



Review

Cone visual pigments[☆]Yasushi Imamoto, Yoshinori Shichida^{*}

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ABSTRACT

Cone visual pigments are visual opsins that are present in vertebrate cone photoreceptor cells and act as photoreceptor molecules responsible for photopic vision. Like the rod visual pigment rhodopsin, which is responsible for scotopic vision, cone visual pigments contain the chromophore 11-*cis*-retinal, which undergoes *cis*–*trans* isomerization resulting in the induction of conformational changes of the protein moiety to form a G protein-activating state. There are multiple types of cone visual pigments with different absorption maxima, which are the molecular basis of color discrimination in animals. Cone visual pigments form a phylogenetic sister group with non-visual opsin groups such as pinopsin, VA opsin, parapinopsin and parietopsin groups. Cone visual pigments diverged into four groups with different absorption maxima, and the rhodopsin group diverged from one of the four groups of cone visual pigments. The photochemical behavior of cone visual pigments is similar to that of pinopsin but considerably different from those of other non-visual opsins. G protein activation efficiency of cone visual pigments is also comparable to that of pinopsin but higher than that of the other non-visual opsins. Recent measurements with sufficient time-resolution demonstrated that G protein activation efficiency of cone visual pigments is lower than that of rhodopsin, which is one of the molecular bases for the lower amplification of cones compared to rods. In this review, the uniqueness of cone visual pigments is shown by comparison of their molecular properties with those of non-visual opsins and rhodopsin. This article is part of a Special Issue entitled: Retinal Proteins – You can teach an old dog new tricks.

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1. Introduction

In the retinas of most vertebrates, there are two types of photoreceptor cells, rods and cones (Fig. 1). Rods are responsible for scotopic vision, the vision working under dim light conditions where cones are not functional, whereas photopic vision, the vision working under daylight conditions is mediated by cones. In agreement with this visual duplicity, rods are more sensitive than cones and can generate a response from even a single photon. Although less sensitive than rods, cones respond and regenerate more rapidly than rods and exhibit considerably greater adaptive ability than rods. Rods contain a single rod visual pigment (rhodopsin), whereas cones use several types of cone visual pigments with different absorption maxima. Integration of the photon signals from cones having cone visual pigments with different absorption maxima enables animals to discriminate the color of materials.

Investigation of visual pigments at the molecular level started in the 1950s using the bovine rhodopsin as a representative visual pigment, and several fundamental properties of rhodopsin were elucidated in the 1950s to 1960s. Physico-chemical and biochemical studies of rhodopsin have been widely performed to elucidate the detailed molecular mechanism of light absorption and G protein activation by rhodopsin

since Prof. George Wald won the Nobel Prize in 1967 [1]. Investigation of the cone visual pigments also started in the 1950s, and multiple types of cones with different spectral sensitivities were identified in primates and fishes by microspectroscopy [2,3] and electrophysiology [4]. However, molecular-level investigations were hampered due to the difficulties of isolation of cone visual pigments from retinas. In the 1980s, our group began to isolate and separate chicken cone visual pigments by using more than ten thousand chicken eyes and succeeded in obtaining absorption spectra of four kinds of cone visual pigments and characterizing their bleaching processes after absorption of a photon [5–7]. We also prepared monoclonal antibodies against chicken red (iodopsin) [8] and determined the amino acid sequences of four kinds of cone visual pigments by cDNA cloning [9,10].

Textbooks at the time we started investigating cone visual pigments stated that photopic and scotopic vision were diversified before the acquisition of color vision (e.g. [11]). That is, it was inferred that rod and cone visual pigments diversified first and then multiple types of cone visual pigments with different absorption maxima were diversified. Therefore, we expected that we might discover new molecular mechanisms, which had not been obtained from the studies of rhodopsin, in a study of cone visual pigments. However, our subsequent cDNA cloning experiments and phylogenetic analysis clearly showed that ancestral vertebrate visual pigments first diverged into four kinds of cone visual pigment groups later [10] (Fig. 2). Furthermore, subsequent investigations on the so-called non-visual opsins such as pinopsin [12], parapinopsin

