Rhodopsin Oligomerization and Aggregation

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Abstract

Rhodopsin is the light receptor in photoreceptor cells of the retina and a prototypical G protein-coupled receptor. Two types of quaternary structures can be adopted by rhodopsin. If rhodopsin folds and attains a proper tertiary structure, it can then form oligomers and nanodomains within the photoreceptor cell membrane. In contrast, if rhodopsin misfolds, it cannot progress through the biosynthetic pathway and instead will form aggregates that can cause retinal degenerative disease. In this review, emerging views are highlighted on the supramolecular organization of rhodopsin within the membrane of photoreceptor cells and the aggregation of rhodopsin that can lead to retinal degeneration.

Keywords G protein-coupled receptor · Quaternary structure · Photoreceptor cell · Retina · Phototransduction · Retinal degeneration

Introduction

Vision is initiated in photoreceptor cells, which are located in the outer retina. The initiation of vision occurs when light activates rhodopsin or cone opsins in photoreceptor cells, initiating a prototypical G protein-mediated signaling cascade called phototransduction. Rod photoreceptor cells are responsible for scotopic vision and contain rhodopsin whereas cone photoreceptor cells are responsible for photopic vision and contain cone opsins. Rod photoreceptor cell biology and rhodopsin's structure and function have been characterized more extensively compared to cone photoreceptor cell biology and cone opsin structure and function, in part, because of the abundance of rod photoreceptor cells in most vertebrate retina. The focus here will be on the quaternary structures formed by rhodopsin.

Rhodopsin is a prototypical G protein-coupled receptor (GPCR), exhibiting a characteristic 7 α-helical transmembrane architecture. Rhodopsin is a two-component system that includes the apoprotein opsin and the covalently bound chromophore 11-*cis* retinal. Light causes the isomerization of 11-*cis* retinal to all-*trans* retinal, thereby activating rhodopsin and initiating phototransduction (Park 2014). The

biosynthesis and activity of rhodopsin occur in distinct compartments of the rod photoreceptor cell. Photoreceptor cells are compartmentalized into an inner segment and an outer segment (Fig. 1). The biosynthetic machinery is in the rod inner segment. Rhodopsin must be synthesized and adopt a proper three-dimensional structure to bypass quality-control mechanisms in the endoplasmic reticulum (ER). If rhodopsin misfolds, it must be discarded or aggregation can occur leading to dysfunction. Several mutations in rhodopsin cause misfolding and aggregation of the receptor. Emerging views on rhodopsin aggregation will be presented here.

After passing quality control, rhodopsin is transported to the base of the connecting cilium, which separates the inner and outer segments, and then transported across the connecting cilium into the rod outer segment (ROS) (Goldberg et al. 2016; Insinna and Besharse 2008; Nemet et al. 2015; Sung and Chuang 2010; Wang and Deretic 2014; Wensel et al. 2016). The ROS contains a structured system of membranes containing stacks of disks, which are double lamellar membranes connect by a rim, that are encased by a plasma membrane (Gilliam et al. 2012; Nickell et al. 2007). Rhodopsin is incorporated into disks at the base of the ROS (Ding et al. 2015; Volland et al. 2015). Rhodopsin is densely packed into the membrane of the disks. Emerging views on rhodopsin supramolecular organization within the disk membrane will be presented here.

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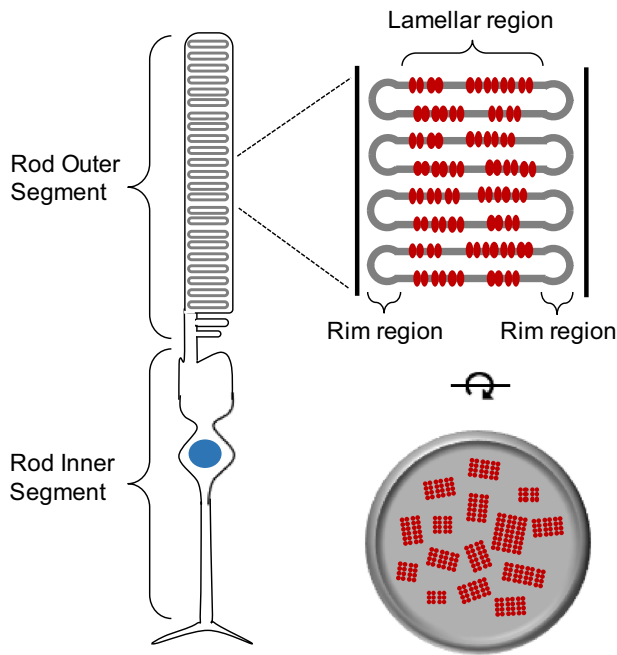
Fig. 1 Cartoon of a rod photoreceptor cell. Rod photoreceptor cells contain a rod outer segment and rod inner segment. The rod outer segment contains stacks of membranous disks, which are shown in a side view and top view. Rhodopsin (red) is densely packed within the membrane of the disks, forming nanodomains of oligomeric receptor (This figure is reprinted from (Rakshit et al. 2017), with permission from Elsevier) (Color figure online)

