Biophysical Analysis of Membrane Proteins

Investigating Structure and Function

Edited by
Eva Pebay-Peyroula

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The Editor

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Preface

Membrane proteins are known to be key molecules in cellular communications, from signal transduction to ion exchanges or transport of metabolites and other molecules. They also participate in the synthesis of ATP, by generating the proton gradient necessary for the rotatory motor of ATP-synthetase to function and to catalyze ATP formation from ADP and inorganic phosphate. Membrane proteins are necessary for the import of soluble or membrane proteins from the cytosol, where they are synthesized into various compartments such as the mitochondrial matrix or outer and inner mitochondrial membranes. Living organisms have also designed efficient machineries that protect cells from toxic elements. Bacteria or eukaryotic cells have, in their membranes, efflux pumps that will clean the cell. The efflux of toxic elements also has drastic consequences for the efficiency of drugs that may find difficulties in penetrating the cell in order to be active. In contrast to soluble proteins, membrane proteins are embedded in a medium which is organized continuously from the atomic level (at the nanoscale) to the micron range. However, the mesoscopic organization of membranes influences, through long-range effects, the properties of the molecules that are embedded in the membranes. Therefore, an understanding of the function of membrane-integrated molecular machineries necessitates a description of the proteins on the atomic level, their various conformations, their specialized organization, as well as their dynamics within the membrane.

Despite attracting great interest, membrane proteins are still difficult to study at the molecular level. Indeed, they are difficult to produce, to extract from their natural environment, and to purify in a native conformation. However, during the past decade efforts have been stepped up worldwide such that several new structures have been resolved at high resolution and their details published within the past two to three years. All of these structures have opened a wide field of discussion about the function and the topology of membrane proteins, their interactions with lipids, the need for such interactions, interactions with ligands or cofactors, and a large number of functional mechanisms could be postulated. At the same time, it has also become clear from the results of many studies that, even with very high-resolution structures, the atomic details were insufficient to understand the function. Further information was needed on the identification and characterization of different conformations, on the dynamics that are necessary for

conformational changes, on how membrane proteins are inserted in their natural environment, and on how they are organized within the membrane. Although, crystallography represents an extremely powerful method by which to describe the atomic structures of proteins, an ensemble of complementary biophysical approaches is essential in order to fully describe the structure–function relationships of proteins in general, and of membrane proteins in particular.

This book will serve as a cutting-edge resource for the biophysical methods that are – or soon will be – the major techniques used in the field. Each chapter is dedicated to a specific approach, describing the method involved, highlighting the experimental procedure and/or the basic principles, and offering an up-to-date understanding of what is measured, what can be deduced from the measurements, as well as the limitations of each procedure. This comprehensive reference book will be helpful to junior scientists whose target is to solve structure–function problems associated with membrane proteins, an will surely guide them in their experimental choices. Indeed, this book will also serve as a resource for anybody who is interested in membranes.

Following a general introduction to membrane protein structures and X-ray crystallography, the book is divided in five sections. Part I (the Introduction) is dedicated to structural approaches, while in Part II, Chapter 2 describes several aspects of electron microscopy either on single particles or on two-dimensional and tubular crystals, and Chapter 3 illustrates the current possibilities of NMR, and their future. Part III is centered on molecular interactions and the study of large molecular assemblies, with Chapter 4 illustrating how analytical ultracentrifugation can be used to address the study of membrane proteins solubilized in detergent micelles. Chapter 5 discusses how surface plasmon resonance – a well-known method used to study molecular interaction with soluble proteins – can also be adapted to membrane proteins. Molecular interactions and the topology of large assemblies of membrane proteins, either in reconstituted systems or in natural membranes, can also be studied by using atomic force microscopy, as shown in Chapter 6. Part IV is focused on dynamics, either by computational or experimental approaches. Here, Chapter 7 illustrates the possibilities of molecular dynamic calculations, while Chapter 8 describes how transport pathways can be followed by free energy calculations and Chapter 9 highlights the power of neutron scattering for studying membrane protein in their natural environment. Part V focuses on spectroscopies of various types. For example, circular dichroism can be extended to membrane proteins, as shown in Chapter 10, whilst infrared or Raman spectroscopy is able to probe either global folding properties or very fine local information, as demonstrated in Chapters 11 and 12, respectively. Finally, Part VI is devoted to functional approaches in whole cells, wherein Chapter 13 explains the possibilities offered by FRET or BRET experiments.

