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Mechanism of activation of sensory rhodopsin I: Evidence for a steric trigger

(phototransduction/*Halobacterium halobium*/signaling state/bacteriorhodopsin/retinal analogs)

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ABSTRACT **Sensory rhodopsin I (SR-I) and bacteriorhodopsin (BR) from *Halobacterium halobium* show broad structural and spectroscopic similarities and yet perform distinct functions: photosensory reception and proton pumping, respectively. Probing the photoactive sites of SR-I and BR with 24 retinal analogs reveals differences in the protein environments near the retinal 13-methyl group and near the β-ionone ring. 13-*cis*-Retinal does not form a retinylidene pigment with the SR-I apoprotein, although this isomer binds to the BR apoprotein even more rapidly than all-*trans*-retinal, the functional isomer of both pigments. The activation of both SR-I and BR requires all-*trans*/13-*cis* isomerization of retinal; however, a steric interaction between the retinal 13-methyl group and the protein is required for SR-I activation but not for that of BR. These results reveal a key difference between SR-I and BR that is likely to be the initial diverging point in their photoactivation pathways. We propose the 13-methyl group–protein interaction functions as a trigger for SR-I activation—i.e., converts photon absorption by the chromophore into protein conformational changes. A similar steric trigger is essential for activation of mammalian rhodopsin, indicating a common mechanism for receptor activation in archaebacterial and vertebrate retinylidene photosensors.**

Retinylidene proteins, consisting of a retinal molecule covalently linked to an intrinsic membrane-embedded apoprotein, are ubiquitously found as photosensory receptors in visual systems throughout the animal kingdom (1, 2). Their characteristic structure is a single polypeptide, which is believed to fold into seven transmembrane helices, forming an internal pocket where the retinylidene chromophore is bound. In vertebrate rhodopsin (M_r 41,000), photon absorption by the chromophore induces its 11-*cis*/all-*trans* isomerization (3). This reaction subsequently produces an altered conformation of the protein, metarhodopsin-II, which activates a signal-transduction cascade (4). Absence of the methyl group at the carbon 9 (C-9) position of retinal prevents photochemical production of metarhodopsin-II and blocks sensory signaling >90%, indicating the coupling of retinal isomerization to molecular changes in the protein requires steric interaction between the 9-methyl group and protein residues (5).

A family of retinylidene proteins also exists in the archaebacterial species *Halobacterium halobium*. The best characterized, bacteriorhodopsin (BR; M_r 26,000, see ref. 6), is a light-driven proton pump, rather than a sensory receptor. Its function requires all-*trans*/13-*cis* retinal isomerization (6, 7). Amino acid sequences of BR and rhodopsin are not homologous. In contrast to rhodopsin, neither of the two polyene-chain methyl groups (C-9 and C-13) (8, 9) nor any of the three methyl groups on the β-ionone ring (10) affect BR photochemical reaction or the number of protons transported per cycle as long as the chromophore is in the active all-*trans* configuration.

Another retinylidene protein in *H. halobium* is sensory rhodopsin I (SR-I; M_r 25,000, see ref. 11), a phototaxis receptor with absorption properties similar to BR (12). The close absorption maxima of SR-I and BR result in cell accumulation in the optimal spectral region for efficient use of solar energy. The retinal-binding pockets of BR and SR-I are closely similar in their electrostatic and hydrophobic interactions with the chromophore, as shown by spectroscopic studies of the BR and SR-I analog pigments reconstituted with retinal analogs (13–16). The SR-I apoprotein gene (*sopI*) has been cloned, and the deduced amino acid sequence (17) exhibits only 26% sequence identity with that of BR but is >80% identical in the putative retinal-binding pocket (17, 18). Activation of SR-I, like that of BR, requires all-*trans*/13-*cis*-retinal isomerization (16).

Photoactivation of BR_{568} (the subscript denotes the absorption maximum) produces a blue shifted photoproduct M_{412} accompanied by proton release from the exterior side of the membrane. The return of M_{412} to BR_{568} ($t_{1/2}$, 5 msec, 23°C) is accompanied by proton uptake from the cytoplasmic side of the membrane (6). Photoactivation of SR-I produces a similar blue-shifted intermediate S_{373} ($t_{1/2}$, 750 msec, 23°C) that possesses a physiologically active conformation at the signal-transmission site of the protein and triggers a signal-transduction chain (19). A key question is the step at which the photochemical reactions of these two proteins diverge to make one a proton pump and the other a sensory receptor. The work presented here identifies a fundamental difference between SR-I and BR in one of the earliest steps of protein activation.