Rhodopsin Oligomers and Nanodomains

Rhodopsin is unique among GPCRs in that it is densely packed within its native membrane. The average density of rhodopsin is estimated to be about 20,000 molecules/μm^2, which is orders of magnitude higher than that of other GPCRs, with estimates ranging from 5 to 30 molecules/μm^2 (Hegener et al. 2004; Herrick-Davis et al. 2015). This high density of rhodopsin within the membrane can be both beneficial and detrimental in facilitating the exquisite sensitivity exhibited by rod photoreceptor cells, which can respond to a single photon of light (Baylor et al. 1979). The high density of rhodopsin can maximize the probability of photon capture, such as it occurs in thylakoid membranes of plants, green algae, and cyanobacteria, which are densely packed with photosynthetic membrane proteins to maximize absorption of sunlight (Kirchhoff 2014). While the high density of rhodopsin is advantageous from the perspective of photon capture probability, G protein signaling is a diffusion-mediated process, which would be adversely affected by a crowded membrane environment densely packed with the receptor. This dichotomy raises an important question: how do you achieve efficient and sensitive signaling within a crowded membrane environment?

A general solution nature has designed for this problem is to impart order within biological membranes (Bethani et al. 2010; Mugler et al. 2013; Radhakrishnan et al. 2012).

What kind of order is present within ROS disk membranes? Historically, rhodopsin was envisioned to be present within the disk membrane as randomly dispersed monomers (Chabre et al. 2003; Chabre and le Maire 2005). More recent studies by atomic force microscopy (AFM) and cryo-electron microscopy (cryo-EM) paint a different picture involving order within the membrane (Gunkel et al. 2015; Liang et al. 2003; Rakshit et al. 2015; Whited and Park 2015). Order within the membrane is achieved by oligomeric rhodopsin, arranged as rows of dimers, dispersed within the membrane forming nanodomains (Fig. 1). This type of order is predicted to overcome the impedance imparted by densely packed rhodopsin and facilitate efficient and sensitive signaling (Cangiano and Dell'Orco 2013; Dell'Orco 2013; Dell'Orco and Schmidt 2008; Gunkel et al. 2015; Schoneberg et al. 2014).

Investigating the organization of rhodopsin within native photoreceptor cell membranes is a challenge, and there are few available methods amenable for such pursuits. AFM has particularly been useful for visualizing membrane proteins under physiologically relevant conditions (Muller 2008; Whited and Park 2014), and a recently developed method utilizing AFM has provided insights to advance our understanding about the packing of rhodopsin in ROS disk membranes (Senapati and Park 2019). Although there are caveats to observations made by AFM, as there are with any method, a similar arrangement of rhodopsin within ROS disk membranes is observed by both AFM and cryo-EM, indicating that observations of oligomeric rhodopsin forming nanodomains is method independent. Moreover, artifacts related to phase separation of lipids due to low sample preparation temperatures, adsorption of samples on a mica substrate, and lateral forces imparted by the AFM tip have been ruled out (Rakshit and Park 2015; Rakshit et al. 2015). Thus, AFM provides a unique window to observe the native organization of rhodopsin within photoreceptor cell membranes. Some of the insights gained by AFM are discussed here.

