Identification of Signaling States of a Sensory Receptor by Modulation of Lifetimes of Stimulus-Induced Conformations: The Case of Sensory Rhodopsin II†

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ABSTRACT: Lifetimes of stimulus-induced conformations of the phototaxis receptor sensory rhodopsin II (SR-II) from *Halobacterium halobium* are modulated with seven receptor analogues. By monitoring the receptor dynamics in vitro and physiological responses of the cell in vivo, we observe receptor signaling efficiency increases with decreasing cycling frequency (turnover number) of the receptor. The results demonstrate that modulating lifetimes of protein conformations at the SR-II photoactivation site with chromophore analogues alters the lifetime of the active conformation at the signaling site. We further explore the relationship between photocycle intermediates and the signaling efficiency by analyzing the time-averaged concentrations of the two long-lived spectral intermediates of the SR-II photocycle: S-II_{350} and S-II_{530}. The results are consistent with the signaling site being activated during formation of S-II_{350}, but not reset by the transition of S-II_{350} into S-II_{530}; rather deactivation appears to require subsequent decay of S-II_{530}. The results indicate the structural changes at the photoactivation site in the S-II_{350} → S-II_{530} transition do not reset the signaling site. The procedure used here, applicable in principle to any photoactivated or ligand-activated receptor, provides an initial approach to identify structural alterations key to the receptor activation process.

Sensory receptors activated by ligand binding or by photon absorption control intracellular processes which lead to physiological responses of the target cell. Postreceptor signal transduction pathways have been elucidated in a number of systems, e.g., phosphotransferase cascades in prokaryotes (Ninfa & Magasanik, 1986; Bourret et al., 1989; Stock et al., 1990) and G-protein and phosphoinositol pathways in eukaryotes (Stryer, 1986; Berridge & Irvine, 1984). But the first step in sensory signaling, the conversion of the extracellular stimulus into an intracellular signal by the receptor, is much less understood. It is generally assumed that stimuli "activate" the receptor protein by inducing structural alterations and converting the molecule from a nonactive state to an active state. Genetic modifications locking receptors into an activated or "signaling state" can provide information regarding domains involved in these structural alterations (Ames & Parkinson, 1988). To complement this approach, time-resolved methods are needed to assess stimulus-induced structural alterations of a receptor protein and their physiological significance.

For receptors containing intrinsic or exogenous spectroscopic reporter groups techniques are available to monitor stimulus-induced receptor dynamics, and a sequence of stepwise protein conformational changes can be resolved. For these receptors it is possible to define the stimulus-induced conformational intermediates which possess the physiologically active conformation at the signaling site and activate postreceptor signal transducing components: signaling states of the receptor. For example, photoexcitation of rhodopsin results in a sequence of five identified protein states characterized by shifts in the absorption spectrum of the protein (Wald, 1968; Birge, 1990). The conformation that exhibits altered affinities for transducin and rhodopsin kinase is not the end product in this sequence but rather an intermediate state (metarhodopsin II; Emeis et al., 1982; Bennett et al., 1982). As kinetics of other sensory receptors containing reporter groups (Zukin et al., 1977) becomes resolvable by kinetic spectroscopy, a method of identifying the receptor signaling states is also needed.

Sensory rhodopsin II (SR-II,[1] also called phoborhodopsin) is a phototaxis receptor in the archaebacterium *Halobacterium halobium* (Takahashi et al., 1985; Spudich & Bogomolni, 1988). Photoexcitation of SR-II (λ_{max} 487 nm) is followed by a sequence of thermal steps which return the molecule to its original state. In this cyclic process (photocycle) three intermediates with distinct absorption maxima have been identified by flash photolysis at room temperature (Tomioka et al., 1986) and low-temperature absorption spectroscopy (Schichida et al., 1988). The first intermediate, S-II_{520} (subscripts denote absorption maxima in nanometers), decays in about 1 ms to form a blue-shifted species S-II_{350}. The decay of S-II_{350} ($t_{1/2}$ 100 ms, room temperature) is paralleled by the rise of a red-shifted species S-II_{530} which thermally returns to SR-II_{487} ($t_{1/2}$ 300 ms), completing the cycle. Photoactivation of the SR-II photocycle mediates a repellent response (a reversal of swimming direction) by the cell.

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[1] Abbreviations: SR-II, sensory rhodopsin II; SR-II(a)–SR-II(g), SR-II analogue pigments reconstituted with chromophore a–g shown in Figure 1; λ_{max}, wavelength of maximum absorption; $t_{1/2}$, half-life; SEM, standard error of the mean; S-II_{520}, S-II_{350}, and S-II_{530}, SR-II photocycle intermediates with λ_{max} at 520, 350, and 530 nm, respectively.

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The basis of the signaling-state analysis is to modulate the concentrations of stimulus-induced conformational intermediates and to measure the subsequent physiological effect. Varying physiological conditions such as temperature or pH (Hazemoto et al., 1983), as well as membrane potential (Manor et al., 1988), can be used to alter receptor kinetics; however, these parameters in general affect postreceptor steps as well. In the case of SR-II, one way to target the modulation to the receptor itself is replacement of the native retinal with retinal analogues. As shown in this report, SR-II analogue pigments reconstituted with retinal analogues altered in the β-ionone ring or the polyene chain portions of the molecule produce apparently native-like long-lived photointermediates, but the decay rates of these intermediates are greatly altered, providing a method of lifetime modulation.

