Effects of modifications of the retinal β-ionone ring on archaebacterial sensory rhodopsin I

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ABSTRACT Ring desmethyl and acyclic analogues of all-*trans* retinal were incorporated into the apoprotein of the phototaxis receptor sensory rhodopsin I (SR-I) in *Halobacterium halobium* membranes. All modified retinals generate SR-I analogue pigments which exhibit "opsin shifts," i.e., their absorption spectra are shifted to longer wavelengths compared with model protonated Schiff bases of the same analogues. Each SR-I pigment analogue exhibits cyclic photochemical reactions as monitored by flash spectroscopy, but the analogue photocycles differ from that of native SR-I by exhibiting pronounced biphasic recovery of flash-induced absorption changes and abnormal flash-induced absorption difference spectra. Despite perturbations in the photochemical properties, the SR-I pigment analogues are capable of both attractant (single photon) and repellent (two photon) phototaxis signaling in cells. Our interpretation is that the hydrophobic ring substituents interact with the binding pocket to maintain the correct configuration for native SR-I absorption and photochemistry, but these interactions are not essential for the physiological function of SR-I as a dual attractant/repellent phototaxis receptor. These results support the conclusion emerging from several studies that the photoactivation process that triggers the conformation changes of SR-I and the related proton pump bacteriorhodopsin is conserved despite the different biological functions of their photoactivation.

INTRODUCTION

Four retinal-containing proteins have been found in *Halobacterium halobium* membranes. Bacteriorhodopsin (BR) and halorhodopsin (HR) are light-driven ion pumps which convert light energy into transmembrane electrochemical potential (Oesterhelt and Stoeckenius, 1973; Stoeckenius and Bogomolni, 1982; Lanyi, 1986). Two phototaxis receptors, sensory rhodopsin I (SR-I) and sensory rhodopsin II (SR-II; also called phobo rhodopsin), have been identified which discriminate color and modulate the motility pattern of the cells to guide them into regions of illumination optimal for BR and HR, while avoiding damaging UV light (Takahashi et al., 1987; Spudich and Bogomolni, 1988). SR-I is an attractant light receptor, and its long-lived photointermediate S_{373} mediates repellent responses (Spudich and Bogomolni, 1984). SR-II is an additional repellent receptor (Takahashi et al., 1985; Tomioka et al., 1986).

Retinal analogues, in which the native retinal is selectively modified, have been used to probe chromophore/protein interaction and structure/function relationships in retinal-containing proteins (Nakanishi et al., 1980; Derguini and Nakanishi, 1986). Studies of pigments containing an analogue modified in the polyene chain with a five-membered ring which prevents all-*trans*/13-*cis* isomerization show that the isomerization is an essential step for the photochemical reaction and proton pumping of BR (Fang et al., 1983; Chang et al., 1985) and also for the photochemical reactions and the phototaxis signaling by sensory rhodopsins in *H. halobium* (Yan et al., 1990). Studies with modified conjugated systems (Spudich, J. L. et al., 1986) and β-ionone ring/polyene chain conformations (Baselt et al., 1989) have shown that the steric and electrostatic properties of the retinal binding sites in SR-I and BR have been conserved despite their different functions.

Ring-modified retinal analogues have been used to study the role of the β-ionone ring in BR and rhodopsin (Gärtner et al., 1983; Courtin et al., 1987; Crouch and Or, 1983; Crouch et al., 1984; Kropf et al., 1973). These studies showed specific interactions between ring methyls and the protein binding site are essential for bovine rhodopsin but not for BR to form stable pigments, and that BR proton pumping is not sensitive to ring portion modifications. Here we report the results of studies of SR-I reconstituted with a series of retinal analogues altered in the β-ionine ring moiety. Properties of SR-I analogues are compared with corresponding results with BR and bovine rhodopsin in previous reports.

