Phodopsins containing 6- to 9-membered rings. The triggering process of visual transduction

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Abstract: A major unsettled problem in the chemistry of vision is clarification of the triggering mechanism that initiates conformational changes in the rhodopsin structure leading to visual transduction. Although it is established that a cis to trans photoisomerization of its chromophore, the protonated Schiff base of 11-cis-retinal, occurs, the more subtle aspects of the mechanism in which photon absorption leads to conformational changes are unclear. Theoretical models propose that charge redistribution or bond polarization in the excited state triggers the conformational changes in rhodopsin leading to enzymatic activation. However, experimentally it remained unknown whether or not excited state polarization is involved at all in the visual transduction process and if so to what extent. In order to clarify this crucial but elusive problem, retinal analogs with 8- and 9-membered rings in the side-chain have been synthesized and incorporated into rhodopsin. Flash photolysis, binding, enzymatic assays and other studies of several such rhodopsin analogs and dihydrorhodopsins have shown that excited state polarization is involved but a complete cis-trans isomerization of the full rigid polyene system is required.

INTRODUCTION

The cross section of the human eye is shown in Fig. 1a. The retina contains 100 million rod cells responsible for scotopic (black/white) vision, whereas the fovea, the area in which images are focused most sharply, contains 3 million cone cells responsible for photopic (color) vision. Each cell contains 500–2000 disks (Fig. 1b), whereas each disk contains 30 million rhodopsin (Rh) molecules; the rhodopsin photoreceptor molecules go through the lipid bilayer of the disks (Fig. 1c). The disks are formed at the stem of the rod and cone outer segments, move towards the tip in about 15 days, after which they are consumed by phagocytes. The rod disks contain Rh absorbing at 500 nm, while the human cone disks contain three kinds of Rhs absorbing maximally in the blue (440 nm), green (530 nm) and red (580 nm); since solar energy is strongest in the 400-700 nm region (“visible” spectral region) humans are taking maximum advantage of light. The kinds of Rh differ from organism to organism: horses, dogs and owls only have rod cells and hence lack color vision, whereas chicken (ref. 1), gold fish (ref. 2), gecko (ref. 3), for example, have multiple Rh species and hence should have superb color vision.

The ratio of rod to cone cells depends on the environment. It is 4000:1 in nocturnal rats, 20:1 in humans, 15:1 in goldfish and 1:1 in frogs. Rhs are insoluble 40 kDa membrane glycoproteins. The primary sequence of bovine Rh consisting of 348 amino acids was determined independently by groups headed by P. Hargrave and the late Y. Ovchinikov in 1982-3 (ref. 4). Site specific mutation pioneered by Khorana for Rh is now a technique used commonly to obtain unnatural Rhs (ref. 5). Rhs are one of the most extensively studied G-protein (transducin) coupled membrane proteins (ref. 6) consisting of 7 transmembrane α-helices, the N and C terminals residing, respectively, in the intradiscal and cytoplasmic sides; the peptidic chain outside the membranes is hydrophilic. The tertiary structure of Rh is unclear since despite the efforts of numerous groups, satisfactory crystals for X-ray analysis have not yet been secured. The sequences of human rod and cone pigments were determined by J. Nathan in 1986-7 (ref. 7); many other Rhs have now been sequenced also by gene cloning techniques.
CHROMOPHORIC CHANGES ACCOMPANYING VISUAL TRANSDUCTION

In most cases the chromophore of Rh is 11-cis-retinal which is linked to a Lys amino group via a protonated Schiff base (PSB); in bovine Rh it is bound to Lys-296. Other chromophoric variations are 3,4-dehydroretinal (salmon, eel, tropical fish, tadpole, salamander), 3-hydroxyretinal (insects) and 4-hydroxyretinal (squid); some organisms have only one kind of retinal whereas others have more than one kind. The brilliant studies led by G. Wald and others established in the late 1960s that irradiation of bovine Rh isomerizes the 11-cis-retinal chromophore to all-trans. For many years, the primary photoproduct was thought to be batho-Rh, λmax 543 nm, discovered by Yoshizawa and Kito in 1958 (ref. 8)(Fig. 2). Since batho-Rh can be sequestered at -140°C it can be subjected to various spectroscopic measurements, which have shown it to possess a distorted all-trans geometry as shown by CD (ref. 9), resonance laser Raman (ref. 10) and Fourier transform IR (ref. 11).

