CMLS, Cell. Mol. Life Sci. 54 (1998) 1299–1315
1420-682X/98/121299-17 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Visual pigment: G-protein-coupled receptor for light signals

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Received 20 July 1998; received after revision 9 September 1998; accepted 23 September 1998

Abstract. The visual pigment present in photoreceptor cells is a prototypical G-protein-coupled receptor (GPCR) that receives a light signal from the outer environment using a light-absorbing chromophore, 11-*cis*-retinal. Through cis-trans isomerization of the chromophore, light energy is transduced into chemical free energy, which is in turn utilized for conformational changes in the protein to activate the retinal G-protein. In combination with site-directed mutagenesis, various spectroscopic and biochemical studies identified functional residues responsible for chromophore binding, color regulation, intramolecular signal transduction and G-protein coupling. Extensive studies reveal that these residues are localized into specific domains of visual pigments, suggesting a highly manipulated molecular architecture in visual pigments. In addition to the recent findings on dysfunctional mutations in patients with retinitis pigmentosa or congenital night blindness, the mechanism of intramolecular signal transduction in visual pigments and their evolutionary relationship are discussed.

Key words. Rhodopsin; G-protein; molecular evolution; color vision; receptor protein; photosignal transduction.

Introduction

The visual transduction process in photoreceptor cells begins with photon absorption by a visual pigment, which is a member of a family of G-protein-coupled receptors (GPCRs). So far, various types of visual pigments have been identified from a variety of photoreceptor cells, and molecular mechanisms of light absorption and G-protein activation have been widely investigated. The advantages of studies on visual pigment in comparison with those on other G-protein-coupled receptors are that visual pigment can be synchronously activated by light and the chromophore can be utilized as an intrinsic spectroscopic probe to monitor structural changes in the protein. These merits enable investigation of each step of the changes in the protein and the process of interaction with the G-protein by various spectroscopies with excellent time and spatial resolutions. Thus visual pigment is one of the model receptors whose activation processes can be elucidated at submolecular or atomic resolution. This review focuses on the mechanism of intramolecular signal transduction in visual pigments based on the experimental results obtained by various biochemical and spectroscopic techniques in combination with site-directed mutagenesis. In addition, the phylogenetic relationship among visual pigments will be discussed to obtain a clue to the difference in the phototransduction cascade among different types of photoreceptor cells.

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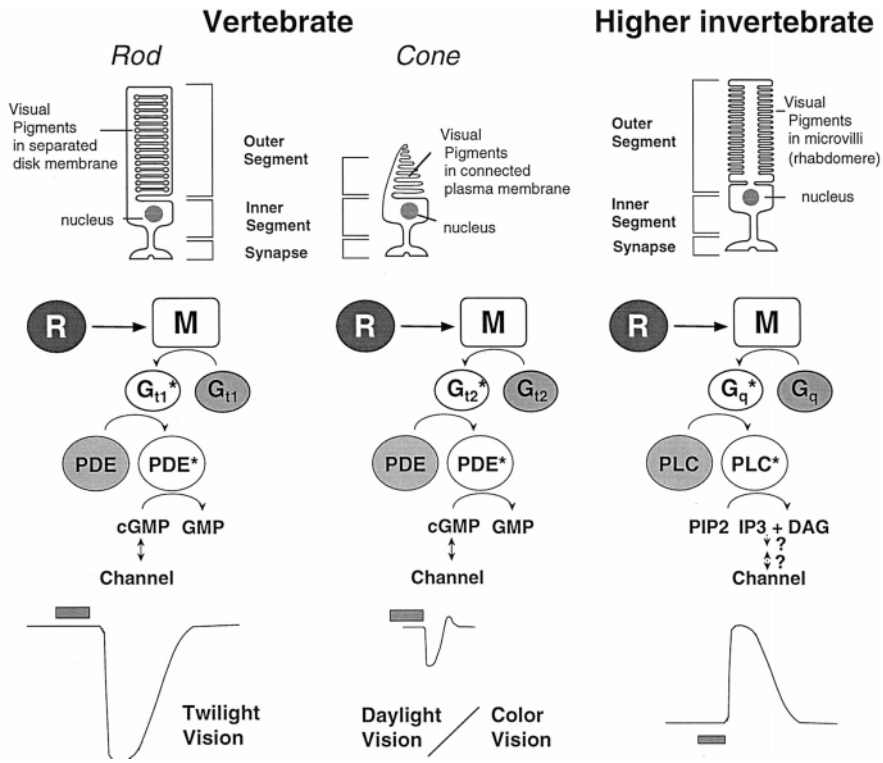

Figure 1. Signal transduction cascade in photoreceptor cells. In vertebrate ciliary photoreceptor cells, such as rods and cones, visual pigments trigger a Gt-mediated signal cascade which eventually leads to hyperpolarization of the cell. In rhabdomeric photoreceptor cells of higher invertebrates such as cephalopods and arthropods, visual pigments trigger a Gq-mediated cascade and lead to depolarization of the cells. Although rods and cones share similar phototransduction cascades, their photoresponse patterns revealed by electrophysiological studies are different. R, visual pigment; M, meta-intermediate; PDE, phosphodiesterase; PLC, phospholipase C.

Other important areas of phototransduction including functional proteins other than visual pigments and electrophysiological investigations are beyond the scope of this article, and the reader is referred to other reviews [1–3].

Visual pigment initiates a G-protein-mediated transduction cascade in photoreceptor cells

The primary role of visual pigment in photoreceptor cells is to receive a photon signal from the outer environment and to transfer the signal to a subsequent enzymatic cascade system through activation of the retinal G-protein (fig. 1). Through cis-trans isomerization of the chromophore, light energy is utilized as chemical free energy to convert the conformation of the protein moiety of the visual pigment from its resting state to an active state. In the photoreceptor cells of most vertebrates, visual pigments activate one of the G-protein subtypes called transducin (Gt) through a guanosine 5′-diphosphate (GDP)-guanosine 5′-triphosphate (GTP) exchange reaction in the α-subunit of transducin [4], which leads to activation of a cyclic guanosine 3′,5′-monophosphate (cGMP) phosphodiesterase [5]. The resulting decrease in concentration of cytosolic cGMP causes closure of the cGMP-gated channel [6] in the plasma membrane, and graded hyperpolarization of the photoreceptor cells results. In contrast, visual pigments present in invertebrate rhabdomeric photoreceptor cells activate the Gq type of G-protein [7], which mediates the so-called inositol 1,4,5-trisphosphate (IP3) cascade involving phospholipase C (PLC)β-4 as an effector enzyme [8]. Although the mechanism leading to the opening of the ion channel in the plasma membrane is not understood, light absorption by the visual pigment ultimately causes depolarization of the photoreceptor cells [9, 10]. Recently, another type of transduction cascade mediated by a novel type of visual pigment and the Go type of G-protein was found in scallop ciliary photoreceptor cells [11] which responds in a hyperpolarizing manner [12]. The subtype specificity of the G-protein required to activate the effector enzyme (or channel) is relatively

strict, and the cascade system elicited by the signal depends on what type of G-protein is activated by the receptor. In other words, the coupling specificity between the receptor and the G-protein subtype is one of the major factors in determining the cascade system utilized (see below).

