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Retention of Native Quaternary Structure in Racemic Melittin Crystals

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Abstract

Racemic crystallography has been used to elucidate the secondary and tertiary structures of peptides and small proteins that are recalcitrant to conventional crystallization. It is unclear, however, whether racemic crystallography can capture native quaternary structure, which could be disrupted by heterochiral associations. We are exploring the use of racemic crystallography to characterize the self-assembly behavior of membrane-associated peptides, very few of which have been crystallized. We report a racemic crystal structure of the membrane-active peptide melittin; the new structure allows comparison with a previously reported crystal structure of L-melittin. The tetrameric assembly observed in crystalline L-melittin has been proposed to represent the tetrameric state detected in solution for this peptide. This tetrameric assembly is precisely reproduced in the racemic crystal, which strengthens the conclusion that the tetramer is biologically relevant. More broadly, these findings suggest that racemic crystallography can provide insight on native quaternary structure.

Graphical Abstract

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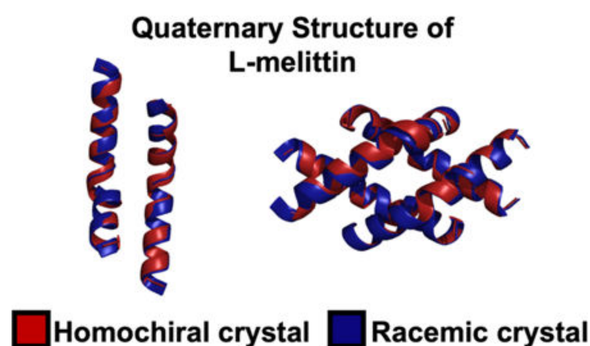
ASSOCIATED CONTENT

Model coordinates and structure factors have been deposited in the Protein Data Bank as entry 6O4M.

Experimental details regarding peptide synthesis and characterization and X-ray structure solution and refinement are available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interests.



α-Helical polypeptides rich in hydrophobic residues play diverse and important roles in biology because of their interactions with membranes. Some are toxic to-ward a broad range of cell types, both eukaryotic and prokaryotic; the honey bee venom melittin is prototypical.[1,2] Membrane-active peptides associated with eukaryotic innate immunity ("host-defense peptides") are selectively toxic toward microbial cells.[3,4] Magainins and ce-cropins are α-helical members of this class.[5,6] Venom peptides and host-defense peptides are water-soluble, because they contain multiple ionizable side chains, but their biological activities arise at least in part from disruption of lipid bilayers, a function which depends on the complement of hydrophobic side chains.[7] Polypeptide segments that are comprised entirely or largely of hydrophobic residues are common within intrinsic membrane proteins. Many of these segments form single-pass transmembrane α-helices that anchor a globular extracellular domain or intracellular domain to the membrane or that connect extracellular and intracellular domains to one another.[8–11] Although the roles and structural contexts of membrane-associated and membrane-embedded α-helical polypeptides differ, in each case function appears to involve specific modes of self-assembly.[12–14] It has been challenging, however, to characterize these quaternary assemblies. X-ray crystallography offers the prospect of atomic resolution structural information, but membrane-associated and membrane-embedded peptides are extraordinarily difficult to crystallize, and few structures are available. We seek to harness racemic crystallization to address this challenge.

Racemates are more prone to crystallization than are the corresponding pure enantiomers, and advances in solid-phase peptide synthesis have allowed racemic crystallization to be applied to many polypeptides since the early 1990s.[15–17] In nearly all cases, however, racemic crystallography has been employed to characterize the tertiary structures of individual protein molecules. A notable exception is the application by Liu et al. of quasiracemic crystallization to characterize ubiquitin oligomers;[18,19] this clever experimental design requires the D polypeptides to assemble non-covalently.

In contrast to the majority of racemic protein crystallography studies, our goal is to elucidate *quaternary* interactions. This distinction is important, because a racemic crystal offers the opportunity for both homochiral and heterochiral associations. Only homochiral associations can be relevant to the behavior of natural polypeptides.

We previously explored racemic crystallography for characterizing quaternary interactions in two membrane-associated polypeptide systems, one based on a host-defense peptide and the

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other based on the transmembrane segment of an intrinsic membrane protein. These studies led to divergent conclusions regarding the ability of racemate structures to elucidate native quaternary interactions.