Rod photoreceptor cells contain 500–2000 stacked disks within a single ROS, depending on the species (Daemen 1973). Although disks within a ROS are often presumed to be identical to each other when describing their properties, they are in fact quite heterogeneous. Different amounts of rhodopsin can be packed into a disk (Haeri et al. 2013; Hsu et al. 2015; Organisciak and Noell 1977; Penn and Anderson 1987), the lipid composition of membranes can be different among disks (Albert et al. 1998; Andrews and Cohen 1979; Boesze-Battaglia et al. 1990; Caldwell and McLaughlin 1985), and the functional properties of the disks can be heterogeneous (Baylor and Lamb 1982; Makino et al. 1990; Mazzolini et al. 2015; Williams and Penn 1985; Young and

Albert 2000). It is not surprising then that properties of the disks visualized by AFM are heterogeneous as well. Heterogeneity has been observed in the size of disks and the size, number, and density of rhodopsin nanodomains (Rakshit and Park 2015; Whited and Park 2015). This heterogeneity points to plasticity in the ROS.

Photoreceptor cells must adapt to the environment of the organism for optimal function and survival of the organism. Adaptations occurring at the level of individual disks have not been studied in much detail. An optimal density of rhodopsin for signaling within ROS disk membranes has been previously proposed (Saxton and Owicki 1989). The packing density of about 20,000 molecules/μm^2 for rhodopsin observed by AFM appears to be optimal for rod photoreceptor cells, at least under normal lighting conditions. Photoreceptor cells appear to adapt to different numbers of rhodopsin incorporated into disks by modulating the size of the disks to maintain an average rhodopsin density of 20,000 molecules/μm^2 (Whited and Park 2015). This packing density is even maintained under conditions where the amount of rhodopsin expressed in photoreceptor cells is reduced by half (Rakshit and Park 2015). Although this packing density of rhodopsin may be optimal under normal conditions, it can be modulated by changes in the environmental lighting condition. Under conditions where animals are housed under constant light or constant dark conditions, the density of rhodopsin decreases or increases, respectively (Rakshit et al. 2017). These adaptations in photoreceptor cells are dependent on the signal from phototransduction and the changes in rhodopsin density within the disk membrane can impact visual function as assessed by electroretinography (Rakshit et al. 2017; Senapati et al. 2018).

The concentration of rhodopsin within the membrane can impact the complement of oligomeric forms that are present within the membrane. Studies on heterologously expressed rhodopsin in cultured cells have demonstrated the existence of an equilibrium of oligomeric forms of rhodopsin (Fig. 2a). A monomer–dimer equilibrium was detected by pulsed-interleaved excitation fluorescence cross-correlation spectroscopy (PIE-FCCS) and a dimer–tetramer equilibrium was detected using Förster resonance energy transfer (FRET) spectrometry (Comar et al. 2014; Mishra et al. 2016). An equilibrium of oligomeric forms has also been detected for other GPCRs as well (Calebiro et al. 2013; Hern et al. 2010; Kasai et al. 2011; Patoway et al. 2013; Stoneman et al. 2017; Ward et al. 2015, 2017), thereby suggesting that oligomerization of all GPCRs can be described by schemes based on chemical equilibria (Fig. 2a). Within such a scheme, the equilibrium constants and concentration of the receptor will dictate the complement of oligomeric forms of the receptor present in the membrane.

The oligomerization of GPCRs is most often investigated in heterologous expression systems, where the receptors are considered to be overexpressed relative to their native expression levels. In the case of rhodopsin, this is not the case. Heterologous expression levels in cells like Chinese hamster ovary (CHO) cells is far below the native expression levels of rhodopsin in photoreceptor cells in the retina (1350 molecules/μm^2 versus 20,000 molecules/μm^2, on average) (Mishra et al. 2016). The difference in concentration of rhodopsin in heterologous expression systems and native photoreceptor cells can explain, at least in part, the difference in oligomeric forms observed in the two systems. Heterologous expression in CHO cells results in a mixture of dimers and tetramers, with higher order oligomers becoming detectable at concentrations of rhodopsin greater than 1150 molecules/μm^2 (Mishra et al. 2016). The size of oligomers in native photoreceptor cell membranes can be estimated from the size of rhodopsin nanodomains detected by AFM (Liang et al. 2003; Senapati and Park 2019). The size of rhodopsin nanodomains/oligomers within ROS disk membranes is heterogeneous, exhibiting a skewed distribution (Fig. 2b, d). The predominant oligomeric species has a size of 335 nm^2 (Rakshit and Park 2015), which corresponds to an oligomer with 24 rhodopsin molecules (i.e., 24-mer). These observations by AFM are consistent with observations in heterologous expression systems suggesting that there is an equilibrium of oligomeric forms of rhodopsin.

