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Primary Events in Dim Light Vision: A Chemical and Spectroscopic Approach Toward Understanding Protein/Chromophore Interactions in Rhodopsin

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ABSTRACT: The visual pigment rhodopsin (bovine) is a 40 kDa protein consisting of 348 amino acids, and is a prototypical member of the subfamily A of G protein-coupled receptors (GPCRs). This remarkably efficient light-activated protein (quantum yield = 0.67) binds the chromophore 11-*cis*-retinal covalently by attachment to Lys296 through a protonated Schiff base. The 11-*cis* geometry of the retinylidene chromophore keeps the partially active opsin protein locked in its inactive state (inverse agonist). Several retinal analogs with defined configurations and stereochemistry have been incorporated into the apoprotein to give rhodopsin analogs. These incorporation results along with the spectroscopic properties of the rhodopsin analogs clarify the mode of entry of the chromophore into the apoprotein and the biologically relevant conformation of the chromophore in the rhodopsin binding site. In addition, difference UV, CD, and photoaffinity labeling studies with a 3-diazo-4-oxo analog of 11-*cis*-retinal have been used to chart the movement of the retinylidene chromophore through the various intermediate stages of visual transduction. © 2004 The Japan Chemical Journal Forum and Wiley Periodicals, Inc. Chem Rec 4: 120–135; 2004: Published online in Wiley InterScience (www.interscience.wiley.com) DOI 10.1002/tcr.20000

Introduction

Figure 1 illustrates the cross-section of a human eye. The estimated 7 million cone cells, responsible for color ("photopic") vision are clustered around the fovea where the incoming image is focused most sharply. The cone 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). A yellow solution absorbs the blue light at 450 nm so that the light that enters our eyes is green and red, which when mixed gives a yellow color. On the other hand, the 100 million rod cells responsible for black and white ("scopotic") vision are mostly distributed in the peripheral area 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, cows and sheep (4 million rods), horses, and dogs only have rods and hence do not have color vision.

The outer segments of rod cells (rod outer segment or ROS) consist of about 1500 thin disks that are formed at the base of the rod visual cells by pinching off sections of the plasma membrane. The disks, which embed the 40 kDa Rh molecules, move towards the tip of the cell where they are phagocytosed by the retinal pigment epithelial cells (RPE), thereby regenerating a photoreceptor cell in about 15 days. Phagocytosis of debris leads to accumulation of orange fluorescent pigments, A2E and others, resulting in photo-toxic

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stress to the RPE which may underlie some incidences of age-related macular degeneration (AMD).[1]

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 from the rod inner segment (RIS) into the rod outer segment (ROS) (see Fig. 1). Upon absorption of one photon of light, the flow of more than a million Na^+ ions is blocked, leading to a 1 mV rise in potential in the rod photoreceptor. The vertebrate visual transuction cascade is outlined in Figure 2. Namely, absorption of a single photon activates ~1000 molecules of G protein (guanyl-nucleotide binding protein; in the case of vision the term transducin is used), which via activation of phosphodiesterase, hydrolyzes 100,000 molecules of cyclic GMP. This drop in cGMP closes the cation-specific ion channels and leads to a build-up of electric potential that is picked up by the optic nerve.[2,3]

There are several unique attributes of the 11-*cis*-retinal that serves as the skeleton for all visual pigments.[4,5] Condensation of the aldehyde group with the ε-amino group of Lys296 rapidly generates a very stable protonated schiff base with a pK_a estimated at ~16.[6] The positive charge on the nitrogen can be delocalized throughout the polyene depending on the distance from the counterion[7] and the local electrostatic field within the protein cavity. The chromophore is non-planar around bonds 6/7 and 12/13 (see figure 3A), the degree of non-planarity depending on the receptor opsin. In turn, the extent of non-planarity allows for the adjustment of the λ_{max} of each individual pigment. The 9-methyl group is needed for hydrophobic binding and stabilization of the rhodopsin groundstate[8] and as a trigger for certain proton transfer events needed to reach the active MII state.[9] The 13-methyl group is also crucial for inducing some out-of-plane distortion around the 12/13 bond which apparently drives the ultra-fast photoi-

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Nina Berova received her Ph.D. in chemistry in 1971 from University of Sofia, Bulgaria. She became in 1982 an Assoc. Prof. at the Univ. Sofia and at the Inst. Organic Chemistry, Bulgarian Academy of Sciences. In 1988 she joined the Department of Chemistry of Columbia University, New York, first as a Visiting professor, and later she accepted her current position of Research Professor at the same Department. She has been recipient of many scholarships, among them, in 1989–1992 a research Fellowship at University of Bochum, Germany, a visiting Professorship in 1994 at Ecole Normal Superieure de Lyon, a Lectureship in 1996 by the Japan Society for Promotion of Science (JSPS), and more recently visiting Professorships at University of Naples, Univ. Santiago de Compostela, Spain, Tokyo Institute of Technology and at the University Louis Pasteur, Strasbourg. Her research is focused on organic stereochemistry and chiroptical spectroscopy, in particular, on the elctronic Circular Dirchroism and its application in structural analysis. In 2000 she was co-editor and co-author of a comprehensive monograph "Circular Dichroism: Principles and Applications" (first edition 1994), co-ed. K. Nakanishi and R.W. Woody, Wiley-VCH. Since 1998 she is a North-American Editor of Whiley-VCH Journal "Chirality". ■

somerization and maintains a high quantum yield in the rhodopsin pigment.[10]

The C_{20} 11-*cis*-retinal, biosynthesized from the C_{40} β-carotene, is the basic structure of all visual pigments. The β-carotene produces C_{20} vitamin A (all-*trans* retinol), the precursor of retinal. However, since humans cannot biosynthesize carotenoids, we have to depend on exogenous sources for vision. The chromophores used by some non-human animals are depicted in figure 3B. The visual chromophore of salmons is 11-*cis*-retinal in fresh water but during their migratory period in the ocean, the chromophore becomes 3,4-dehydro-11-*cis*-retinal to induce a red shift in the vision more suited for the ocean where there is less light. The chromophore of the tadpole is also 3,4-dehydro-11-*cis*-retinal, which again becomes 11-*cis*-retinal in the frog. Squids have an additional 4-α-hydroxyl group[11] while insects have 3β-hydroxy-11-*cis*-retinal,[12] the chromophore possibly responsible for their UV sensitivity.

The rhodopsin visual cycle, the subject of intense study over the years, is outlined in Figure 4 together with the cycling pathway of vitamin A. Irradiation of Rh at 500 nm causes 11-*cis* → *trans* isomerization to yield photo-Rh in less than 200 femtoseconds during which the 11-*cis* becomes a highly distorted *trans* geometry.[13,14] 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,[15] is reflected in the red-shifted absorption maxima of photo-Rh (570 nm) and batho-Rh (543 nm). Batho-Rh was detected as early as 1958[16] and more properly characterized by absorption spectra measured in liquid He, a temperature at which it can be sequestered.[17] Microscopic rate constants coupled with time-resolved spectroscopy led to the finding that a blue-shifted intermediate[18] (BSI, not shown) is in equilibrium with batho-Rh. After batho-Rh the pigment relaxes to lumi-Rh, meta-I Rh, and at the meta-II stage the conformation of extramembrane loops on the cytoplasmic side excites the G protein, thus initiating a cascade of enzymatic reactions.[19]

The bleaching of Rh to the active meta-II Rh allows the protein cavity to become more solvent accessible, and following a reprotonation of the Schiff base (Meta-III Rh[20,21]), imine hydrolysis releases all-*trans*-retinal from opsin. All-*trans*-retinal is then reduced to *trans*-retinol or vitamin A by alcohol dehydrogenase and is carried to the retinal pigment epithelium (RPE), where it is esterified with fatty acids by lecithin retinol acyltransferase (LRAT). Hydrolysis of the retinyl esters provide the uphill 4 kcal/mole energy needed to activate the isomerase leading to 11-*cis* retinol. This is oxidized to 11-*cis*-retinal which then binds to opsin to regenerate Rh.[22]

Rh is one of the most thoroughly investigated G protein coupled receptors (GPCR)[23] consisting of seven hydrophobic transmembrane helices A, B, . . . G (or 1, 2, . . . 7) interconnected by hydrophilic extramembrane loops. Bovine Rh is the most thoroughly studied of all the GPCRs because it is readily accessible from bovine eyes; the majority of studies on Rh are from this source.

