# Extreme Amyloid Polymorphism in *Staphylococcus aureus* Virulent PSMα Peptides

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## Abstract

Members of the *Staphylococcus aureus* phenol-soluble modulin (PSM) peptide family are secreted as functional amyloids that serve diverse roles in pathogenicity and may be present as full-length peptides or as naturally occurring truncations. We recently showed that the activity of PSMα3, the most toxic member, stems from the formation of cross-α fibrils, that are at variance with the cross-β fibrils linked with eukaryotic pathologies. Here, we show that PSMα1 and PSMα4, involved in biofilm structuring, formed the canonical cross-β amyloid fibrils; atomic structures of their amyloidogenic segments featured highly stable steric-zippers, formed by the tight mating of β-sheets. This ultra-high stiffness most likely allows stabilization of the biofilm extracellular matrix. Contrastingly, a truncated PSMα3, found to form reversible fibrils and to have antibacterial activity, revealed two polymorphic and atypical β-rich fibril architectures, which radically differed from both the cross-α fibrils formed by full-length PSMα3, and the cross-β fibrils formed by PSMα1 and PSMα4. Our results point to structural plasticity as the basis of the functional diversity exhibited by *S. aureus* PSMs.

## Main

Amyloids designate peptides and proteins capable of self-assembling into structured oligomers and fibrils, mostly known for their involvement in fatal neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases[1]. Some amyloids are functional, in that they participate in specific physiological activities. In humans, functional amyloids partake in immunity, reproduction and hormone secretion[2-4]. In microorganisms, they act as key virulence factors, and may thus represent novel targets for antibacterial drugs[5,6]. For example, amyloid fibrils are found in the self-produced polymeric matrix that embeds biofilm-forming bacteria, where they act as a physical barrier that increases their resilience and resistance to antimicrobial drugs[5-8] and to the immune system[9]. Functional bacterial amyloids may also act as toxins, killing non-self-cells and thereby increasing virulence[2,6,7,10]. The structural hallmarks of functional amyloids – if any – and how they can be distinguished from disease-associated amyloids remain unclear. To date, only a single atomic resolution structure of a functional bacterial amyloid is available, namely, that of phenol-soluble modulin α3 (PSMα3), the most cytotoxic member of the *Staphylococcus aureus* PSMs peptide family. The structure of the full-length PSMα3 revealed cross-α fibrils[10], a newly discovered mode of amyloid-like assembly, wherein α-helices oriented perpendicular to the fibril axis pile up into sheets, and tightly mate through hydrophobic surface complementarity. The cross-α fibrils[10] thus resemble the canonical cross-β fibrils of pathological amyloids, which form from the tight mating of β-sheets, with strands oriented perpendicular to the fibril axis[11].

*Staphylococcus aureus is* a prominent cause of threatening infections[12] and its PSM family members[13] serve as key virulence factors that stimulate inflammatory responses, alter the host cell cycle, lyse human cells and contribute to biofilm structuring[14,15]. High expression of PSMαs, which are four peptides of about 20-residues in length, increases virulence potential of methicillin-resistant *S. aureus* (MRSA)[16]. Amyloid aggregation plays roles in PSM activities in *S. aureus*. For example, fibrillation of PSMα1 promotes biofilm stability by preventing disassembly by matrix-degrading enzymes and mechanical stress[14]. In addition, fibrillation of PSMα3, enhances its toxicity against human cells[10]. Still, the extent of amyloid fibrillation in all PSM activities remains unclear, especially since PSMs are known to undergo truncation *in vivo* in response to various external stimuli, yielding truncated PSMs with new functions such as antibacterial activities[17-21]. As each of the truncated PSMs may adopt a different type of amyloid architecture, the array of structural species produced by PSMs is much larger than the actual number of PSMs. In the present study, we investigated the structure-function relationships of PSMαs and their short truncations (Supplementary Fig. 1). Our results illuminate the formidable structural plasticity of the multifunctional PSM family.

## Results

We found that in contrast to PSMα3, which forms cross-α fibrils[10], PSMα1 and PSMα4 fibrils, observed by electron microscopy (Fig. 1a&b), exhibit the cross-β signature in X-ray diffraction patterns (Fig. 1c&d), and indeed bind the amyloid-indicator dye thioflavin T[22] (Supplementary Fig. 2). It was previously shown that, in solution, PSMα1 and PSMα4

transition from α-helices to parallel β-sheets within several days, whereas PSMα3 remains helical[22]. To obtain atomic-level insights into the architecture of PSMα1 and PSMα4 fibrils, we computationally identified their amyloidogenic segments (Supplementary Fig. 1), and determined their high-resolution structures by means of X-ray crystallography (Supplementary Table 1). Segments PSMα1-IIKVIK and PSMα4-IIKIIK, both of which are conserved within naturally occurring PSM truncations[18-20], adopt the canonical cross-β fibril architecture, wherein pairs of β-sheets tightly mate through a dry interface, forming a steric zipper (Fig. 2 & Supplementary Fig. 3a). Both fibrils belong to the class 1 of steric zippers, meaning that parallel β-sheets mate face-to-face[23]. Structural indicators of fibril stability of the segments, *i.e.*, buried surface area and shape complementarity between sheets, resemble those of eukaryotic steric-zipper structures (Supplementary Table 2). The similarity between PSMα1 and PSMα4 segments and human pathological amyloids demonstrate that cross-β amyloids are a structural trait shared across species, from bacteria to human.

PSMα3 is toxic to human cells but certain single-point mutations are known to confer antibacterial activity[24]. Indeed, the LFKFFK segment from PSMα3, which forms fibrils (Fig. 3b), demonstrates dose-dependent antibacterial activity against Gram-positive *Micrococcus luteus* and *Staphylococcus hominis*, but is nontoxic to *S. aureus* (Fig. 3a & Supplementary Fig. 4). The observation that the steric-zipper-forming segments PSMα1-IIKVIK and PSMα4-IIKIIK do not elicit antibacterial activity (Fig. 3a & Supplementary Fig. 4) indicates that fibrillation is, in itself, insufficient to confer toxicity. Likewise, toxicity of truncated PSMs cannot be ascribed to charges of their amino-acids, since all three segments feature two basic and four hydrophobic side chains. Contrastingly, antibacterial activity is preserved in the one-residue longer PSMα3 truncation, KLFKFFK (Fig. 3a & Supplementary Figs.

4&5). Thus, the antibacterial activity of the LFKFFK and KLFKFFK segments could stem from the specific fibril architecture they adopt.

We obtained two atomic resolution structures of LFKFFK, both of which revealed a departure from cross-β fibrils and an atypical amyloid architecture (Fig. 4). One polymorph was fundamentally different from steric-zippers, displaying no dry interface between pairs of β-sheets. Instead, hexamers of β-sheets formed cylindrical channels running along the fibril-like structure, effectively yielding nanotubes (Fig. 4a). The second polymorph was composed of out-of-register β-sheets (Fig. 4b), meaning that unlike in canonical cross-β fibrils, β-strands are not perpendicular to the fibril axis[25,26]. Such extreme polymorphism is exceptional within the hundreds of structures of amyloid-like spine segments solved to date[27]. We further found that biofilm-related PSMα4-IIKIIK fibrils are astonishingly more stable than the antibacterial PSMα3-LFKFFK fibrils. While IIKIIK fibrils remain stable at an elevated temperature, explaining their structural role in stabilizing the biofilm and in accordance with their steric-zipper structure, the LFKFFK fibrils dissolve, but reform upon cooling (Supplementary Fig. 6). This resembles the labile hydrogel formation of low-complexity, aromatic-rich, protein segments associated with membraneless assemblies, which form fibrils with kinked β-sheets[28,29]. The reversible LFKFFK fibril formation could thus underlie regulation of a functional role.

