Ligand Binding Mechanisms in Human Cone Visual Pigments

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Keywords: vision, opsin, retinoids, ligand binding site, chromophore regeneration, G protein-coupled receptor, allostery, color blindness

Abstract

Vertebrate vision starts with light absorption by visual pigments in rod and cone photoreceptor cells of the retina. Rhodopsin, in rod cells, responds to dim light, whereas three types of cone opsins (red, green, and blue) function under bright light and mediate color vision. Cone opsins regenerate with retinal much faster than rhodopsin, but the molecular mechanism of regeneration is still unclear. Recent advances in the area pinpoint transient intermediate opsin conformations, and a possible secondary retinal binding site, as determinant factors for regeneration. In this article, we compile previous and recent findings to discuss possible mechanisms of ligand entry in cone opsins, involving a secondary binding site, which may have relevant functional and evolutionary implications.

Vision, Rhodopsin, and Cone Opsins

The first step in human visual perception involves photon absorption by four different visual pigments. These **visual pigments** (see Glossary) include an apoprotein, opsin, covalently bound to the **chromophore** 11-*cis*-retinal (11CR), a vitamin-A derivative that functions as an inverse agonist locking the photoreceptor opsin protein in its inactive state. This inactive conformation corresponds to the resting state of these retinal photoreceptor proteins that serves as a firstline molecule for visual perception which is readily available for photon absorption [1]. Upon illumination, 11CR undergoes a *cis*-to-*trans* isomerization to produce all-*trans*-retinal (ATR), a reaction that displaces the β-ionone ring moiety from the canonical chromophore binding site (Fig. 1A). This photochemical change is the first event in vision and triggers concerted conformational changes on the receptor that lead to its active state which, in turn, initiates subsequent signaling pathways [2]. Rhodopsin is the photoreceptor protein localized in rod cells of the retina, where it is widely distributed, and mediates scotopic vision at low light intensities. **Cone opsins** are found in cone photoreceptor cells, mainly in the fovea region of the retina, and mediate color perception under bright light illumination conditions. Vertebrates with color vision have different types of cone pigments in the retina which evolved from a common non-visual opsin ancestor. They are classified into four gene families, namely L (red), S (purple-UV), M1 (blue), and M2 (green) [3, 4]. Phylogenetic studies have shown that rhodopsin diverged later from the M2 group (see Fig. 2A).

Cone visual pigments evolved to account for the precise **spectral tuning** of different wavelengths of visible light [5]. At the molecular level, this was achieved by selecting distinct amino acids at the canonical binding pocket for the 11CR ligand. (Fig. 2B). Humans and closely related primates procured trichromatic color vision, which involves red-, green-, and blue-sensitive cones [6]. The spectral sensitivity of the visual pigments peaks at approximately 560 nm (red cone opsin), 530 nm (green cone opsin), 420 nm (blue cone opsin), and 500 nm (rhodopsin) [4]. These colors do not always correspond to those of the gene family to which the pigment belongs. Thus, while most vertebrate blue pigments belong to M1, human blue opsin is part of the S group. Similarly, while most green pigments belong to M2, human green and red opsins are part of the L group (see Fig. 2A) [3, 4]. Therefore, the human red and green cone opsin sequences are highly identical (95%), while blue cone opsin sequence identity is much

lower (42% and 44% of red and green cone opsin respectively), and sequence identity between cone opsins and rhodopsin ranges between 45 and 48%.

Opsins belong to the ubiquitous superfamily of G protein-coupled receptors (GPCRs) having the characteristic structural (e.g., seven transmembrane helices) and functional (e.g., G-protein signaling) features of the family [7]. While no cone opsin structure has yet been solved, several rhodopsin structures have already been deposited in the Protein Data Bank (PDB). Rhodopsin was not only the first crystal structure of a GPCR to be solved [8], but also the first GPCR crystallized in different functional states (i.e., active and inactive conformations) [9, 10]. Moreover, rhodopsin has been recently solved in complex with intracellular signaling partners, namely the G protein [11] and visual arrestin [12]. All these structures have provided important clues for understanding the molecular mechanism of rhodopsin photoactivation and have long served as homolog templates to study the structure and activation mechanism of other members of the GPCR superfamily, some of whom undoubtedly possess high pharmacologic and therapeutic interest [13, 14].

