Steric Constraints in the Retinal Binding Pocket of Sensory Rhodopsin I†

Bing Yan,‡,# Aihua Xie,§,∥ G. Ulrich Nienhaus,§ Yuko Katsuta,⊥ and John L. Spudich*,‡

Department of Microbiology and Molecular Genetics, University of Texas Medical School, Houston, Texas 77030, Department of Physics, University of Illinois at Urbana–Champaign, Urbana, Illinois 61801, and Department of Chemistry, Columbia University, New York, New York 10027

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ABSTRACT: Steric constraints in the retinal binding pocket of sensory rhodopsin I (SR-I) are analyzed by studying effects of sample temperature and retinal analogs. The flash-induced yield of the earliest detected intermediate S_{610}, which corresponds to the K intermediate in the bacteriorhodopsin (BR) photocycle, decreases below 220 K and reaches zero at 100 K, while K formation is independent of temperature. The reduced S_{610} formation at low temperatures indicates a more restricted retinal binding pocket in SR-I during primary photochemical events. Introduction of bulky substituents on the retinal polyene chain in four retinal analogs greatly retards or blocks the final step of chromophore binding to the apoprotein of SR-I. Except for the 14-methyl substitution, these modifications exhibit little or no effect on chromophore binding to BR apoprotein. These results corroborate that the retinal polyene chain binding domain in SR-I is more sterically constrained than that of the retinal pocket in BR. Deletion of the β-ionone ring renders the analog SR-I pigments nonfunctional, as does deletion of the 13-methyl group, but the corresponding BR analogs are both photochemically and physiologically active. In contrast to the corresponding BR analog, photolysis of the analog SR-I reconstituted with 13-desmethylretinal does not produce an S_{610}-like intermediate at room temperature. The above results and the previous findings that protein constraints inhibit the accommodation of a stable 13-*cis*-retinal configuration in SR-I suggest a model in which the 13-methyl group functions as a fulcrum to permit movement of one or both ends of retinal to overcome an energy barrier against isomerization.

Sensory rhodopsin I (SR-I) is a phototaxis receptor protein in *Halobacterium salinarium* (also known as *H. halobium*) membranes (Spudich & Bogomolni, 1988). Light activation of its two photochromic forms, SR-I_{587} and S_{373} (Spudich & Bogomolni, 1984), enables cells to migrate into environments favorable for photoactivation of the electrogenic ion pumps bacteriorhodopsin (BR; Oesterhelt et al., 1992; Krebs & Khorana, 1993) and halorhodopsin (HR; Lanyi, 1990) and escape from damaging UV irradiation. From the protein sequence of SR-I and its predicted secondary structure (Blanck et al., 1989), a large (82%) homology in the SR-I retinal binding pocket with that of BR is expected (Henderson et al., 1990). Shifts of absorption maxima of a large number of analog SR-I and BR pigments have been compared and generally agree with overall homologous retinal binding structural domains in these two proteins (Yan et al., 1991). The question remains what structural features are responsible for their diverse functions and different photocycle kinetics.

The complex reactions leading to photosensory transduction and the physiological behavioral responses in *H. salinarium* begins with an *all-trans* to 13-*cis* photoisomerization of retinal in SR-I (Tsuda et al., 1985; Yan et al., 1990a). This event takes place in the SR-I central pocket formed by its seven transmembrane α-helices. Structural changes then presumably propagate to the protein surface as in visual transduction (Stryer, 1986) to activate a signal-transducer protein (Yao & Spudich, 1992). The intramolecular interactions between retinal and protein residues during photoactivation of retinylidene proteins are poorly understood. The process is conventionally monitored by a sequence of flash-induced spectral transients because chromophore isomerization and subsequent protein conformational motions cause changes in the chromophore absorption properties. Photoexcitation of SR-I produces first the S_{610} intermediate (λ_{max} at 610 nm, corresponding to the K intermediate of the BR photocycle) followed by S_{560} (corresponding to the L intermediate of BR) and a long-lived M-like intermediate S_{373}, observed in flash photolysis studies at room temperature (Bogomolni & Spudich, 1987). S_{373} assumes special significance because it is a signal-generating conformation of the receptor (Yan & Spudich, 1991). These photocycle intermediates and the homologous intermediates of BR are comparable in their absorption spectra but different in the kinetics of their transitions. Analogous transitions in the SR-I photocycle are considerably slower than those in BR ($t_{1/2}$ 90 vs 2 μs for K → L; 270 vs 50 μs for L → M and 750 vs 20 ms for M → pigment). More intermediates have been detected in the BR photocycle than in that of SR-I. A dramatic difference between SR-I and BR has been found by low-temperature UV–vis absorption spectroscopy. It was reported that the S_{610} and S_{560} intermediates of SR-I are not trapped at any temperature in the range from 220 to 80 K under photostationary state illumi-

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‡ University of Texas Medical School.

§ University of Illinois at Urbana–Champaign.

∥ Present address: Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, NY 10461.

⊥ Columbia University.

Present address: Sandoz Research Institute, East Hanover, NJ 07936.

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[1] Abbreviations: SR-I or SR-I_{587}, sensory rhodopsin I; BR, bacteriorhodopsin; HR, halorhodopsin; λ_{max}, absorption maximum; S_{560}, S_{610}, and S_{373}, SR-I photocycle intermediates with λ_{max} at 560, 610, and 373 nm, respectively; 13-H-SR-I, SR-I analog pigment reconstituted with 13-desmethylretinal; HPLC, high-performance liquid chromatography; SR-II, sensory rhodopsin II; BOP, bacterioopsin; FWHM, frequency width at half-maximum; 13-Et-SR-I, SR-I analog pigment reconstituted with 13-ethylretinal.

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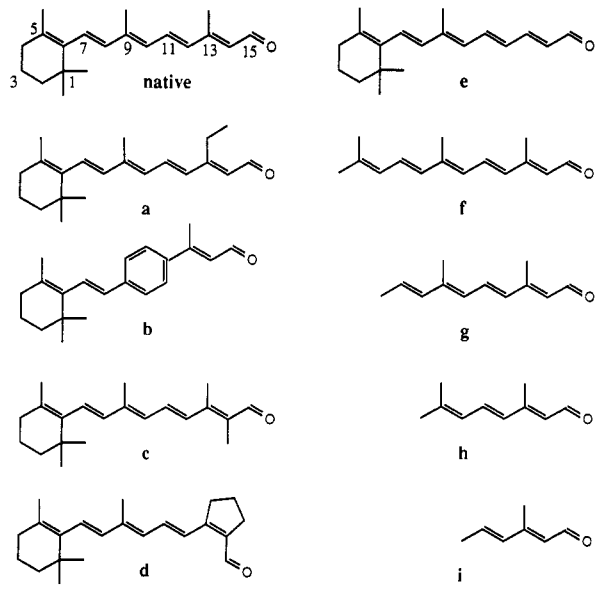


