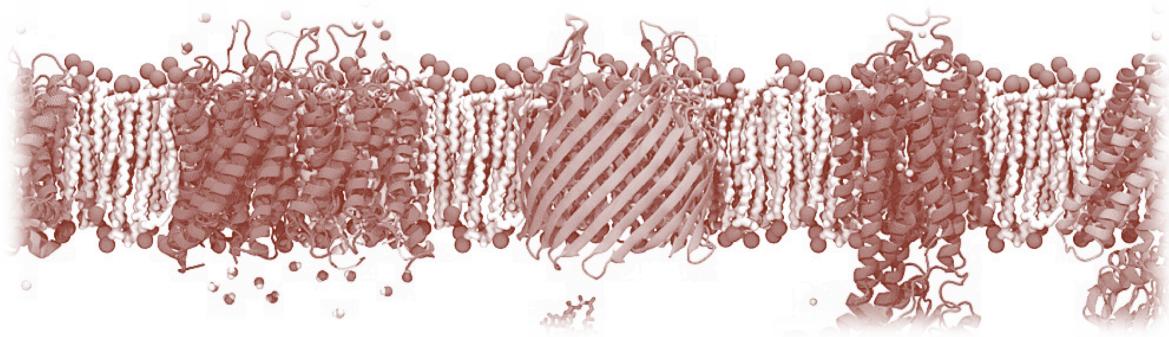


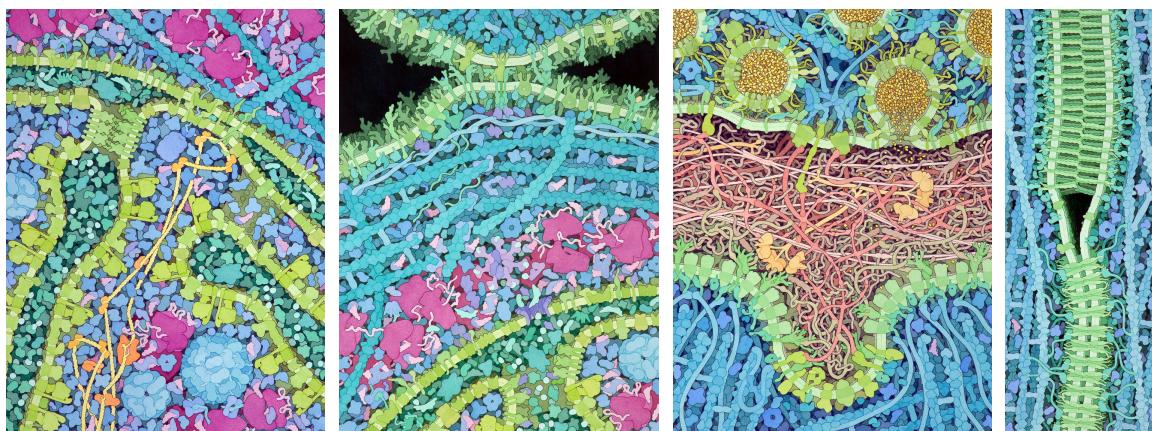


Membrane Proteins: Structural & Functional Challenges

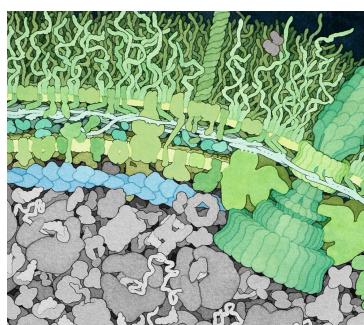
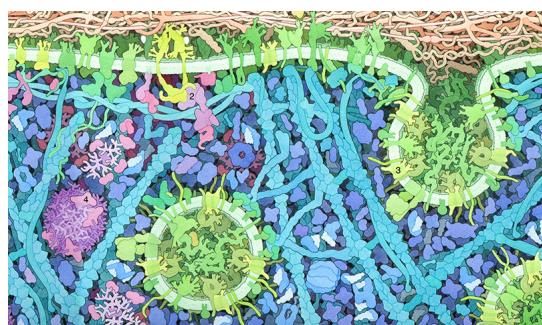


Susana Andrade
Institute of Biochemistry
University Freiburg

Diverse roles and structures



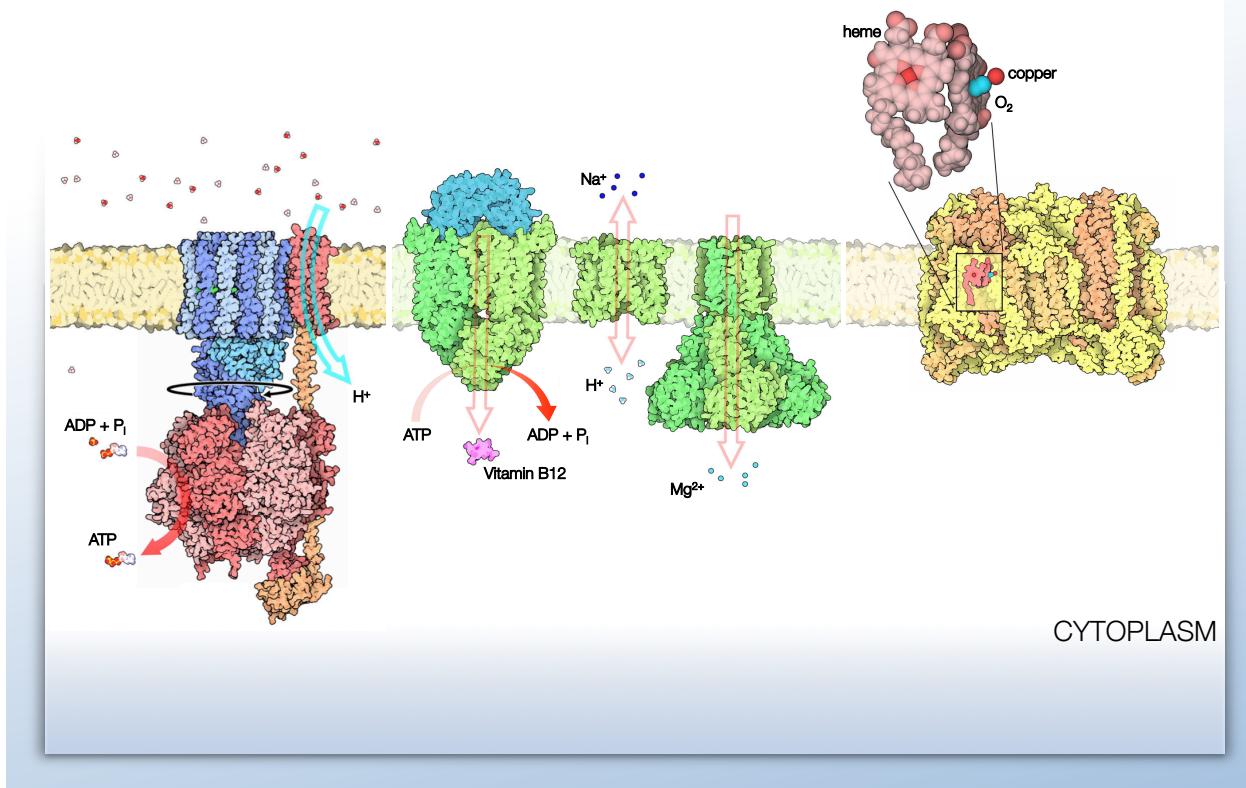
UNI
FREIBURG



Transport
Enzymatic activities
Signal transduction
Intercellular junctions
Cell-cell recognition
Cell shape
Membrane dynamics

