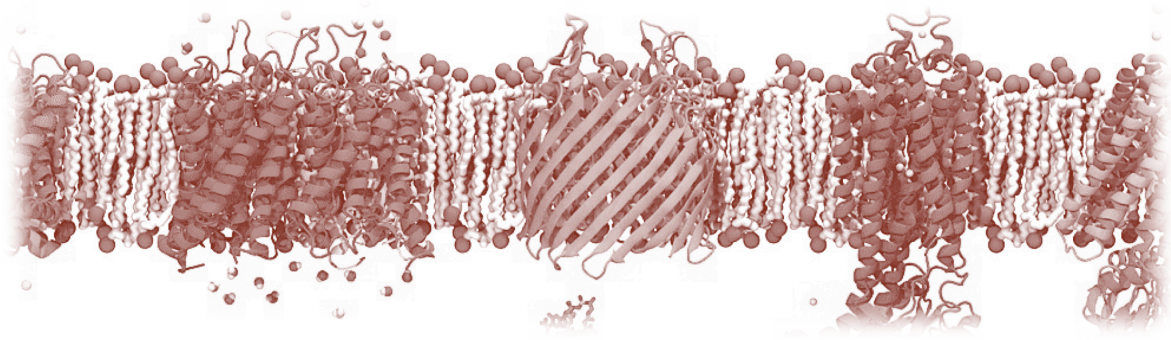
Membrane Proteins:
Structural & Functional Challenges



Susana Andrade
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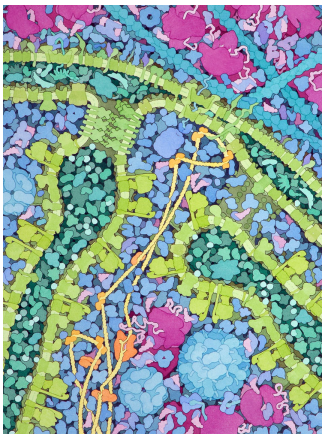
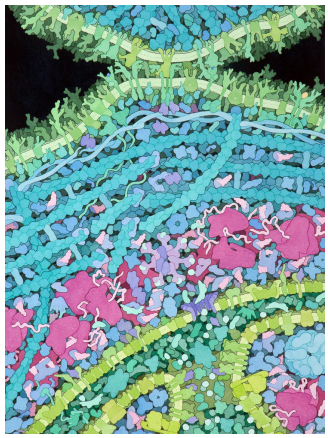
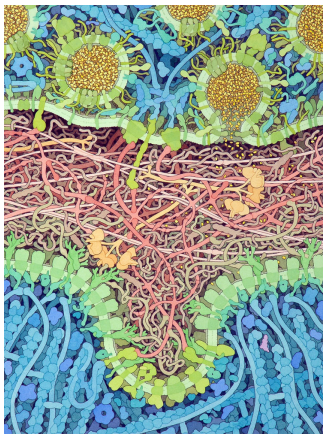
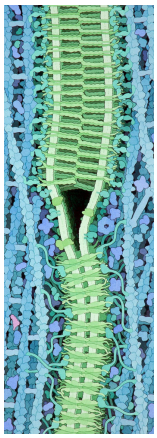
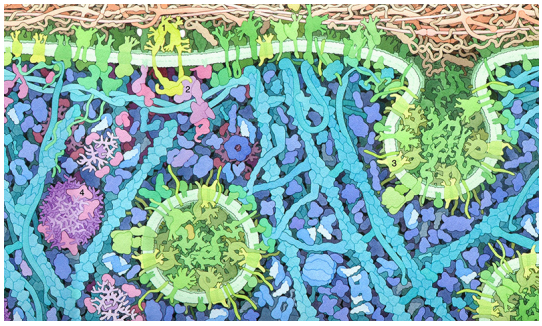
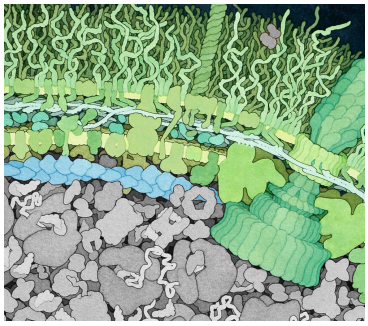
University Freiburg

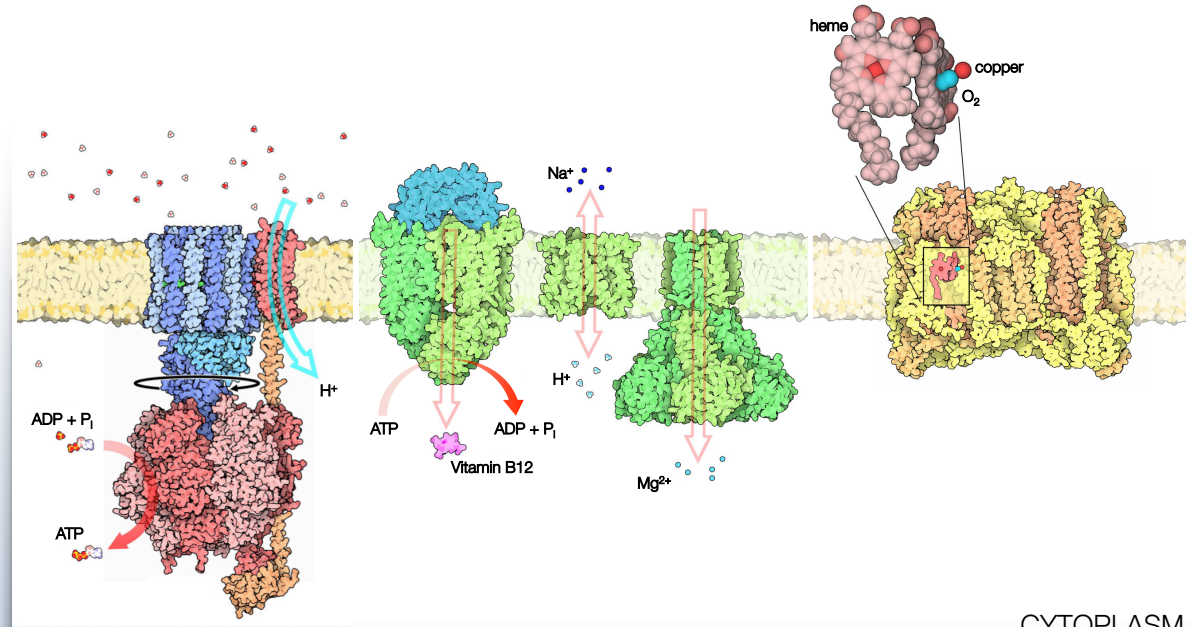
Diverse roles and structures

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

The Machinery of Life, David Goodsell

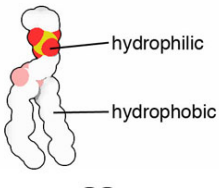
Zooming in



The membrane environment

Composition

- Glycerophospholipids
  - Phosphatidic acid
  - Phosphatidylinositol
  - Phosphatidylserine
  - Phosphatidylcholine
  - Phosphatidylethanolamine
  - Phosphatidylglycerol
  - Diphosphatidylglycerol
  - Variable size and saturation of the aliphatic chains
  - (…)