MATERIALS AND METHODS

Chemicals, Strains, and Culture Conditions. All-*trans*-retinal was purchased from Sigma. Polyene-chain desmethylretinals were synthesized as reported (8). Retinal or analogs were purified and prepared as described (15). Flx5R and Flx3R cells (20), which were used for spectroscopic and behavioral studies, are BR^-, halorhodopsin$^-$, and retina$^-$, and no sensory rhodopsin II apoprotein was detected in our conditions. Culture conditions were as described (16).

Absorption and Flash Spectroscopy. Absorption spectra of Flx5R membrane vesicles were recorded on an Aminco DW 2000 spectrophotometer (SLM Instruments, Urbana, IL) applying the procedures described (21). After absorption spectra measurements, samples were immediately used for

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Abbreviations: SR-I, sensory rhodopsin I; BR, bacteriorhodopsin; M_{412}, BR photoproduct with 412-nm absorption maximum; S_{373}, SR-I photoproduct with 373-nm absorption maximum.

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flash photolysis in a laboratory-constructed cross-beam kinetic spectrophotometer as described (15, 16).

Behavioral Assay. Swimming behavior was monitored at 37°C with nonactinic illumination (730–850 nm), recorded on video tape, and analyzed (5 frames per sec) with the EV1000 software on a SUN 2/120 work station (Motion Analysis System, Santa Rosa, CA). Stimulus intensities were ≈1300 and ≈800 erg·cm^{-2}·s^{-1} at 570 ± 5 nm and 380 ± 5 nm, respectively, at the plane where the light enters the condenser.

Chromophore Extraction. Experimental conditions followed a reported procedure with minor modifications (22). The composition of the extract was analyzed by HPLC (Perkin–Elmer series 4 chromatography coupled with a Spectroflow 773 absorbance detector monitoring at 360 nm, analytical μ-Porasil column, 10% ethyl acetate in hexane, and flow rate of 1 ml/min).

RESULTS

Binding of a Variety of Retinal Analogs to SR-I and BR Apoproteins Reveals General Similarities and Subtle Differences Between Their Photoactive Sites. Retinal analogs (Fig. 1), when bound to the apoprotein, assume different chromophore–protein interactions from the native chromophore, thus shifting the absorption spectra. These spectral shifts are sensitive to the stereo and electrostatic properties of the apoprotein-binding pocket and therefore can be used as spectral probes for comparing SR-I and BR. Absorption maxima of SR-I and BR analog pigments (from the literature and from our studies) are plotted in Fig. 2. Spectral shifts in the analog BR and SR-I pigments correlate highly, and most SR-I and BR analog pigments exhibit similar energy differences (400–900 cm^{-1}), as exhibited by the native pigments (difference of 610 cm^{-1}, **7** in Fig. 2). The parallel trend is broken when the retinal C-13 region (analogs **3** and **10** in Figs. 1 and 2), 5-methyl region (analogs **13** and **14**), and the structure of the β-ionone ring (analogs **2, 16, 17, 19, 21, 24** and, to a lesser degree, in a number of other analogs) were modified.

Unlike in 13-*cis*-BR, 13-*cis*-Retinal Does Not Form a Retinylidene Pigment with the SR-I Apoprotein. BR contains only all-*trans*-chromophore after illumination (light adaptation), and in the dark about half of the chromophores convert to

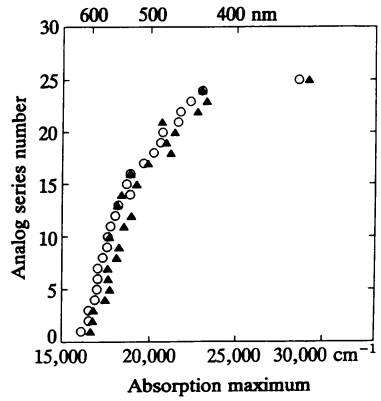

FIG. 2. Absorption maxima of SR-I analogs (○) compared with those of BR analogs (▲). Analogs used are shown in Fig. 1. For SR-I, analogs **3, 15–18,** and **23** are from our unpublished results, and analogs **9–11** are from this study; native retinal **7**, analogs **1, 19–22, 24,** and **25** (also for relevant BR analogs) are from ref. 13; analogs **2** and **4** are from ref. 14; analogs **5, 6, 12–14** are from ref. 15. For BR, analogs **2** and **4** are from ref. 23; analog **6** is from ref. 24; analogs **12, 18,** and **23** are from ref. 25; analogs **9–11** are from ref. 8; analogs **5, 13,** and **14** are from ref. 10; analog **16** is from ref. 26; analog **17** is from ref. 27; analog **3** is from ref. 28, and analogs **8** and **15** are from this study.