FIGURE 1: Formulae of retinal and analogs. Native *all-trans*-retinal and its analogs: **a**, 13-ethylretinal; **b** 3-{4-[2-(2,6,6-trimethylcyclohexenyl)vinyl]phenyl}-2-butenal; **c**, 14-methylretinal; **d**, 13-*cis*-locked retinal; **e**, 13-desmethylretinal; **f**, 3,7,11-trimethyldodecapentaenal; **g**, 3,7-dimethyl-2,4,6,8-decatetraenal; **h**, 3,7-dimethyloctatrienal; and **i**, 3-methylhexadienal.

nation and that the only trappable intermediate is S_{373} above 210 K (Ariki et al., 1987), while the corresponding K and L intermediates of BR are trapped at <110 and <180 K, respectively [as summarized in Birge (1990)]. We report here the findings that the flash-induced yield of the S_{610} intermediate drops to zero from 220 to 110 K; furthermore, retinal analogs with bulky substituents easily enter the central cleft of SR-I yet form the native covalent attachment only very slowly. All these results are in contrast to what is found for BR, indicated a tighter and more rigid retinal binding pocket in SR-I.

In SR-I, *all-trans*/13-*cis* isomerization of retinal is expected to induce transient twists on both sides of the C13-C14 bond as found in BR (Dencher et al., 1989), i.e., isomerization-induced distortion of the Schiff base linked lysine and of the β-ionone ring end. Chromophores without the β-ionone ring and ones with shorter chain lengths are examined in this work to test for steric interaction between the β-ionone ring and the protein residues and to establish the minimal chromophore structure required for SR-I activation. Also, photoreaction of 13-H-SR-I, an analog SR-I pigment reconstituted with 13-desmethylretinal, is studied with better time resolution (10 μs) than previously (500 μs) (Yan et al., 1991). The previous and present results are discussed in term of an SR-I activation model.

MATERIALS AND METHODS

Chemicals, Strains, and Preparations. *All-trans*-retinal was purchased from Sigma. *All-trans*-retinal analogs (Figure 1) were synthesized as previously reported (Gärtner et al., 1983; Zingoni et al., 1986; Fang et al., 1983; Kolling et al., 1984; Chan et al., 1974) and purified by HPLC prior to use. Flx5R and Flx3R are BR^- HR^- Ret^- *H. salinarium* strains in which SR-II apoprotein is not detectable. JW10 is a *H. salinarium* strain producing predominantly bacterioopsin without producing retinal. Pho81BR was produced by transformation of a BR^- HR^- SR-I^- SR-II^- strain, Pho81, into BR^+ by expressing the native *bop* gene (Yan et al., 1992). Purple membrane was isolated and bleached as reported (Becher & Cassim, 1975). Membrane vesicles of Flx5R, JW10, and nicotine-grown Pho81BR were prepared as described (Manor et al., 1988). Membrane preparations were in 20 mM Tris-HCl and 4 M NaCl at pH 7.

Absorption Spectroscopy. Bleached purple membrane and cell membrane vesicles from Flx5R, JW10, and nicotine-grown Pho81BR were used for absorption spectroscopic measurements. Spectra were recorded on an SLM-Aminco DW2000 spectrophotometer (SLM Instruments Inc., Urbana, IL) at 22 ± 0.5 °C. Vesicle suspensions were degassed before measurements. Five microliters of ethanol or an ethanolic solution of *all-trans*-retinal or its analogs was added to the reference and the sample cuvette, respectively. The path length was 10 mm.

Flash Spectroscopy. Suspensions of Flx5R or nicotine-grown Pho81BR membrane vesicles containing reconstituted pigments were used for flash photolysis experiments. To ensure optical transparency at low temperatures, the sample was prepared in 66% glycerol/buffer (v/v). A storage cryostat (Janis 10-DT) was used for low-temperature studies. Sample temperature was controlled with a temperature-sensor diode monitored by a Lakeshore Cryotronics DRC82C controller. The BR sample was light-adapted with green light from a 150-W tungsten–halogen lamp (PTi Model A1010). The actinic flash was provided by a frequency-doubled, Q-switched Nd-YAG laser (Continuum NY61, 6-ns FWHM pulse width, 532 nm, 5–10 mJ/cm^2). Excitation wavelength affects the fraction of K in photochromic equilibrium. From studies on the BR photocycle the BR/K isosbestic point is at 592–594 nm, and 500–520-nm excitation produces the highest fraction of K (Xie, 1991). Since SR-I is 20 nm red-shifted from BR, the 532-nm output of the laser is expected to produce the greatest fraction of S_{610}. The laser energy was sufficient to saturate the sample. Under saturating conditions, the experiments are insensitive to photoselection effects and small fluctuations in laser energy.

The subsequent absorbance changes were monitored at 670 nm for SR-I and 13-desmethyl-SR-I and 650 nm for BR. Even at low temperature, the spectrum of K is broadened due to its distorted chromophore. Estimated from the BR → K difference spectrum (Xie, 1991), K absorbs from 594 to 730 nm. SR-I is 20 nm red-shifted from BR, and 670 nm is the optimal wavelength for monitoring the formation of S_{610}, according to the flash photolysis data of Bogomolni and Spudich (1987). This is confirmed by measurements which showed 670 nm gave the largest signal. Photomultiplier (Hamamatsu 928) signals were digitized with a logarithmic time-base digitizer (Wondertoy II) from 2 μs to 10 s. To minimize actinic effects of the monitoring light at low temperatures, its intensity was kept low, and it was blocked until 2 s before the flash. The sample was warmed to 300 K after each flash to ensure complete recovery of SR-I from photointermediates and then recooled in the dark. Flash-induced photochemical reactions of SR-I and analog pigments were also measured with a cross-beam kinetic spectrophotometer (Yan et al., 1990a) at room temperature in the millisecond to minute range.