The Machinery of Life,
David Goodsell

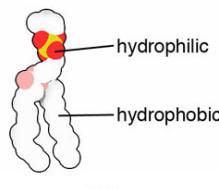
Zooming in



The membrane environment

Composition

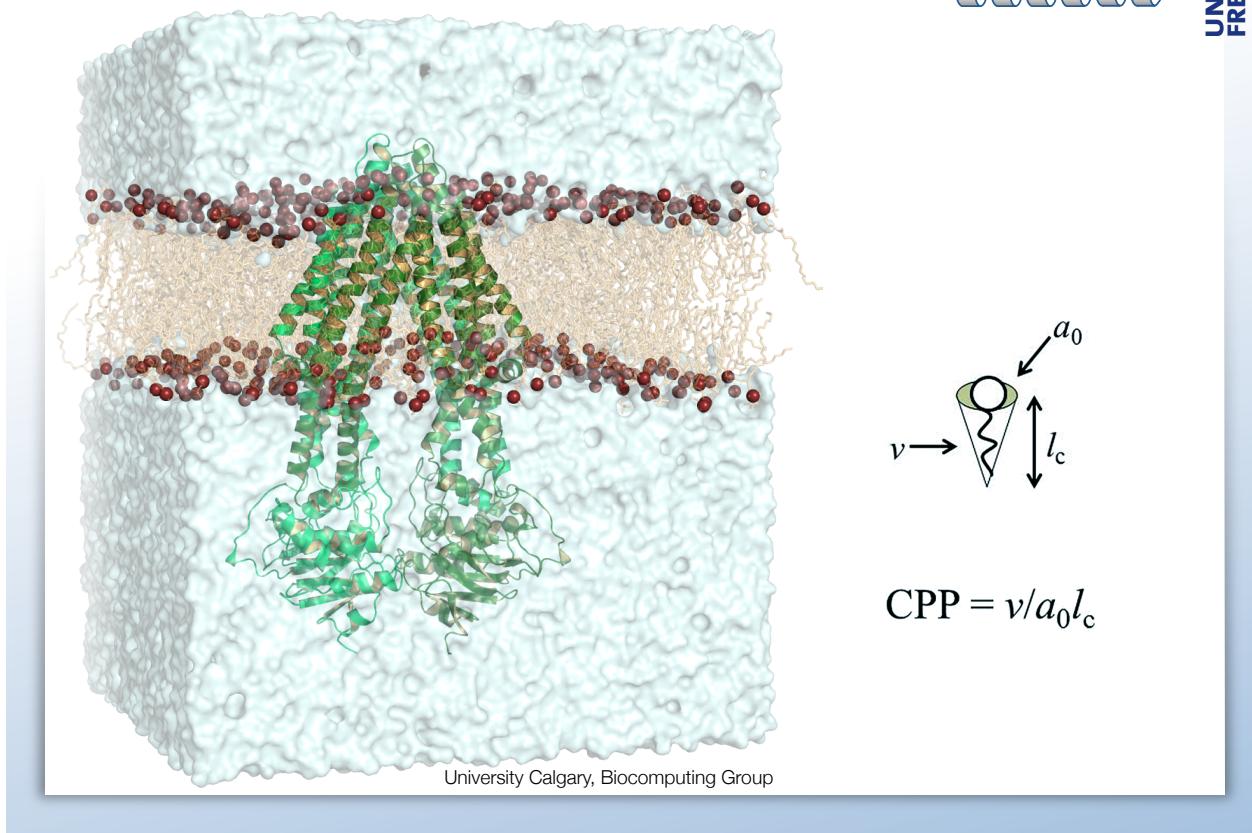
- **Glycerophospholipids**
 - Phosphatidic acid
 - Phosphatidylinositol
 - Phosphatidylserine
 - Phosphatidylcholine
 - Phosphatidylethanolamine
 - Phosphatidylglycerol
 - Diphosphatidylglycerol
 - Variable size and saturation of the aliphatic chains
 - (...)
- **Glyceroxyglycolipids**
 - (...)
- **Sphingophospholipids**
 - (...)
- **Sphingoglycolipids**
 - (...)
- **Sterols**
 - (...)
- **Others**
 - Dolichols
 - (...)



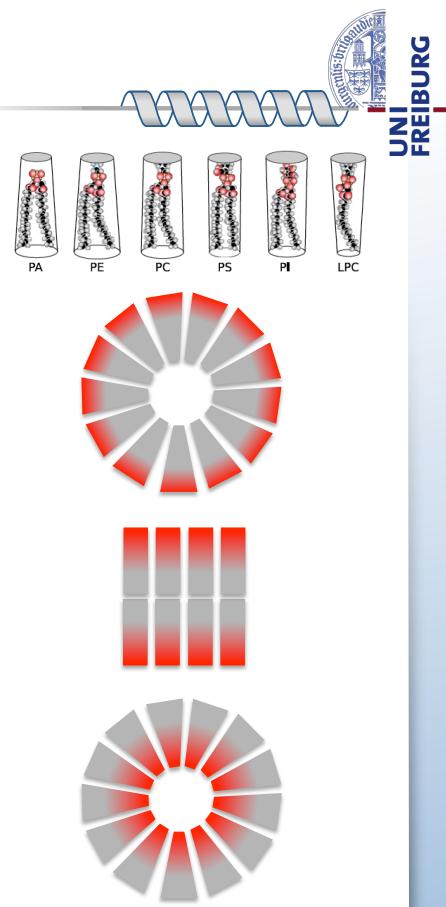
Function

- **Barriers**
 - Create variable compartments
- **Support (for membrane proteins)**
 - Selective barrier, membrane curvature, inner/outer leaflet properties
- **Modulate protein function**
 - Act as cofactors
 - Transverse forces (hydrophobic mismatch)
 - Lateral forces
 - Lipid rafts
- **Signaling**
 - PI, DAG, ceramide, PS, (...)
- **Reservoir of lipids for the cell**
 - Energy, Signal molecules/precursors (...)
- **Others**
 - (...)