Racemic and quasiracemic crystal structures of a derivative of magainin 2 revealed a homochiral dimer assembly featuring a cluster of phenylalanine side chains;[20,21] this assembly mode is consistent with previously reported solution-phase data for other magainin 2 derivatives at interfaces.[22] In a comparable study, Wang, Craik et al. reported the crystal structure of racemic baboon Θ-defensin-2 (BTD-2) and concluded that the homochiral association mode of the β-hairpin peptides in this crystal reflects assembly behavior that underlies the antibacterial activity of L-BTD-2.[23] Although these studies suggest that racemic crystallography can reveal native quaternary structures of membrane-associated peptides, for neither BTD-2 nor the magainin 2 derivatives is there a crystal structure of the L-peptide that would enable direct comparison with the racemic structures. In a first effort to apply racemic crystallography to the transmembrane segment of an intrinsic membrane protein, we determined racemate structures for several peptides derived from the transmembrane portion of the influenza M2 proton channel protein. In each case, how-ever, crystal packing was dominated by heterochiral dimer interactions[24,25] rather than the native homochiral tetramer assembly observed in L-peptide structures.[26–29] Collectively, these prior studies leave unresolved the utility of racemic crystallography for elucidating biologically relevant quaternary interactions. Here we report results for melittin that support the pursuit of racemic crystallography to characterize self-association of membrane-associated peptides.

Melittin (H_2N-GIGAVLKVLTTGLPALISWIKRKRQQ-$CONH_2$) is a 26-residue peptide with an overall charge of +6 at neutral pH.[30–32] Melittin adopts an α-helical conformation that is strongly amphipathic, as indicated by its mean hydrophobic moment value of 0.57 (Figure 1).[33] In dilute aqueous solution L-melittin is monomeric and unstructured, but at higher concentrations L-melittin forms a tetramer and displays extensive α-helicity.[34–36] L-Melittin has been observed to lyse cells via toroidal pore formation,[37–39] but the mode of action varies among different cell types and membrane models.[40] Whatever the mechanism of membrane disruption, peptide association seems to be important.

L-Melittin is one of the very few short, membrane-active peptides lacking disulfide constraints for which a crystal structure has been determined (PDB 2MLT).[41,42] Most residues in the crystalline peptide participate in α-helical secondary structure; a bend between two helical segments occurs at the central proline residue. The crystal contains a tetramer, and most of the nonpolar side chains are clustered in the core of this tetramer. The outer surface of the tetramer is comprised largely of polar side chains. The tetramer observed in the crystal lattice is proposed to correspond to the tetramer that forms in concentrated aqueous solution, e.g., in the form that L-melittin is stored in the honey bee venom sac.[41,42] The apparent correlation between assembly in the crystalline and solution states, and the availability of a crystal structure for L-melittin, attracted us to this peptide as a subject of racemic crystallization.

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Crystallization of racemic melittin was achieved by vapor diffusion from a 1:1 solution of 3 mg/mL peptide in water and 0.05 M ammonium sulfate, 0.1 M BIS-TRIS pH = 6.5, 30% v/v pentaerythritol ethoxylate (see SI for details). Two-fold symmetry is present in both the racemic and L-melittin structures; the racemic structure was solved in space group C2 and the L-melittin structure in space group $C222_1$. Each molecule in the racemic structure has the overall shape of a "bent α-helical rod," as originally noted by Terwilliger et al. for the structure of L-melittin. The original L-melittin structure was re-fined to 2.0 Å, and the new racemic structure was re-fined to 1.27 Å.

The asymmetric unit of the L-melittin crystal contains two independent peptides with slightly different conformations (backbone RMSD = 0.69 Å). Similarly, the asymmetric unit of the new racemic structure contains two independent molecules of L-melittin but also includes two D-melittin molecules (backbone RMSD for each homochiral pair = 1.09 Å). There are four possible pairwise overlay comparisons between one of the independent L-melittin molecules in the homochiral crystal and one of the independent molecules of L-melittin in the racemic crystal. Backbone RMSD values for these comparisons range between 0.53 Å and 1.03 Å; one of these comparisons is shown in Figure 2.

