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A Structure-Function Toolbox for Membrane Transporter and Channels

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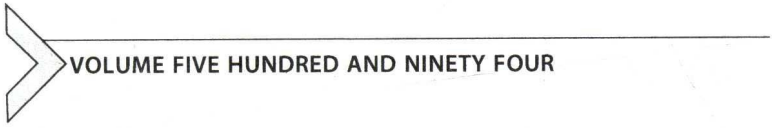
Christine Ziegler



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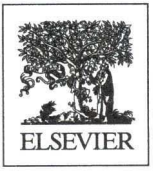

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PREFACE

Membrane transport is fundamental in living cells. However, limited molecular details on function and structure left us with more open questions than answers on this enigmatic biological process. Since solving the first structure of a membrane protein in 1985, X-ray crystallography and single particle cryo-electron microscopy have yielded constantly increasing number of atomic and near-atomic structures, however their amount lingered behind expectations. Similar, dynamical methods like NMR, EPR and single-molecule FRET faced multiple experimental challenges, e.g., the low amount of expressed membrane proteins and the lack of adequate membrane-mimicking environment. In addition, functional descriptions of active and passive membrane transport processes essential to complement and interpret structure and dynamics are likewise difficult to obtain. Undoubtedly, working with membrane transport proteins remains challenging. The overwhelming struggles that might be encountered at each step have figuratively stigmatized this fascinating research field.

New approaches in structure-function methodology and membrane protein handling have recently surfaced, raising hope that we will enter a new era of unprecedented molecular detail in membrane transport. This includes new aspects in membrane protein expression, in thermo-stabilization, in providing more native membrane environment as well as new technical advances in obtaining molecular and functional information.

In this regard, we are proud to present in this volume of Methods in Enzymology a timely overview on cutting-edge techniques that have advanced the structure-function toolbox of membrane transporters and channels. The individual chapters describe innovations from expression over structure determination to dynamical characterization providing creative experimental solutions to typical problems when working with membrane proteins. Hence, this volume is a compilation of knowledge of long-standing membrane protein experts, who have contributed for years with their work to the field of membrane transport.

Stefan Raunser and co-workers begin the volume with a chapter describing a method to study membrane proteins by single particle analysis in a nearly native lipid bilayer using lipid nanodiscs. In case studies, they recount cryo-EM-specific benefits and challenges of this method focusing on membrane proteins.

The second chapter, by Klaus Fendler and colleagues, introduces an electrophysiology method, the Solid Supported Membrane method, for a cell-free functional characterization of electrogenic transporters and channels. The chapter provides in "hands-on" format detailed information on sample and buffer preparation as well as the interpretation of the results.

The groups of Poul Nissen and Jens Frauenfeld report on a complementary method to prepare bilayer nanoparticles using saposin A, a protein that is involved in lipid transport, as flexible and adaptive scaffold protein. The chapter by Dirk Slotboom and co-workers focuses on single-molecule imaging of liposome-reconstituted membrane transporters. Here membrane-reconstituted transporters with fluorescent labels are used to visualize the transport-associated dynamics by total internal reflection fluorescence microscopy at the single-molecule level.

The fifth chapter by members of the group of David Drew covers the important issue of screening for overexpression condition of membrane proteins. In a high-throughput setup in *Escherichia coli* the authors document the advantages of their "one-shot expression approach" approach on several well-known transport proteins.

Eric Geertsma and colleagues take a different approach on the same problem, the overexpression of membrane proteins in in *Escherichia coli*. Their procedure involves transcriptional fusions of small additional RNA combined with a tuneable promoter adjusting the membrane protein expression rate to the downstream folding capacity.

The following chapter by Joseph Mindell and Christopher Mulligan is an outstanding review of the very successful techniques that exploit the chemistry of cysteine to test the structure and mechanism not only of membrane proteins. The authors describe numerous applications reaching from cross-linking and labelling for monitoring conformational changes, to cross-linking strategies coupled to structural studies.