The Editor

Eva Pebay-Peyroula is a professor in the Physics Department at the University of Grenoble. Having gained her PhD in molecular physics in 1986, Prof. Pebay-Peyroula began working the Laue-Langevin Institut, where her interests shifted from physics to biology. Subsequently, after studying the structural properties of lipidic membranes, mainly by neutron diffraction, she moved into the field of protein crystallography, which in turn aroused an interest in membrane proteins. During the past years, Prof. Pebay-Peyroula's main area of study has included light-driven mechanisms achieved by bacterial rhodopsins, membrane proteins from archaeal bacteria and, more recently, the ADP/ATP carrier, a mitochondrial membrane protein. Currently, Prof. Pebay-Peyroula heads the *Institut de Biologie Structurale* in Grenoble and, since 2005, has belonged to the French Academy of Science.

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Part I
Introduction

1
High-Resolution Structures of Membrane Proteins: From X-Ray Crystallography to an Integrated Approach of Membranes

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1.1
Membranes: A Soft Medium?

Membranes delineate cells and cellular compartments, and are efficient barriers that allow the compartmentalization necessary for the functional specificity of each cell or organelle. Membranes are mainly composed of lipids and proteins. As a first approximation, lipids – which spontaneously form bilayers in water – ensure the mechanical properties of the membranes, such as shape, watertightness, robustness and plasticity, whereas proteins are responsible for the communications between compartments or cells, and ensure signaling, channel or transport activities. In fact, membranes are much more complex, and proteins also participate in mechanical properties whereas lipids play a role in the function. Some integral membrane proteins such as the ATP-synthetase located in the inner mitochondrial membrane are described to induce a local curvature of the membrane by dimerization, and could therefore be responsible for the topology of this membrane [1]. Membrane-associated proteins such as clathrin, and associated proteins, by coating the membrane of vesicles formed during endocytosis, may also strongly influence the mechanical properties of the membrane [2]. Likewise, lipids are described now as important players in the function. For example, phosphatidylserine is known to be exposed at the surface of apoptotic cells and used as a signal for the immune system to eliminate the cell [3]. Various sugars participate also both in the mechanical properties and functional aspects of membranes. These play major roles in molecular recognition as illustrated by the role of heparan sulfate molecules [4]. Among all the molecular components of biological membranes, proteins are the only ones to be structured at an atomic level. With the exception of a few individual lipids that are tightly bound to proteins, most of the lipids are organized within a bilayer that can be described at a so-called "mesoscopic" scale by a mean bilayer thickness, a surface area per lipid, lipid order parameters describing chain dynamics and possibly local domain structures [5]. Strong thermal fluctuations of each individual molecule within the membrane make an atomic description irrelevant. Therefore, membranes must be described

at various scales in order to take into account all the molecular components and the high protein concentration of some membranes where proteins account for 50% or more of the membrane [6]. Structure–function analyses of membrane proteins at an atomic level are thus of major importance, and shed light on major cellular processes, signaling pathways, bioenergetics, the control of synaptic junctions, and many others. Indeed, membrane proteins in general – and G-protein-coupled receptors (GPCRs) and ion-channels in particular – are known to be the target for many drugs (60% of drug targets are estimated to be membrane proteins). However, despite high potential interest – both for fundamental understanding and also for pharmaceutical applications such as drug design – very little is still known regarding the structure of membrane proteins compared to soluble proteins. This lack of information reflects the difficulty of producing large quantities of stable membrane proteins and crystallizing them in order to solve their structure by using X-ray diffraction (XRD). In addition, the functional state of membrane proteins is often tightly linked to their natural environment, a lipid bilayer, with some individual lipids bound specifically to the proteins. In order to proceed to structural studies it is first essential to mimic, as best as possible, the natural environment.