Materials and Methods

Chemicals. all-trans-Retinal was purchased from Sigma Chemical Co. (St. Louis, MO) and 3,4-dehydroretinal (analogue a) from Eastman Kodak. Naphthylretinal (analogue) was synthesized as in Iwasa et al. (1984), ring desmethylretinal analogues (analogues b–d) were synthesized as in Courtin et al. (1987), 9-desmethylretinal (analogue f) was synthesized as in van den Tempel and Huismann (1966) and Gärtner et al. (1983), and 13-bromoretinal (analogue g) was synthesized as in Motto et al. (1980). Retinal and analogues were purified by high-performance liquid chromatography on a 10 × 25 mm μ-Porosil column eluted with 8% ethyl acetate in hexane at a flow rate of 3.0 mL/min. The identities of analogues were corroborated by NMR, UV absorption data, and HPLC elution pattern as reported (Iwasa et al., 1984; Courtin et al., 1987; van den Tempel & Huismann, 1966; Gärtner et al., 1983; Motto et al., 1980). Solvent was removed with an argon stream and the retinal or analogues were dissolved in absolute ethanol before addition to membrane preparations.

Strains and Culture Conditions. H. halobium strain Flx3b is $BR^{-}HR^{-}SR\text{-}I^{-}SR\text{-}II^{+}$ (Takahashi et al., 1989). Flx3b was grown to early stationary phase in complex medium and in the presence of 2 mM nicotine to inhibit retinal synthesis as described (Howes & Batra, 1970).

Preparation of Apomembranes and Absorption Spectroscopy. Apomembrane suspensions were prepared as described (Takahashi et al., 1990). The absorption spectra were recorded at 24 ± 1 °C and pH 7 on an SLM-Aminco DW2000 spectrophotometer (SLM Instruments Inc., Urbana, IL) or a Hitachi 110B double-beam spectrophotometer (Mountain View, 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 membrane vesicles at 24 ± 1 °C as in Yan et al. (1990). Monitoring light was provided was provided by a 12-V 50-W tungsten–halogen lamp passed through a monochromator (dispersion 4 nm/mm, 2-mm slit) before impinging on the vesicle suspension in a cuvette (10-mm path length). The detector (photomultiplier Model R928, Hamamatsu Corp., Bridgewater, NJ) was protected from scattered actinic light by narrow-band (peak wavelength ±5 nm) interference filters. Actinic light (duration 250 μs) was provided by an electronic flash (Model 183, Vivitar, Santa Monica, CA). Absorbance transients were captured with a Nicolet (Madison, WI) Model 4562 analog-to-digital converter operating at 1 or 2 ms per point, and 10–20 sweeps were averaged and stored on a Nicolet 4094A digital oscilloscope. Rate constants and amplitudes were obtained by fitting 160 data points evenly distributed over the trace to the time course of

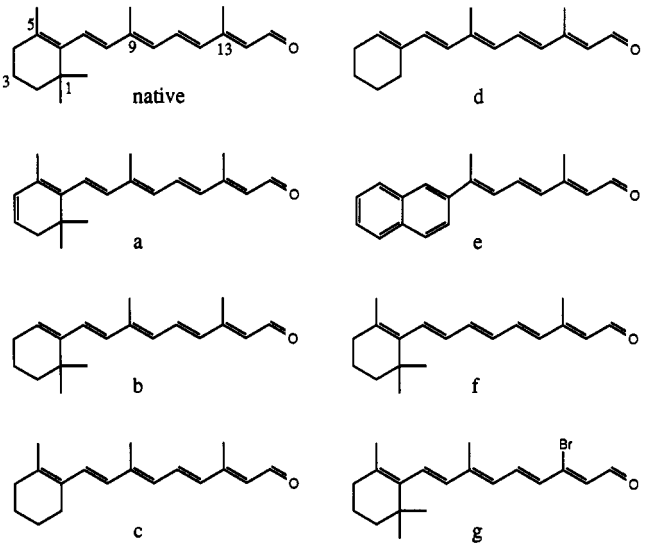

FIGURE 1: The native chromophore and its analogues. *all-trans*-Retinal (native) and its analogues: 3,4–didehydroretinal (a), 5-desmethylretinal (b), 1,1-didesmethylretinal (c), 1,1,5-tridesmethylretinal (d), naphthylretinal (e), 9-desmethylretinal (f), and 13-bromoretinal (g). The retinoid numbering is shown in the native chromophore.

$S\text{-}II_{530}$ or $S\text{-}II_{350}$ concentration. The best fit was decided by the shape of the residual function and the standard deviation of the data from the fit.

Behavioral Assay. Physiological responses of cells were determined as previously described (Yan et al., 1990; Yan & Spudich, 1991). Swimming behavior was monitored at 37 ± 1 °C with nonactinic illumination (730–850 nm), recorded on video tape, and analyzed by using the EV1000 software on a SUN 2/120 work station (Motion Analysis System, Inc., Santa Rosa, CA). Light from a 200-W mercury arc lamp was passed through narrow-bandwidth filters to produce intensities of 800–1000 $erc \cdot cm^{-2} \cdot s^{-1}$ at the plane where the light beam enters the dark-field condenser. The video data were digitized and processed at 5 frames/s, and the reversal frequency was calculated by using a program modified from a previous program (Sundberg et al., 1986).

