Review

Recent Bioorganic Studies on Rhodopsin and Visual Transduction

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Rhodopsin, the pigment responsible for vision in animals, insect and fish is a typical G protein (guanyl-nucleotide binding protein) consisting of seven transmembrane alpha helices and their interconnecting extramembrane loops. In the case of bovine rhodopsin, the best studied of the visual pigments, the chromophore is 11-*cis* retinal attached to the terminal amino group of Lys296 through a protonated Schiff base linkage. Photoaffinity labeling with a 3-diazo-4-oxo-retinoid shows that C-3 of the ionone ring moiety is close to Trp265 in helix F (VI) in dark inactivated rhodopsin. Irradiation causes a *cis* to *trans* isomerization of the 11-*cis* double bond giving rise to the highly strained intermediate bathorhodopsin. This undergoes a series of thermal relaxation through lumi-, *meta*-I and *meta*-II intermediates after which the retinal chromophore is expelled from the opsin binding pocket. Photoaffinity labeling performed with 3-diazo-4-oxoretinal at −196 °C for batho-, −80 °C for lumi-, −40 °C for *meta*-I, and 0 °C for *meta*-II rhodopsin showed that in bathorhodopsin the ring is still close to Trp265. However, in lumi-, *meta*-I and *meta*-II intermediates crosslinking occurs unexpectedly at A169 in helix D (IV). This shows that large movements in the helical arrangements and a flip over of the ring moiety accompanies the transduction (or bleaching) process. These changes in retinal/opsin interactions are necessarily accompanied by movements of the extramembrane loops, which in turn lead to activation of the G protein residing in the cycoplasmic side. Of the numerous G protein coupled receptors, this is the first time that the outline of transduction pathway has been clarified.

Key words transduction path; photoaffinity label; rhodopsin; exciton chirality; Trp2; Ala169

Introduction[1)]

The cross section of a human eye is shown in Fig. 1. The estimated 7 million cone cells, responsible for color ("photopic") vision are clustered around the fovea where the incoming image is focused most sharply. These cells contain three kinds of rhodopsins (Rh) or visual pigments absorbing around 450 nm (blue light), 530 nm (green light) and 560 nm (red light). When light passes through a yellow solution, it absorbs the blue light at 450 nm so that green and red light enter our eyes, leading to the sensation of yellow. On the other hand, the 100 million rod cells responsible for black and white ("scopotic") vision are distributed in the peripheral area of the retina and absorb at 500 nm. Since solar energy is strongest at 400—700 nm, the human eye is taking full advantage of the solar energy distribution. The rod/cone cell ratio depends on the animal: in nocturnal rats it is 4000, humans 20, goldfish 15, frogs 1, while owls only have rod cells. Although not nocturnal, bovine (4 million rods), horse, and dog only have rods and hence lack color vision.

The outer segments of rod cells (rod outer segment or ROS) consists of about 1500 thin membranes called disks that are formed at the basis of the rod visual cell by pinching off sections of the plasma membrane. The disks, which embed the 40 kDa Rh molecules consisting of 348 amino acids, move towards the tip of the cell in about 15 d where they are phagocytosed. A breakdown in phagocytosis by the retinal pigment epithelium cells leads to accumulation of orange fluorescent pigments named A2E and iso-A2E that impair the vision, and in certain cases loss of vision or AMD, *i.e.*, age-related macular degeneration.

Visual transduction is the process by which visual cells convert light into a neural signal, which in turn is transmitted to the brain *via* the optic nerve. In the dark, Na^+ ions flow into ROS. Upon absorption of one photon of light, the flow of >million Na^+ ions is blocked. Namely, absorption of a single photon activates 100 molecules of G proteins (guanyl-nucleotide binding protein, in the case of vision the term transducin is used), which *via* activation of phosphodiesterase, hydrolyzes 100000 molecules of cyclic GMP. This drop in cGMP closes the cation-specific channels and leads to a build-up of electric potential that is picked up by the optic nerves. Rh is one of the most thoroughly investigated G-protein coupled receptors[2)] consisting of seven hydrophobic transmembrane helices A, B,...G (or 1, 2,...7) that are interconnected by hydrophilic extramembrane loops (Fig. 1). The sequence of the 348 amino acids of Rh, a triumph of protein sequencing, was achieved by the groups.[3,4)]

The Chromophore and Transduction Intermediates

Unless otherwise specified, the following refers to studies performed with bovine Rh since the readily available bovine rods provide an excellent source for Rh. The chromophore in the majority of visual pigments is 11-*cis*-retinal (**1**, Fig. 2). In bovine Rh this is linked to the terminal amino group of Lys296.[5)] The fact that absorption maxima of the visual pigments cover the range 350 nm to 680 nm is quite extraordinary considering the fact that this wide range simply arises from the interaction between the retinal chromophore and their receptors. A number of unique aspects of this chromophore makes it ideally suited for controlling the subtle wavelength regulation of visual pigments so as to adjust the vision to suit the environment[6)]:

1. It is a protonated Schiff base (PSB) comprising the terminal moiety of a polyene, and hence the positive charge is delocalized throughout the polyene chain. This delocalization is affected by a various factors, *e.g.*, 2 and 3 in the fol-

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lowing.

2. The counter ion of PSB in bovine Rh is Glu113.[7] In different Rhs, the counterions are different and the distance between the PSB positive charge and counter ion negative charge also differ.

3. The local electrostatic field within the binding site. This concept was originally introduced as the external point charge model.[8]

4. The 1-, 5- and 13-methyl groups are all required for maintaining chromophoric nonplanarity, which in turn is governed by the nature of the Rh binding site.

The 9-methyl group is essential for hydrophobic binding to the Rh receptor which is essential for Rh to change its conformation triggered by the *cis* to *trans* isomerization of the 11-*cis* bond. Finally, retinal is readily available from the ubiquitous carotenoids.

Marine and aquatic animals have 3-dehydro-11-*cis*-retinal as the photopigment (Fig. 2, **2**) in order to shift the vision towards the red, wavelengths more suited for dark vision. Salmon and eel lack the extra double bond when living in fresh water, while the tadpole also loses its 3-ene when it becomes the terrestrial frog. Insects contain an extra 3-hydroxy group,[9] while the squid, *Watasenia scintillans* has three pigments, **1**, **2** and (4*R*)-4-hydroxy-11-*cis*-retinal **3** (Fig. 2).[10]

The visual transduction of Rh is outlined in Fig. 3. Irradiation with 500 nm light isomerizes the 11-*cis*-ene to *trans*, leading to the first intermediate photo-Rh which is formed in the low femto-sec time scale.[11] This is the only light-triggered change that occurs in Rh, the subsequent changes to batho-Rh and others being caused by relaxation of the protein to relieve the high strain caused by the "*trans*" 11-ene of the chromophore/protein complex. The strain energy, estimated to be 30—36 kcal/mol,[12] is reflected in the red-shifted absorption maxima of photo-Rh (555 nm) and batho-Rh (535 nm). Batho-Rh was detected as early as in 1958[13] and more properly characterized by absorption spectroscopy

