molecular dynamics simulations will improve our knowledge of protein folding pathways important in understanding the mechanisms of toxicity due to melittin.^{7,79,80} The experimental approach we employed in enhancing NOE detection could prove to be useful in the structure elucidation of other small peptides in solution. More importantly, the atomic level, 3D-structure determination of melittin presented here will be of considerable significance for our long-term investigations of defining the binding pocket of the substrate mimic, melittin, to the sHSP and molecular chaperone α -crystallin from the ocular lens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Adding glycerol and decreasing temperature increase the intensity of melittin NOESY cross peaks. Panels A and B show representative ω_1 - ω_3 planes of 3D ^1H - ^{15}N NOESY spectra (200 ms mixing time) of 50 μM recombinant melittin in NMR buffer with 30% v/v TFE: (A) at 298 K without glycerol and (B) at 285 K with 10% w/v glycerol. Contour levels of the two spectra (A, B) were kept consistent. One-dimensional projections at the ω_3 (^1H) chemical shift of the overlapping amide protons of Q25 and Q26 (indicated by the vertical broken line) are shown at the right side of the planes. Panel A shows a few NOE signals, whereas (B) shows more NOE signals. Peak amplitudes of off-diagonal peaks in (A) are noticeably lower than those in (B). (C) T_2 time constants for 50 μM melittin with 0% w/v glycerol (orange markers) and 10% w/v glycerol (blue markers) determined within the temperature range 298–285 K using the one-dimensional jump-return Hahn echo experiment.⁴¹ The NMR samples were prepared in 10 mM potassium phosphate buffer at pH 7.0 with 10% v/v D_2O , 30% v/v-deuterated TFE, and either 0- or 10% w/v- deuterated glycerol. Error bars represent the propagated standard deviation of the intensity ratio I_2/I_1 (from eq 1) for peaks in the amide envelope of the melittin ^1H NMR spectrum. The larger values of the standard deviation (error bars) observed for T_2 at higher temperatures and in

the absence of glycerol may be attributed to more extensive fluctuations in the internal dynamics of the peptide backbone under these conditions.³⁶

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Figure 2.

Melittin is helical in low-viscosity and high-viscosity solvents. NMR samples were prepared by dissolving 50 μM [^{13}C , ^{15}N]-melittin in NMR buffer at pH 7.0 with 30% v/v-deuterated TFE and 0- or 10% w/v-deuterated glycerol. (A) Overlaid ^1H - ^{15}N HSQC spectra of melittin recorded under the low-viscosity condition (red profile) and high-viscosity condition (blue profile). Assignments are shown for all residues except G1, I2, and P14. The amide backbone peaks of Q25 and Q26 overlap. The crowded central region of the ^1H - ^{15}N HSQC spectrum is in the inset. Peaks enclosed in Box "a" correspond to the W19 indole. Peaks

enclosed in Box “b” correspond to amide side chains of Q25 and Q26. (B) Helical probability scores (H-prob) plotted against melittin residue number. (C) CSI consensus values plotted against residue number. For (B) and (C), CSI⁴³ version 3.0 was used to calculate the helical propensities and CSI consensus scores from the assigned backbone nuclei of melittin, ¹³C, ¹⁵N, and ¹H. Scores of -1, 0, and +1 correspond to α -helix, random coil, and β -sheet secondary structures, respectively.

Figure 3.

NMR solution structure of recombinant melittin in TFE/water/glycerol determined from NOE data (PDB ID: 6DST) differs from the structure predicted by CS-ROSETTA. For (A) and (B), structure calculation was performed using CYANA⁴⁵ version 3.98. For (C) and (D), structure prediction based on chemical shifts was performed using CS-ROSETTA.⁵⁶ For (B) and (D), structured regions are rendered as thick ribbons, and unstructured regions are shown as cyan-colored tubes; for the structured regions, secondary structural elements were assigned by DSSP^{51,52} and are indicated by color: blue for helix, green for turns, and yellow for bends. (A) Stereoview of 20 conformers showing mostly consistent C^α traces for residues ~4–23, with frayed N- and C-termini. (B) Stereoview of the ribbon diagrams of the 20 conformers from (A). For all conformers, a continuous helical structure spans 4–23, unstructured regions are represented by residues 1 and 25–26, and a turn is assigned to residue 3. Residue 2 may form a turn (in at least 75% of conformers) or be part of an