Measurements of Phototaxis Responses of Cells. Phototactic responses of cells were monitored at 37 ± 0.5 °C with nonactinic illumination (730–850 nm). The data were collected and processed in real time or recorded on video tapes and analyzed later by using EV1000 software on a Sun-SPARC-IPC workstation (Motion Analysis System, Inc., Santa Rosa, CA). Light from a 200-W mercury arc lamp was passed through a filter to select the beam wavelength for

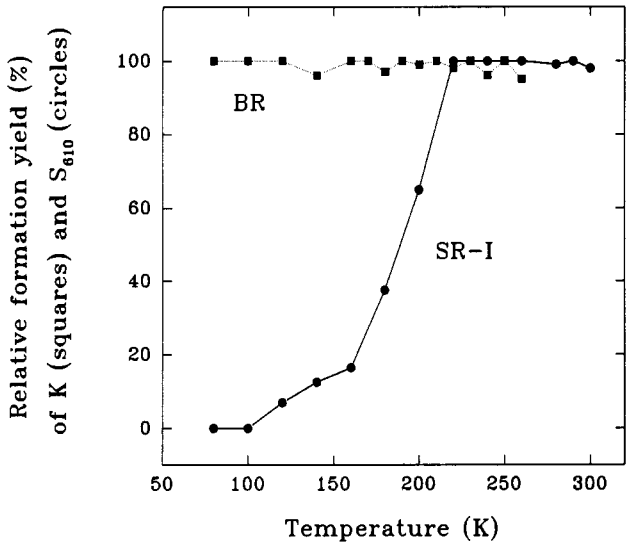


FIGURE 2: Temperature dependence of bathophotoproduct formation yields in SR-I and BR. The flash-induced absorbance changes of SR-I at 670 nm (as in Figure 8) and of BR at 650 nm were measured at various temperatures. The relative amplitudes of the absorbance changes are plotted against the temperature. Both samples were in 66% glycerol/buffer (v/v) and at pH 7.0.

stimulating phototactic response (with a width of 40 nm, FWHM). Video data were digitized and processed at 5 frames/s, and reversal frequencies were calculated by using a program modified from that of Sundberg et al. (1986).

RESULTS

Inhibition of Formation of the S_{610} Intermediate at Low Temperatures. The formation of the S_{610} intermediate of SR-I was measured by flash spectroscopy at various temperatures from 300 to 80 K in 20-K steps. From 300 to 220 K the yield of S_{610} is independent of temperature (Figure 2) even though the decay rate ($t_{1/2}$ 90 ms, 300 K) is slowed down by a factor of 10 with every 20-K decrease. From 220 to 160 K, the amplitude of the flash-induced absorbance change at 670 nm is rapidly diminished with lowering temperature. Below 160 K, it decreases at a slower rate to zero at 100 K (Figure 2). For comparison, we measured the effect of temperature on the formation of the K intermediate of BR under the same conditions. In contrast to the striking temperature dependence in SR-I, the amplitude of the K intermediate is essentially temperature-independent from 260 to 80 K (Figure 2).

Binding of Analogs Containing Bulky Polyene Chain Substituents. To evaluate the steric constraints in the retinal binding pockets of SR-I and BR, we compared the binding rates of SR-I and BR apoproteins with retinal analogs carrying bulky substituents along the polyene chain.

(A) Binding of 13-Ethylretinal. Reconstitution of SR-I and analog SR-I pigment with 13-ethylretinal (13-Et-SR-I) was carried out in Flx5R membrane vesicles which contain SR-I apoprotein (Figure 3A,B,D). When 13-ethylretinal (**a** in Figure 1) was added to Flx5R membranes, a reconstitution intermediate absorbing at ~440 nm with distinct fine structure was formed quickly (Figure 3A), but the conversion of this intermediate to an analog pigment absorbing maximally at 580 nm was slower than that of the corresponding native SR-I process by 30-fold (Figure 3B,D). Since the reconstitution intermediate of the SR-I analog exhibits a red shift and a spectrum with fine structure similar to those found during BR reconstitution, it is likely to correspond to a chromophore in a planar conformation by sandwich-type interactions with

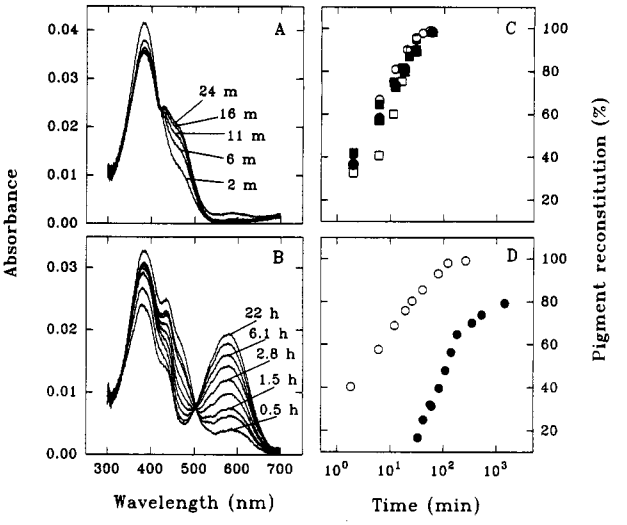


FIGURE 3: Reconstitution of analog SR-I and BR pigments with 13-ethylretinal. Five microliters of an ethanolic solution of **a** (1.2 × 10^{-3} M assuming the same extinction coefficient as *all-trans*-retinal) was added to 3 mL of Flx5R apomembrane suspension prepared by sonication (2–4 mg of protein/mL) of whole cells in 20 mM Tris-HCl and 4 M NaCl at pH 7.0. Absorption spectra were recorded at 2, 6, 11, 16, and 24 min after addition of the analog (A) and at 0.5, 1, 1.5, 2, 2.8, 4.4, 6.1, 11.4, and 22 h after addition (B). Similar procedures were used to reconstitute BR and 13-ethyl-BR analog pigments (C). The apomembranes containing bacterioopsin were (circles) nicotine-grown Pho81BR (Yan et al., 1992) cell membranes prepared by sonication, (squares) bleached purple membrane prepared as in Becher & Cassim (1975), and (triangles) JW10 membranes by sonication. Open symbols are for the native retinal, and solid symbols are for the analog. Due to close reconstitution rates, some open symbols are blocked by solid symbols. Data in panel D are for reconstitution of SR-I and 13-ethyl-SR-I analog pigments in Flx5R apomembranes. Open circles are for the native pigment, and solid circles are for the analog pigment.

protein residues in the central pocket, as has been proposed for BR (Schreckenbach et al., 1977). At this stage of reconstitution, the 13-ethylretinal is already in the proximity of the native retinal binding site. The slow conversion of this intermediate to the pigment indicates a sterically restricted 13-methyl binding site for SR-I.

BR and 13-ethyl-BR analog pigment (13-Et-BR) were reconstituted in three types of apomembranes (Figure 3C): (1) sonicated membrane vesicles of nicotine-grown Pho81BR, which synthesizes BR as the only retinylidene protein (Yan et al., 1992), (2) bleached purple membrane, and (3) apomembranes of strain JW10, which produces bacterioopsin (BO) and is deficient in retinal. *All-trans*-13-ethylretinal (**a** in Figure 1) binds to BO with a rate similar to that of the native chromophore *all-trans*-retinal in each of the three preparations (Figure 3C). Evidently 13-ethylretinal is not sterically hindered as it binds to BO. The light-adapted 13-ethyl-BR analog, in all three preparations, absorbs at 562 nm, which is close to the previous reported value of 559 nm (Gärtner et al., 1988).