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MATERIALS AND METHODS

Retinal and analogs

All-*trans* retinal was purchased from Sigma Chemical Co. (St. Louis, MO). Retinal analogues were synthesized as described elsewhere (Jaya-

0006-3495/90/03/477/7 $2.00

thirtha Rao et al., 1985; Courtin et al., 1987). All retinals were purified by high-performance liquid chromatography (10 × 250 mm μ-Porasil column eluted with 8% ethyl acetate in hexane at a flow rate of 3.0 ml/min) before use.

Strains

H. halobium mutant strain Flx5R (Spudich, E. N., et al., 1986) lacks BR and HR apoproteins (BOP^-HOP^-), overproduces SR-I apoprotein, and furthermore is blocked in retinal synthesis. SR-II apoprotein was not detectable in Flx5R in our preparations. Flx3R is also BOP^-HOP^- and retinal deficient (McCain et al., 1987) and was used for behavioral measurements because of its better motility.

Culture conditions and vesicle preparation

H. halobium were grown aerobically in complex medium (Lanyi and MacDonald, 1979) in the dark and membrane vesicles were prepared by sonication as described (Manor et al., 1988).

Absorption spectra

Absorption spectra were measured in Flx5R vesicles. Ethanolic solutions of all-*trans* retinal or retinal analogues were added to 2.5 ml of Flx5R vesicle suspensions at 1.8 mg protein/ml in 4 M NaCl, 20 mM Tris-HCl, at pH 6.8, and 23–25°C. Spectra were measured on a 110B double beam spectrophotometer (Hitachi Instruments, Inc., Santa Clara, CA) equipped with an integrating sphere. Unreconstituted vesicles were used as reference. Path length was 1 cm.

Flash spectroscopy

Flash-induced absorbance changes were measured in Flx5R vesicles using an experimental system described previously (Spudich, E. N. et al., 1986). For all experiments, an electronic flash (model 283; Vivitar Corporation, Santa Monica, CA) was used for the actinic flash. The actinic light was passed through a wide band interference filter (flash wavelength ± 20 nm). The wavelength of the monitoring beam from a tungsten halogen lamp (12 V, 50W) was selected by a monochromator and the photomultiplier tube was further protected by a narrow band filter transmitting the monitoring wavelength ± 5 nm. Optical filters used were from Ditric Optics Inc. (Hudson, MA). All measurements were made at 25°C. Data points from 20 to 30 flashes were averaged and stored. Each trace was acquired as 3,968 data points, with an acquisition rate of 2 ms/point. Rate constants and amplitudes were obtained by fitting 160 data points evenly distributed over the trace to a single or a double exponential. The best fit was decided by the shape of the residual function and the standard deviation of the data from the fitting curves.

Behavioral assay

Cells were grown to late exponential phase in complex medium and diluted 1:67 into 2 ml fresh medium. Retinal or analogues in 5 μl ethanol were added and the suspension (final concentration 1 μM) was incubated for 2–4 h with shaking at 37°C in the dark before motility measurements. Swimming behavior was monitored at 37°C with nonactinic infrared illumination (wavelength > 700 nm), recorded on video tape and analyzed by using the EV1000 software on a Sun 2/120 work

