BBA - Proteins and Proteomics xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

BBA - Proteins and Proteomics

journal homepage: www.elsevier.com/locate/bbapap

Review

Recent advances in biophysical studies of rhodopsins – Oligomerization, folding, and structure

Leonid S. Brown[a,*], Oliver P. Ernst[b,*]

[a] Department of Physics and Biophysics Interdepartmental Group, University of Guelph, Guelph, Ontario N1G 2W1, Canada
[b] Departments of Biochemistry and Molecular Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

ARTICLE INFO

Keywords:
Rhodopsins
Oligomerization
Folding
Ion transport
Membrane protein structure
Photosensory transduction

ABSTRACT

Retinal-binding proteins, mainly known as rhodopsins, function as photosensors and ion transporters in a wide range of organisms. From halobacterial light-driven proton pump, bacteriorhodopsin, to bovine photoreceptor, visual rhodopsin, they have served as prototypical α-helical membrane proteins in a large number of biophysical studies and aided in the development of many cutting-edge techniques of structural biology and biospectroscopy. In the last decade, microbial and animal rhodopsin families have expanded significantly, bringing into play a number of new interesting structures and functions. In this review, we will discuss recent advances in biophysical approaches to retinal-binding proteins, primarily microbial rhodopsins, including those in optical spectroscopy, X-ray crystallography, nuclear magnetic resonance, and electron paramagnetic resonance, as applied to such fundamental biological aspects as protein oligomerization, folding, and structure.

1. Introduction – rhodopsins as prototypical membrane proteins and testing grounds for biophysical methods development

Rhodopsins constitute a large group of photoactive α-helical membrane proteins which covalently bind retinal as their chromophore *via* lysine Schiff base. The retinal chromophore gives rhodopsins their characteristic color and provides the light-absorbing element, which can store energy of photons after its photoisomerization. The energy stored in the primary photoreaction of rhodopsins can be further used to trigger protein conformational changes in order to perform a number of diverse functions, including photosensory signal transduction, active and passive ion transport, and light-regulated enzymatic activity. There are two very divergent protein classes combined under the common "rhodopsins" name, dissimilar in their primary structure but unified by the presence of retinal chromophore and predominantly seven-trans-membrane helical architecture. The first class of rhodopsins (so-called Type I or microbial rhodopsins) is found in *Archaea*, *Bacteria*, and lower *Eukaryotes* (fungi, algae, and some protists). These microbial rhodopsins mainly have all-*trans* configuration of retinal, which photoisomerizes to 13-*cis* retinal, and perform all of the above mentioned functions. The second class, Type II or animal rhodopsins, are found in *Metazoa*, bind mainly 11-*cis* retinal which photoisomerizes to all-*trans* retinal, and are the main drivers of vision and other photosensory processes in animals. Unlike microbial rhodopsins, animal rhodopsins belong to Class A of G protein-coupled receptors (GPCRs) and interact with a number of partners typical for this class of proteins (G proteins, arrestins, rhodopsin kinases, *etc.*). We refer the readers to the vast literature on both types of rhodopsins for more detail on their structure, functions, and biology (see, for example, references in the following reviews [1–10]).

For almost half a century both microbial and animal rhodopsins have served not only as prototypical α-helical membrane proteins but also as testing grounds for various biophysical methods development. Special contribution of the two most studied rhodopsins should be noted in this respect, that of Type I bacteriorhodopsin (BR), light-driven proton pump of *Halobacteria*, and Type II bovine visual rhodopsin (Rho). Looking back at the massive body of literature on BR, Rho, and their close homologs, one may wonder what made them so popular in the biophysics community. Conceptually, both rhodopsins have been serving as structural models for GPCRs (BR at earlier stages, Rho later on), while BR attracted significant interest as the most primitive and tractable standalone photosynthetic machine for production of trans-membrane proton gradients. Methodologically, among the most