If an equilibrium of oligomeric forms of rhodopsin were present in photoreceptor cell membranes, then the complement of oligomeric forms present within the membrane should be adjustable by changing the concentration of the receptor within the membrane or any factor that alters the equilibrium constant. Evidence for both concentration-dependent and equilibrium constant-dependent changes in the oligomeric status of rhodopsin have been demonstrated in photoreceptor cells (Rakshit et al. 2017). Mice housed in constant light for 10 days have lower concentrations of rhodopsin in the membrane compared to mice housed in constant darkness for 10 days, and mice housed under normal cyclic lighting conditions have intermediary concentrations of rhodopsin (Fig. 2c). Examining histograms of the size of rhodopsin nanodomains/oligomers present in photoreceptor cell membranes from each of these mice reveals that all exhibit a skewed distribution with the predominant oligomeric species corresponding to a 24-mer (Fig. 2b). There is, however, a difference in the proportion of the 24-mer (main peak in histogram) and larger sized oligomers (shown in inset). A lower concentration of rhodopsin (10 days constant light) results in a higher level of the 24-mer and lower level of larger oligomers compared to that at higher concentrations of rhodopsin (10 days constant dark). Thus, lower concentrations of rhodopsin in the membrane shifts the equilibrium more in favor of the 24-mer and higher concentrations of rhodopsin in the membrane
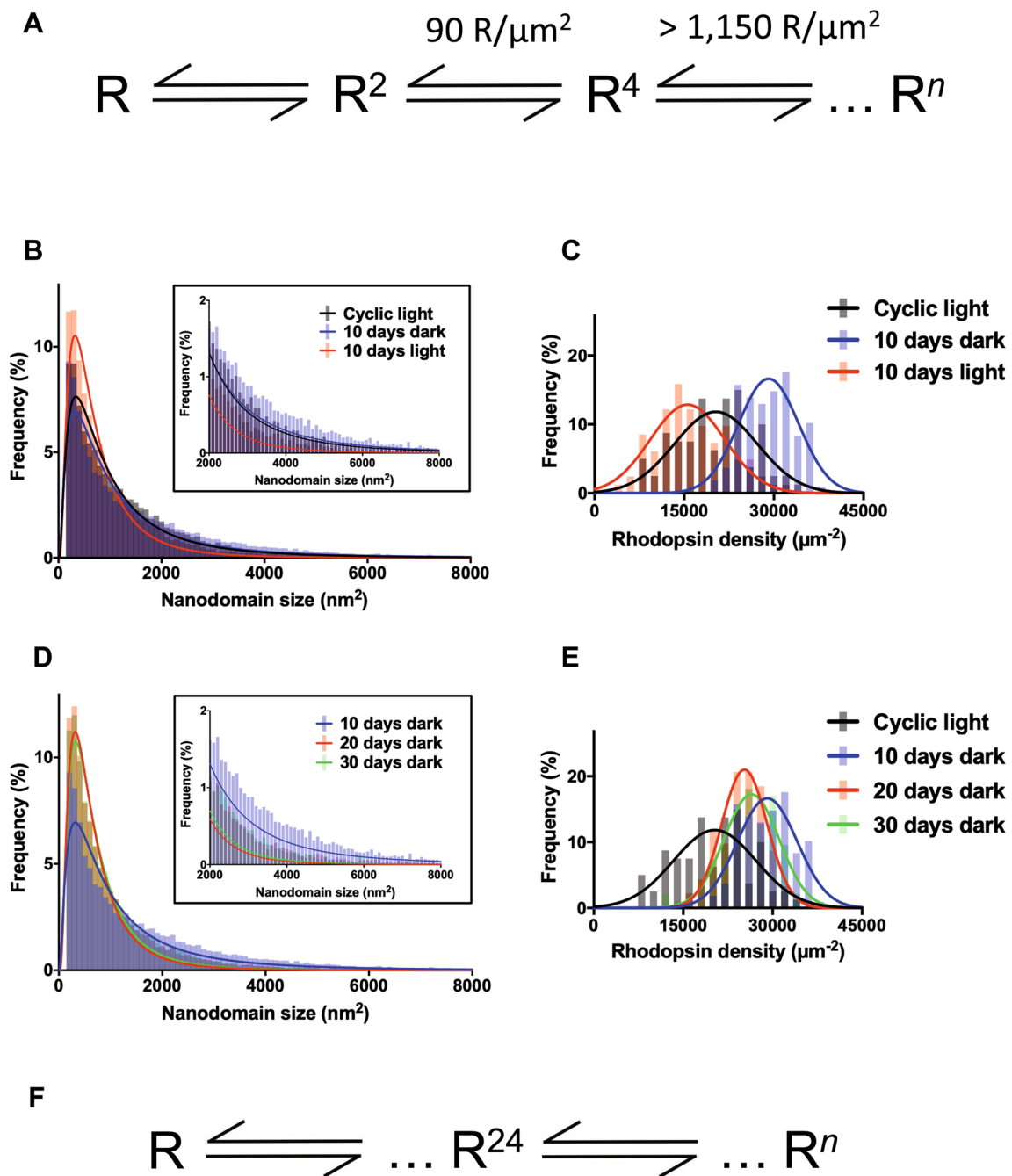
Fig. 2 Equilibrium of different oligomeric forms of rhodopsin. **a** A general schematic showing a chemical equilibrium among differently sized oligomers (denoted by the superscript) of a receptor (R). The equilibrium dissociation constants determined for rhodopsin are denoted (Mishra et al. 2016). **b–e** A summary of analysis of AFM images of ROS disk membranes is shown. Data from mice housed under cyclic light, 10 days constant dark, or 10 days constant light conditions are shown in **b**, **c**, and data from mice housed under 10, 20, or 30 days constant dark conditions are shown in **d**, **e**. Histograms of nanodomain sizes (**b**, **d**) and rhodopsin density within the membrane (**c**, **e**) are shown. The nanodomain size reflects the size of the rhodopsin oligomer. The histograms are reproduced from (Rakshit et al. 2017), with permission from Elsevier. **f** The predominant oligomeric species of rhodopsin in photoreceptor membranes is a 24-mer. The changes in the complement of rhodopsin nanodomains/oligomers illustrated in **b**, **d** can be described in terms of a shift in equilibrium between a 24-mer- and larger-sized oligomers (Color figure online)