Results and Discussion

Photochemical Reactions of Analogue Pigments. Generation of SR-II pigments by each of the chromophores (Figure 1) in Flx3b apomembrane vesicle suspensions was completed in 1–2 h when added at 2-fold excess to SR-II apoprotein. The procedure of regeneration of pigments was described (Takahashi et al., 1990). The formation of pigments was indicated by the time course of their reconstitution monitored spectroscopically (not shown) and by monitoring the restoration of phototaxis responses in vivo in each case (as shown in Figure 3).

Absorption difference spectra of SR-II at 40–0 ms and 400–40 ms are shown in Figure 2B. The difference spectra in these time windows match those previously described and have been attributed to a cyclic reaction scheme with three species, $SR\text{-}II_{487}$ and two intermediates with maximal absorption near 350 and 530 nm, $S\text{-}II_{350}$ and $S\text{-}II_{530}$, respectively (Tomioka et al., 1986). The decay of $S\text{-}II_{350}$ matches the rise of the $S\text{-}II_{530}$ absorption (Figure 2A and Table I) consistent with the proposed photocycle (Tomioka et al., 1986). Rate constants determined from nonlinear least-squares analysis of these transients and absorption transients at other wavelengths are shown in Table I. The major event in the 40-ms difference spectrum (filled circles in Figure 2B) is the depletion of SR-

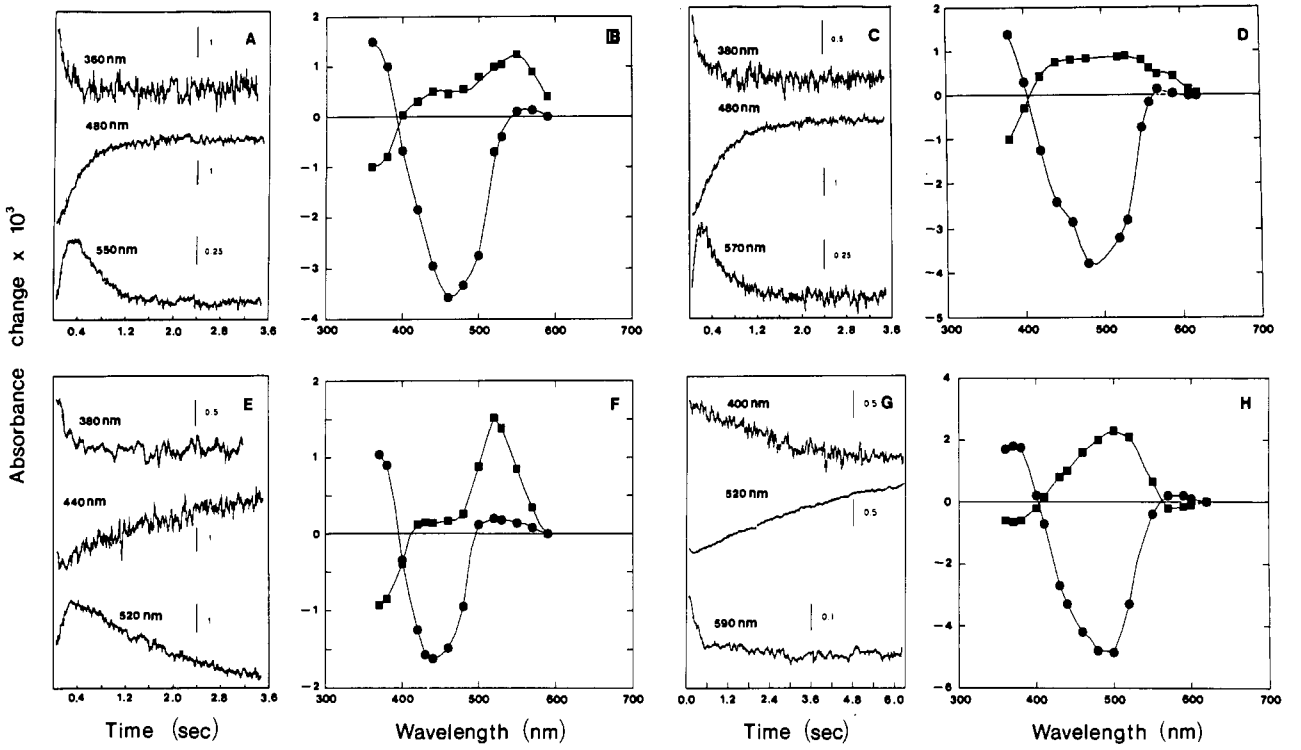

FIGURE 2: Time courses of flash-induced absorbance changes at selected wavelengths and transient absorption difference spectra. SR-II analogue pigments reconstituted with the native chromophore (A, B), and analogues a (C, D), d (E, F), and g (G, H) and other chromophores (not shown), were studied by flash spectroscopy. Absorbance changes induced by a flash (t = 0, flash duration 250 μs) were monitored at various wavelengths. The time courses of absorbance changes at typical wavelengths are shown in panels A, C, E, and G; vertical bars are absorbance units × 10^3. Amplitude differences between t = 40 ms and t = 0 ms for SR-II and its analogue pigments reconstituted from retinal analogues a [SR-II(a)] and d [SR-II(d)] (filled circles in B, D, and F) and between t = 300 ms and t = 0 ms for SR-II(g) (filled circles in H) are plotted against wavelength. Amplitude differences between t = 400 ms and t = 40 ms for native SR-II and SR-II(d) (filled squares in B and F), between t = 200 ms and t = 40 ms for SR-II(a) (filled squares in D), and between t = 2.2 s and t = 300 ms for SR-II(g) (filled squares in H) are also shown. The actinic wavelength was 450 ● 20 nm for panels A, B, and E–H and 500 ● 20 nm for panels C and D. Membrane vesicle suspensions at 3–4 mg of protein/mL contained pigments with maximum absorbance of (1.0–1.5) × 10^{-2}, except for SR-II(d), panels E and F, for which a sample with maximum absorbance of 5 × 10^{-3} was used.

