Movement of Retinal Along the Visual Transduction Path

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Movement of the ligand/receptor complex in rhodopsin (Rh) has been traced. Bleaching of diazoketo rhodopsin (DK-Rh) containing 11-*cis*-3-diazo-4-oxo-retinal yields batho-, lumi-, meta-I-, and meta-II-Rh intermediates corresponding to those of native Rh but at lower temperatures. Photoaffinity labeling of DK-Rh and these bleaching intermediates shows that the ionone ring cross-links to tryptophan-265 on helix F in DK-Rh and batho-Rh, and to alanine-169 on helix D in lumi-, meta-I-, and meta-II-Rh intermediates. It is likely that these movements involving a flip-over of the chromophoric ring trigger changes in cytoplasmic membrane loops resulting in heterotrimeric guanine nucleotide–binding protein (G protein) activation.

Rhodopsin is a seven transmembrane α-helical G protein–coupled receptor (GPCR) composed of 348 amino acids; its chromophore, 11-*cis*-retinal, is bound to Lys^{296} as a protonated Schiff base (*1*). Irradiation of Rh isomerizes the 11-*cis* chromophore to all-*trans*, which triggers a chain of conformational changes in opsin that induces an enzymatic cascade leading to vision (*2*). Figure 1 depicts the intermediates in the visual transduction process, characterized by flash photolysis and various low-temperature spectroscopic measurements (*3*). Irradiation of Rh results in chromophoric 11-*cis* → *trans* isomerization to yield photo-Rh (femtosecond process), which is converted into batho-Rh, a primary product that can be sequestered at −140°C. In contrast to Rh, with a λ_{max} of 500 nm, batho-Rh absorbs at 543 nm wavelength and adopts a highly strained 11-*trans* double bond (*4*). Batho-Rh relaxes thermally to lumi-Rh, which is in equilibrium with the blue-shifted intermediate (BSI) (*5, 6*), and then to meta-I- and meta-II-Rh (Fig. 1).

Photolysis of the pigment incorporating the nonisomerizable 11-*cis*–locked retinal analog **1** (Fig. 2) resulted in cross-linking to Trp^{265} and Leu^{266}, showing that C-3 (carbon in the 3-position) of ionone is close to helix F of Rh in the dark (*7*). In contrast, with the photoreactive retinal analog **2** with an unlocked 11-ene, the C-3 region cross-linked to both helices C and F (*8*). Other studies have also implicated involvement of helices C and F upon activation of GPCR (*9–12*).

By photoaffinity labeling with a Rh analog and identifying the cross-linked amino acids at respective intermediates, we have now traced the changes in retinal/opsin interactions accompanying visual transduction. We used the Rh analog DK-Rh (diazoketo-Rh) generated with 11-*cis*-3-diazo-4-oxo-retinal [DK-**3** (Fig. 3)], a chromophore with two photoactive moieties, the diazoketo group being activated by 254-nm light and the 11-ene activated by 480 nm light. Cross-linking was first performed with restive Rh at −196°C. Transduction intermediates were selectively populated and trapped by raising the temperature from the DK-batho-Rh stage, i.e., batho- (at −196°C), lumi- (−80°C), meta-I- (−40°C), and meta-II-Rh (0°C). Location of the chromophoric β-ionone ring within the protein binding site was then determined by photocross-linking and sequencing performed for each intermediate.

DK-**3** was prepared via semihydrogenation of an 11-yne precursor with activated zinc in an analogous fashion reported for the synthesis of 11-*cis*-retinal (*13, 14*). Incubation of HPLC-purified DK-**3** with rod outer segment membranes regenerated DK-Rh (λ_{max} = 467 nm). Its circular dichroism spectra (CD) exhibited two positive peaks at 306 and 462 nm, closely matching the β and α peaks at 337 and 480 nm, respectively, of native Rh (*15*). The rapid regeneration and spectroscopic data suggest that the binding of DK-**3** to opsin closely matches that of native Rh.