Structural motif of visual pigments

Visual pigment is a 35–55-kDa membrane protein which consists of a single polypeptide 'opsin' and a chromophore, 11-*cis*-retinal [13] (fig. 2). The opsin contains seven transmembrane α-helices, the structural motif typical of the G-protein-coupled receptors [14]. The amino acid sequence of visual pigment was first determined in bovine rhodopsin by three research groups based on sequencing the peptide fragments [15, 16] and complementary DNA (cDNA) cloning [17] (fig. 3). After these pioneering studies, over 50 primary structures of visual pigments, including cone visual pigments and invertebrate rhodopsins, were determined. The amino acid residues which play important roles in the function of visual pigments were then extensively investigated. Hereafter, we denote the amino acid residue by a single-letter code and a number using the bovine rhodopsin numbering system, because the residue numbers counted from the N-terminus are different among various types of visual pigments.

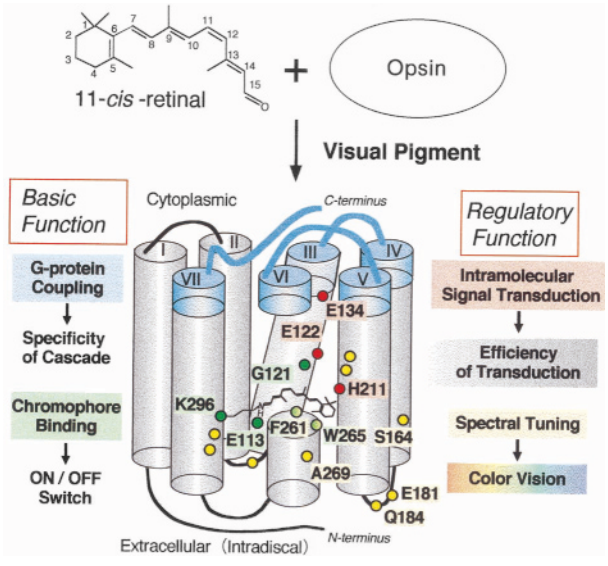

Figure 2. Structural model of bovine rhodopsin. Visual pigment is composed of a protein moiety (opsin) and a chromophore (11-*cis*-retinal). Cytoplasmic domains (blue) are responsible for G-protein coupling, and their amino acid sequences are highly conserved among respective subclasses of visual pigments. Amino acid residues responsible for chromophore binding (green), spectral tuning (yellow) and intramolecular signal transduction (red) are located in helices 3, 5, 6 and 7. According to the projection structure [41], helix 3 is tilted relative to the other helices.

As described above, visual pigment contains 11-*cis*-retinal as a light-absorbing chromophore [13]. The chromophore also acts as an 'inverse agonist' to suppress the ability of the visual pigment to activate the G-protein in the dark [18–20]. Thus the amino acid residue(s) which binds to and interacts with the chromophore is important. Accumulated evidence has now revealed that all of the visual pigments contain a specific lysine residue (K296 in bovine rhodopsin) in the seventh transmembrane helix and that it binds to the chromophore through a protonated Schiff base linkage [15, 21]. In vertebrate visual pigments, the positive charge on the protonated Schiff base is stabilized by a negatively charged E113 which acts as a 'counterion' [22–24].

Replacement of E113 by a residue having no charge affects the K_d value of the Schiff base [22–24] and, more important, makes the protein constitutively active when the chromophore is absent [19]. E113 is therefore thought to keep the visual pigment silent through electric interaction with the protonated Schiff base or the amine group of lysine in the case where the chromophore is absent. However, even in a mutant of rhodopsin having neutral residue at this position, the binding of the chromophore also exhibits little G-protein activity [22, 23]. This fact indicates that the interaction of the chromophore with nearby amino acid residues other than E113 is also important for suppression of G-protein activation. Covalent linkage of the chromophore to the α-helix through the lysine residue is not necessary for pigment function [25].

Like vertebrate visual pigments, invertebrate visual pigments have a protonated Schiff base as their chromophore, but they have no glutamate at position 113. Instead, tyrosine is conserved throughout invertebrate visual pigments with the exception of phenylalanine in place of tyrosine in *Drosophila* ultraviolet (UV) receptors [26, 27]. Thus the mechanism of stabilization of the positive charge on the Schiff base might be different between vertebrate and invertebrate visual pigments. However, recent resonance Raman and Fourier transform infrared (FTIR) spectroscopy revealed that the protonated Schiff base interacts with E113 through a water molecule [28, 29]. The water molecule, which exhibits a similar FTIR signal, is also present in octopus rhodopsin [30]. Thus it could be speculated that the water molecule is essential for the stabilization of the protonated Schiff base and that it is stabilized by either a charged or polarized amino acid residue. The difficulty in the functional expression of invertebrate visual pigments hampers examination of the direct interaction between the protonated Schiff base and the water molecule using site-directed mutagenesis. Thus an

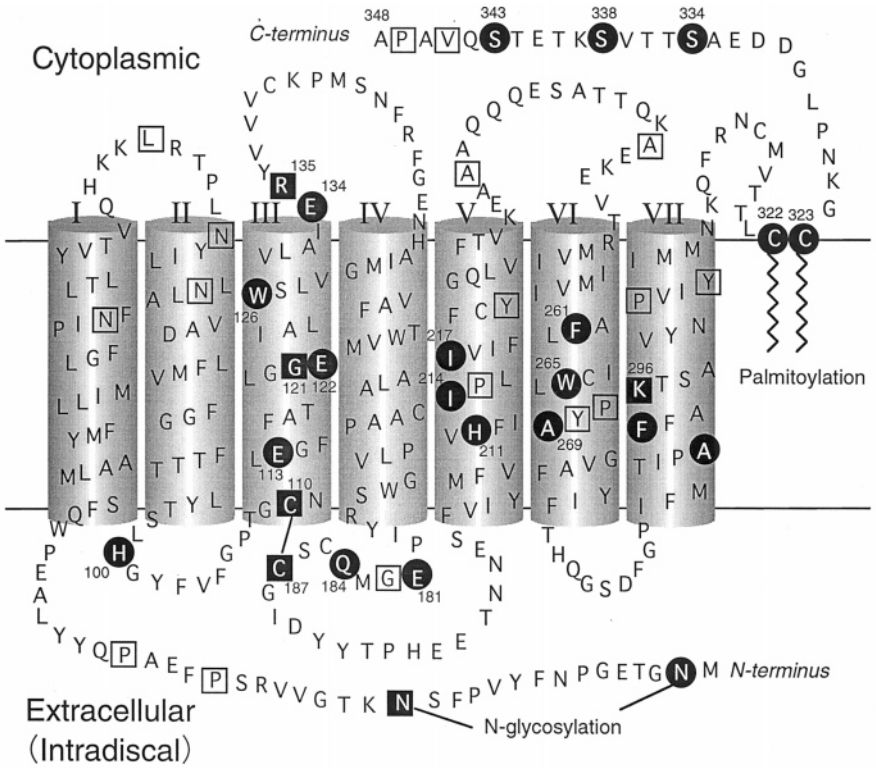

Figure 3. Amino acid sequence of bovine rhodopsin. Amino acid residues conserved in all visual pigments are surrounded by squares. Functional amino acid residues that are confirmed by various experiments are indicated by white letters surrounded by black circles or squares. Two arginines near the N-terminus are confirmed to be substrates of N-glycosylation [147]. Cysteins 322 and 323 are substrates of palmitoylation [148]. Thus the carboxyl-terminal sequence emerging from helix 7 and anchoring to the lipid bilayer via palmitoylcysteins 322 and 323 is thought to be a putative fourth loop of the cytoplasmic side [113].

expression system for invertebrate visual pigments needs to be established.