Chromophore Binding and Dynamics Studies with Photoaffinity Labeling

Our studies toward understanding the mode of binding of the chromophore in the transmembrane region of opsin began in the early 1990's. Diazoketo-ret6 (see Figure 5B) was synthesized with a photo-activatable diazo ketone poised to

Koji Nakanishi was born in Hong Kong in 1925, and brought up in Lyon, London, and Alexandria until 11, He received his B.Sc., Nagoya University in 1947 from Fujio Egami. Following two years of post-graduate work on a GARIOA fellowship (predecessor of Fulbright fellowship) 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, and served as Chairman of the Chemistry Department, 1987–90. He was a founding member and a Director of Research at the International Centre of Insect Physiology and Ecology (ICIPE) in Kenya, 1969–1977. In 1978 he was appointed Director of the Sountory Institute for Bioorganic Research (Sunbor), Osaka, and served until 1991. From April 2001, he was appointed the first director of the chemistry unit at Biosphere 2, Arizona, Columbia Unviersity, until December 2003 when it was closed, Since January 2003 he is Editor-in-Chief of The Chemical Record. ■

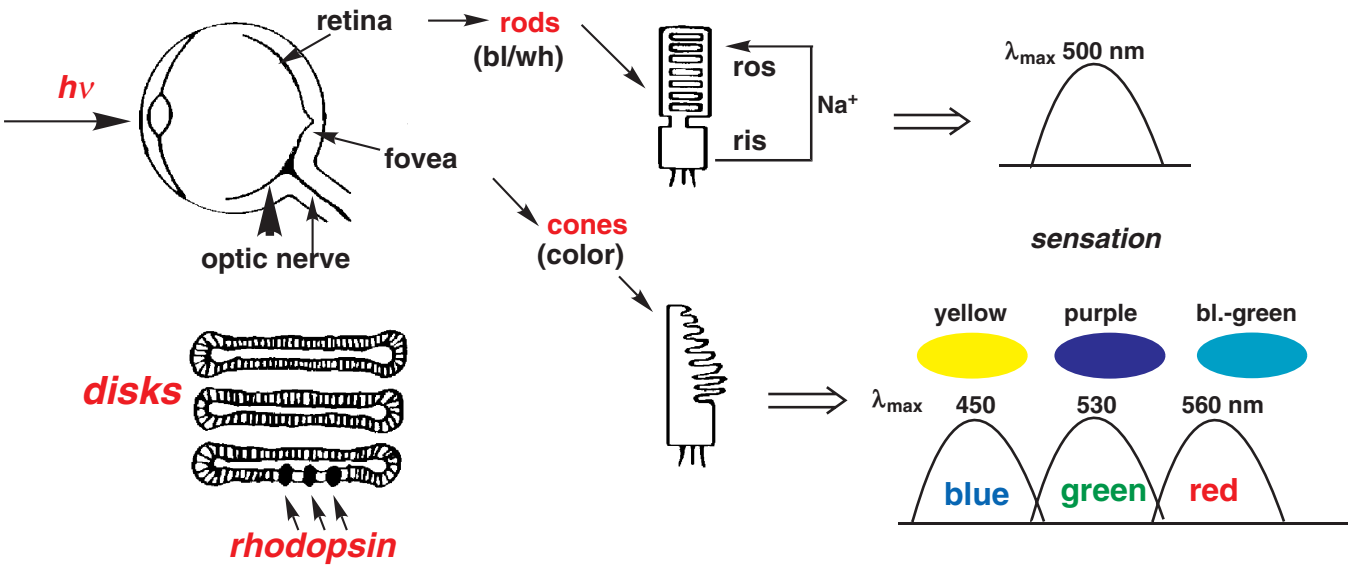

Fig. 1. Cross section of the human eye showing the topology of rods (dim light sensitive) and cones (bright/color light sensitive).

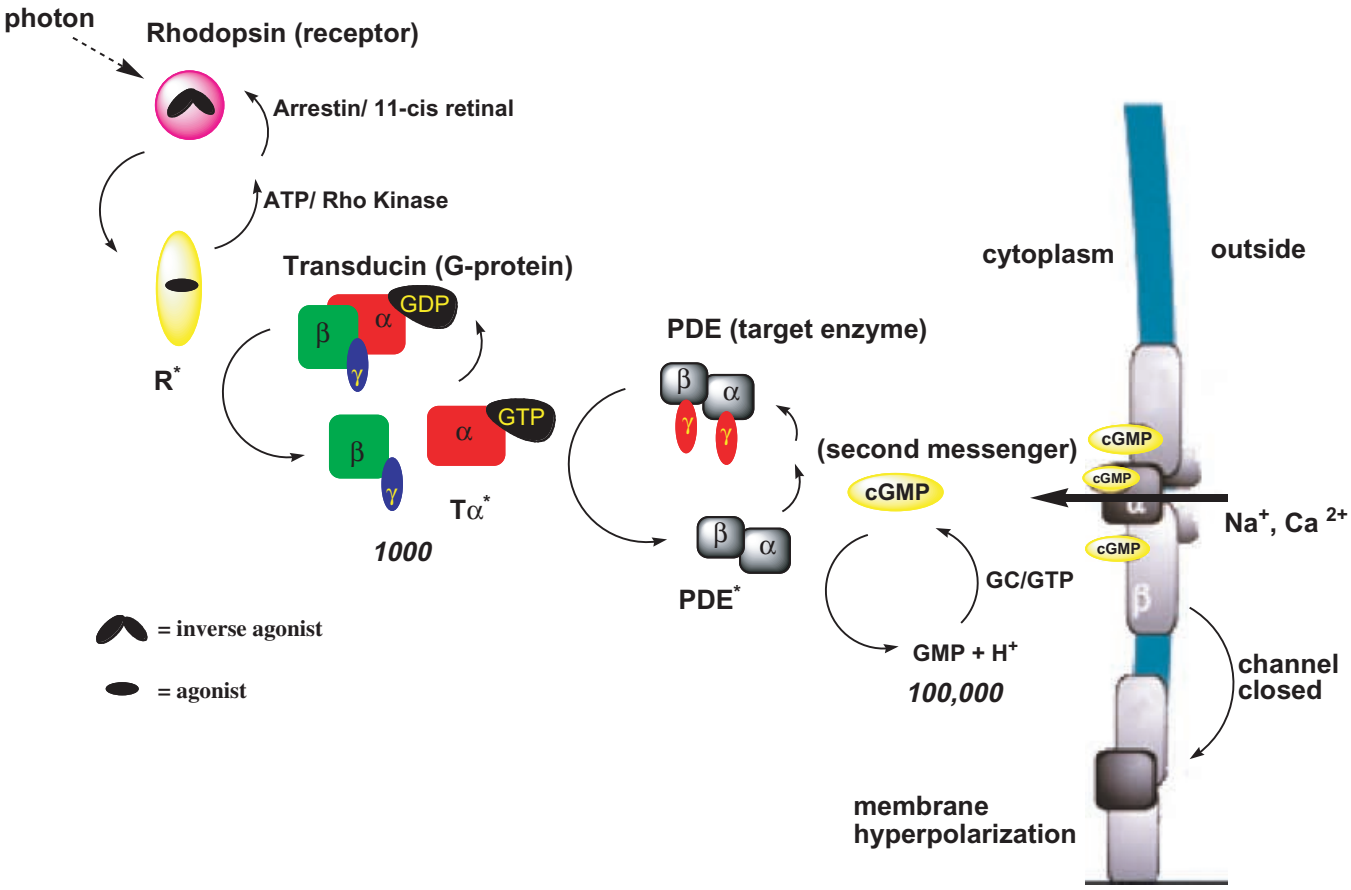

Fig. 2. Schematic overview of the vertebrate visual transduction cascade.

