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**Supporting Material**

# Crystallizing Transmembrane Peptides in Lipidic Mesophases

Nicole Hofer, David Aragao, and Martin Caffrey

# Supplemental Material (S1): Crystallizing Transmembrane Peptides in Lipidic Mesophases

**By**

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## 1) Materials

Monoolein (9.9 MAG, Lots: M-239-A21-Q, M-239-516-5, 356 g/mole) was obtained from NuChek Prep Inc. (Elysian, MN, USA), linear gramicidin (Lot: 1345609 13307134, 1880 g/mole) and 2,2,2-trifluoroethanol (TFE, Lot: S25895-495) were purchased from Sigma-Aldrich (Dublin, Ireland). Crystallization screens PEG/Ion Screen (Lot: 212629), Index (Lot: 214407), Crystal Screen I (Lot: 211094), Crystal Screen II (Lot: 211251), MembFac (Lot: 211423) were obtained from Hampton Research (Aliso Viejo, CA, USA). Wizard I, Wizard II, and Wizard III (Lot: EBS000322200499) were sourced from Emerald BioSystems, Inc. (Bainbridge Island, WA, USA). The crystallization screen MemStart (Lot: 011-121) was obtained from Molecular Dimensions (Suffolk, UK). Water, with a resistivity of >18 MΩ.cm, was purified using a Milli-Q Water System (Millipore, Bedfors, MA, USA), consisting of an Elix 5 UV compartment (Lot: F4HN34349) with a Prograd®2 cartridge (Lot: F9HNO1157) to pre-purify water and a Synergy compartment (Lot: F4EN79695B) with a Simpak®1 cartridge (Lot: F9HN06031) to produce highly purified water followed by sterile filtration through a 0.22 μm MilliPAK®40 filter (Lot: F5PN18060).

## 2) Methods

### 2.1) Gramicidin/Lipid Mixture Preparation

Lipid and gramicidin were mixed in a molar ratio of 20:1, followed by co-solubilization in 2,2,2-trifluoroethanol (TFE). The solution was shaken by hand until the sample was optically clear (approximately 3 minutes.). TFE was then evaporated under a stream of nitrogen gas followed by complete removal under high vacuum using a vacuum pump (Büchi Vac® V500, Büchi, Flawil, Switzerland) at 20 °C for approximately 24 h [1]. Samples were stored at room temperature (RT, 18 – 23 °C) for a maximum of one week until the lipid cubic phase was prepared.

### 2.2) Cubic Phase Preparation and *In Meso* Crystallization

The lipidic cubic phase was prepared by combining the gramicidin/monoolein mixture with Milli-Q water in coupled 100 µl gas-tight Hamilton syringes (Hamilton Company, Reno, Nevada, USA) at a 3/2 weight ratio of monoolein to water as described [2]. All samples were prepared and homogenized to optical clarity at RT [3].

*In meso* crystallization trials were setup by transferring 50 nL of the gramicidin/lipid/water cubic phase onto a 96-well glass crystallization plate which was then covered with 0.8 or 1 µL precipitant solution using an *in meso* robot [3]. Wells were then sealed with a glass coverslide. The glass sandwich plates were transferred into an incubator/imager (RockImager RI1500, Formulatrix, Inc., Waltham, MA, USA) at 20 °C. Crystallization progress was monitored automatically by the RI1500 and manually using normal and polarized light microscopy (Eclipse E 400 Pol, Nikon, Melville, NY, USA). Pyramidal-shaped crystals measuring 30 x 30 x 30 $\mu m^3$ appeared after 3 to 5 days in precipitants containing 20 %(w/v) polyethylene glycol (PEG) 6000, 0.1 M Bicine, pH 9.0.

## 2.3) Harvesting Crystals

Plates with crystals were removed from the RI1500 and transferred to a 20 °C room. Coverglass was scored manually using a tungsten carbide glass cutter (Model: 633657, TCT Scriber & Glass Cutter, Silverline, Yeovil, UK) and gently removed from the well. To minimize changes in composition of well contents, the lipid cubic phase drop was immediately overlaid with approximately 1 µL precipitant solution. Cryo-loops (Micro Mounts, MiTeGen, Ithaca, NY, USA) ranging from 30 to 100 µm were used to harvest crystals. Crystals were directly cryo-cooled in liquid nitrogen and stored in liquid nitrogen until further use [3].