In addition to K296 and E113, some amino acid residues involved in the chromophore binding site have been identified. First, using a photoactivatable analog of 11-*cis*-retinal as a cross-linking reagent, Nakayama and Khorana identified six amino acid residues (F115, A117, E122, W126, S127 and W265) which might be situated near the C3 position of the retinal chromophore [31]. They also showed that mutation of these residues changed the absorption spectrum and the bleaching process of rhodopsin and its ability to activate G-protein [32]. Because they used the retinal analog whose C11=C12 double bond could be isomerized given the experimental conditions under which photo-induced cross-linkings were performed, some of the amino acid residues detected might have resulted in cross-linking reactions after conformational changes in the protein moiety induced by chromophore isomerization. In fact, a similar photoaffinity study using an 11-*cis*-locked retinal analog in which no isomerization occurs identified only W265 and L266 as the reacted amino acid residues [33]. Thus it appears that W265 and L266 are located near the β-ionone ring of retinal in rhodopsin, whereas other residues are located near the chromophore in some intermediate states of rhodopsin (see below). Second, G121, the conserved amino acid residue in all visual pigments, is shown to be situated so as to interact with the 9-methyl group of retinal [34]. This was evidenced by the experimental fact that the replacement of the residue with those having bulky side chains changes the absorption spectrum of the pigment and its ability to activate the G-protein in a volume-dependent manner. Furthermore, similar changes in pigment properties are induced by the reconstitution experiments with native opsin and retinal analogs in which the 9-methyl group was replaced by shorter (desmethyl) or longer groups (ethyl, propyl) [34]. F261 is a counterpart of G121 in the interaction site of the 9-methyl group of retinal because its replacement by alanine compensates the effect of replacement of G121 by a bulky side chain [20]. Our recent investigations

using retinal analogs in which 9-methyl is replaced by larger group (phenyl, benzyl) also show that the 9-methyl group is in the narrow cavity [35] that includes F261 (H. Imai et al., unpublished results). Taken together, the retinal chromophore interacts with amino acid residues at three known interaction sites, the protonated Schiff base to E113 via water molecule(s), the β-ionone ring to W265 and L266, and the 9-methyl to G121 and F261.

There are other identified residues which are important for the function of visual pigments. Among the residues, E134, R135 and Y136, which are situated at the interface between helix 3 and the second cytoplasmic loop, are of special interest because they are homologous with the DRY triad widely conserved in G-protein-coupled receptors [36]. Accumulated evidence has suggested that R135 is essential for G-protein activation and that E134 possibly serves as a regulator by changing its protonation state during the process of activation [37, 38]. The role of Y136 is not strictly determined, although it serves as a binding site for the α-subunit of transducin with subsequent VVVC sequences in bovine rhodopsin. The role of E122, which is strictly conserved only in rod pigments, that is rhodopsins, is in marked contrast to those of the residues described above. While the latter serve as fundamental residues of visual pigments for chromophore binding and G-protein activation, E122 regulates intramolecular signal transduction in rhodopsin, that is, it functions as a determinant in regulating the rate of regeneration from 11-*cis*-retinal and opsin and that of the decay of an enzymatically active intermediate (meta II) [39]. W126, one helical turn apart from E122, is also conserved in vertebrate visual pigments and is shown to be sensitive to the conformational change upon formation of meta II [40]. Our recent investigation using FTIR spectroscopy suggested that W126 interacts with E122, thereby reducing the conformational change in the protein near these residues (T. Oura et al., unpublished results).

Recently, electron cryomicroscopy has provided a map at moderate resolution of the three-dimensional arrangement of the seven α-helices of rhodopsin [41]. In this map, the third helix is considerably tilted from the membrane normal compared with the other helices (fig. 2). Because most amino acid residues described above are situated in the third helix, one might speculate about the relationship between the tilted helix and the presence of functional residues. Recent studies suggested the enlargement of the distance between helices 3 and 6 upon formation of meta II [42, 43]. Thus, exposure of the region which interacts with the G-protein may depend on the tilted third helix.

Molecular evolution of visual pigments

Amino acid sequences of over 50 visual pigments are now available; therefore, one can compare the sequences and construct a phylogenetic tree of the visual pigments to reveal the phylogenetic relationship among visual pigments (fig. 4). The most important findings from the phylogenetic studies could be divided into two parts; one is the phylogenetic relationship between rod and cone visual pigments, including the divergence of cone visual pigments in vertebrate systems. The other is that among all visual pigments in relation to the differ-

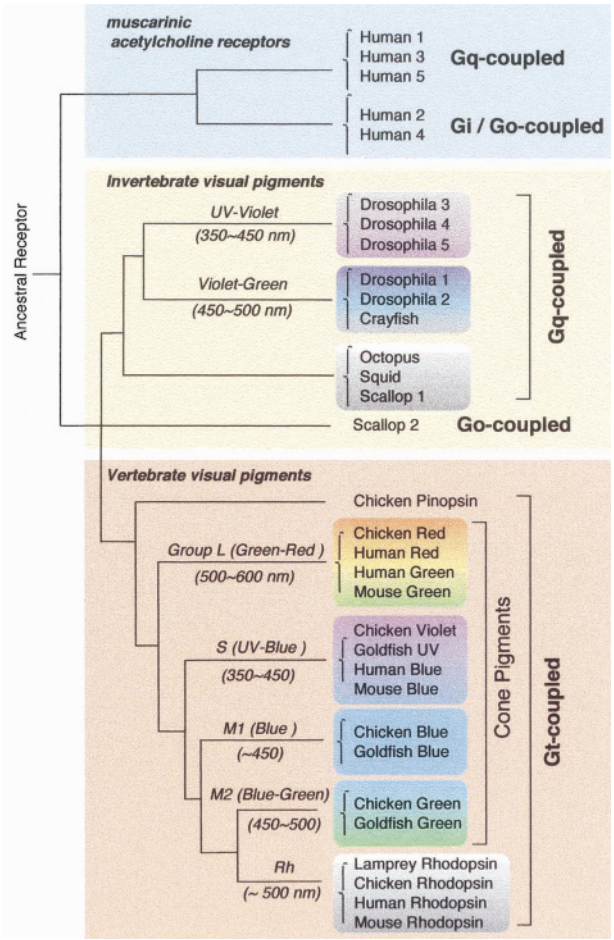

Figure 4. Schematic drawing of the phylogenetic relationship among visual pigments according to the phylogenetic tree based on the amino acid sequences of visual pigments and other GPCRs [11]. Vertebrate and invertebrate pigments are classified into three major groups, each of which is thought to couple with different subtypes of the G-protein. Pigments whose spectral sensitivities are different from each other are diverged independently in vertebrate and invertebrate systems, suggesting the independent evolutionary process of color vision. Rod pigment rhodopsins diverged from one of the groups of cone visual pigments that includes chicken green [46].

ences in the phototransduction cascade among different types of photoreceptor cells.

