Single-molecule biophysics of pharmacological molecules interaction with protein pores and lipids

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Content

- Original results regarding certain AMP's interaction with reconstituted lipid membranes
- Uni-molecular level investigation of interaction between AMP's and Gram-negative bacteria porins
- Stochastic sensing of β-lactam antibiotics with a single protein aggregate

Some introductory info: what are antimicrobial peptides (AMPs) ?

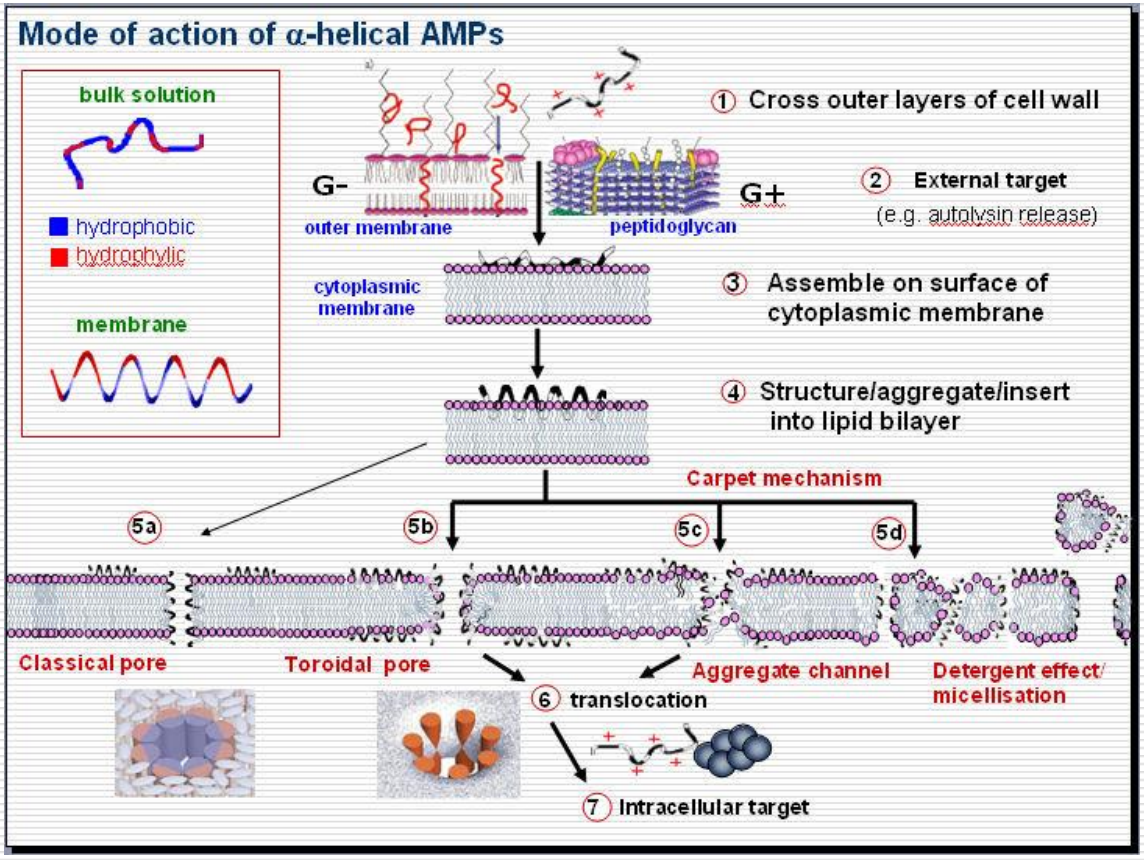
➢Naturally-occurring anti-microbial peptides (AMPs) are basic peptides composed of 12 – 50 amino acids that are distributed throughout all kingdoms of life, ribozomally or non-ribozomally synthesized.

➢AMPs make up one class of a growing number of membrane-active peptides that include anticancer and antiviral peptides, cell-penetrating peptides, viral fusion peptides, and venom peptides. For all these classes of peptides, interactions between the membrane lipid bilayer and the peptide are central to their biological functions.

➢AMPs display extensive sequence heterogeneity; however they do share a number of common characteristics, including a net positive charge of $\geq$ +2 (with +4 to +6 being most common), ~ 50 – 70 % hydrophobic amino acids, and a propensity to fold into amphipathic conformations in the presence of membranes.

➢Importantly, AMPs display a broad spectrum of antimicrobial activity against both Gram negative and Gram positive bacteria, fungi, and enveloped viruses. They retain activity against antibiotic-resistant strains, do not readily elicit resistance and often work in synergy with the innate immune system (http://www.bbcm.univ.trieste.it/).

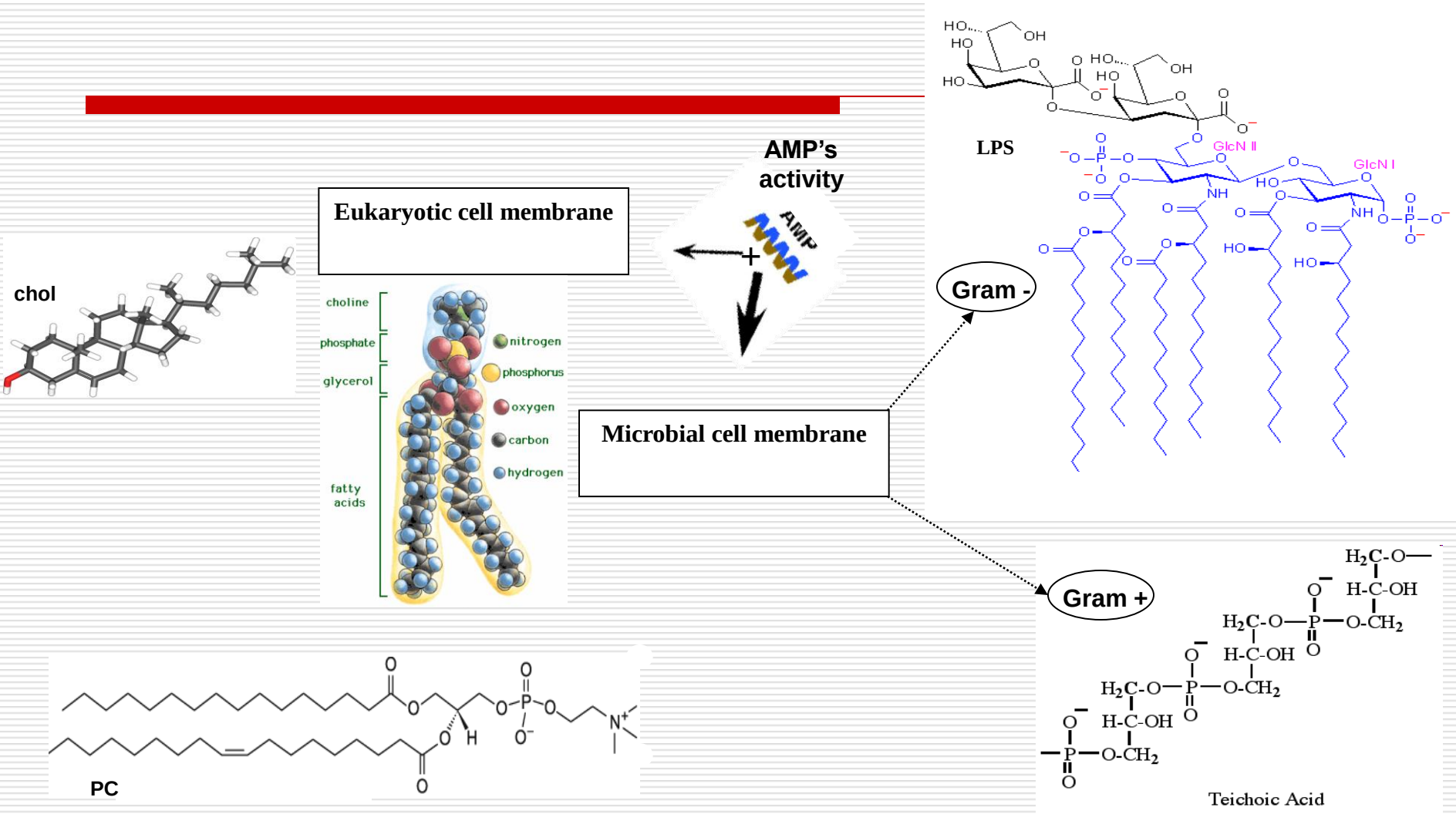
Action mechanisms of AMPs



1. Electrostatically enhanced initial association of a random coil peptide
2. Intercalation into the bilayer, with the peptide long axis parallel to the membrane layer plane
3. A conformational change from a random coil to an amphipathic α helix; ion-pairing of acidic phospholipids with positive residues of the peptide
4. Reorientation and membrane insertion of the peptide, its long axis becoming perpendicular to the monolayer surface. **This step occurs above a threshold peptide-to-lipid ratio, which depends on peptide and type of lipids**. One mechanism of pore formation which would give rise to a threshold concentration is based on the proposition that the adsorption of peptide induces tension within the membrane which is relieved by pore formation. Only when the membrane tension reaches a critical value (corresponding to the threshold peptide concentration) pore formation becomes favorable.
4. Aggregation of the α-helical peptides into oligomers

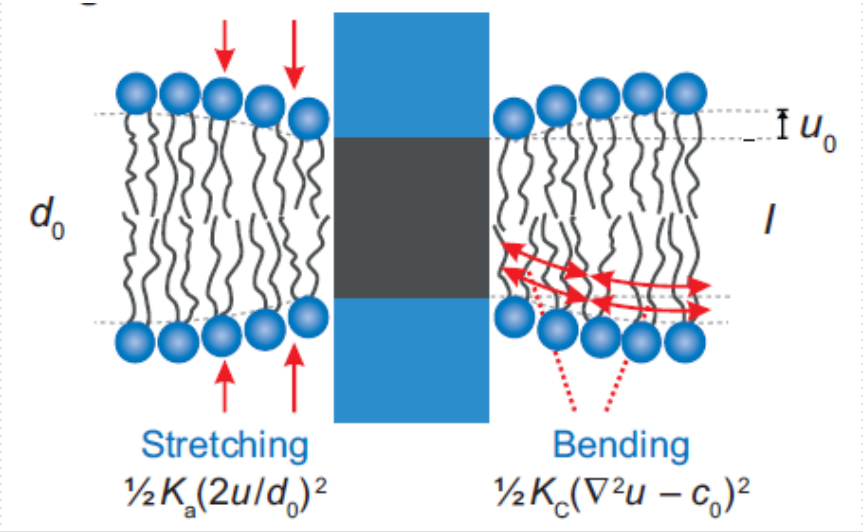
N. Sitaram, R. Nagaraj, Biochimica et Biophysica Acta 1462 (1999) 29-54

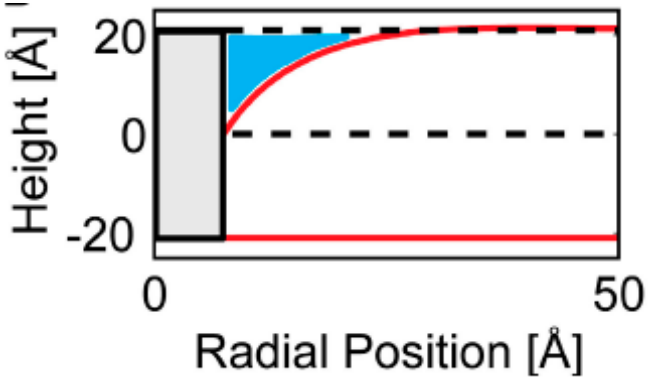
Selective action of AMP's is mainly governed by the net negative charge present on cells membrane

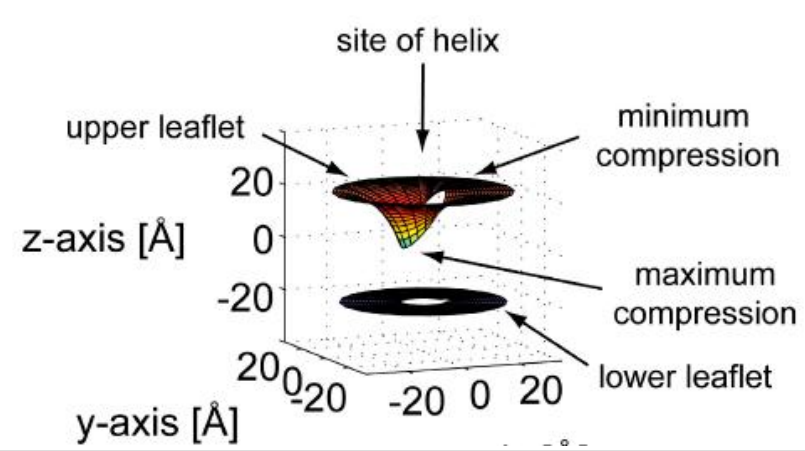


➢Katsumi Matsuzaki, *Control of cell selectivity of antimicrobial peptides*, **BBA - Biomembranes** (**2008**), doi: 10.1016/j.bbamem.2008.09.013