However, about 10 years ago Shichida et al. observed that excitation of cattle Rh with a weak laser pulse by which only one-photon excitation occurred gave rise to an earlier photo-product, photo-Rh, λmax 570 nm, in <3 ps (ref. 12); more recently, the formation of photo-Rh has been found to occur in <200 fs (ref. 13). Batho-Rh is thus the thermally relaxed product of photo-Rh rather than being the primary photoproduct. Clearly photo-Rh must be more highly distorted than batho-Rh. Unlike the batho species, however, it has not yet been possible to quench photo-Rh at low temperatures and hence no other spectral data are available.

The protein continues its thermal relaxation after batho-Rh to give further intermediates, BSI (blue-shifted intermediate, ref. 14) → lumirh → meta-Rh I, in the room temperature time scales shown in Fig. 2; each intermediate can be quenched for spectroscopic measurements at temperatures shown in the Figure. After meta-I, the PSB loses its proton to become an unprotonated Schiff base in Meta-Rh II (hence, the blue shift from 480 nm to 380 nm); however, overall, the protein gains a proton in the transformation of meta-I to meta-II. Meta-II is the critical intermediate, for it is at this stage that the conformation of the Rh molecule becomes suited for the transducin and Rh kinase molecules floating in the extradiscal cytoplasmic side to become activated (Fig. 3)(refs. 4, 5, 6). The activated transducin in turn activates phosphodiesterase (PDE) which hydrolyzes cyclic GMP to GMP. One photon activates 100 molecules of PDE, which then hydrolyze 10^5 molecules of cGMP. This process is called the enzymatic cascade. Since one molecule of meta-II reacts with 100 molecules of transducin, it has to come in contact with this number of transducin, and therefore it should be quite fluid. It is estimated that a Rh molecule rotates in 2x10^-5 sec, and that the fluidity of the membrane is comparable to that of castor oil. By the time that 10^5 molecules of cGMP are hydrolyzed, the Rh kinase phosphorylates the nine Ser and Thr hydroxyl groups on the C terminus and Rh is inactivated (Fig. 3). When cGMP is present in the outer segments (Fig. 1), the sodium gates in the outer segment are kept open and a constant flow of sodium from the inner to the outer segment is maintained. However, hydrolysis of cGMP caused by Rh irradiation closes the sodium gates, and this blockage of sodium ions through the plasma membrane leads to hyperpolarization, which is picked up by the visual nerves, leading to vision. At meta-II, the all-trans-retinal chromophore is expelled from the apoprotein opsin. Since the originally orange-colored Rh becomes pale yellow at this stage, the process in Fig. 2 is called bleaching. The all-trans retinal is then reduced to retinol (vitamin A), esterified, isomerized to 11-cis-retinol through the help of an isomerohydrolase, oxidized to 11-cis-retinal and recombines with opsin to generate Rh (ref. 15). However, details of these crucial steps such as the mechanism of isomerization and turnover rate of vitamin A are still unknown.
Despite clarification of the intermediates leading to visual transduction, a key question that remains to be solved is the mechanistic aspects leading to visual transduction. There is no doubt that an 11-cis to all-trans isomerization has to occur. Thus the Rh analog Rh7 (see Fig. 6 below) was readily formed from the retinal analog ret7 in which the 11-ene is locked into the cis geometry via a side-chain 7-membered ring (Fig. 5)(ref. 16). As expected the nonplanar conformation of the 7-membered ring simulates that of the native chromophore in the binding site as judged from the UV/vis and CD spectra which were similar to those of native Rh. Rh7 was totally stable to light, and was enzymatically inactive, both in vivo (ref. 17) and in vitro (ref. 18), thus showing that 11-cis → trans isomerization is a prerequisite for visual transduction.