Most vertebrates have two types of photoreceptor cells, rods and cones, which are responsible for twilight (scotopic) and daylight (photopic) vision, respectively. Rods are more sensitive to light than cones, whereas cones display rapid photoresponse and rapid adaptation compared to rods [2, 3]. Cones are further classified into at least three subtypes, each of which has a specific visual pigment with a different absorption maximum. Thus, elucidation of the evolutionary relationship between retinal duplicity and color vision has been a long-standing issue. At a visual pigment level, it had been believed that the divergence of rod and cone visual pigments took place earlier than that of subtypes of cone visual pigments, because considerable differences in molecular properties exist between rod and cone visual pigments [44]. In 1986, Nathans et al. [45] determined the amino acid sequences of three types of cone visual pigments in human retina and classified the pigments into two subgroups on the basis of amino acid identities. One is the S group of cone pigments, which includes a blue-sensitive pigment, and the other is the L group, which includes red- and green-sensitive pigments. Human rhodopsin has a sequence less homologous to those of the cone pigments and forms a group distinguishable from these groups. Thus one might speculate that visual pigment genes diverged from a common ancestor of three genes, two of which generated the rhodopsin and blue pigment genes, whereas the third was duplicated in a much more recent evolutionary event to yield the green and red pigment genes [45]. However, the question which diverged earlier, rod and cone pigments or groups L and S of cone pigments, is moot. In 1992 we determined the amino acid sequences of four types of cone visual pigments in chicken retina. The result showed that two of the cone visual pigments, chicken green and blue, exhibit less identity with human red/green and blue, indicating that cone visual pigments should be classified into four groups [46]. More important, we found that chicken green has an amino acid sequence more similar than any other cone pigments to rhodopsin [46, 47], but it displays molecular properties required for a cone visual pigment that are clearly different from those of rhodopsin [48]. We then constructed a phylogenetic tree based on the amino acid identities. The tree indicated that an ancestral visual pigment evolved first into four groups of cone visual pigments and that the group of rhodopsins diverged later from one of the groups of cone visual pigments, including chicken green. These findings showed a definite point of divergence between rod and cone pigments. It should be noted that rhodopsin of a lower vertebrate (lamprey) [49] and that of higher vertebrates diverged much later than did cone visual pigments into four groups. Thus it is evident that animals acquired the ability to distinguish color at least at the stage of the lower vertebrates and acquired scotopic vision later. Recently, we found that a single mutation of the amino acid residue at position 122 exchanges molecular properties of rod and cone pigments (see later) [39]. This fact suggests that single replacement of an amino acid residue is one of the key steps in divergence of scotopic and photopic vision.

The phylogenetic tree of visual pigments including vertebrates and invertebrates provides insight into the divergence of signal transduction cascades in animals. So far, two kinds of phototransduction systems have been reported. One is the Gt-mediated system of vertebrate hyperpolarizing photoreceptor cells, and the other is the Gq-mediated system of invertebrate depolarizing cells, such as those of cephalopods and arthropods. The visual pigments of these systems show sequence homology but are clearly split into two subtypes (Gt- and Gq-coupled) in the phylogenetic tree. These results suggest that the phylogenetic analysis of visual pigments can be an exclusive way to elucidate the evolutionary relationship between phototransduction cascades. In fact, our recent study indicated that a newly identified visual pigment present in scallop hyperpolarizing photoreceptor cells forms a distinct subfamily in the phylogenetic tree and that it couples with the Go type of G-protein but not with Gt and Gq [11].

The phylogenetic tree clearly shows that three subfamilies (Gt-, Gq- and Go-coupled pigments) diverged at a very early time. That is, Go-coupled rhodopsin identified from scallop visual cells diverged from an ancestral visual pigment before the divergence of animals (700 million years ago) into higher invertebrates (deuterostomia) and vertebrates (protostomia). This is also supported by the fact that visual pigment-like proteins (RGR), recently found in mammals [50], clustered with Go-rhodopsin when these extra members were added to the tree [11]. The deepest root of the visual pigments corresponds to the generation of three different genes, not to the divergence of animal species. Thus the multiple phototransduction systems of vision may have emerged before the divergence of animals into vertebrates and invertebrates in the course of evolution. It is likely that, for some time following this divergence, both vertebrates and invertebrates retained each of the multiple phototransduction systems. In fact, recent findings of the Gq-rhodopsin type of visual pigment in photosensitive dermal melanophores of *Xenopus laevis* supported this [51]. This hypothesis could be further extended to incorporate divergence of molecules involved in signal transduction before the formation of signal transduction cascades in the course of molecular evolution [52].

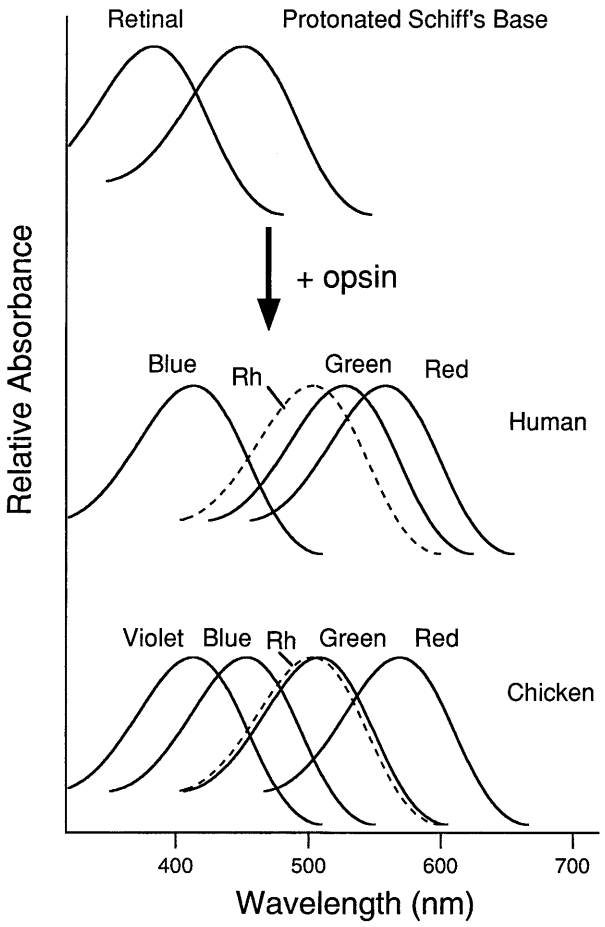

Figure 5. Absorption spectra of model chromophores and visual pigments. Retinal and its protonated Schiff base in organic solution exhibit broad absorption spectra having maxima at ~380 nm and ~440 nm, respectively. On the other hand, the spectra of visual pigments are spread over a wide wavelength and range from ~360 nm to ~630 nm, which reflects the specific interaction of 11-*cis*-retinal or its derivatives with nearby amino acid residues. Humans have three types of cone pigments, human blue, green and red, and a single type of rod pigment, rhodopsin (– – –), whereas chickens have four types of cone pigments, chicken violet, blue, green and red, in addition to the rod pigment, rhodopsin.

Mechanisms of spectral tuning in visual pigments

So far, the absorption spectra of a large number of visual pigments have been determined, and these spectra spread over a wide wavelength range from ~360 nm to ~630 nm (fig. 5). Because all of the visual pigments investigated so far contain 11-*cis*-retinal or its derivatives, the spectral shift would reflect the specific interaction of 11-*cis*-retinal with nearby amino acid residues. As already described, 11-*cis*-retinal binds to the lysine residue in visual pigments through a protonated Schiff base linkage. Thus the mechanism leading to the difference in absorption maximum between a protonated Schiff base in organic solution (about 440 nm) and that in visual pigments (called an 'opsin shift' [53]) has been investigated. On the basis of theoretical calculation and retinal analog studies, three factors which affect the absorption spectrum of vertebrate visual pigments have been implicated [54]: (i) twisting the polyene chain of the chromophore due to steric interaction between the chromophore and bulky amino acid residues, (ii) electrostatic interaction between the chromophore and nearby charged, polarized or polarizable amino acid residues, (iii) distance between the protonated Schiff base and its counterion. While the third mechanism of spectral tuning was clearly demonstrated by retinal analog studies [55] and resonance Raman experiments on bovine rhodopsin [56], the second mechanism should await confirmation until site-directed mutagenesis experiments are performed (fig. 6).