Mechanical features of BM's critically modulate AMP's functioning

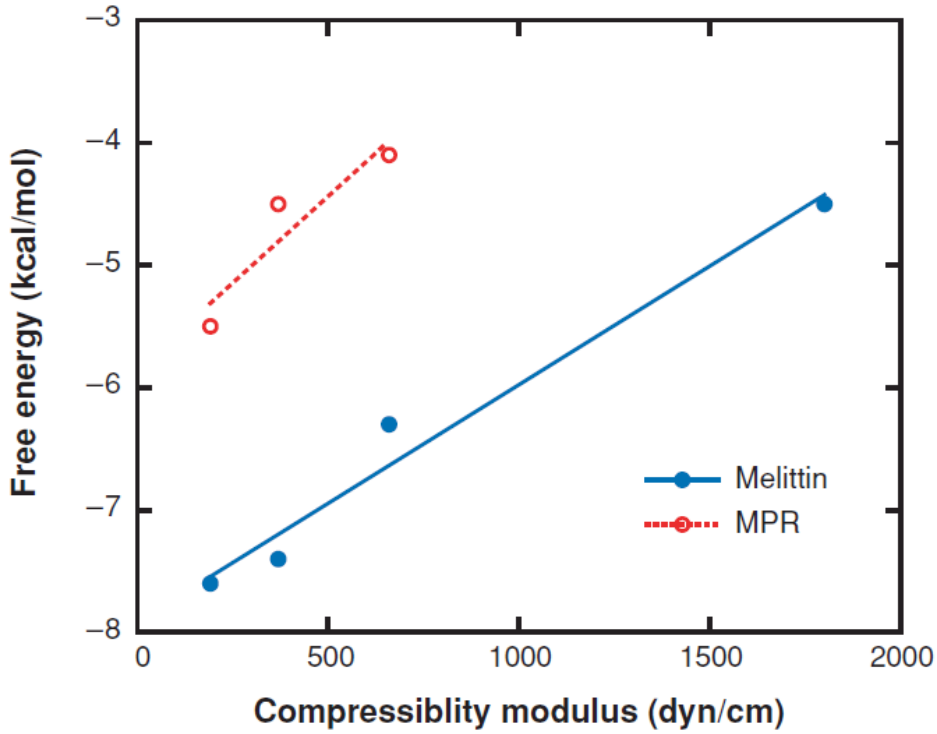






$$\Delta G_{mem} = \int\int \Omega \frac{1}{2}\left(\underbrace{\frac{K_a}{I_0^2}u^2}_{compression} + \underbrace{\frac{1}{2}K_c(\nabla^2 u)^2}_{bending} + \underbrace{\frac{1}{2}\alpha(\nabla u)^2}_{stretch} \right) d\Omega$$

What can lipid properties do to AMP's activity ?



Plot of the free energy of binding versus the bilayer area compressibility modulus for two amphipathic peptides.

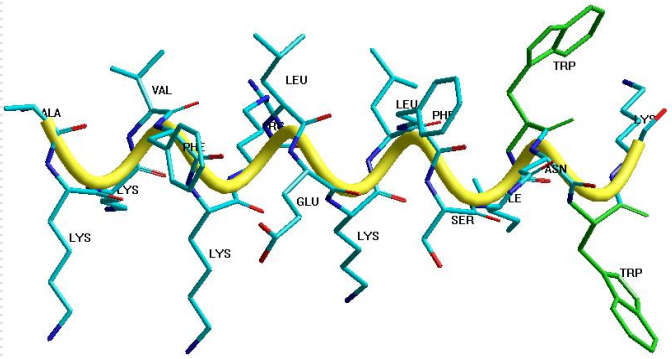
Thomas J. McIntosh and Sidney A. Simon, Annu. Rev. Biophys. Biomol. Struct. 2006. 35:177–98

Single-molecule studies regarding of the interaction between the Helicobacter pylori HP(2–20) antimicrobial peptide analogue HPA3 and artificial lipid membranes

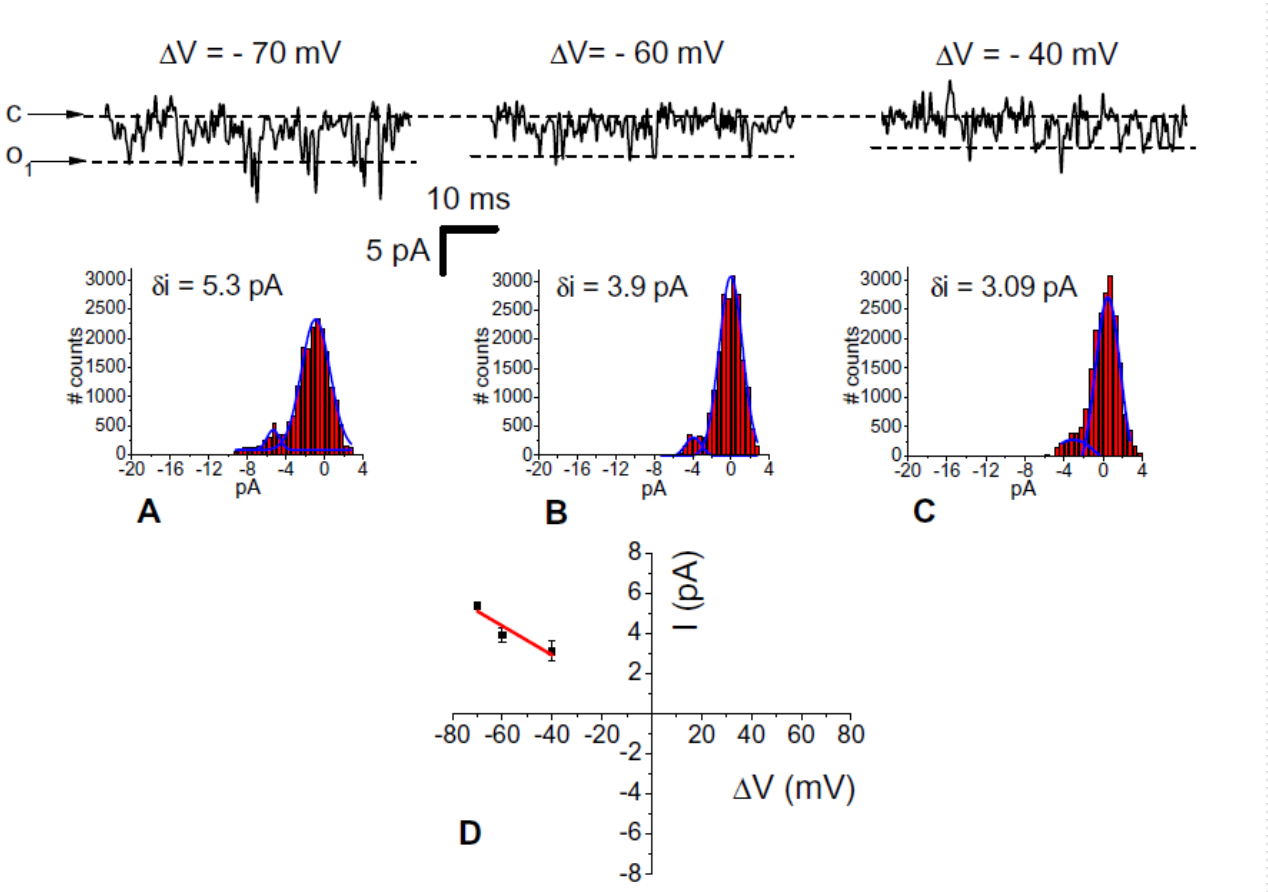
- We investigated the mechanism of action of the HPA3 peptide, an analogue of the linear antimicrobial peptide, HP(2–20), isolated from the N-terminal region of the Helicobacter pylori ribosomal protein.

- The HP(2-20) peptide was shown be bactericidal, and together with its analogues specifically recognize common immune elicitors, including chitin, peptidoglycan, and LPS, thus becoming very promising candidates for the development of new peptide-based antibiotic agents.

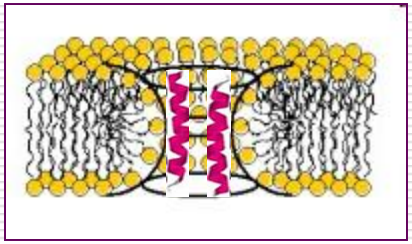
Ala-Lys^{+}-Lys^{+}-Val-Phe-Lys^{+}-Arg^{+}-Leu-Glu^{-}-Lys^{+}-Leu-Phe-Ser-Ile-Trp-Asn-Trp-Lys^{+}-NH2



Selected data which reveal the putative model of HPA3-induced destabilization of lipid membranes

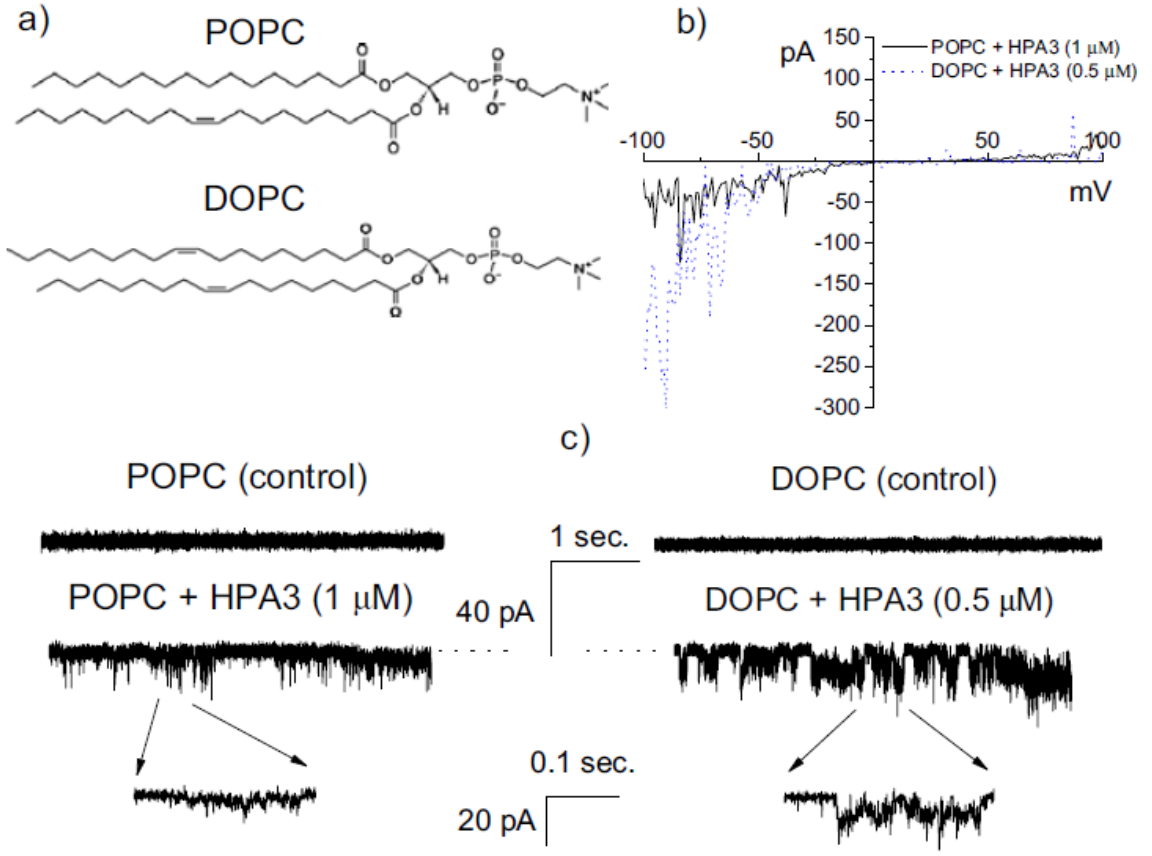


Putative model of HPA3 channels structure



L. Mereuta, T. Luchian, Y. Park, K.-S. Hahm, Biochemical and Biophysical Research Communications 373 (2008) 467–472

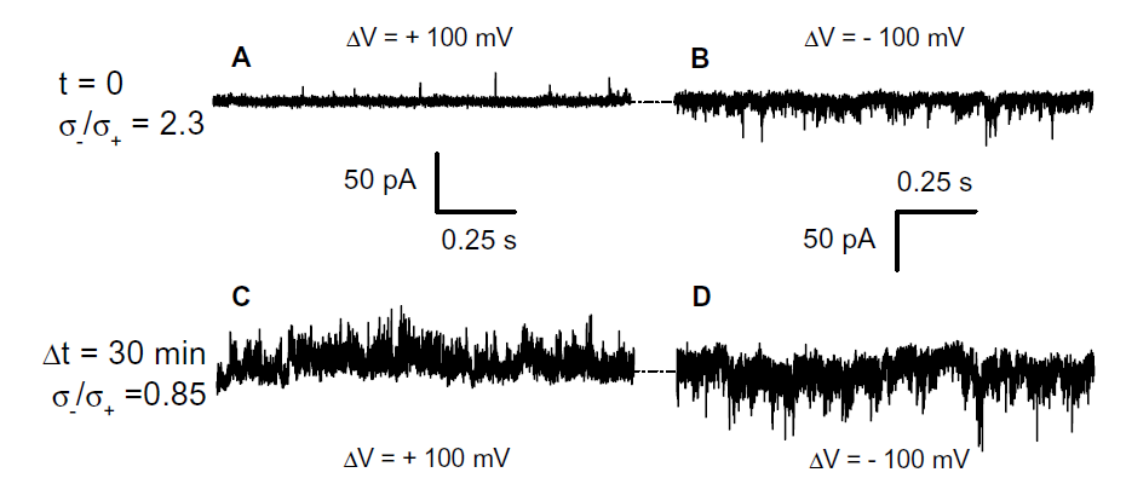
Lipids unsaturation grealty alters the activity of the HPA3 peptide in zwitterionic membranes