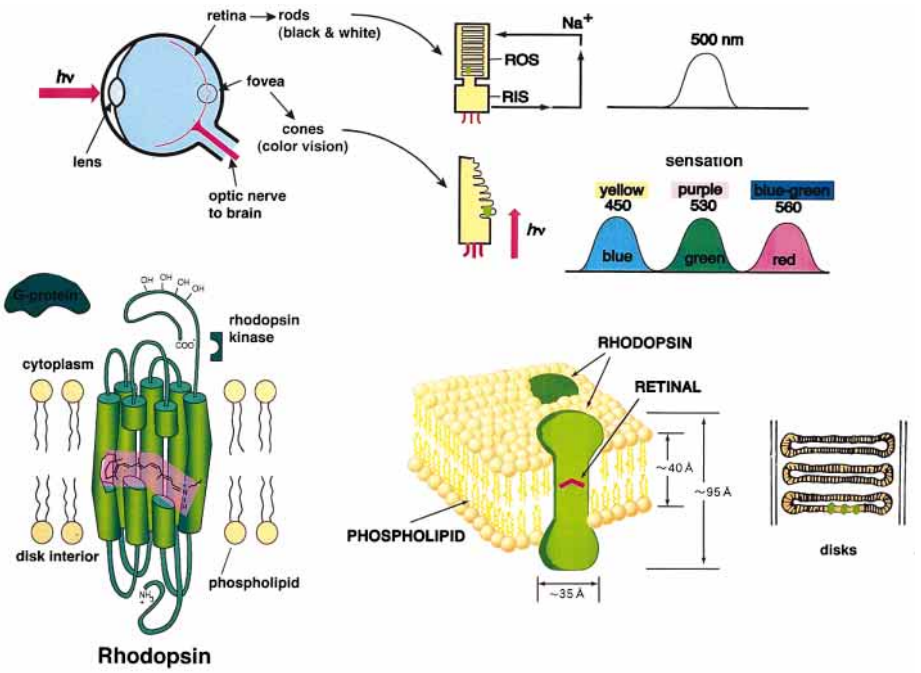

Fig. 1. Cross Section of Eye, Rod and Cone Cells, Disks, and Rhodopsin

Born in Hong Kong, and brought up in Lyon, London, and Alexandria until 11, he received his bachelor's degree in chemistry from Nagoya University, Japan, in 1947 from Fujio Egami. Following two years of post-graduate work with Louis Fieser at Harvard University, he returned to Nagoya University where he completed his Ph.D. in 1954 with Yoshimasa Hirata. He was Assistant Professor at Nagoya until 1958 when he became Professor of Chemistry at Tokyo Kyoiku University. In 1963 he moved to Tohoku University in Sendai and remained there until 1969 when he joined the faculty of Columbia University. In 1980 he became Centennial Professor of Chemistry. He was Chairman of the Chemistry Department, 1987—1990. Dr. Nakanishi was a founding member and a Director of Research at the International Centre of Insect Physiology and Ecology in Kenya from 1969—1977. He also served as the Director of the Suntory Institute for Bioorganic Research in Osaka, 1978—1991. Since 1996 he is assisting the Brazilian government to set up a center of excellence, Institute of Medicinal and Ecological Chemistry centered in São Paulo and Manaus.

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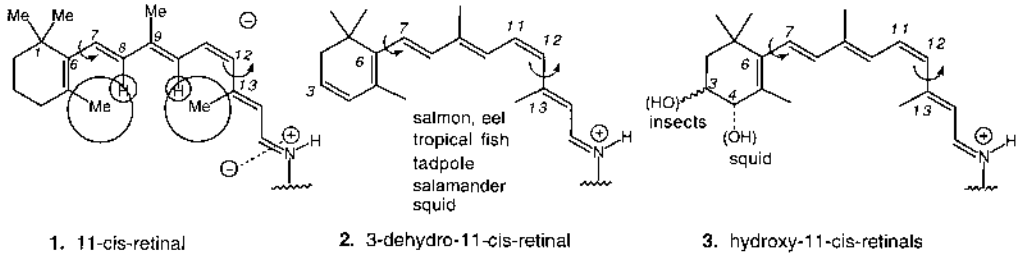

Fig. 2. Rhodopsin Chromophores

They are not planar around 6-*s*-*cis* and 12-*s*-*trans* bonds due to steric hindrance.

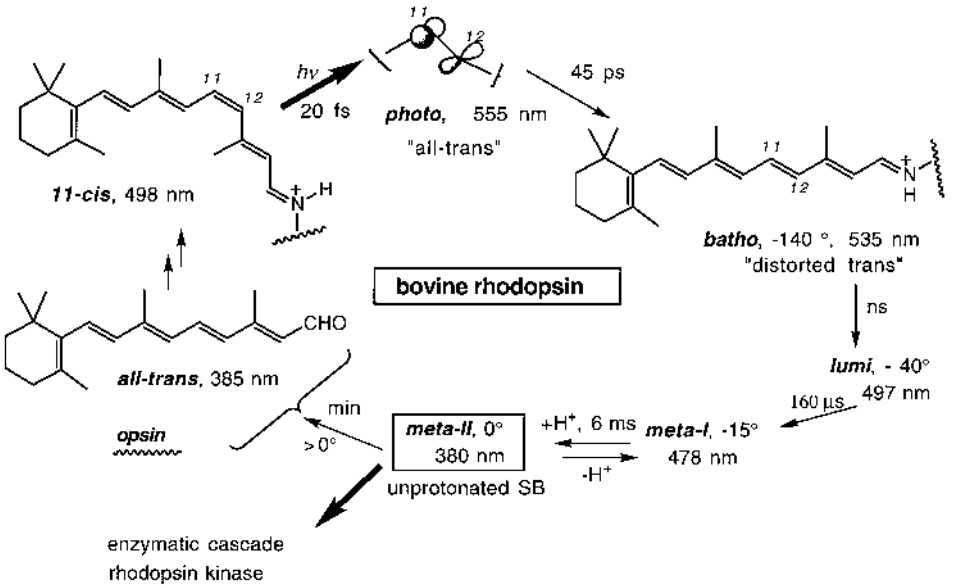

Fig. 3. Rhodopsin Transduction Path

measured in liquid He, a temperature in which it can be sequestered.[14] Microscopic rate constants coupled with time-resolved spectroscopy led to the finding that a blue-shifted intermediate (BSI) is in equilibrium with batho-Rh (not shown). After batho-Rh the pigment relaxes to lumi-Rh, *meta*-I Rh, and it is at the *meta*-II stage that the conformation of the extramembrane loops on the cytoplasmic side leads to excitation of the G protein, thus initiating a cascade of enzymatic reactions.[15]

Photoaffinity Labeling of Restive Rh and Absolute Twist around 12-*s* Bond

Photoaffinity labeling of retinal proteins, particularly rhodopsin, has been hindered by serious factors. Namely, the difficulty in separating the peptidic fragments obtained upon enzymatic or chemical cleavages, a factor that caused problems in the sequencing of the protein,[3] and scrambling of cross-linking upon photoactivation of the photolabel due to isomerization of the *cis*-ene to the *trans*-ene. Although racemic ^{14}C-labeled 9-*cis*-3-diazoacetate[16] and subsequently the 3*R* and 3*S* enantiomers[17] were incorporated into Rh and photo-crosslinked, the results were not published except for a Ph. D. thesis[18] because too many amino acids were affinity labeled. On the other hand, Nakayama and Khorana used the aromatic trifluoromethyldiazirene **1** (Fig. 4) and identified the amino acids in helices C and F to which the diazirene carbon had cross-linked.[19] Their results show that the ionone ring is in the vicinity of helices C and F, but the multitude of cross-linked amino acids and the large distance between some labeled residues (13.5 Å) was presumably caused by *cis*/*trans* isomerization.[19]

In view of these difficulties, analog **2**, [15-^{3}H]-3-diazo-4-oxo-10,13-ethano-11-*cis* retinal or diazoketo-ret6 (Fig. 4), where the 11-*cis* to *trans* isomerization is blocked, was employed. Incubation of analog **2** with bovine opsin gave a pigment in 53% reconstitution yield. Irradiation of the pigment at 254 nm resulted in a 23% cross-link as estimated from the radioactivity of the peptide after separation from the unbound retina by size-exclusion HPLC. The cross-linked apoprotein in membrane suspension was cleaved by V8 protease at Glu239/Ser240 on the cytoplasmic side into large (V8-L) and small (V8-S) fragments; cleavage yield *ca*. 50%. After detergent solubilization and carboxymethylation of cysteine residues, the cleaved V8-L and V8-S fragments were separated by SDS-PAGE. All radioactivity resided in V8-S, while *no radioactivity was associated with the V8-L portion of rhodopsin*, indicating that the cross-linking site resided in helix F or G (Fig. 4). It was fortunate that all radioactivity resided in V8-S since this was the only fraction that showed base-line separation. Cyanogen bromide cleavage showed the radioactivity to reside in BrCN peptide-13, which upon Edman degradation disclosed Trp-265 and Leu-266 to be the crosslinked sites.