The second mechanism of spectral tuning was recently demonstrated by comparative studies of visual pigments belonging to the L group. First, Yokoyama and co-workers proposed the idea that three hydroxyl-bearing amino acid residues (180, 277 and 285 in the human red numbering system) are responsible for the difference in the maximum between fish red- and green-sensitive pigments [57]. Second, Neitz and co-workers reached a similar conclusion regarding the spectra of L group cone pigments in monkeys [58]. Comprehensive studies using chimerical and site-directed mutagenesis in human red and green pigments by Oprian and co-workers then unambiguously identified all the amino acid residues responsible for the spectral shift from green to red pigments [59]. The interesting observation in these studies is that six of seven hydroxyl-bearing amino acid residues are responsible for the spectral red shift, and

	III		IV			V		VI			VII	
Group L	116	129	138	180	197	200	230	233	277	281	285	308	309
Chicken Red (571 nm)	S	E	I	S	H	K	F	A	Y	W	T	A	Y
Human Red (552 / 557 nm)	**S**	E	I	**A/S**	**H**	**K**	**I**	**A**	**Y**	W	**T**	**A**	**Y**
Human Green (530 nm)	**Y**	E	I	**A**	**H**	**K**	**T**	**S**	**F**	W	**A**	A	**F**
Mouse Green (508 nm)	Y	E	I	A	**Y**	K	F	S	Y	W	T	**S**	Y
Group Rh	100	113	122	164	181	184	214	217	261	265	269	292	293
Chicken Rhodopsin (503 nm)	N	E	**E**	A	E	Q	I	A	F	W	A	A	F
Human Rhodopsin (500 nm)	H	E	E	A	E	Q	I	I	F	W	A	A	F
Bovine Rhodopsin (498 nm)	H	**E**	**E**	**A**	E	Q	I	I	**F**	**W**	**A**	A	F
Group M2													
Chicken Green (508 nm)	N	E	**Q**	A	E	Q	I	V	F	W	A	A	**F**
Group M1													
Chicken Blue (455 nm)	Q	E	M	G	E	Q	V	A	F	W	T	S	V
Group S													
Human Blue (425 nm)	N	E	L	G	E	Q	V	S	F	Y	A	S	F
Chicken Violet (415 nm)	R	E	L	G	E	Q	V	S	F	Y	A	A	F

Figure 6. Amino acid residues responsible for the spectral tuning of visual pigment. Amino acid residues which change the absorption spectra of visual pigments confirmed by mutagenesis studies (bold) [32, 39, 59, 149–151] are listed. These residues are numbered by human red (upper) and bovine rhodopsin (lower) numbering systems.

one is responsible for the blue shift. Thus a hydroxyl-bearing group is responsible for either the red or blue shift of the maximum. Recent experiments on mouse green pigment clearly showed that the serine at position 292 caused a blue shift of the maximum [60].

In addition to the above mechanisms, those other than the origin of amino acid residues have been demonstrated. A simple mechanism involves exchanging a retinal chromophore with a related compound having different absorption characteristics. In fact, some amphibians, reptiles and fish have a visual pigment containing 3-dehydro-retinal which has an extra double bond in its conjugated system compared with that of retinal and causes a shift of the maximum to the red [61]. The visual pigment with the greatest degree of red shift ($\lambda_{max} = 630$ nm) identified so far is a 3-dehydro-retinal-containing pigment in fish retina [62, 63]. The other is the binding of chloride observed only in the L group of visual pigments [63–67]. For example, iodopsin, the chicken red-sensitive cone visual pigment, has an absorption maximum at 571 nm which shifts to 530 nm when chloride is removed from the binding site [66, 67]. The binding site of chloride due to the prominent shift of the maximum was originally proposed in the transmembrane region of iodopsin [68], but subsequent mutagenesis experiments on human red and green pigments indicated that chloride binds to H197 (in bovine rhodopsin, E181) and K200 (Q184) residues in the second extracellular loop [69]. Thus the mechanism of the red shift by chloride has been extensively investigated. Accumulated evidence has now revealed that the binding of chloride stabilizes the protein moiety in iodopsin [66, 67], regulates the thermal reactions of intermediates [67, 70] and changes the conformation of the specific site of the chromophore (T. Hirano et al., unpublished), all of which strongly suggests the presence of chloride near the retinal chromophore in the transmembrane region. Thus, it is possible that the second extracellular loop intrudes into the helix region so as to position the chloride binding site near the chromophore. This speculation appears to be supported by the visualization of an electron density region other than helices in the low resolution of the electron density map of frog rhodopsin [41] and the presence of a specific disulfide bond between C110 and C187 which connects between the third helix and the center of the second extracellular loop [69].

Molecular events occurring in the photobleaching process of visual pigments

Electrophysiological studies have demonstrated that rod photoreceptor cells are able to respond to a single photon stimulus, although they exhibit an extremely low level of noise in the dark [3]. At a visual pigment level, these properties originate from the high photosensitivity of rhodopsin, its effective activation of G-protein (transducin) and its inert character in the dark. Thus, the molecular events that occur in photoactivated rhodopsin have been extensively investigated.

After the discovery of cis-trans isomerization of the chromophore as an initial event in photoactivated rhodopsin [13], many attempts were made to elucidate how efficiently the isomerization occurs. The pioneering studies by Dartnall showed that the quantum yield of isomerization in visual pigment is extraordinarily high (0.67) [71] compared with that of the protonated retinylidene Schiff base in organic solution (~ 0.2) [72]. In general, a molecule converts to an electronically excited state when it absorbs a photon and returns to a ground state through a variety of relaxation processes. Because isomerization of the chromophore can be used in rhodopsin as a trapping mechanism of a light signal, it should occur as fast as other processes such as fluorescence, internal conversion and intersystem crossing followed by phosphorescence or internal conversion, all of which cause disappearance of the light signal. Ultrafast spectroscopy using femtosecond laser pulses clearly shows that the isomerization is completed within 200 fs, which is one of the fastest chemical reactions known so far [73, 74]. Theoretical calculation with the aid of absorption spectroscopy then revealed that the isomerization starts at about 60 fs after the photon absorption [75]. Thus, the isomerization occurs as fast as the vibrational motions of the chromophore, which cause the coherent production of the primary intermediate, photorhodopsin [76, 77].

Photorhodopsin then dissociates into all-*trans*-retinal and apoprotein opsin through several thermolabile intermediate states [78]. During this process, conformational changes in opsin as well as changes in interaction between the chromophore and opsin occur. The chromophore acts as an intrinsic spectroscopic probe to monitor the protein structural changes so that each step in the changes has been identified as an intermediate state having a specific absorption spectrum. Historically, low-temperature spectroscopy showed a great advantage in surveying the overall bleaching process of rhodopsin [79], and several distinctive intermediates, such as bathorhodopsin [80, 81], lumirhodopsin [82] and metarhodopsin I–III [83], were identified. Recent kinetic experiments using laser photolysis at room temperature and time-resolved low-temperature spectroscopy showed that other intermediates exist in between the originally identified intermediates [84–86]. Thus the bleaching process of rhodopsin could be summarized in figure 7.