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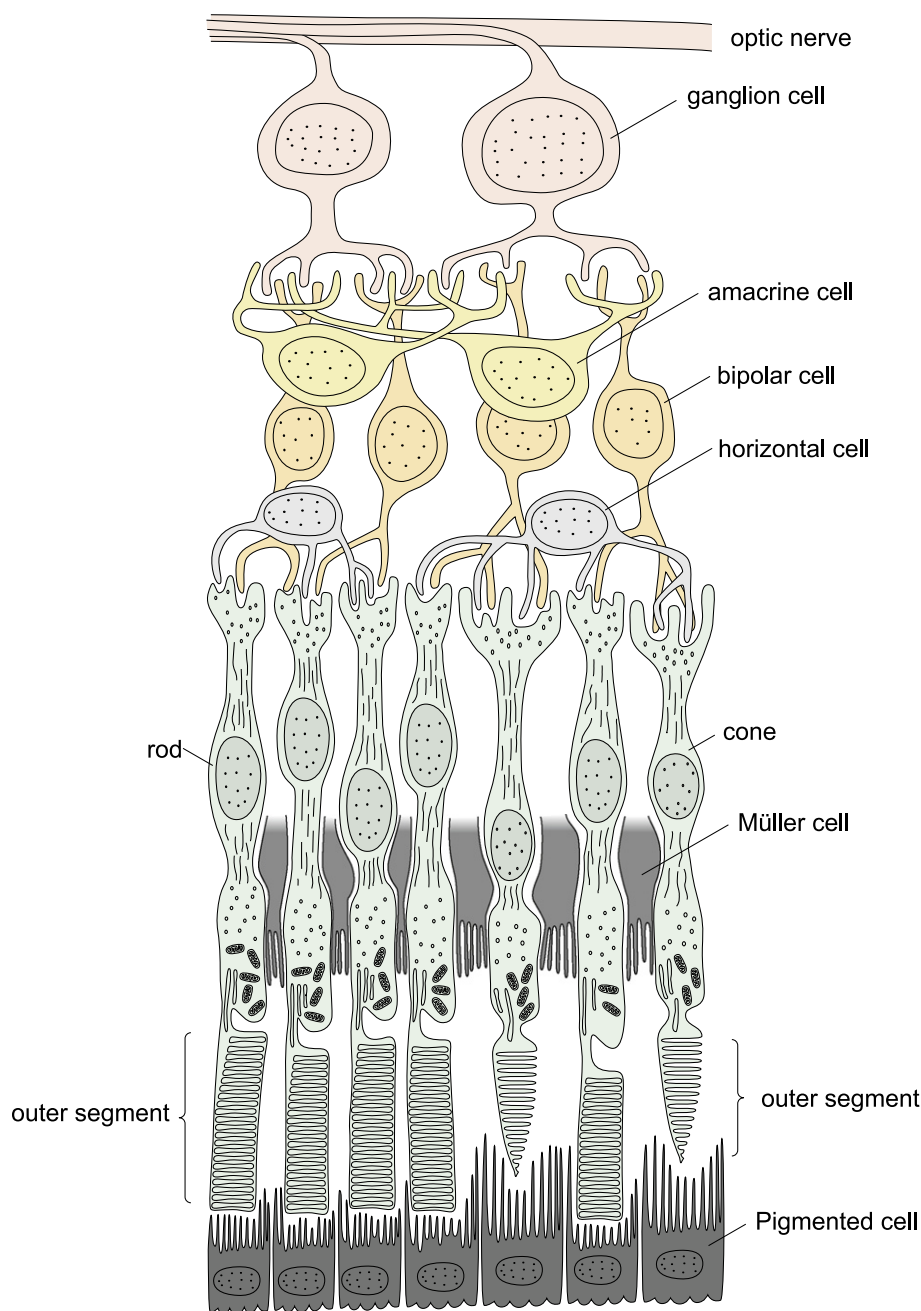


Fig. 1. Schematic drawing of vertebrate retina. In the retinas of most vertebrates, there are two types of photoreceptor cells, rods and cones.

[13], VA opsin [14] and parietopsin [15] showed that visual pigments (visual opsins) were diversified from one of the four kinds of non-visual opsins (Fig. 2). Therefore, it is important to analyze what kind of molecular properties the cone visual pigments have acquired in the course of diversification from non-visual opsins and how rhodopsin evolved from the cone visual pigments. Additionally, the diversification of multiple cone visual pigments with different absorption maxima is also an important issue to be resolved. In the present article, we summarize the molecular properties of cone visual pigments from the viewpoints described above.

2. Molecular evolution of cone visual pigments

As described above, vertebrate rod and cone visual opsins, form a phylogenetic sister group with other opsin groups such as the pinopsin,

VA opsin, parapinopsin, and parietopsin groups [15]. In Fig. 2, we show these groups as having simultaneously diverged, because unambiguous determination of the phylogenetic relationship among these five groups is still difficult based on the available amino acid sequences.

In the course of our previous investigation of the functional divergence of opsins, we found that the position of the counterion was different between vertebrate visual opsins and other opsins [16,17]. Vertebrate visual opsins have a counterion at position 113, whereas many opsins have a counterion at position 181 (in the numbering system of bovine rhodopsin). We also found that the difference in the position of the counterion is correlated with the different spectroscopic and biochemical properties between these pigments. The opsins having the counterion at position 181 exhibit molecular properties of so-called “bistable” pigments in which the resting (dark) state and active state are stable at physiological temperature and are able to revert to each other

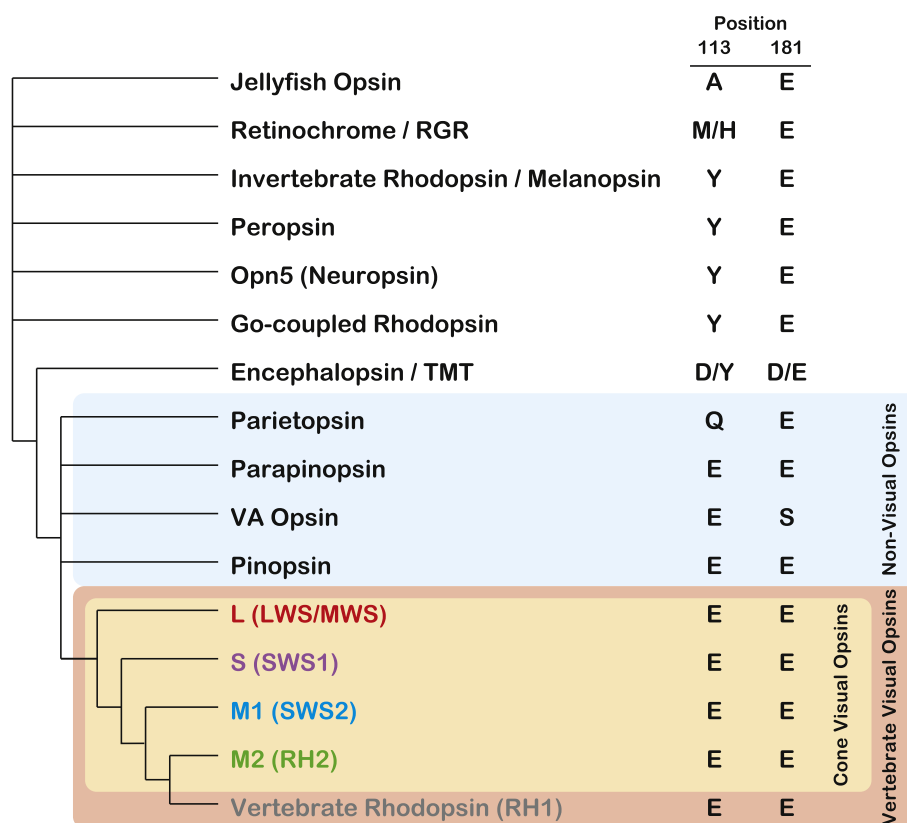


Fig. 2. Phylogenetic tree of opsin family. Modified from [21] and [54].

by absorption of a photon [18]. In contrast, vertebrate visual opsins produce an active state that is thermally unstable and do not revert to the resting state even by absorption of a photon (monostable) [19]. In addition, the G protein activation efficiency of vertebrate visual opsins is significantly greater than that of bistable opsins [17].