1.2
Current Knowledge on Membrane Protein Structures

1.2.1
An Overview of the Protein Data Bank

Currently, amongst more than 40 000 entries, the "Protein Data Bank" (PDB) contains about 250 membrane protein structures, representing at least 120 unique proteins (http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html). Since 1985, when the first membrane protein structure – a photoreaction center from *Rhodopseudomonas viridis* – was resolved [7], the number of structures solved per year has increased almost exponentially, with the progression resembling that of soluble proteins with a slight shift toward lower values [8]. This progression is rather encouraging, and has resulted from the large-scale efforts undertaken recently in several countries. Several programs dedicated to the structural genomics of membrane proteins were started. Some of these are based on large networks and focus on the exploration of various expression systems and on the set up of automated procedures that facilitate these explorations. Smaller networks help to share expertise on membrane protein biochemistry and the physical chemistry of amphiphiles and lipids, and favor interdisciplinary developments that are valuable for structural and functional studies of the proteins in a natural environment.

Most of the structures deposited in the PDB were solved by X-ray crystallography to typical resolutions ranging from 3.5 to 1.5 Å. A few structures were solved by using electron diffraction with two-dimensional crystals. Among these, bacteriorhodopsin – a light-activated proton pump, which is well ordered in two dimen-

sions in the native membrane – was the first membrane protein structure to be determined [9], and was later solved at a resolution of 3.5 Å, or better [10, 11]. Although the nicotinic acetylcholine receptor could never be crystallized in three dimensions, two-dimensional (2-D) tubular arrangements allowed the structure to be solved at 4 Å resolution, revealing the overall topology [12]. Electron diffraction was also used successfully for aquaporins, with AQP4 – a water channel from rat glial cells – being solved to 1.8 Å resolution [13]. As described in Chapter 2, electron microscopy (EM) provides an alternative structural method for membrane proteins, in some cases with a lipidic environment that is close to the native one. This is of particular interest when proteins are present as oligomers in the membrane, possibly in a lipid-dependent manner. EM is also relevant for the characterization of structural modifications that are more likely to be induced in 2-D crystals than in 3-D crystals where crystal contacts might hinder larger movements, as demonstrated for bacteriorhodopsin. More recently, a few structures were reported that had been solved with nuclear magnetic resonance (NMR), including three β-barrel proteins from the *Escherichia coli* outer membrane in dodecylphosphocholine (DPC) or octyl-glucoside micelles [14–16], and one human helical protein, phospholamban, from the sarcoplasmic reticulum [17]. NMR also represents a very useful approach for probing ligand pockets and detecting structural modifications induced by ligand binding [18].

1.2.2 Protein Sources for Structural Studies

The majority of membrane proteins for which structures were solved are derived from bacterial sources, and less than 20% of these are eukaryotic. Indeed, some are specific to bacteria, and solving their structures might create new openings for antibiotics or bioremediations. Others can be used as models for eukaryotic homologues (ion channels, ABC transporters). Unfortunately, even if these models are able to provide the first insights into important structural features, they are certainly not informative enough to provide a full understanding of the functional mechanisms and specifically of functional mechanisms that might achieve an efficient drug design. There remains a broad range of membrane proteins of new classes or different functions and/or different species for which structures are needed. Despite many efforts, the expression of membrane proteins remains a hazardous task, with success relying on the outcome of many different investigations [19, 20]. Obvious restrictions to overexpression result from the limited volume of membranes compared to the cytosol when expressing soluble proteins. Insertion and correct folding in the membranes are also non-trivial issues that must be addressed with appropriate signals within the amino-acid sequence of the protein. Investigations into insertion mechanisms are still under way (e.g., Ref. [21]). Finally, expressing a protein at high level in the membrane causes significant perturbation to the cell, and this often causes a highly toxic effect. However, among the success stories, many bacterial proteins have been expressed in large quantities in *E. coli*; indeed, only recently several eukaryotic proteins were expressed in