The molecular events that occur in the bleaching process have been investigated by detailed analyses of the

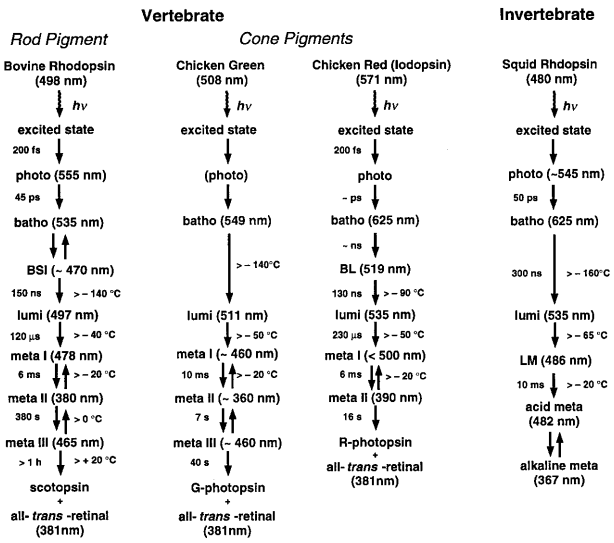

Figure 7. Photobleaching processes of various types of visual pigments. Vertebrate rods [79] and cones [101, 103] and invertebrate [78] visual pigments convert similar intermediates at the early stage of the bleaching process, whereas they convert different meta-intermediates. The absorption maxima of the intermediates are shown in parentheses. Time constants of the transitions between intermediates observed at room temperature and transition temperatures are shown on the left and right sides of the arrows, respectively.

intermediate states. The most important concept regarding the biophysical meaning of cis-trans isomerization was derived from the studies on bathorhodopsin. A calorimetric study showed that about 60% of photon energy ($\sim$30 kcal) is stored as an increase in enthalpy [87] mainly due to the distortion of the chromophore in the restricted chromophore binding site [81, 88]. Thus the primary role of chromophore isomerization is the transformation of the photon energy into chemical free energy, which changes the conformation of the protein moiety into its active state (fig. 8). The extremely inert character of rhodopsin in the dark is also explained by the large activation energy necessary for formation of the active state.

The formation of bathorhodopsin after photon absorption of rhodopsin is extremely rapid (45 ps) [76]; thus, it is easy to believe that only minor rearrangement of the amino acid residues constituting the chromophore binding site could occur. On the other hand, a relatively larger change in chromophore-opsin interaction could occur during the batho-lumi transition via BL(BSI)*-intermediate and the lumi-meta I transition. Accumulated evidence has now revealed that the cyclohexenyl ring portion (β-ionone ring) of the chromophore changes its interaction with nearby amino acid residues during the batho-lumi transition [89], and the interaction between the 9-methyl group of the chromophore and the surrounding protein then disappears in the lumi-meta I transition [90]. These changes in interaction are confirmed to be essential for G-protein activation since removal of the β-ionone ring or the 9-methyl group of retinal results in a decrease in activation of G-protein by rhodopsin [91, 92]. Furthermore, replacement of W265 and G121, which are situated near the β-ionone ring and the 9-methyl group of the chromophore, respectively, affect the conversion process of batho to meta I transition via BL(BSI) and lumi intermediates [93]. In addition to these structural changes, many protein structural changes including water molecules have been suggested by various spectroscopies [94].

Thermodynamic study showed that the transition of lumi to meta I occurs with a decrease in enthalpy and entropy, while that of meta I to meta II occurs with an increase in enthalpy and entropy [95]. These results strongly suggest that the molecular events occurring in the lumi to meta I transition are opposite in nature to those in the meta I to meta II transition. That is, the former transition results in the formation of the specific interaction(s), whereas the latter transition results in the formation of a flexible state at the expense of a loss of interaction(s). Therefore, meta I is in a state in which a thermodynamically stable conformation in the re-

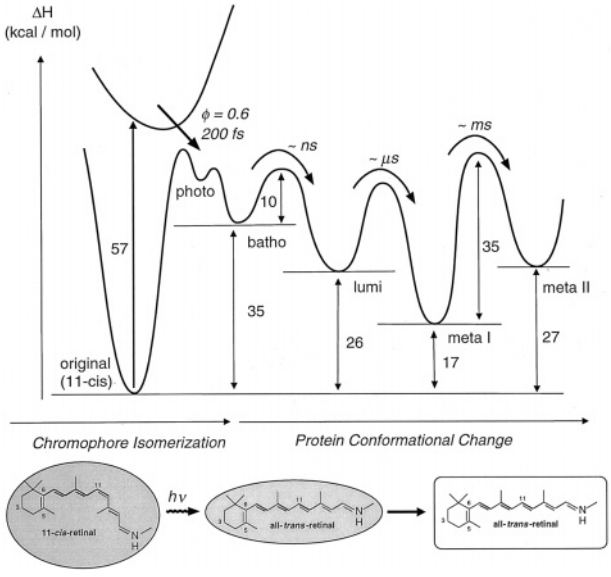

Figure 8. Schematic drawing of the state energy diagram of bovine rhodopsin and its intermediates. The enthalpies and activation enthalpies of rhodopsin and its intermediates are estimated by calorimetric and reaction kinetic experiments [87, 95, 152, 153].

* BL = intermediate between Batho and Lumi; BSI = Blue Shifted Intermediate.

stricted region near the chromophore is established, and the subsequent change to meta II is due to the relaxation of the whole protein into a thermodynamically stable state. In this sense, meta I may be in a state analogous to a precursor of the active state in other ligand-binding receptors.

The most prominent difference between meta I and meta II is their protonation states of the Schiff base chromophore. That is, meta II is the only intermediate whose retinylidene chromophore is deprotonated. Earlier studies indicated that a pH-dependent equilibrium exists between meta I and meta II [83]. However, this equilibrium is not simple, because elevation of the pH caused a shift in equilibrium to meta I, suggesting that deprotonation of the Schiff base in meta II caused incorporation of at least two protons into the protein moiety. The pK value of the pH-dependent reaction is 6.4; thus, a histidine residue was thought to affect the reaction [83]. In fact, a site-directed mutagenesis study clearly showed that H211 affects the reaction [96]. Furthermore, E113, the counterion of the protonated Schiff base, was protonated during the meta I to meta II transition [97]. Because a large conformational change occurs in the meta I to meta II transition, environmental changes in many residues such as D83, D122 and W126 were observed by various spectroscopies [40, 98]. Meta II decomposes into all-*trans*-retinal and opsin via meta III in which the Schiff base is protonated. A pH-dependent equilibrium also exists between meta II and meta III [99].

Comparative studies of cone visual pigments with rhodopsin indicated that photochemical and subsequent thermal reactions of cone visual pigments are basically similar to those of rhodopsin (fig. 8). Like rhodopsin, the cone visual pigments investigated so far have a quantum yield and extinction coefficient similar to that of rhodopsin independent of the absorption maximum [48, 100], suggesting that the difference in photosensitivities between rod and cone photoreceptor cells does not originate from the ability of visual pigment to absorb photons. The most prominent difference is the thermal stabilities of the intermediates, that is, cone visual pigments have intermediates less stable than the corresponding intermediates of rhodopsin [48, 101]. Especially, less stable properties of meta II intermediates of cone visual pigments are important because meta II is the state of visual pigments that activates transducin [102, 103]. The photosensitivities of the photoreceptor cells depend on how many transducins are activated by meta II; thus faster decay of meta II of cone visual pigments may cause less activation of transducin, resulting in lower photosensitivity of cones than rods.

Recently, we found that replacement of the amino acid residue positioned at 122 dramatically changes thermal stability of the meta II intermediate [39]. The residue E122 is highly conserved in vertebrate rhodopsins but is replaced by neutral residues in cone pigments. Replacement of E122 of rhodopsin by the residue containing chicken green- or red-sensitive cone pigment converts the meta II decay rate of rhodopsin into those of the respective cone pigments. Exchange of the residue at position 122 between rhodopsin and chicken green-sensitive cone pigment interconverts their activation efficiency to transducin. It should be noted that the regeneration rates of rhodopsin and cone visual pigments from 11-*cis*-retinal and opsin were also exchangeable by mutation of the residues at position 122. Thus, the amino acid residue at position 122 is one of the determinants of the functional differences between rod and cone visual pigments.