(B) Binding of 9,12-Phenylretinal. In this compound, the polyene chain of retinal is locked into a 10,11-s-*cis* conformation by a phenyl ring (**b** in Figure 1). The bent conformation and the bulky phenyl ring instead of the native straight *all-trans*-retinal would be expected to produce steric interactions along the polyene chain if the binding pocket is narrow. Indeed, this analog binds to SR-I apoprotein (Figure 4B,D) at a rate 12-fold slower than native retinal. In contrast, it binds to BO at the same rate as native retinal (Figure 4A,C).

(C) Binding of 14-Methylretinal and 13-Cis-Locked Retinal. Addition of 14-methylretinal or the five-membered ring 13-

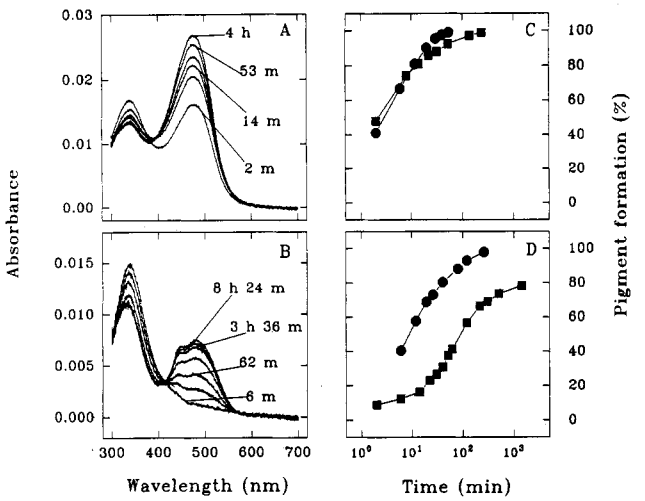


FIGURE 4: Reconstitution of analog SR-I and BR pigments with 9,12-phenylretinal. Five microliters of an ethanolic solution of **b** was added to 3 mL of nicotine-grown Pho81BR membranes containing bacterioopsin (A) or SR-I apomembranes (B). Spectra were taken at 2, 8, 14, 22, and 53 min, 2 h 20 min, and 4 h (A) and 6, 30, and 62 min, 3 h 36 min, 5 h 6 min, and 8 h 24 min (B) after addition of the analog. Rates for reconstitution of bacterioopsin with the native retinal (circles) or the analog (squares) are compared in panel C, and those for reconstitution of SR-I apomembranes are compared in panel D.

cis-locked retinal (**c** and **d** in Figure 1) to SR-I apomembranes produces a reconstitution intermediate absorbing near 420 nm quickly, but it does not convert to a red-shifted pigment (Figure 5A,B). In contrast, the 13-*cis*-locked retinal forms analog BR pigment efficiently despite the 10-fold slower rate than that of native retinal (Fang et al., 1983). The 14-methylretinal does not form a red-shifted BR analog pigment (Schiffmiller et al., 1985).

Binding of Acyclic Retinal Analogs to SR-I Apoprotein. Analogs **f** (Yan et al., 1990b), **g**, and **h** bind to SR-I apoprotein (Figure 6) faster than the native chromophore, presumably due to the lack of the rate-limiting ring/chain coplanarization step. Analog SR-I pigments reconstituted from **g** and **h** exhibit absorption maxima at 497 and 449 nm, respectively. The relative extinction maxima of these two analog SR-I pigments in comparison to that of the native SR-I are similar to those of corresponding BR analogs (Muradin-Szweykowska et al., 1984). Analog **i** with one less double bond than **h**, apparently did not bind to SR-I apoprotein since it did not form a red-shifted pigment with SR-I apoprotein, nor did it inhibit regeneration of SR-I when *all-trans*-retinal was added to apomembranes incubated with this analog for 2 h.

Photochemical Reactions of Analog Pigments. Photon absorption by the chromophore of SR-I and subsequent retinal isomerization energize conformational changes of the protein which have been described in terms of a photocycle model including three intermediates with distinct absorption maxima (Bogomolni & Spudich, 1987). At 1 ms after photoexcitation, the S_{373} intermediate has already formed and decays directly to SR-I with a $t_{1/2}$ of 750 ms at room temperature (Figure 7A). Photochemical activity of SR-I pigment reconstituted from **g** (Figure 7C) or **h** (not shown) is not detectable in our measurements (250-μs time resolution), whereas **g** was reported to generate a photochemically and physiologically active BR analog pigment (Zingoni et al., 1986). 13-Et-SR-I exhibits a much slower photocycle ($t_{1/2}$ = 15 s) with a lower yield (Figure 7B) compared with the native SR-I pigment with equal maximum extinction. The flash-induced absorption difference spectrum of the analog pigment shows depletion of

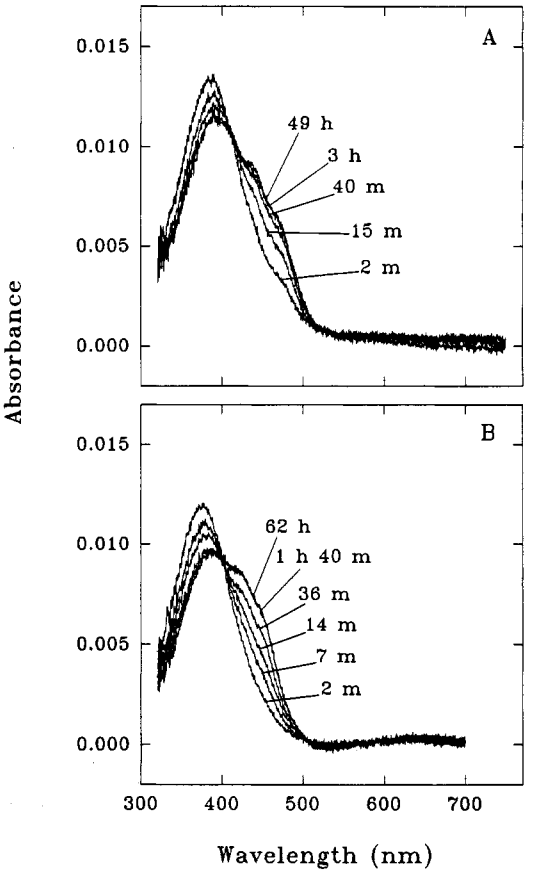


FIGURE 5: Reconstitution of analog SR-I pigments with 14-methylretinal and 13-*cis*-locked retinal. Five microliters of an ethanolic solution of **c** (A) or **d** (B) was added to 3 mL of SR-I apomembranes. Absorption spectra were taken at 2, 15, and 40 min, 3 h, and 49 h (A) and 2, 7, 14, and 36 min, 1 h 40 min, and 62 h (B) after addition of analog.

a pigment with the same absorption maximum as 13-Et-SR-I and the transient formation of a S_{373}-like photointermediate (inset of Figure 7B). On the other hand, the 13-Et-BR was reported to exhibit a slightly faster photocycle due to a faster decay of its M_{412}-like intermediate compared to that of native BR (Gärtner et al., 1988). Flash photolysis of the SR-I analog pigment reconstituted from 9,12-phenylretinal (**b** in Figure 1) did not produce any photochemical changes at 470 and 380 nm (not shown), consistent with the argument that steric hindrance from the phenyl ring inhibits retinal isomerization, as concluded for the corresponding inactive BR analog (Kolling et al., 1984).