The nature of rhodopsin function augments this pigment a prominent model for optogenetic research and for the progressive development of the optogenetic toolbox with a wide range of potential applications [8, 15]. In fact, rhodopsin has been widely studied by biophysical and biochemical methods aimed at characterizing the protein photocycle at the molecular level. During this cycle (Fig 1B), 11CR isomerization to ATR triggers protein conformational changes leading to the Meta I conformation through a series of short-lived photointermediates (photorhodopsin, bathorhodopsin, lumirhodopsin). The Meta I intermediate still features the chromophore covalently bound to the opsin through a protonated Schiff base (PSB) linkage [16]. Subsequent deprotonation of the PSB results in the formation of Meta II with a typical absorbance maximum at 380 nm for all opsins. The Meta II state of the photoactivated visual pigment binds and activates the G protein, transducin, starting the visual phototransduction cascade. This active conformation eventually decays to ATR and free opsin apoprotein by hydrolysis of the Schiff base (SB) linkage. Although the conformational intermediates of photoactivated opsins are characterized, our knowledge of the apoprotein conformations existing prior to retinal uptake is very limited.

Most of our understanding of the specific features of chromophore **regeneration** and activation mechanisms of cone visual pigments, and the cone opsin photocycle, has been extrapolated from comparison with previous rhodopsin studies. However, in order to unravel the specific details of chromophore regeneration and activation mechanisms of cone visual pigments, we need to integrate cone opsin photocycle findings with the wealth of rhodopsin studies. For instance, we know that the Meta II active photointermediate in cone opsins is short lived compared with that of rhodopsin [17-19]. Therefore, identifying the key structural features underlying retinal uptake and cone opsin activation mechanisms is clearly needed to fully understand visual perception at different light intensities and to develop novel therapeutic strategies to treat congenital retinal disorders. This knowledge should contribute, in addition, to broaden our knowledge on the structural information niche and the associated molecular mechanisms of the GPCR repertoire. In this review, we compile and discuss the recent advancements in the understandings of ligand binding (including **allosteric** modulation), stability, and chromophore regeneration of human cone visual pigments.

Transient Cone Opsin Conformations prior to Chromophore Regeneration

GPCRs are dynamic proteins that exhibit multiple functionally distinct conformational states [7]. Such a conformational ensemble provides GPCRs, like opsins, with a particular functional cycle comprising multiple steps (see Fig 1B). In the inactive dark-adapted state of rod and cone opsins, the retinal chromophore covalently binds to these visual pigments through its aldehyde group via a PSB linkage to the ε-amino group of $Lys^{7.43}$ (numbering refers to the general scheme for GPCRs [20]). The PSB is then stabilized by a salt bridge with the carboxylic group of the counterion, $Glu^{3.28}$ [17]. The active Meta II conformation formed upon pigment illumination decays with time yielding the empty opsin ready for free 11CR uptake thus restoring the dark-adapted state during the adaptation mechanism of the visual cycle. The fate of a desensitized receptor -the conformational state of the receptor that has lost its activation ability and that eventually dissociates into free opsin and ATR- (Fig. 1B) includes either (i) receptor turn-over (i.e., regeneration of the apoprotein by chromophore reuptake), or (ii) deactivation (i.e., receptor phosphorylation and subsequent internalization). Therefore, the fast Meta II decay process, in cone visual pigments, requires efficient receptor turn-over (i.e., fast chromophore binding to the

photoactivated visual pigment) for a continuous visual photocycle ensuring an optimal function of the transduction cascade.

11CR uptake and ATR exit mechanisms are both sensitive to conformational selection and existing equilibria among cone opsin intermediates. The Hofmann group proposed the retinal channeling hypothesis to describe the passage and exit of retinal into and out of the protein via specific entry/exit pathways [21]. Further studies suggested that this channel could involve two openings between transmembrane helices (TMs) 1 and 7, and between TMs 5 and 6 (Fig. 3A) [22, 23]. Interestingly, retinal isomers strictly bind to a specific subset of receptor conformations, namely 11CR and ATR can only bind inactive and active opsin conformations, respectively [24].

In our recent studies on cone opsins, we showed that chromophore regeneration also involves dynamic conformations of the apoprotein [25-27]. Key differences with rhodopsin are that cone opsins: i) are more prone to spontaneous dissociation into free opsin and ATR, ii) exhibit a faster Meta II decay, and iii) have more serine and threonine residues in the C-terminus that can become phosphorylated [28].

In an earlier study, we compared the regeneration process of rhodopsin and red cone opsin using retinal analogs added after the time taken for the apparent complete decay of the Meta II conformation. We found that while both 11CR and 9-*cis*-retinal (9CR) could regenerate red cone opsin, only 9CR was able to regenerate rhodopsin after the same time. This evidence suggests that these two opsin subtypes have different conformational dynamics, namely red cone opsin shows higher conformational flexibility than rhodopsin [25]. Similarly, a transient activation model, in which the transient intermediate conformation of ligand-free opsin potentiates the selective interaction between 11CR and ATR, has been proposed for rhodopsin as well [24]. Therefore, such transient opsin conformations may favor stable complexes with specific retinal isomers and this may play a role in cellular rhodopsin homeostasis.