## Discussion

Work on human disease-associated amyloids has shown that the vast majority of amyloids are β-rich and polymorphic by nature[27], which was suggested to encode different level of

neurotoxicity and prion strains[30]. We hypothesize that functional amyloids are even richer in polymorphisms, as they encode highly diverse functions in a reduced number of related sequences. In the present study, we found considerable structural diversity among close homologs (Supplementary Figs. 1&7). While PSMα3 forms cross-α fibrils and enhances toxicity against human cells[10], PSMα1 and PSMα4 form cross-β fibrils (Fig. 1) which likely play a role in stabilizing the biofilm matrix[14]. Biofilms formed by pathogenic *S. aureus* strains displaying robust amyloid fibril formation indeed contained a high level of PSMα1 and PSMα4 fibrils[14,31]. The role of amyloid in biofilm development is evidenced in other β-rich microbial amyloids, e.g., curli CsgA from *Escherichia coli*[7] and FabC in *pseudomonas*[32]. The highly stable steric zipper structures (Fig. 2 & Supplementary Fig. 3), forming the spines of the cross-β fibrils, putatively serve as the building block cementing the biofilm and creating the rigidity that can explain the resistance of amyloid-containing biofilms[8,14].

A noticeable difference between the steric-zipper structures of the PSMα1 and PSMα4 segments, and the two polymorphs of the LFKFFK segment, is the orientation of the β-strands, namely parallel vs. antiparallel, respectively (Figs. 2 & 4). Fibrils with β-sheets arranged in an antiparallel orientation are generally more toxic than those with a parallel arrangement[33-36]. Moreover, the cylindrical architecture of LFKFFK polymorph I (Fig. 4a) is reminiscent of that displayed by a polymorph of amyloid-β fibrils[37], and a HET-s prion fragment[38]. Similarly, stable toxic oligomeric species of α-synuclein[39] and a segment of the human αB-crystalline, named cylindrin[40] display antiparallel β-strand orientation and cylindrical architectures. The second polymorph of LFKFFK is composed of out-of-register β-sheets (Fig. 4b), a morphology that was also suggested to serve as a pathway to toxic amyloid aggregates[25], and the interface between the sheets is reminiscent of the KLVFFA

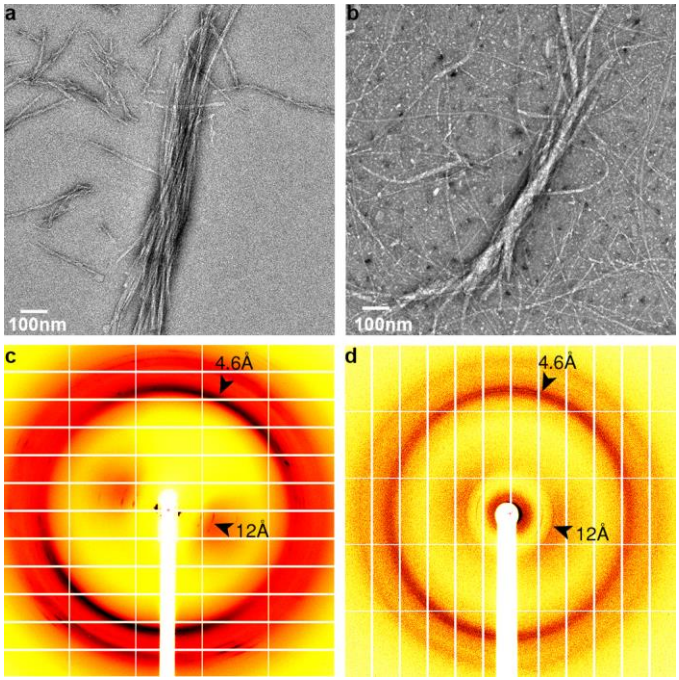
segment of amyloid-β[36]. Thus, the two different polymorphs of the antibacterial peptide LFKFFK display features which, in human disease-associated amyloids, correlate with toxicity. The PSMα3 sequence largely offers a fascinating peek at the complexity of the amyloid fold, populating β-rich amyloid states in the truncated antibacterial LFKFFK form, but forming cross-α fibril in the context of cytotoxic full-length PSMα3[10].

A link between amyloids and antibacterial activity has been previously proposed, following the observations that several human host defense peptides form amyloid-like fibrils[41,42], and that several human pathological amyloids display antimicrobial action[43-46]. LFKFFK presents an additional example, further associating antibacterial activity with atypical and reversible fibril architectures, which differ from those yielding the highly stable fibrils playing a structural role in biofilms. While it remains unclear which (if not both) polymorph(s) is (are) associated with antibacterial activity, our two LFKFFK structures suggest that specific structural features are required to yield such activities. The biological relevance of the LFKFFK segment of PSMα3 is supported by the observation that LFKFFK is nontoxic to the PSMα3-secreting *S. aureus*, but toxic to the closely related *S. hominis* and to *M. luteus* (Fig. 3). This suggests a mechanism by which *S. aureus* is able to adapt to the toxic fold of LFKFFK fibrils.

Together we found that PSMαs demonstrate a vast structural diversity of amyloid-like structures, including cross-α, cross-β, out-of-register β-sheets, and hexameric configurations (Figs. 1, 2, 4 & Supplementary Fig. 7). By simultaneously secreting PSMαs located at the same operon, the *S. aureus* bacterium generates various virulent activities encoded by diverse amyloid morphologies. PSMs have critical and diverse roles during infection and represent a promising target for anti-staphylococcal therapy[15]. The atomic

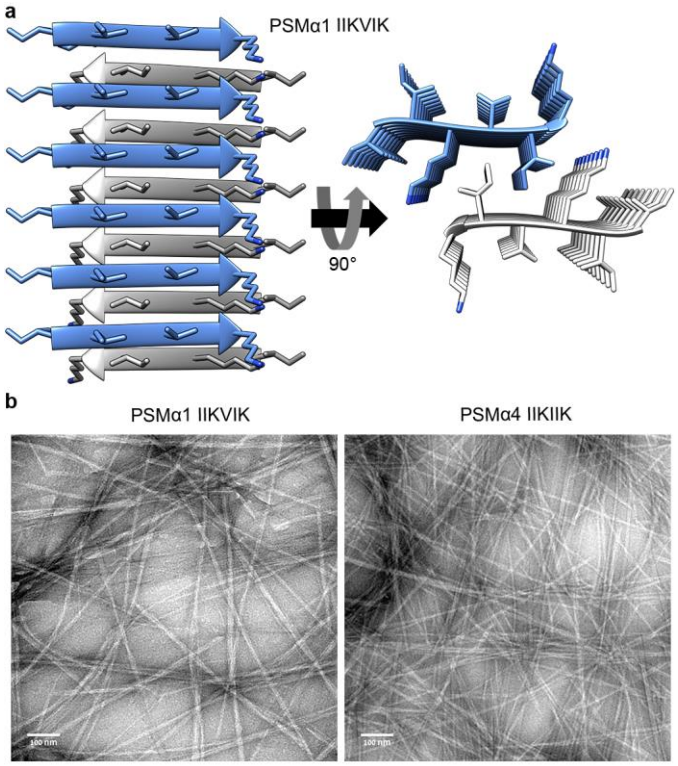
structures of the PSMα1 and PSMα4 derivatives offer templates for the design of anti-biofilm compounds. In addition, the antibacterial activity of LFKFFK and KLFKFFK may be advantageous in the development of antimicrobial peptides based on amyloid segments.