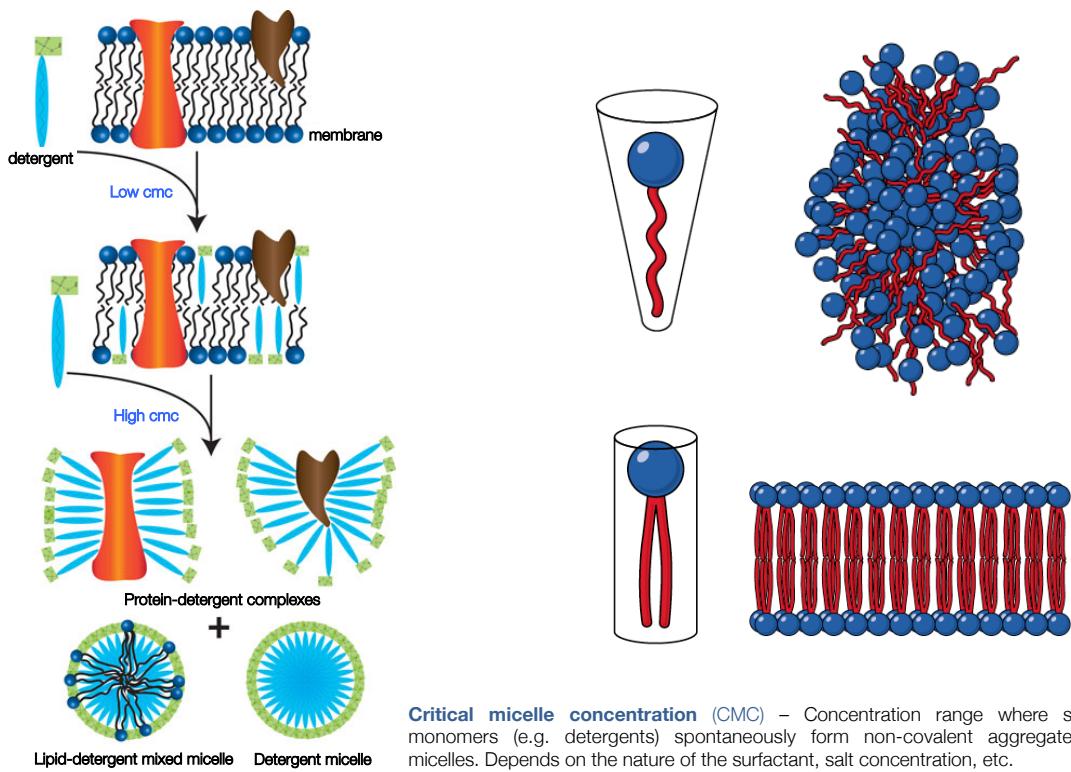
Surfactants as tools



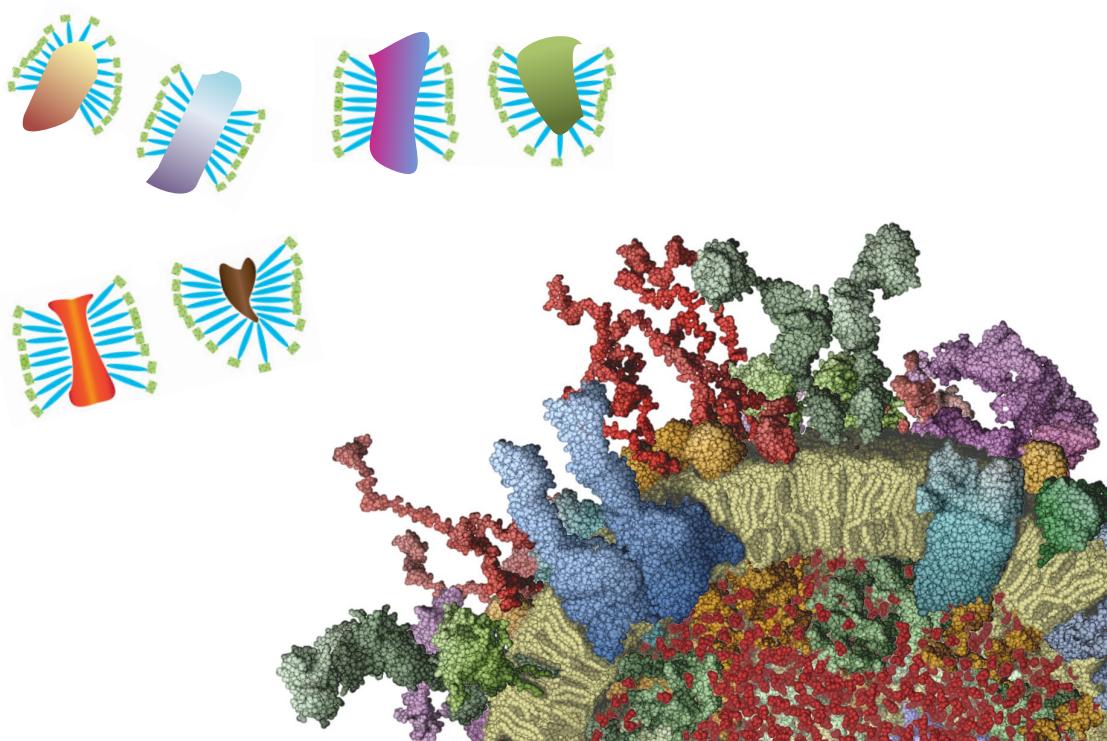
Critical Packing Parameter ($v/a_0 l_c$)	Critical Packing Shape	Structures Formed
$< 1/3$	Cone	Spherical micelles
$1/3 - 1/2$	Truncated cone	Cylindrical micelles Flexible bilayers, vesicles
$1/2 - 1$	Truncated cone	Planar bilayers
~ 1	Cylinder	
> 1	Inverted truncated cone or wedge	Inverted micelles



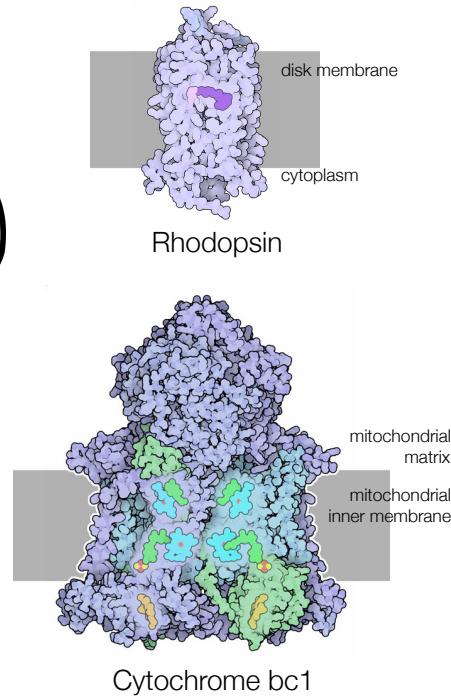
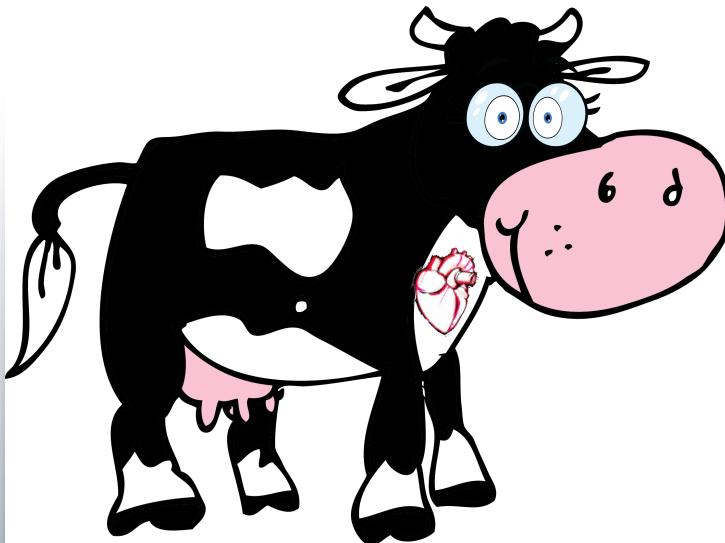
Solubilizing membrane proteins



Purifying membrane proteins



Purifying membrane proteins



Producing (membrane) proteins



To produce the protein encoded by a piece of cloned DNA, expression plasmids/vectors are required:

- **Promoter (inducible vs constitutive)** : allows regulating amount and time of protein expression
- **Antibiotic**: allows selecting cells carrying the plasmid
- **Ori**: origin of replication/replicon is the place where DNA replication begins, enabling a plasmid to reproduce itself
- **Multiple cloning region**: short segment of DNA with multiple restriction sites. It allows inserting a gene at a precise position.
- ...

❖ Common DNA sources and delivery mechanisms are **plasmids**, **viruses** (e.g. baculovirus, retrovirus, adenovirus), **artificial chromosomes** and **bacteriophage** (such as lambda).

Producing (membrane) proteins



Cell-based expression system:

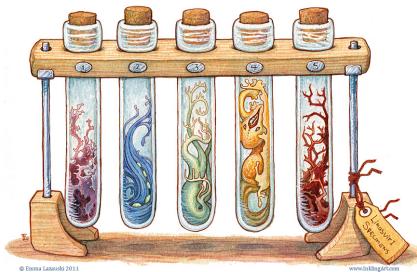
- The plasmid is placed *inside a cell*

Common hosts are **bacteria** (e.g. *E. coli*, *B. subtilis*), **yeast** (e.g. *S. cerevisiae*, *P. pastoris*), **eukaryotic cell lines** (HeLa, HEK), (...).



The best expression system depends on the characteristics of the protein to produce:

- Bacterial – can produce large amounts of protein. Post-translational modifications and folding (inclusion bodies) can be a problem.
- *S. cerevisiae* – when significant post-translational modifications are required.
- Insect or mammalian cell lines – for human-like splicing of mRNA. Glycosylation, (...).
- (...)



Cell-free expression system:

- *in vitro*, with purified RNA polymerase, ribosomes, tRNA and ribonucleotides.

Producing (membrane) proteins



(...)

RNA polymerase will produce mRNA.

Ribosomes translate mRNA into a **protein**.

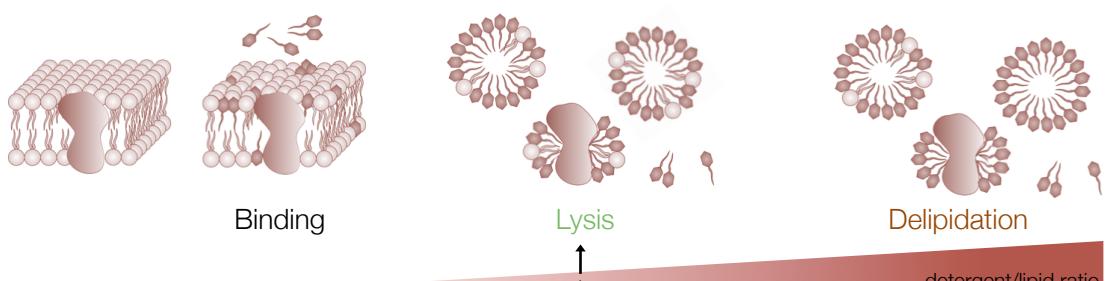
- Clone (homolog genes)
- Clone (plasmid/promoter type)
- Express (host cells, growth conditions)
- Protein targeting, yield
- Protein functionality
- Protein purification scheme



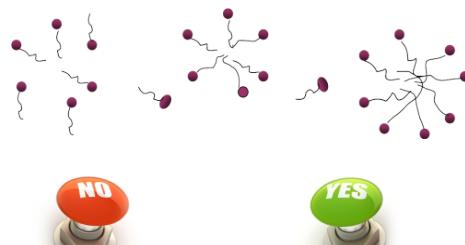
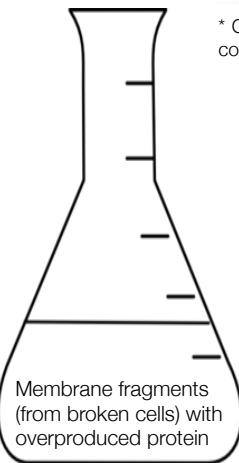
Epitope tags (portion of a molecule where antibody binds) can be added to help visualization by western blot or immunofluorescence.

Peptides can be added to increase solubility and detection (e.g. MBP, GFP)...