In a later study, we compared red versus green cone opsin regeneration by adding the retinal immediately after illumination. Intriguingly, these two highly homologous opsins displayed different regeneration modes likely due to different conformational dynamics prior to ligand binding. Another significant difference was that while photoactivated green cone opsin primarily formed an unprotonated SB linkage with endogenously added 11CR, red cone opsin showed the spectral features of a typical PSB linkage with its natural 11CR chromophore [26]. Interestingly,

a recent study shows how an 11-*cis*-6-membered ring locked retinal analog can regenerate rhodopsin but not green cone opsin, hence suggesting that green cone opsins have a tighter retinal binding site [29]. On the other hand, blue cone opsin appeared to behave in a similar way to red cone opsin in response to canonical 11CR binding [27]. Thus, further studies are required to unveil the physiological significance of the initial unprotonated SB linkage formation and its conversion to 11CR-PSB green opsin in the visual transduction process of green cone opsin.

Evolution has presumably favored a retinal isomeric configuration for coupling to a specific transient conformation of the ligand-free receptor. We may exploit this recognition to identify these conformations and attempt to characterize the process of retinal binding to opsin. 11CR is conserved throughout evolution because of the high quantum yield of its isomerisation reaction into ATR, as well as its fast response to the light stimulus that makes it one of the fastest reactions in nature [30]. In order to elucidate the regeneration mechanism of opsins, other retinal analogs such as 7CR, 9CR, 13CR, and ATR, have been used to study their binding properties to different visual pigments [31-33]. According to one of these studies, the estimated rank order of the binding energy for different retinal isomers has been found to be as follows: 9-*cis* > 13-*cis* > 11-*cis* > all-*trans* [34]. 9CR is often used as an exogenous analog to study the structure and function of visual pigments [35]. Of note, while the binding free energy required by 9CR is lower than that of 11CR [34], the **photoisomerization** of isorhodopsin (9CR-bound opsin) is three times slower than that of rhodopsin (11CR-bound opsin) [36]. These conformational differences are in line with the observed behavior between rhodopsin and red and green cone opsin regenerated with 9CR and 11CR [26, 29].

Overall, these recent findings confirm that opsin regeneration requires the photoactivated ligand-free protein to adopt specific transient conformations. These conformations are distinct for each cone opsin and point to the existence of specific different regeneration pathways. Moreover, in accordance with the general lock-and-key hypothesis of protein-ligand interactions, retinal and retinal analogs seem to entail a combination of accessibility and specificity factors towards a given opsin conformation. Differences in the visual pigment molecular evolution and in associated intragenic epistatic interactions among these structurally similar red, green, and blue cone opsin pigments, may justify the different functionally-relevant conformations observed when studying chromophore regeneration mechanisms [37, 38].

Opsin Stability and the Chromophore Regeneration Process

One important issue in the study of visual pigments is the stability of purified opsins in detergent-containing solutions. Just like any other membrane protein, detergents compromise opsin stability when compared with native membrane environments, an effect that is boosted by the absence of ligand making the crystallization of ligand-free GPCRs, like opsin, a very challenging task. In the particular case of opsin, such instability eventually hampers its regeneration capacity under usual experimental conditions [39]. In rhodopsin, removal of palmitoyl anchors at C-terminal cysteines Cys322$^{8.59}$ and Cys323$^{8.60}$ have a strong inhibitory effect on opsin stability [40]. Previous studies in cone opsins closely related to the human blue, red, and green opsins revealed that these receptors lack palmitoylation [41]. Conversely, different opsin crystal structures have demonstrated that certain detergents can access the orthosteric binding site stabilizing the active state of this protein (Fig. 3B) [42-44]. Similarly, a recent study demonstrated that membrane cholesterol can bind at the same position in the adenosine A_{2A} receptor (see Fig. 3B) [45]. This is noteworthy, because the authors performed tunnel calculations to show that the pathway followed by cholesterol to access the receptor is almost identical to the one suggested for retinal entry/exit into/from opsin. It is becoming more and more apparent that both detergents and membrane lipids play a key role in modulating opsin conformational stability and hence chromophore regeneration. In this regard, the effect of the lipid environment on the energetics of chromophore binding, in the case of rhodopsin, has been recently examined [46].