Among the non-visual opsins mentioned above, parapinopsin and pinopsin have Glu at both positions 113 and 181. However, the molecular properties of these opsins are quite different from each other. Parapinopsin is a typical bistable opsin whose G protein activation efficiency is 1/20 that of bovine rhodopsin [17,18], whereas pinopsin shows monostable behavior and G protein activation efficiency comparable to those of bovine rhodopsin [20]. Therefore, changes of amino acids appear to have changed the interactions involving Glu113 and Glu181 during the divergence into parapinopsin and pinopsin. Therefore, to get insights into the diversification of opsins we recently investigated the molecular properties of additional non-visual opsins, parietopsin [21] and VA opsin [22].

Spectroscopic analysis of parietopsin expressed in cultured cells showed that parietopsin is a visible-light absorbing opsin. Because parietopsin has Glu at 181 but not 113, the counterion of parietopsin should be Glu181, which was confirmed by mutational analysis. In addition, we confirmed that, like invertebrate rhodopsin, parietopsin has a lower molar extinction coefficient than bovine rhodopsin. However, irradiation of parietopsin caused formation of an intermediate having a deprotonated chromophore Schiff base after the formation of another intermediate that absorbs visible light. These intermediates correspond to the photobleaching intermediates of vertebrate rhodopsin called metarhodopsin II and metarhodopsin I, respectively. Metarhodopsin-I-like intermediate (Meta-I) and metarhodopsin-II-like intermediate (Meta-II) of parietopsin are in pH-dependent equilibrium, but the pH dependency follows the pK_a of the chromophore Schiff base, unlike that of visual opsins. Therefore, it is likely that the coupling of hydrogen-

bonding networks around the chromophore and the conserved D(E)RY region in visual opsins [23,24] is not established in parietopsin. In visual opsins, a chromophore proton is transferred to Glu113 in Helix III, resulting in the disruption of the salt bridge between helices III and VII, which triggers the rearrangement of helices. However, the chromophore proton of parietopsin is likely to be transferred to Glu181 in the second extracellular loop, and little helical rearrangement would be induced by this proton transfer. In fact, no in vitro demonstration of G protein activation by parietopsin has been reported, probably because the G protein activation efficiency of parietopsin is very low.

VA opsin also absorbs visible light. Because the amino acid residue of VA opsin at position 181 is Ser instead of Glu, the counterion is likely to be Glu113. On absorption of a photon, VA opsin converts to a metarhodopsin-I-like intermediate. This intermediate is converted to a more blue-shifted intermediate, but the absorption maximum of this intermediate is still in the visible region, indicating that the chromophore of this intermediate is protonated. This implies that no proton transfer from the chromophore to the counterion occurs during the reaction process of VA opsin. Because this intermediate is not converted to the original VA opsin by photon absorption, VA opsin is not a bistable opsin. This intermediate activates Gi-type G protein with activation efficiency one-fifth that of bovine rhodopsin, which is between those of parapinopsin and rhodopsin. Thus, it is likely that VA opsin is an evolutionary intermediate opsin between bistable and visual opsins [22].

Because Glu113 is conserved in cone visual pigments, it is likely that Glu113 acts as a counterion and the chromophore proton is transferred to Glu113 upon absorbing a photon. Additionally, pH dependent equilibrium between Meta-I and Meta-II similar to that of rhodopsin [25] suggests the coupling of hydrogen bonding networks around the chromophore and ERY region, which would induce substantial rearrangement of the helices, resulting in the high Gt-activating efficiency.

3. Molecular properties of cone visual pigments

Cone visual pigments are widely diverged, as shown by their wide variety of absorption maxima, but phylogenetically distinct from the rhodopsin group. In fact, there are several molecular properties that are common among the cone visual pigments but distinct from those of rhodopsins [26–29]. Because the difference in molecular properties of cone visual pigments and rhodopsin is consistent with the difference between the cell responses, the connection between these molecular properties and cell responses has been extensively studied by spectroscopic and biochemical experiments. Nowadays techniques to generate transgenic animals in which a functional protein specific to cones (visual pigment [30,31], Gt [32], or GRK [33]) is expressed in rods are available. Also, more sophisticated techniques to generate knock-in animals in which rod visual pigment in rods is replaced with cone visual pigment is applicable [34]. Electrophysiological studies using these animals have provided increasing insights into the molecular basis of photoresponses of the visual cells. Here, the molecular properties of cone visual pigments are reviewed in comparison with those of rhodopsins.

3.1. General property of cone visual pigments

Because of the relatively lower photosensitivity of cones than rods, it was previously speculated that cone visual pigments would exhibit photosensitivity lower than that of rhodopsin. Since the photosensitivity of a visual pigment is proportional to the product of its extinction coefficient (ϵ) and quantum yield (ϕ) for cis–trans isomerization, we determined ϵ and ϕ of chicken iodopsin [35], and later of chicken green [27], and compared them with those of bovine rhodopsin. The results clearly showed that they are comparable to those of rhodopsin, indicating that the lower photosensitivity of cones than rods could be accounted for by differences in the photobleaching process and/or the Gt activation process (see below).