**Figure 1. PSMα1 and PSMα4 form cross-β amyloid fibrils**



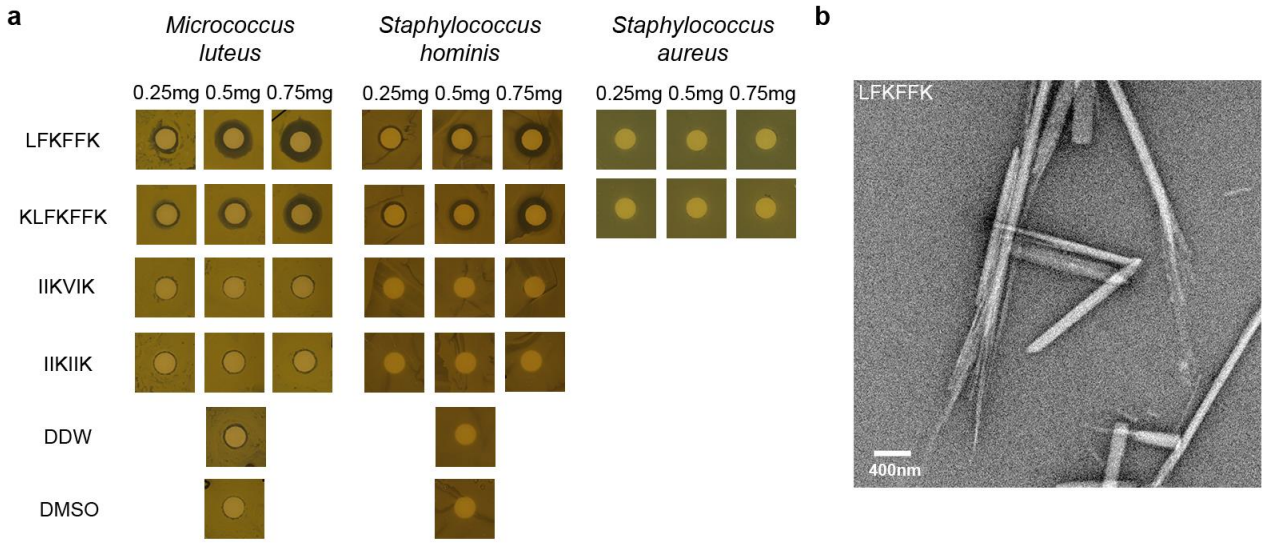

Electron micrographs of PSMα1 **(a)** and PSMα4 **(b)** show elongated fibrils. X-ray fibril diffraction of PSMα1 **(c)** and PSMα4 **(d)** shows major diffraction orthogonal arches at 4.6Å and 12Å spacings, indicative of the cross-β signature[11].

**Figure 2. PSMα1 and PSMα4 spine segments form steric zipper fibrils**



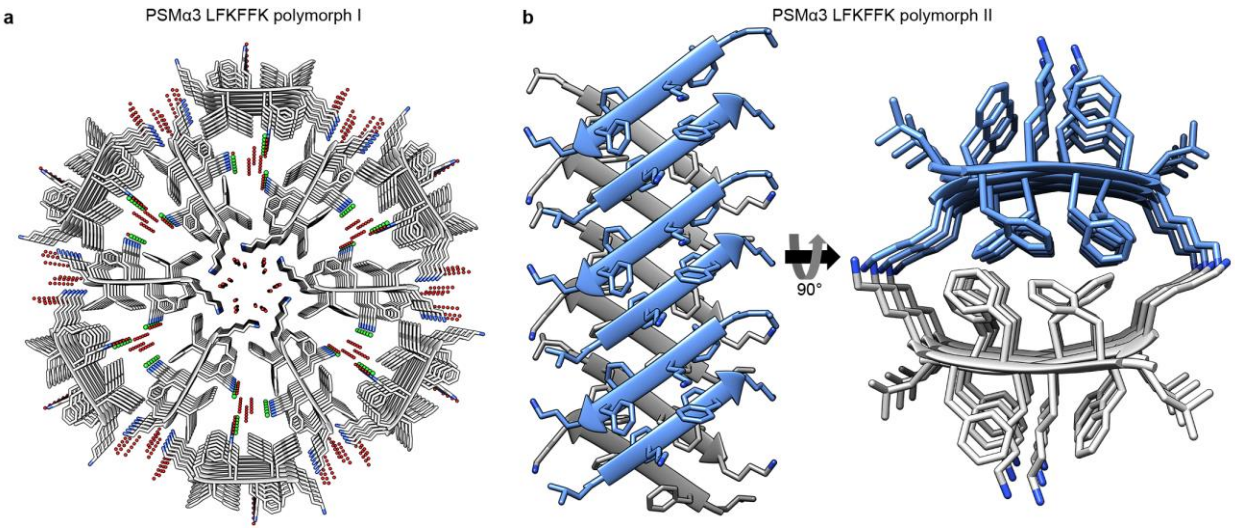

**(a)** Crystal structure of the PSMα1 spine segment IIKVIK determined at 1.1Å resolution, showing the canonical steric-zipper architecture of tightly mated β-sheets composed of parallel β-strands. In the left panel, the view is perpendicular to the fibril axis and the β-strands run horizontally. In the right panel, the view is down the fibril axis. The segments are shown in ribbon representation, with the side chains shown as sticks. The carbons within each β-sheet are colored either gray or light blue, and nitrogen atoms in side chains are colored blue. Here, six layers of β-strands are presented. Theoretically, fibrils can contain thousands of layers. A similar structure of the PSMα4-IIKIIK and its crystal packing are shown in Supplementary Fig. 3. **(b)** Electron micrographs visualizing PSMα1-IIKVIK and PSMα4-IIKIIK fibrils.

**Figure 3. Antibacterial activity of the fibril-forming LFKFFK from *S. aureus* PSMα3**




Disc diffusion assay testing antibacterial activity against different bacteria. In this assay, the antibacterial agent diffuses into the agar and inhibits germination and growth of the test microorganism. **(a)** LFKFFK and KLFKFFK segments from PSMα3, but not the steric-zipper forming segments PSMα1-IIKVIK and PSMα4-IIKIIK, showed dose-dependent antibacterial activity against *M. luteus* and *S. hominis.* LFKFFK and KLFKFFK were not toxic to *S. aureus*, the bacterium which secretes PSMα3. Discs soaked with only DDW or Dimethyl-sulfoxide (DMSO) served as controls. The results of the disc diffusion assay coincide with the antibacterial activity seen in solution (Supplementary Fig. 4). **(b)** An electron micrograph visualizing both fibrils and nano-crystals formed by LFKFFK incubated for one month. Earlier fibril formation is shown in Supplementary Figure 6.

**Figure 4. Structural polymorphism of the LFKFFK segment from *S. aureus* PSMα3**




**(a)** Crystal structure of polymorph I of the PSMα3 spine segment LFKFFK determined at 1.5Å resolution. The structure reveals a unique amyloid-like hexameric architecture, which forms elongated cylindrical cavities along the fibril axis. The view is down the fibril axis. The segments are shown in ribbon representation, with side chains shown as sticks with gray carbons and blue nitrogen atoms. Water molecules (oxygen in red) and chloride ions (green) that counteract the charge of the lysine side chains, are shown as small spheres. **(b)** Crystal structure of LFKFFK polymorph II determined at 1.85Å resolution, revealed a rare amyloid-like architecture of out-of-register β-sheets[25,26], in which each β-strand is at an angle of ~50° from the fibril axis, instead of the close to 90° angle found for in-register sheets. In both polymorphs, the β-sheets are composed of anti-parallel strands. In the left panel, the view is perpendicular to the fibril axis, and in the right panel, the view is down the fibril axis. The segments are shown in ribbon representation, with side chain shown as sticks. The carbons within each β-sheet are colored either gray or light blue, and nitrogen atoms in side chains are colored blue.

## Methods

### Peptides and Reagents

PSM native peptides and shorter derivatives (custom synthesis) at >98% purity were purchased from GL Biochem (Shanghai) Ltd., as well as from GenScript. The short (6-7 residues) PSMα segments were synthesized with capped termini (acetylated in the N-terminus and amidated in the C-terminus) or with unmodified termini for crystallography. PSMα full-length peptides were synthesized with unmodified termini. Hexafluoroisopropanol (HFIP) and Thioflavin T (ThT) were purchased from Sigma-Aldrich. Ultra-pure water was purchased from Biological Industries. Dimethyl-sulfoxide (DMSO) was purchased from Merck.