From our measurements, addition of glycerol (20–66%) to the membrane suspension does not affect the absorption spectrum of SR-I nor the transition from S_{610} to S_{560}. The scattering of the SR-I sample is greatly reduced in the glycerol/buffer solvent. Flash-induced absorbance changes in SR-I and 13-H-SR-I were monitored at 670 nm in 66% glycerol/buffer suspensions at 22 °C. After photoexcitation of SR-I, a transient is formed which decays at a rate much slower ($t_{1/2}$ 90 μs, Figure 8) than the K intermediate (2 μs). In 13-H-SR-I, no such intermediate is detected 10 μs after the flash (Figure 8). The shape and the height of the absorption spectrum of the 13-H-SR-I pigment (λ_{max} 567 nm) were not changed after flashes even with a greater intensity than that used in Figure 8. Therefore, the results show that a S_{610}-like intermediate is not formed after photoexcitation of 13-H-SR-I.

Photosensory Behavioral Measurements. Halobacteria recognize patterns of light and search for optimal illumination

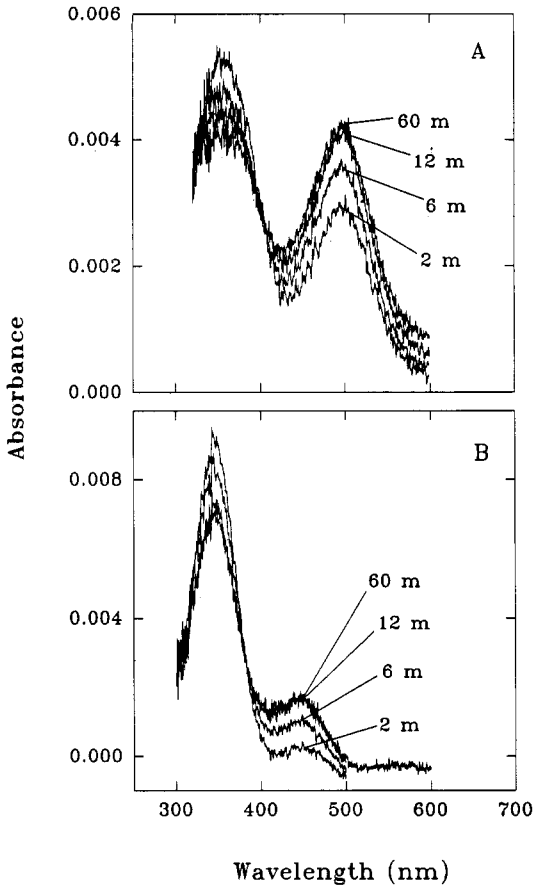


FIGURE 6: Reconstitution of analog SR-I and BR pigments with acyclic retinal analogs. Excess amounts of **g** (A) or **h** (B) in 5 μL of ethanol were added to Flx5R apomembranes. Spectra in both cases were taken at 2, 6, 12, and 60 min after addition.

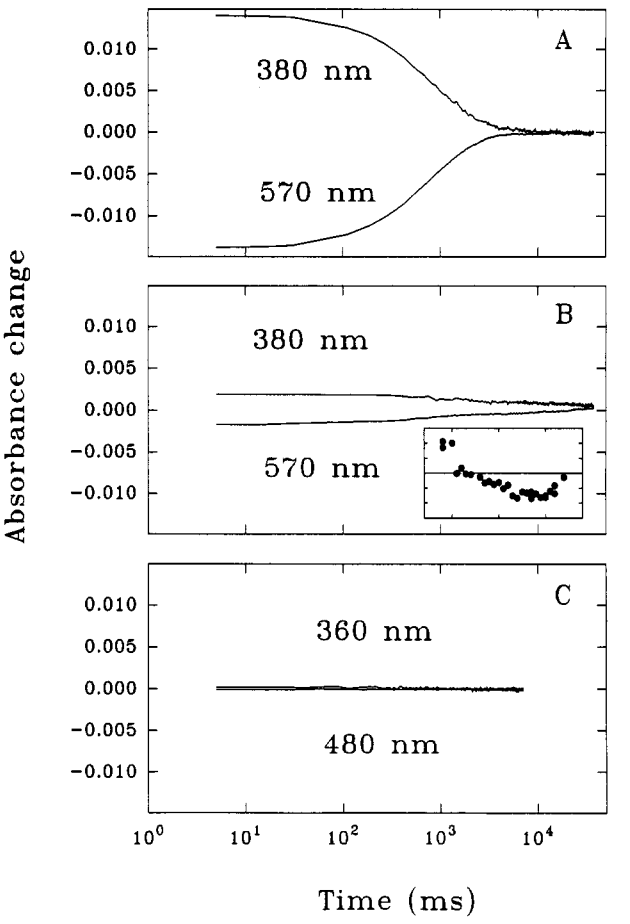


FIGURE 7: Flash-induced absorbance changes and difference spectra of the native and analog pigments. Actinic flashes at 580–620 nm were used for excitation of SR-I (A) and 13-ethyl-SR-I analog pigment (B), and actinic flashes at 480–520 nm were used for analog g reconstituted SR-I analog pigment (C). Flash-induced absorbance changes were monitored at various wavelengths in the near-UV through visible spectral region (only absorbance changes at selected wavelengths are shown). The time 0 is defined as the time of actinic flash in all cases. Inset, panel B: the flash-induced absorption difference spectrum 5 ms after the flash is shown for 13-ethyl-SR-I analog pigment. On the ordinate, tics above and below the zero line are spaced by 1×10^{-3} absorbance units. The three tics on the abscissa from left to right are 400, 500, and 600 nm.

by suppressing their spontaneous reversal frequency when the receptors detect a temporal increase of the attractant light (green-red, Figure 9A) or a decrease in repellent light (blue-UV, not shown) and by enhancing their reversal frequency in response to an increase in repellent light (Figure 9D) or a decrease in attractant light (Figure 9A).

Analogs **g** (Figure 9C,F) and **h** (not shown) do not reconstitute behavioral photoresponses in our assay. Although SR-I analog pigments are reconstituted by these two retinal analogs (Figure 6), these SR-I analog pigments are apparently physiologically inactive, consistent with their lack of photochemical reactivity.