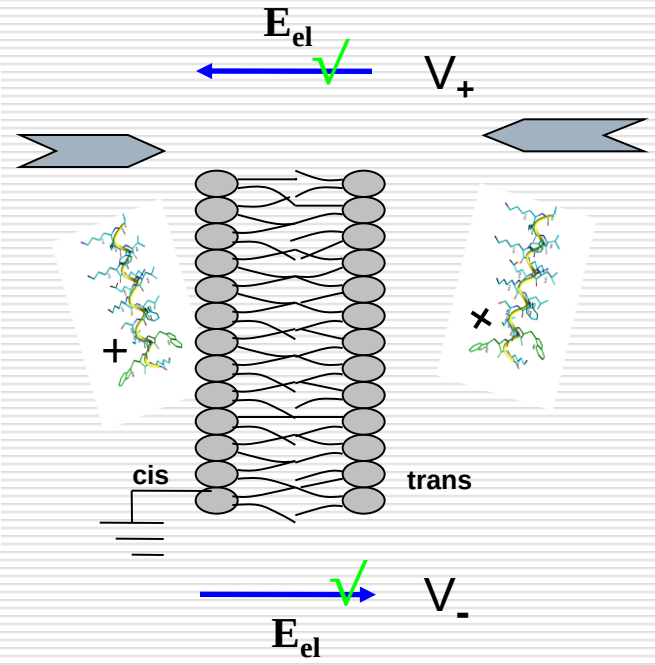


I-V diagrams which characterize transport properties of HPA3 pores inserted in lipid membranes made of lipids containing one monounsaturated acyl chain (POPC) and two monounsaturated acyl chains (DOPC).

L. Mereuta, T. Luchian, Y. Park, K.-S. Hahm, J Bioenerg Biomembr, 41(1), 79 -84, 2009

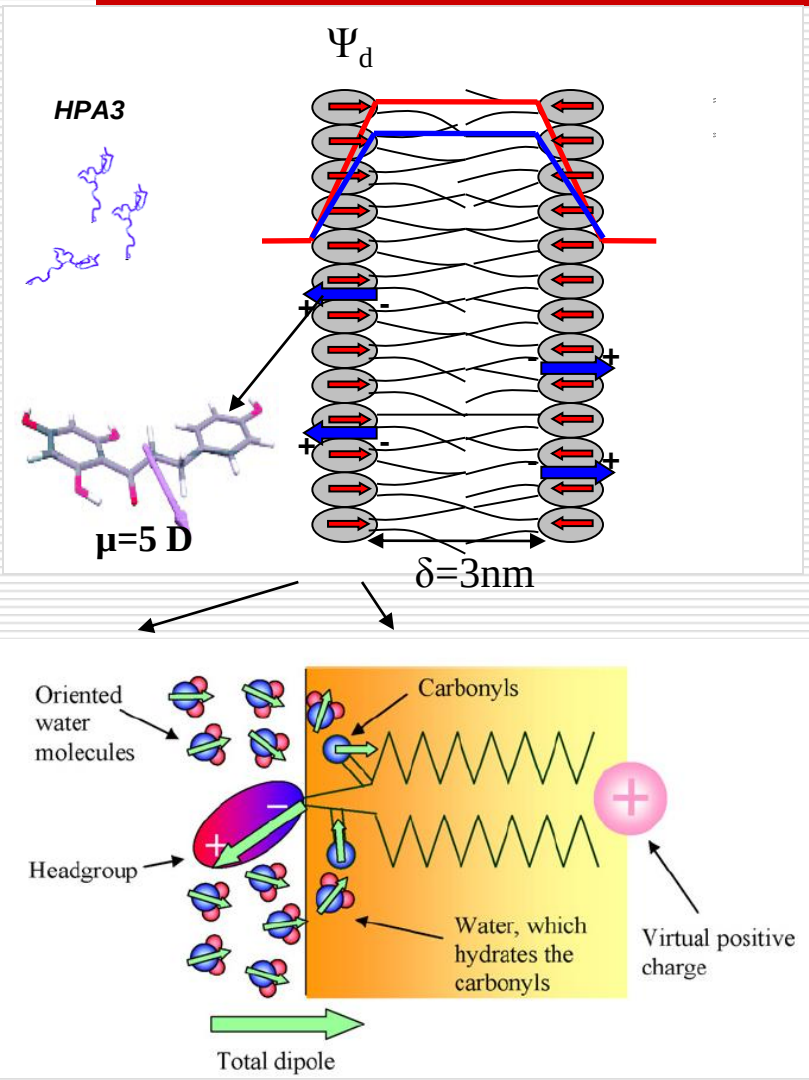
HPA3 peptide is capable of translocation across a zwitterionic membrane

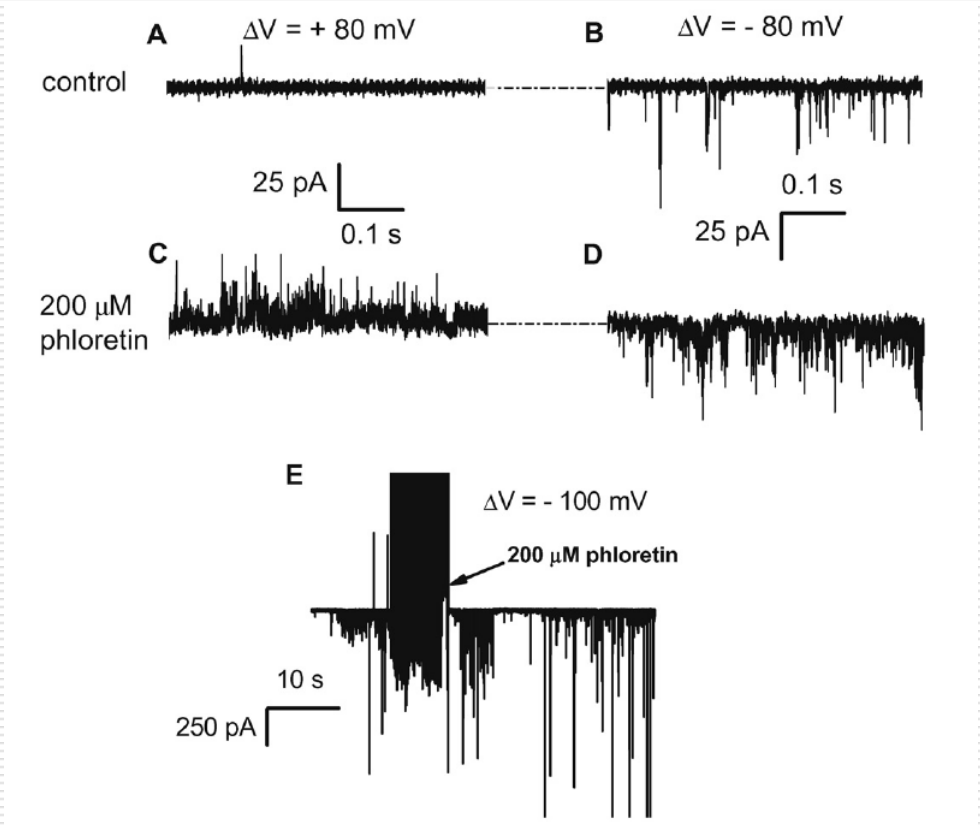




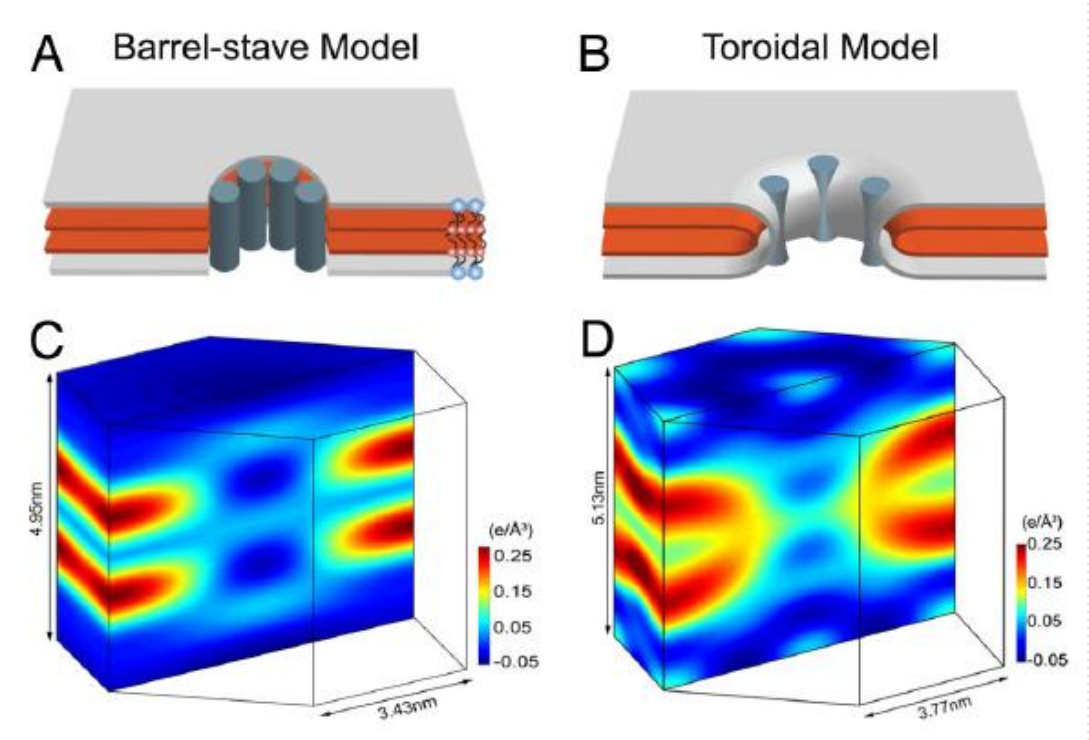
Over time, HPA3's pore-forming ability becomes more apparent at positive-applied potentials as well, which can be ascribed to the increased peptide density in the trans leaflet of the membrane generated by the *translocated peptides across the membrane*

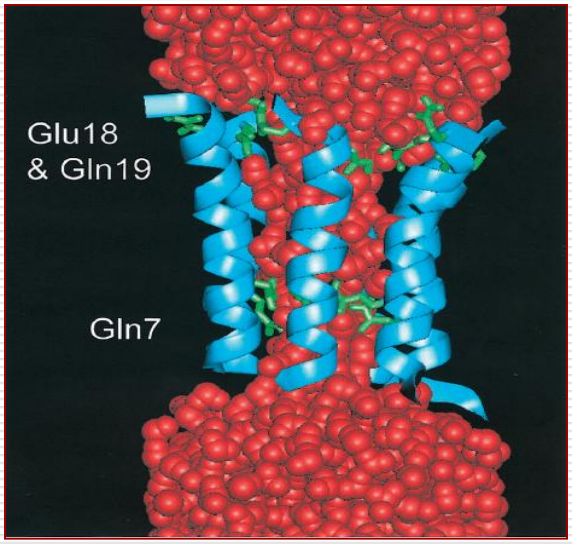
Membrane dipole potential changes greatly alter HPA3 activity in lipid membranes

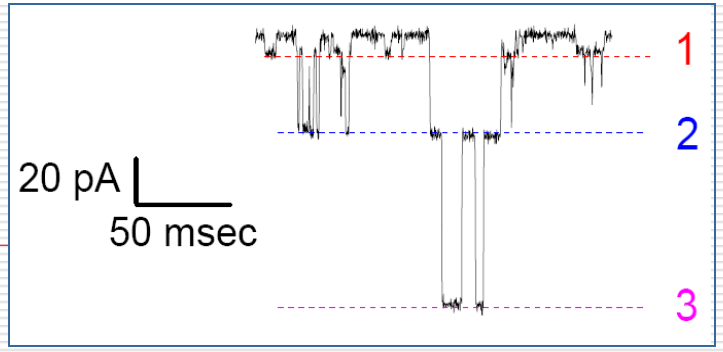




Dipole potential changes inflict pore forming model - dependent alterations on model AMPs