PSMαs wild type (WT) and mutant peptide sequences (Uniprot accession codes are in parenthesis); segments of short derivatives used here are marked in bold:

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PSMα1 (H9BRQ5)         MGIIAGIIKVIKSLIEQFTGK
PSMα1 mutant I7P/K9P   MGIIAGPIPVIKSLIEQFTGK
PSMα3 (H9BRQ7)         MEFVAKLFKFFKDLLGKFLGNN
PSMα4 (H9BRQ8)         MAIVGTIIKIIKAIIDIFAK
PSMα4 mutant I8P/I10P  MAIVGTIPKPIKAIIDIFAK
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### Peptides pre-treatment

Lyophilized synthetic PSMαs were dissolved in HFIP to a concentration of 0.5 mg/ml followed by a 10 minutes sonication in a bath-sonicator at room temperature. The organic solvent was evaporated using a mini rotational vacuum concentrator (Christ, Germany) at 1,000 rpm for 2 hours at room temperature. Treated peptides were aliquoted and stored at -20°C prior to use.

**Computational prediction of amyloid spine segments**

Amyloidogenic propensities of segments from PSMαs were predicted using combined information from several computational methods, including ZipperDB[47], Tango[48,49], Waltz[50] and Zyggregator[47-51].

**Fibril X-ray diffraction**

Pre-treated PSMα1 and PSMα4 peptide aliquots were re-dissolved in DMSO to 20 mM and immediately diluted 2-fold in ultra-pure water. To prepare the sample, 2 µl droplets of the peptide solution were placed between two sealed-end glass capillaries, and incubated at 37°C for 2 days to allow fibril formation. X-ray fibril diffraction of PSMα1 fibrils was collected at the micro-focus beamline P14 operated by EMBL Hamburg at the PETRA III storage ring (DESY, Hamburg, Germany). X-ray fibril diffraction of PSMα4 fibrils was collected at the micro-focus beamline ID23-2 of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France.

**Thioflavin T (ThT) fluorescence fibrillation kinetics assay**

Thioflavin T (ThT) is a widely used "gold standard" stain for identifying and exploring formation kinetics of amyloid fibrils, both in vivo and in vitro[52]. Fibrillation curves in presence of ThT commonly show a lag time for the nucleation step, followed by rapid aggregation. Pre-treated PSMα1, PSMα1 I7P/K9P, PSMα4, and PSMα4 I8P/I10P peptide aliquots were dissolved in DMSO to 10 mM and immediately diluted to 50 µM in Tris buffer pH 7.5 for PSMα1 and PSMα1 I7P/K9P, or in ultra-pure water for PSMα4 and PSMα4 I8P/I10P, containing filtered ThT diluted from stock made in ultra-pure water. Final concentrations for each reaction were 50 µM peptide (PSMα1, PSMα4, or their mutants) and

200 μM ThT. Blank solutions were also prepared for each reaction, containing everything but the peptides. The reaction mixture was carried out in a black 96-well flat-bottom plate (Greiner bio-one) covered with a thermal seal film (EXCEL scientific) and incubated in a plate reader (CLARIOstar) at a temperature of 37°C with 500 rpm shaking for 85 seconds before each reading cycle, and up to 1000 cycles of 6 minutes each. Measurements were made in triplicates. Fluorescence was measured by excitation at 438±20nm and emission at 490±20nm over a period of about 100 hours. All triplicate values were averaged, appropriate blanks were subtracted, and the resulting values were plotted against time. Calculated standard errors of the mean are presented as error bars. The entire experiment was repeated at least three times on different days.

**Transmission electron microscopy**

Fibrillated PSMα1 and PSMα4 samples were collected following the ThT fibrillation kinetics assay (as described above) by combining the contents of 2-3 wells from the plate. Solutions were centrifuged at 21,000xg and the supernatant was discarded. The pellet containing the fibrils was re-suspended in 20 μl of ultra-pure water.

Fibril formation of the short segments, IIKIIK, IIKVIK, LFKFFK and KLFKFFK, was examined for peptides dissolved directly from the powder form. IIKVIK from PSMα1 and IIKIIK from PSMα4 were re-suspended to 20 mM with 75% DMSO in ultra-pure water and incubated for about one week at room temperature (Fig. 2b). LFKFFK and KLFKFFK from PSMα3 were re-suspended in ultra-pure water to 10 mM. LFKFFK was incubated for about a month at room temperature (Fig. 4b). KLFKFFK was incubated for 14 days at room temperature (Supplementary Fig. 5).

Five-microliter samples were applied directly onto copper TEM grids with support films of Formvar/Carbon (Ted Pella), which were charged by glow-discharge (PELCO easiGlow, Ted Pella) immediately before use. Grids were allowed to adhere for 2 min and negatively stained with 5 μl of 2% uranyl acetate solution. Micrographs were recorded using FEI Tecnai G2 T20 S-Twin transmission electron microscope at an accelerating voltage of 200 KeV (located at the Department of Materials Science & Engineering at the Technion, Israel), or Philips CM120 TEM at an accelerating voltage of 120 kV (located at the Russell Berrie Electron Microscopy Center of Soft Matter at the Technion, Israel).

*Fibril thermal-stability assay* (Supplementary Fig. 6) Peptides were dissolved to 10 mM (LFKFFK in ultra-pure water and IIKIIK in DMSO) and incubated at room temperature for three days. Samples were applied directly onto copper TEM grids and stained as described above. The samples were then incubated at 50°C in a pre-heated thermoblock for 5 minutes and immediately applied onto TEM grids. The samples were then allowed to cool down at room temperature for 20 minutes and applied onto TEM grids. Micrographs were recorded as described above.

**Crystallizing conditions**

Peptides synthesized with free (unmodified) termini were used for crystallization experiments. IIKVIK of PSMα1 and IIKIIK of PSMα4 were dissolved in 0.5% acetonitrile in ultra-pure water at 10 mM, and LFKFFK of PSMα3 was dissolved in ultra-pure water at 10 mM, followed by a 10 minutes sonication in a bath-sonicator at room temperature and centrifugation at 17,000xg at 4°C prior to crystallization. Peptide solution drops at 10 nl volume were dispensed by the Mosquito automated liquid dispensing robot (TTP Labtech, UK), to crystallization screening plates. All crystals were grown at 20°C via hanging-drop

vapor diffusion. The drop was a mixture of 10 mM peptide and reservoir solution as follows: IIKVIK: 0.5 M Lithium sulfate; 15% polyethylene glycol 8000. IIKIIK: 0.2 M Ammonium sulfate; 20% polyethylene glycol 3350. LFKFFK polymorph I**:** 0.2 M Potassium thiocyanate, 20% polyethylene glycol 3350. LFKFFK polymorph II**:** 0.1 M Sodium acetate pH 5.1; 45% polyethylene glycol 400; 0.09 M Lithium sulfate. Micro-crystals grew after few days and were mounted on glass needles glued to brass pins[53]. Crystals were kept at room temperature prior to data collection.

**Structure determination and refinement**

X-ray diffraction data was collected at 100°K. The X-ray diffraction data for IIKVIK, IIKIIK, and LFKFFK polymorph I was collected at the micro-focus beamline ID23-EH2 of the European Synchrotron Radiation Facility (ESRF) in Grenoble, France; wavelength of data collection was 0.8729 Å. The X-ray diffraction data for LFKFFK polymorph II was collected at the micro-focus beamline P14 operated by EMBL at the PETRAIII storage ring (DESY, Hamburg, Germany); wavelength of data collection was 0.9763 Å. Data indexation, integration and scaling were performed using XDS/XSCALE[54] or with DENZO/SCALEPACK[55]. Molecular replacement solutions for all segments were obtained using the program Phaser[56] within the CCP4 suite[56-58]. The search models consisted of available structures of geometrically idealized β-strands. Crystallographic refinements were performed with the program Refmac5[58]. Model building was performed with Coot[59] and illustrated with Chimera[60]. There were no residues that fell in the disallowed region of the Ramachandran plot. Crystallographic statistics are listed in Supplementary Table 1.