Phototaxis responses of *H. salinarium* cells were previously reported when 13-ethylretinal was added to a wild-type strain under conditions which inhibit retinal synthesis (Schimz et al., 1983). However, the behavior of the bacteria may have been complicated by the coexcitation of a number of other retinylidene proteins. Here we measure *partial* photoresponses mediated by 13-ethyl-SR-I in a strain lacking apoproteins of BR, HR, and detectable amounts of SR-II. Despite the relatively low flash yield of an S_{373}-like intermediate in the 13-ethyl-SR-I analog pigment (Figure 7B), the fast rise (<1 ms) and slow decay of this intermediate results in its accumulation in continuous illumination conditions. Consistent with the proposed function of S_{373} as the attractant signaling state (Yan & Spudich, 1991), the orange light step-up causes a reversal suppression response (Figure 9B). As expected from the additional function of S_{373} as a repellent receptor (Spudich & Bogomolni, 1984), under continuous orange background light illumination, a near-UV light stimulus causes a repellent response (Figure 9E). These responses are comparable to those of cells incubated with the native retinal (Figure 9A,D). In response to the removal of the attractant light illumination, cells incubated with the native retinal undergo a period of reversal induction (Figure 9A). The attractant light step-down response, however, is not observed for this analog receptor. This result fits the observation from analog SR-I pigments with various decay rates of their S_{373}-like intermediates that the SR-I step-down response is diminished when the decay rate of this intermediate is decreased.

DISCUSSION

Steric Constraints in the Retinal Pocket Indicated by Temperature Effects. Free energy barriers separate individual intermediates and enable their detection within limited ranges of temperature and/or time. All retinylidene proteins other than SR-I studied by low-temperature absorption spectroscopy exhibit the property that early photoproducts can be trapped at low temperatures, e.g., rhodopsin (Yoshizawa & Wald, 1963), BR (Iwasa et al., 1979), HR (Zimanyi et al., 1989), sensory rhodopsin II (SR-II or phoborhodopsin; Imamoto et al., 1991) and *Natronobacterium pharaonis* phoborhodopsin

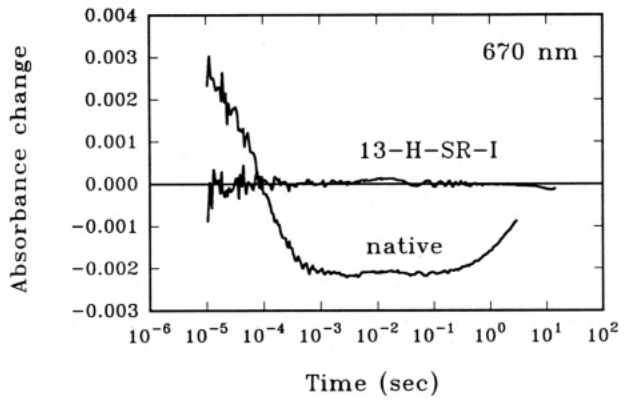


FIGURE 8: Flash-induced absorbance changes of SR-I and 13-H-SR-I at 670 nm. Suspensions of Flx5R membranes were reconstituted with excess *all-trans*-retinal or *all-trans*-13-desmethylretinal for 5 days at room temperature. The pigments formed have absorbance of ~0.02 at their absorption maxima. The membrane suspension was pelleted and resuspended in 1 mL of buffer (Tris-HCl at pH 7.0 and 4 M NaCl) and 2 mL of glycerol. A laser flash (532 nm, 5–10 mJ, 10-ns pulse duration) was delivered at time 0.

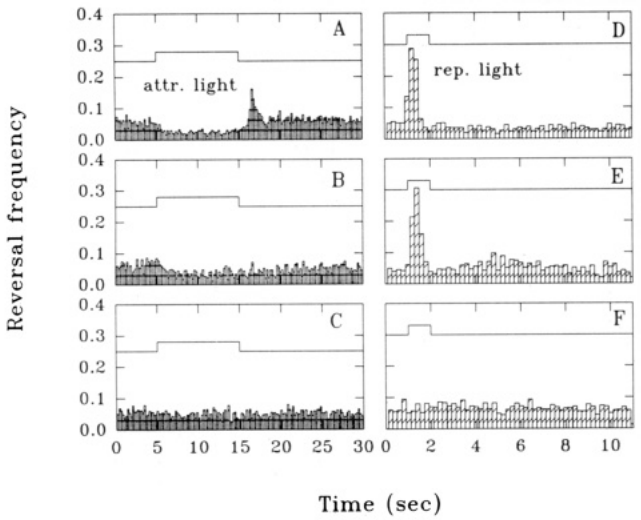


FIGURE 9: Photosensing behavior of cells incubated with the native chromophore, 13-ethylretinal, and **g**. An early stationary-phase culture of Flx3R cells was diluted 1:67 into fresh medium and cell reversal frequency responses to photostimuli were analyzed. Cells were incubated with *all-trans*-retinal (A,D) or 13-ethylretinal (B,E) for 3.5 days or with **g** (C,F) for 2 h. Step-up of a 580–620-nm (75 W/m², panels A and B) or a 480–520-nm (60 W/m², panel C) light from 0 W/m² was delivered as shown. A pulse of 380–420 nm (90 W/m²) was delivered in panels D–F with background light illumination (>550 nm and 150 W/m² for panels D and E, >500 nm and 200 W/m² for panel F).

(Hirayama et al., 1992). However, in SR-I, no photointermediates other than S_{373} were trapped from 300 to 80 K (Ariki et al., 1987) although the transient K- and L-like intermediates were detected by flash photolysis at room temperature (Bogomolni & Spudich, 1987). One possible explanation is that temperature-dependent branching reactions occur in the early half of the photocycle and become dominant as temperature is lowered. An alternative explanation is that photoreactions are blocked at low temperatures. We undertook flash photolysis of SR-I at various temperatures from 300 to 80 K to distinguish these possibilities.

As temperature is lowered from 300 to 220 K, the amount of S_{610} formation is invariant, independent of temperature. Within a similar temperature range, S_{560} formation is not affected but S_{373} formation yield drops dramatically with lowering temperature (Ariki et al., 1987; Ohtani et al., 1989). Thus, a branch reaction from S_{560} back to SR-I was suggested (Ohtani et al., 1988). Below 220 K, the flash-induced formation of S_{610} decreases and is reduced to zero at 100 K. The blocked formation of S_{610} below 100 K is consistent with the previous results of Ariki et al. (1987) that S_{610} was not trapped at these temperatures. At temperatures between 220 and 100 K, the S_{610} is formed in low yield and decays in the dark. The preflash amount of excitable SR-I is recovered within minutes. The branching reaction from S_{560} back to SR-I suggested by Ohtani et al. (1988) or reversion of the bathointermediate to SR-I due to low-temperature inhibition of protein conformational changes as found in iodopsin (Imamoto et al., 1989) may be responsible for the inability to trap S_{610} and S_{560} at these temperatures.