## 2.4) Data Collection

Diffraction data were collected on GM/CA CAT beamline 23ID-B with a MAR 300 CCD detector using 1.033 Å X-rays. A complete 1.70 Å resolution data set was obtained by collecting 227 images with a 1.0° oscillation and a 2.3 s exposure per image, a collimated beamsize of 10 µm [4] and a sample-to-detector distance of 200 mm. Data were indexed, integrated and 172 images were scaled using HKL2000 [5]. Data were then converted to structure factors using the CCP4 program TRUNCATE [6]. Data collection statistics are given in Table S1. The crystals were found to be in space group $P2_1$ with unit cell dimensions a = 30.6 Å, b = 62.6 Å, c = 30.6 Å, β = 100.0°. There are 3 dimers of gramicidin per asymmetric unit giving a total molecular weight of approximately 11.3 kDa. The corresponding Matthews' coefficient is 2.53 $Å^3$ / Da [7] that corresponds to a solvent content of 51 %.

## 2.5) Phase Determination and Refinement

The structure was solved by molecular replacement using Phaser [8] and 1AL4 [9] as model. Molecular replacement was done with a dimer in which all side chains were converted to alanine and by changing the default resolution limit in Phaser from 2.5 Å to 1.8 Å.
Refinement was performed using restrained maximum likelihood as implemented in Refmac [10]. Side chains were modelled in using Coot [11]. Positional and isotropic B-factor parameters were refined for each atom. Alternative conformations for side chains were added where suggested by |Fo|-|Fc| maps with an initial occupancy of 0.5. In those cases where B-factors and maps justified it, occupancy was set to 0.3 and to 0.7 so that maps were clear and neighbouring atoms had similar B-factors. After this stage of refinement, PEG, lipid and water molecules and a sodium ion were added to the model based on electron density and standard geometrical and chemical restraints. Molecules were subsequently deleted if they were not visible at the 1.0 sigma level in 2|Fo| – |Fc| electron density maps. Molecules assigned as PEG or lipid were also deleted if no clear hydrogen bonds could be assigned and no clear lipid head groups were identifiable in the electron density. Electron density in those regions was left un-modelled. At the end of refinement the structure contained three dimers of gramicidin, four PEG molecules, two water molecules, and one sodium ion.

## 2.6) Structure Validation

The quality of the structural model was evaluated, as much as possible, with MolProbity

[12], RAMPAGE [13], and Sfcheck [14]. Because D-amino acids are not in the corresponding libraries and gramicidin includes six D- residues a limited number of quality scores was possible. In MolProbity, for example, the reported model produced a clash score of 3.41 after hydrogens were added. This places the model in the 98$^{th}$ percentile of all structures with comparable resolution. This is on a scale where the 100$^{th}$ percentile is the best and 0$^{th}$ percentile the worst structure model. Furthermore, there are no bond length or angle outliers. Due to the right-handed chirality of D-amino acids, scores for Cβ deviation, rotamers, and Ramachandran do not evaluate this structure properly and were not used.

The coordinates, structure factors, and related data have been deposited in the PDB under code identifier: 2XDC.

**Table S1: Processing and Refinement Statistics**

| | |
|---|---|
| Space group | $P2_1$ |
| Unit cell parameters | a = 30.6 Å, b = 62.6 Å, c = 30.6 Å, β = 100.0 ° |
| X-ray source | 23ID-B (GM/CA CAT, Advanced Photon Source) |
| Wavelength [Å] | 1.033 |
| Resolution range (highest shell) [Å] | 31.3 – 1.7 (1.73 – 1.70) |
| Number of unique reflections | 11779 |
| Completeness (highest shell) [%] | 94.2 (80.8) |
| Redundancy (highest shell) [%] | 3.0 (2.2) |
| R merge (highest shell) [%] | 6.7 (26.5) |
| I / δ(I) (highest shell) | 13.1 (3.3) |
| | |
| Refinement | |
| Number of reflections | |
| Working (highest shell) | 11175 (706) |
| Test (highest shell)[1] | 604 (29) |
| Protein atoms | 816 |
| Solvent atoms | 107 (4 PEGs, 2 water, 1 sodium ion) |
| Average B-factor [Å$^2$] | |
| Protein and Solvent | 19.1 |
| Protein | |
| Chain A | 15.9 |
| Chain B | 16.3 |
| Chain C | 17.4 |
| Chain D | 18.9 |
| Chain E | 17.6 |
| Chain F | 19.4 |