Purifying membrane proteins

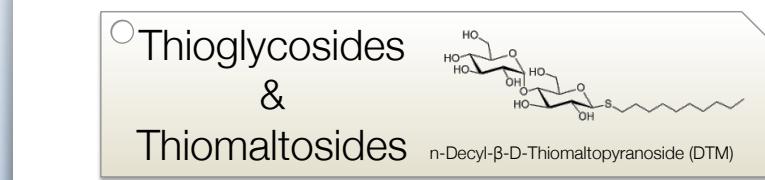
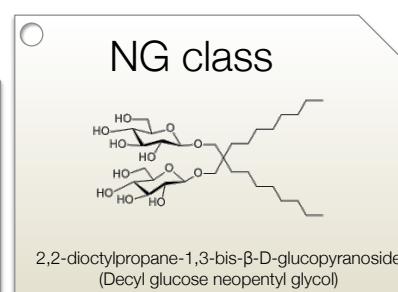
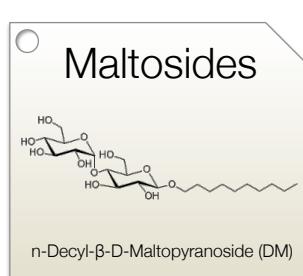
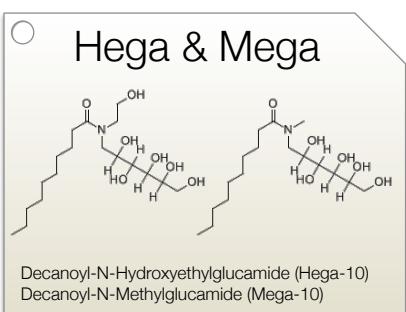
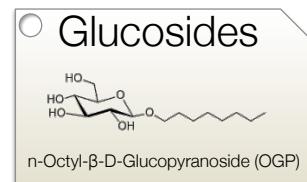
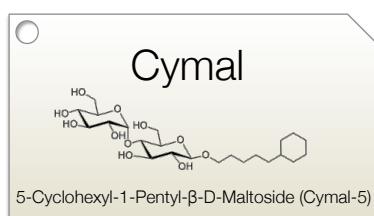
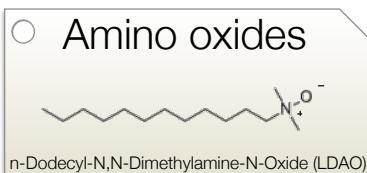


* Critical solubilization concentration – Exact value depends on the nature of the detergent, the nature and concentration of lipids, the protein concentration, temperature, buffer conditions, etc.



Critical micelle concentration (CMC) – Concentration range where surfactant monomers (e.g. detergents) spontaneously form non-covalent aggregates called micelles. Depends on the nature of the surfactant, salt concentration, etc.

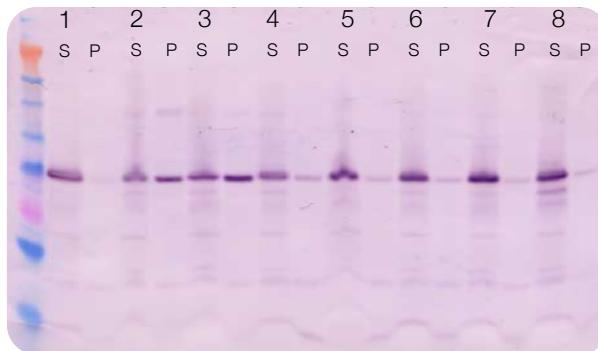
The detergent variable



The detergent variable

Solubilization test:

e.g. Western blot* of solubilized (S) and un-solubilized (P) membranes



- 1: SDS
- 2: HEGA-10
- 3: C₈E₄
- 4: LDAO
- 5: DDM
- 6: Triton X-100
- 7: FOS-CHOLINE-12
- 8: OGP

Epitope tags (portion of a molecule where antibody binds) can be added to help visualization by western blot or immunofluorescence.

Peptides can be added to increase solubility and detection (e.g. MBP, GFP)...

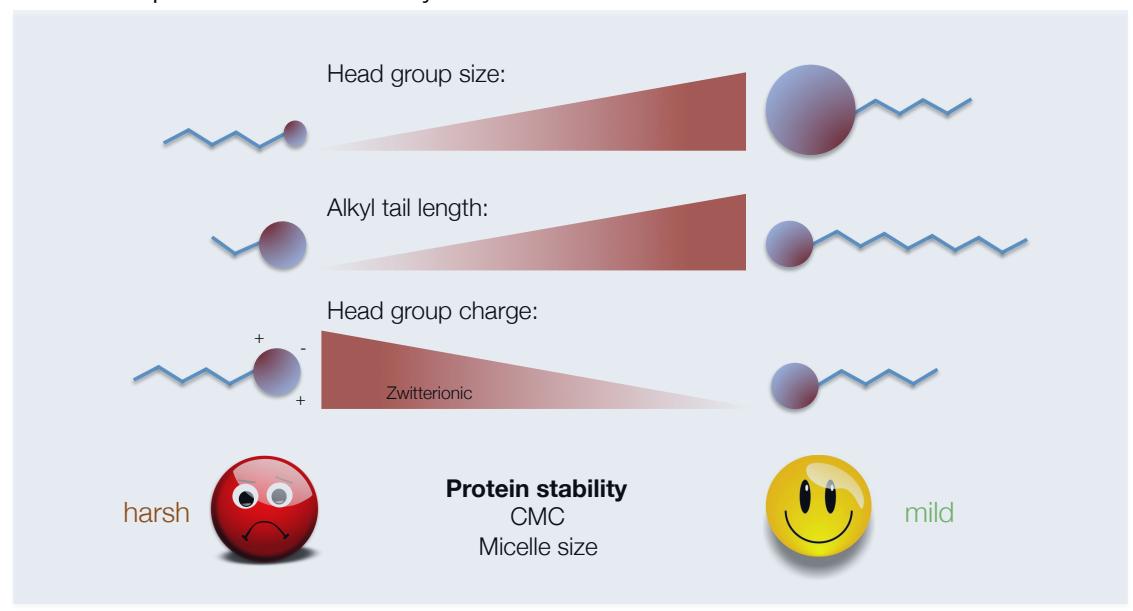
*Rath et al (2007), PNAS, 106:1760

The detergent variable

1. Protein solubilization ✓

GOAL – Extract efficiently the target membrane protein and keep it stable in solution.