Cysteine modifications are also an important tool in Christos Pliotas's chapter; this chapter provides a detailed description of pulsed-EPR methodology using mechanosensitive ion channels as model to investigate oligomerization, conformation, and the effect of lipids on the protein.

The last chapter offers insights into strategies from Christopher Tate's lab for the thermo-stabilization of a GPCR–mini-G protein complex. This chapter summarizes theoretical and practical aspects of the methodology used for stabilizing this complex and provides examples of thermo-stability shifts.

We are convinced that your research will be stimulated by these new approaches and hope that you will enjoy this collection.

CHRISTINE ZIEGLER

CONTENTS

CHAPTER ONE

Lipid Nanodiscs as a Tool for High-Resolution Structure Determination of Membrane Proteins by Single-Particle Cryo-EM

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Contents

Abstract

The "resolution revolution" in electron cryomicroscopy (cryo-EM) profoundly changed structural biology of membrane proteins. Near-atomic structures of medium size to large membrane protein complexes can now be determined without crystallization. This significantly accelerates structure determination and also the visualization of small bound ligands. There is an additional advantage: the structure of membrane proteins can now be studied in their native or nearly native lipid bilayer environment. A popular lipid bilayer mimetic are lipid nanodiscs, which have been thoroughly characterized and successfully utilized in multiple applications. Here, we provide a guide for using lipid nanodiscs as a tool for single-particle cryo-EM of membrane proteins. We discuss general methodological aspects and specific challenges of protein reconstitution into lipid nanodiscs and high-resolution structure determination of the nanodisc-embedded complexes. Furthermore, we describe in detail case studies of two successful applications of nanodiscs in cryo-EM, namely, the structure determination of the rabbit

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ryanodine receptor, RyR1, and the pore-forming TcdA1 toxin subunit from *Photorhabdus luminescens*. We discuss cryo-EM-specific hurdles concerning sample homogeneity, distribution of reconstituted particles in vitreous ice, and solutions to overcome them.

1. INTRODUCTION

It is predicted that ~23% of human genes code for membrane proteins (Uhlén et al., 2015). Because of their surface accessibility and central importance in many cellular mechanisms, they are attractive drug targets. Thus, in 2006, membrane proteins constituted more than 60% of all known drug targets (Overington, Al-Lazikani, & Hopkins, 2006).

Structural information is key for understanding the interaction between membrane proteins, in particular receptors, and small drug molecules in atomic detail. However, structural investigations on membrane proteins are also important to understand essential cellular mechanisms, including signaling, transport, and energy conversion at the molecular level.

While some structures have been obtained by electron crystallography (Raunser & Walz, 2009) and solution NMR (Hiller & Wagner, 2009), the majority of membrane protein structures has been determined by X-ray crystallography (Vinothkumar & Henderson, 2010). Although the structure of the first membrane protein was solved in 1985 (Deisenhofer, Epp, Miki, Huber, & Michel, 1985), membrane proteins have remained a challenge for structural biology over the past decades. Besides difficulties in obtaining ordered crystals in the case of crystallography, major bottlenecks have been the expression, solubilization, and purification of sufficient amounts of recombinant and biologically active membrane proteins. In recent years, significant progress has been made to overcome these chalenges, and many structures of important membrane proteins have been solved. These include transporters, respiratory complexes, G-protein-coupled receptors (GPCRs), ion channels, and adventitious membrane proteins (Vinothkumar & Henderson, 2010).

However, in many cases, in particular for mammalian membrane proteins, crystals of sufficient quality have not been obtained. Recently, the introduction of a new generation of direct electron detectors (McMullan, Faruqi, Clare, & Henderson, 2014) and the parallel development of image-processing software (Brilot et al., 2012; Li et al., 2013) increased the resolution limit of single-particle electron cryomicroscopy (cryo-EM)