Differences in composition and structure of the retinal pocket in retinylidene proteins are expected to affect the temperature dependence of their photoreactions. Formation of bathointermediates in rhodopsin and BR does not depend on temperature from 300 to 77 K (Hurley et al., 1977). This result was interpreted to indicate the efficient channeling of the excited system into a minimum, along a barrierless potential surface connecting the *cis* and *trans* configuration (Hurley et al., 1977). However, isorhodopsin exhibits a temperature-dependent quantum efficiency, and a small barrier at the excited-state potential surface was proposed to interpret this dependence (Birge et al., 1988). The energy barrier is likely introduced by changes in the location of retinal in conversion from the native 11-*cis* to the 9-*cis* configuration. A 9-*cis* (instead of the native 11-*cis*) to *all-trans* isomerization of retinal alters both electrostatic and steric retinal/protein interactions. In a more related case, HR also exhibits a temperature-dependent formation of its K-like intermediate. The amount of K-like intermediate of HR in the photostationary state at 80 K is substantially less than at 140 K under similar illumination conditions. It was suggested that protein relaxations are inhibited at low temperatures and that the quantum efficiency is decreased by relative rigidity of the protein (Zimanyi et al., 1989). The low-temperature flash result for SR-I is an extreme case, namely, very tight retinal/protein interactions prevent the retinal isomerization at low temperature.

Our data are in accord with the presence of an energy barrier for photoisomerization; the height of the energy barrier is greatly increased as the molecule is cooled. In the photoisomerization process, the increased steric hindrance raises the energy barrier for retinal isomerization. The decrease of S_{610} formation with lowering temperature appears to be a complex process (Figure 2). The energy surface of the excited state may be temperature-dependent. Retinal proteins or photointermediates undergo structural alterations at low temperature as shown by spectral shifts from UV–vis spectroscopy (Yoshizawa & Wald, 1963; Iwasa et al., 1979) or by changes in vibrational modes from resonance Raman spectroscopy (Doig et al., 1991). Such structural changes may create a significant energy barrier at the excited state against a complete retinal isomerization. The detection of steric hindrance around the retinal polyene chain (see below) suggests that the SR-I retinal pocket, unlike that of BR, is highly confined. Therefore, protein vibrational motions may be crucially involved in the formation of S_{610}. In this case, freezing out the protein motion by lowering temperature would increase the activation energy barrier for photoisomerization. It is interesting to note that the observed transition temperature for disappearance of S_{610} is in the range of the glass transition temperature of proteins, below which protein motions are arrested (Iben et al., 1989; Nienhaus et al., 1992).

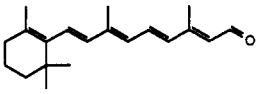
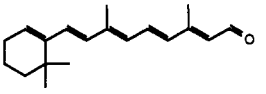
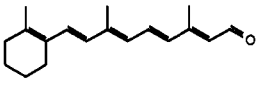
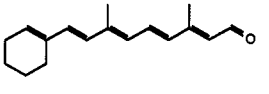
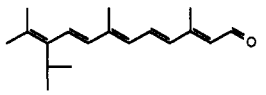
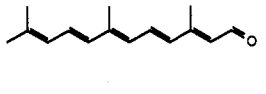
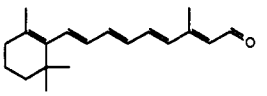
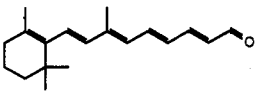
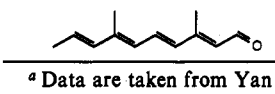
Steric Constraints in the Retinal Pocket Indicated by Analog Effects. The low-temperature flash results indicate a more confined, rigid retinal binding pocket in SR-I compared to that in BR. Unambiguous identification of the structural constraints will probably require a SR-I crystal structure determination. However, the study of interactions of the SR-I apoprotein with retinal analogs carrying bulky substituents provides clues to the location of the constraints, especially when the results are compared with analog effects in BR for which an atomic resolution structural model is available (Henderson et al., 1990). The BR reconstitution reaction is known to proceed by retinal entering the pocket (step 1), being forced to adopt a ring/chain coplanarized conformation near the binding site (step 2) (Schreckenbach et al., 1977, 1978), and forming a covalent protonated Schiff base bond (step 3) (Gärtner et al., 1983). Similar reconstitution processes and intermediates have been detected in SR-I analog reconstitutions (Yan et al., 1990a, 1991). In principle, retarded retinal binding may result from the narrowness in the entrance or in the binding pocket, but retardation on step 3 would strongly indicate steric hindrance in the retinal pocket itself. Conversion of retinal 13-methyl into an ethyl group specifically retards step 3 for SR-I but not for BR (Figure 3C,D). On the other hand, conversion of 13-methyl into a hydrogen retards this step for both proteins (Gärtner et al., 1983; Yan et al., 1991). These observations indicate that in both SR-I and BR the retinal 13-methyl is interacting with a protein residue to facilitate step 3 during reconstitution. However, this residue is flexible in BR (possibly Leu93; Subramaniam et al., 1991) but much more rigid in SR-I.

The differing steric hindrances in the retinal pockets of SR-I and BR probed with **b** and **d** are especially evident. Analog **b** exhibits the same binding rate to BR apoprotein as native retinal (Figure 4A,C), but it binds to SR-I apoprotein 12-fold slower than native retinal (Figure 4B,D). Step 3 of **d** binding to BR apoprotein is 10-fold slower than native retinal (Fang et al., 1983), but this step is completely blocked for SR-I apoprotein (Figure 5B). The third step of pigment reconstitution with 14-methylretinal is blocked for both SR-I apoprotein (Figure 5A) and BO (Schiffmiller et al., 1983), indicating that a rigid protein residue, perhaps tryptophan (Henderson et al., 1990), is positioned similarly near C14 in both SR-I and BR. In line with this conclusion, a retinal analog locked in a 13-*trans* configuration with a five-membered ring across C12 and C14 retards both analog SR-I (Yan et al., 1990a) and BR (Fang et al., 1983) pigment formation to similar extents.

A previous finding (Yan et al., 1991) which suggests additional steric constraints in SR-I is that 13-*cis*-retinal forms a 440-nm-absorbing reconstitution intermediate quickly but does not form a covalent pigment unless it is isomerized to *all-trans*. Steric constraints preventing the binding of 14-methylretinal in both SR-I and BR are not responsible for inhibiting the binding of 13-*cis*-retinal to SR-I apoprotein since 13-*cis*-retinal binds to BR apoprotein even more rapidly than *all-trans*-retinal (Oesterhelt & Schuhmann, 1974). This result indicates additional inhibitory constraints for adoption of a 13-*cis*-retinal configuration exist specifically in SR-I but not in BR (see below). These binding results further support the conclusion that the protein residues forming the retinal polyene chain pocket impose more steric hindrance in SR-I than in BR.