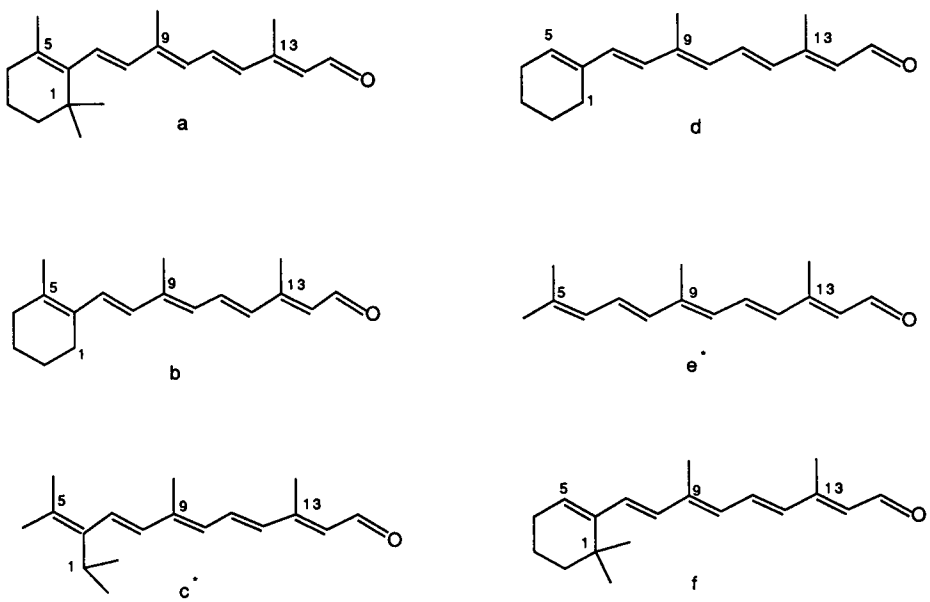

FIGURE 1 Retinal analogues modified at β-ionone ring moiety of all-*trans* retinal. Structure of (*a*) all-*trans* retinal and (*b–f*) retinal analogues used (*b*) 1,1-didesmethyl retinal, (*c*) 3,7,11-trimethyl-10-isopropyl dodeca-2,4,6,8,10-pentaenal, (*d*) 1,1,5-tridesmethyl retinal, (*e*) 3,7,11-trimethyldodeca-2,4,6,8,10-pentaenal, (*f*) 5-desmethyl retinal.
*The retinoid numbering system is used for *c* and *e* for the sake of convenience.

station (Motion Analysis System, Inc., Santa Rosa, CA) as described (Sundberg et al., 1986). Photo-stimuli were delivered with the optical arrangement and interference filters described previously for SR-I activation (600 ± 20 nm for the SR-I attractant form SR-I_{587}, and 400 ± 5 nm for the SR-I repellent form S_{373}). Reversals were assessed with the program described (Sundberg et al., 1986).

RESULTS

Ring analogs form pigments with SR-I apoprotein

SR-I pigments were generated by adding ethanolic solutions of all-*trans* retinal (*a* in Fig. 1) and analogues (*b–f* in Fig. 1) to Flx5R vesicle suspensions in both subsaturating and excess amounts. For all analogues, reconstitution rates were similar to that of native all-*trans* retinal at room temperature. This reconstitution behavior is similar to that of BR apoprotein with ring desmethyl analogues (Gartner et al., 1983; Courtin et al., 1987) and acyclic retinal analogues (J. Rao et al., 1985; Crouch et al., 1984) but differs from the behavior of bovine opsin (Crouch and Or, 1983) whose regenerability is sensitive to ring modifications (Kropf et al., 1973). The absorption maxima of SR-I analogues are shifted to longer wavelengths compared with those of corresponding model protonated Schiff bases measured as previously reported (Arnaboldi et al., 1979) (Table 1). When excess retinal is added, each analogue *b–f* generates less absorption beyond 500 nm than all-*trans* retinal (spectra not shown). Analogue pigment absorption at maxima of the main band equals 86 and 84% of that of native SR-I for *b* and *c*, respectively. These analogue pigments may exhibit lesser extinction coefficients than the native pigment. However, unlike conditions usually used to assess extinction coefficients (e.g., in purple membrane or rod outer segment preparations), the concentration of both retinal and apoprotein is low and we cannot assume saturation of the reconstitution. Additionally, lesser equilibrium constants for analogue/apoprotein binding could also account for the smaller absorbance.