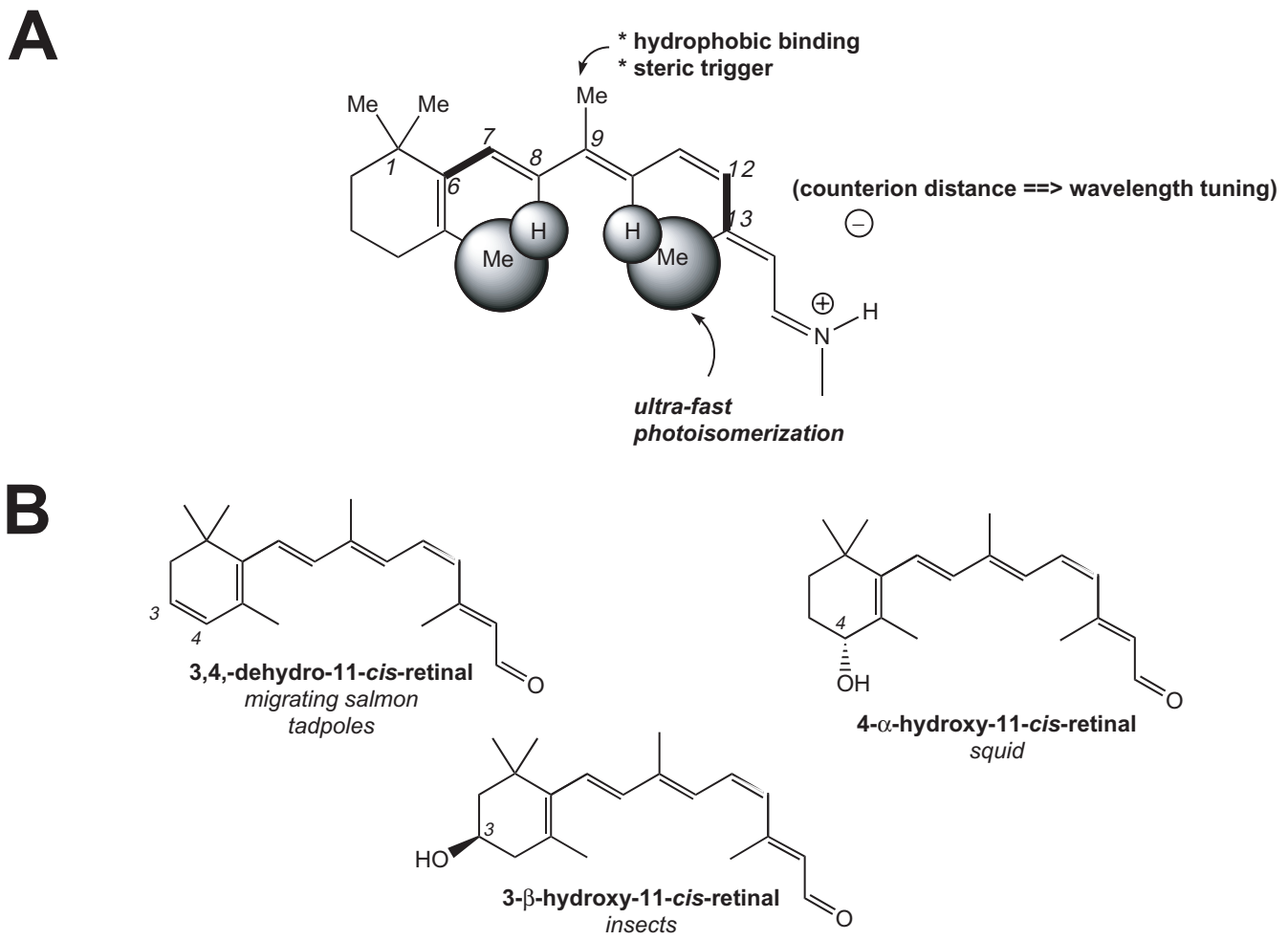

Fig. 3. A) Unique attributes of 11-*cis*-retinal in wavelength tuning B) Three modified chromophores used in squid, salmon, tadpoles, and insects.

crosslink into the β-ionone binding site, while a six membered ring constrained the C10—C13 torsions in order to retain the 11-*cis* geometry in the opsin binding site upon activation of the photolabel.[24] After receptor cleavage with V8 protease and CNBr, Edman sequencing revealed that the 3-position of the ionone ring is proximal to Trp-265 and Leu-266; diazoketo-ret6 was the first to clarify the ground state mode of binding of the chromophore in Rh.

The fact that both the labeled Trp-265/Leu-266 residues and the Schiff base linkage to Lys-296 are in the middle of the lipid bilayer showed that the chromophore in ground-state Rh has the polyene chain essentially parallel to the membrane plane and that the entire chromophore resides near the center of the bilayer, with the polyene long axis slightly tilted relative to the membrane plane. This orientation of the chromophore favors maximal exposure of the polyene chain to the incident light.

After many years of effort by various groups, the first X-ray crystallographic structure of Rh was secured in 2000 by Okada and coworkers.[25] In Figure 5B the chromophore is, indeed, seen with its ionone ring close to Trp265 in helix F.

Two years later, Okada and Shichida generated a higher resolution rhodopsin structure, which succeeded in showing the critical role played by 6 water molecules in the transmembrane region.[26] It was suggested that the water molecules located in the vicinity of highly conserved amino acid residues in the binding site participate in salt bridge stabilization. The formally bound water molecule located between Ser186 and Glu181 has been recently identified as being involved in a hydrogen-bonded network that transfers a proton to the primary counterion Glu113 in the Meta-I state, switching the PSB counterion to Glu 181.[27] These water molecules are also likely involved in the regulation of Rh activity and spectral tuning, i.e., regulation of absorption maxima of visual pigments.

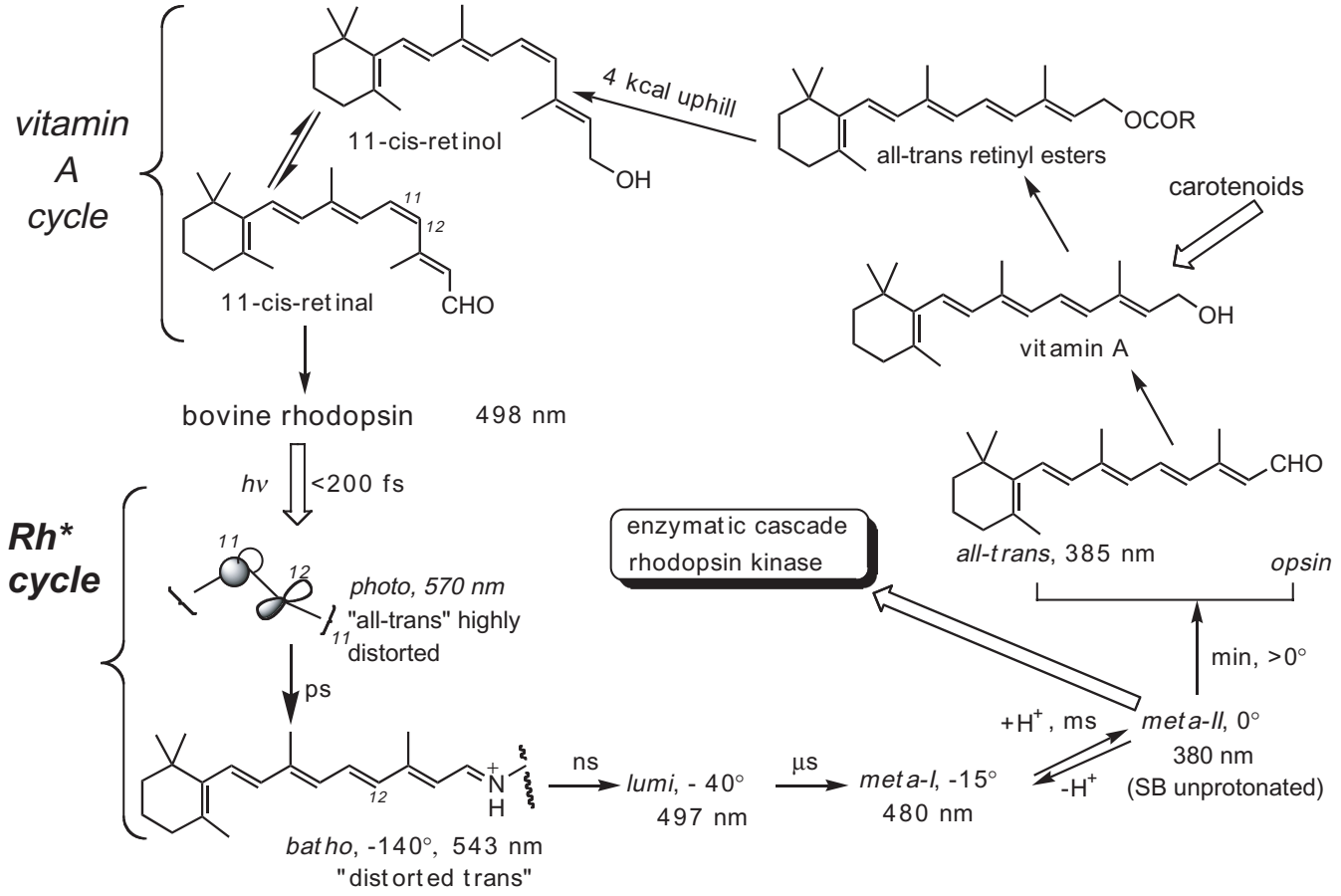

Fig. 4. The visual transduction cycle and vitamin A cycle.

Photocross-Linked Sites Along the Visual Transduction Path

Our next objective was to chart the movement of the chromophore through the various intermediates of the Rh bleaching process, namely batho-, lumi-, meta-I and meta-II Rhs (Figure 4). For this purpose, (11Z)-3-diazo-4-oxo [15-$^{3}H_{1}$]retinal (see Figure 6, structure with —CTO instead of —CHO) containing two photoactive groups, the diazoketo and 11-*cis*-ene, was selected as the retinoid. This unstable retinoid was first prepared by submitting the all-*trans* diazoketone to retinochrome, an enzyme contained in squid eyes that isomerizes all-*trans*-retinal into the 11-*cis* form.[28] Because of the presence of this isomerase in its eye,[29,30] the photocycle of squid vision does not require exogenous replenishment of retinal. Retinochrome indeed performed the expected isomerization but this route was abandoned because the scale was limited to microgram quantities.

An efficient and general protocol for *cis*-hydrogenation of an 11-yne group to 11-*cis*-ene in retinoids was developed employing Cu/Ag activated Zn dust.[31] This route was then employed to make the light sensitive and unstable 11Z-diazoketo retinal.

As depicted in Figure 6, the Rh analog incorporating diazoketo-11-*cis*-retinal, diazoketo-Rh (DK-Rh) has similar UV/VIS and CD spectra to those of native Rh. This suggested that the induced chiral fit of diazoketo-retinal into the binding site, despite presence of the 3-diazo-4-keto functionalities, was similar to that of 11-*cis*-retinal. The leniency of the ionone binding site was not entirely surprising as it was shown earlier that even adamantyl allenic retinoids could be readily incorporated into opsin to give the corresponding Rh analog.[32]

The bleaching intermediates of native Rh are shown together with the corresponding intermediates of DK-Rh (UV difference measurements A-D, Fig. 7). DK-Rh was dissolved in 66% v/v glycerol and irradiated by UV in the glass-like state.

