Structure analysis and MD simulation of the biofilm-inducing peptide TisB from *E. coli*

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Introduction

Nowadays one of the big issues in modern medicine is the fight against chronic bacterial biofilms. The reason for the recalcitrance of these biofilms are so-called persister cells. Persisters are dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant against modern antibiotics [1]. It is known that one protein plays a major role in persister cell formation in *E.coli*: TisB. This 29 amino acid long peptide gets overexpressed upon environmental stress, integrates into the bacterial inner membrane and decreases the proton motive force and ATP levels [2]. The molecular mechanism is still unclear, how this leads cells into dormancy and how it results in the formation of persister cells [3].

We analyzed the structure and function of membrane-bound TisB by CD [4], NMR, MD simulations and a vesicle transport assay. We propose that the formation of salt-bridges between two antiparallel amphiphilic helices allows TisB to insert into membranes and form a proton-conductive path..

Summary and conclusions

- OCD experiments revealed for TisB a transmembrane orientation in lipid bilayers (Fig. A)
- TisB orientation depends on the P/L ratio (Fig. B)
- Transmembrane orientation was confirmed by ^{19}F-NMR (Fig. C)
- When inserted in the membrane, TisB adopts a tilt angle of ~ 30° (Fig. D)
- MD results on pore instability were confirmed by leakage experiments (Fig. E,G-H)
- MD shows a very high likelyhood for an anti-parallel TisB dimer, associated via their complementary charge motifs (Fig. F)

Complementary charge motifs are patterns of charged amino acids in a protein primary sequence that exhibit a complementary mirror symmetry (Fig. I). Two amphiphilic TisB helices could self-assemble via their complementary charge motifs in an anti-parallel way, and thus be able to immerse into the membrane. A water wire along the charged residues could form a proton-conductive pathway across the hydrophobic membrane core, allowing TisB to breakdown the proton gradient over the bacterial membrane (Fig. J). This complementary charge motifs represent a novel concept in protein folding and assembly.

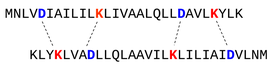


Figure I: *Complementary charges occur along the sequence in TisB. Anionic residues (blue) have a cationic (red) partner to complement their charge in an anti-parallel alignment.*

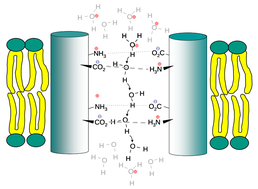


Figure J: *Model of the putative mode of action of TisB. Uprigth TisB monomers form a dimer via their complementary charge motifs. The formed saltbridges allow the generation of a pore which can be filled by a string of water molecules. Proton translocation would then be possible via proton shuttling along the water string (Grotthuß-mechanism) thus leading to the transport of protons into the cytoplasm and subsequently to the breakdown of membrane potential.*

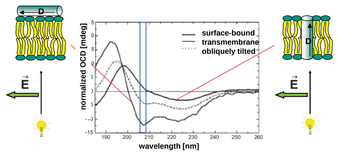
References

[1] Lewis, K. (2010), *Annual Reviews of Microbiology*, **64**:357-372. [2] Vogel *et* al. (2004), *Current Biology*, **14**:2271-2276. [3] Dörr *et* al. (2010), *PLoS Biology*, **8**:e1000317. [4] Bürck *et* al. (2008), *Biophysical Journal*, **95**:3872-3881. [5] Kelly *et* al. (2005), *Biochimica et Biophysica Acta*, **14**:2271-2276. [6] Ellens, H. *et* al. (1984), *Biochemistry*, **23**:1532-1538. [7] Huang *et* al. (2000), *Biochemistry*, **39**:8347-8352. [8] FOX, R.O. jr. *et* al. (1982), *Nature*, **25**:325-330. [9] Wieprecht, T. *et* al. (2000), *Biochemistry*, **39**:442-452.

Results

Oriented CD spectroscopy

OCD experiments were carried out to estimate the tilt angle of the TisB peptide in lipid bilayers. The orientation was found to depend on the peptide-lipid ratio (Fig. A). At a ratio of 1:100 and above the peptide adopts a transmembrane orientation, whereas at 1:150 the peptide adopts a surface-bound state. This behaviour is well-known for pore-forming antimicrobial peptides [7]. The transmembrane orientation was maintained in various lipid moembranes with different thickness (Fig. A).



OCD spectra of a protein helix in a transmembrane orientation, at an oblique tilt angle, and in a surface-bound alignment. The intensity of the CD band around 207 nm (blue box) serves as a „fingerprint" to estimate the orientation of helices in membranes. Modified after [5].

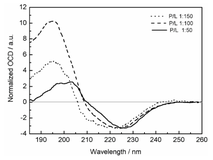


Figure A: *OCD spectra of TisB in DOPC bilayers with different P/L ratios. In a 1:50 and 1:100 ratio TisB integrates into the bilayer, whereas a 1:150 ratio leads to a surface bound state of TisB.*

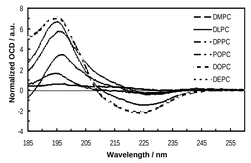
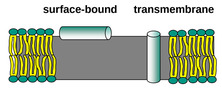


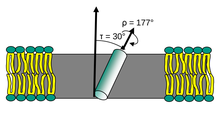
Figure B: *OCD spectra of TisB in different lipid bilayers at a peptide to lipid ratio of 1/100. The transmembrane orientation of TisB is maintained in all lipid bilayers, as represented by the near-zero values of the 207 nm band.*

Solid-state ^{19}F-NMR spectroscopy

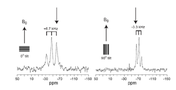
^{19}F-NMR experiments on selectively labelled TisB analogues were performed in oriented POPC bilayers at 308 K to obtain the ^{19}F dipolar splittings (Fig. C). These values were fitted to an α-helical structure to perform an orientational RMSD analysis. The result is summarized in Fig. D, confirming that the amphiphilic TisB has a transmembrane alignment.