Photoexcited visual pigment is phosphorylated, resulting in the reduction of Gt activation efficiency and eventual quenching by the binding of arrestin [36–38]. It was reported that in carp retinas, both the expression level and specific activity of a receptor kinase in cones (GRK7) are 10 times greater than those in rods (GRK1) [39]. Photoexcited pigments are eventually dissociated into all-trans-retinal and opsin, which is then reconstituted into pigment by being supplemented with 11-cis-retinal. The rate of regeneration of cone visual pigments from 11-cis-retinal and photopsin (opsin of cone visual pigment) is much faster (a few hundred times faster) than that from 11-cis-retinal and scotopsin (opsin of rhodopsin) [26,27]. Once photoexcited pigment is decomposed, the Gt activating state is generated by photon only after the regeneration of pigment. Therefore, the rapid regeneration of cone pigments is suitable for functioning in the daylight condition where cones have to process successive light stimuli.

3.2. Early photobleaching process of cone visual pigments

Because of the difficulty of the separation of cone visual pigments and rhodopsin from the extract of the retina, only iodopsin and gecko green (P521) were subjected to biophysical and biochemical analysis before the 1990s. Iodopsin and P521 are present in the chicken or gecko retina in relatively large quantities (30–50% of the level of rhodopsin). Because the characteristics of cone visual pigments were quite different from those of rhodopsin, it was possible to clarify several basic properties of cone visual pigments without using isolated samples.

When 11-cis-retinal is added to the photo-bleached homogenate of chicken retina, iodopsin is first regenerated, and then rhodopsin is regenerated. The rate of regeneration of iodopsin is estimated to be 500-fold greater than that of rhodopsin [26]. Therefore, if a small amount of 11-cis-retinal is added to a homogenate of chicken retina, iodopsin is selectively regenerated. Even if significant amounts of scotopsin are present in the photopsin sample, scotopsin does not

hinder the spectroscopic assay of iodopsin because scotopsin is not photoactive. The spectroscopic study of cone visual pigments in the early stage was carried out using such iodopsin samples [40].

Nowadays, the use of column chromatography to separate visual pigments or expression systems to produce recombinant pigments has made it possible to characterize various kinds of visual pigments. However, in many spectroscopic studies, it was necessary to use low-temperature spectroscopy because only a limited amount of cone visual pigment was available. The results demonstrated that the 11-cis-retinal chromophore of cone visual pigment is isomerized to all-trans form [41,42], resulting in a red-shifted absorption spectrum [43]. The dark state and batho intermediate form a photo-steady-state mixture with iso-pigment having 9-cis chromophore [44]. The formation of a photo-steady-state mixture among 11-cis, all-trans, and 9-cis chromophores but no 7-cis or 13-cis chromophore at liquid nitrogen temperature is common to rhodopsin and all cone visual pigments studied so far. Therefore, it is likely that the stereoselectivity of the chromophore binding site is common to all vertebrate visual pigments, while electrostatic perturbation regulates the specific absorption maximum. Although UV pigment has a deprotonated chromophore in the dark state, its primary photointermediate has a protonated Schiff base [45].

By thermal reactions, bathorhodopsin is successively converted to lumirhodopsin, metarhodopsin I, metarhodopsin II and metarhodopsin III before it dissociates into all-trans-retinal plus scotopsin. Low temperature spectroscopy of iodopsin, however, demonstrated that bathoiodopsin formed at liquid nitrogen temperature thermally reverts to the dark state [40,46]. Such reverse reactions were observed for all the photobleaching intermediates of iodopsin formed at low temperatures [47,48]. Because these reverse reactions are completely suppressed by replacing chloride by nitrate, this reaction is likely to be specific for iodopsin in the chloride-bound form [46,48]. The mechanism of the facilitation of the isomerization from the all-trans to the 11-cis form, which never occurs without protein on this time scale, is unclear, but the delocalization of the π -electron of the red-shifted chromophore would contribute to this phenomenon, because P521 shows no reverse reaction [49]. Flash photolysis experiments of iodopsin showed that no reverse reaction occurred at physiological temperature, and the early photobleaching process of iodopsin is comparable to that of rhodopsin [6,7]. For M1 and M2 group pigments, it was confirmed that photobleaching intermediates corresponding to those of rhodopsin are formed in their photobleaching process [28,29,49].

3.3. Physiologically relevant intermediates and conformational changes

The cis-trans isomerization of the chromophore in a restricted chromophore binding site produces the highly twisted chromophore. In the early stages of the photobleaching process (<1 ms after photoexcitation), the substantial changes in the absorption spectrum are mainly due to the relaxation of the distortion of the chromophore, and the conformational change of the opsin moiety is relatively small [50,51]. After that, rearrangements of the hydrogen bonding network involving the chromophore Schiff base take place, which drive the large conformational changes that produce the Gt-activating state. The Gt-activating state of vertebrate rhodopsin is the UV-light absorbing state called metarhodopsin II having a deprotonated chromophore Schiff base. A similar intermediate (Meta-II) is formed from cone visual pigment, and it then promptly decays into all-trans-retinal and photopsin by the hydrolysis of the chromophore Schiff base [52,53].

The molecular properties of Meta-II may be related to the cell responses [54]. Although it remains a long-standing unresolved issue, it is of importance to characterize the formation and decay kinetics of Meta-II of cone visual pigments. Metarhodopsin I decays to metarhodopsin II, and equilibrium between them is established, followed by slow formation of metarhodopsin III from metarhodopsin I (Fig. 3). Therefore, the sequential formation of metarhodopsin I, metarhodopsin II and metarhodopsin III is seemingly observed in the photobleaching process

of rhodopsin. The sequence of the formation of Meta intermediates of cone visual pigments appears to be different from that of rhodopsin, and to vary among the phylogenetic groups of cone visual pigments [25]. The bleaching process of S group pigment (chicken violet) is qualitatively similar to that of rhodopsin (Meta-I → Meta-I/Meta-II → Meta-III → all-*trans*-retinal/photopsin), but the rate constants are significantly greater than those of rhodopsin. For M1, M2, and L group pigments (chicken blue, chicken green, and monkey green/iodopsin, respectively), parallel conversions from Meta-I to Meta-II (and/or all-*trans*-retinal plus photopsin) and from Meta-III to all-*trans*-retinal plus photopsin are observed. These spectral changes are accounted for by a reaction pathway in which a mixture of Meta-I, Meta-II, and Meta-III is formed from Meta-I, and then these intermediates decay into all-*trans*-retinal plus photopsin. Detailed fluorescence measurements during photopsin formation using chicken green demonstrated that the hydrolysis of the Schiff base chromophore of Meta-II occurs before the decay of Meta-III [53]. These findings imply that the rate of formation of Meta-III is comparable to that of Meta-II and their back-reactions to Meta-I are slow (Fig. 3).