TABLE 1 Absorption maxima of retinal analogues (CHO), their protonated Schiff bases (PSB) in methanol, their analogue SR-I pigments (pigment) and the opsin shifts (OS)

Chromophore	CHO	PSB	Pigment* (OS)
	nm	*nm*	*nm* (cm^{-1})
a	382	445	587‡ (5440)
b	404	476	588‡ (4000)
c	382	445	587‡ (5440)
d	394	465	530§ (2640)
e	400	465	555§ (3490)
f	390	458	550§ (3650)

*Maximum of the main absorption band.
‡Reproducible ±1 nm.
§Reproducible ±3 nm.

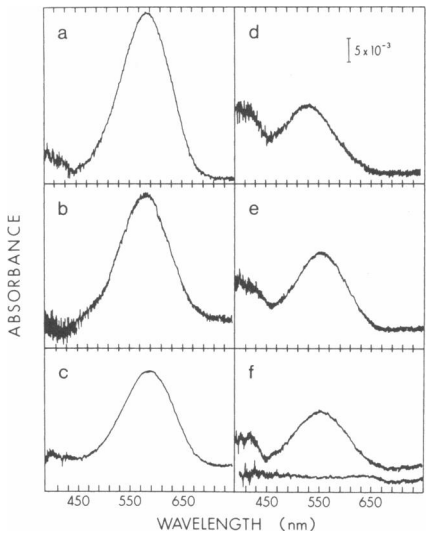

FIGURE 2 Absorption spectra of SR-I and SR-I analogues. 5 μl ethanolic solutions of all-*trans* retinal (*a*) and analogues (*b–f*) (approximate final concentration 480, 390, 330, 340, 330, 280 nM, respectively) was added to Flx5R vesicle suspension at 1.8 mg protein/ml in 4 M NaCl, 20 mM Tris-HCl, pH 6.8, at 23°–25°C and absorption spectra measured as in Materials and Methods. Spectra were at subsaturating concentrations of retinal and were recorded 1 h after retinal addition. A baseline after ethanol addition is shown at the bottom of panel *f*. The absorbance scale for all spectra is shown in *d*.

Analogue pigments *d–f* (Fig. 2) exhibit much lower absorption beyond 500 nm (57, 55, and 48% of native SR-I absorption, respectively, when excess analogue is present, spectra not shown). These pigments, in addition, show broader spectra and absorption below 450 nm even with subsaturating amounts of analogues. The shorter wavelength absorption may be due to the formation of multiple pigment species, or alternatively to the free chromophore (see Discussion).

The binding site of one of the analogues was demonstrated to be the same as that of native chromophore by adding all-*trans* retinal to Flx5R membranes containing the 1,1,5-desmethyl pigment (Fig. 1 *d*). The characteristic native SR-I peak at 590 nm replaces the peak of analogue pigment at 530 nm in 9 h with an isosbestic point at 550 nm (data not shown), confirming the analogue is bound to the same site as native chromophore.