As we have previously shown [25], 11CR could not regenerate photoactivated rhodopsin after 90 min of photoactivation, whereas the 9CR analog could do so under the same experimental conditions. This finding challenged the long-held notion of the instability of ligand-free rhodopsin. More interestingly, both retinal analogs added a long time after photoactivation in solution could regenerate cone opsins more effectively than they did for rhodopsin, despite the former are be less stable in detergent [25] which is evident from the fact that rhodopsin has been found to be ~300 times more thermally stable at 37°C than cone opsins [47]. All in all, there is compelling evidence that there are different opsin stabilities in solution across the opsin repertoire and confirms that this parameter can modulate key molecular events governing opsin

biology such as retinal uptake and release that can be relevant factors in the pathophysiology of retinal degenerative diseases.

Secondary Retinal Binding Site in Cone Visual Pigments

As an alternative to the orthosteric site, the topography of GPCRs offers different binding pockets, where certain allosteric ligands can bind to modulate the response of the orthosteric ligand [48-50]. Some of these allosteric sites exist along the canonical binding pathway and transiently accommodate the ligand before reaching the orthosteric site [51, 52]. This also includes ligands that enter through the membrane [53, 54], such as retinal. Therefore, such sites may also exist along the binding pathway of retinal into opsins. In fact, binding of more than one retinal per opsin was proposed for rhodopsin already 15 years ago [55]. In this study, the authors suggested that a second retinal molecule could interact non-covalently with opsin at two possible sites they named entrance and exit sites [55]. Even before that, non-covalently bound retinal was reported in cone photoreceptors isolated from salamander retinas [56] and also in bovine rhodopsin from rod outer segment (ROS) disc membranes [57]. Later, X-ray crystallography revealed binding of one β-ionone (the ring moiety of the retinal molecule) at the extracellular side of TM7 [58]. X-ray crystallography has also proven that detergent molecules can bind in a cavity between TMs 1 and 7 [59], or even near the orthosteric site [60] (Fig. 3C). All these evidences support the proposal that retinoid molecules can bind to opsins at sites other than their canonical chromophore binding site. By the time of editing this review, recent structures of prostaglandin receptors have revealed that the orthosteric pocket in these receptors locates between TMs 1, 2 and 7, with one antagonist occupying the interface with the lipid interface in a very similar cavity than the one proposed as secondary binding site of retinal [61]. The possible existence of a secondary retinal binding site remained elusive in the case of the human cone visual pigments. Very recently, our regeneration experiments with photoactivated ligand-free cone opsins revealed the existence of such a site, which we suggested would be located between TMs 1 and 7, based on molecular docking (Fig. 3D) [26, 27]. In fact, our predictions positioned the second retinal molecule in a very similar location to that of a detergent molecule recently resolved in rhodopsin (Fig. 3D). In these studies, the proposed ligand binding mechanism involved a two-step mechanism: (i) 11CR binds to a transient opsin conformation at the secondary retinal binding site. This process may cause rapid chromophore regeneration by

incidentally displacing ATR out of the orthosteric binding site; and (ii) the secondary binding site accommodates additional 11CR, this time with slower kinetics. This second slower step would be due to the conformational change induced in the regenerated protein and the reduced availability of bulk 11CR, which would synergistically slow-down 11CR binding to the secondary site [26, 27]. While this secondary binding is not detected in the regeneration of red/green cone pigments by 9CR, this isomer can regenerate blue cone opsin via this mechanism. Such isomer selectivity suggests that the secondary retinal uptake event is not only due to free retinal availability but rather points to specific retinal-opsin interactions in different opsin conformational states**.**

Ligand Binding of Visual Pigments and Congenital Retinal Disorders

As discussed earlier, trichromatic color vision requires the absorption of photons from different visible wavelengths. The functional loss of any one of the cone pigments due to certain congenital disorders limits vision to **dichromacy** and the loss of two cone pigments leads to **monochromacy** [62]. Clinically identified opsin mutations result in retinal disorders ranging from a mild phenotype such as color blindness to a severe condition such as that found in cone dystrophies. The retinoid cycle (i.e., synthesis of 11CR from ATR) involves various retinoid binding proteins including transporter proteins and isomerizing enzymes [63]. Therefore, mutations in these proteins other than visual pigments can lead to vision impairment and is associated with several retinal diseases such as Leber congenital amaurosis (LCA), Stargardt macular degeneration, congenital cone-rod dystrophy, and retinitis pigmentosa (RP) [64].

Altogether these deleterious mutations would restructure the functional conformation of the proteins which would further result in either compromised stability or ineffective retinoid binding. When the retinal binding event is compromised by point mutations that alter opsin stability, introducing an engineered disulfide bond into an opsin model improves the free-state opsin stability [65]. Although this approach is far from a practical implementation as a potential therapeutic strategy, it can be used to study the molecular features of mutant opsins. Opsins reconstituted into lipids [66], or supported by nanodiscs [67], can also be used to study the altered structure–function relationships of the visual pigments. In a therapeutic context, application of chemical chaperones can also counteract the mutation effect by recovering stable and functional conformations of the receptor [68]. In addition, exploring the applications of

retinal analogs is a promising strategy with potential therapeutic impact as it is aimed at ameliorating the disrupted specific retinal–protein interactions in individuals with congenital disorders.