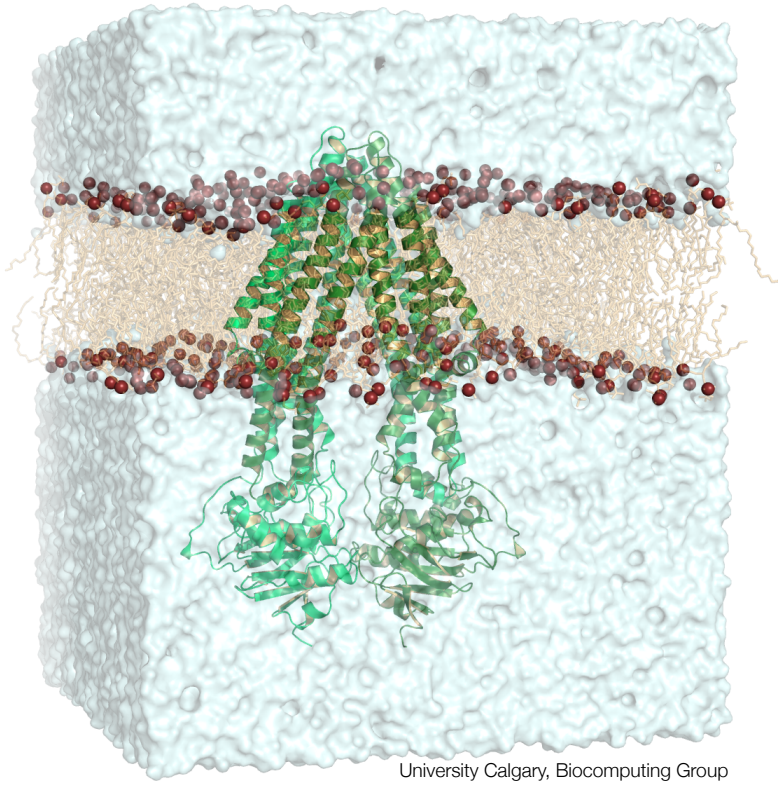


- Glyceroglycolipids
  - (…)
- Sphingophospholipids
  - (…)
- Sphingoglycolipids
  - (…)
- Sterols
  - (…)
- Others
  - Dolichols
  - (…)

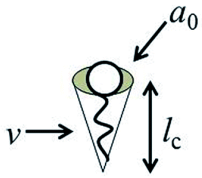
Function

- 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*

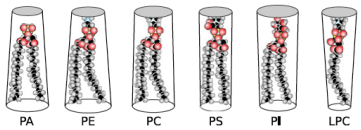


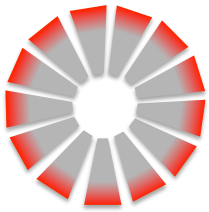
University Calgary, Biocomputing Group

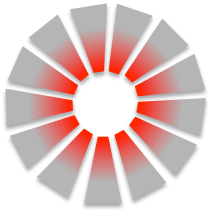


$$\mathrm{CPP} = v/a_0 l_c$$

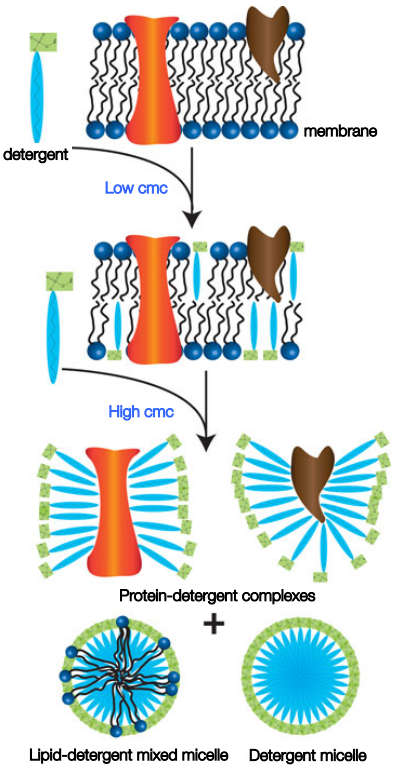
Critical Packing Parameter ($v/a_0 l_c$)	Critical Packing Shape	Structures Formed
< 1/3	Cone	Spherical micelles
1/3 – 1/2	Truncated cone	Cylindrical micelles
1/2 -1	Truncated cone	Flexible bilayers, vesicles
~1	Cylinder	Planar bilayers
>1	Inverted truncated cone or wedge	Inverted micelles

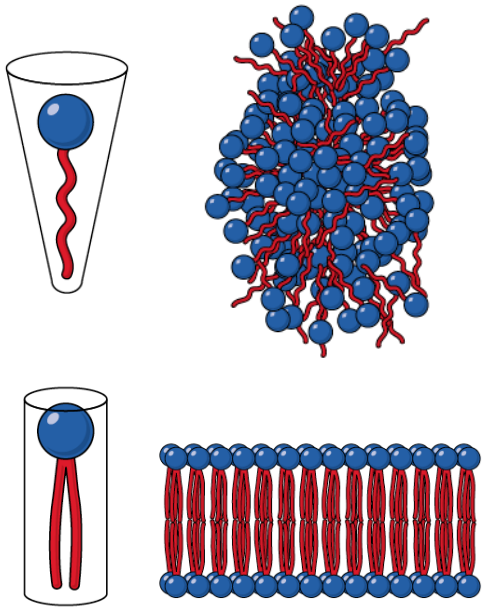






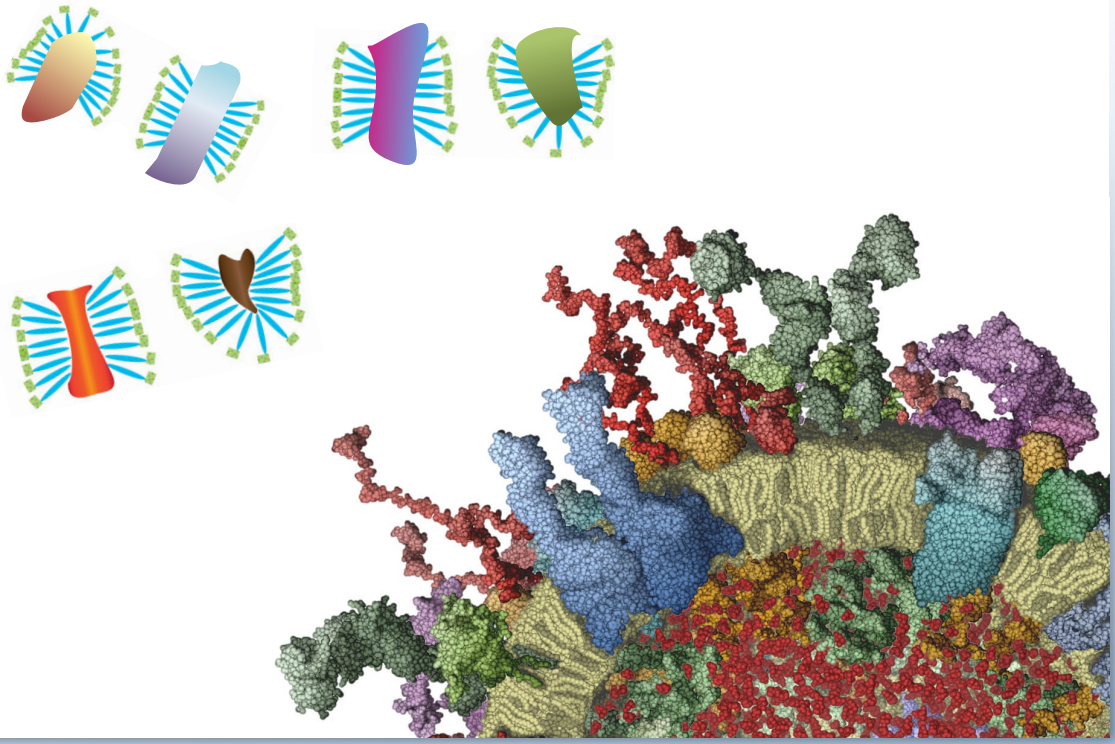
Solubilizing membrane proteins



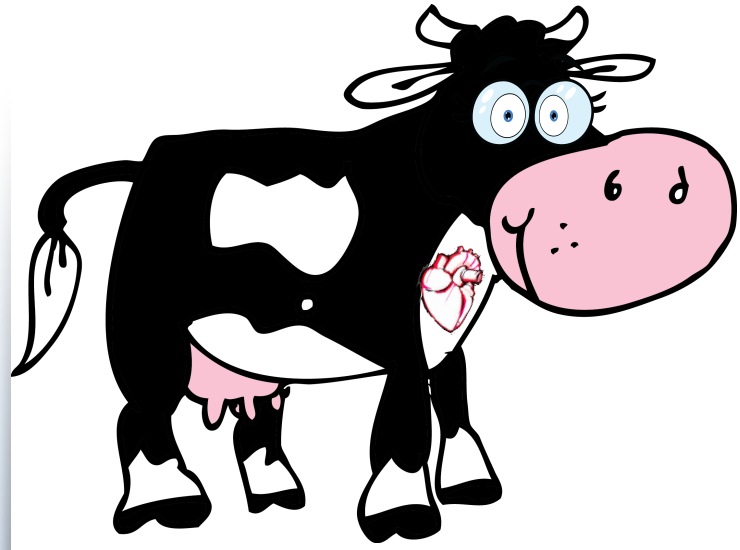


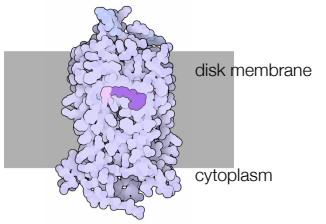
Critical micelle concentration (CMC) – Concentration range where surfactant monomers (e.g. detergents) spontaneously form non-covalent aggregates called micelles. Depends on the nature of the surfactant, salt concentration, etc.