Metarhodopsin II is in equilibrium with metarhodopsin I, depending on pH and temperature [55,56], and the same is also observed for cone visual pigments except for the L group cone visual pigments [25]. However, the pH-dependent equilibrium between the Meta intermediates of L group pigments is restored by replacing chloride by nitrate, suggesting that chloride affects the hydrogen bonding network involving the chromophore Schiff base and ERY region. In metarhodopsin II, helix VI moves outward by 6–7 Å and the long cytoplasmic loop between helices V–VI forms an α -helix, resulting in the elongation of helix V [57–59]. The rigid cluster sticks out from the membrane, and this region is involved in the interaction with Gt. Although the crystal structures are not available for either Meta-II or the dark state of cone visual pigment, the conformational change of cone visual pigment is likely to be comparable to that of rhodopsin, because cone visual pigments activate rod-type Gt, although the efficiency is lower [53,60,61].

To highlight the difference between cone visual pigment and rhodopsin, characterization of cone visual pigment in M2 group, which is phylogenetically the closest to the rhodopsin group, is useful. From this standpoint, chicken green pigment has been well characterized. In the primary structure, the amino acid sequence of the second and third cytoplasmic loops, which are involved in the recognition and interaction with Gt, are well conserved between rhodopsin and chicken green. In contrast,

Glu122 and Ile189 are conserved in rhodopsins, but they are replaced by Gln and Pro, respectively, in many cone visual pigments. Mutational analysis of positions 122 and 189 of chicken green and rhodopsin demonstrated that Glu at 122 and Ile at 189 suppress the hydrolysis of the chromophore Schiff base of Meta-II but slow the regeneration from opsin and 11-*cis*-retinal [53,62–64]. Kinetic analysis of Gt activation using mutants of chicken green and rhodopsin for positions 122 and 189 showed that the rate of chromophore hydrolysis is negatively correlated with the initial velocity of Gt activation [53], suggesting that the amino acid residues at positions 122 and 189 account for not only the resistance to the chromophore hydrolysis in Meta-II but also the conformation of Meta-II for efficient Gt-activation. The long-lived Gt activation state with high efficiency is typical of rhodopsins, implying that rhodopsin acquired these amino acid residues during the evolution for its function.

4. Vertebrate color vision

In vision, as in a camera, color discrimination is performed using a combination of several types of photosensors that have different wavelength sensitivity. Vertebrates that have color vision have several types of cones in their retina (Fig. 4). It is well known that humans and closely related primates have trichromatic color vision, in which red-, green-, and blue-sensitive cones are involved. Most mammals, including new world monkeys have dichromacy, whereas many birds and fishes have tetrachromacy [10,65]. Some fishes and amphibians use 3,4-dehydroretinal (A2 retinal) as the chromophore, which shows a red-shifted absorption spectrum in comparison with A1 retinal [66].

Nathans and coworkers opened the door to understanding the molecular basis of color vision in the 1980s. They cloned and sequenced the genes of three kinds of human rhodopsin-like pigments. In addition, they assigned these genes by comparing them with those of color blind individuals [67,68]. As a result, the amino acid sequences of human red, green, and blue pigments were deduced (human “blue” pigment phylogenetically belongs to S group involving violet and UV pigments, but it is customarily called human “blue” pigment). Because the amino acid identity between red and green cone visual pigments was 96%, whereas those between red pigment and rhodopsin, between blue pigment and rhodopsin, and between red and blue pigments was ~40%, they speculated that humans recently acquired trichromacy by divergence into red and green pigments. These findings heralded the phylogenetic analyses of the molecular evolution of visual pigments.

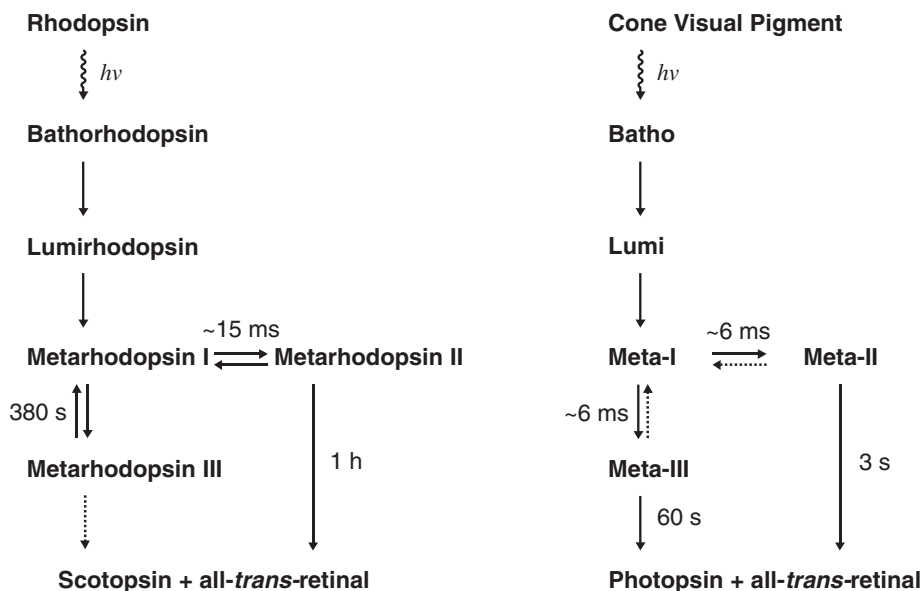


Fig. 3. Photobleaching process of cone pigments and rhodopsin. The time constants for cone pigment are those of chicken green pigment. It should be noted that intermediates detected in both rhodopsin and cone visual pigments are only shown in the figure.