**Calculations of structural properties**

The Lawrence and Colman's shape complementarity index[61] was used to calculate the shape complementarity between pairs of sheets forming the dry interface. Area buried was calculated using AREAIMOL[62,63] with a probe radius of 1.4 Å. Calculations were performed using the CCP4 package[57]. The summation of the differences between the accessible surface areas of one molecule alone and in contact with the other strands on the same sheet or opposite sheets, as indicated in Supplementary Table 2, constitutes the reported area buried.

**Bacterial strains and culture media**

*Micrococcus luteus* (an environmental isolate) was a kind gift from Prof. Charles Greenblatt from the Hebrew university in Jerusalem, Israel. An inoculum was grown in Luria-Bertani (LB) medium at 30°C with 220 rpm shaking overnight. *Staphylococcus hominis* (ATTC 27844) and *Staphylococcus aureus* (ATCC 25923) were purchased from ATCC, USA. An inoculum was grown in brain-heart infusion (BHI) media at 37°C with 220 rpm shaking overnight.

**Disc diffusion assay**

Bacterial cultures were grown overnight as described above and diluted 1000-fold to a fresh media until growth reached to optical density of ~0.4 measured at 595 nm ($OD_{595}$). The refreshed cultures were plated on LB-agar or BHI-agar according to the bacterial strain. The antibacterial activity of PSMα segments was examined. Lyophilized peptides were dissolved in ultra-pure water (LFKFFK and KLFKFFK) or DMSO (IIKVIK and IIKIIK) to a final concentration of 50 mg/ml. The concentrated peptide solutions were loaded on blank antimicrobial susceptibility discs (Oxoid, UK). Discs loaded with ultra-pure water or DMSO

were used as controls. The discs were gently placed on bacteria plated agar and incubated over-night at the appropriate temperatures.

**Antibacterial activity in solution**

Bacterial cultures were grown overnight as described above and diluted 1,000-fold to a fresh media until growth reached to $OD_{595}$ of ~0.4. Peptides were dissolved to a concentration of 10 mM (LFKFFK and KLFKFFK in ultra-pure water, IIKVIK and IIKIIK in DMSO) and diluted to 1 mM in growth media (LB for *M. luteus* and BHI for *S. hominis*). Two-fold serial dilutions of the tested peptides in LB or BHI media ranging from 250 μM to 1 μM were performed in a sterile 96-well plate. Final DMSO concentration was fixed to 2.5% in all samples. Wells containing everything but the peptide served as controls. Bacterial growth was determined by measuring the $OD_{595}$ during a 24 hours incubation at 30°/37°C according to the bacterial strain with 250 rpm shaking. The experiment was performed in triplicates. All triplicate values were averaged, appropriate blanks were subtracted, and the resulting values were plotted against peptide concentration. Calculated standard errors of the mean are presented as error bars. The entire experiment was repeated at least three times on different days.

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**Authors contributions:** N.S. and M.L. conceived the study and designed the experiments. All authors collected X-ray data. A.M. initiated experiments. N.S. performed the experiments. N.S. and M.L. solved the crystal structures with contributions from J-P.C. N.S., J-P.C. and M.L. wrote the paper.

Supplementary Information

# Extreme Amyloid Polymorphism in *Staphylococcus aureus* Virulent PSMα Peptides

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**Supplementary Figure 1: Amyloidogenic segments in *S. aureus* PSMαs**

**a**

PSMα1 (H9BRQ5) MGIIAG**IIKVIK**SLIEQFTGK

PSMα3 (H9BRQ7) MEFVAK**LFKFFK**DLLGKFLGNN

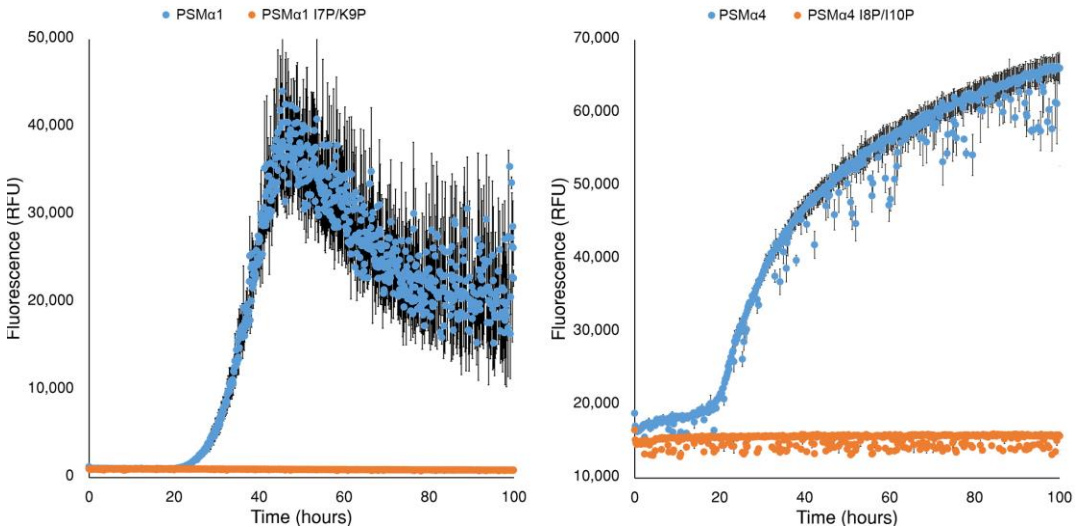
PSMα4 (H9BRQ8) MAIVGT**IIKIIK**AIIDIFAK

**b**

| Peptide pairs | Identity |
|---|---|
| PSMα1 - PSMα3 | 32% |
| PSMα1 - PSMα4 | 48% |
| PSMα3 - PSMα4 | 23% |

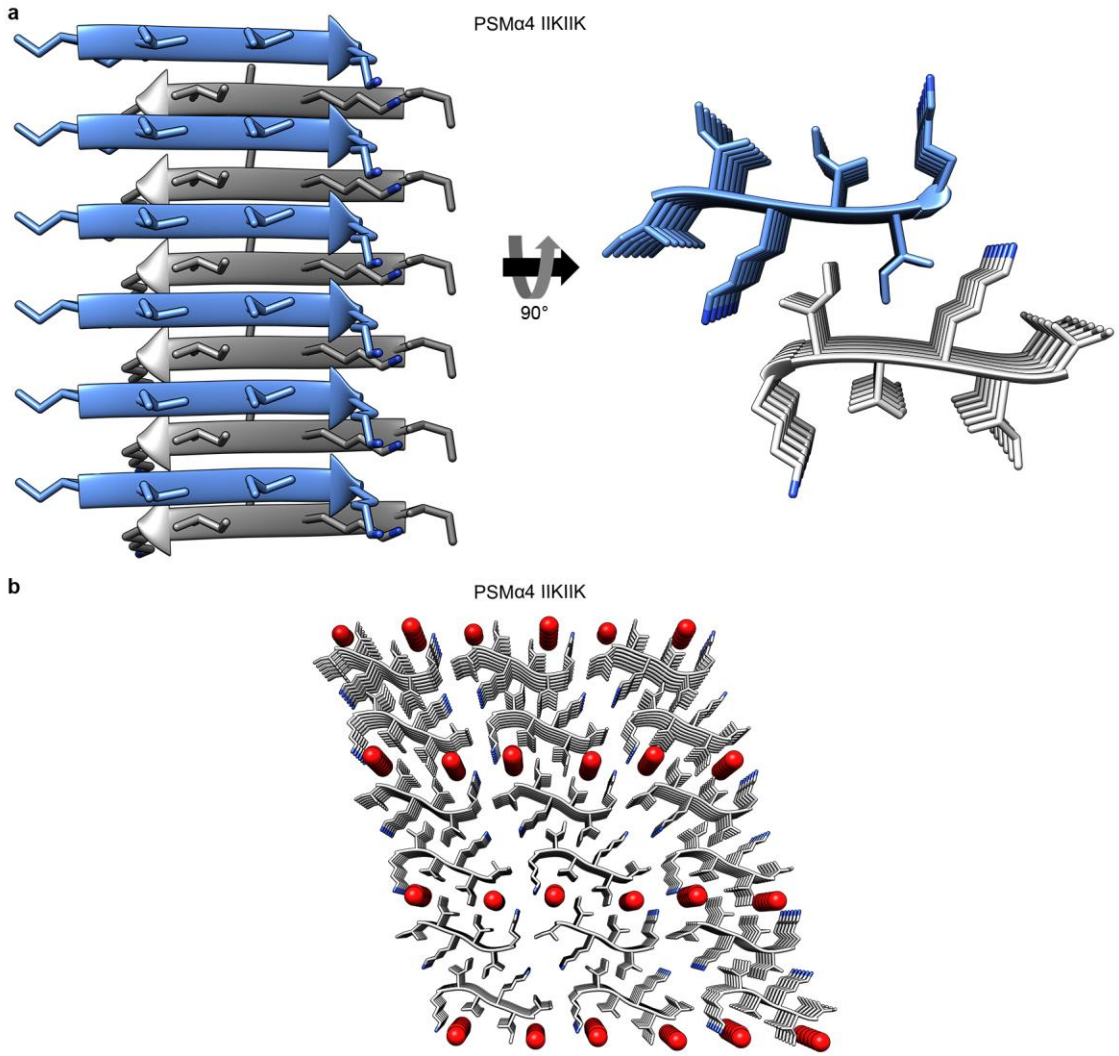
**(a)** The sequences of *S. aureus* PSMα1, PSMα3 and PSMα4 (UniProt accession number is indicated in parentheses). Segments marked in bold were predicted to have amyloidogenic propensity based on computational methods[1-5], and were selected for structural characterization. **(b)** Percentage of sequence identity between pairs of PSMα.