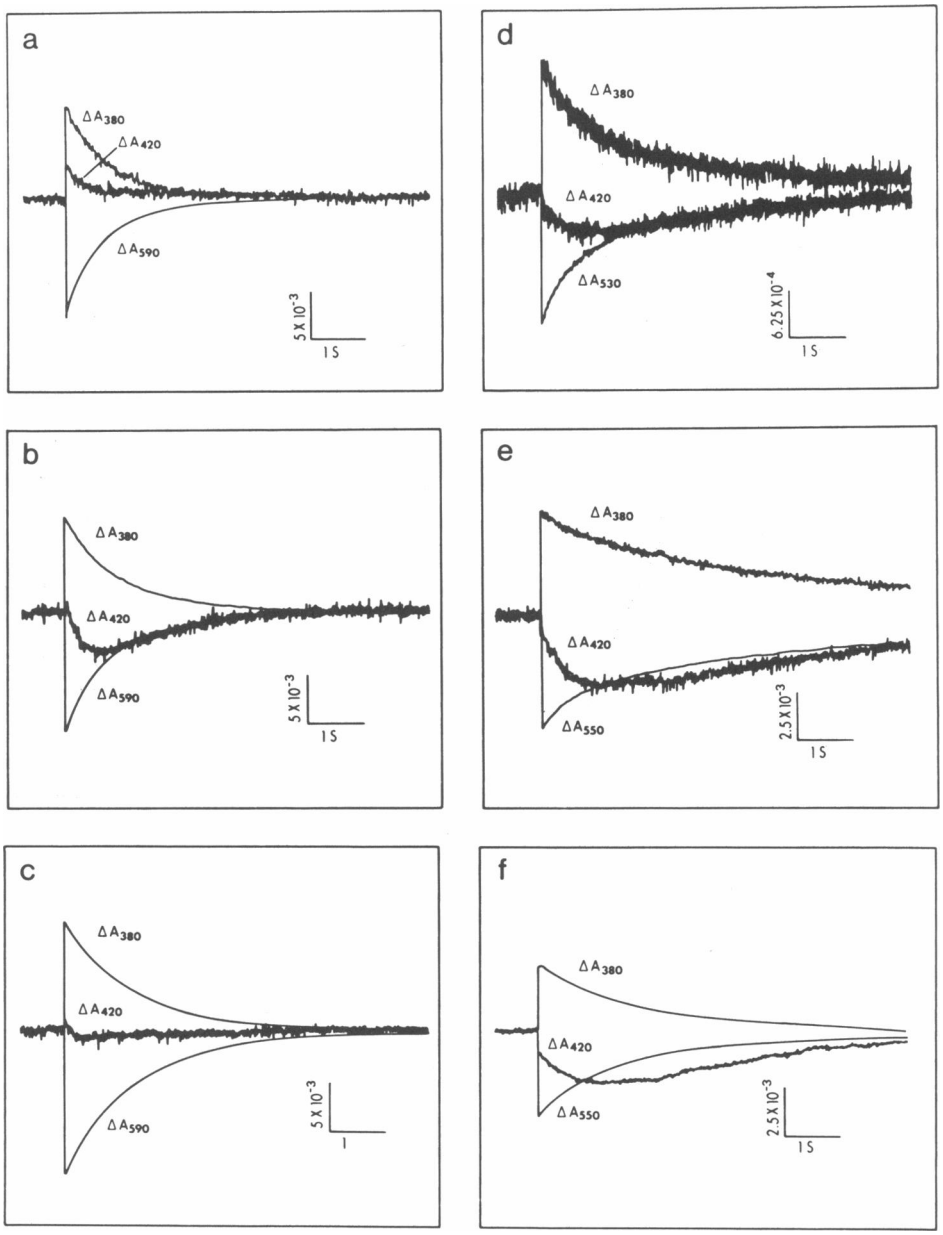

FIGURE 3 Flash-induced absorbance changes. Actinic light of 600 ± 20 nm and 1 ms duration was used and the sample absorbance was monitored with a cross-beam flash kinetic spectrophotometer at the wavelengths indicated on each trace. The scale shown in each panel is for 380 nm and 590 nm transients. In every panel the scale of the 420 nm transients is as in panel *d*.

Ring modifications alter the photochemical reaction cycle of SR-I and flash-induced absorption difference spectra exhibit multiple components

All of the SR-I analogues undergo photochemical reaction cycles which can be monitored by flash photolysis (Fig. 3). Unlike native SR-I (Fig. 3 *a*), pigments from *b–f* generate photochemical cycles with biphasic kinetics, especially evident at 420 nm (Fig. 3), pigments from *d–f* exhibit very slow components in their recovery.