The helices in Fig. 4 are arranged according to a theoretical assignment of the seven transmembrane helices.[20] As the current transmembrane model of the rhodopsin helices

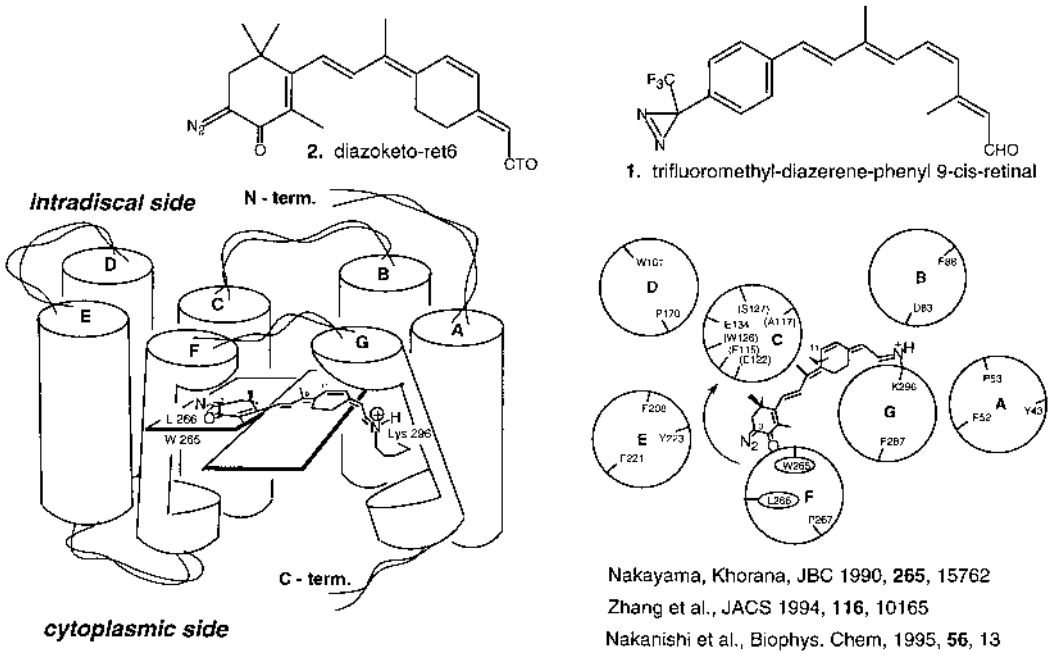

Fig. 4. Photoaffinity Labeling of Rhodopsin with Retinoids **1** and **2**

places both the labeled Trp-265/Leu-266 residues and the Schiff base linkage to Lys-296 in the middle of the lipid bilayer, this shows that the chromophore in dark Rh has its plane of the polyene essentially parallel to the membrane plane and that the entire chromophore must reside near the center of the bilayer, with the polyene long axis tilted only slightly relative to the membrane plane. The crosslinking of diazoketo **2** to only two amino acids is in sharp contrast to that of phenyl analog **1** which crosslinked to multiple residues in helices F and C. Although implication of this difference was not fully realized at the time, our recent results demonstrate that the binding of the ring in **2** is caused by a flip-over of the ionone moiety when the 11-ene isomerizes.

The electron density map[21] of rhodopsin reveals a binding pocket that lacks a straight portion to accommodate the all-*trans* chromophore (Fig. 4, helical wheel); thus only *cis* isomers (except for 13-*cis*) can be incorporated into the protein core which is formed predominantly by helices B, C, F, and G. Therefore, the *cis* to *trans* isomerization of the chromophore could push one or more helices away to generate a more accommodating transoid cavity. This could be the origin of the protein conformational changes leading to *meta*-II stage and which are reversed upon release of all-*trans* retinal.[22] Indeed 11-*cis*-retinal acts as an inverse agonist that retains the pigment in its inactive form that does not activate the G-protein.[23]

As mentioned above, the 11-*cis*-retinal chromophore is twisted around the 6-*s*- and 11-*s*-*cis* bonds due to steric hindrance between the methyl and hydrogen atoms, Fig. 2, (**1**). The UV/Vis and CD spectra of bovine rhodopsin are shown in Fig. 5 (**1**). Native rhodopsin, λ_{max} 500 nm, exhibits two positive CD Cotton effects at 480 nm ($\Delta\varepsilon$+2.8, "α-band") and 337 nm ($\Delta\varepsilon$+9.8, "β-band"). A rhodopsin with a retinal analog in which B/C planes adopt a planar structure *via* a five-membered ring between C-10/C-13 exhibits a CD with a negligible 480 nm α-band. Similarly, the pigment formed from a retinal with planar A/B conformation *via* a five-membered bridge between C-5/C-8 showed a weak 337 nm β-band. Based on these results, Ito and co-workers[24] assigned the origin of the α- and β-bands to distortions around 12-*s*-*trans* and 6-*s*-*cis* bonds, respectively. What is the absolute sense of twist around the 12-*s*-bond in Rh ? Is it as depicted in Fig. 5 (**2**) or is the twist of the B/C planes positive ?

This was determined by application of the CD exciton chirality method[25] to pigments incorporating dihydroretinal chromophores.[26] According to this nonempirical method, a clockwise twist or a positive helicity between two chromophoric electric transition moments interacting through space leads to a positive exciton couplet, *i.e.*, a CD curve with a positive longer wavelength Cotton effect and a negative shorter wavelength Cotton effect (see Fig. 5). Saturation of the retinal 11-ene breaks the chromophore into two independent conjugated systems, *i.e.*, the triene and the protonated Schiff base (PSB) moieties as shown in (**2**) and (**3**) (Fig. 5). If the through-space interaction between these two chromophores in the pigment is sufficiently intense, it could give rise to a couplet, the sign of the split CD reflecting the absolute twist within the pigment binding site. Provided 11,12-dihydroretinal adopts a conformation similar to 11-*cis*-retinal in rhodopsin, the exciton coupling between the triene and PSB should appear as either a positive or negative CD couplet depending on whether planes B/C constitute a positive or negative twist (Fig. 5). Indeed, incorporation of 11,12-dihydroretinal into Rh gave 11,12-2H-Rh that, unlike Rh itself, showed a negative couplet with an amplitude of -4.5, thus showing the twist to be as depicted in (**2**) Fig. 5. Moreover, the pigment with the less flexible 11,12-dihydro-ret7 (retinal with a trimethylene bridge from C-10 to C-13 (**3** in Fig. 5) also showed a more intense negative couplet with A=−11.6. These data define the absolute sense of twist as negative, namely, planes B and C constitute a negative chirality.[26] It was gratifying to find that the interchromophoric through space interaction in these dihydro-retinoids was reflected in the pigments.