**Supplementary Figure 2. Thioflavin T binding and fibrillation kinetics of PSMα1 and PSMα4**



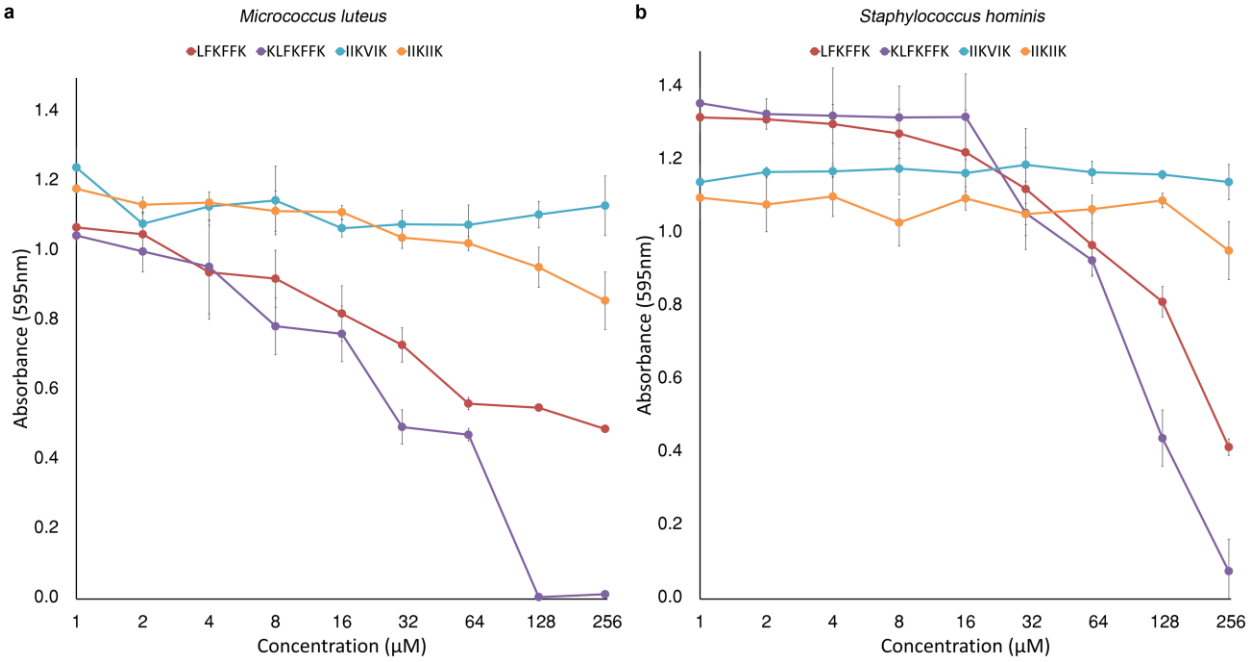

Fibrillation kinetics of PSMα1, PSMα4 and mutants that sustained substitution of two residues within the predicted amyloidogenic region to proline residues (PSMα1 I7P/K9P and PSMα4 I8P/I10P). The fibrillation reaction contained 50 µM peptide and 200 µM Thioflavin T. PSMα1 and PSMα4 showed rapid fibrillation following a ~20 h lag time. The mutants showed no ThT binding. The graph shows mean fluorescence readings of triplicate ThT measurements.

**Supplementary Figure 3. Steric-zipper structure of PSMα4 IIKIIK**




**(a)** The structure of PSMα4-IIKIIK determined at 1.6Å resolution included pairs of β-sheets which are the basic unit of the fibril; side-chains protruding from the two β-sheets intermesh to form a dry, tightly self-complementing steric zipper bonding the sheets. The β-sheets are composed of parallel β-strands. In the left panel, the view is perpendicular to the fibril axis and the β-strands run horizontally. In the right panel, the view is down the fibril axis. The segments are shown in ribbon representation, with side chains shown as sticks. The carbons within each β-sheet are colored either gray or light blue, and nitrogen atoms in side chains are colored blue. The structure of IIKIIK is highly similar to the structure of IIKVIK from PSMα1 (Fig. 2). **(b)** The crystal packing of IIKIIK from PSMα4 is depicted down the fibril axis showing alternating dry and wet interfaces. Residues colored by atom-type are represented as sticks, and water molecules are in red spheres.