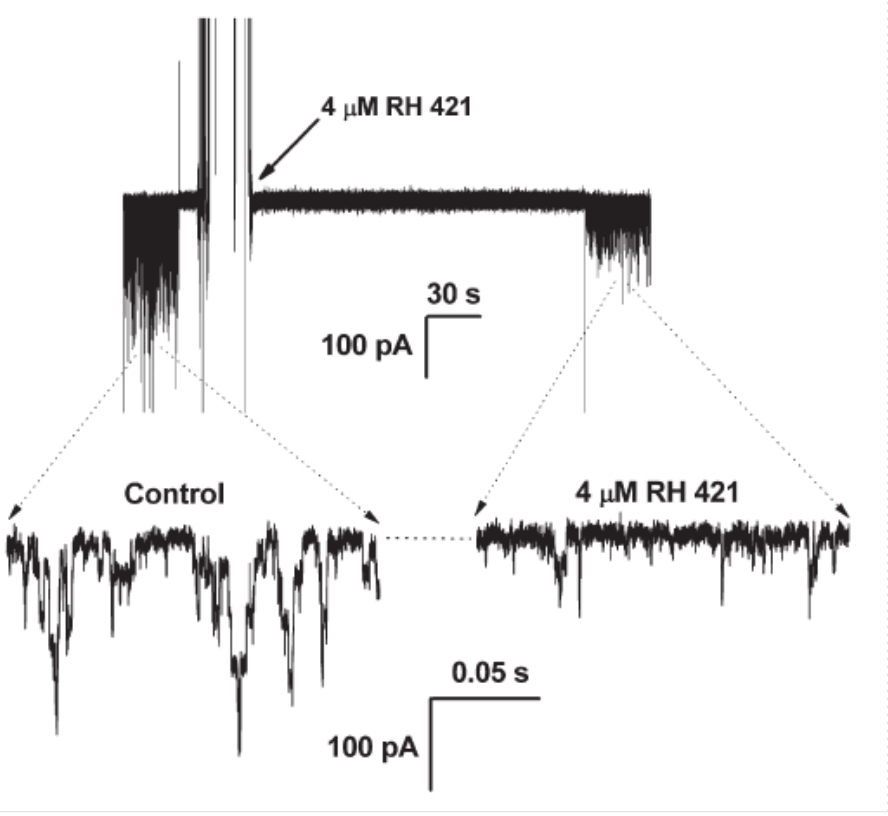


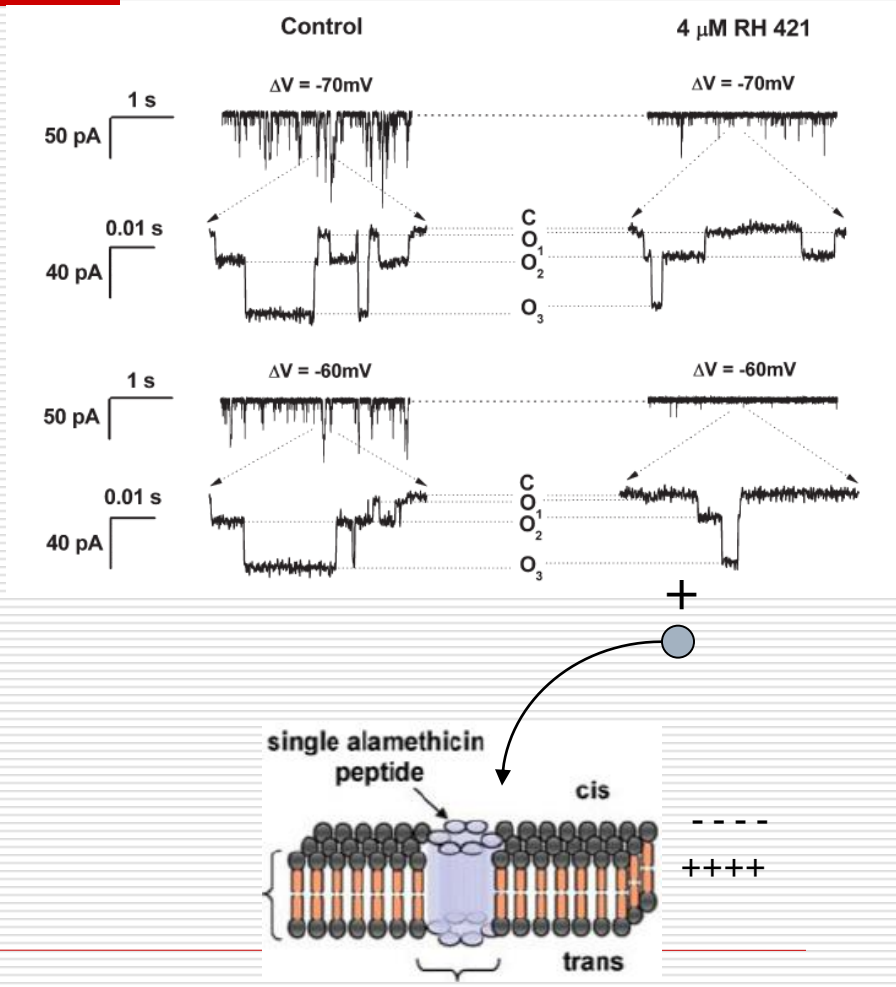




S. Qiana et al., PNAS 105(45), 17379–17383

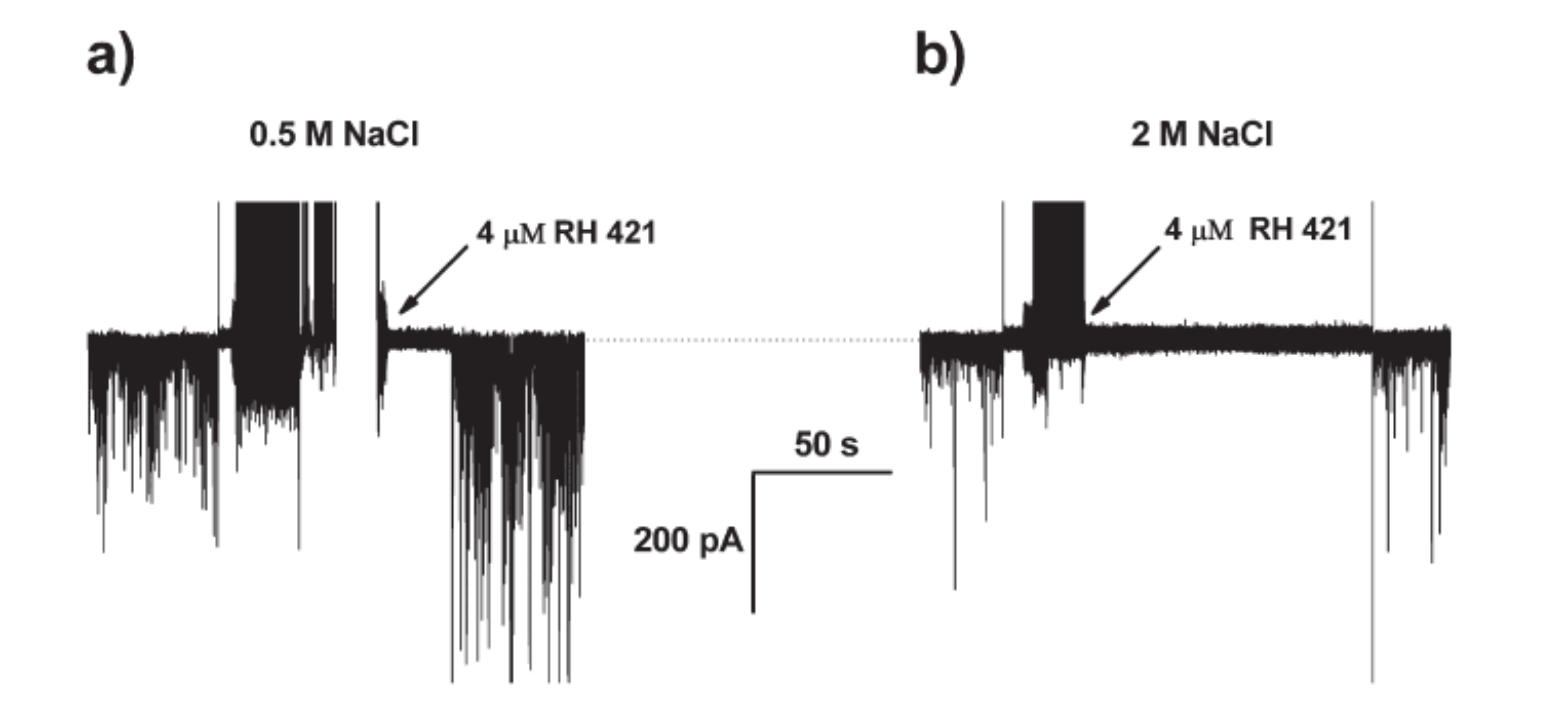
Dipole potential changes inflict pore forming model - dependent alterations on model AMPs (the case for alamethicin)





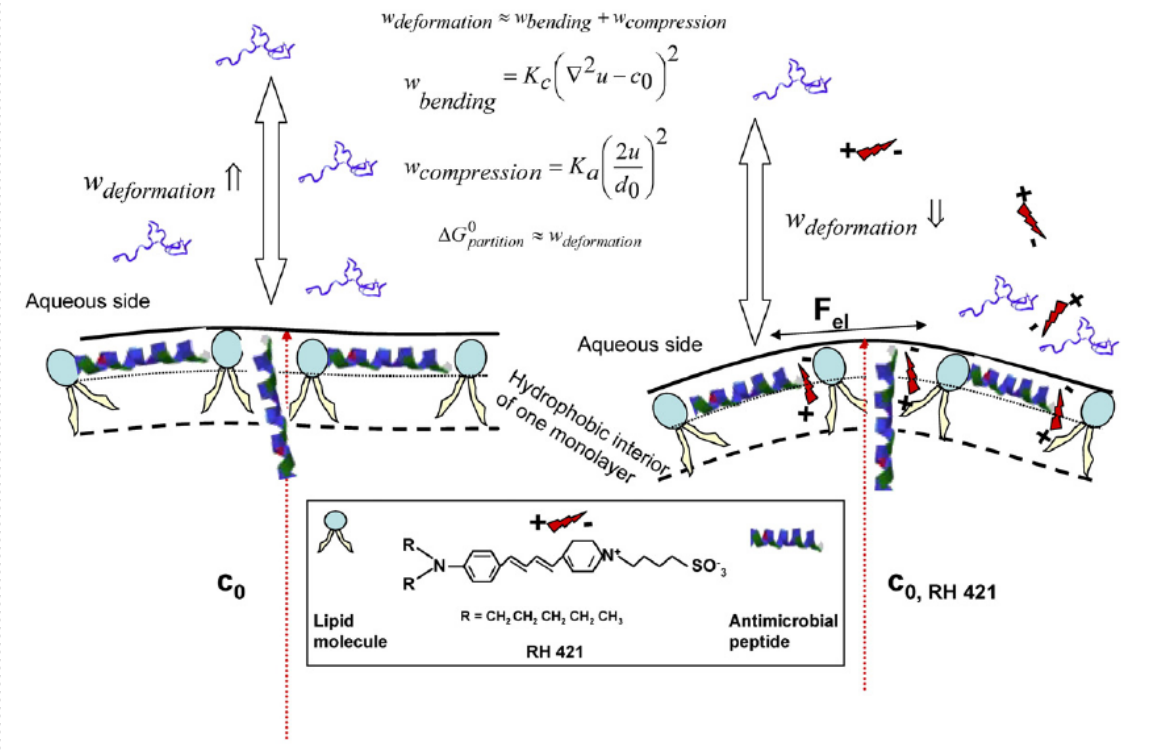
A. Apetrei, L. Mereuta, T. Luchian, Biochimica et Biophysica Acta – General Subjects 1790 (8), 809-816, 2009

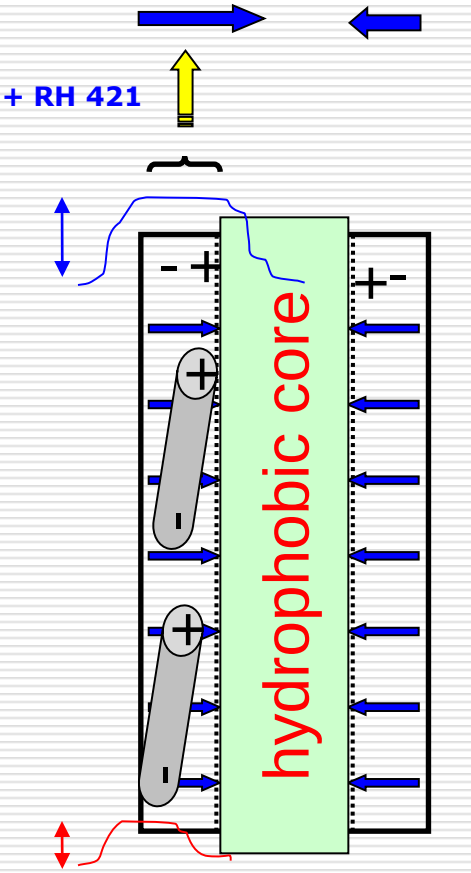
Dipole potential changes inflict pore forming model - dependent alterations on model AMPs (the case for melittin)