sufficient quantity and quality in heterologous systems to allow crystallization and structure determination. For example, a voltage-gated potassium channel, Kv1.2, from *Rattus norvegicus* was expressed in *Pichia pastoris* and its structure solved to 2.9 Å resolution [22]. Elsewhere, a plant aquaporin, soPIP2;1, from spinach was also expressed in *Pichia pastoris* and solved to 2.1 Å resolution in its closed state and 3.9 Å in its open state [23]. A recent breakthrough of heterologous expression was achieved for the sarcoplasmic Ca-ATPase. This protein, which is highly abundant in the rabbit sarcoplasmic reticulum, was purified from the native membrane and extensively studied, leading to the structures of different conformations from which a functional mechanism was postulated. Recently, the protein was overexpressed in yeast, whereupon it could be purified, crystallized and the structure solved, thus opening the way to functional and structural studies of mutants, which serve as an essential link in a complete structure–function analysis [24]. These examples of recent successes in heterologous expression demonstrate that such as approach is possible, and that the rate of success depends not only on the exploration of various expression systems but also on the knowledge of the biochemical behavior of the protein itself.

1.2.3
The Diversity of Membrane Protein Topologies

The only structural motifs of membrane-inserted peptides are α-helices and β-barrels Transmembrane helices (TMH) are identified by hydrophobic scoring from the protein sequence. β-barrels are found in bacterial outer membranes and are more difficult to predict from their amino-acid composition, although recent progress in β-barrel prediction has emerged. Extensive internal hydrogen bondings in α-helices and β-barrels ameliorate the high energetic cost of dehydrating the peptide bonds, which is necessary for the insertion of peptides into membranes [25]. Although very few membrane proteins are known to be structurally organized in multi-domains, the structures currently available in the PDB highlight the diversity of transmembrane arrangements. TMH bundles create various topologies, depending on the tilt and the kinks that are possible for each individual helix. Some examples of overall membrane protein structures are illustrated in Fig. 1.1. Heteromeric or homomeric associations of TMHs also contribute to the variety of membrane protein topologies. Setting apart proteins with a single TMH (for which the TMH is mainly a membrane anchor and in some cases is responsible for signal transduction through protein dimerization), most membrane proteins that have a function in the membrane have more than six TMHs. Channels are constituted by more than eight TMHs (an octamer of one TMH for WZA, tetramer of two TMHs for various potassium channels, pentamer of two TMHs for the nicotinic receptor). In these examples, channels are formed by several TMHs, each of which is derived from one of the monomers, whereas transport pathways can also be formed within a single monomer of several TMHs (seven TMHs for aquaporins, six for the mitochondrial ADP/ATP carrier, and 12 for lactose permease), which in turn form multimers in the native membrane (tetramer for aquaporins, dimer for lactose permease). Currently, DsbB (a component of a periplasmic