Theoretical models suggesting that polarization or charge redistribution occurring in the excited state triggers transduction have been proposed (Fig. 4). The original sudden polarization model (ref. 19) is based on ab initio calculations of the pentadienyl PSB model and originates in the rationalization forwarded by Dauben and coworkers to account for the experimental photocyclization of trans-3-ethylideneoctamethine to a bicyclobutane moiety (ref. 20). It states that charge separation of the 11-ene in excited state pentadienyl PSB leads to the flow of the negative charge towards the positively charged N terminal where they cancel each other; the positive charge is distributed from C-7 to C-11 with a concomitant orthogonal twist of the C-11/C-12 torsional angle. Calculations of charge separation as a function of the torsional twist showed that polarization is negligible at low angles, suddenly develops at 89°, reaches a maximum at 90° and disappears at 91° (hence, sudden polarization). The change in dipole moment from the ground to excited state, calculated to be 33 Debyes, leads to visual transduction. More recent calculations suggest that polarization occurs over a wider range of angle distortion, ranging from 40° to 130° (ref. 21). The charge redistribution models propose that the direct or indirect electrostatic interaction of the excited state dipole moment triggers the protein conformational changes leading to transduction. In fact, determinations of dipole moments in the excited states of all-trans-retinal butylamine PSB (ref. 22a) and bacteriorhodopsin (ref. 22b)
led to changes in dipole moments of 12 D and 13.5 D, respectively. Also, the pKa of 11-cis and all-
trans-retinal PSB in the ground state is ca. 7, while that in the excited state (ref. 23) is ca. 16.6,
indicating an increase in basicity. However, it remained unknown to what extent such excited state
polarization is actually involved in the visual transduction process.

FLASH PHOTOLYSIS OF NATIVE Rh AND Rh5-8 ANALOGS
Pico- and nanosecond flash photolyses of native Rh and Rh analogs regenerated from retinals with fixed
11,12-ene geometry through 5, 6, 7 and 8 membered rings, ret5-8 (Fig. 5, 1-4), have led to
interesting results directly associated with the ring flexibilities (ref. 24). These results together
with enzymatic assays, especially those performed with Rh8 (Rh with ret8-derived chromophore)
have enabled us to clarify the crucial requirements involved in visual transduction.

As mentioned above, the nonbleachable Rh7 or the Rh analog prepared from ret7 (Fig. 5, 3) was stable
to light (ref. 16); however, as discussed below (Fig. 6) it did give rise to a transient photoproduct
although it could not be quenched at low temperatures (ref. 25). Ret8 and ret9 (Fig. 5, 4 and 5) were
synthesized because it was conceived that the increased ring flexibility over ret7 might lead to a
photoproduct with a distorted all-trans structure corresponding to batho-Rh that could be quenched at
a temperature higher than -140°C (Fig. 2) thus facilitating spectroscopic measurements. Although
this was not the case, the eventual incorporation of ret8 into Rh (ref. 27) led to a clearer definition of
the crucial factors involved in the triggering process of visual transduction (ref. 28). The syntheses
of ret8 and ret9 were achieved in the early 1980s but were not published until recently (ref. 26)
because of numerous difficulties in binding them to opsin; despite repeated efforts we have still not
managed to regenerate the Rh9 analog.