Increasing experimental evidence supports the exogenous supplementation of retinal analogs in order to recover degenerative cone photoreceptor cells. In this regard, 9CR administration recovers cone function and improves cone density [69, 70], and administration of 9-*cis*-retinyl-acetate increases the rhodopsin regeneration ratio, and dark adaptation in Rpe65 (an enzyme that acts on ATR) deficient mice [71]. The inherently less stable classical P23H rhodopsin mutant could also be regenerated with 9CR [72]. In other cases, blocking of constitutively active mutant visual pigments can be achieved by locked retinal isomers, that is, retinal analogs which are protected from photoisomerization, such as 11-*cis*-6mr-retinal [29, 73].

LCA is a group of retinal degenerative disorders, in which vision loss is associated with perturbed 11CR synthesis but featuring relatively well-preserved retinal cells [74]. QLT, a 9-*cis*-retinoid analog has been tested as a potential therapeutic agent for the treatment of the LCA clinical phenotype [75]. Our recent findings, with the 9CR isomer, showed improved regeneration at various time-points after photoactivation in the case of red, green, and blue cone pigments as well as in the case of rhodopsin [25-27]. Overall, these results provide novel evidence for the great potential of specific retinal isomers (alone or in combination with other molecules [76] that can act as allosteric modulators), or chemically modified retinoid analogs, as therapeutic tools for the successful treatment of visual disorders associated with mutations in cone photoreceptor proteins.

Concluding Remarks and Future Perspectives

Recent studies on ligand binding mechanisms of cone visual pigments stress the importance of specific conformational changes prior to visual pigment regeneration with 11CR, and the potential role of secondary retinal binding to the protein. The transient cone opsin conformational intermediates discussed here would serve as distinct fine-tuning molecular components modulating receptor activation and retinal release, and diverting the receptor from arrestin-mediated internalization. This effect may show similarities with ligand-biased signaling proposed for other GPCRs of pharmacological importance. Further studies on the kinetics and thermodynamic features of the different opsin conformational states, in membrane-mimicking

environments, would help unravel the structural basis of the visual photocycle and ligand binding mechanisms. The secondary retinoid binding to cone visual pigments may function as a regulatory mechanism of dark adaptation in the phototransduction process. However, one of the fundamental questions (see *Outstanding questions*) is that the physiological role of this secondary retinal binding site has to be pondered and the involvement of the analyzed conformations in the development of retinal degenerative diseases deserves further investigation. The use of retinoid derivatives as potential agents to counteract the conformational defects of disease-causing opsin mutations deserves further investigation. In this regard, the combined use of retinoids and other small molecules appears as a novel strategy for therapeutic intervention.

Acknowledgements

We would like to thank Dr. Eva Ramon for helpful discussions. This work was supported by CIG grant (293796) from EU and Grups de Recerca Consolidats de la Generalitat de Catalunya (2017 SGR 163) to PG, and in part by grants SAF2011-30216-C02-01 to PG and SAF2015-74627-JIN to AC from the Ministerio de Ciencia e Innovación (Spain). SS was the recipient of FI-AGAUR and FPI-UPC Fellowships.

Declaration of Conflicting Interests

The authors declare that there is no conflict of interest.

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Glossary

Allostery; the effect conferred by the binding of a ligand to a protein site other than the canonical (orthosteric) site, resulting in modulation of the function triggered by the orthosteric ligand.

Chromophore; the molecule responsible for light absorption.

Mono/Dichromacy; a color vision disorder in which only one/two among the three types of cone cells are functional resulting in altered color visual perception.

Opsin cycle; in the presence of light, the opsin covalently-bound 11-*cis*-retinal isomerizes to all-*trans*-retinal and triggers protein conformational changes leading to the active state, through a series of photointermediates. The visual pigment eventually loses all-*trans*-retinal and further regenerates with a new 11-*cis*-retinal molecule regenerating again retinal-bound opsin available for the next visual photo-transduction cascade (see Fig. 1).

Opsins; heptahelical transmembrane class-A G protein-coupled receptors localized in membrane invaginations of photoreceptor cells in the retina.

Palmitoylation; covalent addition of a palmitoyl group to specific Cys322 and Cys323 residues in rhodopsin (S-palmitoylation) known to improve opsin stability.

Photoisomerization; the catalytic process by which incident light photons, of a specific wavelength, isomerize the opsin chromophore.