The bleaching process of invertebrate rhodopsin is somewhat different from that of vertebrate rhodopsin (fig. 8). Like vertebrate rhodopsin, invertebrate rhodopsin converts to the early intermediates, photo-, batho- and lumirhodopsins, whose spectroscopic characteristics are similar to those from vertebrate rhodopsin [104–106]. However, subsequent intermediates, called LM(meso)-rhodopsin and acid metarhodopsin, exhibit characteristics different from those of meta-intermediates of vertebrate rhodopsin. Unlike metarhodopsin I, LM-rhodopsin has no circular dichroism (CD) in the visible region [106], suggesting that the chiral cavity of the retinal binding site present in both rhodopsins disappears in LM-rhodopsin but not in metarhodopsin I. Acid metarhodopsin is stable even at room temperature, which causes the unbleached characteristics of invertebrate rhodopsin [107]. Furthermore, a meta II-like intermediate having a deprotonated Schiff base is not produced from invertebrate rhodopsin under neutral conditions, although alkalization of acid metarhodopsin produces an artificial intermediate state, alkaline metarhodopsin [107]. These differences may reflect the absence of a counterion in the chromophore binding site of invertebrate rhodopsin. The important point is that the G-protein is activated under neutral conditions [8, 108] where alkaline metarhodopsin hardly exists. The fact suggest that deprotonation of the Schiff base is not necessary for the activation of the G-protein. These results might provide insight into the molecular events essential for the G-protein activation by visual pigment (see below).

Activation of G-protein

As already described, one of the main functions of visual pigments is to activate their specific G-proteins in a light-dependent manner. Several intermediate states of visual pigments have been identified so far; thus, the first step should be to determine which intermediate(s) binds to and activates the G-protein. Next, the interaction site(s) of visual pigments with G-proteins as well as the structural difference between inactive and active states of visual pigments should be elucidated.

The first implication that meta II could be an active state of rhodopsin came from the biochemical evidence that phosphodiesterase, the effector enzyme of the phototransduction cascade in vertebrate photoreceptor cells, was activated even at low temperatures where meta II did not convert to the subsequent intermediate meta III [109]. Spectroscopic measurements then indicated that a large amount of meta II was accumulated in the presence of transducin [110] and that the accumulation was abolished by GTP. The exchange reaction results in release of transducin from photoactivated rhodopsin [111]. Thus, the spectroscopic experiments clearly showed that meta II forms a complex with transducin and catalyzes the GDP-GTP exchange reaction in transducin. Recently, it was shown that an intermediate other than meta II also interacts with transducin [86, 112]. This intermediate is a precursor of meta II and was called meta Ib because it displays an absorption maximum similar to but about 20 nm blue-shifted from that of the previously identified meta I (now called meta Ia). The interesting observation is that meta Ib can bind to transducin but induces no GDP-GTP exchange reaction, whereas the exchange reaction occurs at the meta II stage. That is, transducin can form a complex with meta Ib, and the subsequent change in conformation of the complex reaches the state (transducin-meta II complex) that induces the exchange reaction. The binding of transducin to meta Ib causes stabilization of meta Ib, but the stabilization is not as prominent as that of meta II, resulting in accumulation of a large amount of meta II in the presence of transducin.

Which regions of the activated rhodopsin interact with transducin? Because rhodopsin and transducin are transmembrane and peripheral proteins, respectively, it is easy to speculate that the interaction sites of rhodopsin with transducin should be in the cytoplasmic loop regions. The competitive inhibition experiments using the peptides of the loop regions of rhodopsin clearly showed that the second and third cytoplasmic loops in addition to a putative fourth loop (310–321) and the carboxyl-terminal sequence emerging from helix 7 and anchoring to the lipid bilayer via palmitoylcyteines 322 and 323 are the candidates for interaction with transducin [113]. Subsequent experiments in combination with rhodopsin mutants have now identified the specific amino acid residues in the second and third loops as the interaction sites [42, 43, 114]. Furthermore, the exposure of the residues to the surface of photoactivated rhodopsin originates from enlargement of the distance between helices 3 and 6 due to the conformational changes in the helices region [42, 43].

Among the molecular events occurring in the bleaching process, the most important event is the cis-trans isomerization of the chromophore, because it is the origin of all the changes in protein. The changes in interaction of the β-ionone ring and the 9-methyl group with nearby proteins (probably W265, G121 and F261 in rhodopsin) are the subsequent events responsible for the transducin activation [34]. The occurrence of these events also indicates that production of a highly twisted all-trans chromophore but not a thermally relaxed one by photon absorption is essential for the conformational changes in the protein.

The proton on the Schiff base in rhodopsin mediates the interaction between the chromophore and a counterion E113; thus, its transfer to the counterion results in the loss of interaction, inducing a flexible conformation near the Schiff base. As a result, it has frequently been suggested that deprotonation of the Schiff base at the meta II stage plays a role in forming an active complex in which a GDP-GTP exchange reaction occurs. This was first examined by a chemically modified rhodopsin, in which the active-site lysine was methylated to inhibit deprotonation of the Schiff base [115, 116]. Oprian and co-workers then showed that replacement of the active-site lysine and the counterion by neutral residues caused the opsin to become constitutively active [19]. Therefore, it was speculated that electrostatic interaction between the protonated Schiff base and the counterion in rhodopsin or that between lysine and the counterion in opsin could keep the protein inactive and the active state could be formed concurrent with the loss of these interactions. Later studies using rhodopsin mutants in which a counterion was moved from the original position indicated that activation of transducin does not correlate with the Schiff base deprotonation [117]. Thus the minimum requirement for the activation of transducin is now thought to be the absence of charge at position 113 [118]. The advantage of this hypothesis is that it also meets the activation process of the G-protein by invertebrate visual pigments which have no charged residue at position 113 and do not convert an intermediate having a deprotonated Schiff base. However, even in the absence of the charged residue at this position, binding of 11-*cis*-retinal caused the protein to become inactive [22, 23]. Taken together, the activation mechanism of transducin by rhodopsin is believed to be as follows: Photoisomerization of the rhodopsin chromophore causes conformational changes in the amino acid residues in helices 3 and 6 situated near the β-ionone ring and the 9-methyl group of the chromophore, resulting in a tilt of helices 3 and 6 to give rise to a specific conformation of the cytoplasmic loop to interact with transducin. The changes in conformation of the rhodopsin-transducin complex including Schiff base deprotonation could then occur to accomplish a GDP-GTP exchange reaction.

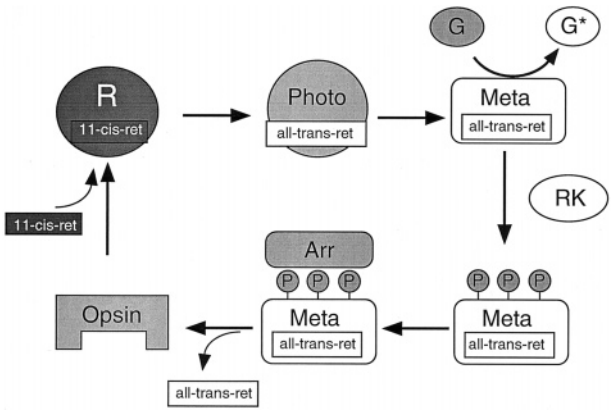

Figure 9. Schematic drawing of activation and inactivation processes of the visual pigments. The photoisomerized chromophore in the photorhodopsin state (photo) induces a conformational change in protein and leads to the formation of active states (meta). C-terminus serines in this state are then phosphorylated by rhodopsin kinase. Arrestin binds to the phosphorylation state to inhibit binding of transducin. After the dissociation of all-*trans*-retinal from opsin, 11-*cis*-retinal binds to opsin to regenerate the inactive dark state.