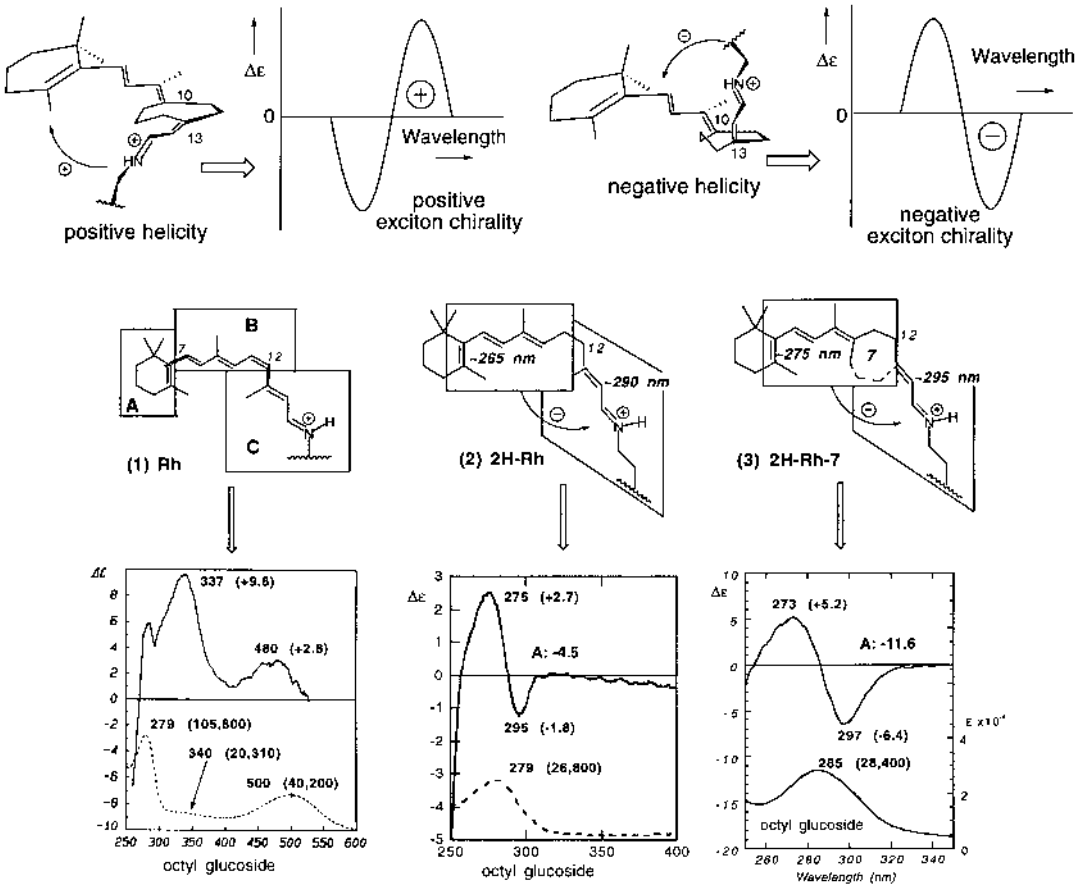

Fig. 5. Exciton Coupled CD Applied to 11,12-2H-Rh and 2H-Rh-7; Also CD of Rh. UV Also Shown

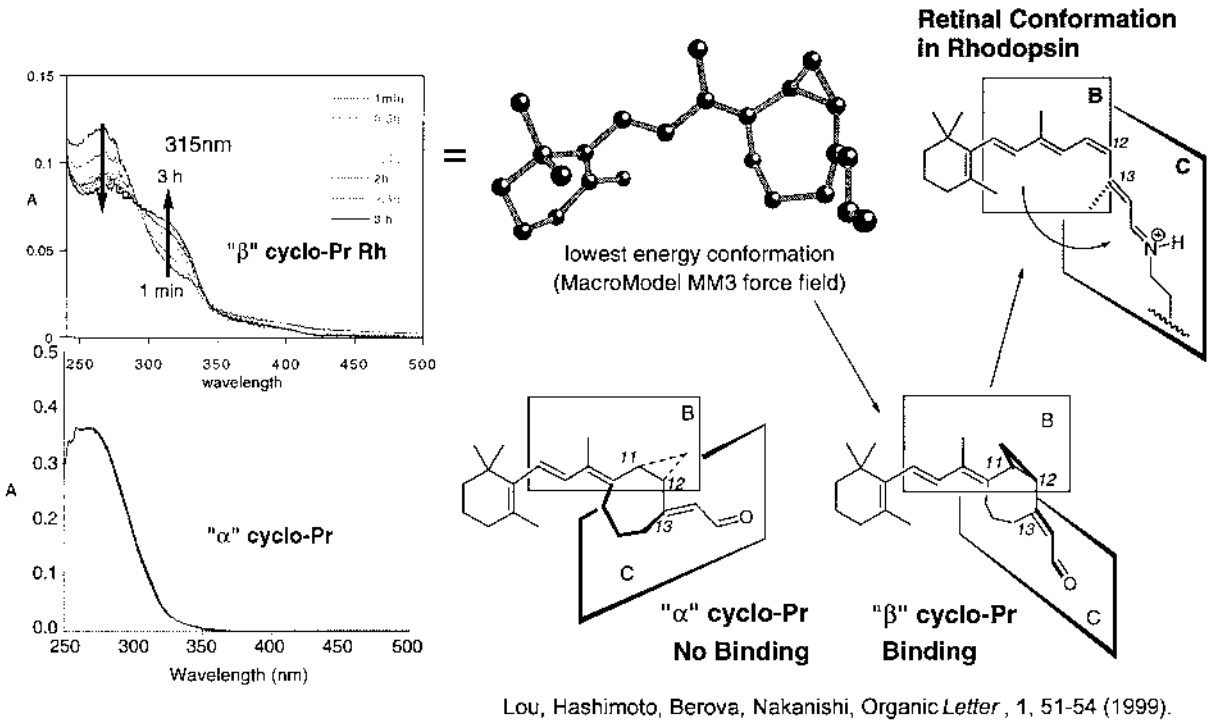

Fig. 6. Stereoselectivity of the Retinal Binding Site in Rh

In addition to the CD studies, an independent bioorganic study to determine the sense of twist, an independent bioorganic study was performed.[27] The two 11-*cis*-locked α-and β-11,12-cyclopropylretinals shown in Fig. 6 were synthesized chemoenzymatically. Molecular modeling showed the lowest energy conformations of the two enantiomers to be as