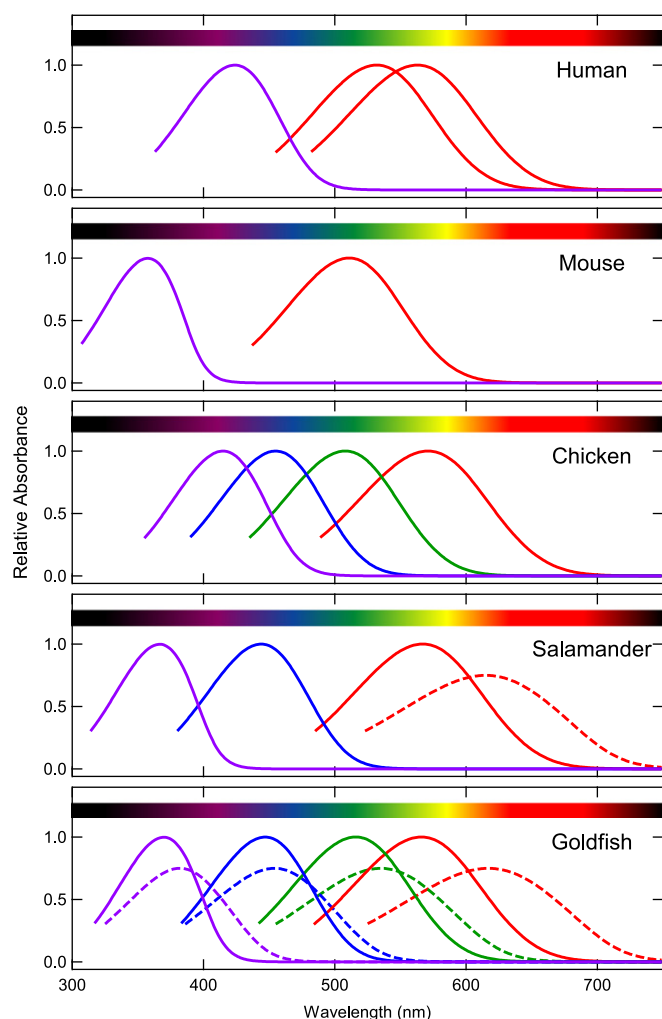


Fig. 4. Absorption spectra of cone visual pigments in representative animals. The spectra of cone visual pigments of human (563, 532, and 424 nm [79,101]), mouse (511 and 358 nm [91,97]), chicken (571, 508, 455, and 415 nm [5]), salamander (615 (A2), 567 (A1), 444, and 367 nm [65,102,103]), and goldfish (566, 516, 447, and 370 nm for A1, and 617, 535, 454, and 382 nm for A2 [104]) are shown. The absorption spectra of the pigments belonging to L, M2, M1, and S group are shown in red, green, blue, and violet, respectively. The spectra of pigments having A2 retinal is shown by broken lines. These spectra were generated using Govardovskii's template [105].

Before the determination of the primary structures of cone visual pigments, attempts to elucidate the biochemical basis of color vision by isolating the cone visual pigments were made using chicken retina, which is rich in cone visual pigments and can be obtained in large quantity. Fager and coworkers reported that the chicken visual pigments can be separated from each other by the combination of concanavalin A affinity column chromatography and DEAE ion exchange column chromatography [69,70]. Then Okano et al. improved the methods by using the dialyzable detergent CHAPS instead of digitonin and obtained sufficient amounts of visual pigments for spectroscopic and biochemical studies [5]. The preparation of native pigments from animal retinas is more advantageous for obtaining a large amount of pigments than isolating recombinant pigments from cultured cells, although mutant proteins cannot be obtained from retina. Native pigments isolated from retinas were widely used in the early stages of the characterization of cone visual pigments, when an expression system for cone visual pigment had not been established.

5. Color tuning mechanism of cone visual pigments

Vertebrate visual pigments are composed of 340–370 amino acid residues. They are folded into a seven-transmembrane structure,

which is common to G protein coupled receptors [71,72], and embedded in the outer segment membrane. To absorb near-UV to visible light, visual pigments bind to the 11-*cis*-retinylidene chromophore at the lysine residue in the middle of the seventh transmembrane helix. It binds via a Schiff base linkage, which is protonated in the pigments absorbing visible light. While the chemical structure of the chromophore of cone visual pigments is identical to that of rhodopsin with the exception that the Schiff base linkage of UV pigment is unprotonated, the absorption maxima of cone visual pigments are expanded ranging from 360 nm to 600 nm.

The mechanism of this opsin shift is a long-standing subject for experimental and theoretical studies of visual pigments. The absorption maximum of free retinal is located at 360–380 nm regardless of its configuration [73,74]. Spectroscopic studies using model compounds such as alkyl-retinal Schiff base demonstrated that the formation of a Schiff base with an amino group does not by itself shift the absorption maximum markedly, but the protonation causes a substantial red shift to 440 nm [75]. Further red shift is explained by (1) interaction between the protonated Schiff base and its counterion, (2) distortion of the conjugated double bond system of the retinal chromophore, and (3) electrostatic perturbation of the π -electron system by polar or charged residues [75,76]. Because the phylogenetic relationship is highly consistent with the absorption maxima of the members [77], the amino acid residues conserved in each group are likely to be involved in the spectral tuning.

