

Evidence for Phenylalanine Zipper-Mediated Dimerization in the X-ray Crystal Structure of a Magainin 2 Analogue

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S Supporting Information

ABSTRACT: High-resolution structure elucidation has been challenging for the large group of host-defense peptides that form helices on or within membranes but do not manifest a strong folding propensity in aqueous solution. Here we report the crystal structure of an analogue of the widely studied host-defense peptide magainin 2. Magainin 2 (S8A, G13A, G18A) is a designed variant that displays enhanced antibacterial activity relative to the natural peptide. The crystal structure of magainin 2 (S8A, G13A, G18A), obtained for the racemic form, features a dimerization mode that has previously been proposed to play a role in the antibacterial activity of magainin 2 and related peptides.

Eukaryotes produce many peptides that inhibit the growth of prokaryotes.^{1–5} A wide range of sequences and bioactive conformations are found among “host-defense peptides”, and diverse mechanisms of action are possible within this family.^{6–14} One large subset of these molecules appears to act via disruption of bacterial membrane barrier function. Multiple mechanisms for membrane compromise have been proposed, including (1) formation of discrete ion channels via specific peptide assemblies,¹⁵ (2) formation of large and variably sized toroidal pores,¹⁶ (3) complete destruction of the lipid bilayer with concomitant formation of peptide–lipid micelles (“carpet mechanism”),¹⁷ (4) induction of phase separation among lipids with concomitant leakage at phase boundaries,¹⁸ and (5) disruption of the hydrophobic barrier via “interfacial activity”.^{3,6} For many membrane-active antimicrobial peptides, the precise mechanism is unclear.

Efforts to understand the basis of polypeptide function typically include structural characterization; however, elucidation of bioactive conformations has been challenging for the large group of antimicrobial peptides that form helices on or within membranes but do not manifest a strong folding propensity in aqueous solution. These peptides are often rich in both charged and hydrophobic side chains, and they are too short to form stable tertiary structures. NMR methods can be useful for structural characterization of this class of molecules since these techniques can be applied to micelle- or vesicle-associated peptides. In contrast, crystallographic characterization, which can often provide structural information of higher resolution relative to NMR, has found only limited application in the study of helical antimicrobial peptides. Crystal structures have been reported for

Peptide	Sequence
magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
magainin 2 (S8A, G13A, G18A)	GIGKFLH AA KKF AK AFV AE IMNS
magainin 2 (F5Y, F16W)	GIGK YL HSAKKFGKAFVGEIMNS
MSI-78	GIGKFL KK AKKFGKAFV GRIL KK

Figure 1. Sequences of magainin 2 and several designed analogues. Positions of amino acid substitutions, relative to magainin 2, are shown in red; magainin 2 (S8A, G13A, G18A) is abbreviated “Ala mag” throughout the text.

several members of the peptaibol family (nonribosomal helix-forming peptides from fungi).¹⁹ These peptides are very hydrophobic and contain many helix-promoting Aib (amino-isobutyric acid) residues. To our knowledge, crystal structures have been determined for only two examples of highly hydrophilic, helical antimicrobial peptides. Melittin, a component of honey bee venom, is toxic to both prokaryotic and eukaryotic cells; this 26-mer bears a net charge of +6 at physiological pH and forms pores in lipid bilayers.²⁰ The crystal structure of melittin reveals an amphiphilic α -helix that is bent at the central proline residue.²¹ Dermicidin, a host-defense peptide, contains 48 residues, 16 of which have ionizable side chains; this polypeptide bears a net charge of –2 at physiological pH. Dermicidin forms a long α -helix that assembles into discrete channels in prokaryotic membranes, and the recently reported crystal structure provides a compelling model for a hexameric channel.²²

We report the crystal structure of a synthetic analogue of a polycationic host-defense peptide from the large group that appears to disrupt membranes by non-channel mechanisms. Specifically, we have determined the structure of magainin 2 (S8A, G13A, G18A), (“Ala mag”) (Figure 1), in which one Ser and two Gly residues have been changed to Ala in order to increase helical propensity.²³ This variant displays enhanced antibacterial activity relative to magainin 2 itself. Both Ala mag and magainin 2 contain 23 residues and should bear a net charge of +3 near neutral pH (four Lys residues and one Glu). The crystal structure of Ala mag reveals a dimeric association mode that is generally consistent with NMR-based conclusions previously reported for a double mutant of magainin 2 (F5Y, F16W) bound to phospholipid vesicles²⁴ and for the more highly modified analogue MSI-78 bound to micelles.²⁵ However, NMR analysis of magainin 2 itself and of Ala mag in the presence of