Purifying membrane proteins

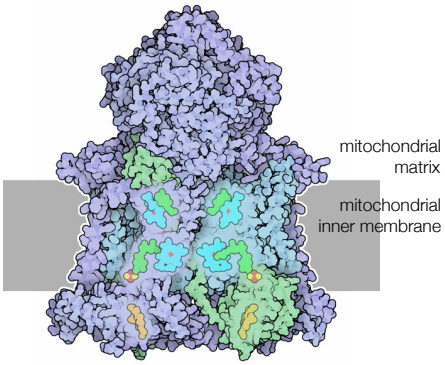


Purifying membrane proteins





Rhodopsin



Cytochrome bc1

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.
- …

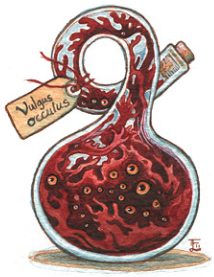
❖ Common DNA sources and delivery mechanisms are **plasmids**, **viruses** (e.g. baculovirus, retrovirus, adenovirus), **artificial chromosomes** and **bacteriophage** (such as lambda).

Producing (membrane) proteins

Cell-based expression system:

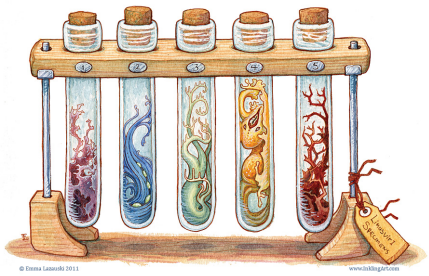
- The plasmid is placed *inside a cell*

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.

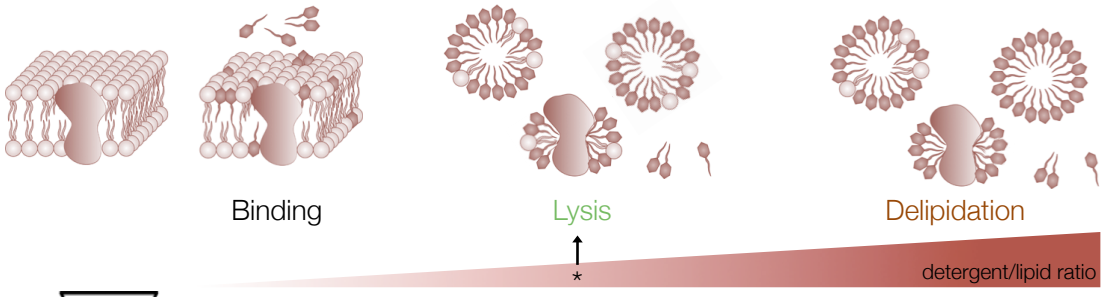
Producing (membrane) proteins

(…)
RNA polymerase will produce mRNA.
Ribosomes translate mRNA into a **protein**.

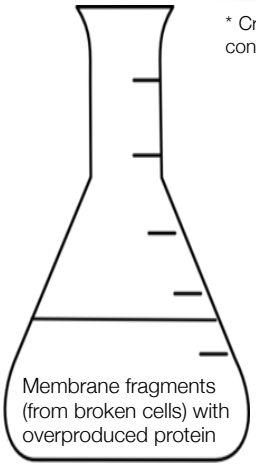
- Clone (homolog genes)
- Clone (plasmid/promoter type)
- Express (host cells, growth conditions)
- Protein targetting, yield
- Protein functionality
- Protein purification scheme

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)…

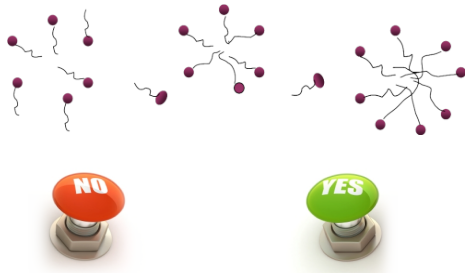
Purifying membrane proteins



* 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.



Membrane fragments (from broken cells) with overproduced protein



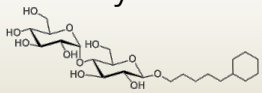
Critical micelle concentration (CMC) – Concentration range where surfactant monomers (e.g. detergents) spontaneously form non-covalent aggregates called micelles. Depends on the nature of the surfactant, salt concentration, etc.

The detergent variable

Amino oxides

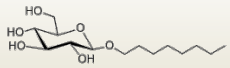
n-Dodecyl-N,N-Dimethylamine-N-Oxide (LDAO)

Cymal



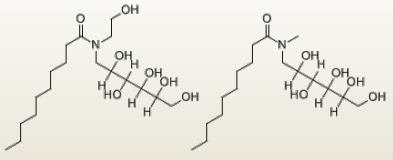
5-Cyclohexyl-1-Pentyl-β-D-Maltoside (Cymal-5)

Glucosides



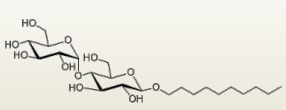
n-Octyl-β-D-Glucopyranoside (OGP)

Hega & Mega



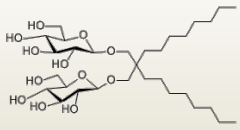
Decanoyl-N-Hydroxyethylglucamide (Hega-10)
Decanoyl-N-Methylglucamide (Mega-10)

Maltosides



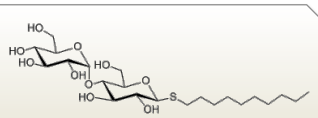
n-Decyl-β-D-Maltopyranoside (DM)

NG class



2,2-dioctylpropane-1,3-bis-β-D-glucopyranoside
(Decyl glucose neopentyl glycol)

Thioglycosides & Thiomaltosides

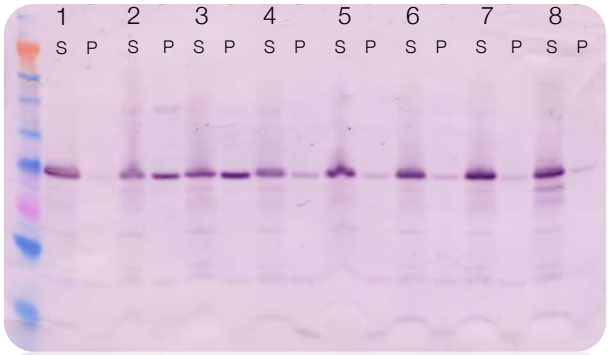


n-Decyl-β-D-Thiomaltopyranoside (DTM)

The detergent variable

Solubilization test:

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



1: SDS
2: HEGA-10
3: C_8E_4
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)…