In the crystal structure of L-melittin, the two independent molecules pack side-by-side with antiparallel orientation (Figure 3A).[41,42] The resulting dimer has a concave face dominated by nonpolar side chains and a convex face dominated by polar side chains (Figures 3B and S5). Pairs of dimers pack against one another via their concave surfaces; the peptides in one dimer have an oblique orientation relative to the peptides in the other dimer (Figure 3C). The resulting tetrameric assembly features a hydrophobic core. Terwilliger et al. pro-posed that the tetramer observed in the L-melittin crystal corresponds to the tetrameric form of L-melittin that occurs in concentrated solution.[41,42] The new racemic crystal structure reveals a homochiral tetrameric association very similar to that observed in the L-melittin structure (Figure 3). This similarity suggests that racemic crystallography can capture the intrinsic self-association propensity of this peptide toxin.

In both the L-melittin and racemic melittin crystal structures, packing between asymmetric units is in part aided by the tetrameric assembly resulting from interactions between the concave surfaces of the melittin dimers. However, packing between the convex surfaces of the dimers is quite different between the two crystalline forms. In the L-melittin structure, the convex (polar) surface of each side-by-side dimer displays an almost perpendicular orientation relative to the convex surface of a side-by-side dimer from a neighboring tetramer (Figure 4A). In the racemic structure, however, each molecule in the side-by-side L-melittin dimer makes close contact with an antiparallel D-melittin molecule, and the two D-melittin neighbors for a given L-melittin dimer come from different dimers (Figure 4B). One of the two heterochiral contacts includes four intermolecular hydrogen-bonds (Figure 4C). The packing between layers within the L-melittin and racemic melittin crystals is compared in Figures 5 and S6.

Dutta et al. have recently evaluated the role of absolute configuration in the toxicity manifested by the Aβ42 peptide, which forms fibrils in the brains of Alzheimer's Disease patients. These workers found that L-Aβ42 was quite toxic toward neuron-like cells;

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however, racemic Aβ42 was significantly less toxic, apparently because the racemate was more prone to aggregation than was L-Aβ42.[43,44] This discovery motivated us to ask whether racemic melittin would display reduced toxicity relative to L-melittin; the biological activity of racemic melittin has not previously been evaluated. No reduction in either antibacterial or hemolytic activity was observed for the racemate relative to the enantiomerically pure peptide (Table 1). For both Aβ42 and melittin, toxicity is believed to result from soluble species (monomers or oligomers), and the divergent stereochemical trends could arise because racemic Aβ42 appears to be far less soluble than racemic melittin. The solubility difference presumably reflects distinctions in self-assembly mode: Aβ42 forms intermolecular β-sheets that are potentially infinite in size, while melittin adopts α-helical secondary structure and favors a discrete, closed assembly. Consistent with previous reports by Wade et al.,[45] we observed that L-melittin and D-melittin display very similar antibacterial and hemolytic activities (Table 1).