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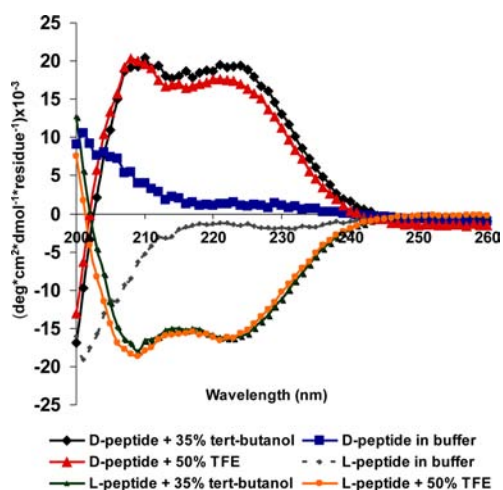


Figure 2. Ala mag is α -helical under the crystallization condition. Circular dichroism spectra of L- and D-Ala mag peptides. All solutions contained 0.1 M sodium citrate, pH 5.6.

micelles did not reveal evidence of dimer formation.^{26,27} Matsuzaki et al. have proposed that magainin 2 dimerizes upon binding to the surface of a lipid bilayer,²⁸ but the inconsistency among NMR studies leaves this hypothesis in question.

Our efforts to crystallize enantiopure forms of magainin 2, Ala mag, or MSI-78 were unsuccessful, and we therefore prepared racemic versions of each peptide. It has been suggested that racemic polypeptides are more susceptible to crystallization than the corresponding pure enantiomers because of the availability of additional space groups that contain inversion symmetry operations and are therefore inaccessible to homochiral samples.²⁹ Additionally, it has been noted that determination of phase angles for mixtures of peptides that form centrosymmetric or pseudo-centrosymmetric crystals is greatly simplified relative to homochiral samples.³⁰ A number of polypeptides have been crystallized in racemic form over the past two decades,^{30b,31} including the peptaibol trichogin A IV.^{19e}

Racemic crystallization was not successful in our hands for magainin 2 or MSI-78; however, we identified two crystallization conditions for racemic Ala mag via sparse-matrix screening. One condition contained 20% ethanol and the other contained 35% *tert*-butanol. Introduction of small, aliphatic alcohols to aqueous peptide solutions is known to promote α -helix formation relative to purely aqueous conditions.³² Indeed, circular dichroism (CD) data show that introduction of 35% *tert*-butanol has a dramatic α -helix-promoting effect for enantiopure Ala mag relative to aqueous buffer (Figure 2). In this example, *tert*-butanol proved to be comparable to the most popular helix-promoting co-solvent, 2,2,2-trifluoroethanol (TFE). It must be noted that the conditions used to crystallize Ala mag are not representative of the membrane environment; however, crystals generally do not reflect a polypeptide's native environment.

Optimization of the *tert*-butanol conditions, but not the ethanol conditions, provided crystals suitable for diffraction measurements (Figure S4). The structure of Ala mag was solved and refined to 1.75 Å resolution (Table S1). Racemic Ala mag crystallized in space group $I42d$, which is non-centrosymmetric but contains symmetry operators that relate the L- and D-polypeptides. The asymmetric unit contains one L-peptide, while the unit cell comprises eight L- and eight D-polypeptides. The Ala mag racemate crystals contained an estimated 43% bulk solvent.

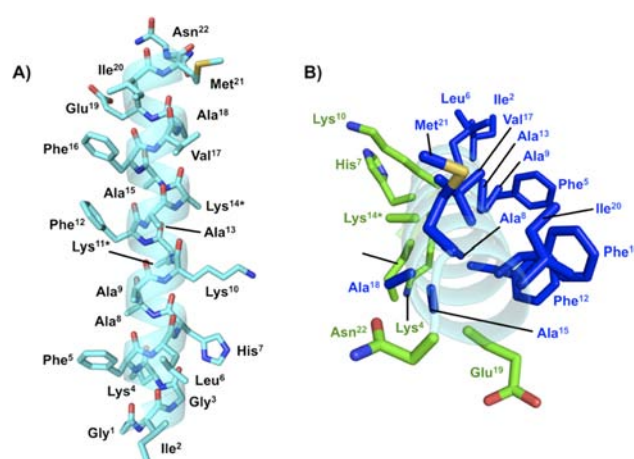


Figure 3. Crystal structure of Ala mag. (A) Residues 1–22 of Ala mag are incorporated into a single α -helix. (B) The helical conformation of Ala mag is globally amphiphilic. All hydrophilic side chains (green) are clustered along one side of the helix, and nearly all of the hydrophobic side chains (blue) are clustered on the opposite side of the helix.