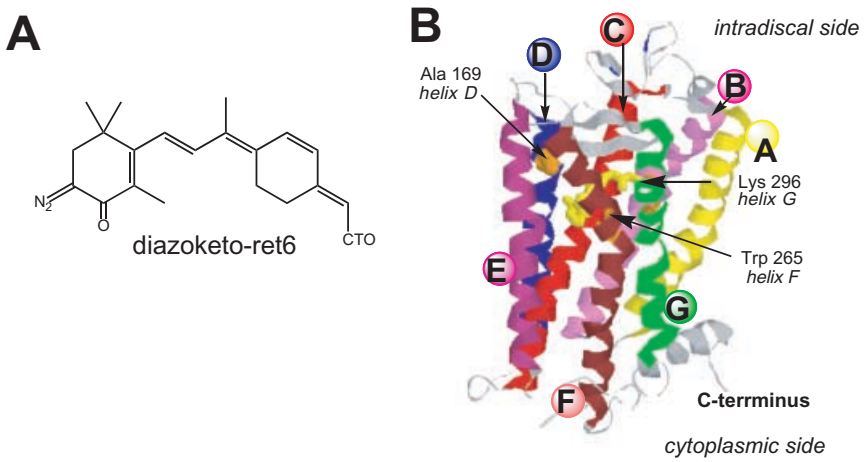

Fig. 5. A) Diazoketo-ret6 used to label the ground state ionone ring binding site in rhodopsin B) 2.8 Å resolution x-ray structure of bovine rhodopsin showing the ionone ring proximal to Trp265.

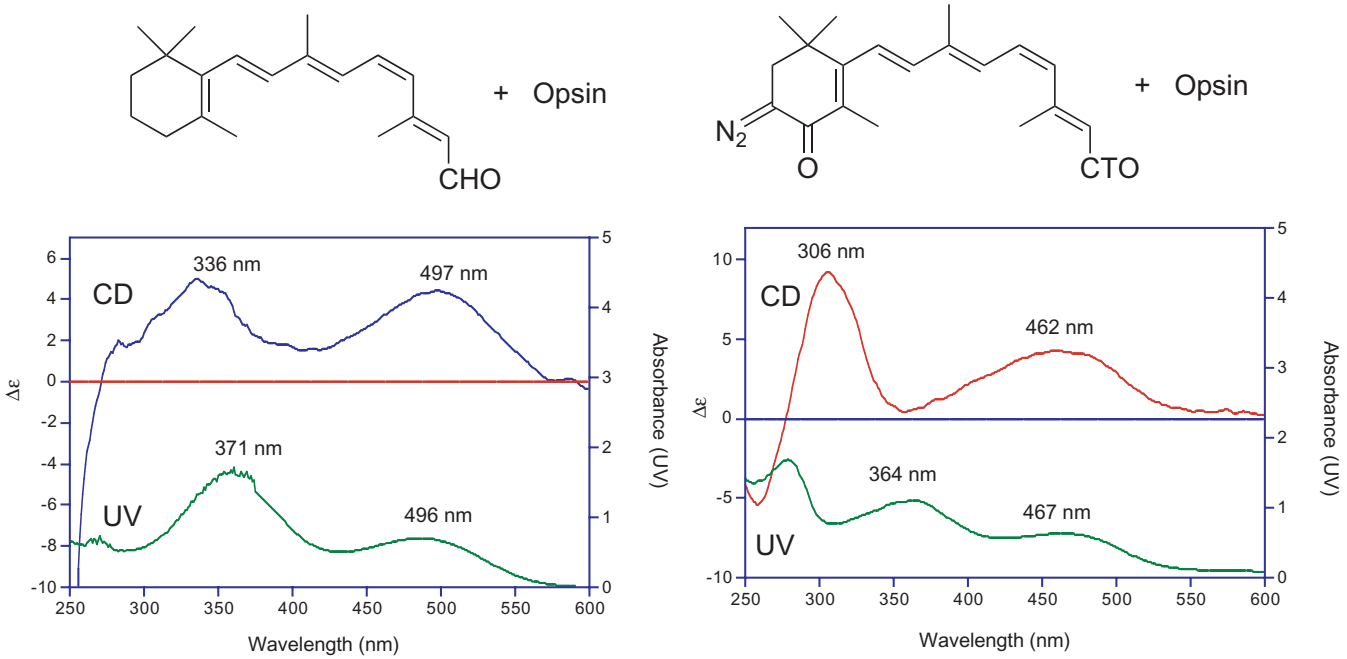

Fig. 6. UV/VIS and CD of Rh and Diazoketo-Rh (in CHAPSO / HEPES, pH 7.0).

Irradiation with 436 nm light at −196°C caused a red-shift in the absorption leading to a batho-Rh intermediate. Gradual temperature elevation showed formation of the three additional intermediates, Lumi-, Meta I- and Meta II-Rh. It should be noted that in order to trap the analogous intermediates of DK-Rh, lower temperatures were required than for the native rhodopsin intermediates.

The photocross-linking was performed with the four intermediates at the temperatures indicated. For example, photocross-linking of DK-lumi-Rh, with an optimal temperature of −80°C, was performed as follows: 1) the (11Z)-DK retinoid was incubated in the dark in 67% glycerol with opsin at room temperature for 10 min to give DK-Rh, λ_{max} 467 nm; 2) DK-Rh was irradiated with 480 nm light for 5 min at −196°C to yield DK-batho, 510 nm; 3) the temperature was raised to −80°C to populate the DK-lumi state; 4) the lumi-Rh conformation was then maintained by freezing to −196°C and irradiated with 254 nm light for photocross-

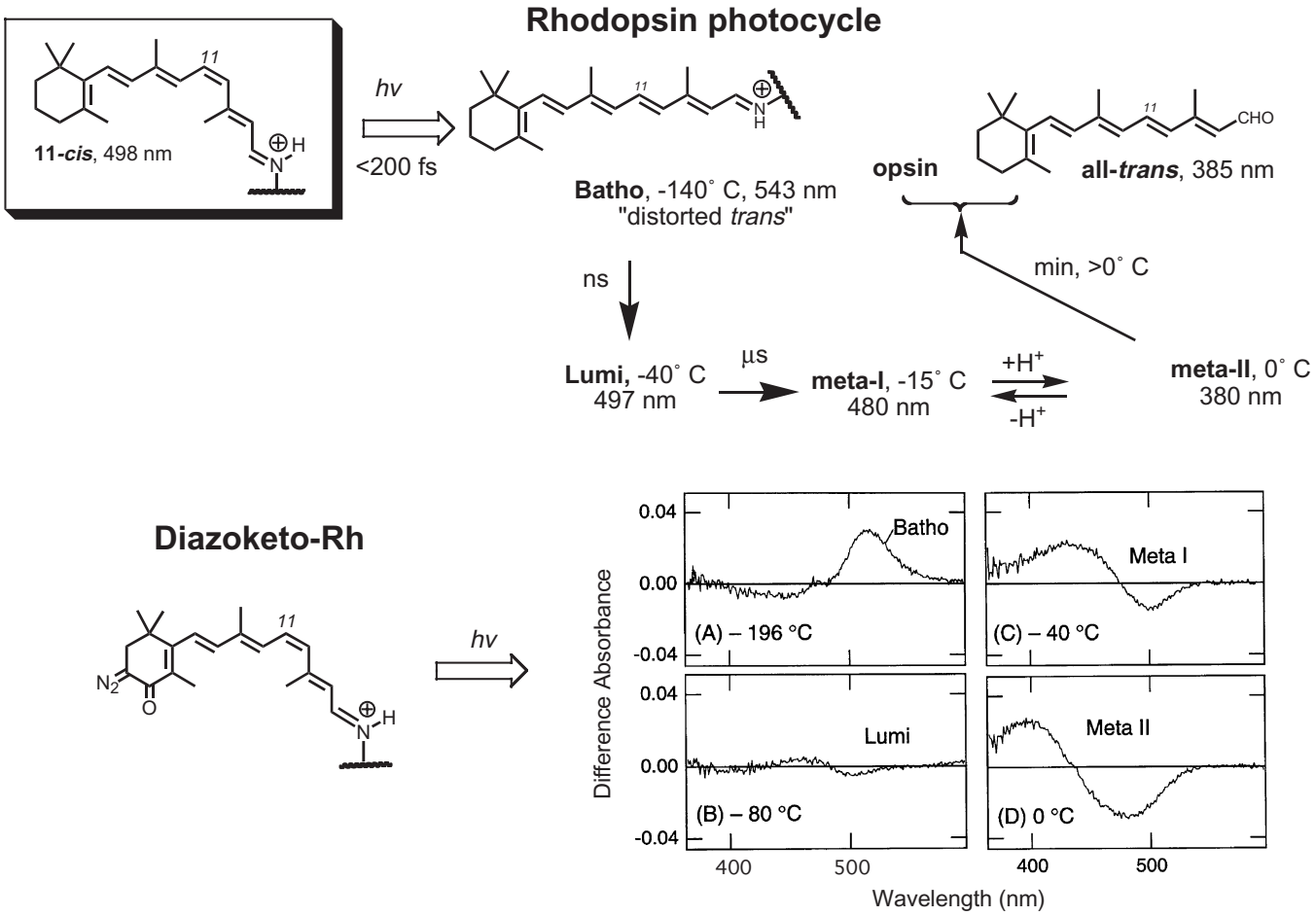

Fig. 7. Rhodopsin photocycle and the corresponding bleaching intermediates of Diazoketo-Rh.

linking. The glycerol was removed by precipitating the receptor with trichloroacetic acid. The Rh analog was submitted to cyanogen bromide cleavage and the fragments were analyzed by HPLC.[33]

Figure 8 shows a representative HPLC trace from the digestion of crosslinked DK-Rh. Small peaks with vertical bars around 33 min and 58 min in the HPLC trace were radioactive and indicated the CNBr-cleaved peptide fragments that contained the tritiated retinoid. The radioactive peptide at 58 min, resulting from crosslinking at the Rh and Batho-Rh stages comprised part of helices F/G, while the radioactive fraction at 33 min from the lumi, meta-I and meta-II-Rh experiments resulted from helix D. Edman degradation of each radioactive peptide showed that the cross-linked site in Rh and batho-Rh was cleanly only one amino acid, Trp265, while that in lumi, meta-I and meta-II-Rh was only Ala169.[33] As depicted in the schematic drawing in Figure 8, this suggested that in the transition from batho-Rh to lumi-Rh, helix C moves out and helix D rotates so that Ala169 is presented to the ionone ring in the binding site; simultaneously the 11-*cis* to *trans* isomerization of the retinoid is accompanied by the flip-over of the ionone ring as depicted so that C-3 now becomes close to Ala169.