Table I: Decay Rate Constants of S-II_{350}-like (k_1) and S-II_{530}-like (k_2) Intermediates in Photocycles of SR-II and Its Analogues (T = 25 ● 1 °C, pH 7)

chromophore	k_1 (1/s)[a]	k_1 (1/s)[b]	k_2 (1/s)[c]
native	7.3	7.0 ± 0.1	2.3 ± 0.1
a	6.5	9.4 ± 0.2	3.0 ± 0.1
b	7.1	7.5 ● 0.1	1.2 ± 0.1
c	8.3	9.0 ± 0.1	0.7 ± 0.1
d	10.0	8.8 ± 0.1	0.34 ± 0.02
e		6.7 ± 0.1	0.08 ± 0.01
f		3.1 ± 0.1	0.12 ● 0.01
g	0.27 ± 0.01		–[d]

[a] Determined from the decay of S-II_{350}-like intermediates. The value for g is from three wavelengths, and the rest are from single-wavelength measurement. [b] Determined from the rise of S-II_{530}-like intermediates; mean ± SEM of 4–10 values. [c] Mean ± SEM of 4–7 values. [d] No accumulation of an S-II_{530}-like intermediate was observed. Simulation studies assuming an S-II_{530}-like intermediate with a k_2 similar to or greater than the k_2 value of the native pigment (2.3 s^{-1}) fit the experimental data.

II_{487} and formation of S-II_{350}. The 400-ms difference spectrum (filled squares in Figure 2B) is dominated by the transition from S-II_{350} to S-II_{530} and accompanied by some decay of the later species to SR-II_{487}.

The absorption transients and difference spectra of SR-II analogues reconstituted with analogues a, d, and g (Figure 2C–H) fit well the photocycle scheme of the native pigment. The relative spectral shifts of photocycle intermediates in analogue pigments indicate the formation of native-like conformational intermediates; however, the decay rates of these intermediates are altered. The decay of the S-II_{530}-like intermediate (shown as k_2 in Table I) is accelerated by 23% in SR-II(a) (Figure 2C, Table I) compared to the native pigment and decreased to 15% of the rate of the native SR-II in the case of SR-II(d) (Figure 2E, Table I). The smaller k_2 results in a higher accumulation of the S-II_{530}-like intermediate of SR-II(d) (Figure 2F) as compared to native SR-II and SR-II(a) (Figure 2B,D). In the case of SR-II(g), the decay of S-II_{350} is slowed down by a factor of 25 (Figure 2G). Generally, retinylidene protein photochemical reactions produce a blue-shifted intermediate state which has a protein conformation with a deprotonated chromophore, such as metarhodopsin II_{380} in rhodopsin, S_{373} in SR-I, and M_{412} in BR. Most probably, S-II_{350} is a chemically similar intermediate state. Reisomerization and reprotonation of the chromophore as well as the protein relaxation are required for the return of this blue-shifted intermediate to the pigment in a photocycle. In BR (Stoeckenius & Bogomolni, 1982), N- or O-intermediates are formed during this return. Correspondingly, S-II_{530} is an O-like intermediate in the SR-II photocycle. In some cases, these processes appear to take place in a concerted manner and no O-like intermediate is observed [e.g., in the SR-I photocycle (Spudich & Bogomolni, 1988)]. In SR-II(g), an S-II_{530}-like intermediate may be produced but decays too rapidly to be accumulated (Figure 2G,H). Similar analysis of four other analogue pigments (analogues b, c, e, and f in Figure 1) showed similar good fits to the native reaction scheme with various rates of decay of their S-II_{350}- and S-II_{530}-like intermediates (Table I).

Phototaxis Signaling by Analogue Receptors. Cells were incubated with excess of chromophores (Figure 1), and the

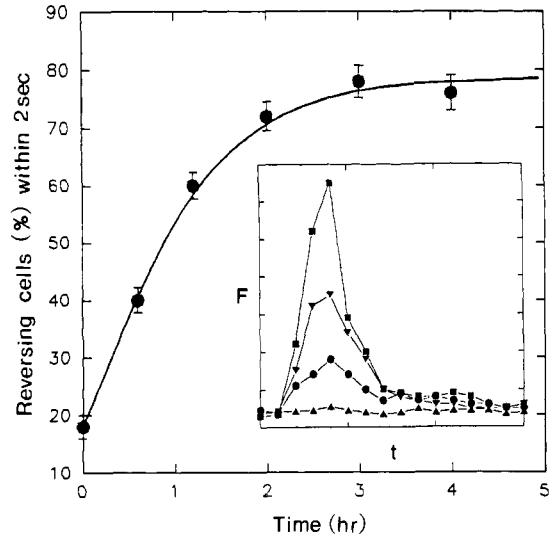

FIGURE 3: Restoration of phototaxis responses of cells. Nicotine-grown Flx3b cells at early stationary phase were diluted 1:50 into fresh medium, and swimming behavior was analyzed by computerized cell-tracking and motion analysis. Up to 10 μL of ethanol solutions of different chromophores was added to diluted cells a final concentration of $\sim 5 \times 10^{-7}$ M, and the responses were determined during incubation (shown here for native chromophore). Light-induced reversal frequency changes at different times after addition of *all-trans*-retinal are shown in the inset (F, reversal frequency; t, time; the reversal frequency was measured for 3 s starting from 0 s when the stimulus was given). The filled upward triangles are responses before retinal addition, and filled circles, downward triangles, and squares are responses at 35 min, 72 min, and 3 h, respectively, after addition of retinal. Reversing cells (%) within 2 s was used as a measure of phototactic response (R) of the cell population and is plotted against time in the figure.