Indications for Concerted Steric Interactions at the C4–C6 Unit of the Retinal β-Ionone Ring. Critical steric interactions of various components on the retinal with the protein have been tested *in vitro* and *in vivo* by reconstitution of SR-I analogs with selective deletions (Table I). The two methyl groups at C1,5-methyl and most parts of the β-ionone ring (Yan et al., 1990b), as well as the 9-methyl group (Yan et al., 1991), can each be removed without diminishing SR-I function. The loss of photochemical and photosignaling activities of the SR-I analog formed with 13-desmethylretinal provided the first evidence for the involvement of steric interactions in SR-I activation (Yan et al., 1991). If the crucial 13-methyl/protein interaction is intact, the SR-I analogs are active as long as the chromophore retains the C4-C6 part of the β-ionone ring (**f**). Analog **f** represents the minimal chromophore skeleton required for a functional photoreceptor since **g**, with further deletion of the C4–C6 part (following retinoid numbering), though forming an analog SR-I pigment (Figure 6A), fails to generate photochemical and physiological functions (Figure 7C, Figure 9C,F, and Table I). On the other hand, the corresponding BR analog has been shown to be functional (Zingoni et al., 1986). Steric confinements in the SR-I retinal pocket predict that removing retinal substituents would liberate retinal from steric hindrances and facilitate the photoreaction if those substituents are not actively involved in the process. While most retinal substituents can be removed without affecting the pigment activity as shown in Table I, the 13-methyl and C4–C6 portion of the β-ionone ring are indispensable. We take this as support for the requirement of concerted steric retinal/protein interactions from these distinct portions of retinal during SR-I photoactivation.

Table I: Properties of SR-I Analog Pigments with Chromophore Substituent Deletions[a]

chromophore	λmax of pig. (nm)	photoproduction of S_{373}-like int.	phototaxis signaling
	587	Yes	Yes
	550	Yes	Yes
	588	Yes	Yes
	530	Yes	Yes
	587	Yes	Yes
	555	Yes	Yes
	569	Yes	Yes
	567	No	No
	497	No	No

[a] Data are taken from Yan et al. (1990b, 1991) and this work. int. = intermediate; pig. = pigment; photoaxis signaling was measured as the reversal frequency suppression response to orange light and induction response to near-UV light with orange background light.

Role of the 13-Methyl Group in Photoisomerization. Red-shifted intermediates are commonly observed as the first detected intermediates following retinal isomerization in known retinal-based pigments. The red shift is believed to be caused by isomerization-induced charge separation between the protonated Schiff base and its counterion(s) and can be used as an indicator for the formation of an isomerized intermediate. 13-H-SR-I absorbs at 567 nm and contains an *all-trans* chromophore (Yan et al., 1991). Photolysis of this pigment does not produce an S_{610}-like intermediate (Figure 8), indicating *all-trans*/13-*cis* photoisomerization of retinal in SR-I requires 13-methyl. We do not exclude the possible transient formation of a partially isomerized species more rapidly than our time resolution. However, the formation of a complete isomerized chromophore in this analog pigment can be ruled out by our data because thermal double-bond isomerization cannot occur within our time resolution (microseconds).

Unlike BR, the 13-*cis* configuration of retinal is not accommodated in the SR-I binding site in the dark (Yan et al., 1991). The restrictions inhibiting thermal isomerization of *all-trans*-retinal to 13-*cis* or binding of 13-*cis*-retinal may be steric in origin. In SR-I, helix A and helix G, which contains the lysine for binding retinal, are subject to more severe steric interactions because up to nine small residues on these helices in BR (Phe27, Gly31, Gly218, Gly220, Leu221, Ile222, Leu223, Leu224, and Ser226) are changed to bulkier ones in SR-I (Trp23, Arg27, Pro207, Val209, Tyr210, Phe211, Phe212, Tyr213, and Arg215). Greater steric restrictions in SR-I were also predicted through constructing an SR-I structural model based on its similarity to BR by energy minimization calculations (S. L. Lin and B. Yan, unpublished). Comparison of SR-I and BR models predicts greater steric constraints on movement of Lys205 in SR-I while the corresponding Lys216 in BR is much less confined. The experimental results and these structural considerations suggest more steric inhibitory constraints resist the adoption of a 13-*cis*-retinal configuration in SR-I but not in BR. Steric interactions between parts of the β-ionone ring and protein residues, as shown above, are involved in SR-I activation but not in that of BR. In both BR (Dencher et al., 1989) and SR-I, an isomerization-induced twist at both ends of retinal mimics a lever-like movement. The beam of the lever would include the Schiff base linked lysine side chain on one side of the fulcrum and the β-ionone ring on the other. Steric retinal/protein interactions therefrom may be so restrictive in SR-I that a fulcrum provided by the retinal 13-methyl is indispensable. In this interpretation, our result would imply that the chromophore in 13-H-SR-I cannot isomerize without the function of this fulcrum. Alternatively, removal of the 13-methyl may alter the structure of the retinal binding cavity in a manner which increases the energy barrier against isomerization.

Conclusions. We observe an unusual temperature dependence for S_{610} intermediate formation from photoexcitation of SR-I: flash-induced S_{610} formation decreases markedly below 220 K and is totally blocked at 100 K. The result suggests there are steric constraints from the rigid retinal binding pocket in SR-I and the frozen protein side chains block retinal photoisomerization at 100 K. Consistent with this interpretation, the retinal polyene chain binding domain of SR-I appears highly sterically confined since retinal analogs carrying extra sterically demanding substituents on the polyene chain are difficult to fit into the retinal pocket. In contrast, the formation yield of the K intermediate of BR does not depend on temperature from 300 to 9 K and the polyene chain binding site in BR is not as sterically constrained as in SR-I according to comparative analog binding studies.

The C4–C6 portion of the β-ionone ring and 13-methyl are required for SR-I activation but not for that of BR. Therefore, steric constraints in the retinal polyene chain binding site of SR-I, interactions between the retinal β-ionone ring and protein residues, and steric restrictions on the movement of the side chain of Lys205 for adopting a 13-*cis* configuration appear to impose an unconquerable energy barrier for isomerization below 100 K. Even at room temperatures, to accommodate the transient twist at both ends of the chromophore induced by the *all-trans*/13-*cis* isomerization of retinal, the 13-methyl may play an indispensable role as a fulcrum to overcome steric constraints produced therefrom. Consistent with this interpretation, photolysis of 13-H-SR-I failed to form an S_{610}-like intermediate at room temperature.

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