**Supplementary Figure 4. Antibacterial activity of LFKFFK and KLFKFFK in solution**

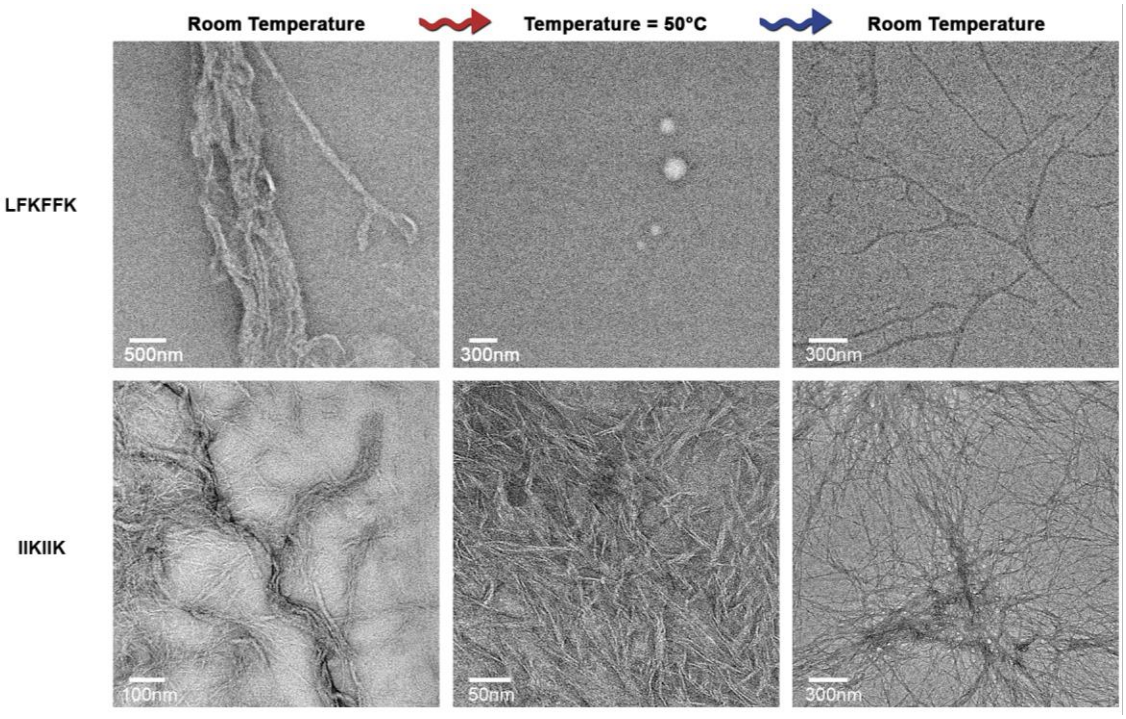



The LFKFFK and KLFKFFK peptides from PSMα3 inhibited the growth of *M. luteus* **(a)** and *S. hominis* **(b)** in a dose-dependent manner. The graphs show the maximal optical density (OD) of *M. luteus* culture at t=18 hours **(a)**, or *S. hominis* culture at t=8 hours **(b)** in the presence of different peptide concentrations. The steric-zipper-forming segments, IIKVIK from PSMα1 and IIKIIK from PSMα4, did not show a significant antibacterial effect on either species.

**Supplementary Figure 5. Electron micrograph of the KLFKFFK segment from PSMα3**

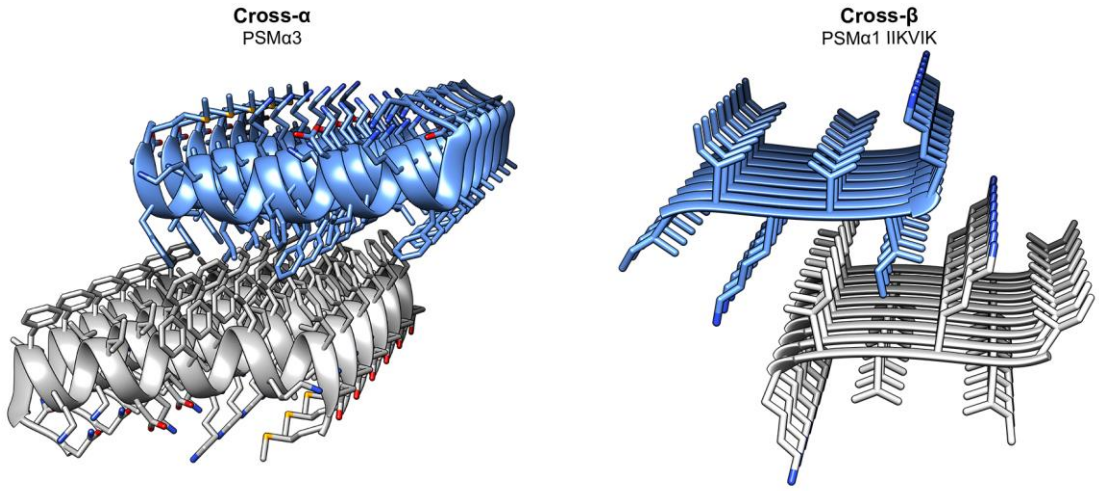
Electron micrograph of a twisted crystalline fibril of KLFKFFK after fourteen days of incubation at room temperature.

**Supplementary Figure 6. LFKFFK forms thermo-reversible fibrils compared to the stable IIKIIK fibrils**




LFKFFK formed fibrils at room temperature, which dissolved upon heating to 50°C and reformed after cooling, albeit with a different morphology. In contrast, IIKIIK fibrils were thermo-stable. Both peptides were incubated for three days at room temperature before fixation and heating-cooling cycles. The same sample was assayed following temperature change. Thermostability of the fibrils corresponded to the crystal structures; IIKIIK showed the highly stable steric-zipper architecture, while LFKFFK formed atypical amyloid-like structures.

**Supplementary Figure 7. Similar structural architecture of cross-α and cross-β fibrils**




The cross-α amyloid-like fibrils of PSMα3[10] (left panel) as well as the cross-β steric-zipper fibrils of PSMα1-IIKVIK (right panel) form pairs of tightly mated sheets running along the fibril axis. Here only six layers are shown. Individual molecules are oriented perpendicular to the fibril axis. The peptides are shown in ribbon representation with side chains shown as sticks. The carbons within each sheet are colored either gray or light blue, with heteroatoms in side chains colored by atom type (nitrogen in blue, oxygen in red, and sulfur in yellow).

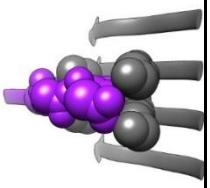
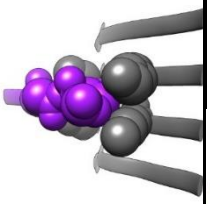
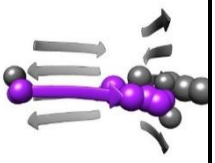
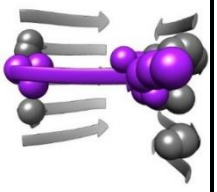
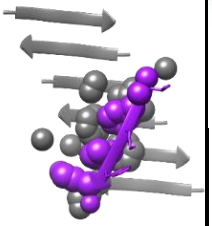
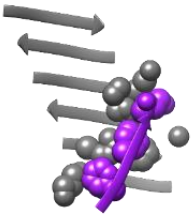
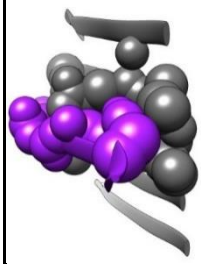
**Supplementary Table 1. Data collection and refinement statistics (molecular replacement)**