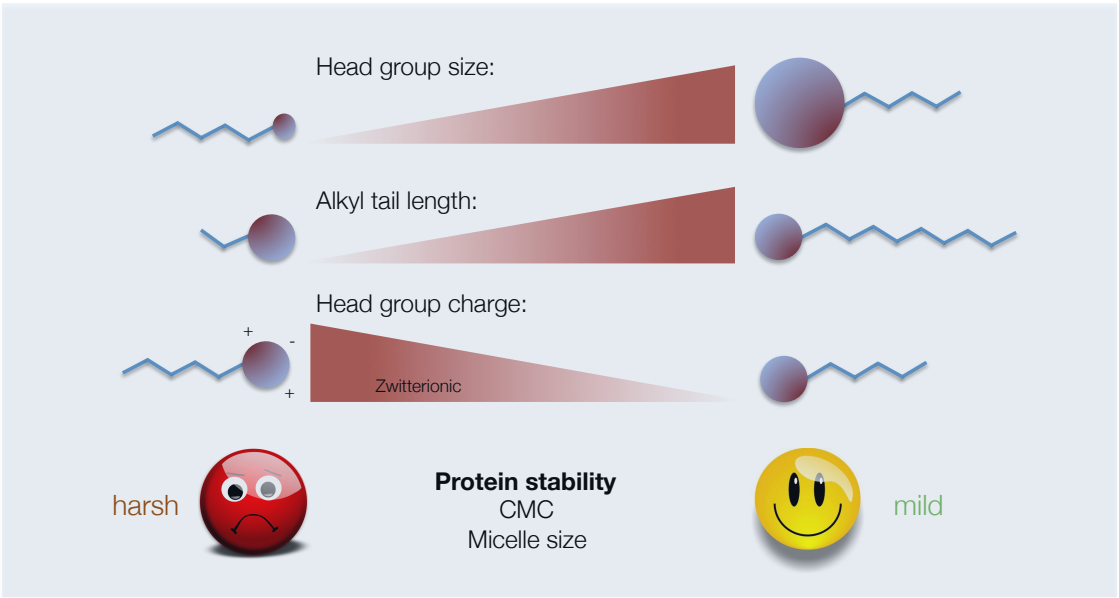
*Rath *et al* (2007), *PNAS*, **106**:1760

The detergent variable

1. Protein solubilization ✓

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

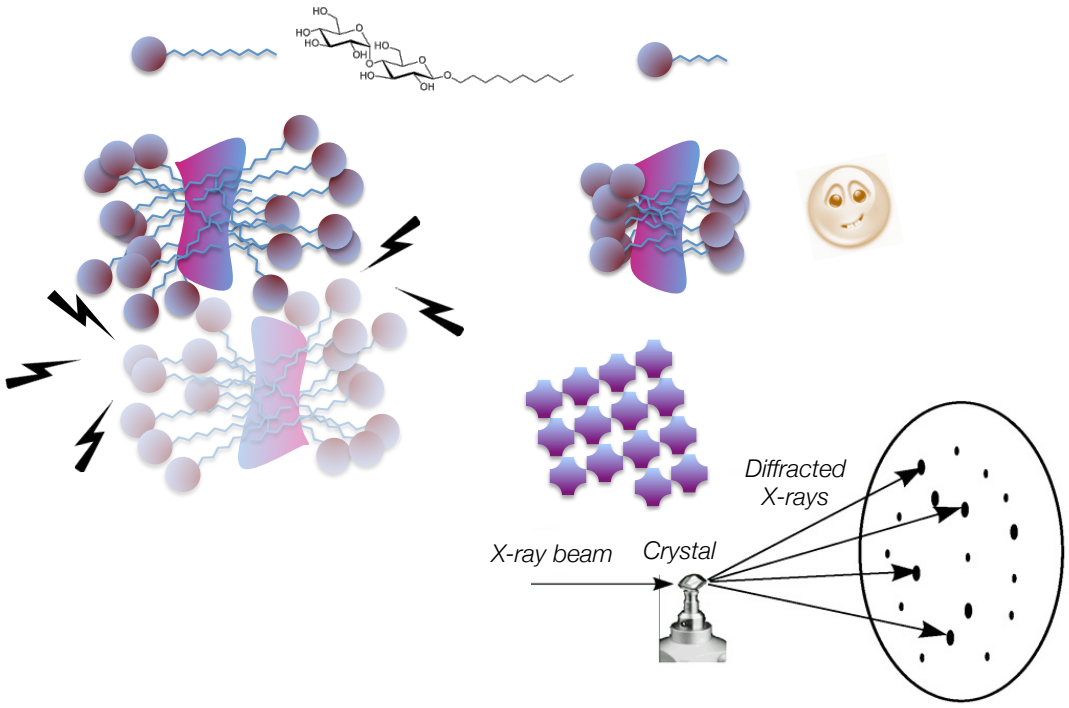
2. Protein purification & stability



The beauty and the beast

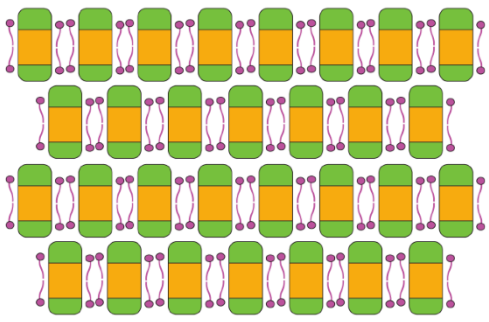
3. Protein characterization

- *X-ray crystallography*

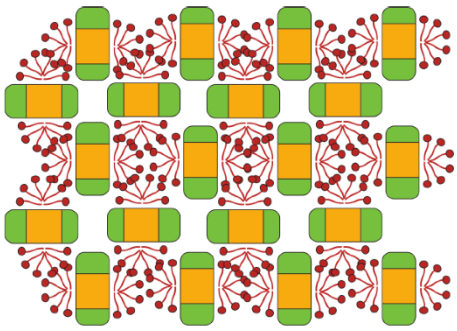


Types of membrane protein crystals

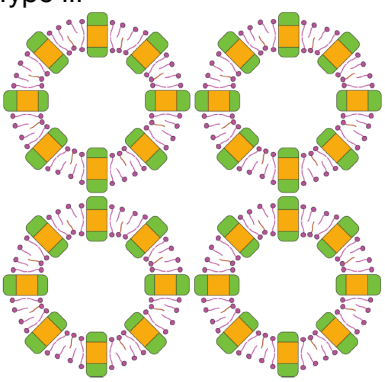
- Type I



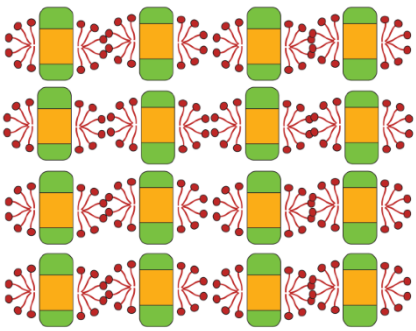
- Type II



- Type III

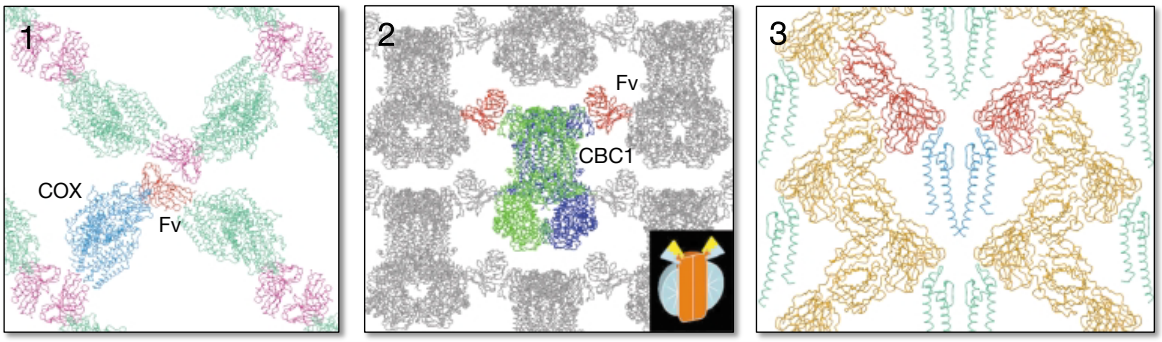


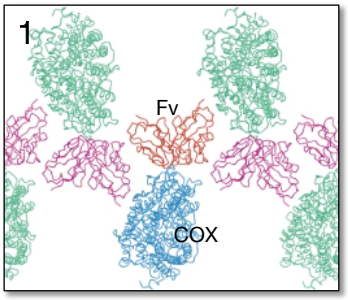
- Type IV



Never give up

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





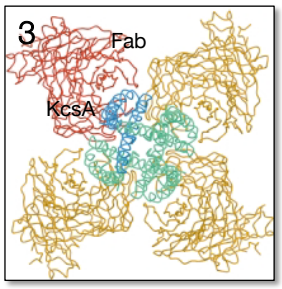
Crystal lattices of:

(1) four cytochrome c oxidase subunits with recombinant Fv fragment;