Recent molecular dynamics calculations performed by Ishiguro[34] support our experimental photolabeling result that the major relaxation or elongation of the chromophore takes place in the batho to lumi transition. However, the calculated model of the lumi intermediate of native rhodopsin showed that Ala169 was actually opposite the cyclohexenyl binding site and not close to C-3 at all. Ishiguro suggests that the diazoketone group used in our studies caused a steric interaction with helix D. The calculated model of the lumi state with a 3-diazo-4-oxo group on the ionone ring showed deformation of the Cys167-Pro171 region of helix D, and in the deformed helix,

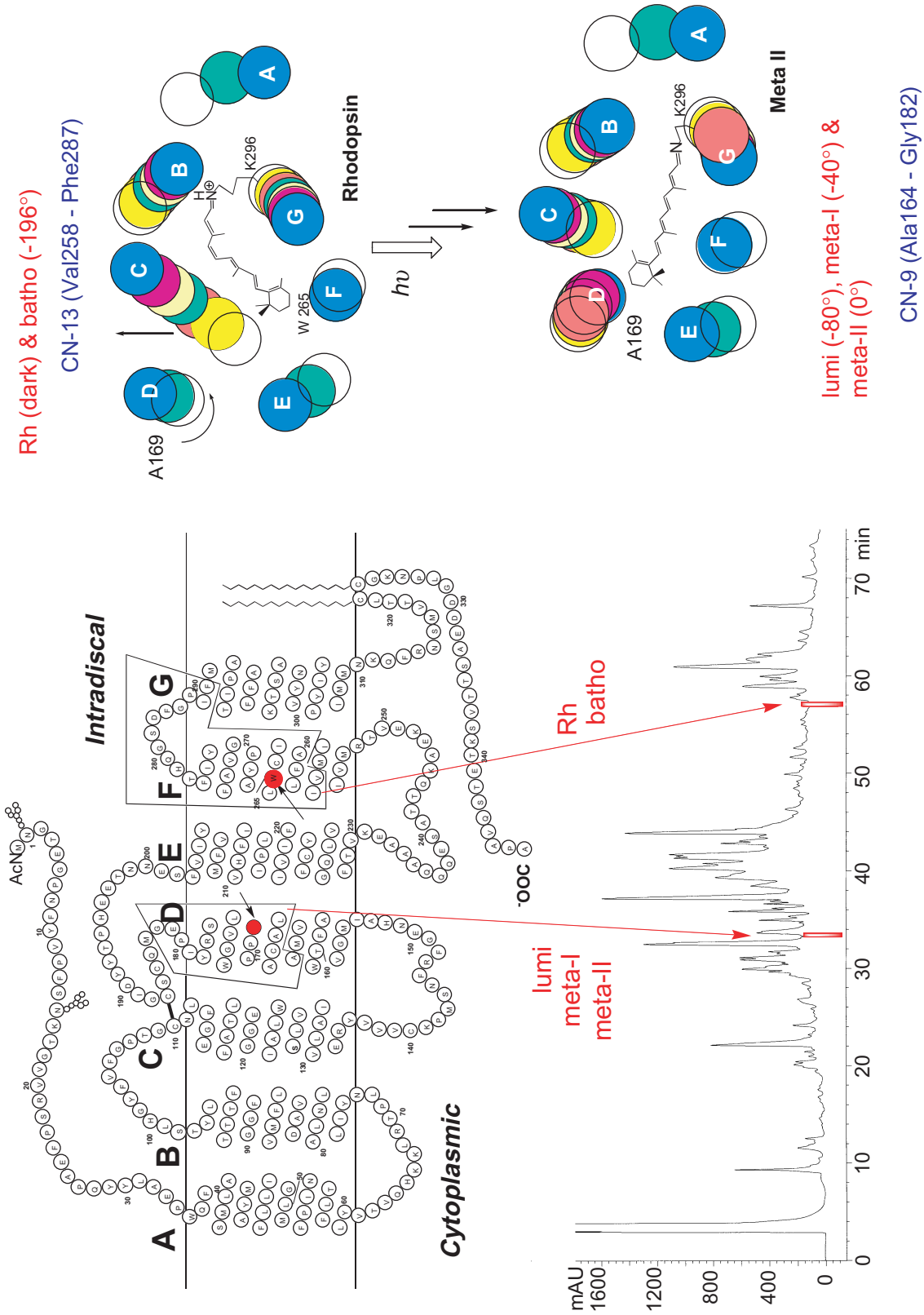

Fig. 8. Reverse phase HPLC profile showing the radioactively labeled peptide fragments at each stage of the visual transduction path.

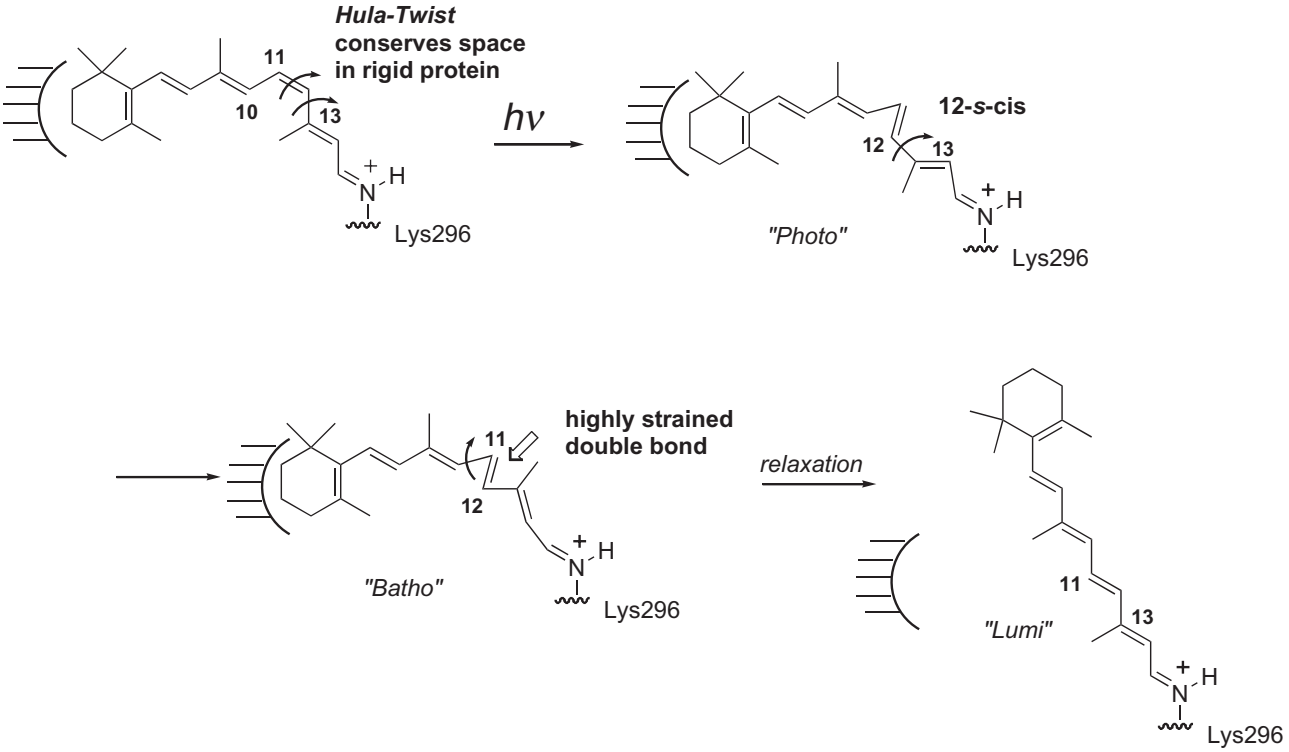

Fig. 9. Putative movements of the rhodopsin chromophore during the various photo-intermediates. Adapted from ref. 32. Arrows show the direction of motion in the indicated bonds.