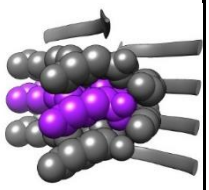
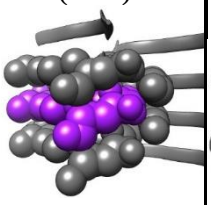
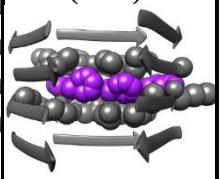
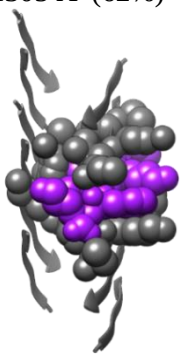
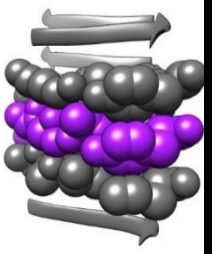
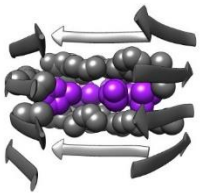
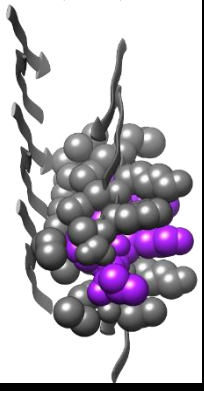
| | IIKVIK (PSMα1) | IIKIIK (PSMα4) |
|---|---|---|
| PDB accession code | 6FG4 | 6FGR |
| Beamline | ESRF ID23-2 | ESRF ID23-2 |
| Date | October 8th, 2014 | October 8th, 2014 |
| **Data collection** | | |
| Space group | C 1 2 1 | P 1 |
| Cell dimensions | | |
| *a*, *b*, *c* (Å) | 45.27 4.80 22.90 | 4.83 22.38 23.06 |
| α, β, γ (°) | 90.00 107.65 90.00 | 107.00 90.01 96.20 |
| Wavelength (Å) | 0.8729 | 0.8729 |
| Resolution (Å) | 21.8-1.1 (1.13-1.10) | 22.1-1.5 (1.56-1.50) |
| [a] R-factor observed (%) | 13.3 (75.2) | 20.6 (62.2) |
| [b] $R_{meas}$ (%) | 14.0 (82.4) | 21.7 (69.4) |
| *I* / sigma | 10.1 (2.1) | 7.2 (2.4) |
| Total reflections | 23104 (876) | 13816 (770) |
| Unique reflections | 2070 (136) | 1397 (158) |
| Completeness (%) | 93.9 (80.0) | 95.0 (89.3) |
| Redundancy | 11.2 (6.4) | 9.9 (4.9) |
| [c] $CC_{1/2}$ (%) | 99.8 (96.7) | 99.4 (74.4) |
| **Refinement** | | |
| Resolution (Å) | 18.4-1.1 (1.23-1.10) | 18.2-1.5 (1.54-1.50) |
| Completeness (%) | 94.0 (83.5) | 95.1 (93.9) |
| [d] No. reflections | 1863 | 1257 |
| [e] $R_{work}$ (%) | 15.9 (21.2) | 18.3 (26.4) |
| $R_{free}$ (%) | 19.4 (26.4) | 22.1 (26.1) |
| No. atoms | 77 | 127 |
| Protein | 65 | 102 |
| Ligand/ion | 10 | 22 |
| Water | 2 | 3 |
| *B*-factors | | |
| Protein | 8.8 | 7.7 (Chain A)<br>8.2 (Chain B) |
| Ligand/ion | 14.5 ($SO_4$) | 37.3 ($SO_4$)<br>6.7 (EDO) |
| Water | 33.6 | 26.9 |
| R.m.s. deviations | | |
| Bond lengths (Å) | 0.010 | 0.010 |
| Bond angles (°) | 1.973 | 1.903 |
| Clash score [6] | 0.00 | 0.00 |
| Molprobity score [6] | 1.33 | 0.5 |
| Molprobity percentile [6] | 85th percentile | 100th percentile |
| Number of xtals used for scaling | Four spots from one crystal were used. | Four spots from one crystal were used. |

| | **LFKFFK polymorph I (PSMα3)** | **LFKFFK polymorph II (PSMα3)** |
|---|---|---|
| PDB accession code | 6FHC | 6FHD |
| Beamline | ESRF ID23-2 | EMBL P14 PETRA III |
| Date | July 24th, 2014 | May 2nd, 2016 |
| | | |
| **Data collection** | | |
| Space group | P 6 5 | C 1 2 1 |
| Cell dimensions | | |
| *a*, *b*, *c* (Å) | 35.79 35.79 9.63 | 41.03 11.73 24.61 |
| α, β, γ (°) | 90.00 90.00 120.00 | 90.00 121.89 90.00 |
| Wavelength (Å) | 0.8729 | 0.9763 |
| Resolution (Å) | 100-1.5 (1.55-1.5) | 20.9-1.8 (1.96-1.85) |
| [a] R-factor observed (%) | 8.2 (67) | 28.4 (54.7) |
| [b] $R_{meas}$ (%) | 13.1 (83.1) | 29.2 (56.3) |
| *I* / sigma | 20.6 (2.3) | 9.1 (5.6) |
| Total reflections | 16288 | 16102 (2590) |
| Unique reflections | 1179 (118) | 913 (148) |
| Completeness (%) | 97.4 (98.3) | 96.3 (91.9) |
| Redundancy | 13.8 (9.6) | 17.6 (17.5) |
| [c] $CC_{1/2}$ (%) | 96.5 (97.6) | 99.3 (94.6) |
| | | |
| **Refinement** | | |
| Resolution (Å) | 31.0-1.5 (1.55-1.51) | 19.3-1.8 (1.90-1.85) |
| Completeness (%) | 97.8 (98.9) | 97.3 (98.8) |
| [d] No. reflections | 1057 | 821 |
| [e] $R_{work}$ (%) | 11.8 (27.7) | 17.1 (29.7) |
| $R_{free}$ (%) | 16.2 (21.2) | 18.7 (43.6) |
| No. atoms | 86 | 136 |
| Protein | 60 | 120 |
| Ligand/ion | 21 | 11 |
| Water | 5 | 5 |
| *B*-factors | | |
| Protein | 10.2 | 9.6 (Chain A)<br>11.3 (Chain B) |
| Ligand/ion | 25.1 ($CO_3$)<br>18.3 (SCN)<br>23.7 (PE5)<br>46.5 (Cl) | 29.1 ($SO_4$)<br>25.9 (Na) |
| Water | 49.9 | 23.5 |
| R.m.s. deviations | | |
| Bond lengths (Å) | 0.016 | 0.017 |
| Bond angles (°) | 1.995 | 1.893 |
| Clash score [6] | 6.1 | 0 |
| Molprobity score [6] | 1.33 | 1.20 |
| Molprobity percentile [6] | 94th percentile | 99th percentile |
| Number of xtals used for scaling | One crystal | Three spots from one crystal were used. |

Values in parentheses are for highest-resolution shell.
(a) Rfactor square.
(b) R-meas is a redundancy-independent R-factor defined in[7].
(c) $CC_{1/2}$ is percentage of correlation between intensities from random half-datasets[8].
(d) Number of reflections corresponds to the working set.
(e) Rwork corresponds to working set.

**Supplementary Table 2. Features of the PSMα structures compared to the NNQQNY steric zipper structure.**

| | **PSMα1 IIKVIK** | **PSMα4 IIKIIK** | **PSMα3 LFKFFK Polymorph I** | **PSMα3 LFKFFK Polymorph II** | **NNQQNY** |
|---|---|---|---|---|---|
| **Shape complementarity** | 0.89 | 0.89 | 0.79 | 0.82 | 0.86 |
| **Inter-strand distance along the sheet** | 4.80 Å | 4.83 Å | 4.82 Å (between antiparallel β-strands) | 4.80 Å (between antiparallel β-strands) | 4.87 Å |
| **Area buried** | One molecule against an opposite sheet: 532 Å$^2$ (28%)[a]  | One molecule against an opposite sheet: 510 Å$^2$ (27%)[a]  | One molecule against two opposite sheets in the trimer: Strand 1[b]: 223 Å$^2$ (11%)[a]  Strand 2[b]: 281 Å$^2$ (13%)[a]  | One molecule against an opposite sheet: Strand 1[b]: 414 Å$^2$ (20%)[a]  Strand 2[b]: 438 Å$^2$ (21%)[a]  | One molecule against an opposite sheet: 469 Å$^2$ (27%)[a]  |

| | One molecule within pairs of sheets: 1343 Å$^2$ (72%)[a] | One molecule within pairs of sheets: 1338 Å$^2$ (71%)[a] | One molecule within a trimer: Strand 1[b]: 1342 Å$^2$ (62%)[a] | One molecule within pairs of sheets: Strand 1[b]: 1305 Å$^2$ (62%)[a] | One molecule within pairs of sheets: 1268 Å$^2$ (75%)[a] |
|---|---|---|---|---|---|
| |  |  |  |  |  |
| | | | Strand 2[b]: 1380 Å$^2$ (64%)[a]  | Strand 2[b]: 1329 Å$^2$ (63%)[a]  | |

The values of shape complementarity and surface area buried calculated for the PSMα structures are compared with those of the NNQQNY segment from yeast prion Sup35 (PDB code 1YJO)[9] steric zipper structure. The NNQQNY was chosen for this comparison as it shows one of the highest values of shape complementarity and surface area buried among steric zipper structures[10].

[a] The number depicted is the percentage of the total accessible surface area of one strand (colored purple) that is in contact with the surrounding depicted strands (colored grey).

[b] LFKFFK assemble into antiparallel β-sheets in both forms, hence each of the antiparallel β-strands forms a difference interface that was calculated separately.

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