Low-temperature ultraviolet (UV) studies of DK-Rh, performed as outlined in Fig. 3, revealed that the DK-batho, DK-lumi, DK-meta-I, and DK-meta-II species can be maximally populated at −196°C, −80°C, −40°C, and 0°C, respectively. The two photosensitive moieties in DK-**3** have to be selectively activated, first to isomerize the 11-*cis* double bond and subsequently to photoactivate the diazoketo group at the appropriate intermediate species.

The 11-*cis* → *trans* isomerization of the polyene, triggered by irradiation of the sample with a 1000-W tungsten lamp equipped with a 480-nm band-pass filter, initiates movement of the chromophore within the

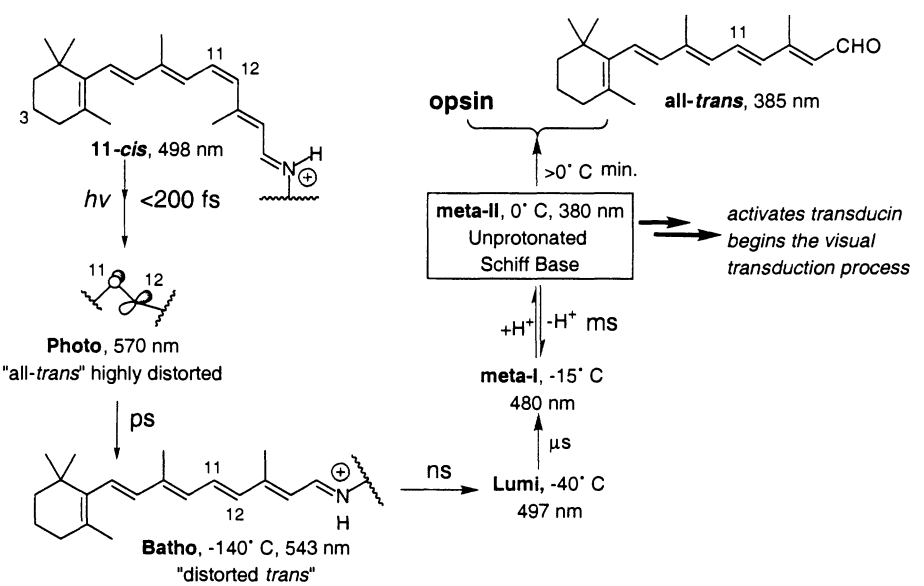

Fig. 1. Spectroscopically detected intermediates in the photoisomerization of Rh. Initially, the 11-*cis* double bond is isomerized to a highly distorted *trans* intermediate (photo-Rh, cannot be isolated). Batho-Rh, a distorted 11-*trans* conformer, can be trapped at −140°C, which upon warming to −40°C yields lumi-Rh. A blue-shifted intermediate (BSI) is in thermal equilibrium with the lumi-Rh intermediate but is not observed under cryogenic conditions; however, it can be observed with flash photolysis at ambient temperatures (*5, 6*). Meta-I-Rh, sequestered at −15°C, is in equilibrium with meta-II-Rh at 0°C. Therefore, it is possible to trap each intermediate at specific temperatures and study the orientation and movement of the chromophore with respect to the protein.

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protein-binding pocket. Because the respective photointermediates can be trapped at specific temperatures, movement of the β-ionone ring can be tracked, provided the extent of cross-linking is reasonable to allow sequencing of the specific site, using the tritium label. Cross-linking was initiated by irradiation with low-pressure mercury lamps, which were fitted with a 254-nm band-pass filter in order to filter out other wavelengths. For all photoaffinity experiments, DK-Rh solution in 66% glycerol was cooled to −196°C.