time courses of the response restoration were measured (data for native retinal is shown in Figure 3). Light-induced inductions of cell reversal frequency at different times after retinal addition are shown in the inset of Figure 3. After full reconstitution with chromophores, fluence–response curves were determined by using monochromatic light at the isosbestic point of the pigment analogue and the native SR-II (see inset in each panel of Figure 4 except panel H). Photostimulation of cells containing each analogue in Figure 1 also produces similar reversal induction peaks as in the inset of Figure 3 except at different ranges of light intensity. The rates of phototaxis responses restored by addition of these analogues are comparable to in vitro pigment reconstitution rates. The percentage of reversing cells within 2 s after stimulation was used as a measure of phototactic response (R) of the cell population for determining the response restoration time course (Figure 3) and the fluence–response curves (Figure 4).

Phototaxis Sensitivity of the Cell and the Relative Signaling Efficiency of the Receptor. According to Grotthus-Draper's law, only absorbed photons can cause a photochemical reaction and thereby mediate a biological response. The number (N) of photons absorbed by a single cell can be calculated as $N = KI(dt)\epsilon n$, where K = constant, I = light intensity, dt = duration of pulse, ϵ = extinction coefficient of the receptor molecule, and n = the number of receptors. The biological response $R = f(N,\Phi)$, where Φ = the quantum efficiency of a photoreceptor molecule.

The number of analogue receptors (n) is nearly the same as the native receptors according to our spectroscopic observation that retinal analogues a–g bind to apomembranes quickly and completely. Φ is difficult to measure in membrane preparations; however, our flash photolysis measurements disfavor the possibility that any analogue can reconstitute a

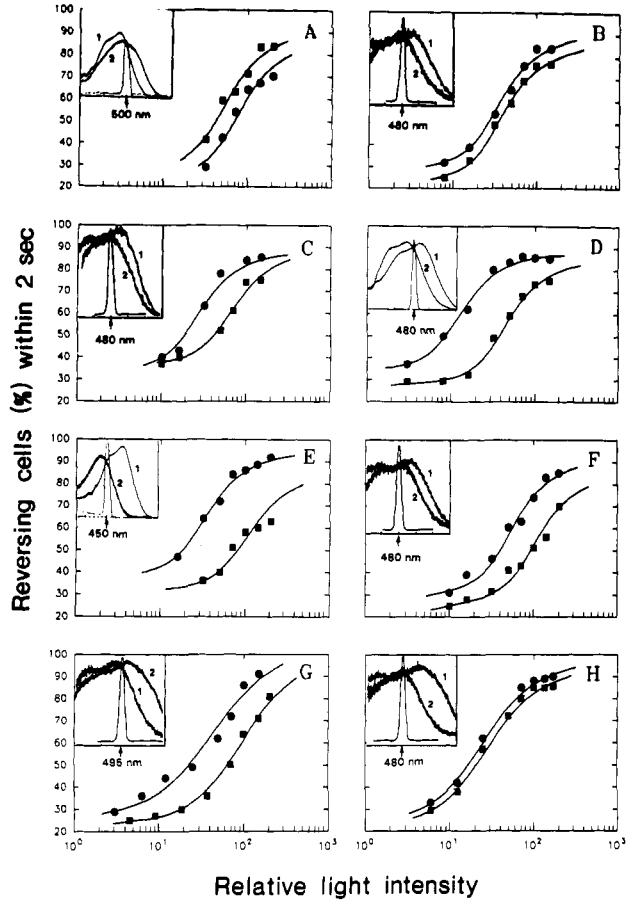

FIGURE 4: Fluence–responses of cells incubated with retinal analogues compared with those incubated with the native chromophore. Spectra of the analogue and the native pigments as well as the transmittance spectrum of the filter for delivering the stimulating light are shown in each inset. The spectra labeled 1 in each inset are for the native SR-II, and those labeled 2 are for SR-II(a)–SR-II(g) (A–G), except panel H in which 1 is for SR-II(g) and 2 for SR-II(f). Curves with filled squares in each panel are for cells incubated with the native chromophore, and filled circles are for analogues a–g (panels A–G). Apomembranes contained the same amount of SR-II apoproteins in each case, and the reconstitution was saturated when the spectra were taken. Both analogues f and g, although reconstituting pigments with longer lived $S\text{-}II_{530}$ and $S\text{-}II_{350}$, respectively, generate higher sensitivities than the native chromophore (see panels F and G). Cells incubated with these two analogues are directly compared in panel H (filled circles for analogue f and squares for g). Stimulus durations were 100 or 200 ms in order to reach the near-saturating responses. The reversal integral without stimuli was nearly constant (23–28% within 2 s).

pigment which exhibits a higher quantum efficiency than that of the native pigment. Therefore, we tentatively assume the quantum efficiencies for SR-II analogues are equal to that of SR-II. Higher sensitivity was observed for all receptor analogues except for SR-II(a), even though we expect them to exhibit quantum efficiencies smaller than or equal to that of native SR-II. To ensure equal absorption by analogue receptors with different absorption maxima compared to native SR-II, we used monochromatic filters for delivering stimulating light at the isosbestic point of each analogue pigment and the native SR-II as shown in the insets of Figure 4, except Figure 4H which compares effects of two analogues. From these considerations we expect responses to depend only on the photon fluence and the intrinsic properties (photocycle kinetics) of the individual receptor.