shifts the equilibrium more in favor of the larger oligomers (Fig. 2f).

The equilibrium between the 24-mer and larger oligomers can still be shifted even when the concentration of rhodopsin is similar. Mice housed for 10 days, 20 days, or 30 days in constant darkness all exhibit similarly elevated concentrations of rhodopsin in the membrane compared to mice housed under normal cyclic lighting conditions (Fig. 2e). Yet, the complement of oligomeric forms is different in mice housed for 10 days in constant darkness versus mice housed for longer periods in complete darkness. The equilibrium appears to be shifted more in favor of the 24-mer than the larger oligomers for mice housed for longer periods of constant darkness compared to that for mice housed for 10 days in constant darkness (Fig. 2d, f). Thus, in this instance, it is not the concentration responsible for shifting the equilibrium but, rather, some factor that appears to have changed the equilibrium constant. It is unclear what this factor can be at the moment. An obvious source of change may be the lipids in the membrane.

Lipids in the membrane appear to play a role in driving oligomerization or stabilizing the oligomeric complex (Botelho et al. 2006; Jastrzebska et al. 2006; Periole et al. 2007; Soubias et al. 2015). It is unclear, however, which membrane lipids can alter the equilibrium constants. Docosahexaenoic acid (DHA) is highly abundant in ROS disk membranes and can affect the structure and function of rhodopsin (Boesze-Battaglia and Albert 1989; Brown 1994; Bush et al. 1994; Grossfield et al. 2006; Mitchell et al. 2001; Niu et al. 2001, 2004; Wiedmann et al. 1988). Although modulation of DHA in the membrane of photoreceptor cells of mice impacts function and leads to some adaptations in ROS disk membranes, the complement of oligomers in the membrane is not significantly altered (Senapati et al. 2018). Further studies are required to better understand the interplay of specific lipids and oligomerization, and determining what factors can change the equilibrium constants that underlie rhodopsin oligomerization.