unstructured region, and residue 24 is assigned to a bend (in 50% of conformers) or an α -helix. The average bend angle of the NMR structure is $131 \pm 8^\circ$. (C) Stereoview of 10 CS-ROSETTA-calculated conformers showing mostly consistent C^α traces. (D) Stereoview of the ribbon diagrams of the 10 conformers from (C). All conformers have two helices spanning residues ~ 3 –10 and ~ 14 –23, which are separated by turns and unstructured regions on residues ~ 11 –13. The average bend angle of the CS-ROSETTA-predicted structure is $26 \pm 10^\circ$. The global C^α RMSD between the representative NOE-derived NMR structure (model 10) and the CS-ROSETTA-predicted structure (model 1) is 8.0 Å (residues 1–26). The global C^α RMSD for the structured regions (residues 3–21) after removal of the frayed ends is 16.8 Å.

Figure 4.

NOEs from ^1H - ^{15}N NOESY and ^1H - ^{13}C NOESY spectra constrain the bend angle of melittin in TFE/water/glycerol. (A) Backbone trace and 3D model of the representative structure of melittin (model 10 from Figure 3A,B) showing the central “hinge” region (residues 7–13) enclosed in the black box and inter-residual NOEs represented as the black lines. Secondary structural elements assigned by DSSP^{51,52} are indicated by the color of the backbone trace: green for turns, yellow for bends, and cyan for unstructured regions. The helical region is represented by blue (α -helical regions with only $\text{CO}_i\text{--NH}_{i+4}$ hydrogen bonds) and red (noncanonical 3_{10} -helix, with both $\text{CO}_i\text{--NH}_{i+4}$ and $\text{CO}_i\text{--NH}_{i+3}$ hydrogen bonds). P14, shown in stick models, is colored magenta. (B) Selected $\omega_2(^1\text{H}^{\text{N}})\text{--}\omega_3(^1\text{H}^{\text{NOE}})$ strips at the $\omega_1(^{15}\text{N}^{\text{H}})$ chemical shifts of T10, T11, and G12 are shown, and the corresponding positions of amide protons are indicated in the 3D model. The d_{NN} connectivities in the NOESY strips are indicated by the red broken lines. (C) The $\omega_2(^1\text{H}^{\text{N}})\text{--}\omega_3(^1\text{H}^{\text{NOE}})$ strip at the T11 $\omega_1(^{15}\text{N}^{\text{H}})$ chemical shift shows a medium-range NOE between H^{N} of T11 and H^{α} of V8. The $\omega_2(^1\text{H}^{\beta})\text{--}\omega_3(^1\text{H}^{\text{NOE}})$ strip at the T11 $\omega_1(^{13}\text{C}^{\beta})$ chemical shift also shows a medium-range NOE between H^{β} of T11 and H^{α} of V8. The $\omega_2(^1\text{H}^{\alpha})\text{--}\omega_3(^1\text{H}^{\text{NOE}})$ strip at the T11 $\omega_1(^{13}\text{C}^{\alpha})$ chemical shift shows medium-range NOEs involving P14 protons and H^{α} of T11. Inter-residual NOEs are labeled in red, and intraresidual NOEs are labeled in black.

Figure 5.