Ala169 can be exposed to the reactive photolabel. This proposed perturbation of helix D in the later photo-intermediates of the DK-Rh pigment may explain why DK-Rh crosslinked in the physiologically active meta-II state shows a 70% initial rate increase in transducin activation over the non-crosslinked sample. (N. Fishkin, T. Furuta, K. Nakanishi, unpublished). Covalent attachment of Ala169 to the chromophore may trap this perturbed conformation of rhodopsin, leading to the additional agonism we observed.

Ishiguro's simulation derived chromophore movements (CVFF parameters in Discover 3) involved in the 11-*cis* to all-*trans* photo-isomerization from ground state rhodopsin to lumi are shown in Figure 9. The initial photo-isomerization is now believed to involve σ-bond rotation along C12—C13 in addition to the C11—C12 double bond isomerization, a space conserving "Hula-Twist" mechanism proposed initially by Liu.[35] Work by several groups is under way to experimentally determine the structures of the various photo-intermediates of rhodopsin using x-ray crystallography, and perhaps soon we will have a more detailed picture of how retinal moves inside the binding site.

Defining the Absolute Twist Around 6-*s*-cis Bond in the Retinylidene Chromophore

The 6α- and 6β-Locked (11Z)-Retinals

In the last few years, we have successfully exploited the phenomenon of enantioselective binding in opsin in order to determine the absolute sense of twist of the retinal chromophore in the ground state pigment.

The two conformationally locked retinals **1** and **2** shown in Figure 10 were synthesized in order to determine the absolute sense of twist around the C6—C7 bond in the ground state pigment.[36] As shown in Fig. 10-A, the α-enantiomer **1** was smoothly incorporated into opsin and gave rise to a CD spectrum (Fig. 10-C) similar to that of native Rh (Fig. 10-D). In contrast, the β-enantiomer **2** (which has a positive C6—C7 torsion angle) did not bind (Fig. 10-B). From these results, it was concluded that the 6-*s* bond in the native chromophore is twisted as depicted in the stereo drawing of the lowest energy conformation of **1** (Fig 10-E). Namely, the C5—C8 dihedral is −35° in the locked analog used in our study which is comparable to the angle of −52.8° later found for the chromophore in the refined crystal structure of

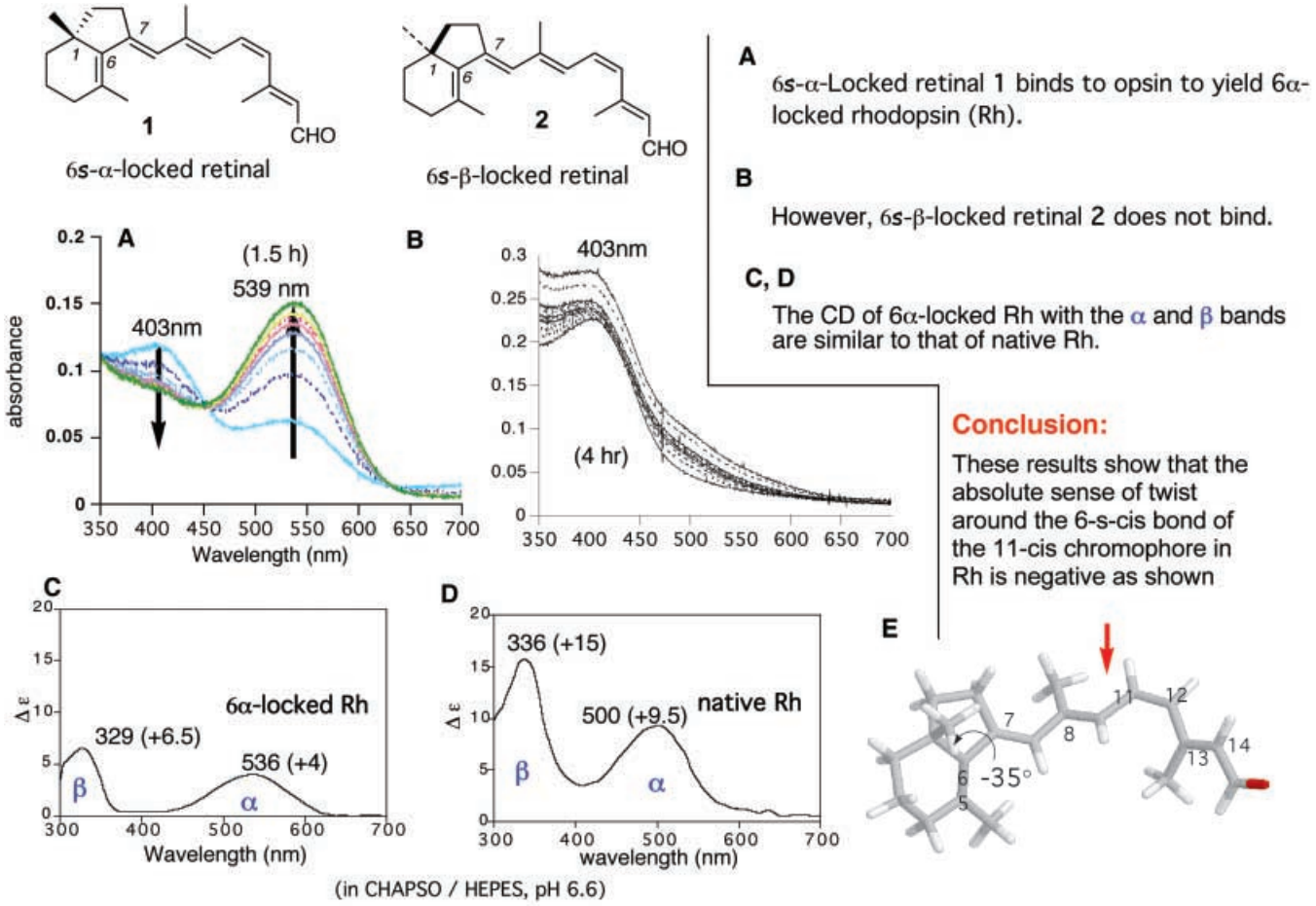

Fig. 10. A) UV binding curve upon incubation of 6s-α-locked retinal **1** with opsin recorded over 1.5 h B) UV binding curve upon incubation of 6s-β-locked retinal **2** with opsin over 4 hours C) CD spectrum of 6-α-locked Rh pigment D) CD spectrum of native Rh.

rhodopsin.[37] While the sense of twist was defined by 6α-locked retinal **1**, the magnitude of the twist angle for this analog is smaller than that of the native chromophore, which is reflected in the larger degree of polyene planarity and the red shifted absorption for **1** in solution and bound to opsin (403 nm in EtOH, 539 nm in opsin). During this study, we also gained information on the conformation around the C6/C7 bond in the Meta-II state. Namely, a transducin activation assay showed that the rhodopsin pigment containing the 6α-locked compound **1** exhibited 80% activity of the native chromophore, implying that the conformation around the 6-s-*cis* bond is not required to change (i.e. to s-*trans*) in the steps leading up to visual transduction.

(11Z)-Locked Cyclopropyl Retinal and its Binding to Rh: Determining the C12—C13 Twist

The chromophore of (11Z)-locked cyclopropyl retinal-7 (CPR ret7)[38] consists of a cyclopropyl group flanked by polyene and enal moieties, and hence was expected to clarify the little known conjugative properties of the cyclopropyl group (CPR). As seen in Figs. 11-A and 11-B, the CD were of the exciton coupled bisignate type thus showing that the conjugative effect of CPR was minimal. Calculations also showed that two distinct conformations around the C6—C7 bond exist for this molecule and the observed CD is actually a superimposition of opposite sign exciton couplets originating from two diastereomeric populations.

Only the depicted 11β,12β-CPR ret7 enantiomer (Fig. 11-A), but not the corresponding α-CPR, binds to opsin (Fig.

