Investigating the size and stoichiometry of pore-forming toxins using native ESI-MS

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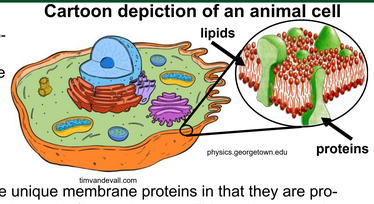
Introduction

Pharmaceuticals, pathogens target membrane components
Our cells are surrounded by membranes, which act as gatekeepers, allowing certain molecules to enter and exit the cell. Membranes are composed largely of lipids and proteins, and because of their important role in protecting our cellular contents, many different pathogens have evolved to attack or even hijack these membrane components. In fact, more than half of new pharmaceutical drugs target membrane proteins.

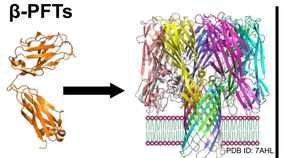
Cartoon depiction of an animal cell



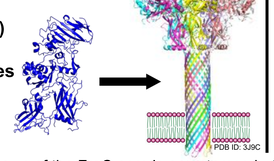
Pore-forming toxins (PFTs) are unique membrane proteins
PFTs are produced by organisms in all kingdoms of life. They are unique membrane proteins in that they are produced as water-soluble monomers that transition into membrane-inserted oligomeric pores. This action can activate cell survival, membrane repair, and immune responses in the host cells. Despite their importance in a variety of interesting biological processes, PFTs and other membrane proteins are quite challenging to study using traditional methods that work well for water-soluble, cytosolic proteins, such as X-ray crystallography, NMR, and cryo-EM. Membrane proteins require a membrane-like environment (lipids or detergent micelles) in order to retain their stability and protect their transmembrane regions. Owing to the challenge of studying PFTs in their native-like transmembrane pore forms, the crystal structures shown here are just four out of approximately a dozen that have been solved to date, while thousands of crystal structures have been solved for cytosolic or water-soluble proteins.

β-PFTs

α-hemolysin (*Staph. aureus*) forms heptameric pores

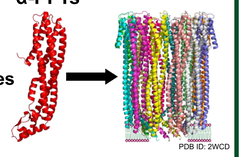


Anthrax toxin (*Bacillus anthracis*) forms heptameric and octameric pores in a manner dependent on pH

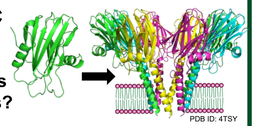


α-PFTs

Cytolysin A (*E. coli*) forms dodecameric pores



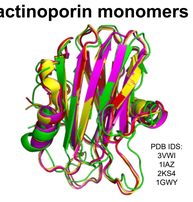
Fragaceatoxin C (FraC) from the strawberry sea anemones forms octameric pores? Depends on environment?



The crystal structure shown of the FraC pore is an octamer, but there is disagreement in the field about the final stoichiometry of the pore, and several other different oligomeric states have been proposed. That other PFTs, such as anthrax toxin, have been shown to form pores of variable stoichiometry according to environmental factors raises the question of whether the stoichiometry of FraC pores might also vary in different environments.

Why FraC and sea anemone toxins?
FraC is just one of many similar kinds of sea anemone toxins, called actinoporins. These sea anemone toxins have been discovered in over 30 different sea anemone species so far, and despite being produced by different species found in very different locations across the world, these toxins have remarkably similar amino acid sequences. The crystal structures of the water-soluble monomeric form of four different actinoporins have been solved and are almost identical. However, despite the high sequence identity and structural similarity, actinoporins have exhibited quite different pore-forming activity when assayed in model membrane systems. Thus, actinoporins present a unique opportunity to examine how small differences in sequence might affect the oligomeric state, lipid interactions, and activity of the transmembrane pores.

Overlay of the four solved crystal structures of actinoporin monomers

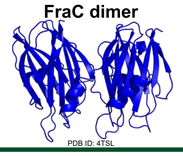


FraC was selected because it is one of the most well-studied actinoporins so far. In addition to information about its pore-forming activity in different environments, crystal structures of the monomer, dimer, and an octameric pore form have been solved. Different research groups have also been able to express FraC recombinantly in bacteria.

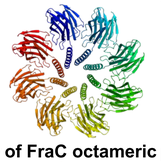
***Actinia fragacea*, strawberry sea anemone**

FraC monomer

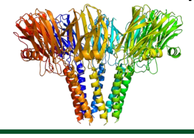
FraC dimer



Top view of FraC octameric pore



Side view of FraC octameric pore

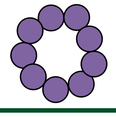


Project Questions

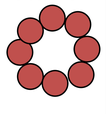
Oligomeric state(s) of FraC pores?

Tetramer?

Nonamer?



Octamer?



Mechanism of pore formation?

1) Embed, then oligomerize?

or

2) Oligomerize, then embed?