Leu13–Pro14 peptide bond in melittin is in a *trans* configuration. NOEs between P14 and neighboring residues are shown in NOESY strips and in the representative NMR structure of melittin in TFE/water/glycerol (model 10 from the ensemble in Figure 3A,B). P14, shown in magenta, is part of an α -helical structure (the α -helix to which P14 belongs is represented by the blue backbone trace), whereas the noncanonical 3_{10} -helix preceding Pro14 is shown with the red backbone trace). (A) $\omega_2(^1\text{H}^{\text{N}})-\omega_3(^1\text{H}^{\text{NOE}})$ strip from the 3D $^1\text{H}-^{15}\text{N}$ NOESY spectrum of melittin at the $\omega_1(^{15}\text{N}^{\text{H}})$ chemical shift of L13 showing d_{NH} connectivity. (B) $\omega_2(^1\text{H}^{\beta})-\omega_3(^1\text{H}^{\text{NOE}})$ strip from the 3D $^1\text{H}-^{13}\text{C}$ NOESY spectrum of melittin at the $\omega_1(^{13}\text{C}^{\beta})$ chemical shift of L13 showing $d_{\beta\beta}$ connectivity. (C) $\omega_2(^1\text{H}^{\alpha})-\omega_3(^1\text{H}^{\text{NOE}})$ strip from a 3D $^1\text{H}-^{13}\text{C}$ NOESY spectrum of melittin at the $\omega_1(^{13}\text{C}^{\alpha})$ chemical shift of P14 showing $d_{\alpha\text{N}}(i, i+3)$ and $d_{\alpha\text{N}}(i, i+4)$ connectivities with I17 and S18, respectively. In all strips, intrareidual NOEs are indicated by the black labels and inter-residual NOEs with the red labels.

Figure 6.

Structural comparison of melittin PDB entries. As a visual guide, Pro14 is colored magenta and is shown as stick models in (A) and (B). Pro14 is sticking out of the plane in the conformer from PDB ID: 6DST. The complete list of hydrogen bonds is given in Table S1. (A) Backbone hydrogen bonds (CO_I-NH_j) in the crystal structure of tetrameric melittin (2MLT), the solution NMR structure in methanol (PDB ID: 2MW6), the crystal structure of centrin-bound melittin (PDB ID: 3QRX), and the solution NMR structure in TFE/water/glycerol (PDB ID: 6DST). The secondary structural elements determined by DSSP^{51,52} are indicated by the color of the backbone traces. For the solution NMR structures, the secondary structure assignments shown apply to at least 50% of conformers, and the hydrogen bonds are only shown if they are present in at least 70% of the conformers. Shifted hydrogen bonding partners are mostly observed in the region enclosed in the box. (B) Hydrogen bonds (shown as the dashed lines) in the boxed region (residues ~7–13) are

mapped onto 3D models. $\text{CO}_I\text{-NH}_{i+4}$ bonds are shown in green, $\text{CO}_I\text{-NH}_{i+3}$ bonds are colored red, and $\text{CO}_I\text{-NH}_{i+5}$ bonds are colored black. Note that 2MW6 lacks hydrogen bonds in this region. (C) Overlaid backbone traces of representative structures of melittin from PDB entries 2MLT, 3QRX, and 6DST.

Table 1.

Structural Statistics of the Ensemble of 20 Melittin Conformers

parameter	value
NOE upper distance limits ^a	369
intraresidual ($i = j$)	104
short-range [$(i - j) = 1$]	154
medium-range [$1 < (i - j) \leq 5$]	111
long-range	0
dihedral angle (ψ and ϕ) constraints ^a	252
residual target function value ($\text{\AA}^2$) ^a	1.19 ± 0.03
residual NOE violations ^a	
number 0.1 $\text{\AA}$	1 ± 1
maximum ($\text{\AA}$)	0.25 ± 0.03
residual dihedral angle violations ^a	
number 5°	1 ± 1
maximum (deg)	6.28 ± 0.35
residual van der Waals violations ^a	
number 0.2 $\text{\AA}$	2 ± 0
maximum ($\text{\AA}$)	0.26 ± 0.01
RMSD to the mean coordinates ($\text{\AA}$) ^a	
backbone (N, C $^\alpha$, C')	0.66 ± 0.23
heavy atoms	1.17 ± 0.22
Ramachandran plot statistics (%) ^a	
most favored regions	92.1
additional allowed regions	7.9
generally allowed	0.0
disallowed	0
clash score ^b	8

^aThe average values for the 20 conformers with the lowest residual target function are given. These values were calculated using CYANA⁴⁵ version 3.98, following the 7th cycle of the automated NOE assignment. RMSD stands for root-mean-square deviation.

^bCalculated using the PDB validation server (<http://deposit.pdb.org/validate>).