13-*cis* so that an equilibrium mixture of all-*trans* and 13-*cis* isomers is formed (dark adaptation). SR-I does not exhibit such light–dark adaptation, and chromophore extraction from SR-I in the dark with or without prior illumination yielded ≥95% all-*trans*-retinal (29). The 13-*cis*-BR is generated by adding 13-*cis*-retinal to bacterioopsin in the dark (30), and 13-*cis*-retinal binds to bacterioopsin even faster than the all-*trans*-isomer (31). When 13-*cis*-retinal was added to membranes containing SR-I apoprotein, a reconstitution intermediate with a structured absorption at 430–450 nm (Fig. 3*A*) was formed, which over hours converts to a pigment with an absorption spectrum (Fig. 3*B*) and photocycle (data not shown) indistinguishable from those of native SR-I. Extraction of the chromophore during reconstitution reveals that the 13-*cis*-retinal-reconstituted pigment actually has an all-*trans* configuration (Fig. 3 *Inset*).

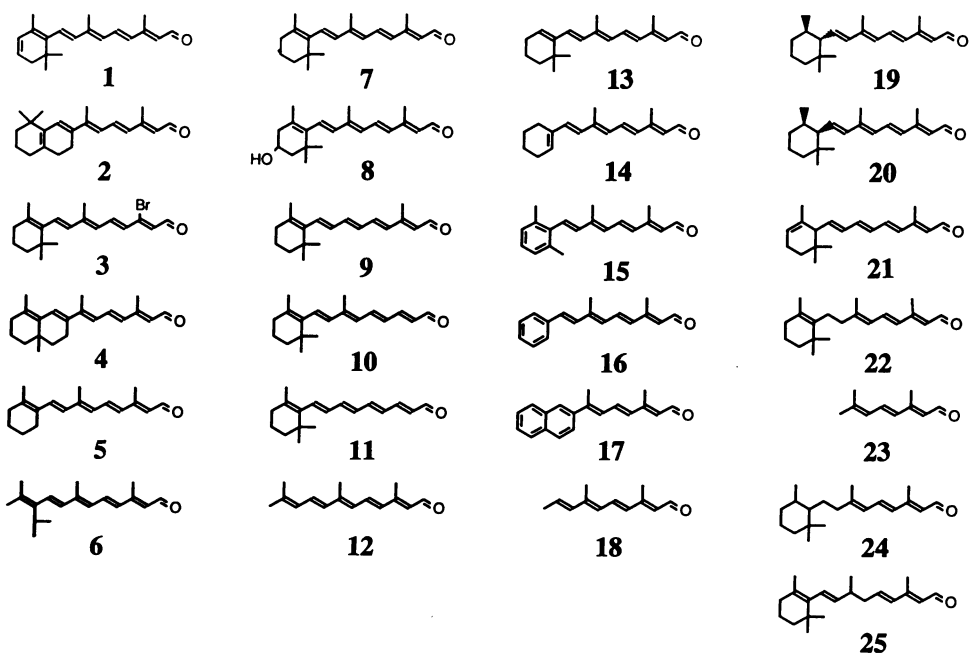

FIG. 1. Native retinal **7** and analogs used for probing SR-I and BR retinal-binding sites.