Regeneration; the covalent binding of retinal to opsins by Schiff base formation with a $Lys^{7.43}$ residue at the orthosteric site.

Retinal channeling hypothesis; the retinal molecule passes through a specific groove of the apoprotein to form retinal-bound visual pigment. It proposes specific entrance/exit sites for 11-*cis*-/all-*trans*-retinal into opsins.

Spectral tuning; the phenomenon of molecular variance around the protein bound chromophore of visual pigments modulating the photoisomerization of the visual pigment to photons of specific wavelength.

Visual pigments; opsins, with their covalently bound chromophore, which are at their dark state and readily available for photoactivation and visual transduction.

Figure Legends

Figure 1: Photoactivation of Visual Pigments. (A) 11CR is covalently bound to a Lys residue of opsin, apoprotein of visual pigments, at their canonical chromophore binding site via a PSB linkage. The protonation is stabilized by a Glu residue which is known as the "counterion". Photoactivation (hν) activates the visual pigments by isomerizing 11CR to ATR to form the Meta II state. Further release of ATR empties the retinal binding site leaving free opsin ready for regeneration**. (B)** The dark-state visual pigment (opsin bound with 11CR) gets activated by light (hν) and the 11CR isomerizes to ATR in *fs*, initially with unaffected protonation of the SB. After a series of short-lived photointermediates, the Meta I intermediate is formed that still harbours a PSB. Meta I conformation is converted, in the *ms* time range, to the Meta II active conformation, where the SB becomes deprotonated, the retinal linkage is eventually hydrolyzed, and the isomerized ATR is released from the opsin binding pocket. Free opsin can become phosphorylated, and processed for arrestin-mediated internalization, or it can be regenerated with a fresh 11CR molecule through a dynamic transient conformational state. (hν, quantum of light; fs, femtosecond; Meta I, metarhodopsin I; ms, millisecond; Meta II, Metharhodopsin II; 11CR, 11-*cis*-retinal; ATR, all-*trans*-retinal).

Figure 2: Phylogenetic Tree and Sequence Alignment of Visual Pigments. (A) Phylogenetic tree of visual pigments of human, mouse, chicken, salamander, and goldfish constructed using the UPGMA method in MEGA [77]; (*) despite their names, these proteins have been considered to be red opsins [78, 79]. **(B)** Multiple sequence alignment of human rhodopsin (Rh), blue (B), green (G), and red (R) cone opsins computed using ClustalO [80]. Boxes with dashed lines indicate the reference residue for each helix (.50 according to Ballesteros and Weinstein numbering scheme [21]); boxes with full lines indicate the position of $Lys^{7.43}$ (the anchoring point for retinal) and the glutamic residues ($E^{3.28}$ and E^{ECL2}) that act as counterions.

Figure 3: Entrance/Exit Openings for the Orthosteric Retinal Pocket and the Possible Secondary Binding Site in Cone Opsins. (A) Superposition of the active G-protein bound rhodopsin complex (PDB id 6CMO; in different colors for each TM helix: 1: cyan, 2: yellow, 3: red, 4: gray, 5: green, 6: blue, 7: pale-red) and dark-state rhodopsin (PDB id 1GZM; in white). ATR and 11CR are shown as sticks and with a dotted surface in green and red, respectively. The G protein is shown in gray with a transparent surface**. (B)** Examples of detergent or lipid

molecules superposed at the retinal binding pocket. Shown with ball-and-sticks are two β-octylglucosides and one β-nonylglucoside molecules from rhodopsin structures with PDB ids 4PXF (magenta), 4J4Q (orange) and 4X1H (dark-cyan), respectively, and one cholesterol molecule from a representative snapshot of a simulation of the A_{2A} receptor taken from reference [47]. ATR and 11CTR are shown in sticks as reference. **(C)** A non-retinal small molecule (S-RS01) binding near the TM5/6 opening that, in addition, occupies the space of the β-ionone ring of both ATR and 11CR (PDB id 6FK6). **(D)** The putative secondary retinal binding pocket proposed in reference [26]. As a representative, one of the obtained docking poses that can coexist with ATR or 11CR is shown in orange. An octylglucoside molecule (in magenta) has been crystallized in a very similar location in one rhodopsin crystal structure (PDB id 6FK6). (PDB id, Protein Data Bank identification code consisting of four characters combining letters and numbers and specific for the structure of a given protein; ATR, all-*trans*-retinal: 11CR, 11-*cis*-retinal; TM, transmembrane helix; G protein, guanine nucleotide-binding protein, named transducin in the case of visual pigments).