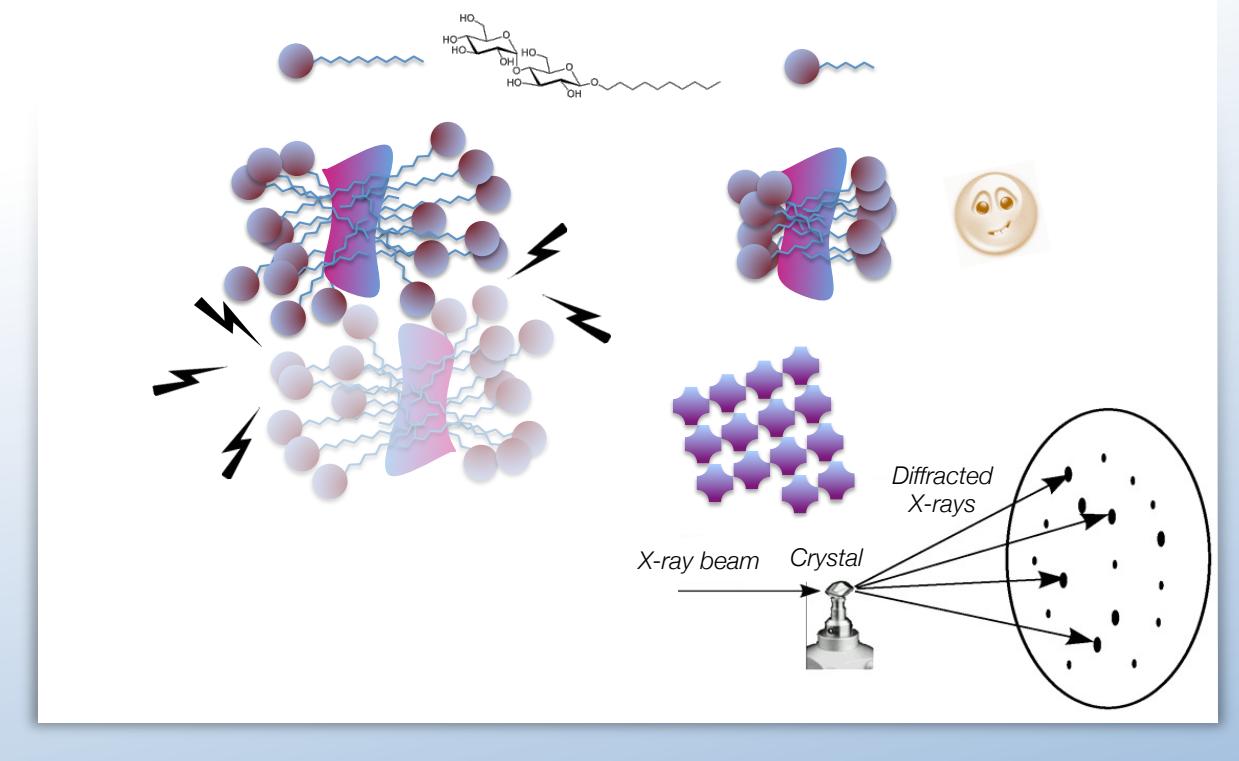
2. Protein purification & stability



The beauty and the beast

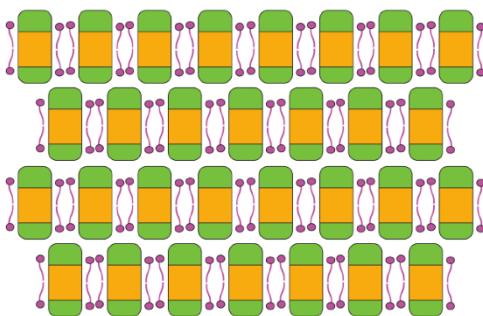
3. Protein characterization

- *X-ray crystallography*

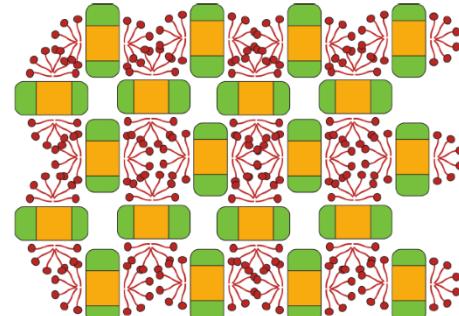


Types of membrane protein crystals

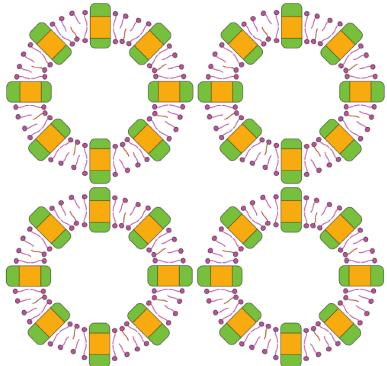
- Type I



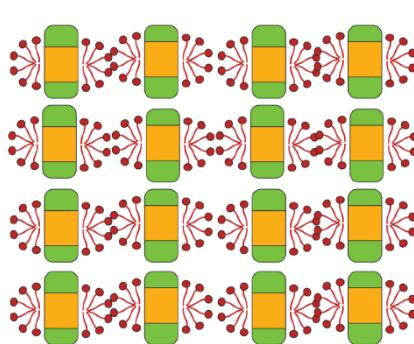
- Type II



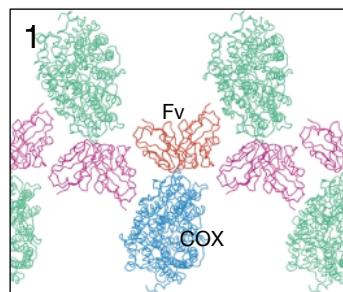
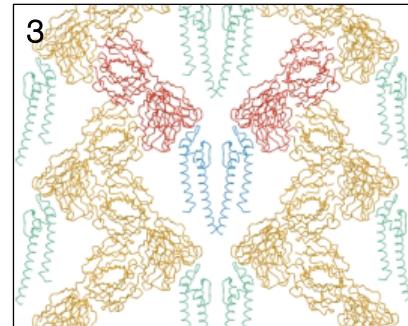
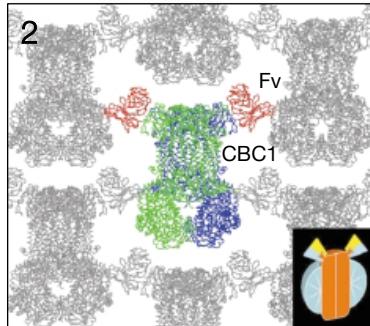
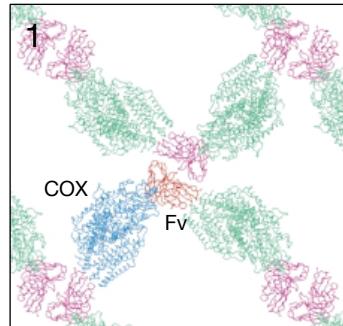
- Type III



- Type IV

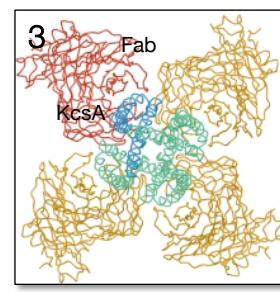


Monovalent antibody fragments can be generated recombinantly as **Fv** (fragment variable ~28 kDa) or **Fab** (fragment antibody binding ~56 kDa) or by proteolytic cleavage.

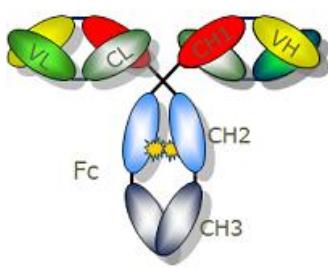


Crystal lattices of:
 (1) four cytochrome c oxidase subunits with recombinant **Fv** fragment;
 (2) (2) cytochrome bc1 complex with **Fv** fragment bound to the catalytic Rieske protein subunit;
 (3) (3) KcsA in complex with a **proteolytic Fab** fragment

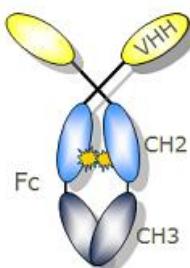
In: Hunte & Michel (2002) *Cur Op Struct Biol*, 12:503



Conventional antibody



Camel Heavy-Chain antibody



VHH or Nanobody



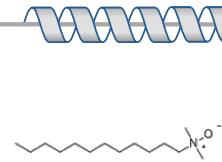
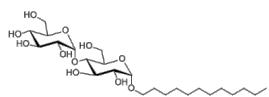
Recombinant Nanobodies are small (15kDa), monomeric, bind target with nM affinity, are stable, easy to manipulate and are well expressed in bacterial expression systems so that they are cheaper and easier to produce in all kind of formats than standard monoclonal antibodies.

Nanobodies often bind to epitopes that are less immunogenic for conventional antibodies, such as the active sites of enzymes.

Due to their small size, they also target areas that are not accessible to standard antibodies.

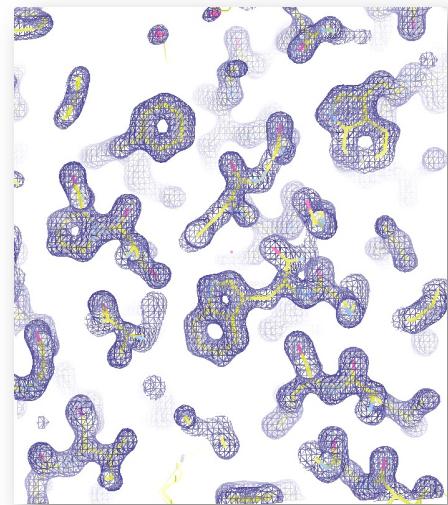
Another advantage is that they generally bind conformational epitopes

High-resolution protein crystal structures



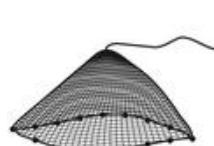
- Identify residues and elements
- Detect conformational changes
- Discuss molecular interactions
- Visualize pores/channels/cavities
- Reveal lipid/detergent binding sites
- Identify functional residues & locations (active site, selectivity filter, gating, ...)

- High level of disorder due to high flexibility



Electron density map of Af-Amt1 at 1.3 Å resolution.

Never give up



Proteins are dynamics entities!