Misfolding and Aggregation of Rhodopsin

GPCRs are highly hydrophobic proteins and must adopt a proper tertiary structure to avoid the fate of misfolding and aggregation. Heritable mutations in GPCRs can cause receptor misfolding and a range of human disease (Tao and Conn 2014). The rhodopsin gene is a hot spot for inherited mutations causing retinal disease (Mendes et al. 2005; Nathans et al. 1992; Stojanovic and Hwa 2002). There are over 100 reported mutations in the rhodopsin gene in patients with inherited retinal disease, most of them causing retinitis pigmentosa (RP)—a progressive retinal degenerative disease. Over half of the mutations in rhodopsin with

known biochemical defect result in receptor misfolding and aggregation, which lead to autosomal dominant RP (adRP) (Athanasiou et al. 2018). Rhodopsin mutations are the largest cause of adRP (Dalke and Graw 2005; Hartong et al. 2006).

Rhodopsin synthesis requires a network of coordinated processes in the ER that maintains protein homeostasis or proteostasis (Athanasiou et al. 2013; Gorbatyuk and Gorbatyuk 2013; Griciuc et al. 2011; Kroeger et al. 2012). These processes include a chaperone system that aids in the proper folding of rhodopsin and a quality-control system that eliminates improperly folded proteins via the ubiquitin–proteasome system or autophagy. Stresses on the ER perturbing proteostasis activate the unfolded protein response, which restores proteostasis in the short term but can lead to photoreceptor cell death in the long term if chronically activated (Walter and Ron 2011). The precise mechanisms that lead to photoreceptor cell death in retinal disease are still being uncovered (Adekeye et al. 2014; Arango-Gonzalez et al. 2014; Chiang et al. 2014; Sizova et al. 2014). Misfolded rhodopsin forms nonnative oligomers (i.e., aggregates), which can disrupt proteostasis and cause photoreceptor cell death (Bence et al. 2001; Illing et al. 2002). Inhibiting rhodopsin aggregation appears to reduce retinal degeneration (Athanasiou et al. 2012; Gorbatyuk et al. 2010; Parfitt et al. 2014; Vasireddy et al. 2011), establishing a direct link between aggregation and retinal degeneration. The mechanism by which aggregates cause cell toxicity is still unclear. Little is known about the process of rhodopsin misfolding and the nature of aggregates formed.

Mutations in rhodopsin that cause misfolding and aggregation are present within the transmembrane helices and extracellular surface of the receptor (Fig. 3). Since the discovery of the first mutation in rhodopsin causing adRP, the P23H mutation (Dryja et al. 1990), misfolding mutants of rhodopsin have been characterized biochemically and by microscopy (Garriga et al. 1996; Hwa et al. 1997; Kaushal and Khorana 1994; Sung et al. 1991a, b). Based on these characterizations, misfolding mutants of rhodopsin have been classified as either complete or partial misfolding mutants (Kaushal and Khorana 1994; Krebs et al. 2010; Sung et al. 1991a; 1993). Complete misfolding mutants cannot bind or be rescued by 11-*cis* retinal and are predominantly retained in the ER rather than trafficking correctly to the plasma membrane. Partial misfolding mutants can variably bind and be rescued by 11-*cis* retinal and are variably retained in the ER. The complete misfolding mutations tend to be located in close proximity to the binding pocket for 11-*cis* retinal whereas partial misfolding mutations are more distal (Fig. 3b).

The biochemical and microscopy-based methods used to classify rhodopsin mutants as complete and partial misfolding mutants do not directly assess the aggregation of