The final structure contains nearly all of the peptide backbone (Figure 3A); the C-terminal residue, S23, could not be reliably modeled in the electron density because of disorder. Residues 1–22 are incorporated into a single α -helix, with 21 residues located in the “preferred” α -helical region of the Ramachandran plot and one residue (N22) located in an “allowed” α -helical region. Only weak electron density was observed for the long, flexible side chains of K11 and K14; consequently, only some of the side-chain carbon atoms in these residues were included in the crystallographic model. The helical conformation of Ala mag is globally amphiphilic, as expected (Figure 3B): all hydrophilic side chains are clustered along one side of the helix. Nearly all of the hydrophobic side chains are clustered on the opposite side of the helix (those of A15 and A18 are the only exceptions).

Pairs of homochiral peptides pack against one another in antiparallel side-to-side fashion (Figure 4A). The interface between these paired helices displays a side-chain packing arrangement that is somewhat reminiscent of coiled-coil association. Coiled coils comprise two or more α -helices that wind around each other.³³ Interactions between neighboring helices involve “knobs-into-holes” (KIH) interdigitation of side chains: the knob side chain from one α -helix fits into a hole generated by four side chains from the adjacent α -helix.^{34,35} Sequences that form coiled coils display a heptad repeat pattern; the heptad positions are designated *abcdefg*. The knob side chains occur at positions *a* and *d*, and the hole side chains occur at *a*, *d*, *e*, and *g*. Use of the program SOCKET³⁴ to analyze potential KIH side-chain packing within our structure indicated that the Ala mag dimer is a “marginal coiled coil”. The helical interface seems to be centered on two heptads formed by the segment F5–A18 (Figures S1–S3). Phe occurs at the two *a* positions (F5 and F12), while Ala occurs at the two *d* positions (A8 and A15). The *a* position Phe side chains project away from the core of the interface; the steric bulk of the phenyl rings cannot be accommodated within a typical KIH packing arrangement, which presumably explains the deviation from a classical coiled-coil association mode. Indeed, neither of the dimer structures deduced by NMR for magainin 2 analogues^{24,25} corresponds to a coiled coil according to SOCKET.

In the Ala mag crystal, the aromatic residues within the antiparallel homochiral dimer display an interaction mode

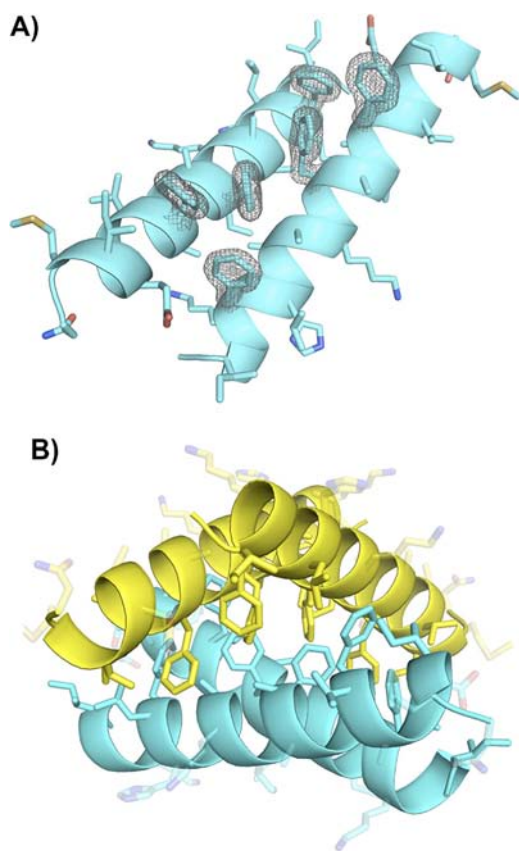


Figure 4. (A) Dimeric association of L-Ala mag and intercalated phenylalanine side chains. $2F_o - F_c$ electron density corresponding to side-chain atoms is shown at a contour of 1.5σ . (B) Crystal packing between dimers of L- and D-Ala mag (blue and yellow, respectively) results in burial of hydrophobic surfaces.