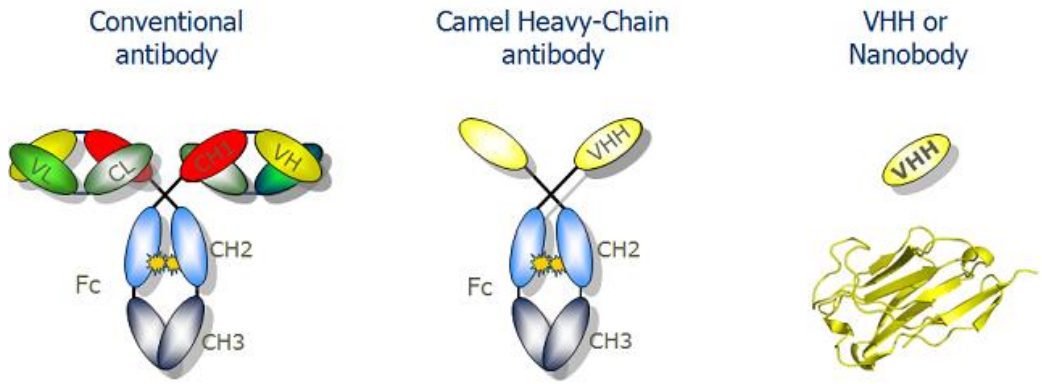
(2) (2) cytochrome bc1 complex with Fv fragment bound to the catalytic Rieske protein subunit;

(3) (3) KcsA in complex with a proteolytic Fab fragment

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



Never give up



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.

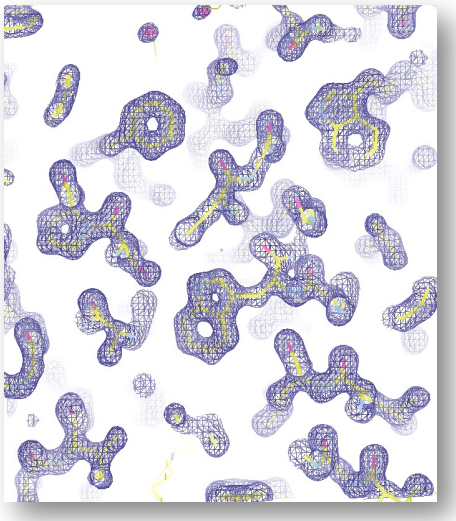
Another advantage is that they generally bind conformational epitopes

http://www.structuralbiology.be/chaperones

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, …)

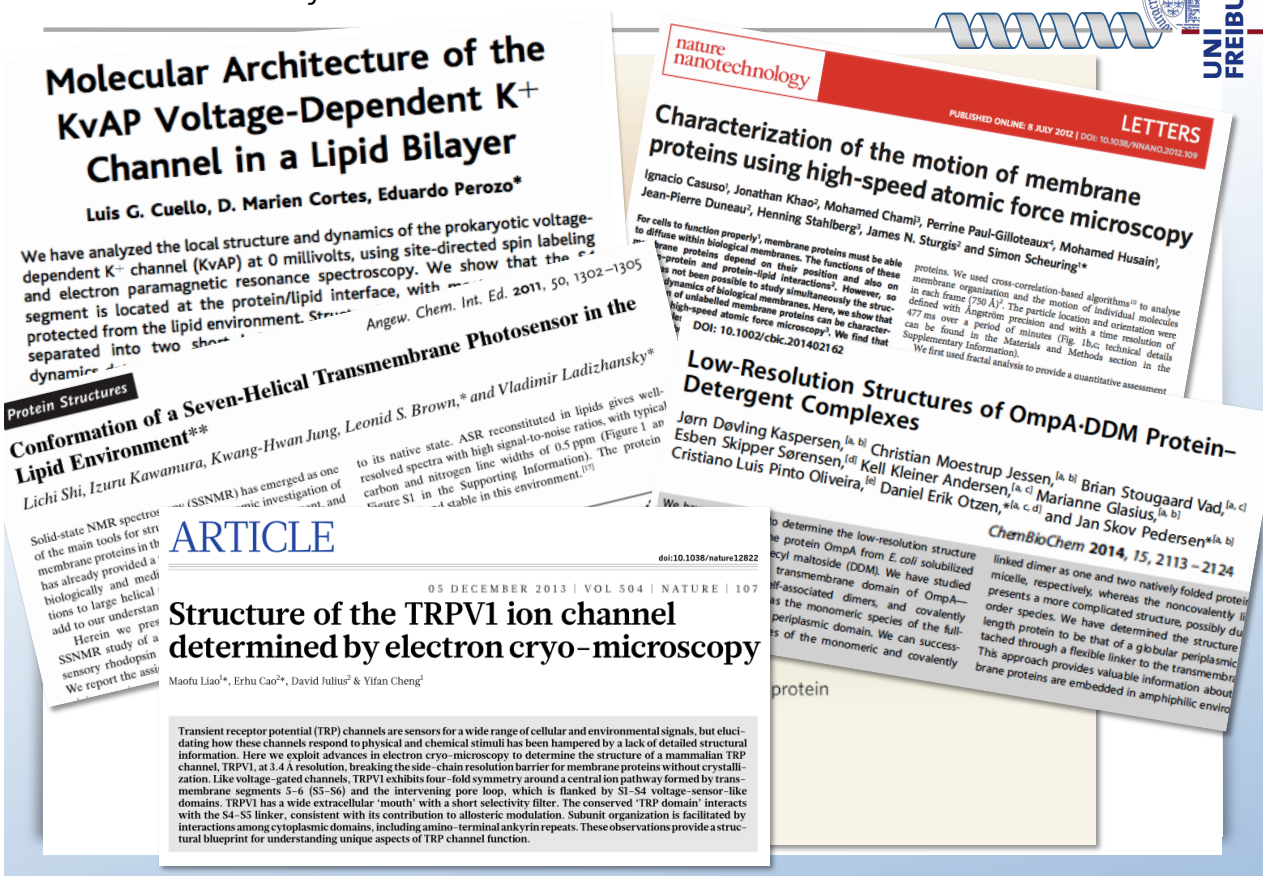
- High level of disorder due to high flexibility



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

Never give up

Proteins are dynamics entities!