to atomic resolution. Cryo-EM of the post-"resolution revolution" era, as Werner Kühlbrandt coined it (Kühlbrandt, 2014), allows now for the direct structure determination of proteins to near-atomic resolution (between 3 and 4 Å) without crystallization. Consequently, this new development has had a major impact on structural biology, and many structures of mammalian protein complexes that resisted crystallization so far have been determined in the last 3 years. Good examples are γ-secretase (Bai et al., 2015), respiratory complex I (Fiedorczuk et al., 2016; Zhu, Vinothkumar, & Hirst, 2016) and supercomplexes (Letts, Fiedorczuk, & Sazanov, 2016; Wu, Gu, Guo, Huang, & Yang, 2016), ATP synthase (Allegretti et al., 2015), different ions channels (Liao, Cao, Julius, & Cheng, 2013; Wu, Yan, et al., 2016), and transporters (Kim et al., 2015).

As for X-ray crystallography, pure protein samples are needed for single-particle cryo-EM studies. Typically, purification protocols for membrane proteins start with extracting the transmembrane proteins from membranes by solubilizing them in detergents. Protein purification by means of different techniques, mainly affinity and size-exclusion chromatography (SEC), are also performed in the presence of detergent to prevent the precipitation of the protein. Detergent-solubilized membrane proteins can be directly used for single-particle cryo-EM. Since cryo-EM allows observing individual unrestrained protein molecules embedded in a thin layer of amorphous ice, there are no restrictions on choosing a suitable detergent, which is usually not the case in crystallography. For the same reason, detergent-free systems can also be used to study the structures of membrane proteins. Among these systems are polymers such as amphipols (Popot et al., 2011) and styrene–maleic acid (SMA) copolymers (Scheidelaar et al., 2015), which can substitute detergents, or lipid-based systems, such as lipid nanodiscs (Schuler, Denisov, & Sligar, 2013), a saposin-lipoprotein nanoparticle system (e.g., Salipro®, EP 2745834 B1) (Frauenfeld et al., 2016), and even liposomes (Wang & Sigworth, 2010).

Since detergents must be used at concentration levels above the critical micelle concentration (CMC) to prevent precipitation of the protein, their concentration in the solution is high enough to decrease the contrast of cryo-EM images (Schmidt-Krey & Rubinstein, 2011). This is not the case when reconstituting membrane proteins in amphipols. Therefore, amphipols have been successfully used for the determination of the first high-resolution structures of the TRPV1 channel (Liao et al., 2013) and γ-secretase (Bai et al., 2015). In general, amphipols are popular polymers for reconstituting membrane proteins in single-particle cryo-EM (Popot

et al., 2011). Although this process is more straightforward than reconstituting proteins into lipid environments, amphipols as well as detergents have the disadvantage that the system does not contain lipids. There are a number of reasons why studying the structure of membrane proteins in a lipid environment has advantages over examining them in detergent.

First of all, the lipid bilayer is the native environment for membrane proteins, and it has significantly different physical properties than its mimetic detergent micelle (Zhou & Cross, 2013). In contrast to planar lipid bilayers, detergent micelles typically have a spherical shape and differ considerably in their dielectric constant and electrostatic potential. In some cases these differences can have severe consequences, resulting in structures of nonnatively folded membrane proteins when crystallized from detergent solution (Cross, Murray, & Watts, 2013; Zhou & Cross, 2013). However, the effects are normally milder and while the proper fold is preserved when the membrane protein is solubilized in detergent or reconstituted in polymers, the protein dynamics, which are more sensitive to the environment than the structure, are very often influenced or altered. For example, the absorption spectrum of bacteriorhodopsin shifts upon solubilization in detergent and its photocycle kinetics are changed (Lam & Packer, 1983), even if its structure remains virtually unaltered. A more recent vivid example is related to the functional dynamics of the rabbit ryanodine receptor 1 (RyR1). Solubilized in Tween-20 (Yan et al., 2015) or CHAPS (Zalk et al., 2015), the structure of the channel is similar to the one determined in a lipid environment (Efremov, Leitner, Aebersold, & Raunser, 2015). However, in Tween-20 it is locked in a closed state and does not open under conditions that induce the full opening of the channel in lipid membranes (Bai, Yan, Wu, Li, & Yan, 2016).