Molecular Architecture of the KvAP Voltage-Dependent K⁺ Channel in a Lipid Bilayer

Luis G. Cuello, D. Marien Cortes, Eduardo Perozo*

We have analyzed the local structure and dynamics of the prokaryotic voltage-dependent K⁺ channel (KvAP) at 0 millivolts, using site-directed spin labeling and electron paramagnetic resonance spectroscopy. We show that the segment is located at the protein/lipid interface, with the side chains protected from the lipid environment. *Structure* 2011, 19, 1302–1305. *Angew. Chem. Int. Ed.* 2011, 50, 1302–1305. *doi: 10.1002/anie.201005212*

Protein Structures

Conformation of a Seven-Helical Transmembrane Photosensor in the Lipid Environment**

Lichi Shi, Izuru Kawamura, Kwang-Hwan Jung, Leonid S. Brown,* and Vladimir Ladizhansky*

(SSNMR) has emerged as one

of the main tools for struc-

ture and dynamics of mem-

brane proteins in the

biologically and medi-

cal environments. Herein we

add to our understand-

ing of the structure and

function of membrane pro-

teins by reporting the first

solid-state NMR study of a

sensory rhodopsin

in its native state. ASR reconstituted in lipids gives well-

resolved spectra with high signal-to-noise ratios, with typical

carbon and nitrogen line widths of 0.5 ppm (Figure 1 and

Figure S1 in the Supporting Information). The protein

is stable in this environment. *Angew. Chem. Int. Ed.* 2011, 50, 1302–1305. *doi: 10.1002/anie.201005212*

ARTICLE

05 DECEMBER 2013 | VOL 504 | NATURE | 107

Structure of the TRPV1 ion channel determined by electron cryo-microscopy

Maofu Liao*, Erhu Cao*, David Julius² & Yifan Cheng¹

Transient receptor potential (TRP) channels are sensors for a wide range of cellular and environmental signals, but elucidating how these channels respond to physical and chemical stimuli has been hampered by a lack of detailed structural information. Here we exploit advances in electron cryo-microscopy to determine the structure of a mammalian TRP channel, TRPV1, at 3.4 Å resolution, breaking the side-chain resolution barrier for membrane proteins without crystallization. Like voltage-gated channels, TRPV1 exhibits four-fold symmetry around a central ion pathway formed by transmembrane domains 1–4 (residues 185–360) and a flanking pore-forming loop, which is flanked by the voltage-sensing and nucleotide-binding domains. TRPV1 has a wide cytoplasmic linker with a short selective patch and a long “linker” domain. The linker domain interacts with the S4–S5 linker, consistent with its contribution to allosteric modulation. Subunit organization is facilitated by interactions among cytoplasmic domains, including amino-terminal ankyrin repeats. These observations provide a structural blueprint for understanding unique aspects of TRP channel function.

nature nanotechnology

PUBLISHED ONLINE: 8 JULY 2012 | DOI: 10.1038/NNANO.2012.89

LETTERS

Characterization of the motion of membrane proteins using high-speed atomic force microscopy

Ignacio Casuso¹, Jonathan Khao², Mohamed Cham³, Perrine Paul-Gilloteaux⁴, Mohamed Husain¹, Jean-Pierre Duneau², Henning Stahlberg⁵, James N. Sturgis² and Simon Scheuring^{1*}

For cells to function properly, membrane proteins must be able to diffuse within biological membranes. The function of these proteins depend on their position and also on protein–protein and protein–lipid interactions¹. However, we have not been possible to study simultaneously the structure and dynamics of biological membranes. Here, we show that high-speed atomic force microscopy² can be characterized by unlabelled membrane proteins can be characterized by high-speed atomic force microscopy³. We find that

proteins. We used cross-correlation-based algorithms^{4,5} to analyse membrane organization and the motion of individual molecules in each frame (750 Å)². The particle location and orientation were defined with Angström precision and with a time resolution of 477 ms over a period of minutes (Fig. 1b,c; technical details can be found in the Materials and Methods section in the Supplementary Information).

We first used fractal analysis to provide a quantitative assessment

Low-Resolution Structures of OmpA-DDM Protein-Detergent Complexes

Jørn Døvling Kaspersen,^[a, b] Christian Moestrup Jessen,^[a, b] Brian Stougaard Vad,^[a, b] Esben Skipper Sørensen,^[a, d] Kell Kleiner Andersen,^[a, c] Marianne Glasius,^[a, b] Cristiano Luis Pinto Oliveira,^[a, d] Daniel Erik Otzen,^[a, c, d] and Jan Skov Pedersen^[a, b]

ChemBioChem 2014, 15, 2113–2124

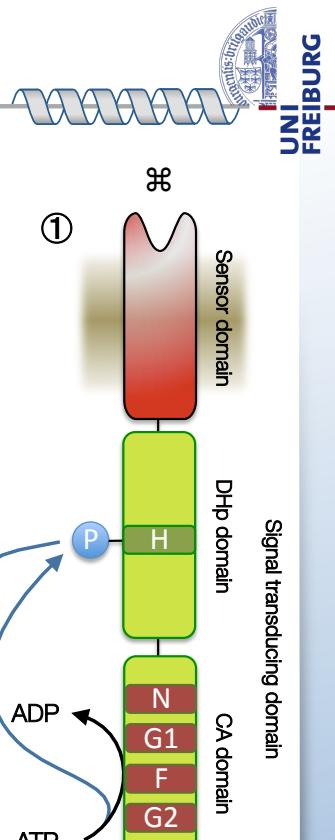
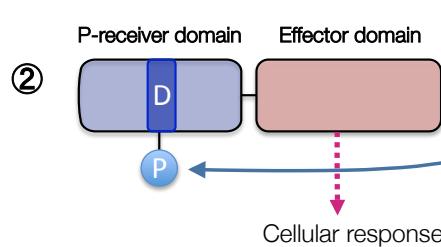
doi:10.1002/cbic.201402162

Highly flexible proteins ...

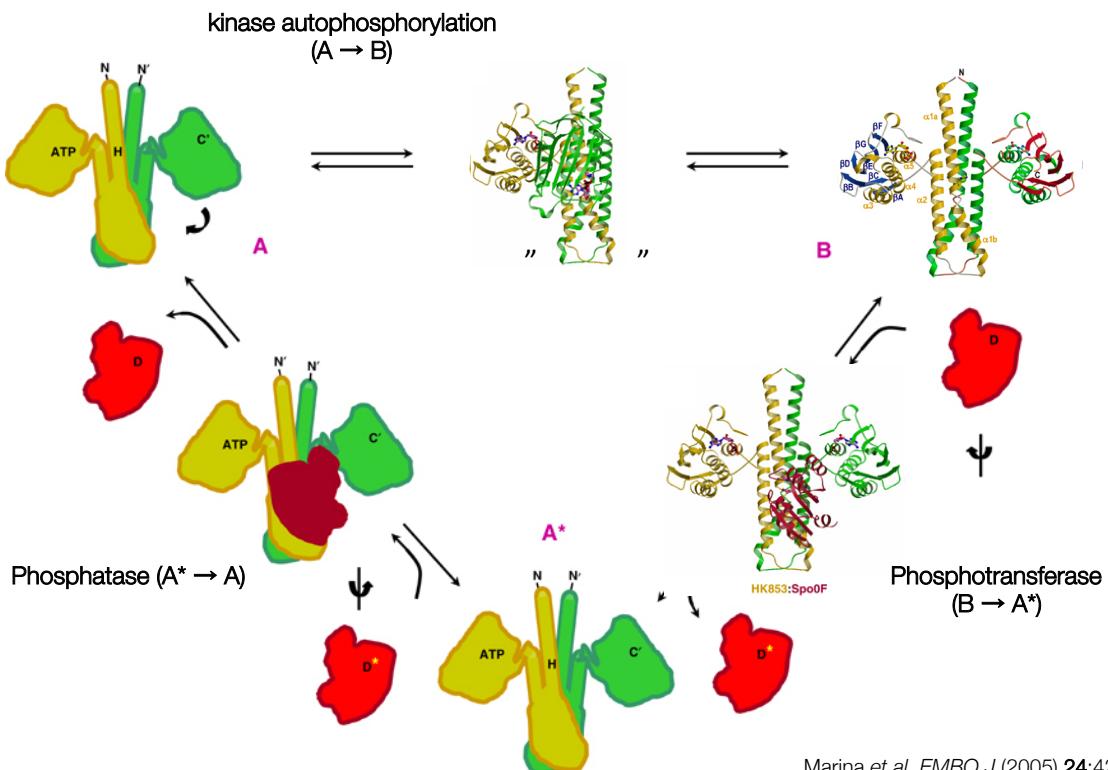
Two-component signal transduction systems (TCS):