The resting state of the pigment was investigated by keeping the DK-Rh glycerol solution at −196°C in complete darkness and irradiating it with 254 nm UV light. The cross-linked protein was pyridyethylated in glycerol before separation of the protein from the membrane. After cyanogen bromide cleavage, peptidic fragments were purified and sequenced. The labeled amino acid was identified as Trp^{265} in fragment 13, i.e., CN-13 (Val^{258} to Phe^{287}) of helix F. In a previous photoaffinity study using a nonisomerizable 11-*cis*-retinal analog, DK-ret6 **1** (Fig. 2), Trp^{265} and Leu^{266} were the cross-linking sites (*7*). The additional cross-linking to Leu^{266} with DK-ret6 **1** probably originates in the difference in shapes of DK-**3** and DK-ret6 **1**. The latter has a planar conformation around the critical 11,12-ene as opposed to DK-**3**, which adopts a conformation similar to native chromophore, i.e., a negative twist around the 12-*s*-bond (Fig. 2) (*16*). The resting conformation near the β-ionone ring has thus been established using a photoaffinity analog very closely resembling the natural chromophore.

Although the 11-*cis*-bond in DK-**3** is not locked and the molecule is flexible, clean labeling occured only at Trp^{265}, indicative of a rigidly bound chromophore. The result shows that Trp^{265}, a highly conserved residue among GPCRs (*17*), is directly involved in ligand binding. The close proximity of the ionone ring and Trp^{265} restricts the geometry of 11-*cis*-retinal to a shape that functions as an inverse agonist (antagonist) for opsin activation. This finding agrees with theoretical three-dimensional (3D) models of Rh, in which Trp^{265} is very close to the β-ionone ring (Fig. 4A) (*18–22*).

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-Rh stage. The photoisomerized sample was then subjected to photocross-linking conditions (254 nm, –196°C), and the labeled amino acid was determined as described above. Cross-linking again occurred only at Trp^{265}. Thus, interaction between the β-ionone ring moiety and the opsin hardly changes at the batho-Rh stage (*23*), for which an all-*trans* chromophoric geometry is suggested by resonance Raman spectroscopy (*24, 25*). Carbon-13 nuclear magnetic resonance (^{13}C NMR) of batho-Rh incorporating ^{13}C-enriched retinals at specific carbons have also shown the absence of large chemical shift differences between Rh and batho-Rh (*26*). Similarly, Fourier transform infrared spectroscopy (FTIR) and resonance Raman spectra show that the C=N stretching frequency remains unchanged at around 1655 cm^{-1} in batho-Rh (*27–29*). The present photocross-linking results, together with NMR and vibrational spectroscopic data, demonstrate that the 11-*cis* → *trans* isomerization occurs with minimal movement of the β-ionone ring or minimal changes in the ligand/receptor (opsin) interaction, thus leading to a very large strain in batho-Rh. The conversion of Rh to batho-Rh gives rise to a 520- to 543-nm bathochromic shift, accompanied by inversion of the positive CD 480-nm α band in Rh to an intense negative CD band at 520 nm in batho-Rh (*4*). Such changes could be accounted for by the high strain, especially in the "*trans*" 11-ene of the chromophore/protein complex, estimated to be 30 to 36 kcal/mol of the photon energy absorbed by Rh (*30, 31*).

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. 3), −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 photocross-linking.

The amino acid labeled in the lumi-Rh stage was identified in fragment CN-9 (Ala^{164} to Gly^{182}) of the D helix as Ala^{169}. There was no evidence for any labeling in fragment CN-13, which was labeled in DK-Rh and DK-batho-Rh. At −80°C, the temperature at which lumi-Rh cross-linking was performed, the pigment consists of a 1:1 mixture of Rh and lumi-Rh and not 100% lumi. The composition of the photosteady state mixture was estimated by conventional protocols (*32*). The fact that only Ala^{169} was cross-linked at the lumi-Rh intermediate could be due to a more efficient cross-linking to Ala or to a closer proximity of the ionone ring to