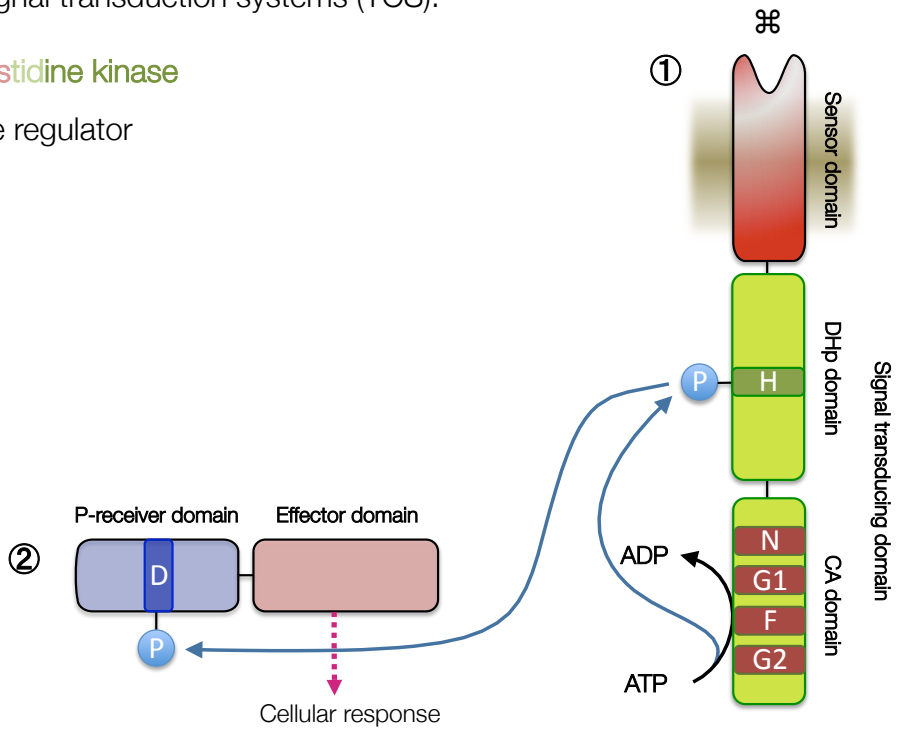


Highly flexible proteins …

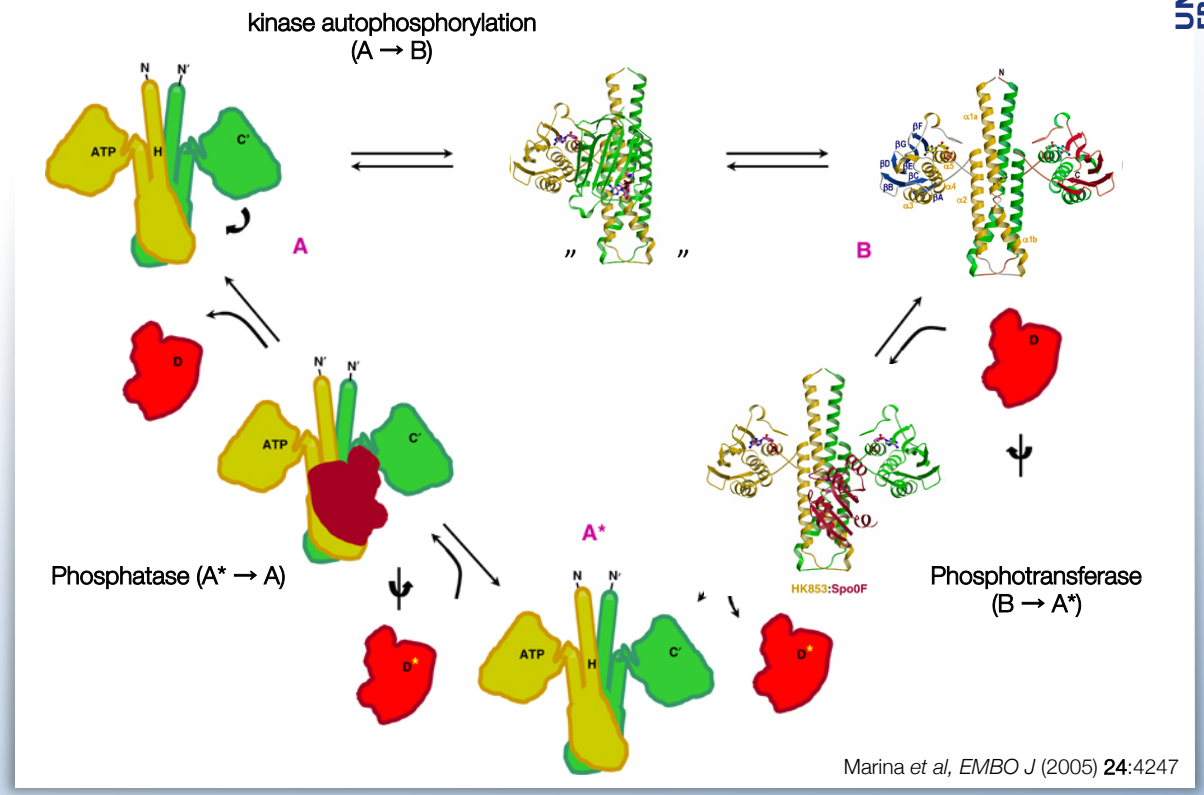
Two-component signal transduction systems (TCS):

① - Sensor histidine kinase

② - Response regulator



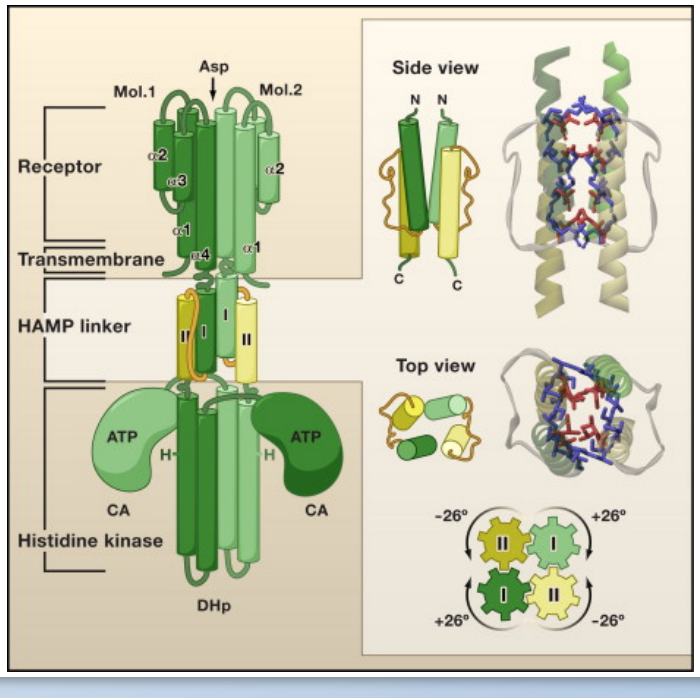
Structure-based scheme of the HK reactions

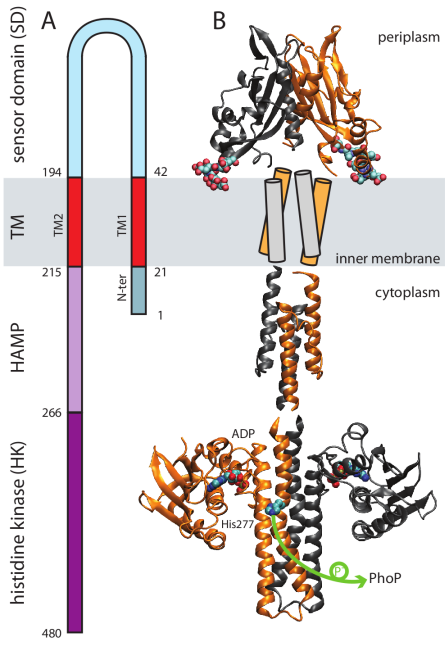


Marina *et al, EMBO J* (2005) **24**:4247

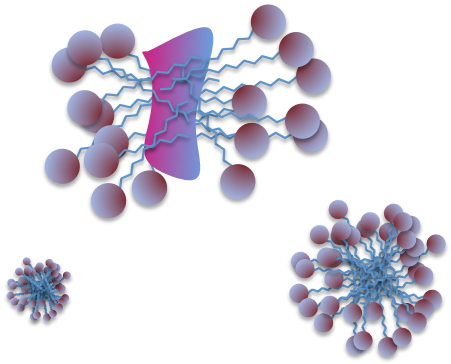
How is signal transmitted after reception?

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.

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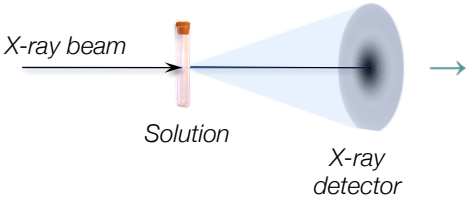
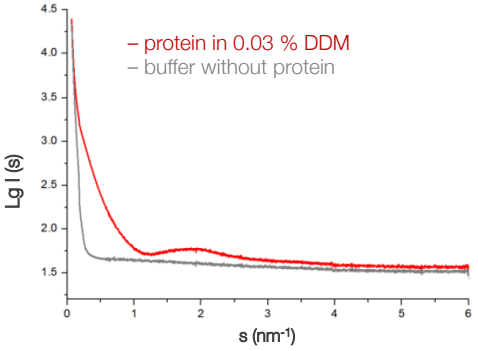
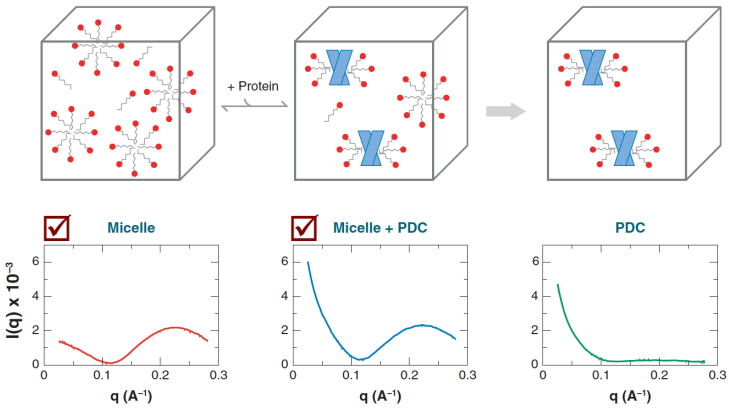




The beauty and the beast

How to get rid of detergent backscattering from protein scattering?