With the results reported here, we have provided strong evidence in two independent systems, a host-defense peptide[20,21] and the toxin melittin, that racemic crystal structures can offer atomic-resolution information valuable for assessing quaternary assemblies relevant to biological function of L-peptides. In contrast, heterochiral associations have been observed in several M2-TM racemate structures rather than the biologically relevant homochiral tetramer assembly.[24,25] The high similarity between tetrameric motifs observed in the new racemic melittin structure reported here and the previously reported L-melittin structure is a significant finding; comparison between racemic and L-peptide structures was not possible in previous studies of magainin-2 derivatives[20,21] or BTD-2.[23] Collectively, these studies encourage further exploration of racemic crystallization as a potentially general source of high-resolution information on the intrinsic assembly preferences of natural L-peptides for which function requires self-assembly and interaction with membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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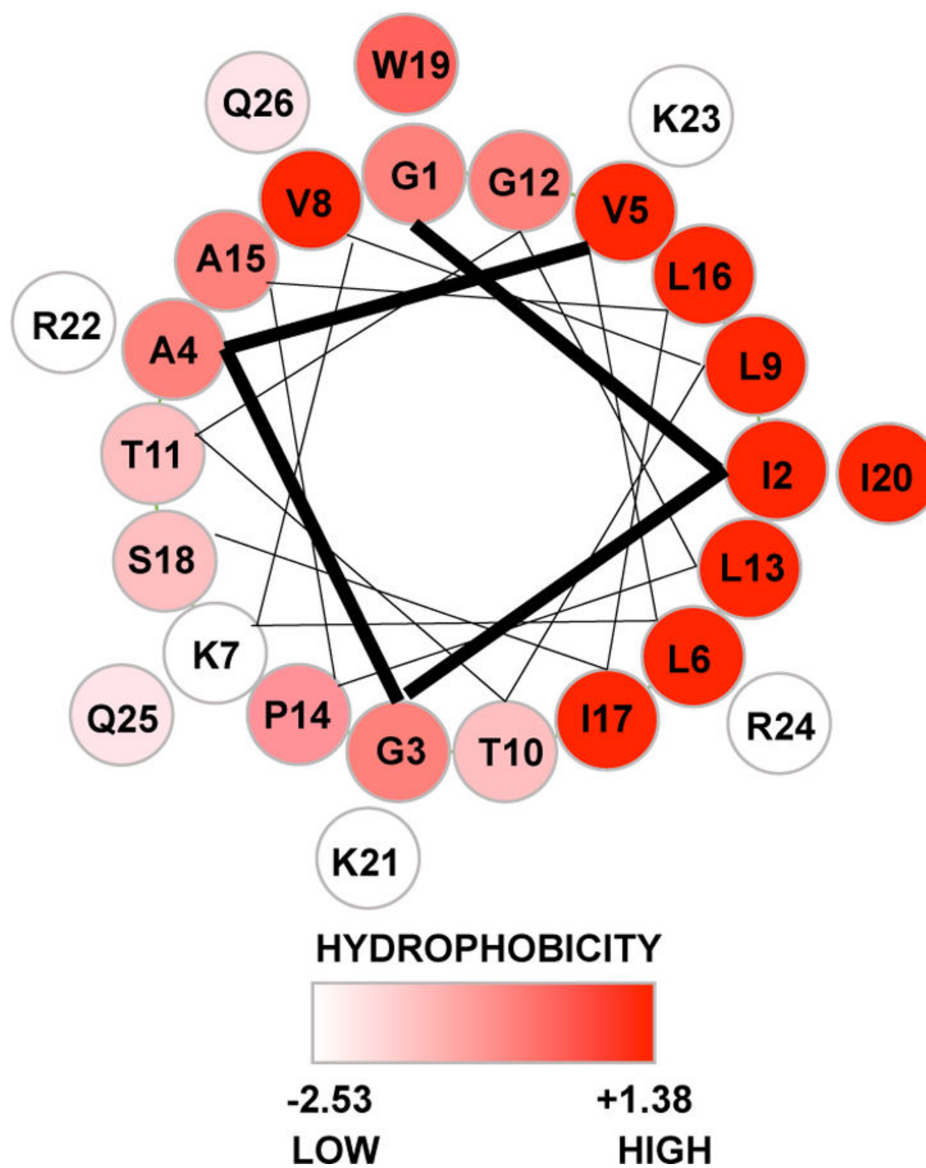

Figure 1.

The helical wheel of melittin is shown here colored according to the Eisenberg scale, which quantifies the hydrophobicity of the 20 proteinogenic residues.[33]