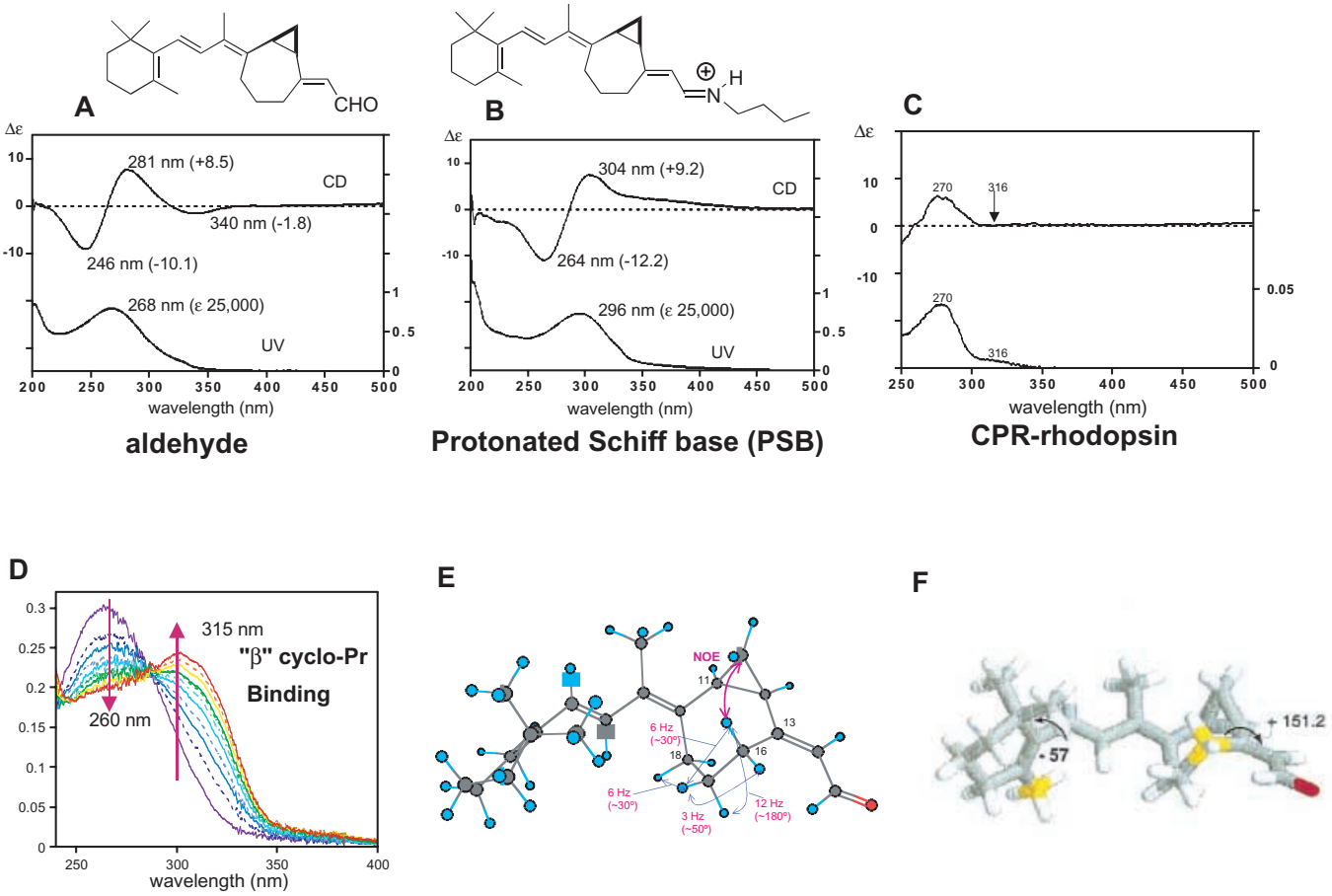

Fig. 11. UV/CD spectra of A) β-CPR-ret7 aldehyde B) protonated schiff base and C) CPR-rhodopsin D) UV binding data for β-CPR-ret7 incubated with opsin E) NMR derived constraints for β-CPR-ret7 F) Jaguar 4.1 optimized conformation, showing a positive C12—C13 dihedral.

11-D). The UV of CPR ret7-Rh (Fig. 11-C) showed extrema at 270 nm (mainly opsin absorption) and a shoulder at 316 nm from the protonated Schiff base in the protein, which is only a modest red-shift from that of the protonated Schiff base formed with butyl amine (PSB, Fig. 11-B, 304 nm). This is unlike the case of other retinoids that exhibit their pigment extrema at much longer wavelengths than the PSB in solution, e.g., the PSB between retinal and butylamine absorbs at 440 nm whereas Rh absorbs at 500 nm. This result further supports the finding that the presence of the β-CPR moiety causes a significant break in conjugation in the chromophore. In addition, the lack of any CD band at 316 nm suggests that once the β-CPR-ret7 passes through the binding cleft, it may undergo a compression effect in the transmembrane binding site which flattens the chromophore, thereby reducing its optical activity.

Detailed NMR measurements by DQF-COSY, NOESY and HSQC, and energy optimizations by B3LYP/6–31G** (Jaguar 4.1) led to solution conformations E and F (Fig. 11) in which the C12/C13 bond is slightly positively twisted. Namely, the opsin binding cleft accommodates the β-CPR retinoid with a slightly positive twist or with the 13-Me in front of the retinoid plane (see the stereo view in Fig. 11-F). The calculated C11—C14 dihedral angle for the lowest energy twisted chair conformation shown in Figure 11-F is 151.2° which is almost identical to that determined for the native chromophore in the x-ray structure of rhodopsin (+151.6°).[26,37] This suggests that the native 11-*cis*-retinal enters the binding cleft with a positive 12-*s*-bond twist and that its conformation in the binding site may also be positively twisted around the 12-*s*-bond.[39]

Overall Chromophore Conformation in Rh

Results with the 6α-locked (11Z)-retinal (Figure 10) and (11Z)-locked CPR ret7 (Figure 11) now define, respectively,

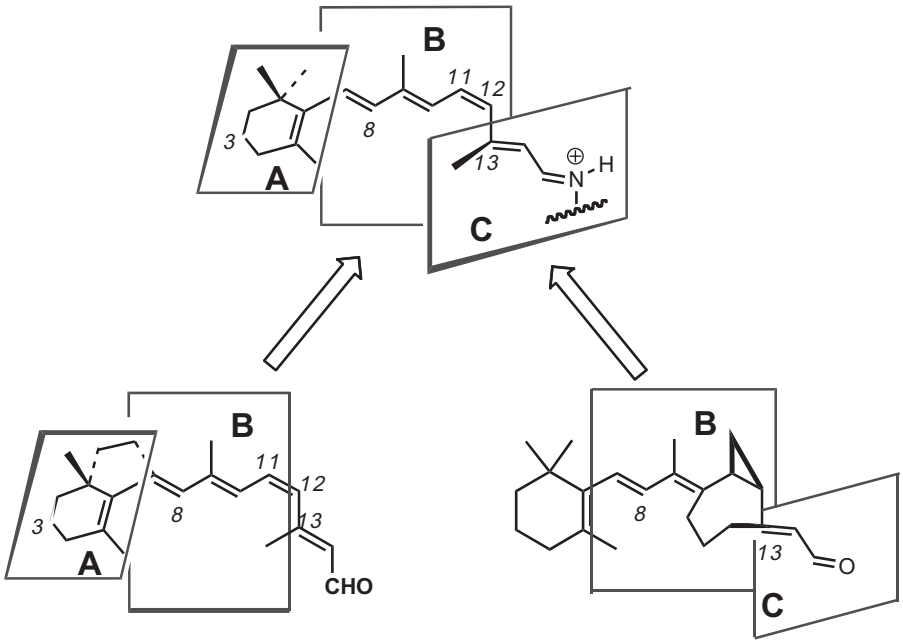

Fig. 12. Chromophore conformation in dark Rh based on combined data from binding studies with 6α-locked retinal and β-CPR-ret7.

the biologically relevant conformation of (11Z)-retinal within the Rh binding site, as depicted by the twist between planes A/B and B/C (Figure 12).

Studies in Transient Dark Activity: Insights into the Mechanism of 11-*cis*-Retinal Entry into Opsin

In 1980, Ebrey and coworkers observed that 13-demethylretinal (13dmRet, see Fig. 13) incubated with opsin induced phosphodiesterase activity (second messenger in visual cascade; see Figure 2) even in the absense of light.[40] It was shown much later that this phenomenon, termed dark activity, is transient, and eventually results in formation of a ground state, inactive pigment.[41] The following mechanism of this dark activity was proposed based on our studies with 13dmRet, its 11,12-dihydro analog, and a further analog, ret6.[42]

The opsin cavity is divided into three domains, site I for the ring moiety, site II for the polyene chain, and site III for the protonated Shiff base (PSB).

Step I: The β-ionone ring of 11-*cis*-retinal settles into opsin site I first. This is based on the observation that pretreatment of opsin with β-ionone completely blocks any formation of a PSB when 11-*cis*-retinal is applied.

Step II: Transient dark activity requires schiff base formation, but protonation of the imine nitrogen immediately destroys the transient activity. When SB formation is blocked by dimethylation of Lys296 there is no transient activity.[41] Moreover, it was found that as PSB formation in the 13dmret pigment increases, there is a corresponding decrease in transient transducin activation (see Fig. 13-A).

Step III: As the chromophore enters the binding site with its cyclohexene ring in site I and the unprotonated Schiff base in site III, the polyene enters site II and adjusts its torsional angles. It is at this intermediate stage when the flexible polyene chain is about to settle into site II that transient activity is detected (Fig. 13-C). Interesting, the 13dmRet pigment shows a characteristic meta-II-like CD (negative band around 280 nm[43]) during the dark activity period. The ring-locked and rigid ret6 cannot achieve this and hence induces no dark activity (see Fig. 13-B). In addition, no dark activity has been reported for 11-*cis*-retinal; an explanation for this is that since the binding of 11-*cis*-retinal is 10-fold faster than 13dmRet, the retinal and opsin rapidly attain the inactive state.