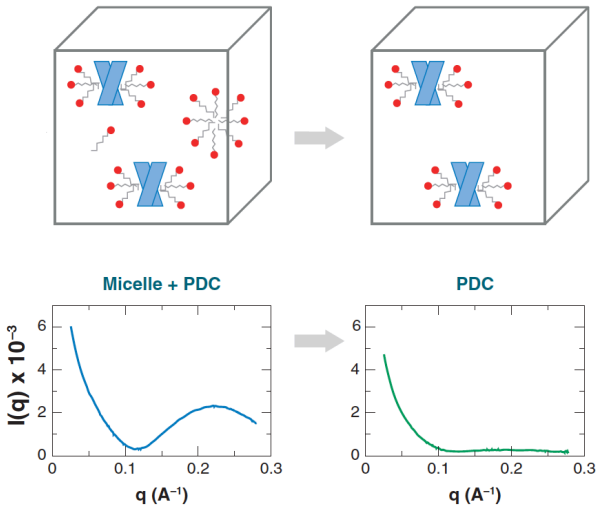
Adapted from:
Lipfert & Doniach (2007)
Ann Rev Biophys Biomol Struct 36:307.

- "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..

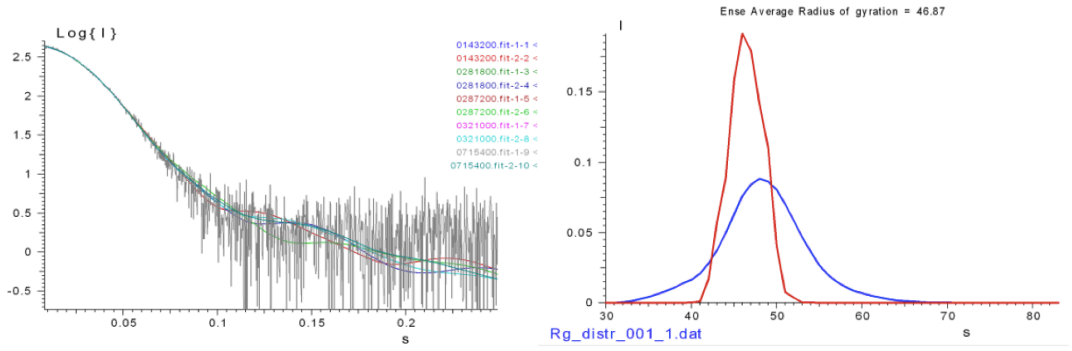
The beauty and the beast

How to get rid of detergent backscattering from protein scattering?

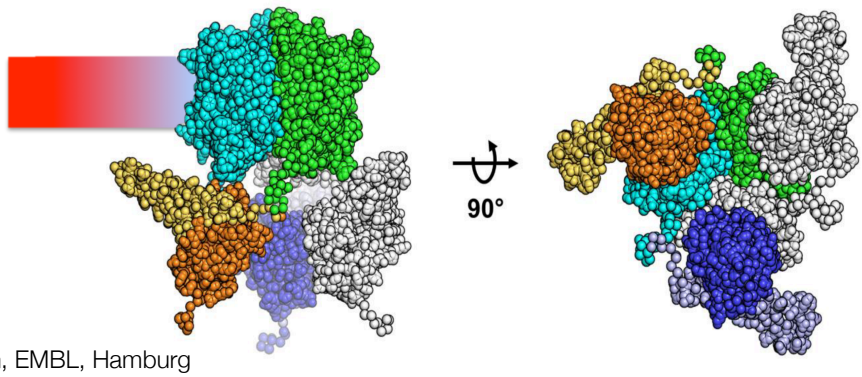
- *Dialysis* – Detergents with high CMCs are easily removed by dialysis. So that micelles disintegrate into monomers that easily pass through dialysis tubing over time.
- *Hydrophobic beads* – Detergents with low CMCs are typically removed by adsorption to hydrophobic beads (bio-beads) followed by filtration 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):

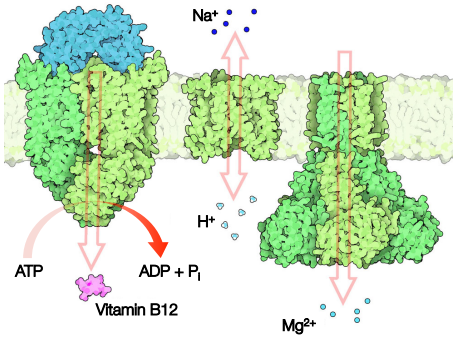


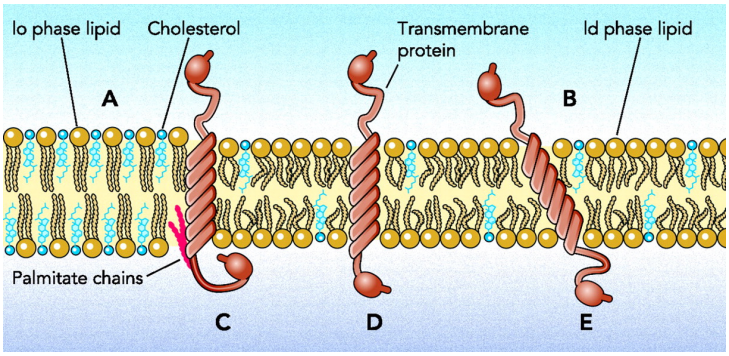
Dmitri Svergun, EMBL, Hamburg

The beauty and the beast (…)

The lipid environment can severely influence the protein

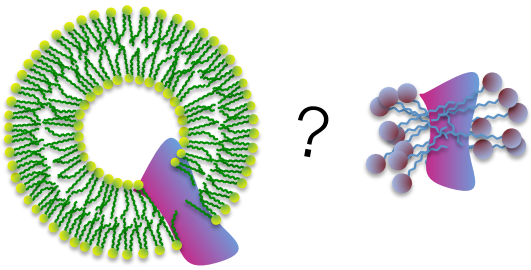
- fold
- activity

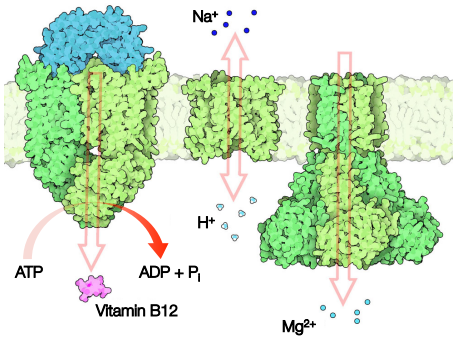


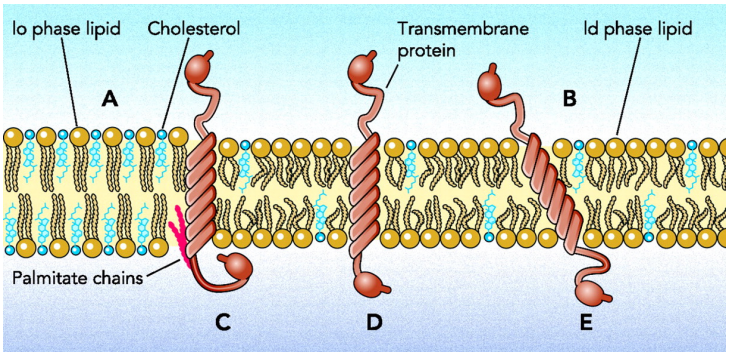


The beauty and the beast (…)