① - Sensor histidine kinase

② - Response regulator



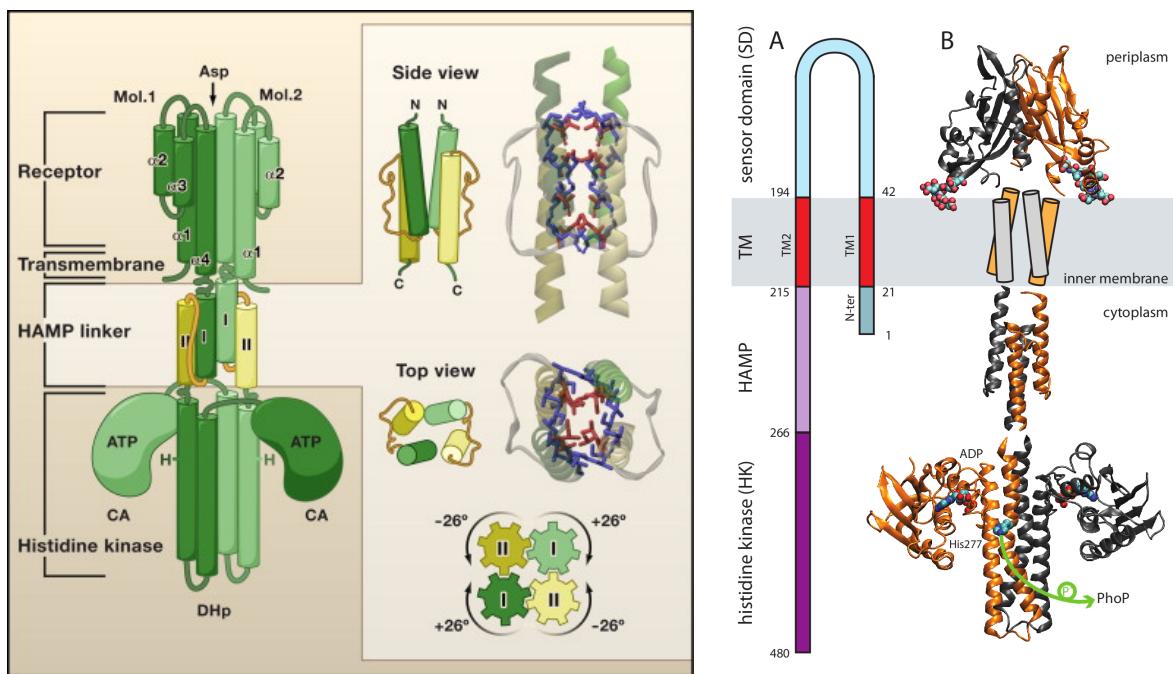
Structure-based scheme of the HK reactions



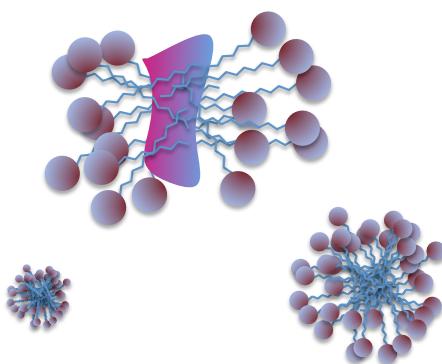
Marina et al, *EMBO J* (2005) 24:4247

How is signal transmitted after reception?

E.g. – HAMP domains are typically associated with membrane domains and relay extracellular signals into intracellular responses. A unifying mechanism for HAMP domain signal transduction has yet to emerge, mainly due to lack of structural information.

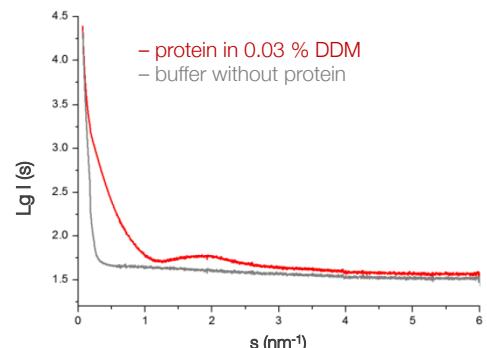
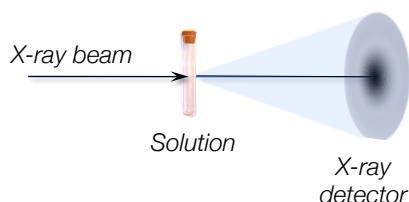


SAXS of the full-length sensor histidine kinase



Protein is pure, stable and reveals an homogeneous trimeric form in buffer containing 10 % glycerol and 0.03 % DDM.

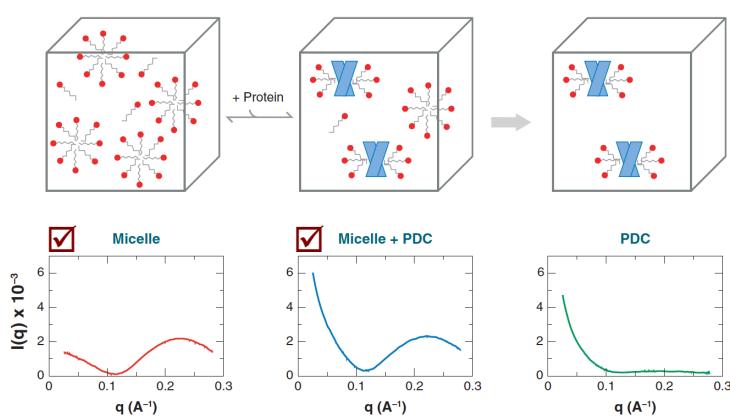
Dmitri Svergun, EMBL, Hamburg



The beauty and the beast



How to get rid of detergent backscattering from protein scattering?



Adapted from:
Lipfert & Doniach (2007)
Ann Rev Biophys Biomol Struct 36:307.

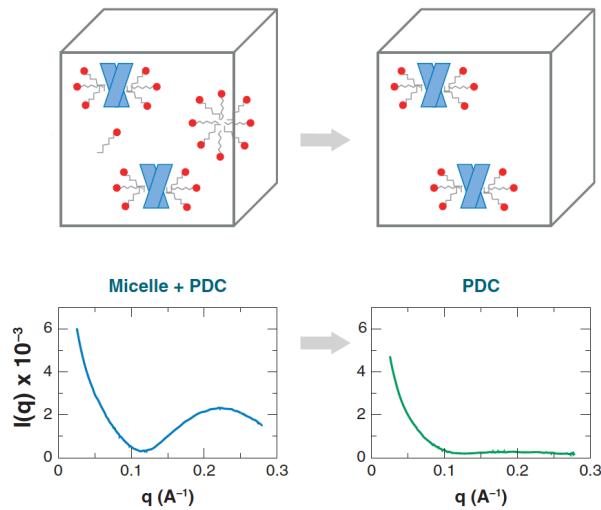
- ➡ “Density matching” – Match the scattering density (contrast) of the solvent to that of the detergent..
- ➡ “Subtracting micellar scattering” – Separate the contributions of the detergent micelles in the presence and absence of protein..
- ➡ “Singular value decomposition” – Collect data at various protein:detergent ratios and apply a global fitting procedure..

The beauty and the beast

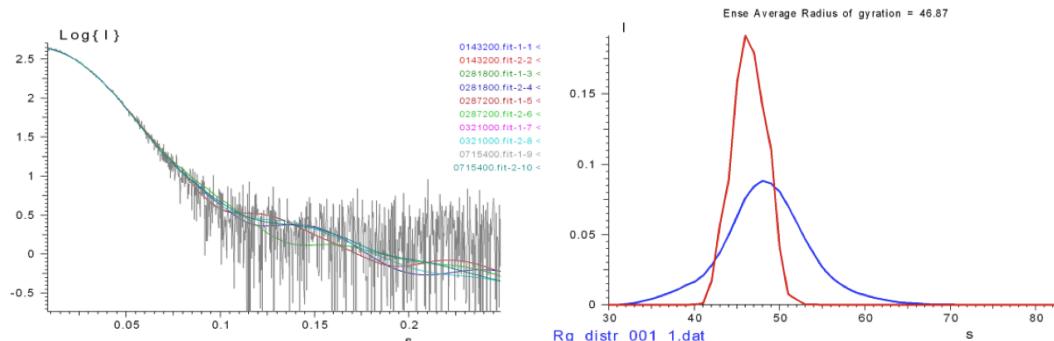
How to get rid of detergent backscattering from protein scattering?



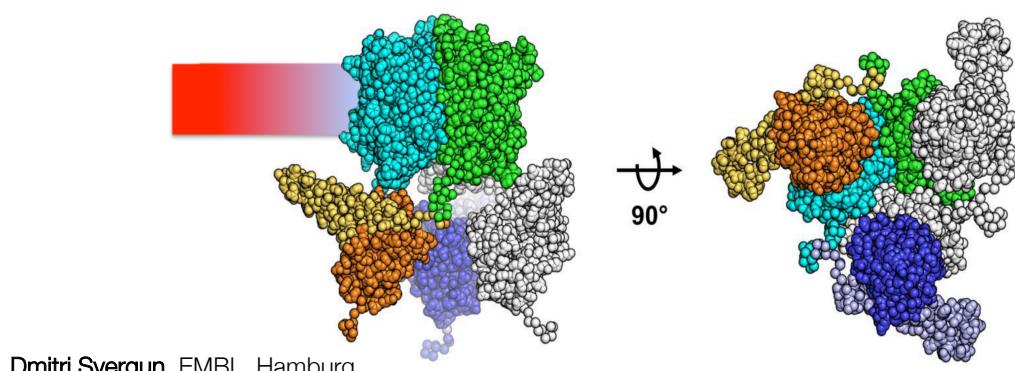
- **Dialysis** – Detergents with high CMCs are easily removed by dialysis. So that micelles disintegrate into monomers that easily pass through dialysis tubing over time.
- **Hydrophobic beads** – Detergents with low CMCs are typically removed by adsorption to hydrophobic beads (bio-beads) followed by filtration or centrifugation.
- **Chromatography** – Gel filtration can be used to separate detergent micelles from protein-detergent complexes and free protein based on size differences. Detergents can also be removed or exchanged by affinity chromatography.