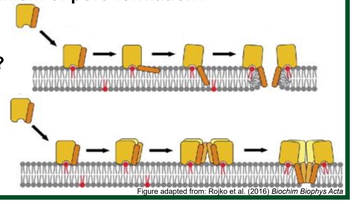


Figure adapted from: Rojko et al. (2016) *Biochim Biophys Acta*

Methods

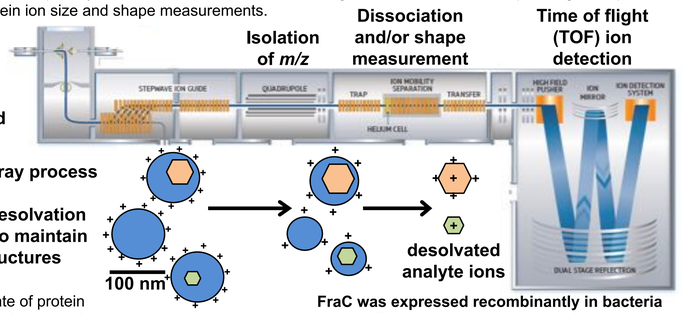
Native mass spectrometry allows us to study protein complexes in their native-like conformation
In native electrospray ionization mass spectrometry (native ESI-MS) experiments, the protein sample is first gently ionized by spraying it from an aqueous buffer directly into the mass spectrometer. At the end of the instrument is a detector which outputs a value of *m/z*, or the mass-to-charge ratio of the ions. Our Waters Synapt G2-S*i* mass spectrometer also has the capability to select for ions of certain *m/z* ranges, dissociate or break apart large complexes, and to obtain protein ion size and shape measurements.

Gentle ionization, conditions of solution easily varied

Isolation of *m/z*

Dissociation and/or shape measurement

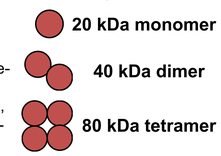
Time of flight (TOF) ion detection



The electrospray process

Controlled desolvation parameters to maintain native structures

The oligomeric state of protein complexes can be determined directly from the mass spectrum. Once the mass of the monomer is known, it is relatively easy to determine the oligomeric state of a larger complex, as the mass will be some multiple of the monomer mass.

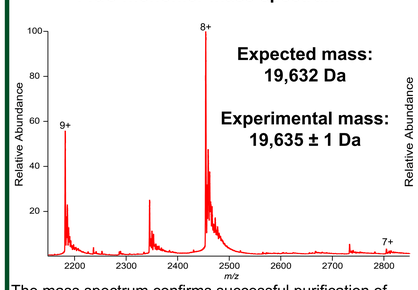


FraC was expressed recombinantly in bacteria and purified via cation exchange chromatography
We obtained a plasmid containing the FraC gene (pFraC) from Dr. Koldo Morante. pFraC was transformed into *E. coli* BL21(DE3) cells and expressed. FraC monomers were purified via cation exchange chromatography with an increasing linear salt gradient, and native ESI-MS was used to confirm the identity of the protein.

Results: Oligomeric states

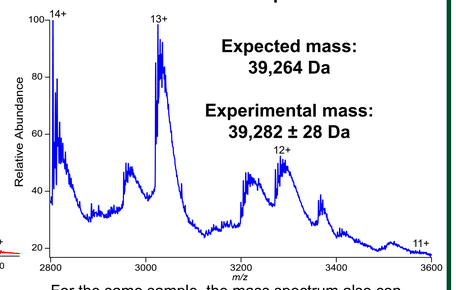
FraC exists in the monomeric and dimeric states in solution

FraC monomer mass spectrum



The mass spectrum confirms successful purification of FraC monomers. The labeled peaks in the mass spectrum correspond to the 7+, 8+, and 9+ charge states of FraC monomers, and the *m/z* values of these peaks were used to determine the experimental mass of the protein.

FraC dimer mass spectrum



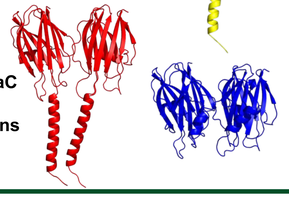
For the same sample, the mass spectrum also contained peaks corresponding to the 11+, 12+, 13+, and 14+ charge states of FraC dimer species.

Results: Mechanism of pore formation

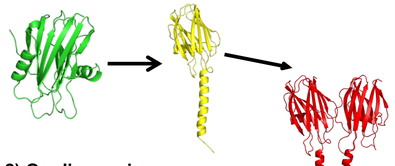
Having identified two different oligomeric states of FraC, we can begin to approach the question regarding the mechanism of pore formation. In line with the two simplistic models proposed for pore formation, there are two possible conformations each of FraC dimers and monomers that could exist: one with the helix extended and one that is still compact. Thus, the two simplistic mechanisms of FraC pore formation can be modeled as shown here.

Possible FraC monomer conformations

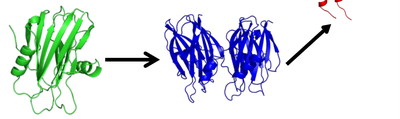
Possible FraC dimer conformations



1) Embed, then oligomerize?