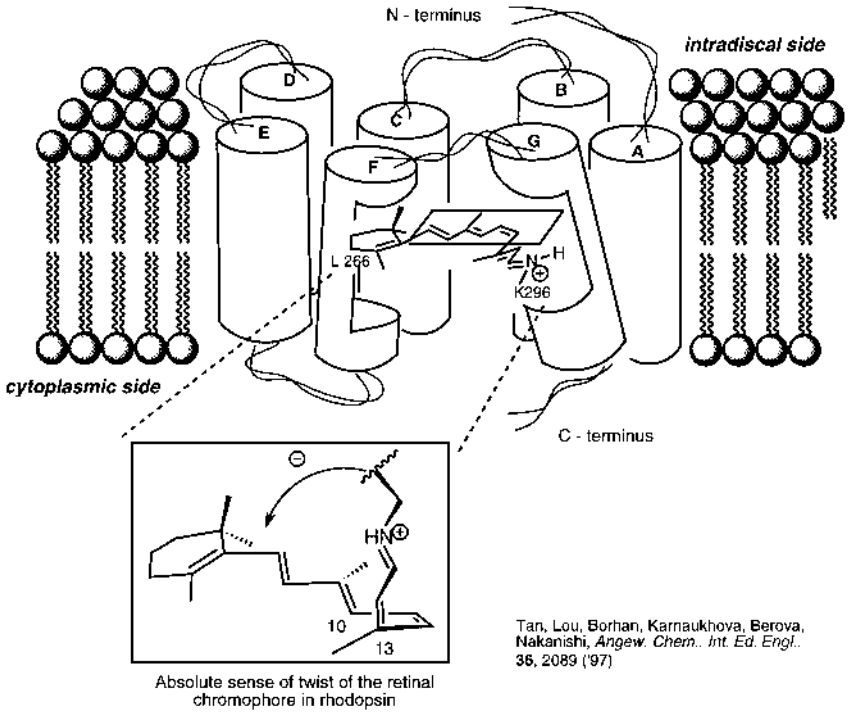

Fig. 7. Absolute Sense of Twist around the 12-*s*-Bond in the Rh Chromophore

depicted (only one enantiomer shown). It was a matter of checking the selectivity, if any, of the incorporation of the two enantiomers in Rh. It turned out that upon incubation, only the β-cyclopropyl analog bound to give the Rh analog absorbing at 315 nm. The binding experiments with the two enantiomers demonstrate the steric preference of the retinal binding site; as depicted, the twist between planes B and C is in agreement with the results deduced by the CD exciton chirality approach. It is thus concluded that the absolute twist of planes B and C is as shown in Fig. 7.

This negative helicity of the retinal chromophore agrees with theoretical calculations by Kakitani and Kakitani[28] and solid-state NMR studies by Han and Smith.[29] However, in a recent *ab initio* study,[30] the chiroptical data of PSB formed from methylamine and 11-*cis*-retinal with four arbitrarily assigned geometries were calculated, whereupon a positively twisted C12/C13 geometry gave rise to a positive CD which implied a positive helicity around the C12/C13 bond. This is opposite to the results obtained by CD and binding studies. However, the effect of the protein, such as the PSB counter ion and electrostatic field within the retinal binding site was not considered in this calculation.

Preparation of 3-Diazo-4-oxo-11-*cis*-retinal (DK 11-*cis*-Retinal).

The next target was to determine which side of the 11-*cis*-ene flips over upon photoisomerization of the 11-*cis* to all-*trans* chromophore in Rh (see structure at bottom right, Fig. 8). In order to solve this aspect, it became necessary to prepare 3-diazo-4-oxo-11-*cis*-retinal carrying two photolabile moieties. The synthesis of 11-*cis*-retinal analogs in general are complicated by their unusual instability to light and temperature and their facile isomerization under conditions which most *Z* double bonds survive. Moreover, most schemes geared towards introducing the 11-*cis* geometry, especially those close to the end of the synthetic scheme, have either failed or resulted in low yields and formation of complex isomeric mixtures. Also, the more common photoisomerization strategy of all-*trans*-retinoids and HPLC purification of the 11-*cis*-isomer is not compatible with analogs containing light sensitive functional groups. The unusual difficulty encountered in generating the 11-*cis*-geometry is associated with the steric hindrance between 10-H and 13-Me which leads to a nonplanar conformation that could easily isomerize and relieve stress under most experimental conditions.

The title compound was first successfully prepared by a chemoenzymatic synthesis utilizing the unique properties of retinochrome contained in octopus and squid eyes (Fig. 8).[31] Retinochrome, a protein folded into seven α-helices, is a photo-isomerase that catalyzes the conversion of all-*trans*-retinal to 11-*cis*-retinal. The water-soluble retinal carrier protein incorporates 11-*cis*-retinal upon incubation with metaretinochrome; further incubation with opsin containing membranes yields Rh. Thus the retinal carrier protein appears to act as a light-activated shuttle that transfers the retinal molecules between Rh and retinochrome. Although the conversion of all-*trans* 3-diazo-4-oxoretinal could be carried out in 50% yield, and the enzyme could theoretically be recycled, this approach was not suited for securing sufficient material (*ca.* 10 mg) that would be needed for conducting photocrosslinking studies.

Numerous attempts to synthesize the title compound by conventional retinoid syntheses all gave unsatisfactory results including semihydrogenation of the 11-yne.[32] The synthesis was finally achieved in high yield and with 100% *cis* stereoselectivity by performing the yne-reduction with Cu/Ag activated zinc dust in methanol/water at room temperature (Fig. 9).[32]