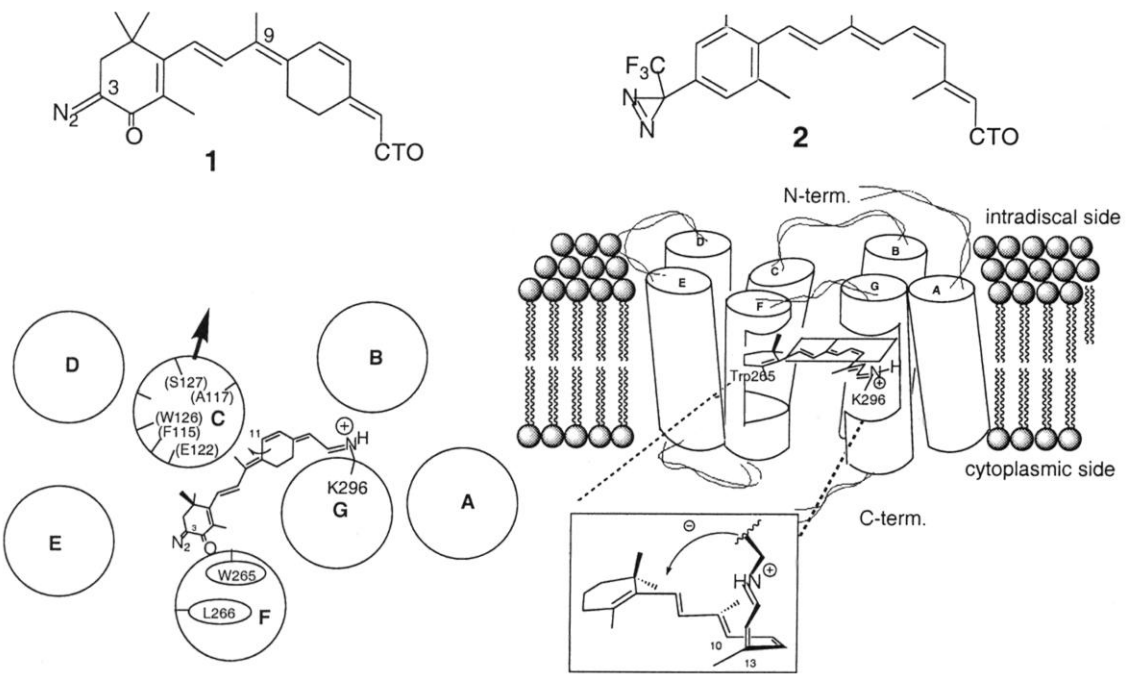

Fig. 2. Distribution of cross-linked amino acids resulting from photolabeled retinal analogs **1** (*7*) and **2** (*8*). View is from the intradiscal side. Trp^{265} and Leu^{266} in helix F are results from analog **1** (*7*), whereas Trp^{265} in helix F and the five additional amino acids in helix C are results from photocross-linking studies with analog **2** (*8*). The 3D view incorporates the absolute sense of twist around the chromophore 12-13 single bond (*16*).

Ala^{169} in lumi-Rh than to Trp^{265}. In any event, 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 substantial blue-shift (543 nm → 497 nm). The cross-linked amino acid in meta-I and meta-II is again only Ala^{169}.

It is difficult to rationalize the site of cross-linking at the lumi-, meta-I-, and meta-II-Rh stages based on available 3D models of Rh. According to the 3D models for restive Rh, helix D does not appear to be close to the position occupied by the isomerized retinal β-ionone ring (Fig. 4B) (*18–22*). The results indicate that photoisomerization triggers a gross structural change in the protein, which moves helix D to the vicinity of the photolabel. Photoisomerization separates the ionone ring from Trp^{265} in helix F and eliminates the interaction between 9-Me and Phe^{261}, a highly conserved residue in all GPCRs one-pitch below Trp^{265}; 9-Me is also close to Gly^{121} in helix C and Phe^{261} in helix F (*33*). This induces separation of helix C from helix F and approach of the ionone ring to Ala^{169} in helix D. Although the ionone ring remains close to Ala^{169} in lumi-, meta-I, and meta-II Rh, the ligand/opsin interactions are clearly changing as seen by changes in the respective CD (*4, 34*).