Laser flash photolysis of native Rh and Rh5, 7 and 8 were performed with pigments solubilized in 1-
2% digitonin; the results are summarized in Fig. 6, which schematically depicts the ground- and
excited-state potential surfaces along the 11-ene torsional coordinates (refs. 24, 25). The excited
state of Rh gives rise to the two ground state species, the primary photoproduct photo-Rh (ref. 12)
within 200 fs (ref. 13) with a high quantum yield of 0.67, and the initial Rh. Since the Rh
chromophore is fixed at both ends via the β-ring binding site (ref. 29) and the lysine residue (ref.
30) the photon energy is absorbed by the flexible middle portion centered around the cis-11-ene to
isomerize it to the transoid configuration. The protein cannot change its conformation in the short time
scale of 200 fs, thus giving rise to a highly distorted 11-ene. The protein and the 11-ene conformations thermally relax in 6 ps to yield batho-Rh; this thermal relaxation continues to give subsequent intermediates shown in Fig. 2, terminating in meta-II in which the conformation of the cytoplasmic loops activates the enzymes (Fig. 3). In Rh5, the rigid five-membered ring does not allow the 11-ene to isomerize so that excitation led only to an excited singlet state which decays to the ground state by emitting fluorescence, i.e., only vertical photophysical changes, and no conformational/configurational photochemical changes occur (ref. 25). Fukada et al. made the interesting observation that although Rh5 is nonbleachable, prolonged irradiation for 60 min at 0 °C shifted the 495 maximum to 466 nm, yet upon re-extraction of the chromophore the starting ret5 was recovered; they attributed this to a photochemical isomerization of the delocalized C-8/C-9 “single” bond (ref. 31).

The UV/vis and CD spectra of Rh7 are similar to those of native Rh; thus the chromophoric binding site in Rh7 closely resembles that of native Rh, i.e., both ends of the chromophore are fixed in the binding site. Picosecond flash photolysis showed that the nonbleachable Rh7 absorbing at 490 nm yielded in 7 ps a pigment with a maximum at 580 nm (P580) corresponding to photo-Rh; this decayed to the original Rh7 in 44 ns (ref. 24). Since it has been shown that a one-bond cis-trans photoisomerization occurs in 1,3-cyclooctadiene (ref. 32) and 1,3-cycloheptadiene (ref. 33), both with four annular sp² carbons, it is reasonable to assume that irradiation causes the relatively flexible central 11-ene in the cycloheptatrienylidene moiety to photoisomerize to the trans configuration (Fig. 6). However, the quantum yield of P580 formation was only 7% of that of native photo-Rh, the low value reflecting the preferred tendency of excited state Rh7 to relax to the original cis form, due to the high distortion of transoid cycloheptatrienylidene in photo-Rh7.

In the case of Rh8 with increased flexibility in the chromophore, the highly distorted photo-Rh-like intermediate P585 can relax one step further to a batho-like intermediate P577 in 1 ns before returning to the original ground state Rh8 (ref. 24). Importantly, the quantum yield of P585 formation is high and similar to that of native Rh. We can therefore speculate that formation of early intermediates is dependent on the flexibility of the 11-ene containing ring, and that photoisomerization is followed by stepwise relaxation of the opsin containing the highly strained 11-ene. In native Rh the change, leading to Meta-II, is irreversible; in ring analogs it is not. The crucial aspect to note from these flash photolysis experiments is that since irradiation of Rh7 and Rh8 affords photo-Rh, in quantum yields of ca. 13 % and 100 %, respectively, of that of native Rh, it can be concluded that charge redistribution or “sudden polarization” has occurred in the excited state. Then is Rh8 enzymatically active, i.e., functional?