3. Protein characterization (cont.)
   - *Functional assays*

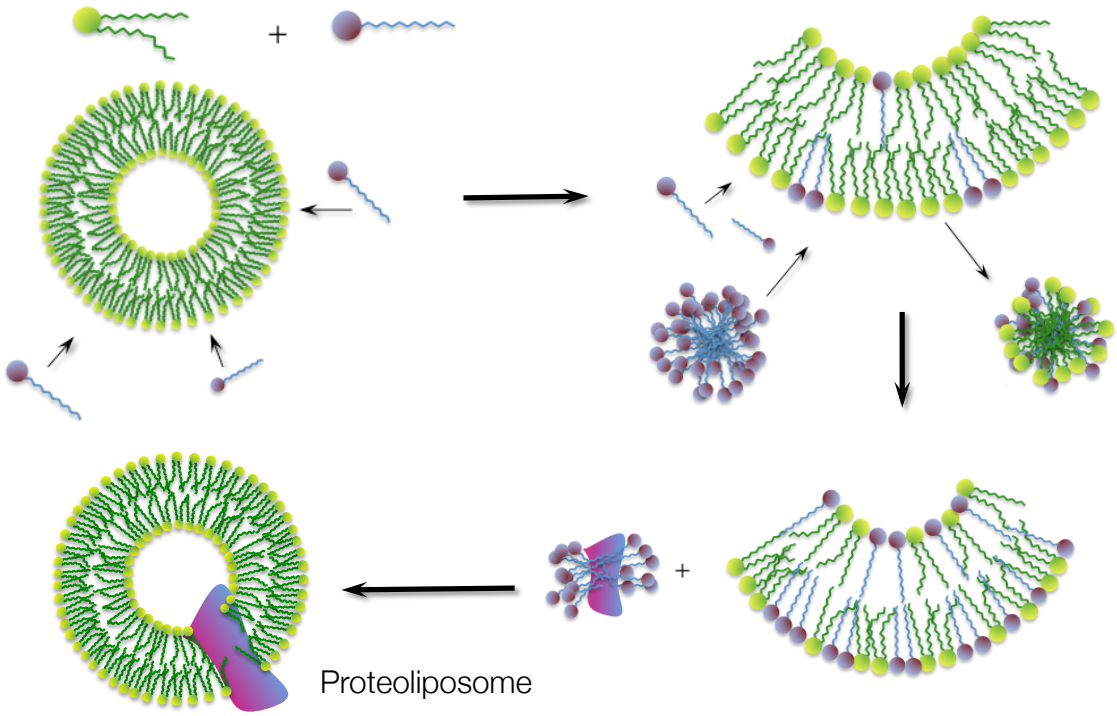






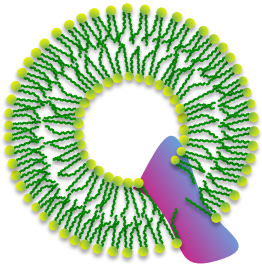
Reconstitution of membrane proteins

Liposome solubilization:

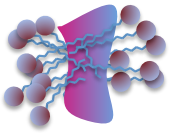


NEVER GIVE UP

3. Protein characterization

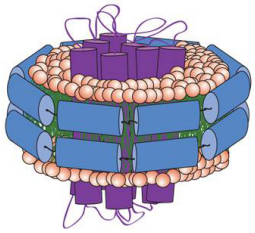


Lipids



Detergents

Lipid-like detergents

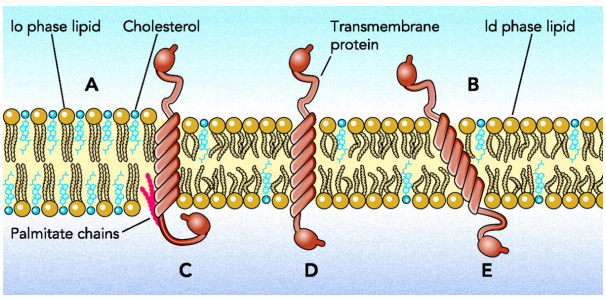


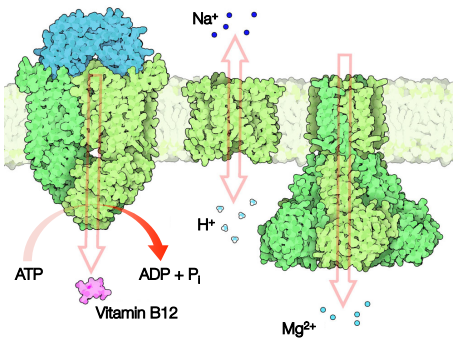
Nanodiscs

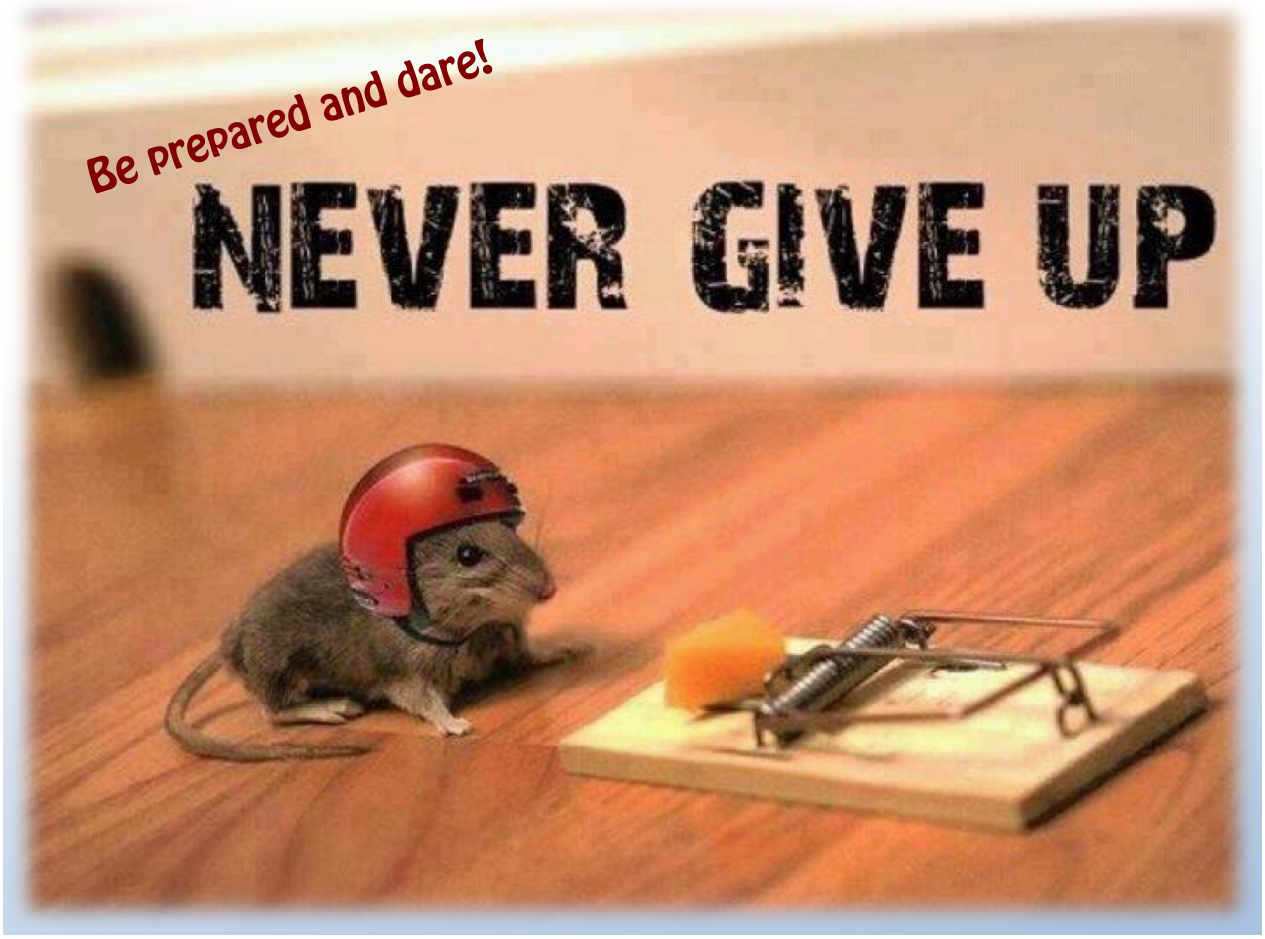
Bicelles

Lipidic-cubic phases

(…)






Be prepared and dare!
NEVER GIVE UP