| | |
|---|---|
| Solvent | |
| PEG | 31.3 |
| Water | 36.8 |
| Sodium ion | 41.0 |
| R factor [%] | 17.9 |
| R free [%] | 21.2 |
| R.m.s.d bond distance [Å] | 0.02 |
| R.m.s.d bond angles [°] | 1.9 |

[1] Test represents 5.1 % of total

### 2.7) Existing Structures of Gramicidin in the Protein Data Bank

As of 8th February 2010 the Protein Data Bank includes 22 relevant entries under the search word gramicidin. These are summarized in Table S2.

**Table S2. Structures of linear gramicidin in the Protein Data Bank**

| **Method (resolution, space group, cell dimensions)[1]** | **Dispersing Medium[2]** | **Conformation (arrangement, packing)[3]** | **PDB ID[4]** | **Ref.** |
|---|---|---|---|---|
| MX (1.13 Å; $P2_12_12_1$; a = 32.4 Å, b = 32.5 Å, c = 24.2 Å, $\alpha = \beta = \gamma = 90.0°$) | n-Propanol | DSDH (AP, LH) | 1AL4 | [9] |
| MX (1.20 Å; $P2_1$; a = 14.9 Å, b = 26.0 Å, c = 31.9 Å, $\beta = 92.0°$) | Methanol | DSDH (AP, LH) | 1ALX | [9] |
| MX (0.86 Å; $P2_12_12_1$; a = 31.6 Å, b = 32.4 Å, c = 24.2 Å, $\alpha = \beta = \gamma = 90.0°$) | Ethanol | DSDH (AP, LH) | 1ALZ | [9] |
| MX (1.4 Å; $P2_12_12_1$; a = 31.1 Å, b = 31.9 Å, c = 52.1 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Cesium Chloride) | DSDH (AP, RH) | 1AV2 | [15] |
| MX (1.7 Å; $P2_12_12_1$; a = 20.6 Å, b = 27.9 Å, c = 52.0 Å, $\alpha = \beta = \gamma = 90.0°$) | Glacial acetic acid | DSDH (AP, RH) | 1BDW | [15] |
| MX (2.0 Å; $P2_12_12_1$; a = 32.1 Å, b = 52.1 Å, c = 31.2 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Cesium Chloride) | DSDH (AP, LH) | 1C4D | [16] |
| MX (2.5 Å; $P2_12_12_1$; a = 32.1 Å, b = 51.8 Å, c = 31.0 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Potassium Thiocyanate | DSDH (AP, LH) | 1GMK | [17] |
| MX (1.14 Å; $P2_12_12_1$; a = 30.1 Å, b = 31.3 Å, c = 51.7 Å, $\alpha = \beta = \gamma = 90.0°$) | Ethanol (+Rubidium Chloride) | DSDH (AP, RH) | 1W5U | [18] |

| **Method (resolution, space group, cell dimensions)[1]** | **Dispersing Medium[2]** | **Conformation (arrangement, packing)[3]** | **PDB ID[4]** | **Ref.** |
|---|---|---|---|---|
| MX (0.80 Å; $P2_12_12_1$; a = 30.1 Å, b = 31.3 Å, c = 51.6 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Potassium Iodide) | DSDH (AP, RH) | 2IZQ | [19] |
| MX (1.7 Å; $P2_1$; a = 30.6 Å, b = 62.6 Å, c = 30.6 Å, $\beta = 100.0°$) | Lipidic mesophase | DSDH (AP, LH; Type I) | 2XDC | This work |
| NMR | SDS micelle | HHSH (AP, RH) | 1GRM | [20] |
| NMR | SDS micelle | HHSH (AP, RH) | 1JNO | [21] |
| NMR | SDS micelle | HHSH (AP, RH) | 1JO3 | [21] |
| NMR | SDS micelle | HHSH (AP, RH) | 1JO4 | [21] |
| NMR | Benzene/acetone 10:1 (by vol.) | DSDH (P, LH) | 1KQE | [22] |
| NMR | Hydrated DMPC bilayer | HHSH (AP, RH) | 1MAG | [23] |
| NMR | Methanol (+Calcium Chloride) | DSDH (P, LH) | 1MIC | [24] |
| NMR | SDS micelle | HHSH (AP, RH) | 1NG8 | [25] |
| NMR | Dodecyl phosphocholine micelle | HHSH (AP, RH) | 1NRM | To be published |
| NMR | Dodecyl phosphocholine micelle | HHSH (AP, RH) | 1NRU | To be published |
| NMR | SDS micelle | HHSH (AP, RH) | 1NT5 | [25] |
| NMR | SDS micelle | HHSH (AP, RH) | 1NT6 | [25] |
| NMR | d-Chloroform / $d_3$-methanol (1:1 (by vol.)) (+Cesium Chloride) | HHSH (AP, RH) | 1TKQ | [26] |