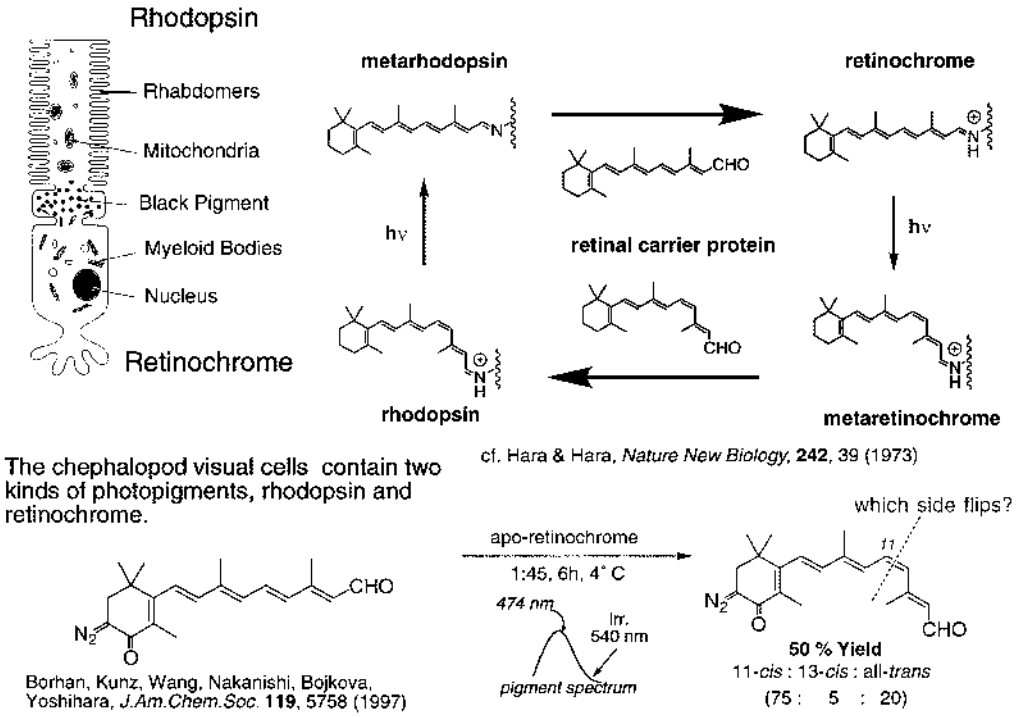

Fig. 8. Retinochrome Catalyzes Isomerization of all-*trans*- to 11-*cis*-Retinal

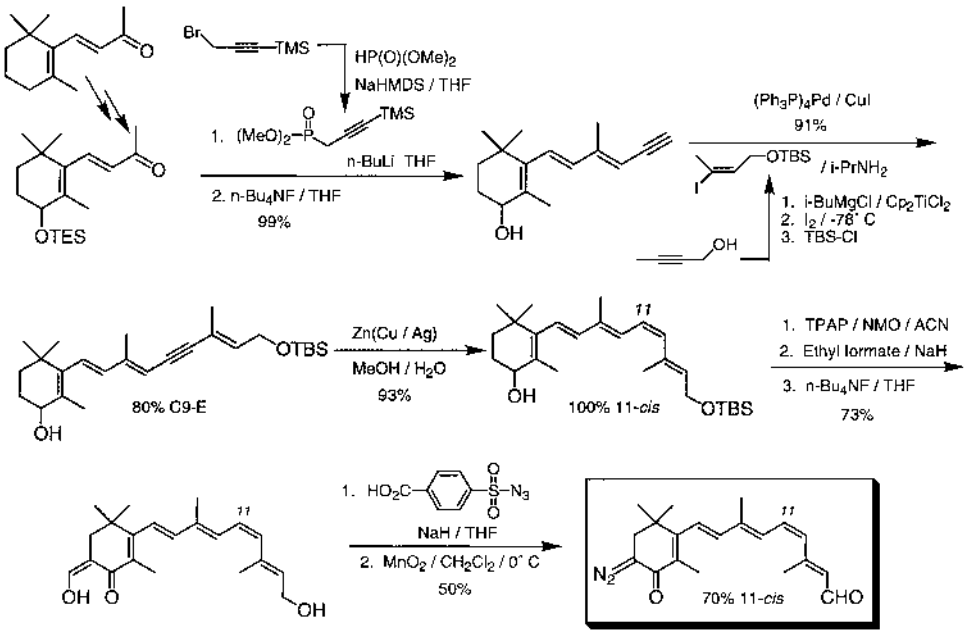

Fig. 9. Synthesis of 11-*cis*-3-Diazo-4-oxoretinal

Changes in Chromophore/Receptor Interactions along the Visual Transduction Path

The photoaffinity labeling experiments with DK-11-*cis* retinal ("DK") was performed using the analog with the tritiated aldehyde function. Incubation of HPLC-purified DK with rod outer segment (ROS) membranes regenerated DK-Rh, λ_{max} 467 nm. Its CD exhibited two positive peaks at 306 nm and 462 nm, closely matching the β and α peaks at 337 and 480 nm, respectively, of native Rh. The rapid regeneration and spectroscopic data suggest that the binding of DK to opsin closely matches that of native Rh. Furthermore, measurement of the photo-intermediates of DK-Rh upon dissolving it in 66% v/v glycerol and irradiation at low temperatures showed that intermediates corresponding to those of native Rh were formed, except at generally lower temperatures (Fig. 10). The changes in chromophore and opsin interactions were then tracked by: (i) performing photoaffinity labeling at the respective optimal temperatures, (ii) pyridiethylate the crosslinked protein prior to separation of the protein from the membrane, (iii) BrCN cleavage, (iv) separate peptidic fragments by HPLC, (v) collect the tritiated HPLC peak and sequence by Edman degradation.[33)]

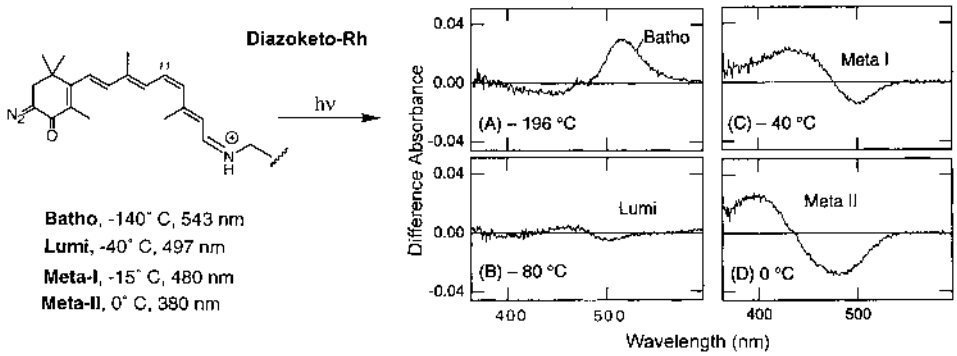

Fig. 10. Photo-intermediates of DK-Rh

The corresponding intermediates of native Rh are also shown together with respective temperatures at which they can be sequestered and absorption maxima.

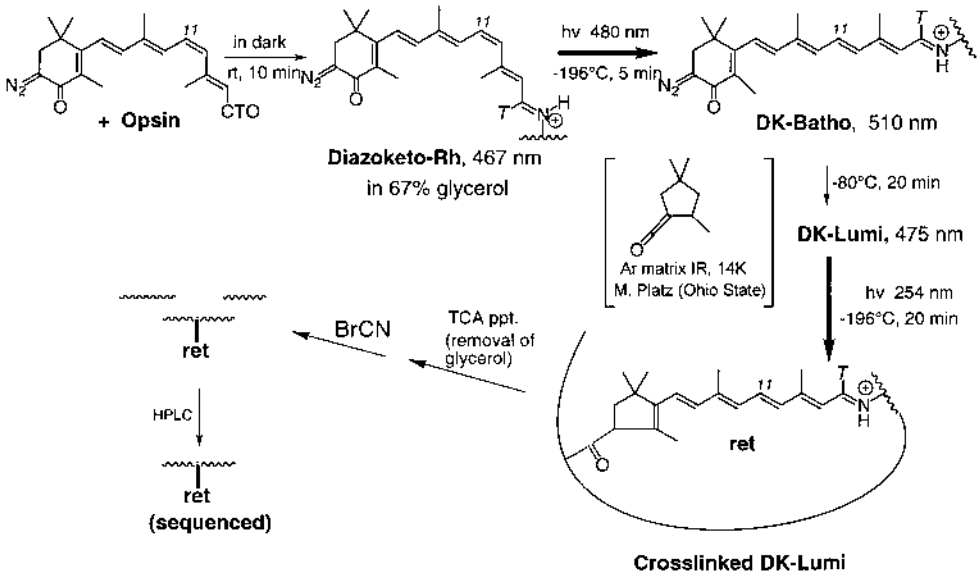

Fig. 11. Photoaffinity Labeling Protocol

DK lumi-Rh with a sequestering temperature of −80°C is taken as example.

The resting state of the pigment was investigated by keeping the DK-Rh glycerol solution at −196 °C in complete darkness and irradiating with 254 nm UV. The labeled amino acid was identified as Trp265 in fragment 13, *i.e.*, CN-13 (Val258 to Phe287) of helix F. In a previous photoaffinity study using non-isomerizable 11-*cis*-retinal analog, DK-ret6 **2** (Fig. 4), Trp265 and Leu266 were the crosslinking sites.[22] The additional crosslinking to Leu266 with DK-ret6 **2** probably is due to difference in the structures of the two DK retinals. Although the 11-*cis*-bond in DK-3 is not locked and the molecule is flexible, clean labeling occurred only at Trp265, indicative of a rigidly bound chromophore. The result shows that Trp265, a highly conserved residue among GPCRs is directly involved in ligand binding. The close proximity of the ionone ring and Trp265 restricts the geometry of 11-*cis*-retinal to a shape that functions as an inverse agonist (antagonist) for opsin activation.