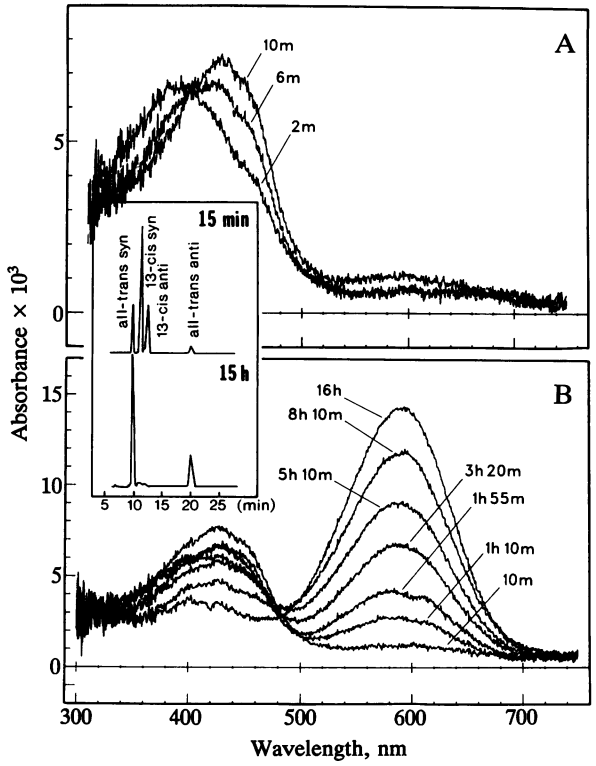

FIG. 3. Pigment reconstitution from 13-*cis*-retinal and membrane-vesicle suspensions containing SR-I apoprotein. Spectra were recorded at various times [reconstitution intermidiate (*A*) and later pigment (*B*)] after adding 13-*cis*-retinal. (*Inset*) HPLC analysis of chromophore oximes extracted at indicated times after 13-*cis*-retinal addition. h, hr; m, min.

Polyene-Chain Desmethylretinal Analogs Reconstitute SR-I Analog Pigments. Previous studies of phototaxis signaling by SR-I analogs revealed that removal of ring methyls or portions of the ring from retinal does not prevent SR-I activation (15). For completeness, whether removal of polyene-chain methyl groups affect the function of SR-I needed to be addressed.

In native membrane-vesicle suspensions, analog **9** associates with the SR-I apoprotein to form an analog pigment absorbing at 569 nm at a similar rate as that of the native retinal **7** (data not shown). With analogs **10** and **11** we observe reconstitution intermediates absorbing at 430–460 nm with evident structured spectra similar to the spectrum of the BR reconstitution intermediate with native retinal (32). The structured intermediates convert to analog pigments absorbing at 567 and 563 nm with a rate 30 times slower than that of native SR-I formation. This behavior is similar to analog BR pigment reconstitution with analogs **10** and **11** (data not shown, also see ref. 8) in which the rate-limiting step is the covalent bond (protonated Schiff base) formation (8).

Unlike 13-H-BR, the Chromophore in 13-H-SR-I Is in the All-*trans* Configuration. 13-H-BR generated from all-*trans*-13-desmethylretinal (analog **10**, Fig. 1) contains >85% 13-*cis*-chromophore (8). In contrast, extraction and HPLC analysis of the chromophore from 13-H-SR-I shows that ≈97% (calculated from the peak area by assuming equal extinction for different isomers) is present as the all-*trans*-isomer (Fig. 4).

Unlike 13-H-BR (*trans*), 13-H-SR-I (*trans*) Is Photochemically Inactive in the msec–sec Time Window. SR-I undergoes a cyclic reaction upon photoactivation, with a rate of cycling considerably slower than that of BR (11). At 2-msec time resolution, a flash induces a transient depletion of absorbance in the SR-I-absorbing region (Fig. 5*A*, trace 2) accompanied

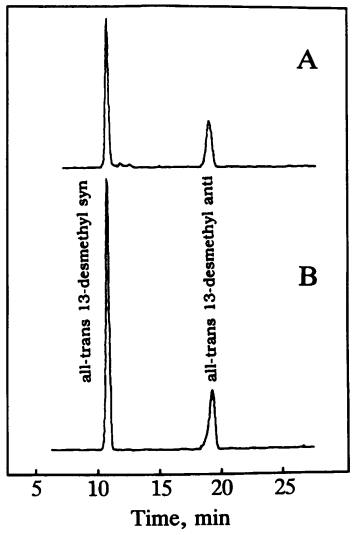

FIG. 4. Chromophore extraction from 13-H-SR-I. (*A*) Analog **10** was added to SR-I apoprotein in native membranes at a 1:2 chromophore-to-apoprotein ratio. After 5 days in the dark, reconstitution was nearly complete. The chromophore was extracted, converted into oxime, and analyzed by HPLC. (*B*) HPLC elution of authentic all-*trans*-13-desmethylretinal oxime with syn (retention time of 10.5 min) and anti (19 min) configurations. Identity of all-*trans*-13-desmethylretinal is based on its typical HPLC retention time and NMR data, as has been characterized (33).