Lys^{296} involved in the protonated Schiff base link (helix F), Trp^{265} (helix G), and Ala^{169} (helix D) are all located around equal heights from the top of the membrane in their respective helices (Fig. 4C). Therefore, the chromophore that stretches between Trp^{265} in helix F and Lys^{296} in helix G runs parallel to the membrane plane; the *cis* → *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. The theoretical models for restive Rh have Ala^{169}, the cross-linking site in the three intermediates lumi-, meta-I-, and meta-II-Rh, exposed to the lipid bilayer and opposite the helical bundle cavity (Fig. 4B). Ala^{169} is not a conserved residue in the GPCR family of proteins. However, Ala^{164} residing one turn down from Ala^{169} in helix D is involved in regulation of absorption maxima, and thus is close to the chromophore (*35*). Therefore, it is not surprising that the all-*trans* chromophore reaches Ala^{169} in lumi-Rh and subsequent intermediates. Although the highly conserved Trp^{161} located two helical turns below Ala^{169} is not implicated in ligand binding, it could be essential for signal transfer. This has also been suggested by site-directed mutagenesis studies that have demonstrated that alteration of Trp^{161} leads to a different meta-II intermediate (*36*). The close proximity and similar spatial orientation of Ala^{169} and Trp^{161} might indicate that the chromophore makes contacts in this vicinity in order to force protein conformational changes that activate transducin.

In conclusion, we have demonstrated that the chromophore in batho-Rh does not move significantly and that a large translation occurs during transition of the batho-Rh to the

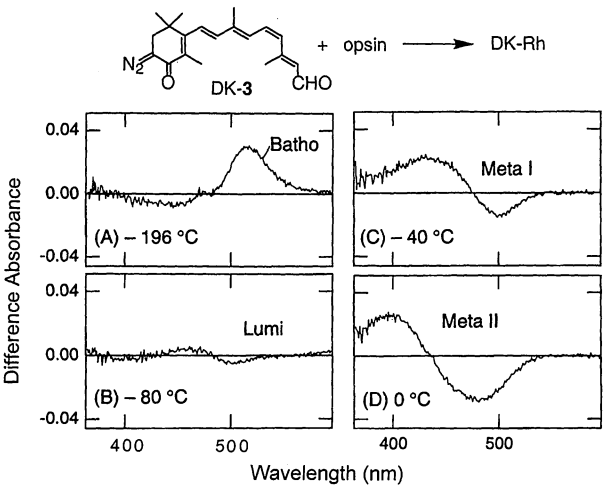

Fig. 3. DK-Rh was dissolved in glycerol (66% v/v), and its photochemical reactions were investigated by low-temperature UV. Irradiation with blue light from a 1000-W tungsten-halogen lamp fitted with a band-pass filter (436 nm) at –196°C caused a red-shift of the spectrum, indicating formation of a batho-Rh intermediate (**A**). Prolonged irradiation (10 min) finally produced a photosteady state mixture containing mainly DK-batho-Rh. Subsequent irradiation of the mixture with orange light (>520 nm) resulted in formation of a blue-shifted iso-product. This product was irradiated with blue light, yielding a spectrum identical with that previously obtained by irradiating DK-Rh directly with blue light (436 nm). These photoreactions indicate that, similar to the native pigment Rh, DK-Rh and its batho- and iso-products are interconvertible by light at –196°C. The photosteady state mixture containing mainly the batho-Rh intermediate was warmed to estimate the number of intermediates that exist in the bleaching process of DK-Rh. Three additional intermediates, lumi-Rh (**B**), meta-I-Rh (**C**), and meta-II-Rh (**D**) were identified. The difference spectrum between each intermediate and DK-Rh was obtained by subtracting the spectrum of DK-Rh from that irradiated at –196°C, followed by warming to the temperatures indicated in the figure. All spectra are similar in shape to the corresponding intermediate in Rh except for their spectral positions.