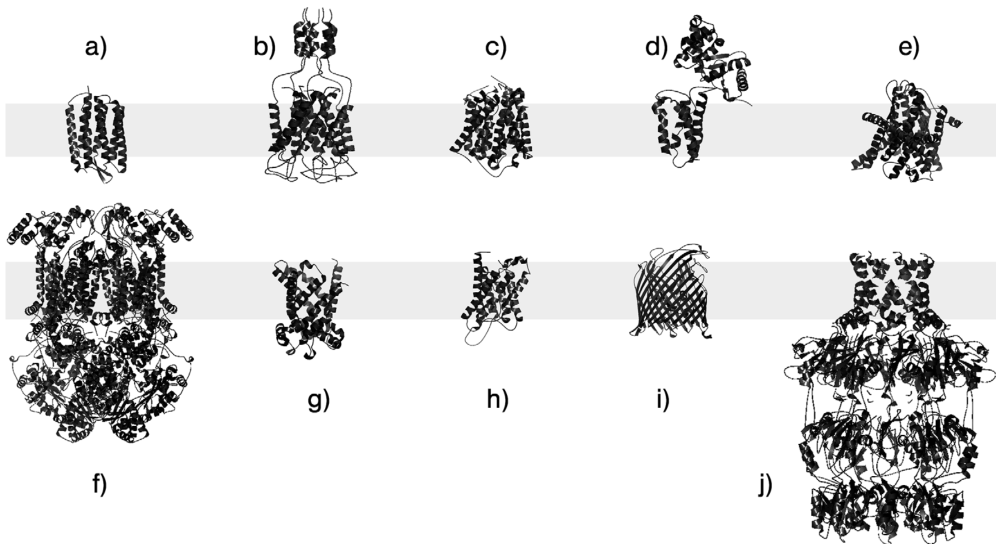

Fig. 1.1 Various topologies of membrane proteins. The Fig. depicts several α-helical proteins showing the diversity of transmembrane helices, and one β-barrel protein. (A) Monomer of bacteriorhodopsin (BR), BR forms a trimer (1qhj). (B) Mechanosensitive channel, a homopentamer with 10 TMHs (1msl). (C) Monomer of the Ammonium transporter AmtB, 11 TMHs per monomer, forms a dimer (1u77). (D) DsbB, four TMHs (2hi7). (E) The protein-conducting channel SecY, heterotrimer with 12 TMHs in total (1rhz). (F) The cytochrome bc1 complex from bovine heart mitochondria, 11 subunits and 12 TMHs per monomer, forms a dimer (1bgy). (G) The ADP/ATP carrier from bovine heart mitochondria, six TMHs (1okc). (H) Monomer of the AQP1 water channel from bovine blood, a homotetramer with seven TMHs per monomer (1j4n). (I) FptA, a pyocheline receptor from the *Pseudomonas aeruginosa* outer membrane, representative for β-barrel structures (1xkw). (J) WZA, the first α-helical protein characterized from the *E. coli* outer membrane (2j58).

oxidase complex with four TMHs) is a membrane protein of known structure, which has the smallest number of TMHs. However, this protein is known to interact with another membrane protein, DsbC, and therefore in the native membrane the total number of TMHs present in the functional complex might be higher. The structure of membrane proteins in a lipidic environment might be energetically more favorable to a larger number of TMH helices. Indeed, it was proposed that helix associations are probably driven by van der Waals interactions through helix–helix interactions rather than hydrophic effects such as those which lead to the folding of soluble proteins [26]. Such stabilizing van der Waals interactions could thus be favored by a larger number of TMHs.

The functional properties of membrane proteins, when driven by dynamic properties, will also constrain the topology. The main role of α-helices in the transmembrane domains of photosynthetic complexes is to locate precisely all of the pigments necessary for the efficiency of photon absorption and their conversion into an electron transfer. The dynamics of such helices must therefore be limited. In contrast, transporters which have to shuttle large metabolites in a very

specific manner over the membrane, must undergo large conformational changes that necessitate the molecule to be highly dynamic. Based on these extreme examples, it is easy to imagine that the number of TMHs of the functional entity within the membrane will play a crucial role.

1.2.4 Genome Analyses

What can be learned from the genome data available so far? The analyses of the genomes were performed in order to identify membrane proteins and to classify them into families. A recent analysis showed that membrane proteins cluster in fewer structural families than do their soluble counterparts [27]. However, because of the physical constraints of the lipidic environment, this smaller number of families is rather logical; indeed, some authors have even proposed that membrane proteins have 10-fold fewer families [28]. For example, Oberai et al. estimate that 90% of the membrane proteins can be classified into 1700 families and are structured with 550 folds, while 700 families structured in 300 folds cover 80% of the membrane proteins. This study is based on the search of the TM segment defined by hydrophobic sequences, and is therefore appropriate to helical rather than to β-barrel proteins. Furthermore, these authors also noted that their estimate was based on a limited number of known structures, and may have been biased by present knowledge. Today, new features continue to emerge from recent experiments. For example, TMHs were characterized in a bacterial outer membrane protein, WZA, the translocon for capsular polysaccharides in *E. coli* [29]. The eightfold repeat of a single TM of the octameric protein forms a 17 Å pore in the outer membrane, showing the first α-helical-barrel in the outer membrane of *E. coli*. Unfortunately, this example clearly illustrates that our current structural knowledge is still limited, and that further experimentally determined structures will provide new data for global genome analyses.

Further interesting information has emerged from the comparison between the size of the families and current structural knowledge (see Table 1 in Ref. [27]). For the most important families – rhodopsin-like GPCRs (5520 members) and major facilitators (3680 members) – only one and three structures, respectively, have yet been determined. Moreover, the situation is no better for other families– some are completely absent from the PDB, and even if a few representatives of a family are structurally known, the overall fold might not be sufficient to provide an understanding of the functional mechanism and to help derive structure-based drug designs.

1.3 X-Ray Crystallography

This section will briefly describe some general aspects of crystallization and crystallography, after which attention will be focused on those features more specific