Analogues b–g generated higher sensitivities in pairwise comparisons of the fluence–response curves to native SR-II; i.e. their fluence–response curves are shifted to lower photon

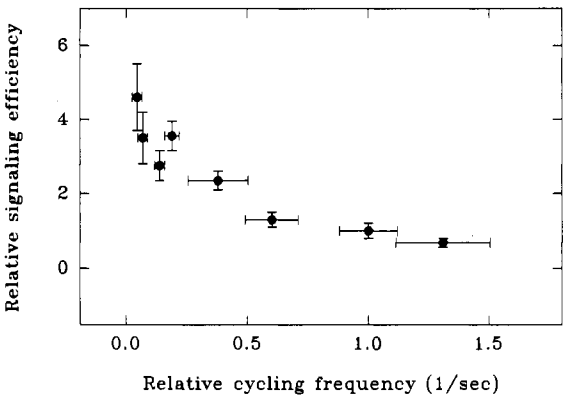

FIGURE 5: Relationship between the relative signaling efficiency of the analogue receptor and their photocycling frequency (turnover number). The relative signaling efficiency is the reciprocal of the number of activated receptors required for 55% reversal for analogue SR-II relative to that for native SR-II (see text). The photocycling frequency (f) of receptors is calculated as $f = (k_1^{-1} + k_2^{-1})^{-1}$, normalized to f of the native SR-II, and plotted. Horizontal error bars are ± the standard error of the mean of the calculated values. Vertical error bars are calculated from the standard deviation values of the fluence–response curves used to determine the relative signaling efficiency.

doses compared to that of the native system (Figure 4B–G). Analogue a formed a receptor analogue giving lower sensitivity (Figure 4A). Both analogues f and g reconstituted more sensitive analogue receptors (Figure 4F,G), though they prolonged the lifetimes of S-II_{530} and S-II_{350}, respectively. They are compared directly in Figure 4H. For quantitative assessment of the various analogue photoreceptors, the reciprocal of the number of photons required for 55% reversals mediated by the analogue SR-II relative to that by the native SR-II is defined as the relative signaling efficiency of the receptor.

Comparison of Receptor Signaling Efficiency and Photocycling Frequency (Turnover Number). Photon absorption by the chromophore of SR-II mediates a fast photochemical event (retinal isomerization) which triggers a cyclic process of protein conformational changes (photocycle) and the physiological response of the cell. Retinal analogues a–g reconstitute SR-II analogue pigments with altered cycling frequency (i.e., altered photocycle transit time). These analogue receptors alter greatly the signaling efficiency of the cell (Figure 4). Receptor signaling efficiency increases as cycling frequency decreases (Figure 5). Our interpretation is that lifetimes of the signaling states have been prolonged and signal transduction enhanced.

Comparison of Receptor Signaling Efficiency and Concentrations of Photocycle Intermediates. It is of interest to know whether signaling efficiency is correlated with a conformational state of the protein. Two long-lived intermediates, S-II_{350} and S-II_{530}, can be distinguished on the basis of their different absorption spectra. Despite the difficulty that perturbing the lifetime of one of these states in general alters the concentration of the other, it is important, especially for sensory receptor studies, to attempt to assess their individual contributions to signaling efficiency to dissect further the signaling process.

(A) Time-Averaged Concentrations of Photocycle Intermediates. As determined by flash photolysis, the observed reactions for all analogue pigments fall into the same scheme as native SR-II in the time domain of our experiments (1–2-ms resolution): SR-II_{487} → S-II_{350} → S-II_{530} → SR-II_{487}. Absorption transients (e.g., bottom traces in Figure 2A,C,E) in the S-II_{530}-like intermediate absorption region fit well the time course of S-II_{530} concentration derived from the rate equation of consecutive reactions assuming that both S-II_{350} to S-II_{530} and S-II_{530} to SR-II_{487} transitions are unidirectional first-order reactions with rate constants k_1 and k_2, respectively:

$$[\text{S-II}_{530}] = K(e^{-k_1 t} - e^{-k_2 t})$$

where

$$K = k_1[\text{S-II}_{350}]_0/(k_2 - k_1)$$

Rate constants obtained from curve fitting reveal that analogue pigments with ring-modified chromophores (analogues a–e) give a similar k_1 as that of native SR-II but varied k_2 values (Table I). These results show that modifications on the β-ionone ring primary alter the kinetics of the S-II_{530} decay step, suggesting that interactions between the ring and the protein are involved in the S-II_{530} decay process. In these analogue pigments, a much smaller effect on the S-II_{350} → S-II_{530} transition rate constant is observed (Table I). SR-II(f) exhibits both small k_1 and k_2 values compared to that of SR-II (Table 1), and SR-II(g) has a 25 times smaller k_1, suggesting that modifications at the polyene chain portion of the retinal affect considerably the first half of the photocycle.