Outstanding Questions

- ✓ What is the underpinning physiological relevance of the transient conformations in photoactivated ligand-free cone opsins prior to chromophore regeneration?

- ✓ Considering the fate of ligand-free GPCRs susceptible to arrestin-mediated internalization, is there a molecular mechanism protecting the inactive ligand-free receptors from entering in the receptor down-regulation pathway?

- ✓ More specifically, do the fine-tuning conformational changes in cone opsins play a significant role in receptor down-regulation?

- ✓ What is the correlation between kinetics of secondary retinal binding and release of the photoisomerized retinal from the activated cone opsins?

- ✓ Can the novel conformations proposed, and/or their alterations by mutations in the opsin genes, play a role in the pathophysiology of retinal disorders?

Highlights

- ✓ Recent reports on the ligand binding mechanisms of cone visual pigments identified transient conformations with concerted dynamics prior to regeneration with 11-*cis*-retinal.

- ✓ A secondary retinal binding site has been identified in cone visual pigments. The retinal at this allosteric site could act as a buffer facilitating the maintenance of ligand-bound species.

- ✓ The novel results support the view that the actual stability of opsins may be the result of the combined effect of the conformational dynamics of the ligand-free opsins and the potential regulatory effects of the second retinal binding.

- ✓ Retinal analogues, like 9-*cis*-retinal, have a clear differential effect on the regeneration mechanism of cone opsins. The combination of retinal analogues with other small molecules can be an interesting approach for the treatment of congenital retinal disorders.