A. Apetrei, L. Mereuta, T. Luchian, BIOCHIMICA ET BIOPHYSICA ACTA-GENERAL SUBJECTS 1790 (8), 809-816, 2009

Oversimplified sketch of the molecular mechanism via which the inclusion of RH 421 molecules leads to changes in AMP's activity which depends also upon the pore-forming model of AMP's



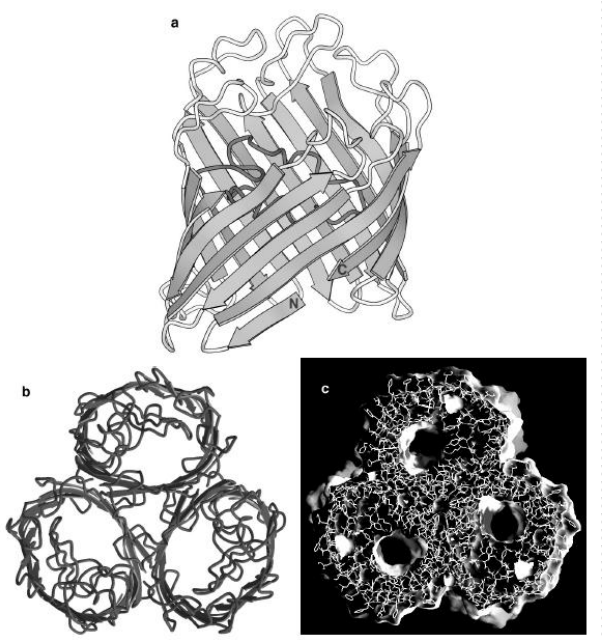


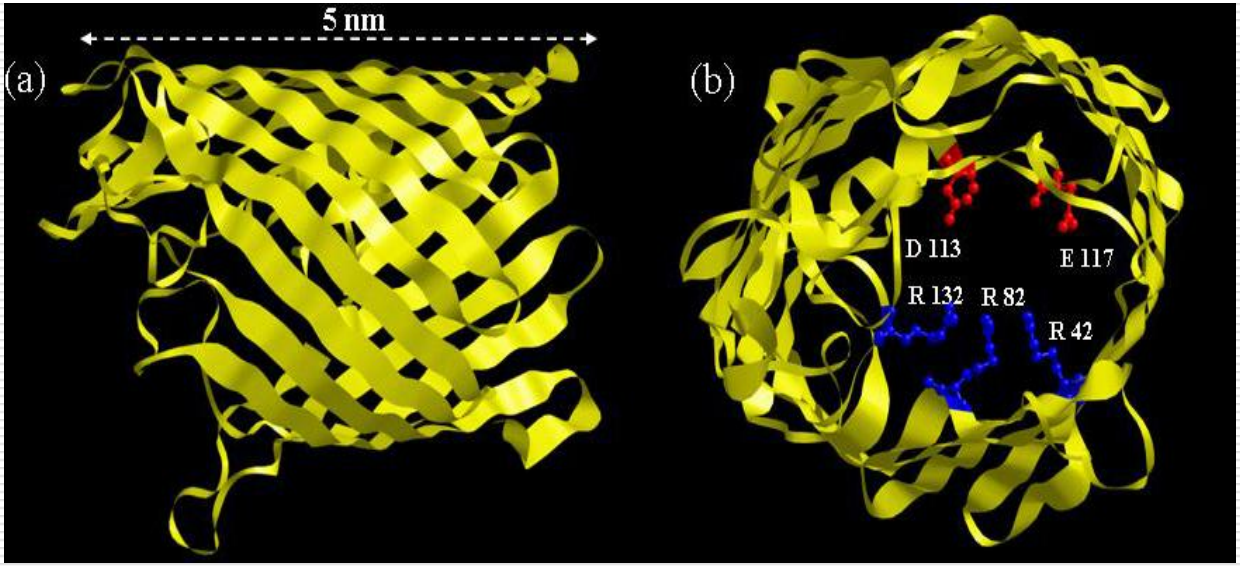
Electrostatic repulsion interactions manifested mostly between charged sulfonate moieties of RH-421 molecules (denoted by Fel), ensue in a local alteration of the lipids packing density, which gives rise to possible changes in the spontaneous curvature of the cis monolayer ('c0, RH 421'N'c0') and possibly its bending modulus, in addition to a negative contribution to the membrane surface tension

2. Uni-molecular level investigation of interaction between AMP's and Gram-negative bacteria porins

- Do selected AMP's interact w/ OmpF ?
- If so, could AMP's translocate through OmpF ?
- Describe in certain details the molecular aspects of AMP's – OmpF interaction

Topological views of the OmpF & geometry



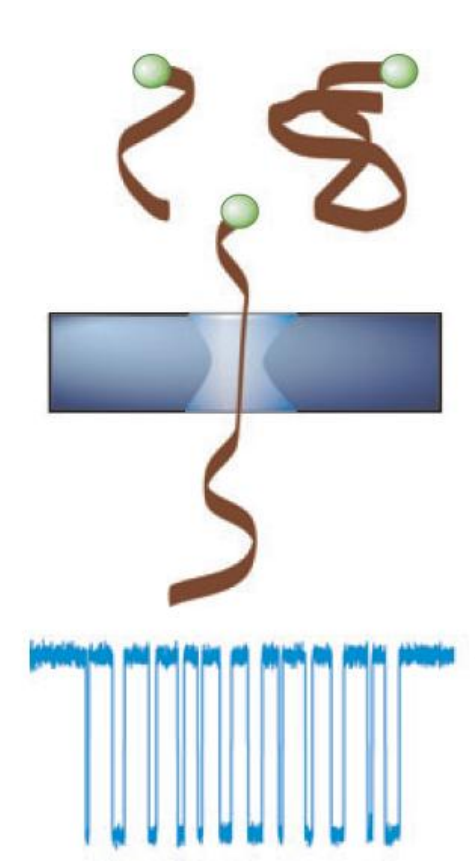


- **The monomeric OmpF is water-filled pore with an elliptical cross-section of 2.7 x 3.8 nm^2 between the main chain atoms in its walls**

- **Due to the presence of loop L3 in the β-barrel, the OmpF pore has an hourglass shape, with a narrow constriction (0.7 x 1.1 nm^2) at approximately half the height of the channel. The solute exclusion limit of the channel, as well as its ion selectivity, to a great extent is determined by this constriction.**

- **OmpF allows the permeation of hydrophilic molecules of molecular weight less than 600 Da; the general porin OmpF (outer membrane protein F) is one of the major outer membrane proteins of *Escherichia coli,* reckoned to be the main gateway for β-lactam antibiotics into periplasmic space.**

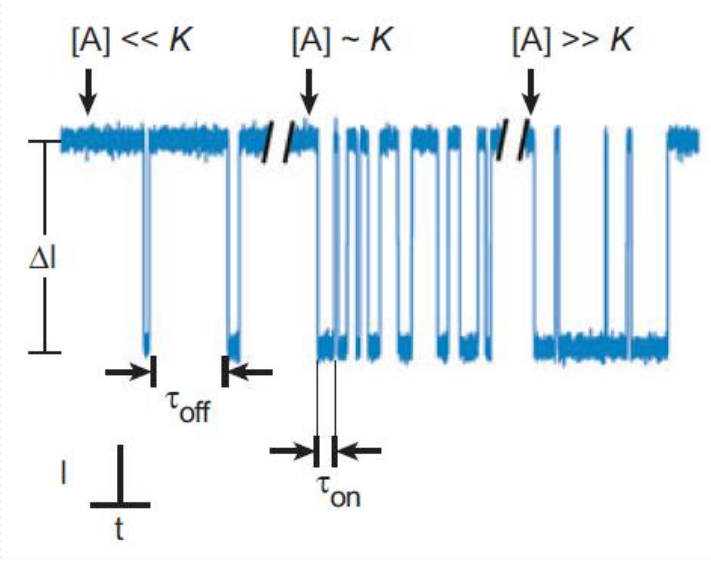
Kinetic analysis of a single porin – ligand interaction, not so hard, in the end



O

C

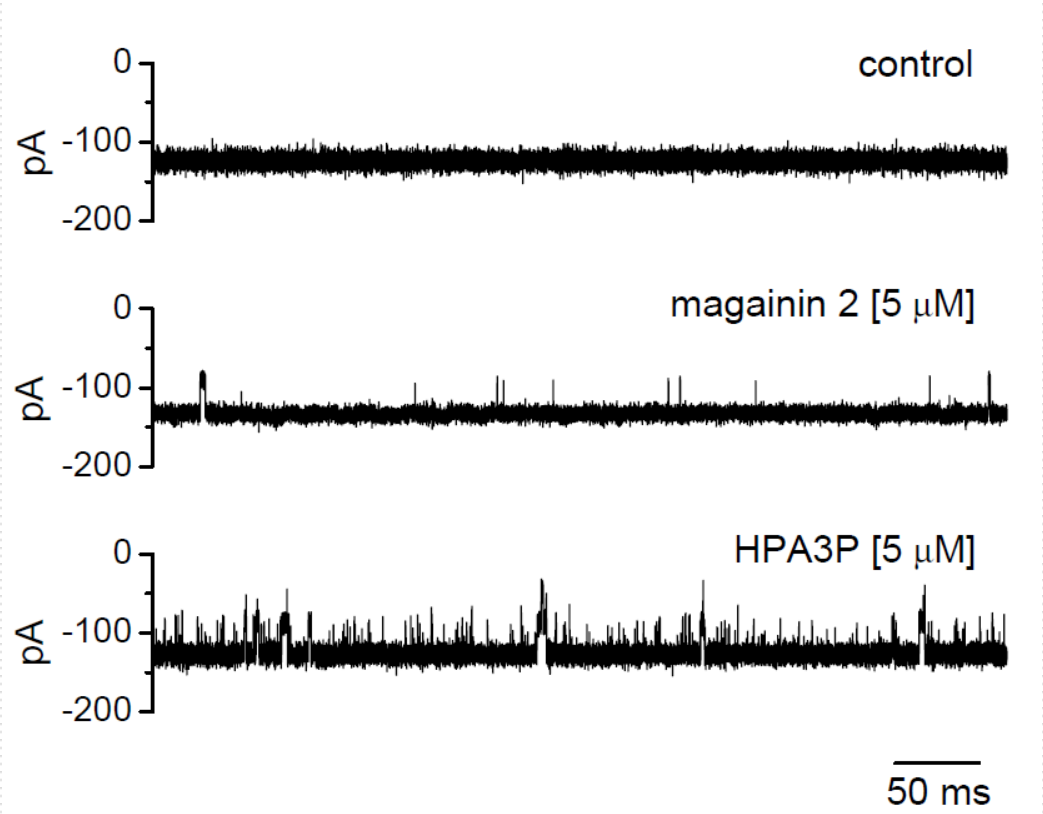
$$O + L \underset{\alpha}{\overset{\beta}{\Leftrightarrow}} C$$



$$\overline{\tau}_{on} = \frac{1}{\beta}$$

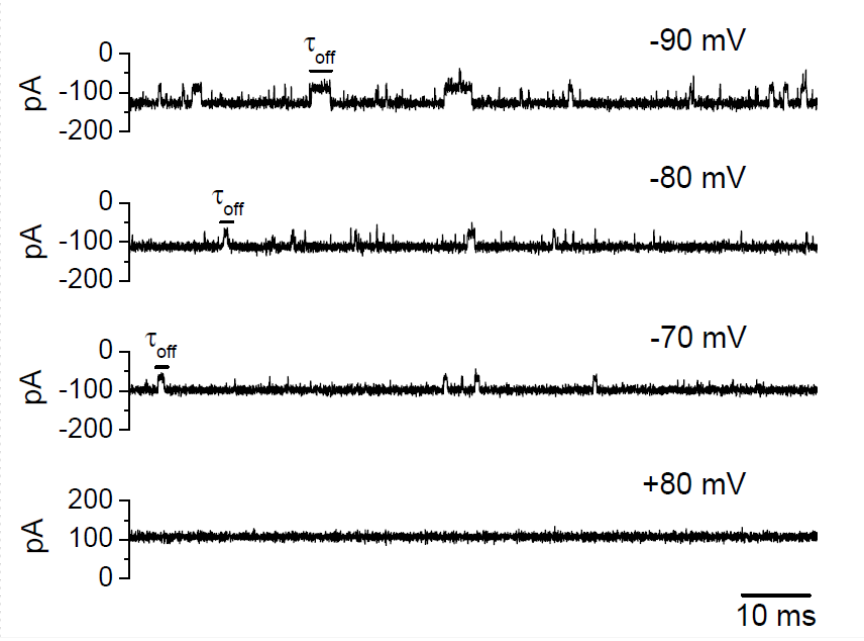
$$\overline{\tau}_{off} = \frac{1}{[L]\alpha}$$

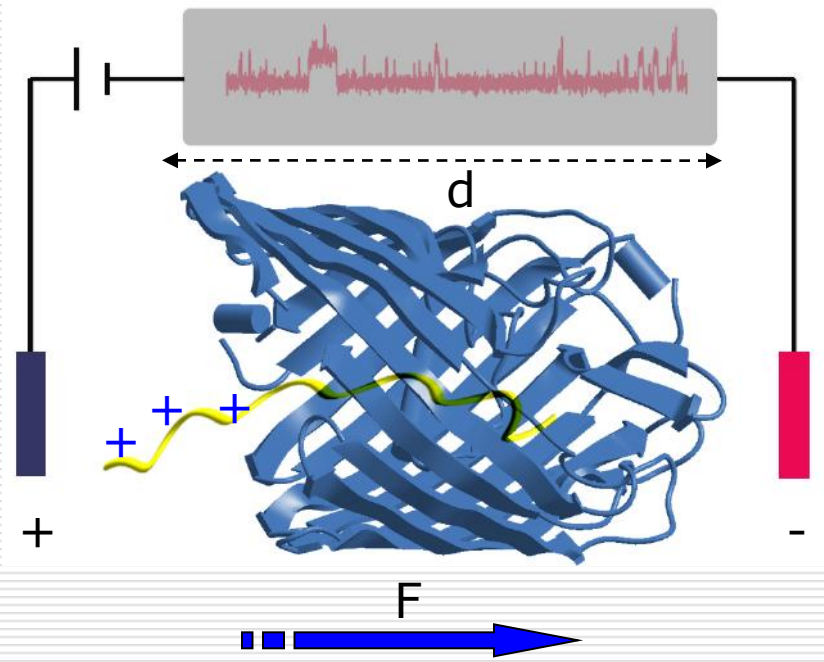
Facts: the AMP's used in this study interact reversibly w/ OmpF



Representative electrical recordings measured at -90 mV through a single OmpF channel, in the absence of added peptide (control) and presence in the cis side of the membrane of either magainin 2 (GIGKFLHSAKKFGKAFVGEIMNS) or HPA3P (AKKVFKRLPKLFSKIWNWK) antimicrobial peptides.