by a transient increase in the near-UV region (Fig. 5*A*, trace 1) due to the formation of the long-lived intermediate S_{373}. S_{373} decays to SR-I_{587} slowly ($t_{1/2}$, 750 msec), and this decay matches the absorbance recovery in the SR-I-absorbing region (450–650 nm). Pigment from analog **9** (9-H-SR-I) exhibits a similar formation of an S_{373}-like intermediate that decays much more slowly ($t_{1/2}$, 9 sec, Fig. 5*B*, traces 1 and 2) than native S_{373}. Flash photolysis of pigments from analogs **10** (13-H-SR-I, Fig. 5*C*, traces 1 and 2) and analog **11** (data not

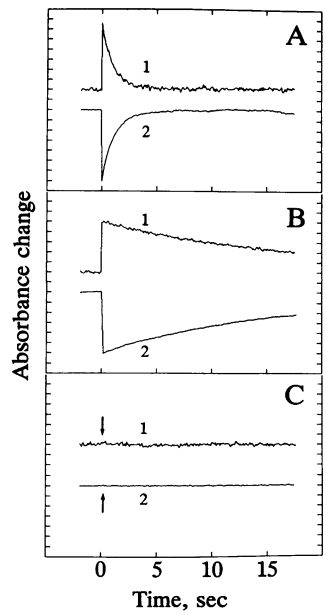

FIG. 5. Flash-induced absorbance changes. Actinic light of 600 ± 20 nm was given at time 0 (indicated by arrows for *C*), and the sample absorbance changes were monitored at 370 and 570 nm. The samples were completely reconstituted SR-I (*A*) and 9-H-SR-I (*B*) and 13-H-SR-I reconstituted for 5 days at 24–26°C (*C*). Trace 1 for each panel is at 370 nm, and trace 2 is at 570 nm. Each spacing along the ordinate represents 2×10^{-3} absorbance units.

shown) does not elicit any photochemical changes at this time resolution and sensitivity. To verify pigment integrity, we measured the absorption spectrum of 13-H-SR-I immediately after the flash photolysis assay. The absorption spectrum and its amplitude were unchanged from those measured immediately before flash photolysis.

Unlike 13-H-BR (*trans*), 13-H-SR-I (*trans*) Is Physiologically Inactive. Addition of all-*trans*-retinal to retinal-deficient cells restores the phototaxis responses mediated by the photointerconvertible (photochromic) attractant and repellent forms (SR-I_{587} and S_{373}, respectively) of SR-I (12). Cells decrease their swimming reorientation (reversal) frequency in response to attractant light (yellow-orange light, Fig. 6*A*) and transiently increase reversal frequency in response to repellent light (near-UV light, Fig. 6*D*). Both attractant and repellent photostimuli used were nonsaturating for native SR-I. Analog **9** fully restored activities of both photochromic receptor systems (Fig. 6 *B* and *E*). Analog **10** did not generate responses from either of these photosystems to these stimuli (Fig. 6 *C* and *F*) nor to photostimulation with >10-fold greater light intensities. Analog **11** also did not generate responses to photostimulations (data not shown), further confirming the essential role of the 13-methyl group. All-*trans*-retinal was added to reference Flx3R cells and cells reconstituted with analog **10** after behavioral measurements, following the reported procedure (16). Reconstitution rates of SR-I in both cell suspensions were measured by monitoring flash-induced absorbance changes at 570 nm. Retardation of SR-I formation by a factor of 4 was observed in the cells preincubated with analog **10** (i.e., those used in behavioral measurements), indicating entry of this analog into the SR-I retinal-binding pocket in these physiologically inactive cells.

DISCUSSION

Differences Between SR-I and BR Retinal-Binding Pockets. A variety of retinal analogs bind to both SR-I and BR apoproteins. Most analog SR-I and BR pigments show a constant difference (400–900 cm^{-1}) between their absorption maxima, which has been attributed to the weaker association of the positively charged Schiff base nitrogen proton with its protein "counterion" in SR-I (34). In general, an approximately parallel trend of absorption shifts indicates a mostly conserved retinal-binding pocket in both proteins, as has been suggested from previous absorption studies (13–16), resonance Raman studies (34), and amino acid sequence comparison (17, 18). From Fig. 2, deviations from the parallel trend are observed when the retinal 13-methyl region, 5-methyl region, and the structure of the ring are modified. These findings indicate different retinal–protein interactions in these particular regions that may arise from different protein residues or their different orientations. The structural difference near the isomerization site (C-13) of retinal is especially of interest because, as discussed below, the C-13 methyl group plays fundamentally different roles in SR-I and BR activation.