Inactivation and recovery processes of visual pigments

Electrophysiological studies demonstrated that the response of the retinal rod photoreceptor cells to a dim flash lasts about 1 s, whereas the intrinsic lifetime of meta II, one of the active states of rhodopsin, is the order of several minutes. These facts indicate the presence of a shutoff mechanism which regulates the active states of rhodopsin (fig. 9). In 1972 photoactivated rhodopsin was found to be phosphorylated [119, 120]. A newly identified 48-kDa protein [121], now called arrestin, bound to the phosphorylated intermediate and inhibited its binding to transducin [122]. The physiological significance of the phosphorylation of the active state and its binding to arrestin were confirmed by recording photoresponses from transgenic mice that have a rhodopsin whose phosphorylation sites (see below) were truncated [123] and that have no arrestin [124]. The phosphorylation is mediated by rhodopsin kinase which is a member of a G-protein-coupled receptor kinase family [125]. Although about 10 possible phosphorylation sites are present in rhodopsin, the sites phosphorylated under physiological conditions are now known to be S334, S338 and S343 near the C-terminus [126, 127]. The activity of rhodopsin kinase is regulated by one of the calcium-binding proteins, S-modulin (recoverin), which is one of the molecular bases of light adaptation [128].

Recently, a splice variant of arrestin (p44) has been found to bind to both phosphorylated and nonphosphorylated intermediates of rhodopsin with an affinity higher than that of the originally identified arrestin [129]. It also inhibits light-dependent photosignal transduction. The presence of receptor kinase [130] and arrestin [131] in cone photoreceptor cells suggests a similar shutoff mechanism in cone cells, although the intrinsic decay of meta II of cone pigments is more than 20 times faster than that of rhodopsin. Invertebrate photoreceptor cells also have a receptor kinase and an arrestin, but their characteristics are somewhat different from those in vertebrate photoreceptor cells. The arrestin is similar to the splice variant of vertebrate arrestin, because it binds to a nonphosphorylated metarhodopsin [132] and inhibits its ability to activate the G-protein in vivo. Invertebrate rhodopsin kinase has a homologous sequence known to be the binding site of $\beta\gamma$-subunits of G-protein in β-adrenergic receptor kinases [133], whereas vertebrate kinase has not. Thus, the shutoff system in invertebrates may be similar to those of the hormonal GPCR family.

The recovery process of rhodopsin from the phosphorylated intermediate has been extensively investigated by Hofman, Palczevsky and their co-workers [134]. Based on their scheme, the phosphorylated intermediate to which arrestin binds is first attacked by retinal dehydrogenase. This enzyme converts all-*trans*-retinal in the protein into all-*trans*-retinol, resulting in release of the chromophore from the protein. This reaction causes a release of arrestin from the protein. The protein is then dephosphorylated by phosphatase 2A, and regenerated by binding with newly synthesized 11-*cis*-retinal. The detailed regeneration mechanism of rhodopsin was also elucidated by Matsumoto and Yoshizawa [135], who demonstrated that regeneration occurs by sequential reactions: the β-ionone ring of the 11-*cis*-retinal first binds to the hydrophobic region of the chromophore binding site, and then the Schiff base linkage between the aldehyde group of 11-*cis*-retinal and the amino group of K296 forms. The rate of regeneration of cone visual pigment is more than 200 times faster than that of rhodopsin [44, 48]; thus, it is thought to be one of the molecular mechanisms of dark adaptation. Recently, we found that the amino acid residue at position 122 regulates the regeneration rate of rod and cone visual pigments. That is, the mutation of E122 of rhodopsin to the corresponding residues of chicken red (I) and green (Q) accelerated the regeneration rate by tens of times [39]. Because all of the rhodopsins investigated so far have a glutamate at position 122, how the residue controls the reaction is of interest. The fact that replacement of glutamate by aspartate caused no acceleration of the regeneration rate clearly showed the importance of the carboxyl group. Thus the regeneration rate be-

comes slower when a carboxyl group is present at position 122, although there might be other residues which regulate the regeneration rate of visual pigment [136]. It should be noted that this kind of mutation is categorized by a change in function, whereas loss-of-function mutants are found in the pigment genes of patients with retinitis pigmentosa (see later). Thus, various types of visual pigments would have evolved out by a change-in-function mutation in the course of animal evolution.

Pigment dysfunction in patients with retinitis pigmentosa

Since the discovery of point mutation in the rhodopsin gene of patients with retinitis pigmentosa [137], over 50 natural mutations have been reported in the genes of patients with genetic retinal diseases [138]. The mutations can be classified into at least three categories based on expression studies using cultured cells [139, 140]. Two of these categories affect pigment regeneration and/or transfer of rhodopsin to the proper sites of photoreceptor cells. It is reasonable to assume that these mutations cause a dysfunction in visual pigment because they would not be able to receive a light signal and/or activate the G-protein properly. Some of the mutations are indeed demonstrated to cause retinal degradation in transgenic mice [141, 142]. The mutations of the amino acid residues that are essential for the chromophore binding and stabilization, G-protein activation, and phosphorylation are also included in these categories. Mutation of the residue (R135) was reported to affect the activation of transducin and/or phosphorylation by rhodopsin kinase [19, 139, 140, 143]. An interesting observation was obtained by the mutation of active-site lysine (K296), by which constitutive activation of transducin was observed in vitro [19]. This is a phenomenon equal to the continuous activation of rhodopsin by light; thus it was hypothesized that continuous activation would cause retinal degradation by a mechanism similar to that in degradation under continuous lights [19]. However, the mutation in K296 did not exhibit constitutive activation in transgenic mice due to the inhibition by constitutive phosphorylation and arrestin binding, although retinal degradation was demonstrated [143].

In addition to the mutations found in patients with retinitis pigmentosa, mutations that disturb suitable arrangements of the chromophore and functional amino acid residues in the protein moiety of visual pigment have been reported. Mutations of the amino acid residues near the ion pair between K296 and E113 [144] that also caused constitutive activation of transducin were found in the patients with congenital night blindness. Detailed analysis of these mutations would further our understanding of the mechanism of intramolecular signal transduction in visual pigments.

Concluding remarks

Since its discovery in the 1870s [145, 146] visual pigment has been extensively studied from various standpoints. The early stage of investigations focused on elucidating its photoreceptive mechanism [13]. Nowadays, visual pigment has been confirmed as a prototypical G-protein-coupled receptor, and over the last several years a remarkable amount of information on the primary sequences of visual pigments and their structure-function relationships has been obtained using the techniques of molecular biology. Furthermore, comprehensive data regarding the structural changes in visual pigment that occur following photoreception were frequently referred to when inferring intermolecular changes in the other G-protein-coupled receptors [36]. Thus, visual pigment studies may be entering a new era. In addition to recent progress in the analysis of the three-dimensional structure of rhodopsin, visual pigments as photoactivatable receptors are generally relevant to understanding the molecular mechanisms of signal transduction by G-protein-coupled receptors.

Acknowledgments. We would like to thank Dr. H. Kandori, A. Terakita and S. Tachibanaki for valuable discussions. We would also like to extend our sincere thanks to Emeritus Professor T. Yoshizawa for his continuous encouragement. This work was supported in part by Grants-in-Aid for Scientific and Cooperative Research from the Japanese Ministry of Education, Science, Sports and Culture. H.I. is supported by JSPS Fellowships for Japanese Junior Scientists and Research Aid from the Inoue Foundation for Science.

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