For the behavioral analysis, a pulse (100 or 200 ms) of relatively weaker stimulating light compared with that in flash photolysis was used. Therefore, S-II_{350} would form more gradually. The total concentration of the S-II_{350}-like intermediate during the illumination period can be calculated as

$$[\text{S-II}_{350}]_{t_1} = (V/k_1)(1 - e^{-k_1 t_1})$$

where V is proportional to the product of the quantum yield of the receptor and the photon flux and t_1 is the illumination period. The time-averaged concentration of S-II_{350} is calculated as

$$\overline{[\text{S-II}_{350}]} = \tfrac{1}{2}\left([\text{S-II}_{350}]_{t_1} + [\text{S-II}_{350}]_{t_1}\int_{t_1}^{2} e^{-k_1 t}\,dt\right)$$

We integrated over 2 s starting from time 0 to match the interval over which physiological responses were assessed. The time-averaged concentration of S-II_{530} was calculated as

$$\overline{[\text{S-II}_{530}]} = \tfrac{1}{2}\int_0^2 [k_1[\text{S-II}_{350}]_0/(k_2 - k_1)](e^{-k_1 t} - e^{-k_2 t})\,dt$$

(B) Comparison of the Relative Signaling Efficiencies of Receptors and Relative Time-Averaged Concentrations of Photocycle Intermediates. No correspondence is found between the relative receptor signaling efficiency and the relative concentration of S-II_{350} (Figure 6A). Figure 6B shows a good correlation between the signaling efficiency and the S-II_{530} intermediate in all analogues with the exception of analogue g. In SR-II(g), no S-II_{530}-like intermediate accumulates and SR-II(g) has a 25-fold longer lived S-II_{350}-like intermediate compared to the native SR-II. This analogue receptor exhibits a signaling efficiency 2.7-fold greater than that of native SR-II. Relative $\overline{[\text{S-II}_{350}]}$ of SR-II(g) and $\overline{[\text{S-II}_{350}]} + \overline{[\text{S-II}_{530}]}$ of the other analogue pigments correlate much better with the receptor signaling efficiency (Figure 6C) than either intermediate alone (Figure 6A,B). Although g is the only analogue which significantly perturbs $\overline{[\text{S-II}_{350}]}$ and from our data it is not possible to establish firmly that the SR-II(g) pigment exhibits a photocycle with exactly the same intermediates as the other SR-II pigments, the best interpretation from these data is that both S-II_{350} and S-II_{530} possess a physiologically active conformation at the signaling site of the receptor protein; i.e., they are signaling states of SR-II_{487}. Kinetic analysis cannot distinguish whether (i) SR-II_{487} acquires an active conformation in S-II_{350} which persists until S-II_{530} decays or (ii) SR-II_{487} loses an inhibitory activity upon S-II_{350} formation which is not

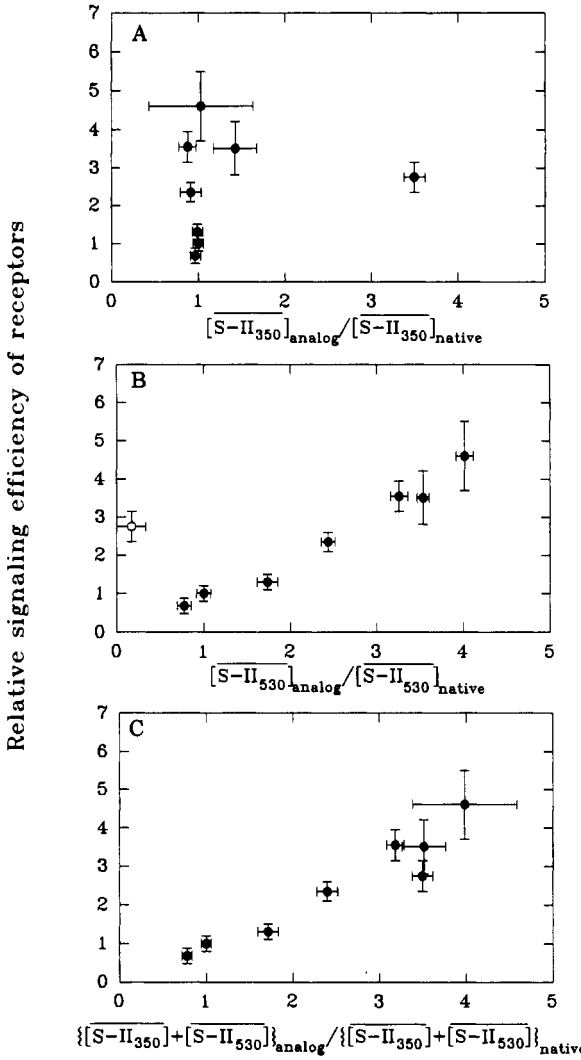

FIGURE 6: Relationship between the relative signaling efficiency of the analogue receptor and the relative time-averaged concentrations of long-lived intermediates. The time-averaged concentrations of intermediates were determined as described in the text. The relationship between signaling efficiency and the relative time-averaged concentrations of intermediates S-II_{350} and S-II_{530} is shown in panels A and B. The point for SR-II(g) in panel B (open circle) is based on the assumption that an S-II_{530}-like intermediate is produced upon the S-II_{350}-like intermediate decays (see text). Panel C is the correlation between signaling efficiency and relative [S-II_{350}] of SR-II(g) and relative [S-II_{350}] + [S-II_{530}] of other analogue pigments. Horizontal error bars are estimated from the effect of errors in determining rate constants on the integral calculations. The horizontal error bar for SR-II(g) is based on assuming a decay rate for the S-II_{530}-like intermediate between 2.3 s^{-1} and an infinite value.

regained until S-II_{530} decays. Whichever the mechanism, S-II_{350} and S-II_{530} can be though of as altered and therefore physiologically "active" conformations of SR-II_{487}.