The function of membrane proteins often depends on the direct interaction with lipids (Phillips, Ursell, Wiggins, & Sens, 2009). A layer of annular lipids surrounds the proteins. These lipids are in direct contact with the hydrophobic surface of the protein and tightly seal the membrane at the protein–membrane interface. Often they take distorted arrangements to adapt to the rough surface of the protein (Lee, 2004) and play an important role in stabilizing membrane protein oligomers (Gupta et al., 2017). However, most of the annular lipids are removed during the purification process, and in most cases they are not reintroduced afterward for crystallography or single-particle cryo-EM with detergents or polymers. In many crystal structures (Yeagle, 2014) and also recent cryo-EM structures (Liao et al., 2013) though, some very tightly bound lipid molecules remain bound during purification and can be resolved in the structures.

The optimal way to purify a membrane protein together with its natural annular lipids is to use detergent-free purification procedures that make use of SMA copolymers (Dörr et al., 2014, 2016) or nanodiscs (Civjan, Bayburt, Schuler, & Sligar, 2003). The development of these techniques is still in its infancy, and it has not yet been broadly applied. The most common approach is thus to purify the protein using detergents and reintroduce lipids afterward. In most cases, lipid composition can be varied and adjusted to mimic the natural membrane environment or to identify key lipids and lipid compositions. This has been done, for instance, for bacteriorhodopsin (Lee et al., 2015), the ABC transporter MbsA (Kawai, Caaveiro, Abe, Katagiri, & Tsumoto, 2011), or cytochrome P450 reductase (Das & Sligar, 2009).

Among the different available options for reconstituting membrane proteins in a lipid bilayer, the lipid nanodisc system currently presents the best-understood and -characterized method. Lipid nanodiscs were developed in the laboratory of Stephen Sligar (Bayburt & Sligar, 2003). They are composed of a patch of lipid bilayer, which is surrounded by membrane scaffold protein (MSP). MSP is a derivative form of apolipoprotein A-1 (Apo-A1) (Ritchie et al., 2009) and composed of short amphipathic helices. In an assembled nanodisc, two MSPs arrange in a parallel or antiparallel manner, forming a belt around the hydrophobic region of the lipid bilayer that includes a few hundred lipid molecules (Fig. 1). Constructs of MSP with

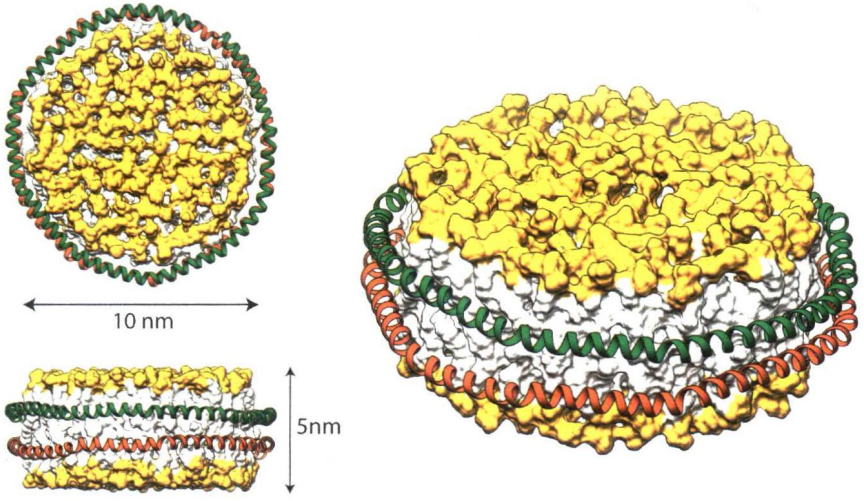

Fig. 1 Model of MSP1–POPC nanodisc structure. The two copies of MSP, surrounding the phospholipid bilayer, are shown in *green* and *brown*. Lipid headgroups are colored in *yellow*. The model was produced using the CHARMM-GUI server.