To achieve photoaffinity labeling at the batho-Rh stage, the glycerol DK-Rh solution was irradiated with 480 nm light at −196 °C to populate the batho stage. The photoisomerized sample was then subjected to photo-crosslinking conditions (254 nm, −196 °C) and the labeled amino acid was determined as described above. Crosslinking again occurred only at Trp265. Thus, interaction between the β-ionone ring moiety and the opsin hardly changes at the batho stage.[34] The protocols for photoaffinity labeling of further intermediates were similar except for the temperature at which each state was populated. After cooling the glycerol DK-Rh solution to −196 °C in complete darkness, it was photoisomerized to batho-Rh by 480 nm irradiation. The protein solution was then warmed in darkness to the appropriate temperatures (Fig. 10), −80 °C for lumi, −40 °C for *meta*-I, and 0 °C for *meta*-II, cooled to −196 °C to freeze the protein conformation, and then irradiated with the 254 nm light to achieve photo-crosslinking.

The amino acid labeled in the lumi stage was Ala169 (helix D) in fragment CN-9 (Ala164 to Gly182) (Fig. 12). There was no evidence for any labeling in fragment CN-13, which was labeled in DK-Rh and DK-batho-Rh. At −80°, the temperature at which lumi-Rh crosslinking was performed, the pigment consists of a 1 : 1 mixture of Rh and lumi-Rh and not 100% lumi. The fact that only Ala169 was crosslinked at the lumi intermediate could be due to a more efficient crosslinking to Ala or to a closer proximity of the ionone ring to Ala169 in lumi-Rh than to Trp265 in Rh. Relaxation of the protein/substrate complex accompanies a large movement of the chromophore between the batho and lumi states, leading to a relieve in torsional strain and a sub-

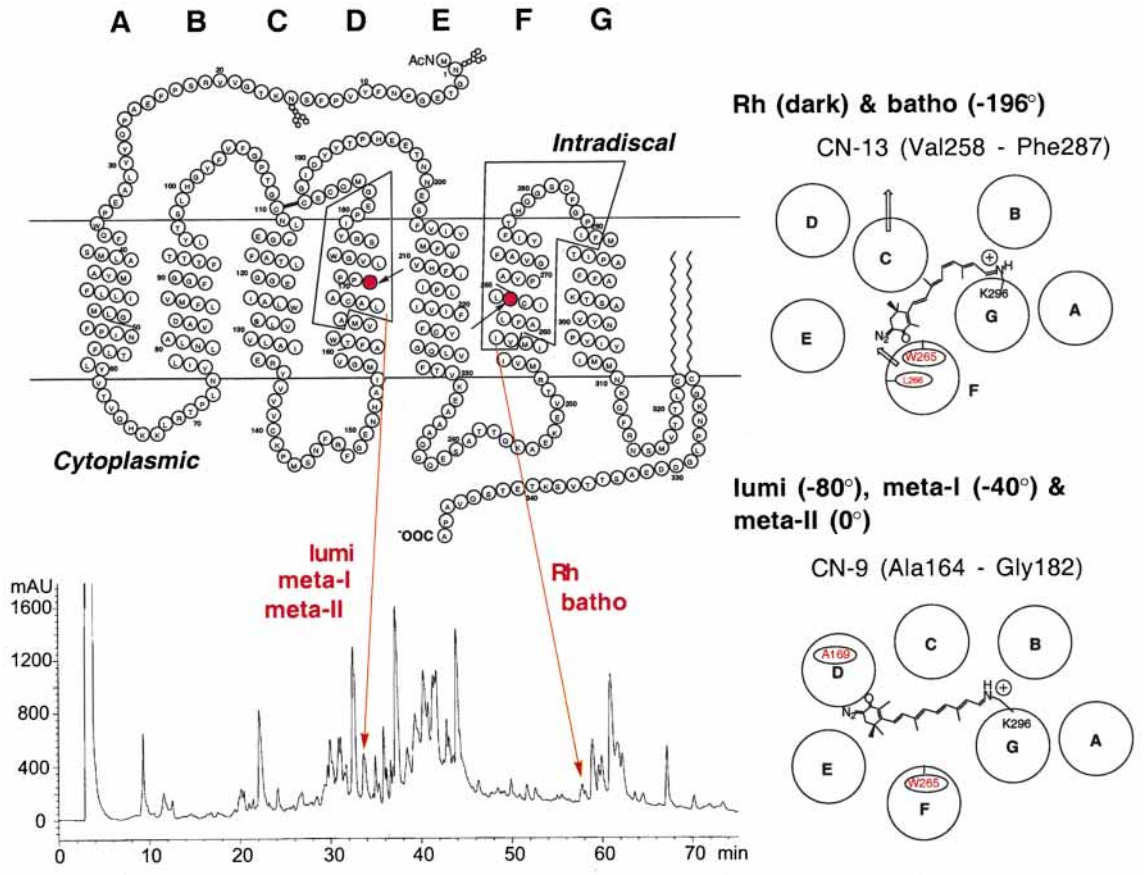

Fig. 12. Photo-crosslinking Sites in Rh (Dark), Batho-, Lumi-, *Meta*-I and *Meta*-II-Rh

The two small HPLC peaks shown by red arrows, the only radioactive peaks in the chromatogram, were sequenced by the Edman method. The crosslinked sites, Trp265 (in dark Rh and batho) and Ala169 (in lumi, *meta*-I and *meta*-II) are highlighted in red in the Rh sequence.

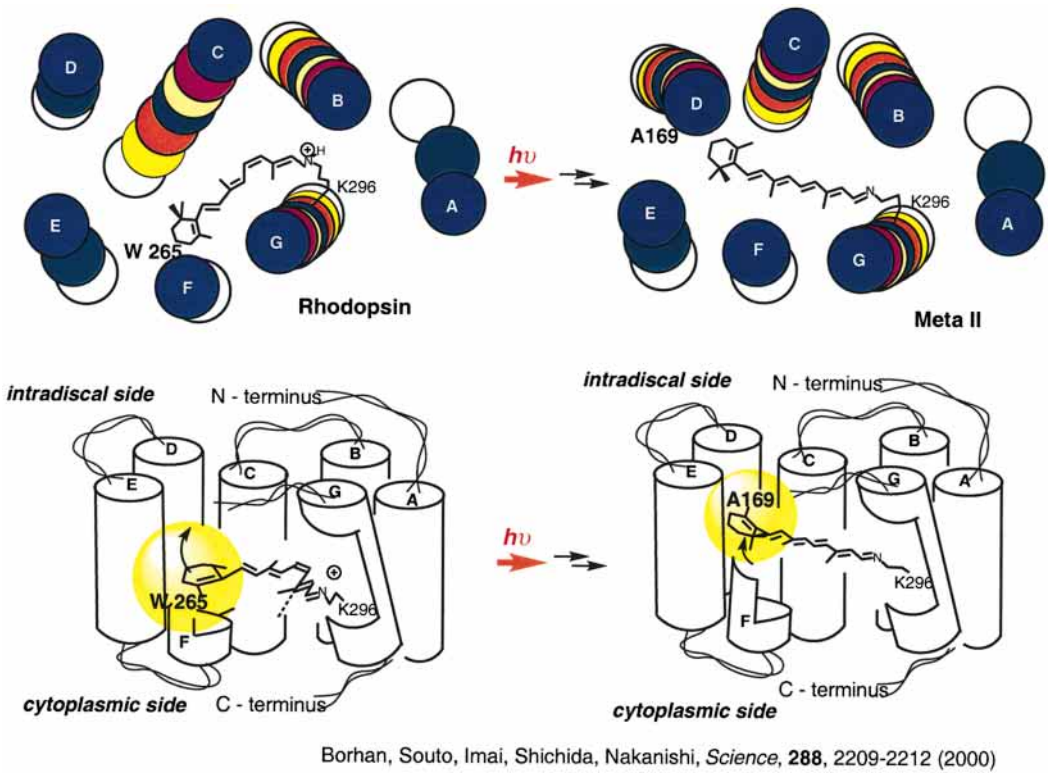

Fig. 13a. Qualitative Depiction of Opsin Conformational Movements in Visual Transduction

In Rh and batho the ionone ring is close to Trp265 in helix F. In lumi the ring flips over with concomitant changes in helical structure and resides close to Ala169 in helix D. The conformation of the extra membrane loops connecting helices C/D and E/F activates the G protein.