Flash-induced absorbance transients as in Fig. 3 monitored at various wavelengths fit well the sum of two exponential terms. After curve fitting, amplitudes 2 ms after flash corresponding to the two rate constants for each transient were plotted against wavelength (Fig. 4). The fast component in each case is similar to native SR-I

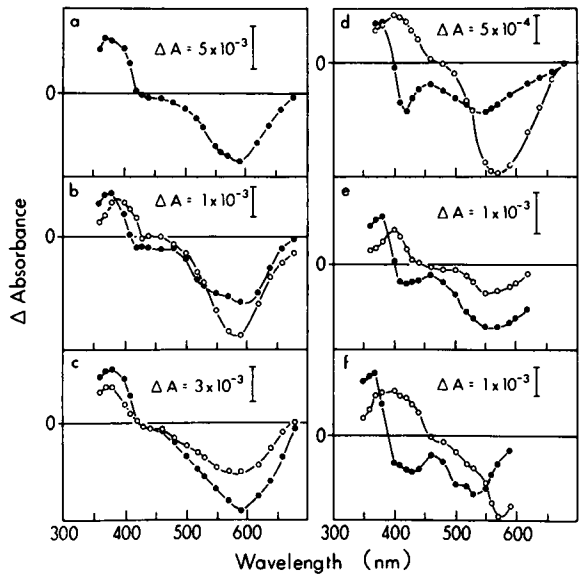

FIGURE 4 Absorption difference spectra. Flash-induced absorbance changes as in Fig. 3 monitored at various wavelengths were fitted with the sum of two exponential terms and the amplitudes 2 ms after the flash corresponding to the two rate constants plotted against wavelength. The actinic wavelength was 600 ± 20 nm for *a*, *b*, *c*, and *d*, 640 nm long-pass (15–31965, Ditric Optics Inc.) for *e* and 600 nm long-pass (15–21945, Ditric Optics Inc.) for *f*. In *a*, a single exponential fits the data. In *b–f* solid symbols represent the slow component ($0.2 \leq k \leq 0.5$ 1/s) amplitudes, and open symbols represent the fast component ($1.0 \leq k \leq 2.2$ 1/s) amplitudes.

in both the rate constant and the shape of its difference spectrum. The slow components of pigments generated with analogues *d–f* exhibit a second depletion minimum near 420 nm.

β-ionone ring-altered analogs reconstitute full phototaxis function of SR-I

All-*trans* retinal and analogues were added to retinal-deficient cells and behavioral responses to stimuli were determined by motion analysis. Addition of all-*trans* retinal to SR-I apoprotein generates the species SR-I_{587}. This form of the pigment, present in the dark-adapted cells, functions as an attractant receptor which suppresses reorientation behavior (swimming reversals) when photoactivated. The motility assays show that each of the analogues generates a red-shifted form of the SR-I receptor, which is effective in attractant signaling at a level similar to (or greater than [Yan, B., T. Takahashi, K. Nakanishi, J. Spudich, unpublished observations]) that of native SR-I_{587} (Table 2). Continuous illumination of native SR-I_{587} generates a photosteady state in which a blue-shifted form of SR-I (designated S_{373}) accumulates.

TABLE 2 **Phototaxis responses (percent reversing in 2 s)***

Chromophore	No stimuli	600 *nm* step-up	400 *nm* step-up‡
a	33 ± 3	6 ± 2	66 ± 5
b	33	6	69
c	31	0	70
d	31	1	62
e	34	5	71
f	32	4	72

*Each determination based on computerized tracking of >120 cells. Determined 0.5–2.5 s after 600 ± 20 nm stimulus, infrared background light.
‡Determined 0.0–2.0 s after 400 ± 5 nm stimulus, 600 ± 20 nm background light.
Mean ± standard deviation of four determinations.

In the native membrane, S_{373} functions as a repellent receptor, photoactivation of which transiently enhances swimming reversals. The motility analysis shows that despite the altered photocycles of the SR-I analogue pigments, each of them generates a blue/near-UV-absorbing long-lived photointermediate which functions as a photorepellent receptor in vivo (Table 2).