SAXS of the full-length sensor histidine kinase



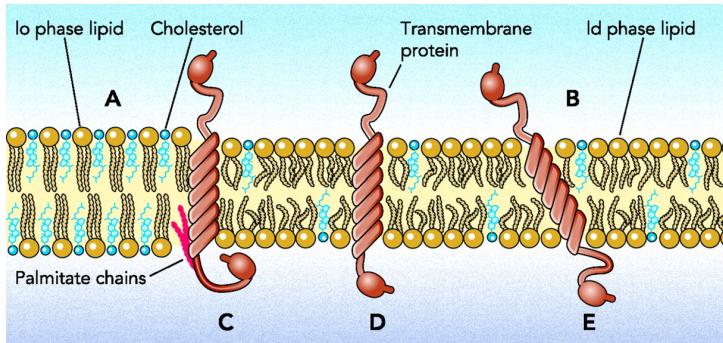
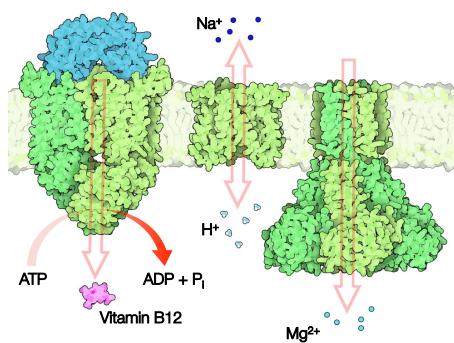
Model obtained using crystal structure models and the ensemble optimization method (EOM):



The beauty and the beast (...)

The lipid environment can severely influence the protein

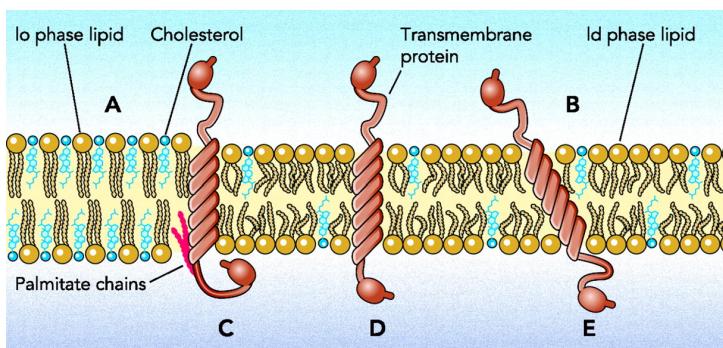
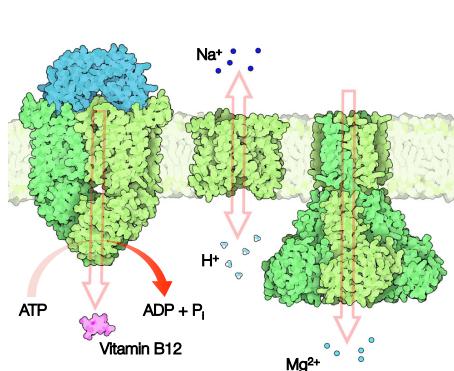
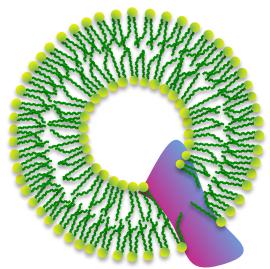
- fold
- activity



The beauty and the beast (...)

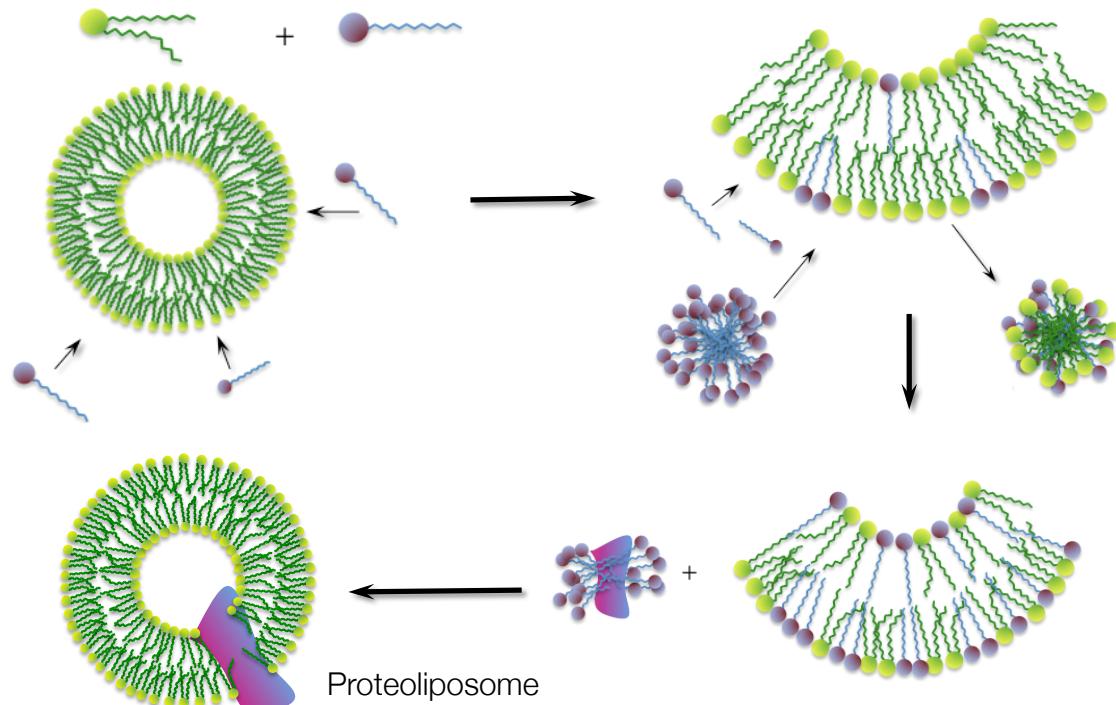
3. Protein characterization (cont.)

- *Functional assays*



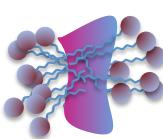
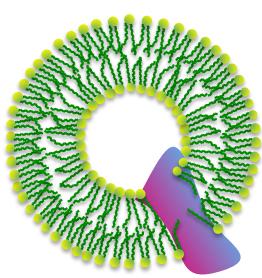
Reconstitution of membrane proteins

Liposome solubilization:

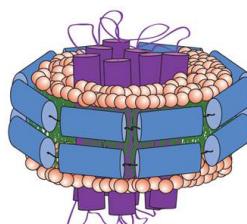


NEVER GIVE UP

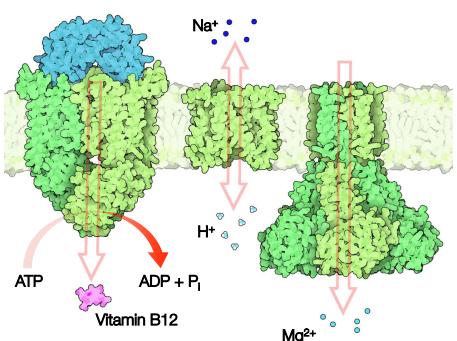
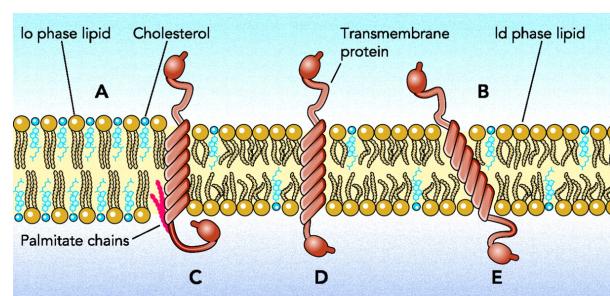
3. Protein characterization



Lipid-like detergents



Bicelles
Lipidic-cubic phases
(...)



Be Prepared and dare!

NEVER GIVE UP