Differences in Binding Affinity Between Ret-7 and Ret-8 Reveal Specificity in Opsin Binding Cleft

Figure 14A depicts three synthetic retinoids **3–5** that have been incorporated into bovine Rh. Ret-7 **3** binds smoothly and gives UV/Vis and CD spectra closely resembling those of native

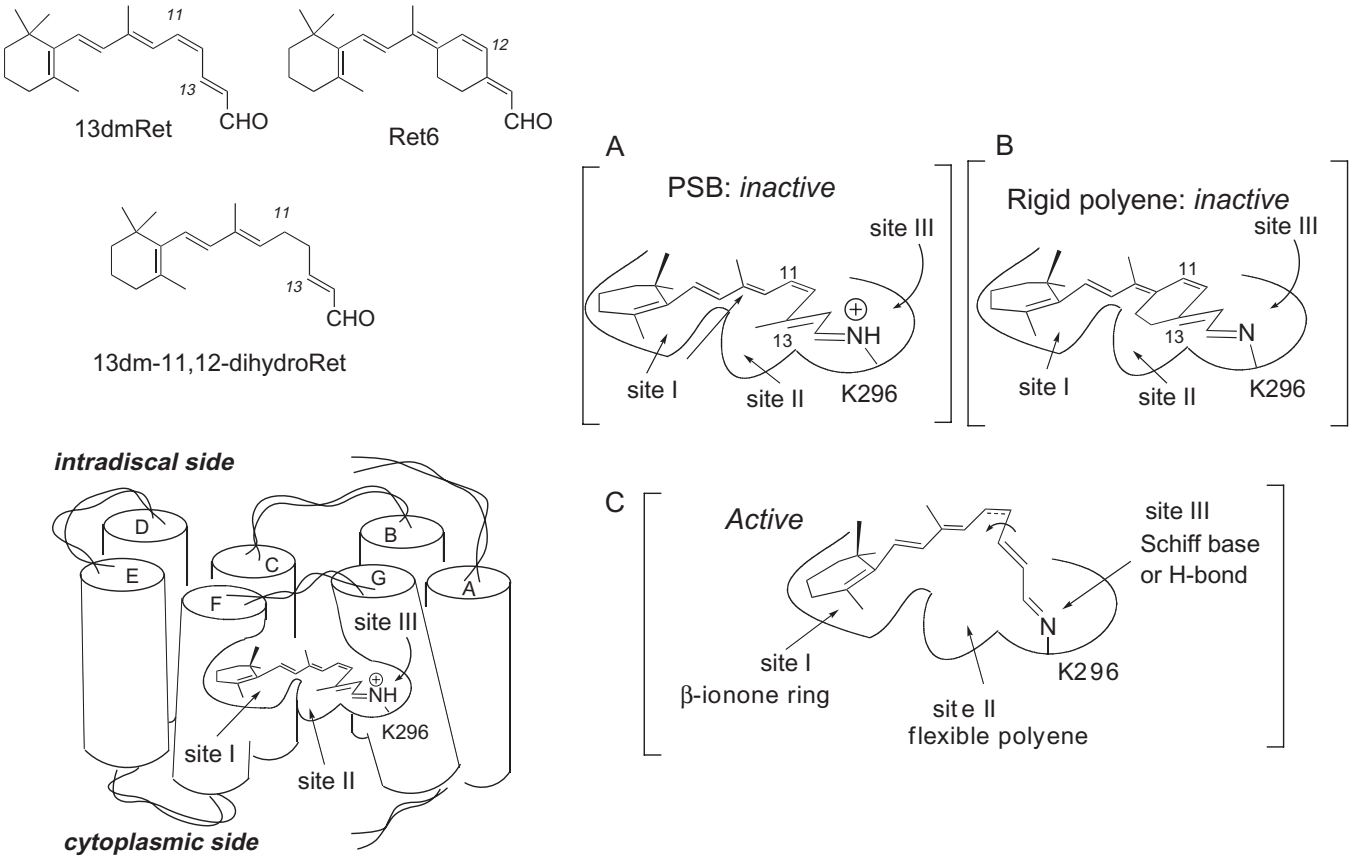

Fig. 13. Transient dark activity in opsin studied with 13dmRet, Ret 6, and 11, 12-dihydro-13dmRet. Sites I, II, and III shown bound to A) native chromophore (inactive) B) Ret 6 (inactive) C) 13dmRet/ 11,12-dihydro-13dmRet (active).

Rh.[44] This smooth entry showed that opsin has a potential cleft that readily incorporates ret-7. In contrast, ret-8 **4** was not readily incorporated; it bound to extramembrane Lys groups to give confusing results that took a few years to resolve. It was only after the extramembrane Lys groups were permethylated to leave only Lys296 free for reaction that a pigment was successfully obtained.[45] This suggested that the conformation of the 8-membered ring was hindering the entrance and hence the other surface-lying Lys residues had to be inactivated before the entry of ret-8 was possible. Since the addition of only one extra methylene unit disrupted smooth entry into opsin, we infer that the bottom portion of the retinal chromophore is a crucial determinant for successful passage into the transmembrane region. We therefore propose that 11-*cis*-retinal enters opsin from the side closer to the C-5 and C-13 methyl groups as depicted in Figure 14-B.[39] In contrast, adamantyl allenic retinal **5** seems to enter opsin easily, suggesting that site I (Figure 13) of the binding cleft is very lenient.[32] Recently, Palczewski and Hofmann proposed an entrance site for 11-*cis*-retinal on opsin based on intrinsic fluorescence measurements and analysis of binding sites for heptane-1,2,3-triol in the refined x-ray structure of rhodopsin.[46] Docking studies showed that 11-*cis*-retinal may occupy a hydrophobic binding pocket that is made up of Phe52, Pro53, Phe56, Leu57, Gln64, and Tyr60 on helix A (see Figure 5-B). In this model, the C-5 and C-13 methyl groups do point inward toward the transmembrane region.

Conclusions

Using a photolabeling approach, we have successfully charted the movement of the ionone ring portion of the rhodopsin chromophore through the intermediate stages of the protein. This is the first of such studies performed on a GPCR. Our conclusion regarding the flip over of the ionone ring in the batho to lumi stage (see Figure 14-B) is now supported by high-level theoretical calculations.[34] Enantioselective binding studies with β-CPR-ret7 and 6-α-locked retinal defined the

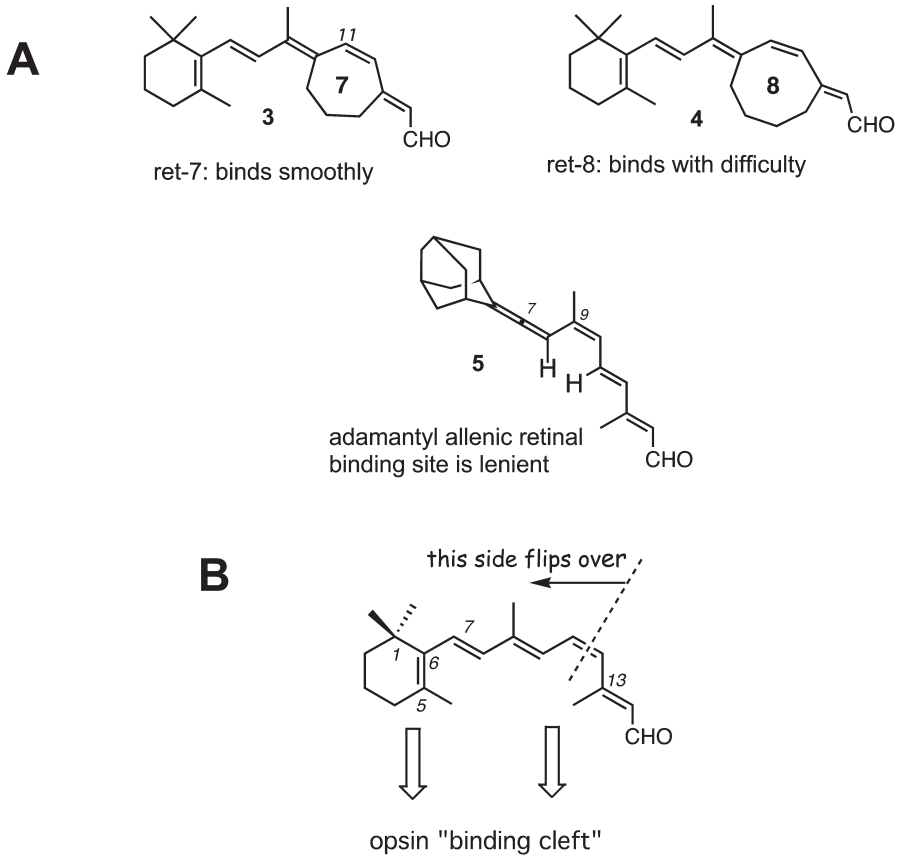

Fig. 14. A) Ret-7 **3**, Ret-8 **4**, and adamantyl allenic retinal **5** B) model showing mode of entry of 11-*cis*-retinal chromophore into opsin binding cleft.

twist sense of the retinylidene chromophore in dark adapted rhodopsin (see Figure 12), and our results generally are in agreement with those found from the crystal structure of rhodopsin.[26,37] Analysis of transient dark activity in pigments containing 13dmRet, 11,12-dihydro-13dmRet, and ret6 reveal a potential mechanism for entry of retinoids into opsin which involves three discrete binding sites (see Figure 13). Moreover, our work with ret-7 and ret-8 seems to suggest the existence of a potential binding cleft on opsin which can detect slight changes in the bottom portion of the 11-*cis*-retinal chromophore. In the future, we also hope to clarify the controversial issue of rhodopsin oligomerization by following CD changes in rhodopsin molecules derivatized with porphyrins. We hope that the studies outlined here demonstrate that a chemical approach for the study of dynamics and 3-D structural interactions in rhodopsin is a powerful companion to biophysical methods.

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