Activation Site vs Signaling Site. In cell surface receptors, ligand binding sites are spatially separated from the signal transmission sites which are usually in the cytoplasmic or membrane-spanning surface of the receptor molecule. Analogously, in retinylidene proteins, photoisomerization of retinal in the central pocket of the protein induces a propagation of conformational changes to the signaling site within about 1 ms (e.g., formation of Meta-II_{380} for rhodopsin or S-II_{350} for SR-II_{487}).

Our results indicate that the activation site and the signaling site of SR-II are distinct and sequentially coupled. The structural alterations at the activation site (in S-II_{520}) propagate to the signaling site at the stage of S-II_{350}. During the receptor reset process, the chromophore is thermally driven to recover by the stage of S-II_{530}, but the signaling site still remains in its physiologically active conformation. The signaling state decays only when the protein conformation at the signaling site recovers (the return to the original pigment SR-II_{487}). The activation site and the signaling site are likely to be coupled by intramolecular allosteric interactions described as "domino effects" (Milburn et al., 1990).

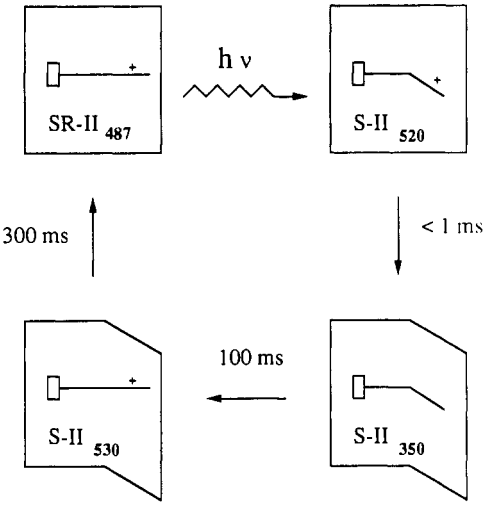

FIGURE 7: Model incorporating our results into the photocycle of SR-II. Photocycle intermediates have been identified by room temperature flash spectroscopy (Tomioka et al., 1986) and low-temperature ultraviolet–visible absorption spectroscopy (Shichida et al., 1988). The simplified retinal structure in the center represents the chromophore structure and chromophore/protein interactions in the photoactivation site (retinal-binding or spectrum-determining site). Straight and bent indicate all-trans and 13-cis chromophore configurations, respectively; + shows the most likely protonation state of the Schiff base nitrogen. The box represents the protein structure, and the change in its shape depicts the conformational changes at the signal transmission site (signaling site) of the receptor.

In summary, the following results were obtained: (i) Decreasing the rate of the photocycle with analogue receptors increases the signaling efficiency. This result provides compelling support for the existence of signaling states, i.e., conformational intermediates of the receptor protein with a physiologically active conformation at the signaling site. (ii) The correlation of signaling efficiency with $\{[\overline{S\text{-}II_{350}}] + [\overline{S\text{-}II_{530}}]\}_{analogue}/\{[\overline{S\text{-}II_{350}}] + [\overline{S\text{-}II_{530}}]\}_{native}$ indicates that both S-II_{350} and S-II_{530} possess an active conformation at the signaling site and, therefore, are signaling states of SR-II_{487}.

A model incorporating this result into the SR-II photocycle is presented (Figure 7). Reasoning from the similarity between the SR-II_{487} and BR photocycles and the all-trans/13-cis isomerization requirement for SR-II activation (Yan et al., 1990), retinal isomerization and the resulting retinal/protein interactions produce protein alterations which lead to the chromophore deprotonation and the propagation of the protein conformational changes to the signaling site: the formation of S-II_{350}. The receptor is reset thermally at the consumption of the protein deformation energy: the retinal is reprotonated and most probably reisomerized to yield S-II_{530}. S-II_{530} still possesses an active conformation at the signaling site. The signaling state disappears when the protein conformation is completely recovered as the photocycle returns to the original pigment SR-II_{487}.

Modulation of Lifetimes of Stimulus-Induced Conformations as a General Tool. Our approach to identifying signaling states of SR-II is generally applicable to other sensory receptors. The strategy is to (i) define stimulus-induced protein

conformations by monitoring structural alterations with spectroscopic reporter groups; (ii) modulate the lifetime of these conformational intermediates by modification of the receptor or its effector; (iii) assess receptor signaling efficiency by physiological measurements; and, finally, (iv) compare signaling efficiency with the time-averaged concentrations of stimulus products (determined over the interval of time in which the physiological response is assessed), in order to determine which of these products are signaling conformations of the receptor.

Photoreceptors can be activated with temporal precision by photostimuli. Photoreleased caged ligands provide a method for transient activation of ligand-activated receptors (McCray & Trentham, 1989). In general, to apply this method, one needs to have spectroscopic reporter groups in positions which sense functionally important transitions in the protein. In the case of SR-II (as in photoreceptors in general) the chromophore itself provides one such reporter group positioned at the photoactivation site.

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