P425 AND PREPARATION OF MeRh8

The solutions of Rh8 in the detergent digitonin used for the flash photolysis experiments cannot be used to answer the crucial question pertaining to the enzymatic activity of Rh8. In contrast to flash photolysis measurements, the pigment has to be embedded properly in lipid bilayer membranes for the formation of Meta-II to be able to activate transducin and rhodopsin kinase. Insurmountable difficulties were encountered in both preparing Rh8 in reconstituted vesicles or in reconstituting Rh8 into vesicles. This was due to the bulkiness and instability of ret8 (Fig. 5, 4) during incubation, and formation of a pigment absorbing at 425 nm (P425) which was found to be totally unrelated to rhodopsin (ref. 27). Phosphatidylethanolamine and phosphatidylserine, which constitute 51-57% of the total phospholipids in rhodopsin disk membrane are aligned in the lipid bilayer so that the free amino groups are directed toward the aqueous phase. When bleached ROS is suspended in phospholipids, these peripheral amino groups function as an “amino wall” and block entrance of the bulky ret8 into the binding site. This leads to binding of ret8 with random amino groups such as those stemming from: phosphatidyethanolamine and phosphatidylserine in disk membrane, the numerous Lys residues of the water-soluble transducin, PDE, and kinase, the nine Lys in the cytoplasmic carboxyl terminal of opsin, etc. Straightforward evidence for the nonspecific origin of P425 was its formation upon incubation of ret8 with a crude enzyme extract from the rod outer segment (ref. 27) or with heat-denatured opsin (ref. 24). When incubated with opsin, P425 quickly formed in 3 hours; was bleached completely upon 10 min irradiation with >420 nm light, and then underwent regrowth after standing in the dark for 100 min; such a peculiar “regeneration” phenomenon was totally misleading at the outset and led us to believe that P425 was a genuine visual pigment for two years.

The problem of making a Rh8 pigment suited for functional assays was finally solved by using methylated Rh (MeRh) in which the exposed free amino groups of Rh and the lipid are methylated while leaving only the crucial Lys-296 within the helix unmethylated (refs. 34, 35). As shown in Fig. 7a, incubation of 11-cis-ret8 with Me-opsin led to the disappearance of the 290/370 nm retinal peaks with concomitant formation of the genuine 502 nm MeRh8 peak, accompanied by an isosbestic point. The UV/vis and CD spectra (Figs. 7b, 7c) are comparable to those of native Rh, thus showing that the chromophore is embedded properly in the binding site. The aldehyde ret8 has two intense absorption
bands at 276 and 346 nm (in hexane) whereas 11-cis-retinal has only one peak at 365 nm; thus the two aldehydes have different conformations when free, but within the opsin binding site the chromophoric conformations become similar due to the protein. In Rh6 and Rh7, all four double bond isomers (11-cis; 11,13-dicis; 9,11-dicis; 9,11,13-tricis) form pigments, whereas with Rh8, only the 11-cis ret8 yields a pigment. When the rings are small and close to planar, the opsin needs to undergo relatively small conformational changes to accommodate the different retinals. The fact that MeRh8 was formed shows that the opsin binding site is spatially lenient at the concave side of the bent 11-cis retinal so that, presumably with some expansion, it can accept the bulging tetramethylene bridge of 11-cis ret8 (ref. 36). However, none of the four ret9 isomers could be introduced into MeRh.

PHOSPHODIESTERASE AND RHODOPSIN KINASE ASSAYS OF MeRh8
As mentioned above, one molecule of activated Rh, i.e., MetaRh-II, leads to the hydrolysis of 100,000 molecules of cGMP to GMP, which is accompanied by release of protons and hence lowering of the pH. Thus the assay for the activated transducin-mediated phosphodiesterase (PDE) enzyme consists of continuous monitoring of the decrease in pH that occurs upon irradiation of a mixture of enzymes and the pigment in buffer (ref. 37). Repeated assay results with MeRh8 clearly showed that it was totally inactive (ref. 28). Further assays with a series of 11-cis-dihydroretinals (ref. 38) gave the following results (relative to Rh): Rh (100 %), 5,6- and 7,8-dihydro (20 %), 9,10 and 11,12-dihydro (nil). Closer the saturated bond to the 11-ene, lower the activity. 11,12-Dihydro-Rh, in which the crucial 11-ene is saturated, is totally inactive, in vivo (ref. 39).