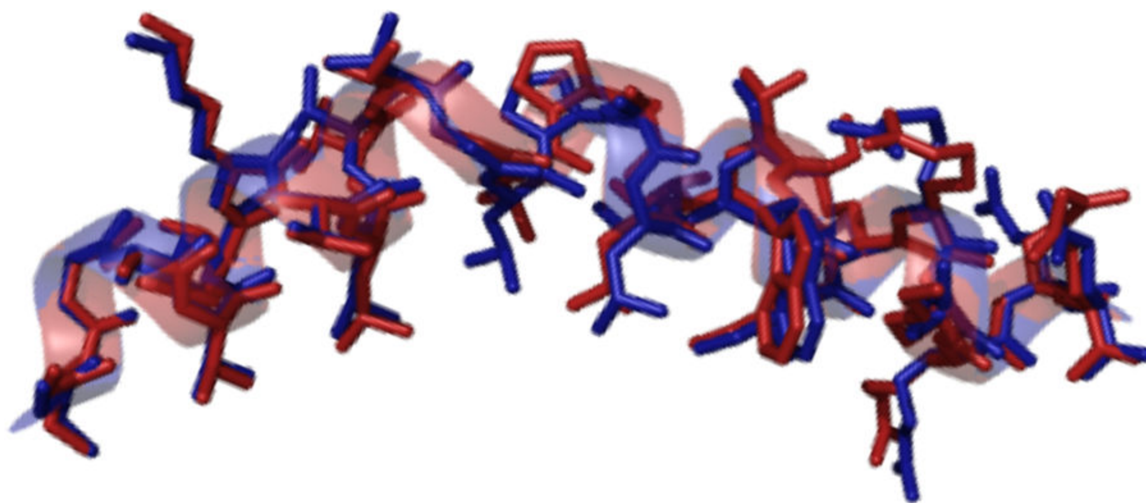

Figure 2.
One of the four possible overlays for an L-melittin molecule from the homochiral crystal structure (blue; PDB 2MLT) and an L-melittin molecule from the racemic crystal structure (backbone RMSD = 0.53 Å, overall RMSD = 1.13 Å).

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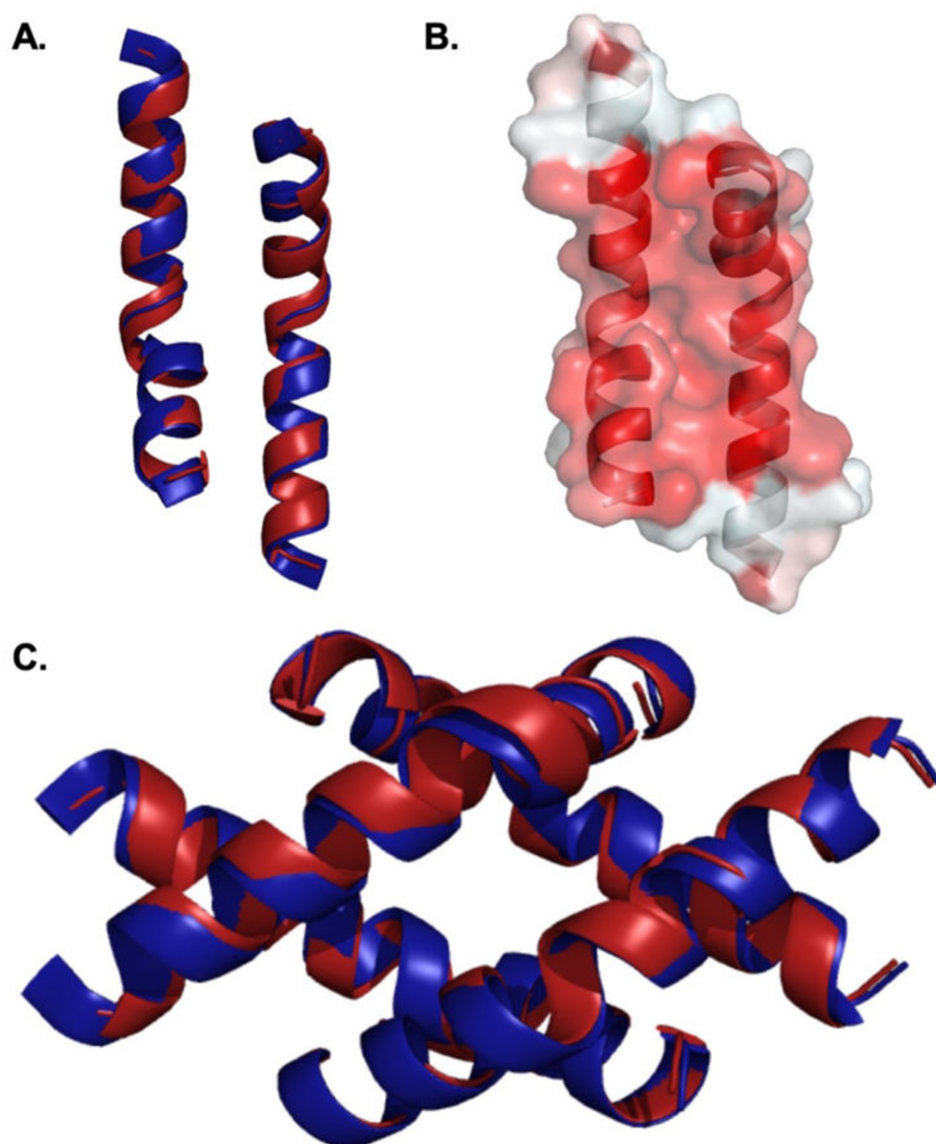

Figure 3.