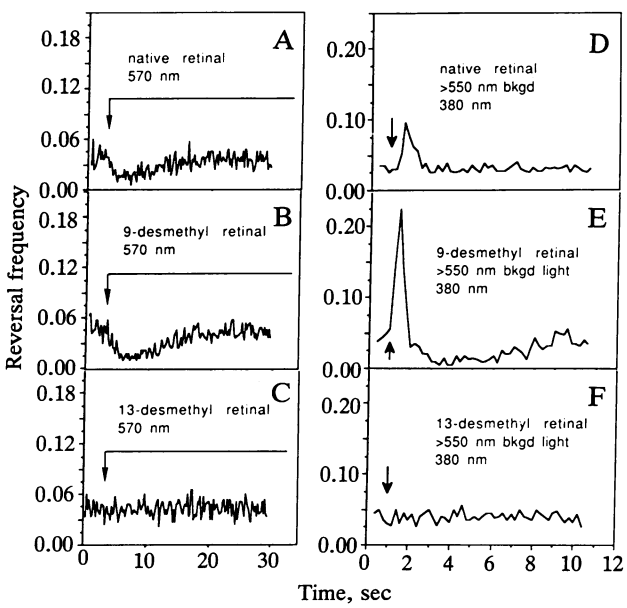

FIG. 6. Native and analog SR-I mediated responses to photostimuli. At times indicated by arrows, a stimulating light of 570 ± 5 nm was delivered for 30 sec (column 1) or of 380 ± 5 nm was delivered for 1 sec (column 2) with a background (bkgd) light (>550 nm). Flx3R cells were grown to early stationary phase with chromophore (10^{-6} M) in the dark with shaking at 37°C. Cells were then diluted 1:67 into 2 ml of fresh medium with the same concentration of chromophore and incubated for 2–4 hr with shaking at 37°C before motility measurements. Data are for cells incubated with native chromophore **7** (*A* and *D*) and analogs **9** (*B* and *E*) and **10** (*C* and *F*). Video data were processed for 30 sec (*A–C*) or for 11 sec (*D–F*).

Photochemical and Photophysiological Properties of 9-H-SR-I. Removal of 9-methyl from the native retinal does not prevent either the photochemical or the physiological activities of the analog pigment (Figs. 5*B*, and 6 *B* and *E*). These observations combined with our previous findings on β-ionone ring modifications (15) demonstrate that neither the 9-methyl nor the intact β-ionone ring is crucial for SR-I activation. But the 9-methyl group and, to a lesser degree, ring methyl groups affect the recovery of photocycling SR-I. The extremely long-lived S_{373}-like intermediate in the 9-H-SR-I photocycle increases the photoattractant sensitivity (19), indicating that S_{373}, in addition to its role as a repellent receptor, is the signaling state of the attractant receptor form SR-I_{587}.

All-*trans* Binding Specificity in SR-I. In contrast to 13-*cis*-BR, the 13-*cis*-isomer does not form a retinylidene pigment in the SR-I retinal-binding pocket in native membranes (Fig. 3). Moreover, even though lack of the retinal 13-methyl group in 13-H-BR causes >85% of the chromophore to be trapped in a 13-*cis* configuration (8), 13-H-SR-I contains ≈97% all-*trans*-chromophore (Fig. 4). Thus, unlike BR, the retinal-binding pocket of SR-I is specific for the all-*trans*-isomer. These facts explain both the lack of light–dark adaptation in SR-I (29) and the absence of the 13-*cis*-chromophore in 13-H-SR-I.