Facts: Voltage plays a crucial role for the interactions between the AMP's and the porin (*in an unexpected way*)



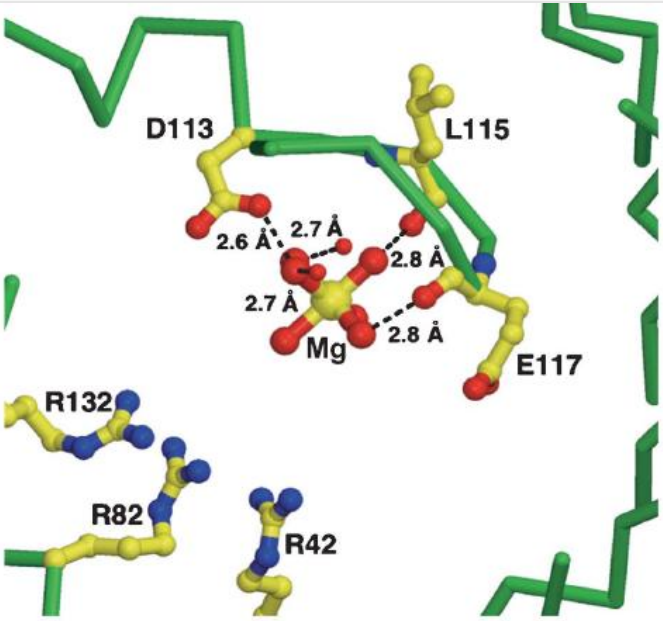


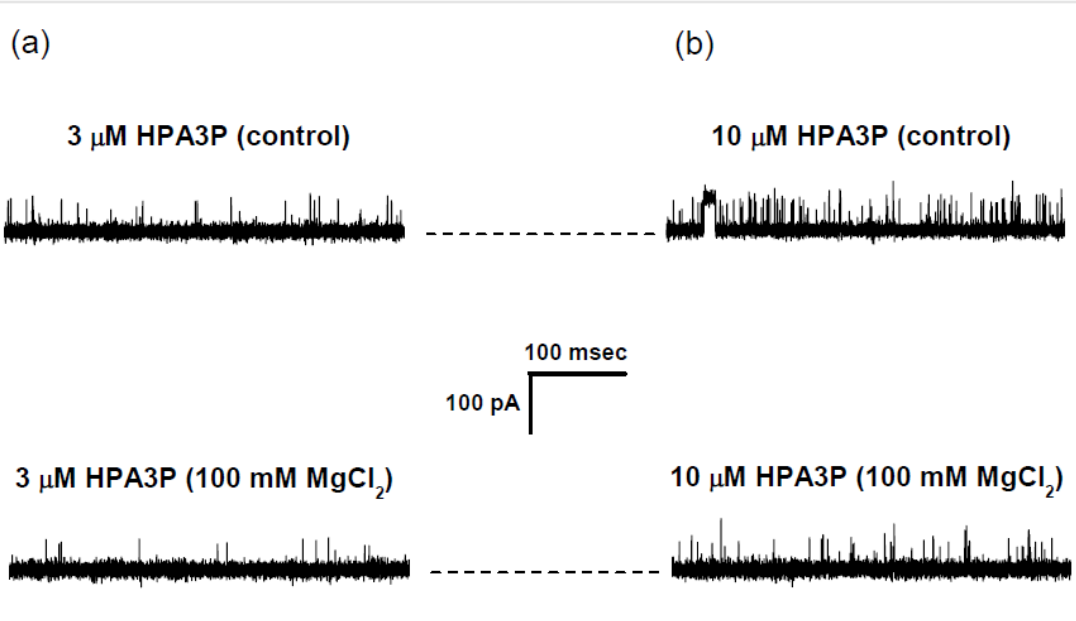
Representative traces showing at the single-molecule level the voltage-dependent reversible block of OmpF by HPA3P (10 mM), as seen in electrical recordings.

$$F = q\frac{\Delta V}{d}$$

$$F = \frac{x_{transit}}{\tau_{off}}$$

Facts: Mg $^{2+}$ ions bind to the same site inside OmpF as the HPA3P does

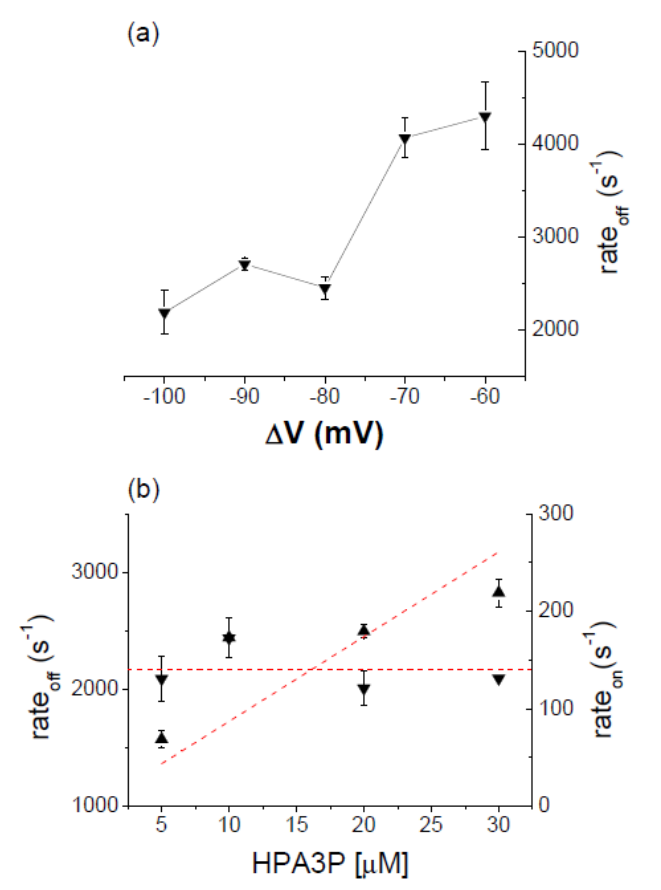




Hydrogen-bonding pattern of $(H_2O)_6$–Mg $^{2+}$ to Asp113, Leu115 and Glu117 (*E. Yamashita et al., The EMBO Journal (2008) 27, 2171–2180*)

The extent of Mg $^{2+}$ - induced decrease in HPA3P binding activity decreases once HPA3P concentration triples

Other numbers which characterize HPA3P – OmpF interaction

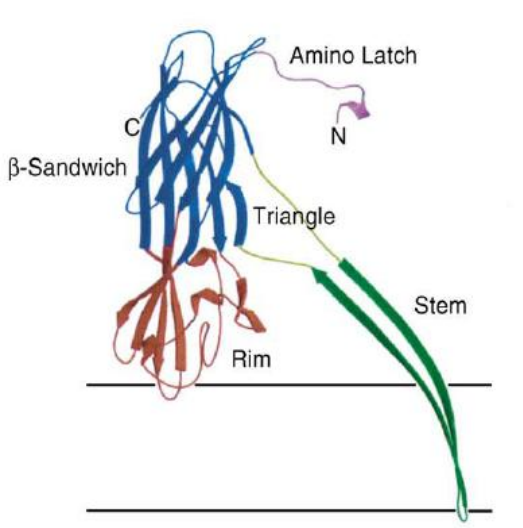


Voltage-dependence of the dissociation rate (rate off;) of HPA3P measured at a 10 μM and (b) the concentration-dependence of the association (rate $_{on}$;) and dissociation rates (rate $_{off}$;) measured at -80 mV

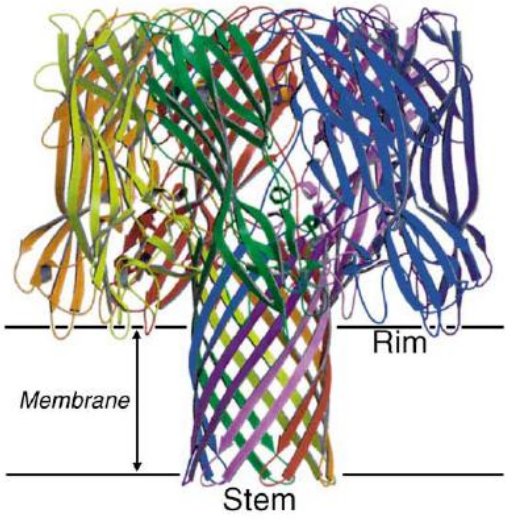
The rate constants of association k_{on} = 8.7 0.9 $\mu M^{-1}s^{-1}$, and k_{off} = 2170 5 s^{-1}).

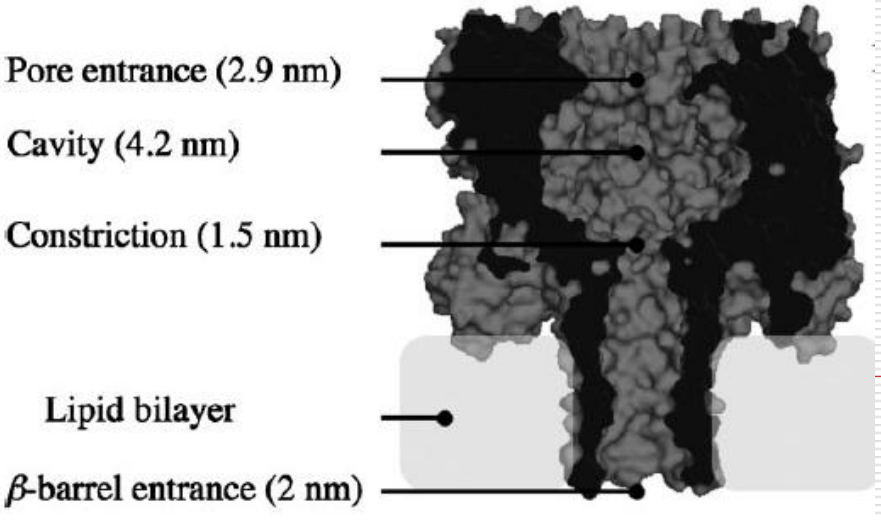
A. Apetrei, A. Asandei, T. Luchian, et al., Journal of Bioenergetics and Biomembranes 42 (2),173-180, 2010

3. Stochastic sensing of β-lactam antibiotics with a single protein aggregate

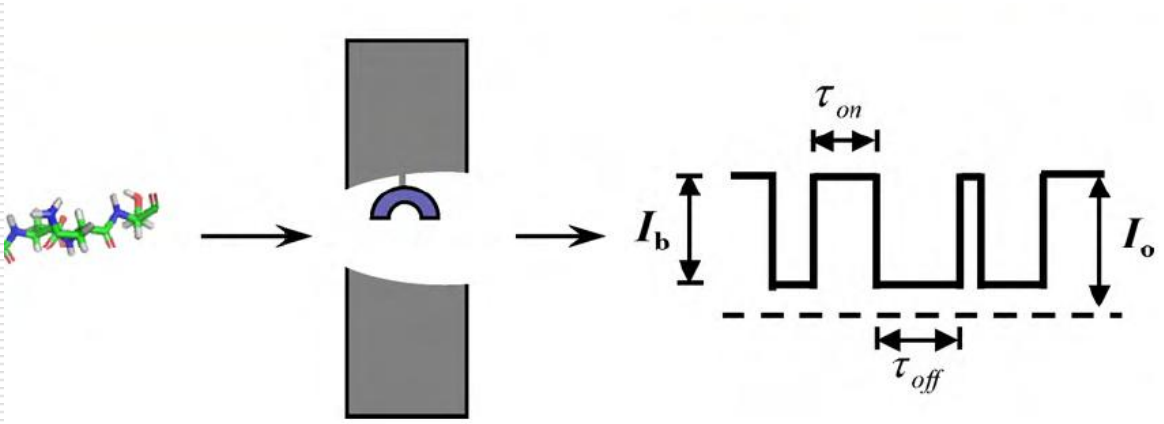


α-HL – a monomeric 293-AA polypeptide that can self-assemble on lipid bilayers to form a heptameric pore.