unrelated to coiled-coil packing. The side chains of F5 and F12, along with those from F16 (ϵ positions), form an array of six phenyl groups that align along one side of the homochiral dimer (Figure 4A). Comparable arrays have been observed or deduced in a variety of peptide assemblies and have been loosely referred to as “phenylalanine zippers”, whether they occur in the interior or on the exterior of an assembly.^{36–39} Phenylalanine residues found in other antimicrobial peptides appear to play an important role in mediating interactions of these peptides with bacterial or mammalian membranes.^{40–42} It has been suggested that the NMR-based models for magainin 2 analogue structures^{24,25} could be inconsistent with dimer binding to biological membranes, since most of the hydrophobic surface area is buried at the helical interfaces in these models.⁴³ In contrast, the homochiral Ala mag dimer found in our crystal structure displays a large hydrophobic surface area comprising side chains from Phe and other residues. This surface may help to anchor the Ala mag dimer to a lipid bilayer.

In the racemate crystal, there is a close association between an antiparallel dimer of L-peptides and an antiparallel dimer of D-peptides (Figure 4B). The two dimers pack against one another along their Phe-rich surfaces, and the core of this unusual four-helix assembly is dominated by aromatic rings. In order to assess whether this heterochiral association mode might influence biological activity, we determined the minimum inhibitory concentrations (MIC) of L-Ala mag, D-Ala mag, and racemic Ala mag for four bacteria: laboratory strains of *Escherichia coli*⁴⁴ and *Bacillus subtilis*⁴⁵ and clinical strains of *Staphylococcus aureus*

Table 1. Antimicrobial Activity of Ala mag with Varying Stereochemistry

Ala mag	MIC, $\mu\text{g}/\text{mL}$ ^a			
	<i>E.c</i>	<i>B.s</i>	<i>S.a</i>	<i>E.f</i>
L-enantiomer	6	3	50	50
D-enantiomer	6	3	50	50
L+D-racemic mixture	6	3	50	50

^aMIC results for *E. coli* (*E.c*), *B. subtilis* (*B.s*), *S. aureus* (*S.a*), and *E. faecium* (*E.f*).

(methicillin-resistant)⁴⁶ and *Enterococcus faecium*⁴⁷ (vancomycin-resistant). The enantiomers display identical antibacterial activities, as has previously been observed for other host-defense peptides and synthetic analogues (Table 1).⁴⁸ The racemic mixture is equipotent with the enantiopure peptides. In addition, the hemolytic activity of the racemic mixture is indistinguishable from that of either pure enantiomer (Figure S5). These observations suggest that the packing of L- and D-dimers observed in the racemate crystal (Figure 4B) is not relevant to the biological activity of racemic Ala mag.

We have shown that racemic crystallization can enable high-resolution structural elucidation of a peptide that is flexible and bears a high density of ionized side chains, a type that has traditionally been very difficult to crystallize. Our experience suggests that racemic crystallization is not a panacea: only one of the three peptides we evaluated provided crystals. Nevertheless, it seems likely that further application of this strategy to medium-sized hydrophilic peptides that have only modest folding propensities will fill a significant structural void. The homochiral association observed in our crystal structure of racemic Ala mag is consistent with the antiparallel helix-dimer motifs previously deduced for other magainin 2 analogues from NMR data.^{24,25} Thus, our structure offers indirect support for the hypothesis that peptides in this family form dimers on bacterial membrane surfaces,²⁸ even though previous NMR studies of magainin 2 and Ala mag did not provide evidence for such dimerization.^{26,27} Moreover, our structure provides a high-resolution view of an interhelical association mode that deviates from an archetypal coiled-coil motif.

■ ASSOCIATED CONTENT

📄 Supporting Information

Experimental details for peptide synthesis, X-ray crystallography, circular dichroism, antibacterial assays, and hemolytic assays. This material is available free of charge via the Internet at <http://pubs.acs.org>. Model coordinates and structure factors have been deposited in the Protein Data Bank as entry 4MGP.

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📝 Notes

The authors declare no competing financial interest.

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