The activated rhodopsin becomes deactivated upon multiple phosphorylation of the Ser and Thr residues residing near the C-terminal by Rh kinase (RK). The assay is performed by phosphorylating Rh in the presence of rhodopsin kinase and ATP-γ-32P, carrying out gel electrophoresis and counting the radioactivity of the 40 kDa band of Rh (ref. 40). Again, relative to the Rh-32P04 activity of MeRh (100 %), that of MeRh7 and MeRh8 was nil. The activities of the dihydro series also decreased sequentially as follows: 5,6-dihydro 43 %; 7,8 dihydro 36 %; 9,10- and 11,12-dihydro 0 %. Ret6 (Fig. 5, 2) yields Rh6 with two absorption maxima at 340 nm and 510 nm; upon prolonged irradiation the latter shifts to 495 nm (refs. 40, 41). The CD behavior however is unique; Rh6 only exhibits a single Cotton effect at 340 nm corresponding to the shorter maxima; upon prolonged irradiation the 340 nm Cotton effect (CE) disappears and a single CE at 493 nm develops (27). Thus although it has been reported that Rh6 shows marginal enzymatic activity, it is likely that this is due to effects quite different from those operating in native Rh, and that the unusual spectral observations first have to be clarified for a better understanding (ongoing).

THE TRIGGERING PROCESS OF VISUAL TRANSDUCTION
Flash photolysis has demonstrated that excitation of Rh7 yields a primary photoproduct corresponding to the native photo-Rh, in a low quantum yield that is only ca. 20 % that of photo-Rh. More important is the fact that Rh8, the Rh analog incorporating ret8, gives a photoproduct P585 similar to native photo-Rh with a high quantum yield similar to photo-Rh, and that the increased ring flexibility of the eight-membered ring allows P585 to thermally relax to P577 corresponding to batho-Rh before reverting to the starting MeRh8. Thus, clearly charge redistribution accompanied by the development of increased dipole moment in the excited state is occurring. However, Rh7 as well as Rh8 are enzymatically inactive i.e., nonfunctional. Furthermore, assay results of the dihydro-Rh series have demonstrated that the entire pi-bond system is required for efficient activity. Increased flexibility of the side-chain resulting from substitution of a single bond for a double bond leads to reduced activity, especially when the single bond approaches the critical 11-ene. Namely, a long and rigid delocalized pi-system is necessary to efficiently change the conformation of native Rh when it absorbs a photon.

Experiments that led us to this conclusion have not been straightforward. For a few years, assays with "Rh8" seemingly gave positive results, which meant that although cis \rightarrow trans isomerization does occur in native Rh, it is not essential and that excited state charge redistribution suffices; these clearly would have been far more dramatic and exciting conclusions! To demonstrate the extent of involvement of the excited state in transduction triggering is such a subtle point, difficult to prove experimentally.
The outcome of present studies is that of the two mechanisms shown in Fig. 4, excited state charge translocation is occurring, but this itself does not lead to activity; full cis-trans isomerization around the 11-ene involving the entire polyene moiety is necessary.

Finally, the 9-methyl, and probably other methyl groups, also play a central role in inducing conformational changes in the protein through their hydrophobic bonding. Ganter et al. have shown that the GTPase activity of 9-desmethyl-Rh was only 8% that of Rh; according to difference FTIR measurements, the pigment did form an intermediate after Meta-I, but unlike genuine Meta-II it was still protonated (ref. 42). This shows that proper conformational changes of the pigment are necessary for the proton of the PSB group to be transferred to the designated amino acid (proline?). In addition to the hydrophobic anchoring effect, the methyl groups at C-1, C-5 and C-13 are necessary to maintain nonplanarity around the 6-s and 12-s bonds of the chromophore. This nonplanarity can subtly adjust the chromophoric conformation so that its absorption maximum becomes optimal for the environment in which the organism lives (ref. 43). Thus the entire retinal molecule with all its methyl groups and the entire pi-system is required for optimal environmental adjustment and for efficient visual transduction. This extremely cleverly designed 11-cis-retinal can be secured readily from β-carotene, a pigment abundantly distributed in Nature (ref. 43).

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REFERENCES