Figure 1

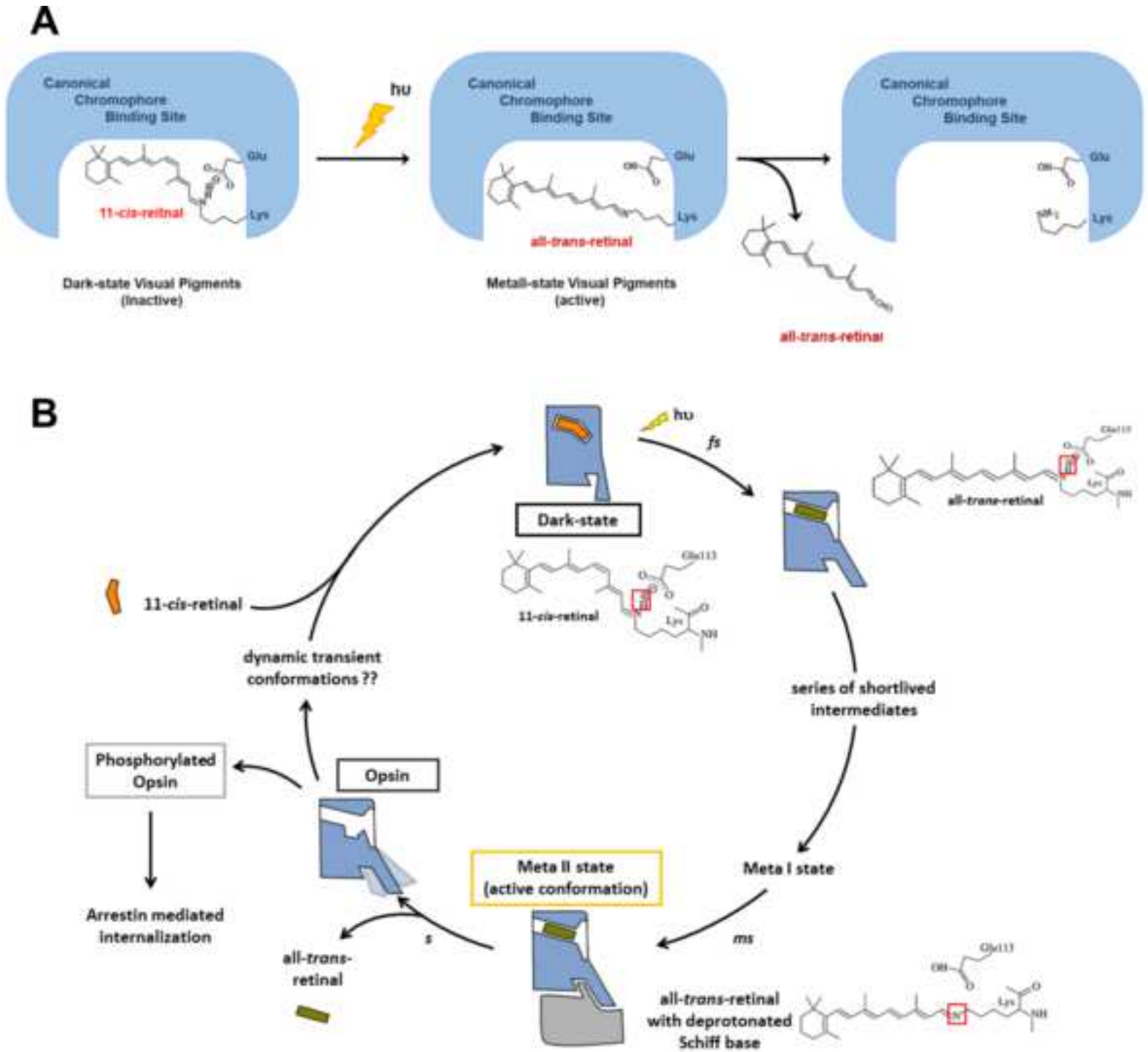

Figure 2

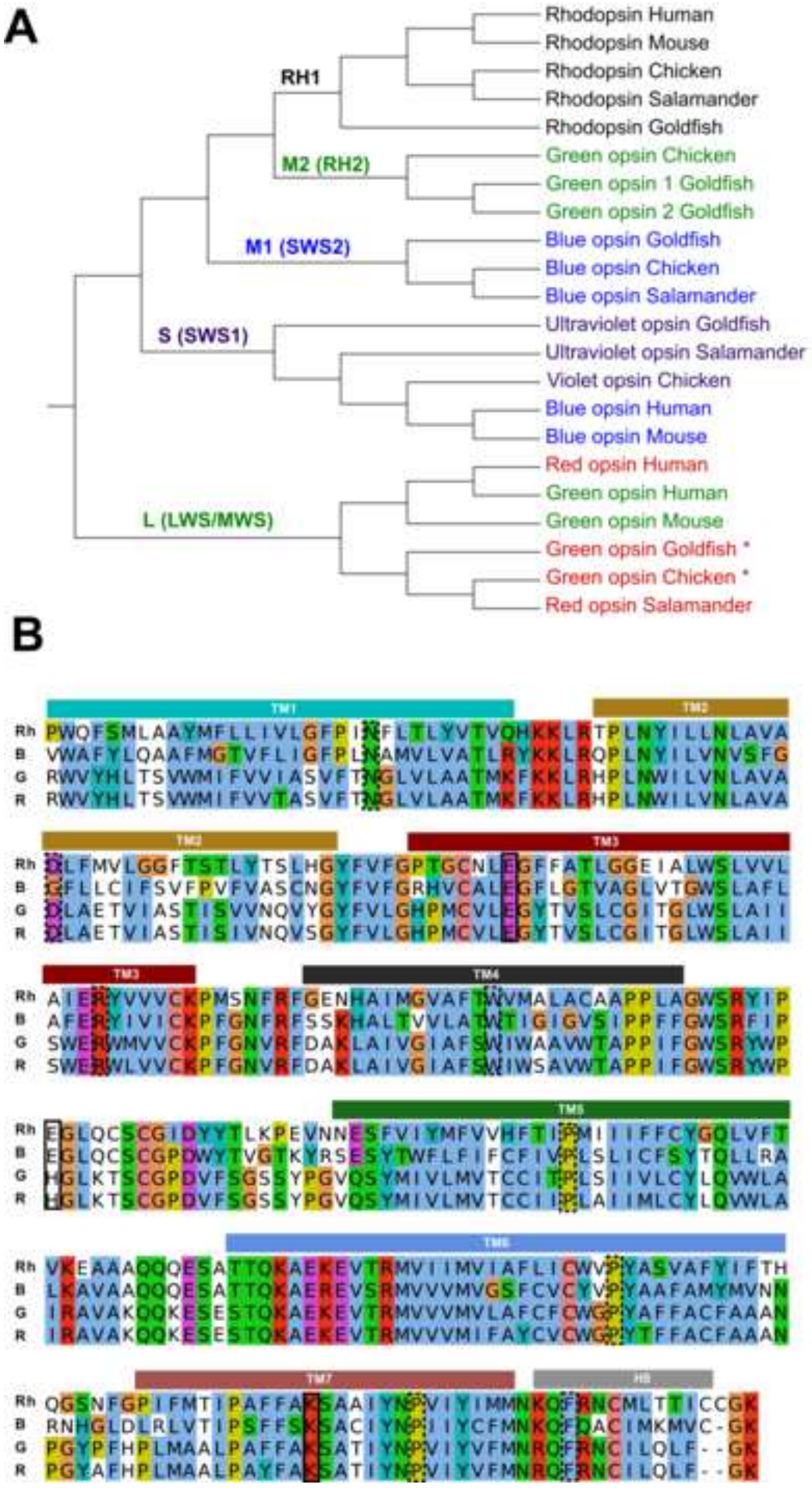

Figure 3