DISCUSSION

The opsin shift measures the energy difference between the absorption maximum of the protonated Schiff base as a free chromophore in a methanol solution and that of the corresponding pigment (Nakanishi et al., 1980). Opsin shifts produced by all analogues except *c* are smaller than that of native SR-I (Table 1). This indicates that modifications at the β-ionone ring site perturb the wavelength regulation mechanisms present in native SR-I. Two factors involving ring interactions have been suggested to contribute to the opsin shifts of SR-I and BR: (*i*) A greater degree of ring/chain coplanarity in the pigments than in the free retinal (Harbison et al., 1985; Baselt et al., 1989); and (*ii*) electrostatic interactions between the β-ionone ring and the protein binding site (Nakanishi et al., 1980; Spudich, J. L. et al., 1986). Ring desmethyl analogues *d* and *f* (Fig. 1) have a larger extent of ring/chain coplanarization than retinal in solution because of the absence of steric interactions between the ring methyl groups and C-8 proton. This factor would shift the protonated Schiff base absorption to the red, thereby decreasing the opsin shift of the pigment. Interactions between the retinal and some charged protein residues near the β-ionone ring also may be perturbed in pigment analogues.

All SR-I analogues exhibit cyclic photochemical reac-

tions demonstrating that the ring substituents are not required for the photochemical reactivity and thermal recovery of SR-I. However, removal of ring substituents does effect the SR-I photocycle as monitored by flash-induced absorption changes. All analogue pigments in this study exhibit biphasic decay of phototransients while that of native SR-I is monophasic. There are several possibilities to explain the observed biphasic kinetics. One is the accumulation of additional photointermediates on the thermal decay path of the S_{373}-like intermediate analogous to the *N* or *O* intermediates in the BR photocycle. The second possibility is that each analogue generates two or more different ground-state species, which has been observed in analogue reconstitution of BR (Sheves et al., 1984; Maeda et al., 1984; Iwasa et al., 1984). The greater absorption below 450 nm and the broader spectra of the analogue pigments in the cases of *d–f* (Fig. 2) compared with native SR-I are consistent with the presence of multiple pigments. Although the present data do not discriminate between these possibilities, ring substituents seem to be required to maintain the native retinal/protein interactions essential for the kinetics of the SR-I photocycle. The C-5 methyl group appears to be most important in maintaining these interactions (Fig. 2 and Fig. 4). Ring substituents could stabilize the native chromophore structure by hydrophobic interactions with protein residues, thereby anchoring the chromophore in the binding pocket, supporting the correct conformation of the chromoprotein to overcome the free energy barrier on the thermal decay pathway of S_{373}. In this regard, it is interesting to note that at ambient temperatures the thermal decay of S_{373} exhibits a shallow temperature dependence indicative of the contribution of entropic processes (Hazemoto et al., 1983).

Despite the perturbations in the spectroscopic and photochemical properties of SR-I, the analogues reconstitute both of the SR-I signaling forms. Therefore, the function of SR-I as a dual attractant/repellent photochromic mixture is a protein property relatively insensitive to the retinal/apoprotein interactions at the β-ionone ring.

The results above extend the close similarity in chromophore/protein interaction in BR and SR-I reported previously (Spudich, J. L. et al., 1986; Bogomolni and Spudich, 1987; Baselt et al., 1989). The data show that the structural requirements in the retinal binding sites of BR and SR-I are closely similar and that the β-ionone ring is not essential in photoactivation of either chromoprotein. A general conclusion from these studies is that the retinal/protein interactions required for the photoactivation process which triggers the conformational changes of BR and SR-I are similar despite the different biological functions of their photoactivation.

We wish to thank E. N. Spudich and L. A. Amici for their assistance and F. Derguini for discussion.

This work was supported by the Public Health Service grants GM27750 (Dr. Spudich) and GM36564 (Dr. Nakanishi) from the National Institutes of Health and grant N-00014-89-j-1629 from the Office of Naval Research (Dr. Spudich).

Received for publication 13 July 1989 and in final form 26 October 1989

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