The Requirement for a Steric Trigger in SR-I Activation. In contrast to removal of each of the three β-ionone ring methyls or the 9-methyl from the retinal, removal of the 13-methyl prevents protein activation—i.e., formation of the SR-I attractant signaling state: S_{373}. All-*trans*-retinal is the functional isomer for both SR-I and BR. The 15% all-*trans*-pigment in 13-H-BR exhibits a similar formation rate of its M_{412}-like intermediate as native BR (35). Proton pumping is expected to be reduced by this low percentage of all-*trans*-pigment. A 5-fold slower decay of the M_{412}-like intermediate in all-*trans*-13-H-BR further reduces its proton-pumping ability to 3% compared with native BR (35), but the number of protons pumped per cycle by all-*trans*-13-H-BR is the same as that by native BR (9). All-*trans*-13-H-SR-I (Fig. 4) does not show any photochemical activity (Fig. 5*C*) in the time window in which functionally important photochemical activity of the native SR-I is observed (Fig. 5*A*). Correspondingly, no phototaxis signaling is detected for 13-H-SR-I (Fig. 6 *C* and *F*). Our results exclude the possibility that the chromophore in 13-H-SR-I, like in 13-H-BR, is trapped in a 13-*cis* configuration and indicate the lack of photochemical and physiological activities in 13-H-SR-I is due to the loss of a crucial interaction between the all-*trans*-retinal 13-methyl group and the protein residues upon photoactivation. Another possibility is that isomerization in SR-I, unlike BR, is sensitive to the 13-methyl group because of configurational or conforma-

tional differences between the SR-I and BR chromophores. However, resonance Raman studies of SR-I have established that the SR-I-chromophore is, as in BR, an all-*trans*, C=N anti, protonated Schiff base (34). Moreover, the C—C single-bond fingerprint modes in SR-I are very similar to those in BR, indicating that they have similar retinal chain structures and environments (34).

In conclusion, we interpret our results as compelling evidence for the requirement of a specific steric interaction between the retinal 13-methyl group and protein residues for SR-I activation. We suggest this interaction functions as a trigger for generating protein conformational changes to produce the signaling state of the receptor S_{373} (19). In BR, the activation driving force appears to be the isomerization-induced pK_a changes in the retinal Schiff base and protein residues (18, 36, 37). These pK_a changes and the formation of M_{412} (the S_{373}-like intermediate of BR) do not require 13-methyl–protein interaction. Activation of bovine rhodopsin requires a steric interaction between the retinal 9-methyl and the protein residues (5). Lack of this interaction in 9-H-rhodopsin leads to an abnormal photochemical reaction that does not produce a metarhodopsin-II-like intermediate and greatly diminished biological function (5). The steric trigger in rhodopsin uses the 9-methyl group, which is close to the isomerizing 11,12-double bond. Analogously, our results indicate the 13-methyl group, which is close to the isomerizing 13,14-bond in SR-I, functions analogously in SR-I activation—i.e., converts photon absorption by the chromophore into protein conformational changes. Relevant to this observation, photo-induced conformational changes in SR-I as monitored by Fourier transform infrared spectroscopy differ from those in BR. In particular, signals indicating lipid disordering accompany the SR-I → S_{373} transition (38) as observed also in the rhodopsin → metarhodopsin-II_{380} transition.

Summary. Photoactive site mapping reveals evident differences despite broad similarities between SR-I and BR. The two proteins share many common features, such as comparable molecular weights, similar secondary structures, and highly conserved retinal-binding pockets, the requirement for a specific all-*trans*/13-*cis* isomerization of retinal and homologous photocycles. But differences are also evident. The M_{412}-like intermediate of SR-I, S_{373}, is 40-nm blue-shifted, and its decay rate is greatly reduced, compared with M_{412} ($t_{1/2}$, 750 msec vs. 5 msec). Moreover, the expected O-like intermediate in the SR-I photocycle must be a fast-decaying intermediate because no accumulation of this intermediate is seen on the S_{373} decay pathway in contrast to BR. There are two dramatic differences.

(*i*) Unlike the retinal-binding pocket of BR, in which both all-*trans* and 13-*cis* retinals form retinylidene linkages, SR-I, in native membranes, has an exclusive all-*trans*-chromophore-binding pocket.

(*ii*) The 13-desmethyl-BR analog, when photostimulated in its all-*trans* form, produces an M_{412}-like intermediate at a normal rate and is functional in proton pumping, but all-*trans*-13-desmethyl-SR-I analog exhibits no photochemical production of an S_{373}-like intermediate or any physiological activity.

The different roles of the 13-methyl group provide a clue to how SR-I and BR, two apparently homologous proteins, have been modified by nature to perform their distinct functions. Finally, it is worth noting that the activation of SR-I, an archaebacterial photoreceptor, is closely similar to the activation of mammalian rhodopsin in requiring steric retinal–protein interactions for generating their signaling states: S_{373} and metarhodopsin-II_{380}, respectively.

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