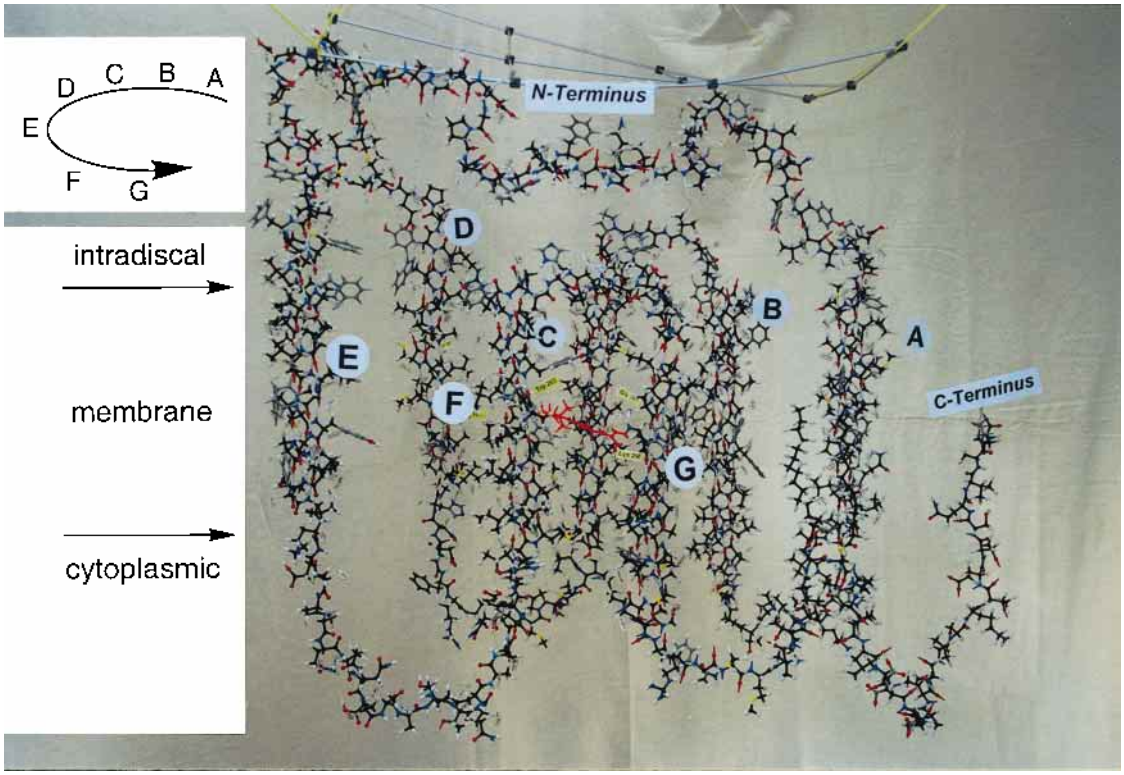

Fig. 13b. A Molecular Model of Rhodopsin Hanging from the Ceiling

The intradiscal side with the N-terminus is at the top as in Fig. 13. The helices are arranged as shown by the circular arrow. The two straight arrows define the boundaries of the membrane.

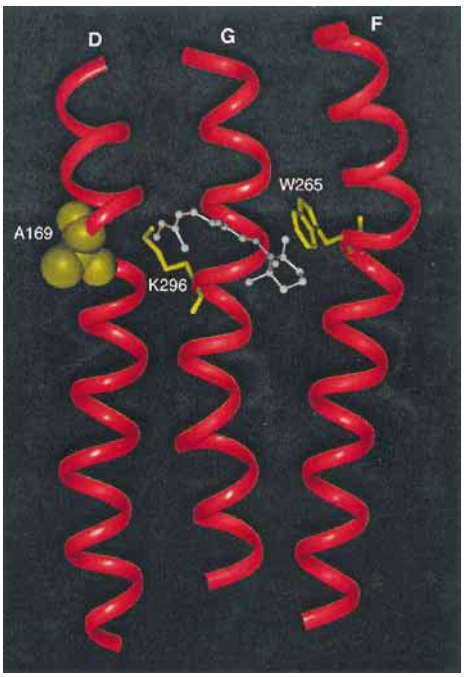

Fig. 14. Side View of a Theoretical 3D Model of Rh by Baldwin *et al.* (1997)

Helices A, B, C and E are omitted. Lys296 anchors the protonated Schiff base. The ionone ring is close to Trp265 in dark-Rh and batho Rh. Upon 11-ene isomerization, the ionone moiety flips over in going from batho to lumi and approaches Ala169. In subsequent *meta*-I and *meta*-II intermediates, the ionone C-3 still crosslinks to Ala169, and hence there is no change in the relative positions of the ionone ring and Ala169. However, the protein conformation changes, as reflected in the CD changes, and the extramembrane loops in *meta*-II lead to activation of the G protein. Note that Lys296, Trp265 and Ala169 are in the same relative heights from the top of the membrane lipid layer.

stantial blue shift (543 nm to 497 nm, Fig. 10). The crosslinked amino acid in *meta*-I and *meta*-II is again only Ala169. The results indicate that photoisomerization, triggered by a flip-over of the chromophoric ring, triggers a gross structural change in the protein, which moves helix D to the vicinity of the photolabel (Fig. 13). Photoisomerization separates the ionone ring from Trp265 in helix F and eliminates the interaction between 9-Me and Phe261, a highly conserved residue in all G protein-coupled receptors one-pitch below Trp265; 9-Me is also close to Gly121 in helix C.[35] This induces separation of helix C from helix F and approach of the ionone ring to Ala169 in helix D. Although the ionone ring remains close to Ala169 in lumi-, *meta*-I and *meta*-II-Rh, the ligand/opsin interactions are clearly changing as seen by changes in the respective CD.[36]

Lys296 involved in the protonated Schiff base link (helix G), Trp265 (helix F) and Ala169 (helix D) are all located around equal heights from the top of the membrane in their respective helices (Fig. 14). Therefore, the chromophore that stretches between Trp265 in helix F and Lys296 in helix G runs parallel to the membrane plane. The *cis* to *trans* isomerization of retinal involves a 180° rotation of the chromophore about the 11-ene to the all-*trans*-chromophore, which retains a similar parallel orientation with the membrane plane.

These results demonstrate that the chromophore in batho does not move significantly, and that a large movement occurs during the batho to lumi conversion. Ala169 in helix D is cross-linked in the lumi-, *meta*-I and *meta*-II stages, leading to movements schematically depicted in Fig. 13. This necessarily leads to conformational changes of cytoplasmic loops connecting helices C/D and E/F. It is proposed that

Trp265 keeps 11-*cis*-retinal as an inverse agonist. It is thus not surprising to see Trp265 in close proximity to the ionone ring in both the resting stage Rh and in batho-Rh, the first high-energy intermediate, while the ring moiety has flipped over in the more relaxed lumi and further intermediates.

Current efforts are directed towards defining the absolute sense of twist around the 6-*s* bond (twist between planes A and B) and clarifying the binding between the G protein and the cytoplasmic loops.

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