Fig. 3 Mutations in rhodopsin that cause misfolding and aggregation. Partial (blue) and complete (yellow) misfolding mutations in rhodopsin are illustrated on the secondary structure (a) and tertiary structure (b) of rhodopsin. Mutation of proline at position 267 (green) can be either partial or complete, depending on the specific mutation. The chromophore 11-*cis* retinal is shown as pink spheres (The figure is reprinted from (Gragg and Park 2019), with permission from Elsevier) (Color figure online)
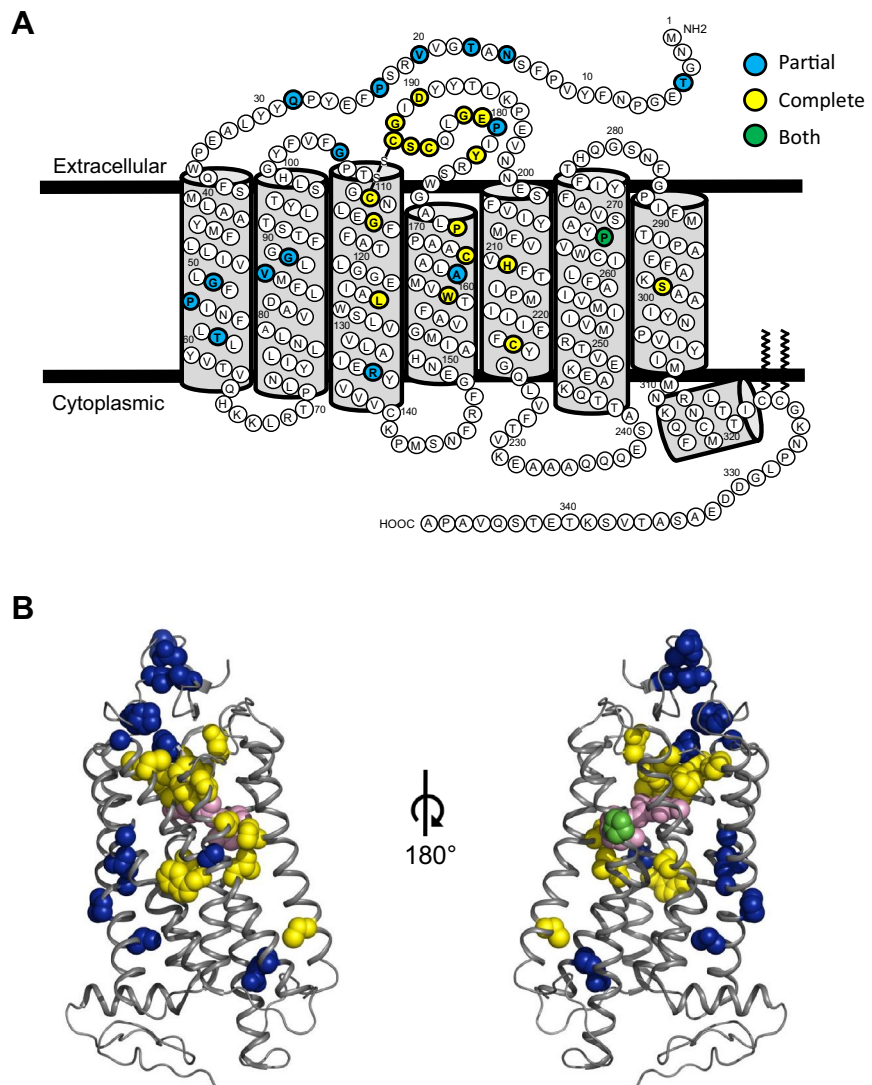
the misfolded rhodopsin mutants. Aggregation of misfolding rhodopsin mutants have, in large part, been assessed by light microscopy [e.g., (Illing et al. 2002; Saliba et al. 2002)]. The types of aggregates examined by light microscopy are inclusion bodies or aggresomes (Kopito 2000), which are relatively large structures identifiable by light microscopy. These types of structures, however, are not detected in knockin mouse models of adRP expressing the P23H mutant of rhodopsin (Price et al. 2011; Sakami et al. 2011). A recently developed method utilizing FRET has detected aggregates of misfolded rhodopsin that appear diffusely dispersed in the ER and are indistinguishable from the wild-type receptor (Gragg and Park 2019; Miller et al. 2015). Thus, the pathogenic aggregates in adRP may be smaller aggregates not discernible by light microscopy rather than larger inclusion bodies or aggresomes. The discussion here will focus on these smaller aggregates.