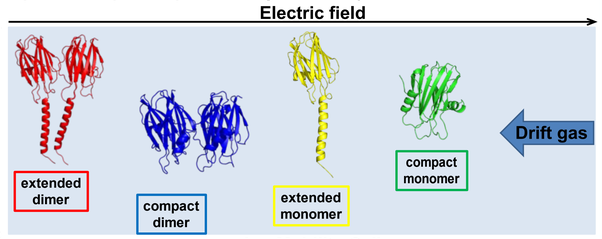
2) Or oligomerize, then embed?



However, the different conformations of FraC dimers and of FraC monomers cannot be distinguished from one another solely based on mass, as they will each have masses of ~39 kDa and 19.6 kDa, respectively.

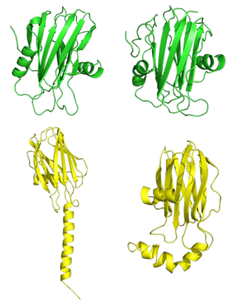
Results: Mechanism of pore formation

We can measure information on protein complex shape/size using ion mobility data
Ion mobility can be used to distinguish between the two possible conformations of FraC dimers and between the two possible conformations of FraC monomers based on the difference in size/shape due to drag force created by flowing a drift gas into the ion mobility cell. The end output of this process is a collisional cross-section (CCS), which is essentially a measurement of the size/shape or surface area of the analyte.

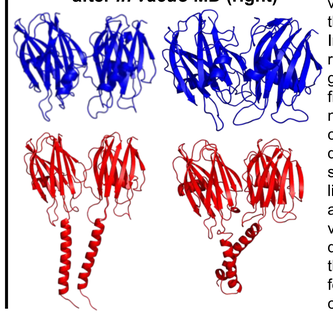


***In vacuo* MD allow us to reproduce gas-phase compaction, compute more accurate expected CCS values**

FraC monomer structures before (left) and after *in vacuo* MD (right)



FraC dimer structures before (left) and after *in vacuo* MD (right)

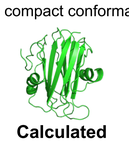


Expected CCS values can be calculated based on a protein's crystal structure, but experimental CCS values are often quite smaller than these due to gas-phase compaction. In order to calculate the most accurate expected CCS values using our group's program Collidoscope, we first perform *in vacuo* molecular dynamics (MD) simulations on FraC crystal structures. We first tested five different force fields for these MD simulations on a range of well-established ion mobility calibrant proteins and selected the one that gave CCS values closest to the experimental data, thus allowing us to reproduce the gas-phase compaction of the different FraC conformations before calculating expected CCSs.

FraC monomers are compact, helix not extended
Comparing the calculated expected CCS values of both conformations of the FraC monomer to the experimentally determined CCS reveals that FraC monomers in these native ESI-MS experiments are most likely in the compact conformation without the helix extended.

Monomer experimental CCS: 20.1 nm²

Calculated CCS: 19.9 nm²

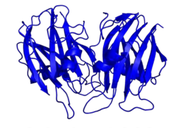


Calculated CCS: 21.1 nm²

FraC oligomerizes into dimers, does not extend helices
The experimentally determined CCS of FraC dimers most closely matches the calculated expected CCS value of the more compact conformation of the FraC dimer. Thus, FraC most likely oligomerizes into dimers before extending its helices into the membrane.

Dimer experimental CCS: 32.0 nm²

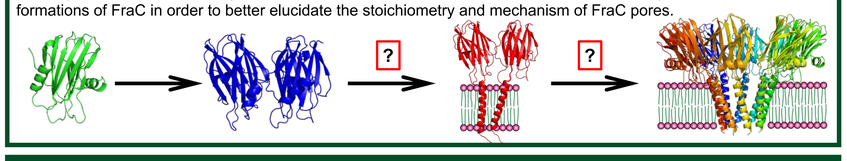
Calculated CCS: 32.3 nm²



Calculated CCS: 35.5 nm²

Conclusions

FraC oligomerizes first before inserting into the membrane
Native ESI-MS was used to identify monomeric and dimeric FraC species. Experimental ion mobility data compared to calculated expected CCS values of *in vacuo* MD simulated FraC structures revealed that both FraC monomers and dimers likely exist in the more compact conformations without their helices extended. Based on the two different possible mechanisms of pore formation, FraC likely oligomerizes first before inserting its helices into the membrane to form pores. However, all of these experiments were performed using water-soluble forms of FraC. The next steps of this project will be to add detergents and lipids to FraC and determine how this affects the oligomeric states and conformations of FraC in order to better elucidate the stoichiometry and mechanism of FraC pores.



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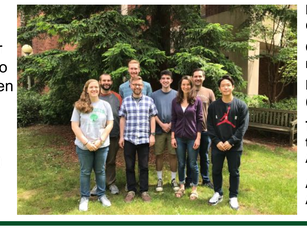

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