Dimeric and tetrameric assembly observed in the L-melittin and racemic melittin crystal structures. Molecules from the L-melittin structure (PDB 2MLT) are shown in blue, and L-melittin molecules from the racemic structure are shown in red. A. An overlay of the side-by-side antiparallel dimer motif looking toward the concave face (backbone RMSD = 0.71 Å). B. A view of the hydrophobic surface of the L-melittin dimer that is color-coded based on the Eisenberg scale (see Fig. 1).[33] C. An overlay of the tetramer motif (backbone RMSD = 0.77 Å).

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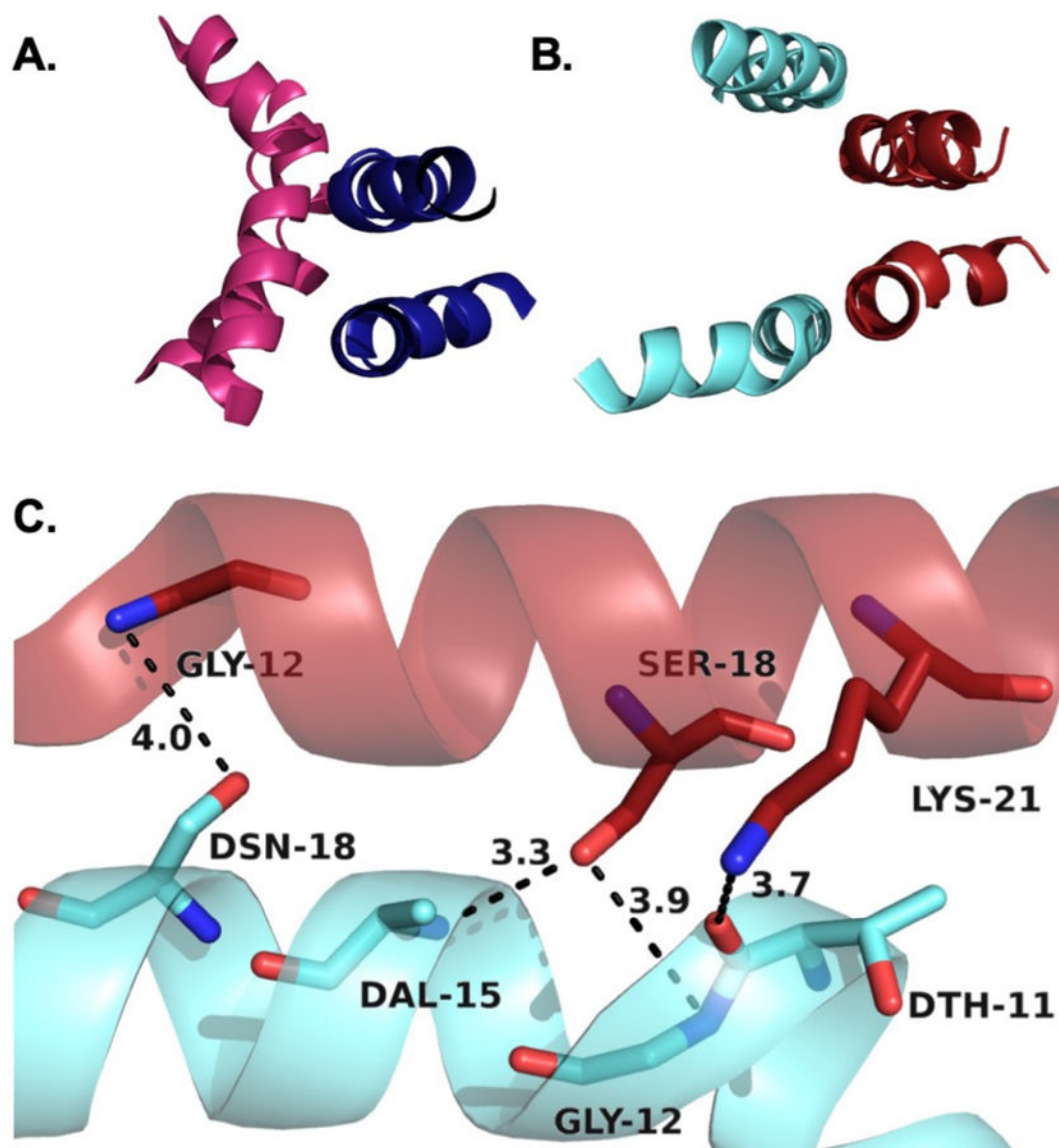

