EMBO Practical Course 2016

Membrane Proteins: Structural & Functional Challenges

Susana Andrade
Institute of Biochemistry
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
  - Phosphatidyglycerol
  - Diphosphatidylglycerol
  - Variable size and saturation of the aliphatic chains
  - (...)

- **Glyceroglycolipids**
  - (...)

- **Sphingophospholipids**
  - (...)

- **Sphingoglycolipids**
  - (...)

- **Sterols**
  - (...)

- **Others**
  - Dolichols
  - (...)

- Sphingophospholipids
  - hydrophilic
  - hydrophobic
The membrane environment

**COMPOSITION**

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- **Glyceroglycolipids**
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  - (...)

- **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**
  - (...)

**Diagram**

- Hydrophilic
- Hydrophobic
The beauty and the beast – I

University Calgary, Biocomputing Group
Solution: Surfactants as *tools*
<table>
<thead>
<tr>
<th>Critical Packing Parameter ( (v/a_0l_c) )</th>
<th>Critical Packing Shape</th>
<th>Structures Formed</th>
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</thead>
<tbody>
<tr>
<td>&lt; 1/3</td>
<td>Cone</td>
<td>Spherical micelles</td>
</tr>
<tr>
<td>1/3 – 1/2</td>
<td>Truncated cone</td>
<td>Cylindrical micelles</td>
</tr>
<tr>
<td>1/2 -1</td>
<td>Truncated cone</td>
<td>Flexible bilayers, vesicles</td>
</tr>
<tr>
<td>~1</td>
<td>Cylinder</td>
<td>Planar bilayers</td>
</tr>
<tr>
<td>&gt;1</td>
<td>Inverted truncated cone or wedge</td>
<td>Inverted micelles</td>
</tr>
</tbody>
</table>

PA, PE, PC, PS, PI, LPC
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

- Cytochrome bc1
- Mitochondrial matrix
- Mitochondrial inner membrane
- Rhodopsin
- Disk membrane
- Cytoplasm
- Cytochrome bc1
- Mitochondrial matrix
- Mitochondrial inner membrane
Over-expression solutions

To produce the protein encoded by a piece of cloned DNA (codon optimized?) adequate expression systems 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).
Over-expression solutions

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, ribonucleotides, lipids, nanodics, detergents,...
Strategies to produce (membrane) proteins

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

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

*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 solution

* 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

- **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

<p>| | | | | | | | |</p>
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<tbody>
<tr>
<td>1</td>
<td>S</td>
<td>P</td>
<td>2</td>
<td>S</td>
<td>P</td>
<td>3</td>
<td>S</td>
</tr>
<tr>
<td>4</td>
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<td>5</td>
<td>S</td>
<td>P</td>
<td>6</td>
<td>S</td>
</tr>
<tr>
<td>7</td>
<td>S</td>
<td>P</td>
<td>8</td>
<td>S</td>
<td>P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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)…

The detergent variable

1. Protein solubilization ✓
   GOAL – Extract efficiently the target membrane protein and keep it stable in solution.

2. Protein purification & stability

   - **Head group size:**
   - **Alkyl tail length:**
   - **Head group charge:**
     - +
     - -
     - Zwitterionic

   - **Protein stability**
     - CMC
     - Micelle size

   harsh → mild
The beauty and the beast – II

3. Protein characterization

- X-ray crystallography

Diffracted X-rays

X-ray beam

Crystal
Types of membrane protein crystals

- Type I
- Type II
- Type III
- Type IV
More solutions

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) cytochrome bc1 complex with Fv fragment bound to the catalytic Rieske protein subunit;
(3) KcsA in complex with a proteolytic Fab fragment

Recombinant nanobodies are small (15 kDa) and stable monomeric molecules that are easy to manipulate, cheaper and easier to produce in all kind of formats than standard monoclonal antibodies (e.g. in bacterial systems).

They can bind epitopes with nM affinity that:

- are less immunogenic for conventional antibodies e.g. active sites of enzymes;
- are small targeting areas that are not accessible to standard antibodies;
- are 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!

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 0.1 millivolts, using site-directed spin labeling and electron paramagnetic resonance spectroscopy. We show that the S6 segment is located at the protein/lipid interface, protected from the lipid environment. Hence, it is not possible to study simultaneously the structure of S6 and proton-lipid interactions. However, we can separate structural information on S6 from that on the S6-ββ loop and on the S6-S5 linker.