The maximal sensitivity of L group pigments shows a wide variety in wavelength (530–570 nm). It should be noted that this variance is not merely a difference among species, but rather is directly relevant to the physiological function in primate color vision. Color vision of human and related primates is trichromatic based on red, green, and blue cone visual pigments. Red and green pigments maximally absorb 560 and 530 nm light, but both of them belong to the L group. In fact, only 15 out of 364 amino acid residues differ between them [68]. Because the malfunction of red and/or green pigments results in color blindness, the amino acid basis of the spectral tuning mechanisms of L group pigments have been extensively studied [67]. Careful comparison of the amino acid sequences of L group pigments suggested that the three amino residues at positions 164, 261, and 269 (in the numbering system of bovine rhodopsin) regulate the absorption maximum [78]. Thereafter, comprehensive mutational analysis demonstrated that seven amino acid residues at 100, 164, 214, 261, 269, and 293 are involved in the spectral difference between red and green pigments [79]. Resonance Raman measurements of the $C_{15}=N$ mode of the chromophore showed that the weakened interaction between the protonated Schiff base and counterion accounts for the red-shifted absorption spectrum of L group pigment as compared to rhodopsin [42,80], but the $C_{15}=N$ modes of human red and green pigments were comparable. This suggests that hydroxyl-bearing amino acids near the conjugated double bond system contribute to the further red shift. This concept was generalized and refined, and is known as the “five site rule” [81]. In this model, the spectral tuning of L group pigments is almost fully explained by the hydroxyl-bearing amino acid residues at positions 164, 181, 261, 269, and 292. This model was examined by theoretical work, and it was shown that the hydroxy group of Tyr and Thr lowers the energy level of excited state or elevates that of the ground state, resulting in the red-shift of the absorption maxima [82].

Notable characteristics of L group visual pigments include their halide-dependent spectral tuning (chloride effect) [83–86]. The absorption spectra of most L group pigments are significantly blue-shifted in the absence of chloride. Because the affinity of chloride for the L group pigments is quite high ($K_D = 0.1$ mM [87,88]), it is likely that all the pigments are present in chloride-bound form in the physiological condition. In vitro experiments, chloride can be replaced by bromide, but not by fluoride or iodide. In contrast, the binding of lyotropic anions such as NO_3^- and ClO_4^- blue-shifts the absorption maximum.

Based on the studies of mutants of human red pigment for positively charged amino acid residues near the chromophore, the chloride binding sites were proposed to be His197 and Lys200 (positions 181 and 184 in the bovine rhodopsin numbering system, respectively) [89]. These residues are positioned in the second extracellular loop, forming the chromophore binding pocket. While the mutation of His181 significantly reduces the chloride effect, H181M and H181N of monkey green show a chloride-dependent red-shift of 15 nm [90], indicating that His181 by itself functions as a chloride binding site. Recently we reported that mutations for Ala289 and Ala292 also abolish the chloride effect [91]. The crystal structure of bovine rhodopsin suggests that these Ala residues are clustered with His181 near the chromophore Schiff base (Fig. 5). FTIR measurements of iodopsin demonstrated that an intense hydrogen-out-of-plane mode of C₁₄H of the chromophore is observed for the batho intermediate, which is significantly reduced by the replacement of chloride by nitrate [92,93]. The changes in vibrational

mode of hydrogen-bonded water upon the photoisomerization of the chromophore are altered by the replacement of chloride by nitrate [93]. These findings strongly suggest that chloride is accommodated among His181, Ala289, and Ala292 (Fig. 5) and the hydrogen-bonding network is perturbed by chloride.

Some animals have UV-sensitive cone visual pigments. These pigments belong to S group, but S group pigments also show a wide variety of absorption maxima ranging from 360 to 430 nm [94]. However, the distribution of the absorption maximum of S group pigments is not continuous but rather is separated into a UV-absorbing group (360–380 nm) and a violet-absorbing group (410–430 nm). The absorption maximum of the former group is close to that of the unprotonated retinal Schiff base, and thus this difference is likely to arise from the protonation state of the chromophore. It is proposed that 8 sites (positions 46, 49, 52, 86, 90, 93, 114, and 118) are involved in the divergence of UV and violet absorption [95] (Fig. 5). However, unlike the five-