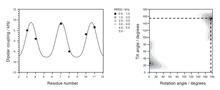
Different alignment states of ^{19}F-labeled peptides produce different NMR spectra.



The peptide alignment is described by the tilt angle τ and the rotation angle ρ.



The solid-state ^{19}F-NMR spectra show well-resolved ^{19}F dipolar splittings.



An NMR wave-plot and RMSD analysis yield the desired τ and ρ angles.

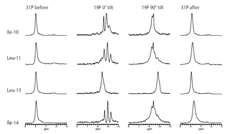


Figure C: *^{19}F-NMR spectra of several ^{19}F-labelled TisB analogues in POPC bilayers at a peptide to lipid ratio of 1/100. Residues 10, 11, 13 and 14 were selectively labeled with CF_3-L-Bpp, one-by-one. Peptides were measured at 0° and 90° sample tilt with respect to the magnetic field, showing that peptides are mobile and uniformly structured.*

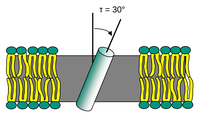


Figure D: *Observed orientation of TisB in lipid membranes. Analysis of the experimental ^{19}F-NMR splittings yielded a tilt angle (τ) of 30° and a rotational angle of 177°. The molecular order parameter was 0.9, with an RMSD of 0.25.*

MD simulations

Molecular dynamics simulations of the membrane insertion process of TisB were carried out using the Gromacs MD package, and the Martini coarse-grained force field was applied to describe the peptides and lipids. Free energy changes were computed using umbrella sampling techniques on 100 simulation windows of 30 ns length each. Starting from a TisB 16mer pore, it turned out that a pore arrangement is not very favourable. Instead dimerization is very likely (Fig. E). Therefore an all-atom MD simulation approach with a TisB anti-parallel dimer was carried out (Fig. F). An anti-parallel dimer is stable and adopts an orientation similar to the angle found by ^{19}F-NMR spectroscopy. Furthermore, the dimer interface is seen to be stabilized by salt bridges between charged residues.

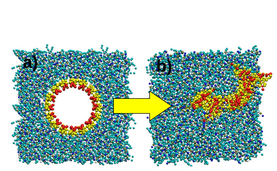


Figure E: *Coarse-grained MD analysis of pore formation by TisB in membranes. Self-assembly as an oligomeric pore is not favourable, but dimer formation is very likely. a) TisB 16mer, t = 0 s, b) t = 1 µs*

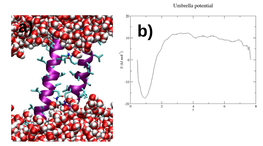


Figure F: ***a)*** *All-atom MD simulation of a putative anti-parallel TisB dimer in membranes. The monomers interact via their complementary charges (blue/red residues, respectively).* ***b)*** *Free energy (umbrella plot) calculated for the energy differences when pulling the monomers apart. The energy minimum suggests that dimerization is very likely. Furthermore, within the dimeric interface a string of water molecules can be found.*

Leakage assay

We used a leakage assay established in the literature [6]. Large unilamellar lipid vesicles were prepared in which a fluorophore/quencher pair is entrapped. Upon peptide addition the fluorescence will rise immediately if the peptide causes membrane damage, because an efflux of entrapped fluorophore leads to a dequenching. Classical pore-forming peptides like Alamethicin and PGLa/Magainin cause a rapid and total (100%) leakage within seconds, whereas weakly perturbing peptides cause hardly any leakage over minutes to hours. Therefore, this assay was used to compare quantitatively the peptide induced membrane destabilization of TisB with known pore-forming peptides Alamethicin and PGLa/Magainin [8-9]. TisB showed almost no effect, thereby confirming our MD result on pore instability (Fig. G and H).

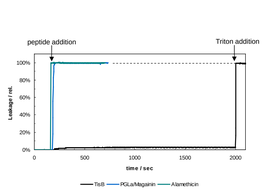


Figure G: *After adding different peptides to fluorophore/quencher containing vesicles, the leakage signal was monitored. After 30 min Triton-X 100 was added to solubilize the vesicles and to reveal 100% leakage level. It is clearly shown that TisB does not behave like known proe-forming peptides. It induces less pronounced leakage. Experiments were carried out at a P/L ratio of 1:100 in DOPC unilamellar vesicles at 30 °C.*

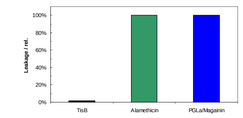


Figure H: *Evaluation of leakage experiments. Compared to the typical pore-forming peptides PGLa/Magainin (1:1) or Alamethicin, TisB induces essentially no leakage (~2%).*