Angew. Chem. Int. Ed. 2011, 50, 1302-1305

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

Enrico Casu,*, Jonathan Khoo, Mohamed Chemli, Perrine Paul-Gilloteaux, Mohamed Husain, Jean-Pierre Dunæu,*, Henrik Stahlsberg, James N. Storøe* and Simon Scheuring*

For cells to function properly, membrane proteins must be able to diffuse within biological membranes. The functions of these proteins depend on their position and their interactions with other proteins both on the surface and within the bilayer. However, it is not possible to study simultaneously the structural and functional information on S6. Here, we show that the dynamics of S6 and S6-ββ loop can be characterized using high-speed atomic force microscopy.

DOI: 10.1038/nnano.2012.304

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

Jørn Davling Kaspersen,*, Christian Moestrup Jensen,*, Brian Stoegvad Vad,*, Ebbern Skippennie,*, Kell Kleiner Andersen,*, Marianne Galsius,*, and Jan Skov Pedersen,*,

Low-resolution structures of the OmpA protein from E. coli solubilized in detergents were obtained. The structures were used to determine the low-resolution structure of OmpA in detergent and in the membrane. This approach provides valuable structural information about protein-protein interactions and the role of detergent molecules.

ChemBioChem 2014, 15, 2113-2124

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

Maofu Liu*, Erhao Cao*, David Jolliv & Yifan Cheng*

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 1.4 Å resolution, revealing the channel domain and the transmembrane segments S5–S6 and the intervening pore loop.

 ARTICLE

05 DECEMBER 2013 | VOL 504 | NATURE | 107
Highly flexible membrane proteins … a case study

Two-component signal transduction systems (TCS):

1. **Sensor histidine kinase**
2. **Response regulator**

![Diagram showing the two-component signal transduction system]

- **Sensor domain**
- **Signal transducing domain**
- **DHp domain**
- **CA domain**
- **P-receiver domain**
- **Effector domain**

**Cellular response**

- **P**
- **ADP**
- **ATP**

**Abbreviations**

- ATP: Adenosine Triphosphate
- ADP: Adenosine Diphosphate
- P: Phosphorylated state
Structure-based scheme of the HK reactions

kinase autophosphorylation

(A → B)

Phosphatase (A* → A)

Phosphotransferase

(B → A*)

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.
A case study...

Electron density maps at 1.9 Å resolution

Contour at 0.7 sigma level

100 μm

Sensor domain

Signal transducing domain

DHp domain

CA domain

P

N

G1

G2

F
Soaking & co-crystallization solution

Electron density maps at 4.5 Å resolution

3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrroline-2,5-dione
**Low-resolution X-ray diffraction solution**

**Manfred Baumstark**, Univ. Klinikum Freiburg, 
**Vladimir Lunin**, Russian Acad Sci, Puschino, RU  
Beamline ID-29 ESRF, Grenoble

*Ab initio* low-resolution phase calculations of highly complete datasets from ≈ 100 – 8 Å:

Other cases:

The beauty and the beast... again

In buffer containing 10 % glycerol and 0.03 % DDM the protein is:

- Pure
- Stable
- Homogeneous
- Trimeric

Dmitri Svergun, EMBL, Hamburg
The beauty and the beast.. again

How to get rid of detergent backscattering from protein scattering?

“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..

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

Detergent variation and removal optimization led to better results!

- protein in 0.01 % DDM
- protein in 0.2 % D9M
- protein in 0.09 % Cymal-5

- buffer without protein

- protein in 0.09 % Cymal-5
- buffer without protein

- protein in 0.03 % DDM
- buffer without protein
SAXS of the full-length sensor histidine kinase

Haydyn Mertens, EMBL, Hamburg
The beauty and the beast (…)

The lipid/detergent environment can severely influence the protein:

- **fold**
- **activity**
Reconstitution solution

Liposome solubilization:

Proteoliposome
Multiple options!

3. Protein characterization

- Lipids
- Detergents
- Lipid-like detergents
- Nanodiscs
- Bicelles
- Lipidic-cubic phases

![Diagram showing various protein structures and lipid environments](image)
Be prepared and dare!

NEVER GIVE UP