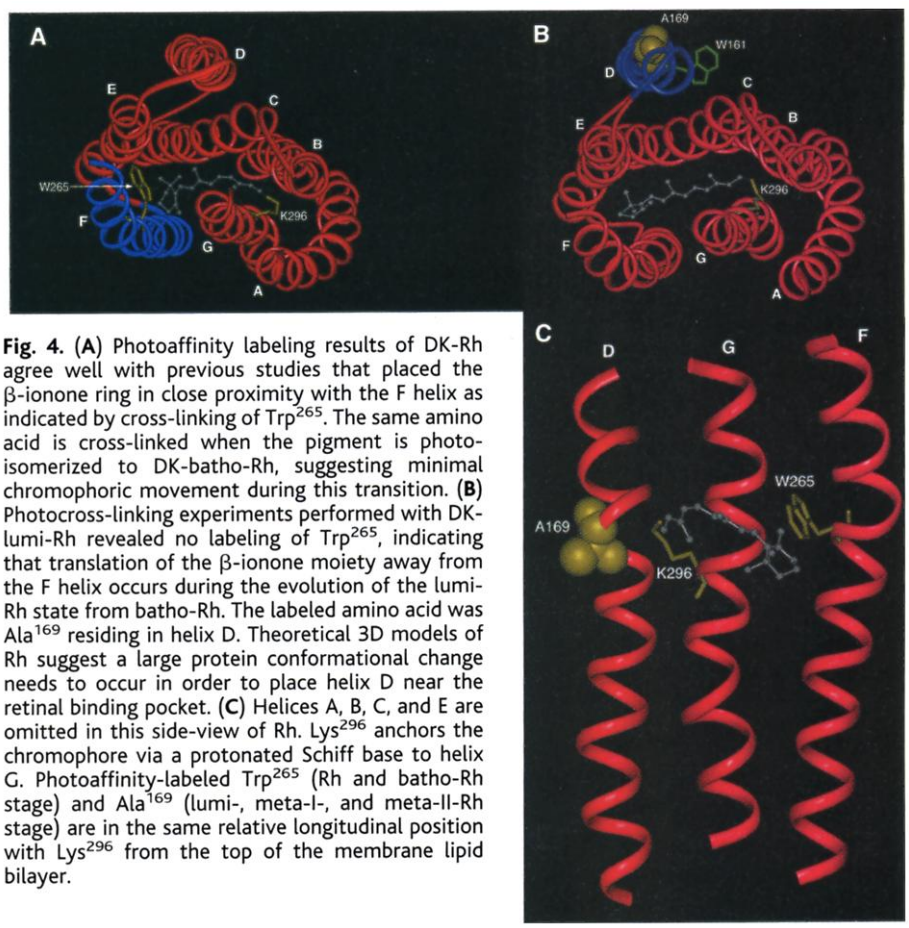

Fig. 4. (**A**) Photoaffinity labeling results of DK-Rh agree well with previous studies that placed the β-ionone ring in close proximity with the F helix as indicated by cross-linking of Trp^{265}. The same amino acid is cross-linked when the pigment is photoisomerized to DK-batho-Rh, suggesting minimal chromophoric movement during this transition. (**B**) Photocross-linking experiments performed with DK-lumi-Rh revealed no labeling of Trp^{265}, indicating that translation of the β-ionone moiety away from the F helix occurs during the evolution of the lumi-Rh state from batho-Rh. The labeled amino acid was Ala^{169} residing in helix D. Theoretical 3D models of Rh suggest a large protein conformational change needs to occur in order to place helix D near the retinal binding pocket. (**C**) Helices A, B, C, and E are omitted in this side-view of Rh. Lys^{296} anchors the chromophore via a protonated Schiff base to helix G. Photoaffinity-labeled Trp^{265} (Rh and batho-Rh stage) and Ala^{169} (lumi-, meta-I-, and meta-II-Rh stage) are in the same relative longitudinal position with Lys^{296} from the top of the membrane lipid bilayer.