[1] Structures have been determined by macromolecular crystallography (MX) and nuclear magnetic resonance (NMR). The resolution, space group and unit cell dimensions associated with MX structures are reported in parenthesis.
[2] Dispersing medium refers to the milieu in which gramicidin was crystallized for MX or dispersed or dissolved for NMR.
[3] The two major conformations found include the double stranded inter-twined double helix (DSDH) and the head-to-head single stranded helix (HHSH). Strands can run parallel (P) or anti-parallel (AP) to one another and helices can be right (RH) - or left (LH) - handed. Where crystal packing is layered it is indicated as Type I [27].
[4] PDB IDs are hyperlinked to the corresponding record entry in the Protein Data Bank.

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score of 3.41 after hydrogens were added. This places the model in the 98$^{th}$ percentile of all structures with comparable resolution. This is on a scale where the 100$^{th}$ percentile is the best and 0$^{th}$ percentile the worst structure model. Furthermore, there are no bond length or angle outliers. Due to the right-handed chirality of D-amino acids, scores for Cβ deviation, rotamers, and Ramachandran do not evaluate this structure properly and were not used.

The coordinates, structure factors, and related data have been deposited in the PDB under code identifier: pending.

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| Redundancy (highest shell) [%] | 3.0 (2.2) |
| R merge (highest shell) [%] | 6.7 (26.5) |
| I / δ(I) (highest shell) | 13.1 (3.3) |
| | |
| Refinement | |
| Number of reflections | |
| Working (highest shell) | 11175 (706) |
| Test (highest shell)[1] | 604 (29) |
| Protein atoms | 816 |
| Solvent atoms | 107 (4 PEGs, 2 water, 1 sodium ion) |
| Average B-factor [Å$^2$] | |
| Protein and Solvent | 19.1 |
| Protein | |
| Chain A | 15.9 |
| Chain B | 16.3 |
| Chain C | 17.4 |
| Chain D | 18.9 |
| Chain E | 17.6 |
| Chain F | 19.4 |
| Solvent | |
| PEG | 31.3 |
| Water | 36.8 |
| Sodium ion | 41.0 |
| R factor [%] | 17.9 |

| | |
|---|---|
| R free [%] | 21.2 |
| R.m.s.d bond distance [Å] | 0.02 |
| R.m.s.d bond angles [°] | 1.9 |

[1] Test represents 5.1 % of total

### 2.7) Existing Structures of Gramicidin in the Protein Data Bank

As of 8th February 2010 the Protein Data Bank [15] includes 22 relevant entries under the search word gramicidin. These are summarized in Table S2.