Abbreviations: AFM, Atomic Force Microscopy; apolipoprotein A-I, apolipoprotein A1; ASR, *Anabaena* sensory rhodopsin; BN-PAGE, blue native - polyacrylamide gel electrophoresis; BPR, blue-absorbing proteorhodopsin; BO, bacterioopsin; BR, bacteriorhodopsin; CD, Circular Dichroism; DEER, double electron-electron resonance; DNP, Dynamic Nuclear Polarization; EM, electron microscopy; EPR, electron paramagnetic resonance; FTIR, Fourier-transform infrared; GPCR, G-protein coupled receptor; GPR, green-absorbing proteorhodopsin; HR, halorhodopsin; LILBID, laser-induced liquid bead ion desorption; MAS, magic-angle spinning; NMR, nuclear magnetic resonance; PR, proteorhodopsin; PRE, paramagnetic relaxation enhancement; Rho, bovine visual rhodopsin; SAXS, small angle X-ray scattering; SSNMR, solid-state NMR

* Corresponding authors.
E-mail addresses: lebrown@uoguelph.ca (L.S. Brown), oliver.ernst@utoronto.ca (O.P. Ernst).

http://dx.doi.org/10.1016/j.bbapap.2017.08.007
Received 20 May 2017; Received in revised form 6 August 2017; Accepted 11 August 2017

plausible technical factors are their ready availability, due to extremely high levels of expression and packing of BR in *Halobacterium salinarum* and Rho in bovine rod cells, combined with ease of isolation of rhodopsin containing membranes (the purple membrane for BR and disks of rod outer segments for Rho). Observable color of rhodopsins (more relevant for BR as Rho should be protected from light to avoid bleaching), along with their high thermal and chemical stability are also very helpful in working with these proteins. Additionally, for BR and its homologs, the cyclic nature of their photoreactions (so-called photocycle) is conducive to application of biophysical techniques requiring highly repetitive measurements, while the photoactivatable nature of both types of rhodopsins is highly advantageous for time-resolved measurements where precise synchronization is needed. Finally, inherent 2D crystallinity of BR in the purple membrane served well both diffraction-based techniques and symmetry-based methods.

There are a number of notable biophysical and biochemical methods development milestones involving BR and Rho. Starting with groundbreaking experiments on co-reconstitution of BR and ATPase in support of the chemiosmotic theory [11] and its pioneering use in the development of electron crystallography [12] in mid-seventies, rhodopsins have continued to excel ever since. In structural biology, BR was a model system for development of several techniques (see Section 4 below for more detailed description). Key examples are the lipidic cubic phase and bicelle crystallization methods [13,14]. In addition, Rho was the first GPCR whose structure was solved crystallographically [15]. Also notable is the role of BR in the development of high-resolution and high-speed Atomic Force Microscopy (AFM) [16,17], in the use of neutrons for studies of protein dynamics [18,19], and, most recently, in time-resolved X-ray crystallography [20]. For spectroscopic techniques, rhodopsins played a very important role in the development of time-resolved Fourier-transform infrared (FTIR) spectroscopy [21–23] and femtosecond stimulated Raman spectroscopy [24]. In the field of ultrafast optical spectroscopy, BR and Rho have been prominent in fundamental studies on the mechanism of photoisomerization, including those on coherent control [25,26] and determining the speed limit of the primary photochemistry of vision [27]. Finally, rhodopsins have been instrumental for the advancement of solution and solid-state nuclear magnetic resonance (SSNMR) of membrane proteins [28–31], including such novel exciting development as Dynamic Nuclear Polarization (DNP) used for the dramatic enhancement of NMR sensitivity [32].

In this review, we will address some of the latest developments in biophysical approaches to retinal-binding proteins, including those in optical spectroscopy, X-ray crystallography, nuclear magnetic resonance (NMR), and electron paramagnetic resonance (EPR), as applied to such fundamental biological aspects as protein oligomerization, folding, and structure.