Figure 4.

Lattice neighbor interactions involving the convex surfaces of side-by-side melittin dimers. A. From the L-melittin structure, with one dimeric asymmetric unit shown in blue and its convex-facing dimeric neighbor in magenta. B. From the racemic structure, with an L-melittin dimer shown in red and its convex-facing D-melittin neighbors shown in light blue. C. Interactions between the L-and D-melittin molecules in the lower region of panel B. The residues are labeled with their PDB 3-letter codes. The 3-letter codes for D-amino acid residues begin with D; for example, the PDB 3-letter code for D-alanine is DAL.

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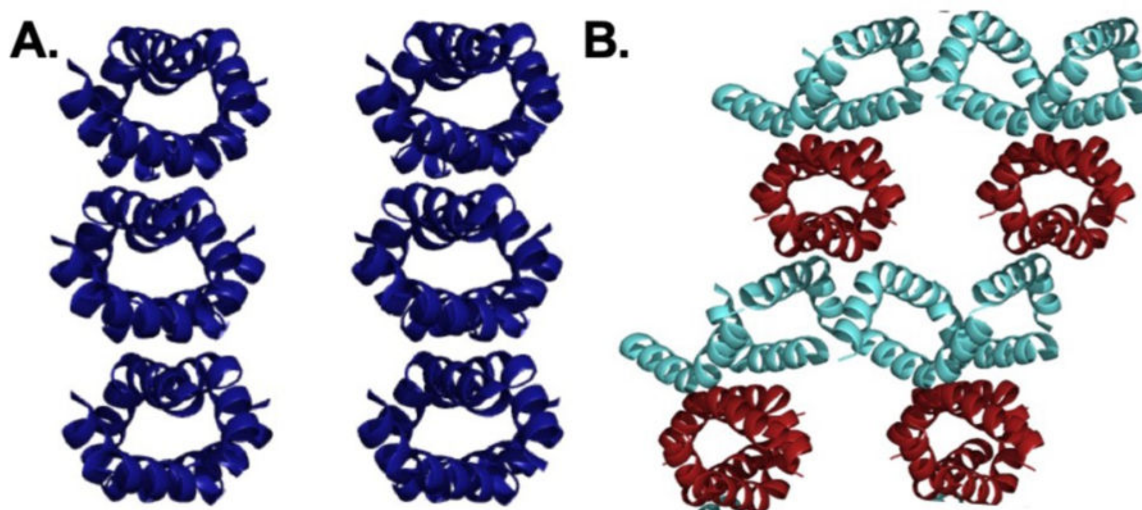

Figure 5.

Packing diagrams. A. L-Melittin (PDB 2MLT). B. Racemic melittin. In panel B the L-melittin peptides are shown in red and the D-peptides in light blue. The racemic crystal displays alternating layers that consist of L-melittin or D-melittin.

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Table 1.

Biological activities of L-, D- and racemic melittin. Values are the median data points of three biological replicates, with three technical replicates each (see SI for minor exception.)

	MIC[A] (μM)			
Peptide	***B. subtilis***	***S. aureus***	***E. faecium***	**HC_{10}[B] (μM)**
L-melittin	4	1	1	1
D-melittin	0.5	1	0.5	1
Racemic melittin	1	1	0.5	2

[A] Minimum inhibitory concentration (MIC) refers to the lowest concentration of peptide that prevents observable bacterial growth. The measurements are conducted with serial two-fold dilutions of peptide; the uncertainty in these measurements is one serial dilution above or below the indicated MIC value.

[B] HC_{10} refers to the concentration of peptide at which 10% of human red blood cells are lysed.