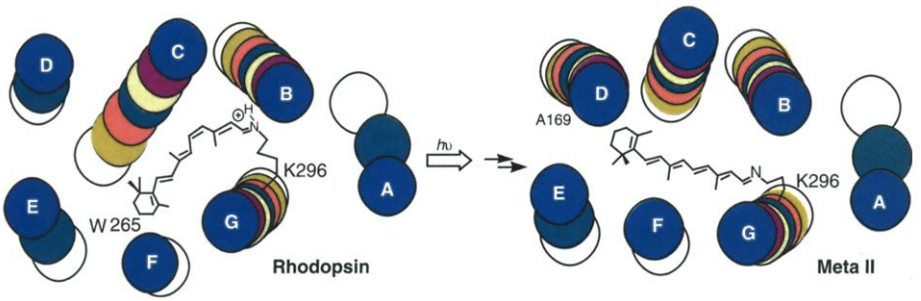

Fig. 5. A highly qualitative depiction of opsin conformational movements triggered by 11-*cis* to all-*trans* photoisomerization of the chromophore. In Rh and batho-Rh, the ionone ring is close to Trp^{265} in helix F, but in Lumi-Rh it flips over with concomitant changes in helical structures and resides close to Ala^{169} in helix D.

lumi-Rh state. Ala^{169}, an amino acid in helix D presumed not to be close to the retinal binding site, is cross-linked in the lumi-, meta-I-, and meta-II-Rh stages. This requires a movement of helices (Fig. 5) and would necessarily alter conformations of the cytoplasmic loops connecting helices C/D and E/F, which have been implicated in transducin activation (*9, 12*). It is proposed that Trp^{265} keeps 11-*cis*-retinal as an inverse agonist; thus, it is not surprising to see Trp^{265} in close proximity to the β-ionone ring both in Rh, the resting stage, and in batho-Rh, the first high-energy intermediate, while the ring moiety has flipped over in the more relaxed lumi-Rh and further intermediates.

The femtosecond photoisomerization of 11,12-ene leading to photo-Rh (Fig. 1) (*37, 38*) and then to batho-Rh does not involve movement of the ionone ring moiety; the highly strained all-*trans* polyene system gives rise to the red-shifted absorption. In the batho- to lumi-Rh step, the stored energy is dissipated by flip-over of the β-ionone ring as well as relaxation of the opsin so that helix D and Ala^{169} are situated near C-3 of the chromophore. In subsequent steps, lumi- to meta-I- to meta-II-Rh, the helical movements change conformations of the extramembrane loops connecting C/D and E/F helices, but the environment surrounding the β-ionone does not change. The present photocross-linking studies where the visual transduction pathway has been traced in a temperature-resolved rather than a time-resolved manner should be applicable to other GPCRs.

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Bacterial Mode of Replication with Eukaryotic-Like Machinery in a Hyperthermophilic Archaeon

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Despite a rapid increase in the amount of available archaeal sequence information, little is known about the duplication of genetic material in the third domain of life. We identified a single origin of bidirectional replication in *Pyrococcus abyssi* by means of in silico analyses of cumulative oligomer skew and the identification of an early replicating chromosomal segment. The replication origin in three *Pyrococcus* species was found to be highly conserved, and several eukaryotic-like DNA replication genes were clustered around it. As in Bacteria, the chromosomal region containing the replication terminus was a hot spot of genome shuffling. Thus, although bacterial and archaeal replication proteins differ profoundly, they are used to replicate chromosomes in a similar manner in both prokaryotic domains.

Recently, comparative genomics has revealed that most archaeal informational processes are similar to those in eukaryotes (*1*). This is especially striking in the case of DNA replication because all putative archaeal DNA replication proteins have eukaryotic homologs only or, alternatively, are more closely related to their eukaryotic counterparts