Misfolded rhodopsin mutants are retained in the ER; however, retention of rhodopsin in the ER itself does not cause aggregation (Miller et al. 2015). The receptor must be misfolded to induce aggregation. The biosynthesis of wild-type rhodopsin results in mostly properly folded rhodopsin with little or no misfolded protein because of proteasomal degradation. When the proteasome is inhibited, the misfolded wild-type rhodopsin that would have been degraded persists and then forms aggregates (Gragg et al. 2016). Secondary structure changes are associated with misfolded rhodopsin mutants. Rhodopsin has a high α-helical content because of its 7 α-helical transmembrane architecture. In misfolding mutants of rhodopsin, this α-helical content is not completely abolished. Only a partial reduction of α-helical structure occurs that is accompanied by an increase in β-sheet structure (Liu et al. 1996; Miller et al. 2015). Interestingly, increased β-sheet structure is a hallmark of

amyloid aggregates, and this secondary structure mediates the protein–protein interactions within the aggregates (Chiti and Dobson 2006; Knowles et al. 2014). Although further studies are required to determine the role that β-sheets have in the aggregation of misfolded rhodopsin mutants, the formation of this secondary structure raises the possibility that they may contribute to the formation of aggregates and that there is specificity in the aggregation process.

A couple observations suggest that the aggregation of rhodopsin does not occur randomly in a nonspecific manner, but rather, is mediated by specific protein–protein interactions. Misfolded rhodopsin does not aggregate with other proteins known to aggregate, including an unrelated misfolded membrane protein (Rajan et al. 2001). Moreover, misfolded rhodopsin mutants do not aggregate with properly folded wild-type rhodopsin (Gragg et al. 2016; Gragg and Park 2018). Thus, aggregation appears to be mediated by specific interactions, likely requiring the β-sheet structures that form in misfolded rhodopsin. The nature of the protein–protein interactions that underlie the formation of aggregates differs from those of oligomers formed by properly folded rhodopsin. The aggregates formed by rhodopsin are resistant to the mild detergent n-dodecyl-β-D-maltoside (DM) but can be disrupted by the harsher detergent sodium dodecyl sulfate (SDS) (Gragg and Park 2019). This behavior contrasts to those exhibited by wild-type rhodopsin that normally forms oligomers, which can be disrupted by DM (Jastrzebska et al. 2004; Miller et al. 2015). Thus, the protein–protein interfaces within aggregates and oligomers are different in nature.

Pharmacological chaperones have been proposed as a therapeutic approach to combat against the detrimental effects of misfolded GPCR mutants (Beerepoot et al. 2017; Tao and Conn 2014), including misfolded rhodopsin mutants. Retinoid-based pharmacological chaperones, such as the endogenous chromophore 11-*cis* retinal, have been shown to aid in the folding and proper cellular trafficking of misfolded rhodopsin mutants (Chen et al. 2015; Krebs et al. 2010; Mendes and Cheetham 2008; Noorwez et al. 2004). The therapeutic effectiveness of retinoid-based chaperones, however, is questionable. Retinoid-based chaperones are only effective for partial misfolding rhodopsin mutants and will have no effect on complete misfolding mutants. Moreover, it is unclear how therapeutically beneficial these chaperoning effects will be since the chaperoned misfolded mutants are unstable (Chen et al. 2014; Opefi et al. 2013). Since misfolding mutants of rhodopsin cause adRP, most patients will express both mutant and wild-type rhodopsin. Partial misfolding mutants do not aggregate with wild-type rhodopsin when coexpressed in the absence of a retinoid chaperone; however, in the presence of a retinoid chaperone, aggregation between the mutant and wild-type receptor is surprisingly observed (Gragg and Park 2018). Thus,

retinoid-based chaperones are not predicted to be beneficial in these instances, but rather, are predicted to be detrimental, making the condition worse. It is unclear whether or not other types of chaperones will exhibit similar negative effects. Further studies will be required to better understand how to prevent or disrupt aggregates of rhodopsin to combat disease.

Concluding Remarks

Two types of quaternary structures formed by rhodopsin have been discussed here, oligomers and aggregates. Oligomers form under native conditions and are involved in the normal function of photoreceptor cells, whereas aggregates form under pathological conditions and are detrimental to the normal function of photoreceptor cells. The hydrophobic nature of rhodopsin makes it difficult to study oligomers and aggregates under physiologically relevant conditions. Methods that can overcome this barrier are beginning to reveal important insights into the nature of these quaternary structures. Further advances are required to better understand the fundamental properties of these quaternary structures, which will reveal further details about the mechanism of action of rhodopsin in phototransduction and the mechanism by which misfolded rhodopsin mutants cause retinal degeneration.

Compliance with Ethical Standards

Conflict of interest The author declares that he has no conflicts of interest to disclose.

Ethical Approval This article does not contain any studies with human participants or animals performed by the author.

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