How does stochastic sensing work ? (a multi-million dollar question)

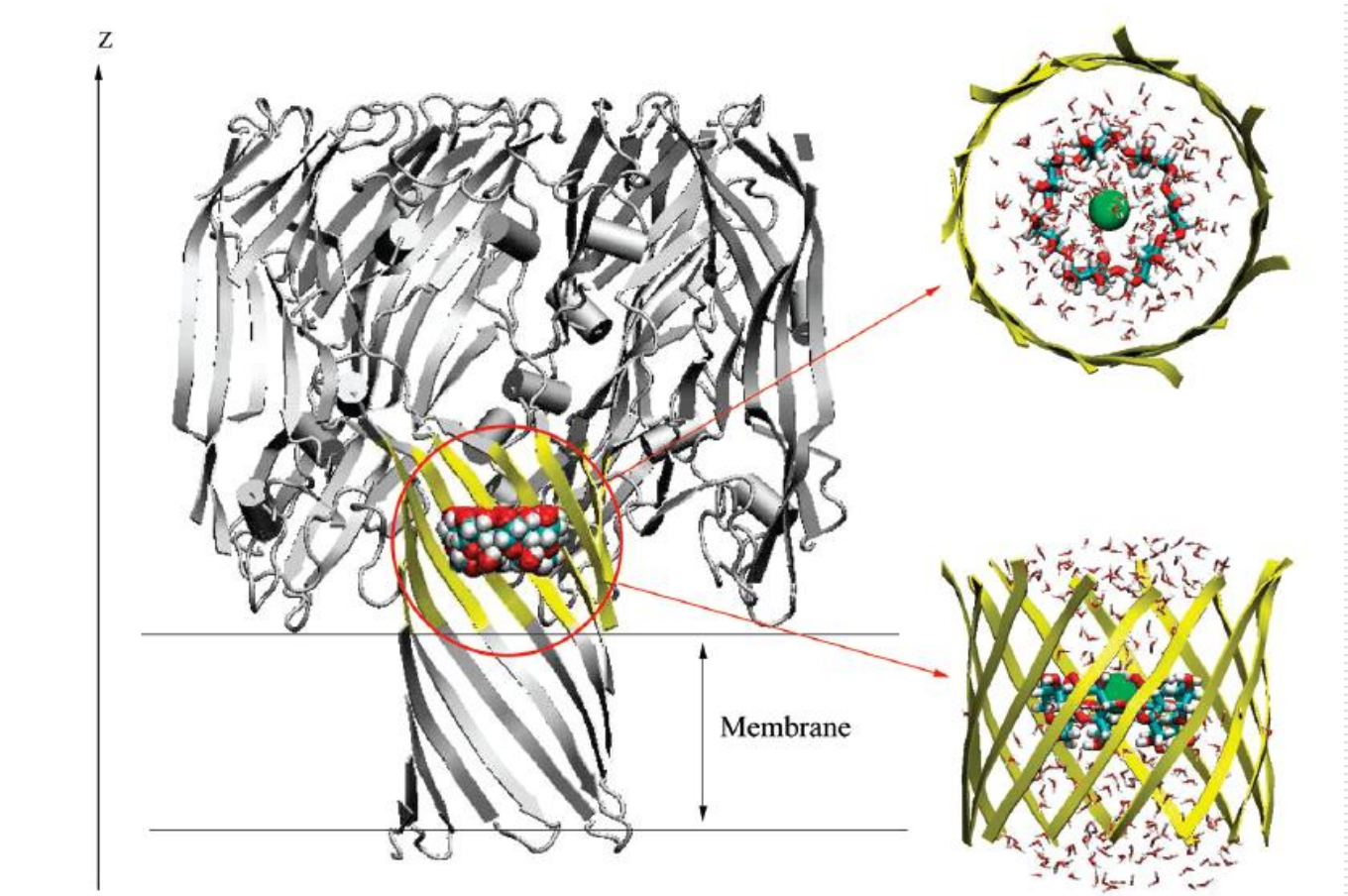


- A protein-nanopore stochastic sensor works by monitoring the ionic current modulations induced in real time by the diffusion of analytes of interest through a single pore, which can be constructed from a biological ion channel - usually α-HL - self-assembled on a fabricated lipid membrane (***note that nanopore experiments have a measurement noise of ~1 pA and a sub-millisecond time resolution in kHz bandwidths***).

- The frequency of occurrence of the current fuctuations reveals the concentration of the analyte, whereas its identity can be deduced from the characteristic magnitude and/or duration of the current fluctuations.

- The use of such biological nanopores for biosensing spans the detection of terrorist agents including explosives, organophosphorus nerve agents, nitrogen mustards, organoarsenic compounds, toxins, viruses, etc.

Molecular graphics view of the simulation system for the $(M113N)_7$ α-HL channel with a cyclodextrin molecule inside

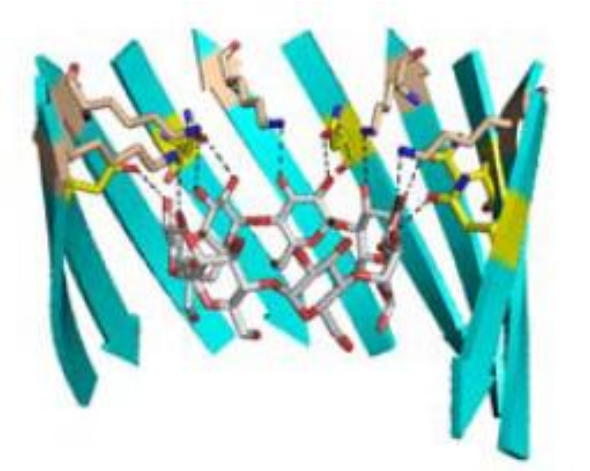


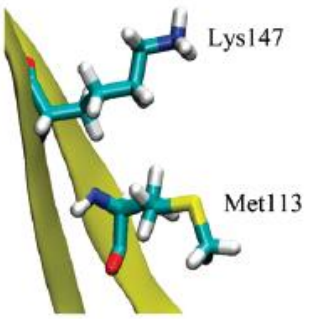
Adapted from Luo Y et al., *J. Phys. Chem. B* 2010, *114,* 952–958

Molecular details about the interaction situs between β-cyclodextrin and the inside of α-HL protein

Site-directed mutagenesis suggest that β-cyclodextrin binds near Met 113 in wild type α-HL and $(M113N)_7$ releases β-cyclodextrin about 104 times more slowly (Gu L-Q. et al., 1999. Nature 398: 686–690).

In the case of M113N7, β-cyclodextrin is bound with the secondary hydroxyl face "upward" and in each glucose unit of the βCD, the 2-hydroxyl is hydrogen bonded to the side-chain amide of an Asn-113 and the 3-hydroxyl is hydrogen bonded to the ϵ-amino group of Lys-147. (Banerjeea A. et al., PNAS Early Edition, 2010)

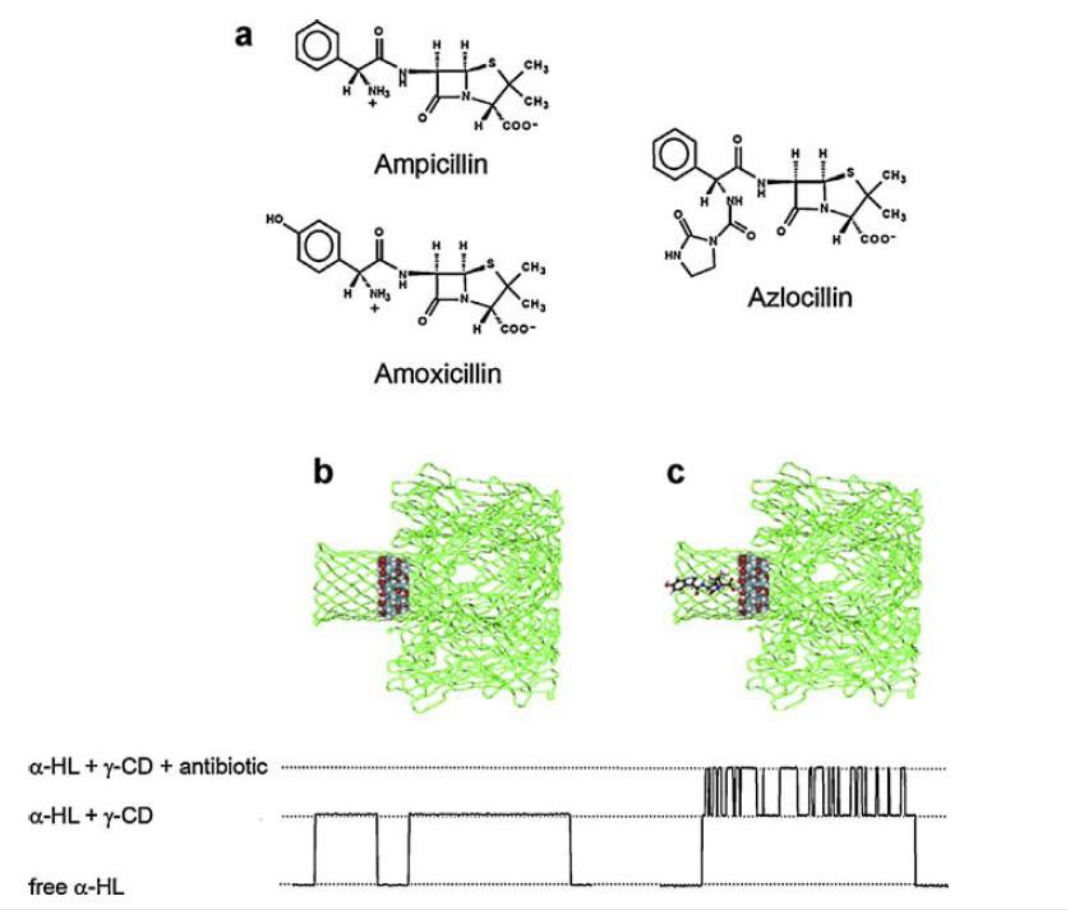




wt-αHL

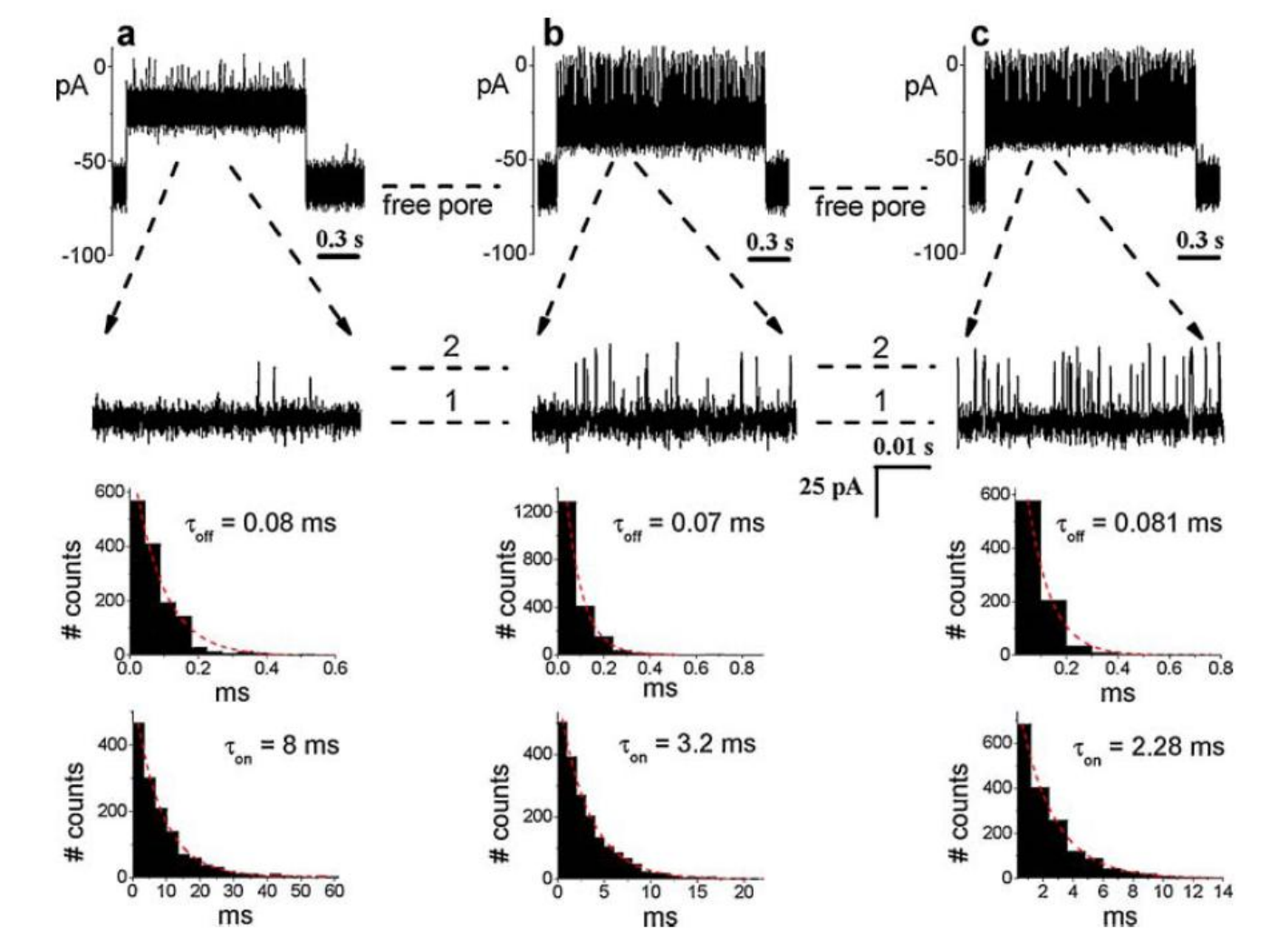
$(M113N)_7$

How stochastic sensing can be achieved: sketchy representation of the uni-molecular interaction between the γ-CD, a bilayer-inserted α-HL protein and antibiotic molecules