2. Biophysical studies of oligomerization of microbial rhodopsins

Microbial rhodopsins can form stable oligomers of varying stoichiometry (from dimers to hexamers) and are also prone to forming 2D crystals. Early electron microscopy (EM) studies of the purple membrane showed that BR forms trimers which are assembled in 2D hexagonal lattice [12], and later electron crystallography, X-ray, and neutron diffraction work revealed the role of specific lipids in stabilization of this supramolecular structure [33–35]. It was soon realized that BR monomer is the functional unit and that the trimers can be easily destroyed by detergents or by lipid dilution [36,37]. On the other hand, more recent studies with BR incorporated into nanodiscs of varying size suggest that there is a minimal number of lipids required to maintain the trimeric assemblies [38]. Even though monomeric BR is functional and has tertiary structure similar to that of oligomeric BR in the purple membrane, its thermal stability is dramatically decreased in monomers, suggesting the importance of intermonomer interactions [39,40]. In the course of these studies it was found that circular dichroism (CD) spectroscopy in the visible wavelength range can serve as a convenient spectroscopic tool to test oligomeric state of BR, due to its sensitivity to excitonic coupling of the retinal chromophores which produces a characteristic bilobed shape of the spectrum [41,42]. More recently, this technique proved to be successful in detecting the presence of oligomers of other microbial rhodopsins, such as dimeric arrangement of algal channelrhodopsins [43], trimeric structure of chloride pump halorhodopsin (HR) [44], *Anabaena* sensory rhodopsin (ASR) [45], proton-pumping *Gloeobacter* rhodopsin [46], *Thermus* rhodopsin [47], and *Salinibacter* xanthorhodopsin [48]. It should be mentioned that in the latter case the CD spectrum may be dominated by excitonic coupling of the carotenoid antennae, rather than the retinal chromophores themselves, and also have contributions from intramolecular coupling of the carotenoid and the retinal [49].

While CD spectroscopy is convenient for detecting oligomers of microbial rhodopsins, it cannot provide details of the molecular architecture and stoichiometry of the assemblies and does not give atomic resolution of the intermonomer interfaces. AFM and EM, on the other hand, have been successful in revealing (or confirming) oligomeric state and lattice arrangement for some microbial rhodopsins, as was the case for dimers of channelrhodopsin-2, trimers of BR and *Parvularcula* rhodopsin, trimers of dimers of archaeal sensory rhodopsins, and pentamers and hexamers of green-absorbing proteorhodopsin (GPR) [17,50–53]. Unfortunately, one cannot always rely on X-ray crystallography, as the same microbial rhodopsin may crystallize in different oligomeric forms depending on the crystallization conditions, so that it may be hard to figure out the native intermonomer contacts of the protein. Among the well-known examples of multiple crystal forms are dimers and trimers of BR, pentamers and hexamers of blue-absorbing proteorhodopsin (BPR), and monomers and pentamers of sodium-pumping *Krokinobacter* rhodopsin (KR2) [14,54,55]. Recent room-temperature serial X-ray crystallography of BR showed that details of monomer-monomer interactions may be somewhat different from those observed at cryogenic temperatures, especially in the more flexible interfacial regions [56]. In view of this, it appears that combination of multiple alternative biophysical and biochemical techniques, including SSNMR and EPR, may be beneficial for revealing native oligomeric states of microbial rhodopsins, as we will discuss using examples of GPR and ASR.

Proteorhodopsins (PRs) are a large subfamily of microbial rhodopsins originally found in marine picoplankton and thought to have mainly proton-pumping function [57]. Green-absorbing and blue-absorbing species of PR are likely adapted to the light quality at different depths [58]. The first glimpse into oligomeric structure of lipid-reconstituted GPR has been provided by AFM, which showed that it exists predominantly in a radially symmetric hexameric form, with occasional pentamers, when it forms 2D crystals *in vitro* [50]. The plasticity of oligomeric state in the solubilized state has been confirmed by LILBID (laser-induced liquid bead ion desorption) mass spectrometry and BN-PAGE (blue native - polyacrylamide gel electrophoresis), which showed that GPR can exist as a monomer or various oligomers, depending on the detergent type and concentration used, and that Triton X-100 and OG promote formation of monomers and dimers, while *n*-dodecyl-β-D-maltoside (DDM) detergent favors formation of pentamers [59,60]. It should be noted that there seem to be no consensus on the oligomeric state of GPR in DDM micelles (300–340 kDa size), as earlier gel-filtration chromatography suggested a trimeric form [61], while later FPLC followed by SEC-LS/UV/RI analysis proposed hexamers independent of DDM concentration [62], both in contradiction to the mass spectrometry and BN-PAGE results [60]. Estimates of intermonomer distances in the DDM micelles, performed by continuous wave EPR of nitroxide spin-labeled GPR and by double electron–electron resonance (DEER) EPR of Gd^{3+} labels agree with the hexameric arrangement [62,63]. A recent crosslinking study indicates that GPR in *E. coli* membranes exists in hexameric form as well, and the oligomers are largely preserved upon solubilization in DDM, but to a much smaller extent in short-chain