**Table S2. Structures of linear gramicidin in the Protein Data Bank**

| **Method (resolution, space group, cell dimensions)[1]** | **Dispersing Medium[2]** | **Conformation (arrangement, packing)[3]** | **PDB ID[4]** | **Ref.** |
|---|---|---|---|---|
| MX (1.13 Å; $P2_12_12_1$; a = 32.4 Å, b = 32.5 Å, c = 24.2 Å, $\alpha = \beta = \gamma = 90.0°$) | n-Propanol | DSDH (AP, LH) | 1AL4 | [9] |
| MX (1.20 Å; $P2_1$; a = 14.9 Å, b = 26.0 Å, c = 31.9 Å, $\beta = 92.0°$) | Methanol | DSDH (AP, LH) | 1ALX | [9] |
| MX (0.86 Å; $P2_12_12_1$; a = 31.6 Å, b = 32.4 Å, c = 24.2 Å, $\alpha = \beta = \gamma = 90.0°$) | Ethanol | DSDH (AP, LH) | 1ALZ | [9] |
| MX (1.4 Å; $P2_12_12_1$; a = 31.1 Å, b = 31.9 Å, c = 52.1 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Cesium Chloride) | DSDH (AP, RH) | 1AV2 | [15] |
| MX (1.7 Å; $P2_12_12_1$; a = 20.6 Å, b = 27.9 Å, c = 52.0 Å, $\alpha = \beta = \gamma = 90.0°$) | Glacial acetic acid | DSDH (AP, RH) | 1BDW | [15] |
| MX (2.0 Å; $P2_12_12_1$; a = 32.1 Å, b = 52.1 Å, c = 31.2 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Cesium Chloride) | DSDH (AP, LH) | 1C4D | [16] |
| MX (2.5 Å; $P2_12_12_1$; a = 32.1 Å, b = 51.8 Å, c = 31.0 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Potassium Thiocyanate | DSDH (AP, LH) | 1GMK | [17] |
| MX (1.14 Å; $P2_12_12_1$; a = 30.1 Å, b = 31.3 Å, c = 51.7 Å, $\alpha = \beta = \gamma = 90.0°$) | Ethanol (+Rubidium Chloride) | DSDH (AP, RH) | 1W5U | [18] |
| MX (0.80 Å; $P2_12_12_1$; a = 30.1 Å, b = 31.3 Å, c = 51.6 Å, $\alpha = \beta = \gamma = 90.0°$) | Methanol (+Potassium Iodide) | DSDH (AP, RH) | 2IZQ | [19] |
| MX (1.7 Å; $P2_1$; a = 30.6 | Lipidic mesophase | DSDH (AP, LH; Type I) | 2XDC | This work |

| | | | | |
|---|---|---|---|---|
| Å, b = 62.6 Å, c = 30.6 Å, β = 100.0°) | | | | |
| NMR | SDS micelle | HHSH (AP, RH) | 1GRM | [20] |
| NMR | SDS micelle | HHSH (AP, RH) | 1JNO | [21] |
| NMR | SDS micelle | HHSH (AP, RH) | 1JO3 | [21] |
| NMR | SDS micelle | HHSH (AP, RH) | 1JO4 | [21] |
| NMR | Benzene/acetone 10:1 (by vol.) | DSDH (P, LH) | 1KQE | [22] |
| NMR | Hydrated DMPC bilayer | HHSH (AP, RH) | 1MAG | [23] |
| NMR | Methanol (+Calcium Chloride) | DSDH (P, LH) | 1MIC | [24] |
| NMR | SDS micelle | HHSH (AP, RH) | 1NG8 | [25] |
| NMR | Dodecyl phosphocholine micelle | HHSH (AP, RH) | 1NRM | To be published |
| NMR | Dodecyl phosphocholine micelle | HHSH (AP, RH) | 1NRU | To be published |
| NMR | SDS micelle | HHSH (AP, RH) | 1NT5 | [25] |
| NMR | SDS micelle | HHSH (AP, RH) | 1NT6 | [25] |
| NMR | d-Chloroform / $d_3$-methanol (1:1 (by vol.)) (+Cesium Chloride) | HHSH (AP, RH) | 1TKQ | [26] |

[1] Structures have been determined by macromolecular crystallography (MX) and nuclear magnetic resonance (NMR). The resolution, space group and unit cell dimensions associated with MX structures are reported in parenthesis.
[2] Dispersing medium refers to the milieu in which gramicidin was crystallized for MX or dispersed or dissolved for NMR.
[3] The two major conformations found include the double stranded inter-twined double helix (DSDH) and the head-to-head single stranded helix (HHSH). Strands can run parallel (P) or anti-parallel (AP) to one another and helices can be right (RH)- or left (LH) - handed. Where crystal packing is layered it is indicated as Type I [27].
[4] PDB IDs are hyperlinked to the corresponding record entry in the Protein Data Bank.

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