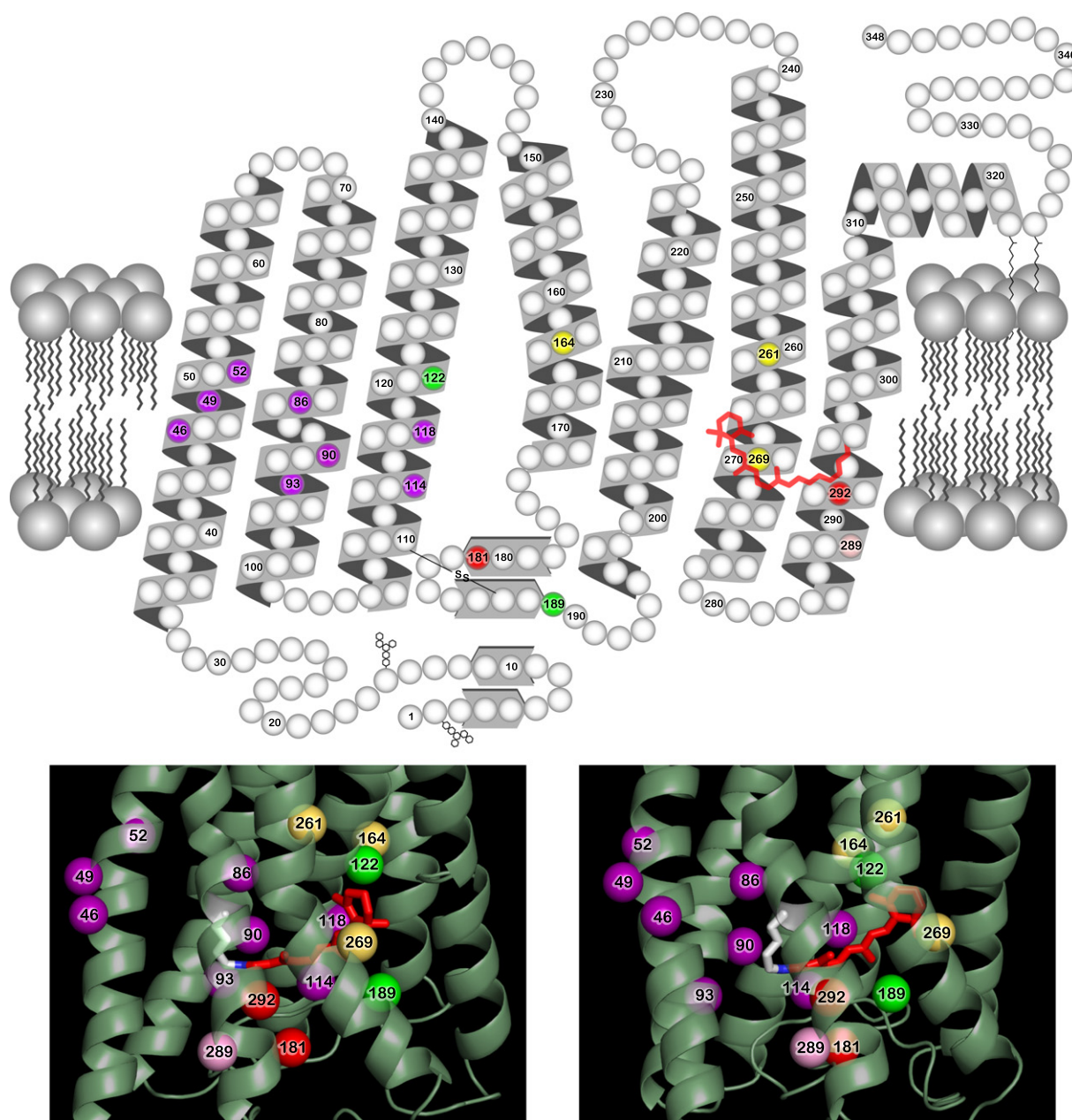


Fig. 5. Positions of amino acids that determine the properties of visual pigment. Five sites of L group pigments are shown in yellow or red, where His181 (red) and Ala292 (red) form a chloride binding site together with Ala289 (pink). Eight sites of S group pigments are shown in violet. Glu122 and Ile189 contribute to the slow decay of Meta-II that is characteristic of rhodopsin (green). The secondary structure of visual pigments is based on the crystal structure of bovine rhodopsin (1U19).

site rule for L group pigment, no clear relationship between the amino acid residues at these 8 sites and the absorption maximum was found, suggesting independent evolution to the UV-absorbing pigments in these different classes. However, in avian S group pigments, it was suggested that Ser90 and Cys90 are responsible for the protonated and unprotonated Schiff base, respectively [95].

It should be noted that Glu113, which functions as a counterion for the protonated Schiff base, is conserved in UV pigments. Tsutsui et al. demonstrated that the photosensitivity of UV pigment (efficiency of photoisomerization) is enhanced in the presence of counterion [96,97].

6. Cone visual pigments and cell function

Recent progress in the techniques to generate transgenic or knock-in animals enabled electrophysiological assays of rods in which cone visual pigment is expressed. The photoresponse of the visual cells should be governed by not only the subclasses of the functional proteins but also their concentration and localization, and the morphology and dimensions of the cells. Therefore, characterization of a rod in which cone visual pigment is expressed provides the most direct comparison between cone visual pigment and rhodopsin in the cell [31,34].

Biochemical assays of isolated visual pigment and Gt have demonstrated that the amount of Gt activated by rhodopsin is 50–100 times greater than that activated by cone visual pigment [53,61]. Because Michaelis–Menten analysis of Gt activation showed that the initial velocity for chicken green was about half of that for rhodopsin, this significant difference is mainly due to the fast decay of Meta-II intermediate of cone visual pigment [53]. The difference in initial velocity between chicken green and rhodopsin is comparable to that in photosensitivity between wild-type rods and rods containing cone visual pigment [31,34]. The small difference between these results is probably due to the difference in the environment of visual pigment between in vivo and in vitro assays or the lack of shutdown mechanisms such as phosphorylation and arrestin binding in the in vitro assay.

The most notable characteristics of rods in comparison with cones are the low level of dark noise of the former [98]. Because the signal generated by activation of a single rhodopsin molecule is so weak, a low noise level is essential for a rod to function as a single-photon detector. A lot of factors might generate dark noise, but the thermal isomerization of the chromophore of the visual pigment is likely to be involved in the noise. In fact, rods show increased dark noise when cone visual pigment is present [30].

To demonstrate the origin of dark noise of the visual cells directly, quantitative analysis of the thermal isomerization from the 11-*cis*- to all-*trans* forms of the chromophore of the visual pigment is essential. However, the rate of thermal isomerization is very low (10^{-11} s^{-1}), and attempts to evaluate it by spectroscopic or biochemical assays have not succeeded so far. In contrast, the tiny signal generated by a few activated receptor molecules is amplified by the enzymatic cascade system in the cell, and it grows to an electrophysiologically detectable dark event. Thus, the contribution of the cone visual pigments to generating the dark noise of the visual cell has mainly been studied by electrophysiology using rods expressing cone visual pigments. One interesting finding is that the level of dark noise correlates with the absorption maximum [99], suggesting that the dark noise arises from isomerization of the chromophore which goes over the same potential barrier as that of photoisomerization.

However, it is clear that the transduction system of the cell is complicated and the molecular basis of the dark noise should be clarified by characterization of the cone visual pigment in comparison with rhodopsin. The crystal structures of cone visual pigments would provide many insights. In rhodopsin, it was proposed that the conformation which facilitates the thermal isomerization is transiently generated in the structural ensemble of rhodopsin [100]. Therefore, it is possible that the difference in the conformational dynamics between cone visual pigment and rhodopsin accounts for the difference in the noise level.

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