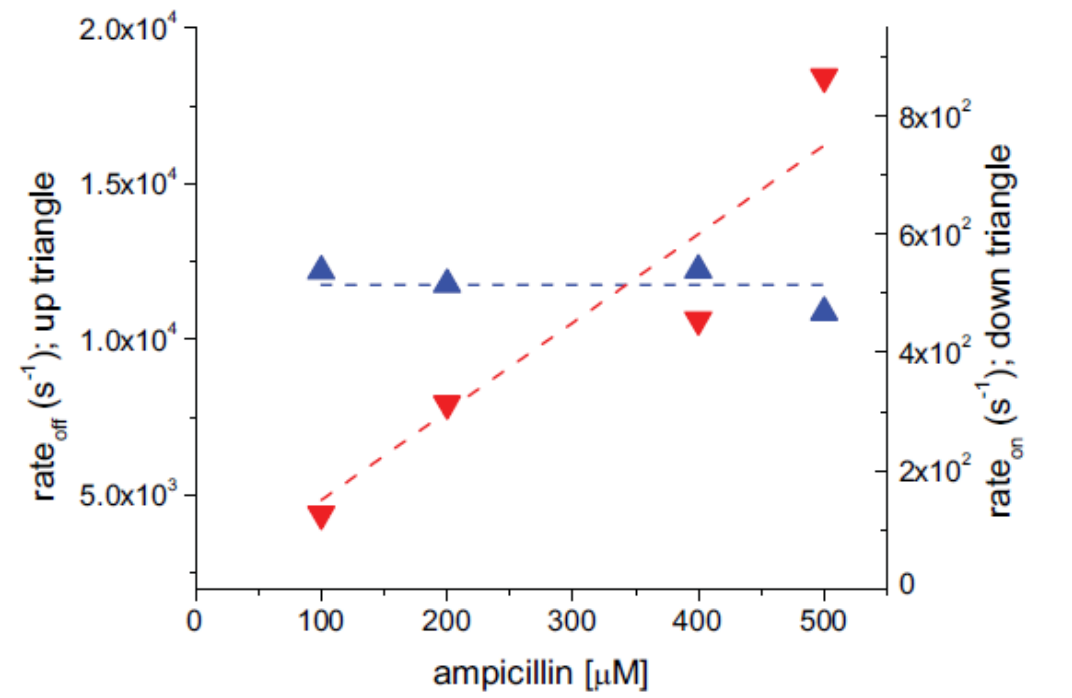


A. Asandei, A. Apetrei A, and T. Luchian, Journal of Molecular Recognition, 2010, in press

Representative current traces and dwell-times histograms for the response of the α-HL–γ-CD complex to (a) 100 μM, (b) 200 μM, and (c) 400 μM ampicillin, recorded at -90 mV



Quantitative kinetic description of ampicillin interaction with the α-HL–γ-CD complex

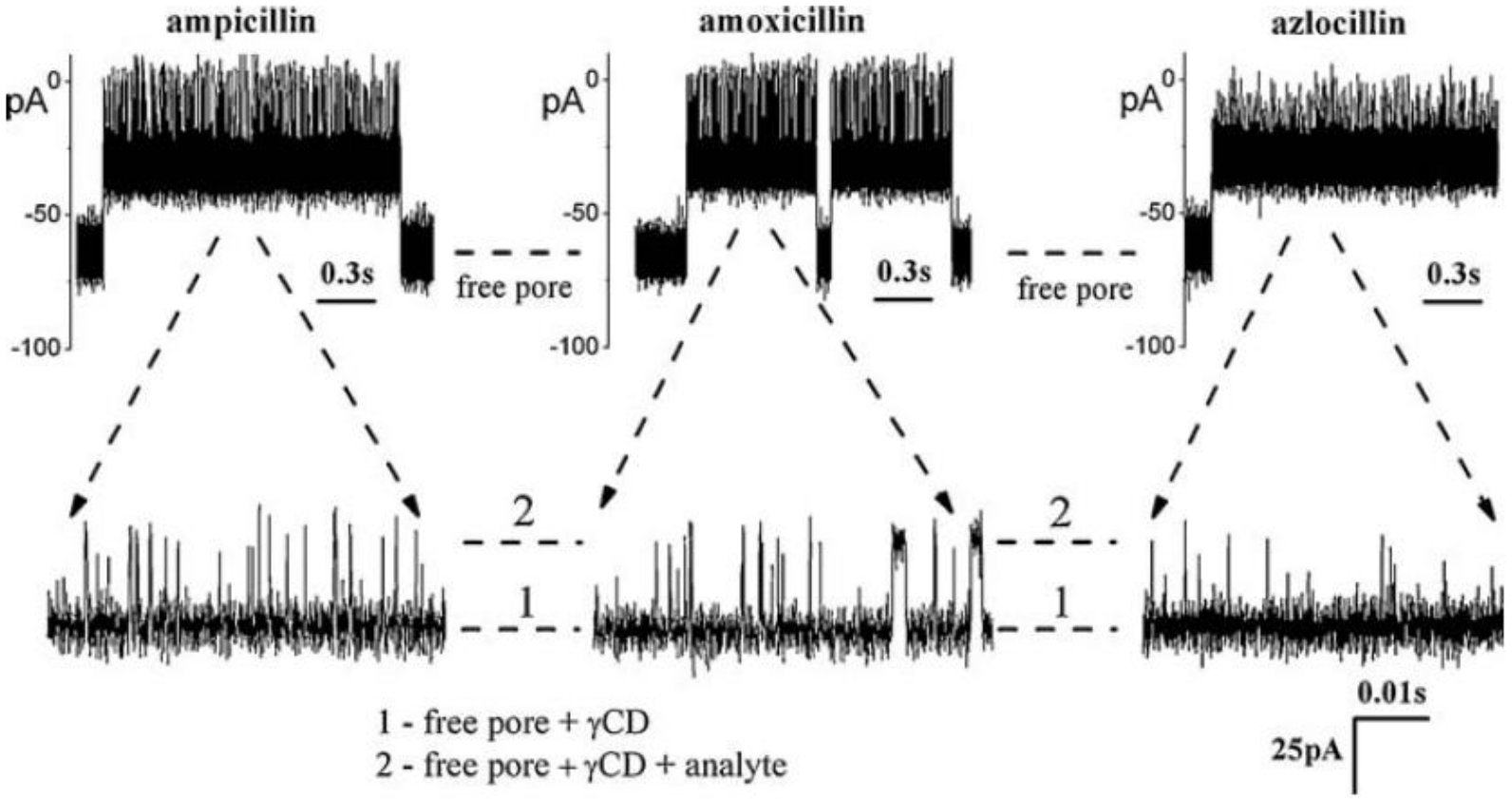


The rate constant k_{on} was obtained from the slope of the linear fit of $rate_{on}$ versus ampicillin concentration.

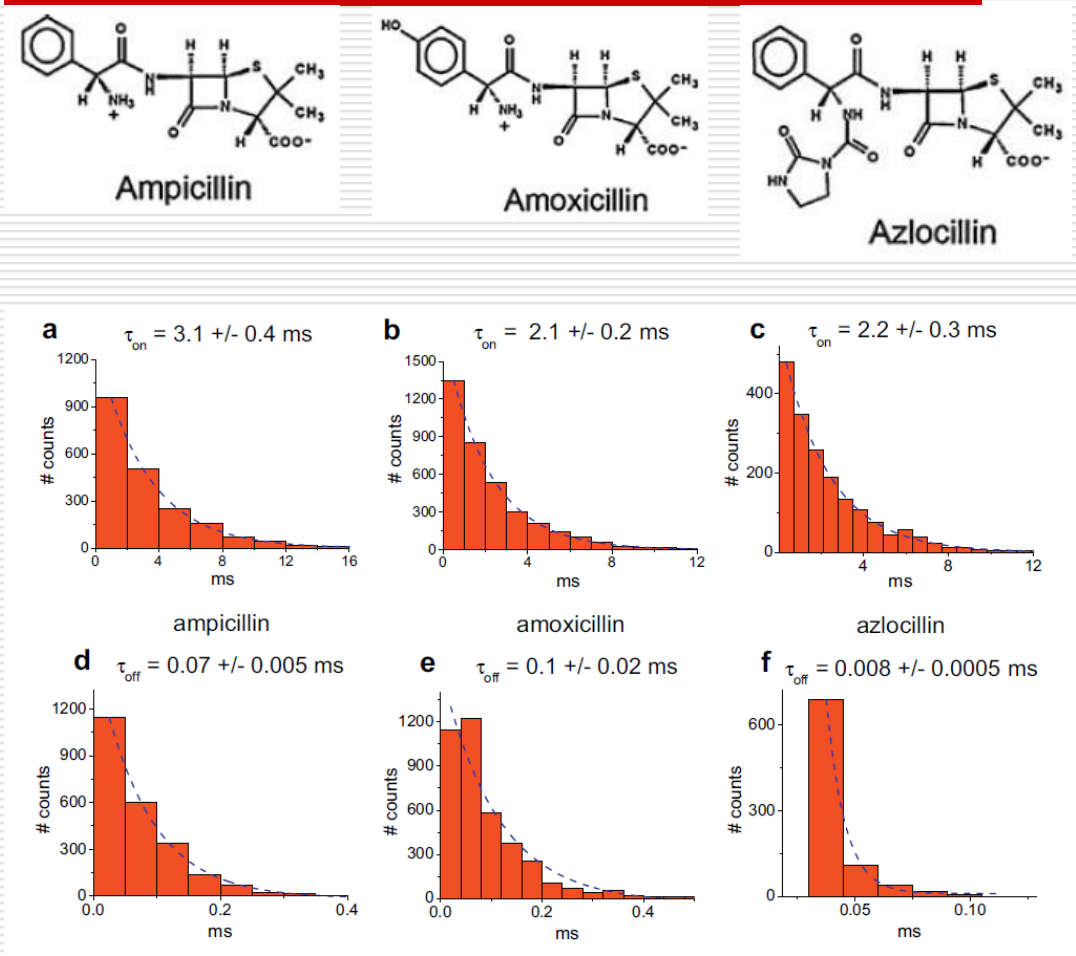
The rate constant k_{off} is independent of ampicillin concentration, and by virtue of the simple bimolecular model taken into account it equals $rate_{off}$.

$$\Delta G = RT \ln\left(\frac{k_{on}}{k_{off}}\right)$$

Stochastic sensing of 200 μM of ampicillin, amoxicillin, and azlocillin added on the trans side of the membrane, by employing a γ-CD molecule temporarily lodged into a single wild-type α-HL protein, at an applied potential of -90 mV.



Representative dwell-time histograms used to characterize the distinct kinetic signature of β-lactam antibiotics interacting reversibly with a single α-HL–γ-CD complex



The fact that the association average time (τ_{on}) of amoxicillin and azlocillin is smaller that that of ampicillin might reflect γ–CD-imposed steric hindrance or an energetically more expensive desolvation step for the binding site access inside the γ-CD nanocavity.

$$CD \cdot gH_2O + H \cdot hH_2O \rightleftharpoons H \cdot CD \cdot (g+h-i)H_2O + iH_2O$$

What about τ_{off}–? The stronger the interaction of the analyte within the nanocavity, the shorter its residence time !

A. Asandei, A. Apetrei A, and T. Luchian, Journal of Molecular Recognition, 2010, in press

…almost there…

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The team involved in the bench-work

In Person: A Dream Lab in Romania, *Science (Science Careers)*, 326(5959), 2009