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Comparative Study on the Chromophore Binding Sites of Rod and Red-Sensitive Cone Visual Pigments by Use of Synthetic Retinal Isomers and Analogues†

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ABSTRACT: A comparative study on the chromophore (retinal) binding sites of the opsin (R-photopsin) from chicken red-sensitive cone visual pigment (iodopsin) and that (scotopsin) from bovine rod pigment (rhodopsin) was made by the aid of geometric isomers of retinal (all-trans, 13-cis, 11-cis, 9-cis, and 7-cis) and retinal analogues including fluorinated (14-F, 12-F, 10-F, and 8-F) and methylated (12-methyl) 11-*cis*-retinals. The stereoselectivity of R-photopsin for the retinal isomers and analogues was almost identical with that of scotopsin, indicating that the shapes of the chromophore binding sites of both opsins are similar, although the former appears to be somewhat more restricted than the latter. The rates of pigment formation from R-photopsin were considerably greater than those from scotopsin. In addition, all the iodopsin isomers and analogues were more susceptible to hydroxylamine than were the rhodopsin ones. These observations suggest that the retinal binding site of iodopsin is located near the protein surface. On the basis of the spectral properties of fluorinated analogues, a polar group in the chromophore binding site of iodopsin as well as rhodopsin was estimated to be located near the hydrogen atom at the C_{10} position of the retinylidene chromophore. A large difference in wavelength between the absorption maxima of iodopsin and rhodopsin was significantly reduced in the 9-cis and 7-cis pigments. On the assumption that the retinylidene chromophore is anchored rigidly at the α-carbon of the lysine residue and loosely at the cyclohexenyl ring, each of the two isomers would have the Schiff-base nitrogen at a position altered from that of the 11-cis pigments. Thus, the remarkable red-shift in the absorption spectrum of iodopsin as compared with that of rhodopsin could be attributed to a difference between the two pigments in the location of a negative charge directly hydrogen bonded to the Schiff-base nitrogen.

In contrast with the extensive works on rod visual pigment (rhodopsin), far less studies have been conducted on cone pigments, which are responsible for photopic vision. This is because of the difficulty in obtaining large quantities of cone cells without contamination of rod cells and because of the lower stability of the cone pigments (Wald et al., 1955; Okano et al., 1989). Among several cone visual pigments, a red-sensitive pigment in chicken retina is relatively well characterized and named iodopsin[1] (Wald et al., 1955). Iodopsin is different in physical and chemical properties from rhodopsin as follows: First, the absorption maximum of iodopsin is located at a longer wavelength [562 nm, Wald et al. (1955);

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[1] In a previous paper (Okano et al., 1989), the term chicken red was used to indicate the chicken red-sensitive cone visual pigment for contrast with other chicken cone pigments. Instead of chicken red, the specific name iodopsin was used in this paper because iodopsin is the only cone pigment used in this experiment and because this is convenient for designating the isomers and analogues, for example, 9-*cis*-iodopsin.

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569 nm, Bowmaker and Knowles (1977); 572 nm, Fukada and Yoshizawa (1982); 571 nm, Okano et al. (1989)] than that of chicken rhodopsin [509 nm, Wald et al. (1955); 506 nm, Bowmaker and Knowles (1977); 503 nm, Okano et al. (1989)]. Second, the rate of regeneration of iodopsin from R-photopsin (the opposin moiety of iodopsin) and 11-*cis*-retinal is about 500 times greater than that of rhodopsin (Wald et al., 1955), probably accounting for the high rate of the dark adaptation. Third, unlike the retinylidene Schiff-base of rhodopsin, that of iodopsin is easily attacked by reagents of low molecule weight such as hydroxylamine, alum (Wald et al., 1955), and sodium borohydride (Matsumoto et al., 1975; Fager et al., 1975) even in the dark. These characteristics suggest that the location (or the shape) of the retinal binding site and the protein–chromophore interaction in iodopsin are remarkably different from those in rhodopsin, though they are not exclusively elucidated.

The present paper is devoted to a study on the retinal binding site of iodopsin with the aid of several retinal analogues and isomers. A series of fluorinated 11-*cis*-retinals (8-F, 10-F, 12-F, and 14-F) were chosen for probing a possible cause for the red-shifted absorption maximum of iodopsin due to the chromophore–opsin interactions. Several retinal isomers (all-trans, 13-cis, 11-cis, 9-cis, and 7-cis) and a methylated 11-*cis*-retinal (12-methylretinal) were also used for probing the shape of the retinal binding site in R-photopsin. Experimental data presented here demonstrate common or different characteristics of the binding site between iodopsin and rhodopsin.

Experimental Procedures

Buffers. The pH of each buffer was adjusted with NaOH at 4 °C. Buffer A is 50 mM HEPES,[2] 140 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 0.6% CHAPS, 0.8 mg/mL PC, 1 mM DTT, 0.1 mM $PhMeSO_2F$, 50 kallikrein inhibitor units/mL aprotinin, and 4 μg/mL leupeptin, pH 6.6. Buffer A-20 is buffer A containing 20% (w/v) glycerol. Buffer A-40 is buffer A containing 40% (w/v) glycerol. Buffer B is 50 mM HEPES, 140 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 2% CHAPS, 1 mg/mL PC, 1 mM DTT, 0.1 mM $PhMeSO_2F$, 50 kallikrein inhibitor units/mL aprotinin, and 4 μg/mL leupeptin, pH 6.6. ROS buffer is 10 mM MOPS, 30 mM NaCl, 60 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM $PhMeSO_2F$, 50 kallikrein inhibitor units/mL aprotinin, and 4 μg/mL leupeptin, pH 7.5.

Preparation of Chicken R-Photopsin. Iodopsin was purified from freshly dissected chicken retinas under dim red light (>680 nm) according to the method of Okano et al. (1989), from which R-photopsin was isolated. Briefly, all the pigments were extracted from chicken rod and cone outer segment membranes with 0.75% CHAPS in the presence of 1.0 mg/mL PC. Then, the extract was diluted with buffer A without CHAPS and PC to give CHAPS and PC concentrations of 0.6% and 0.8 mg/mL, respectively. The diluted extract was loaded on a concanavalin A–Sepharose column, from which iodopsin was eluted with 1.5 mM methyl α-mannoside. The eluate containing iodopsin (usually 100–200 mL) with a high purity (greater than 95% as determined spectrophotometrically) was mixed with the same volume of buffer A-40 to give a final glycerol concentration of 20% (w/v), which significantly improved the stability of iodopsin. Then it was concentrated to 10–20 mL by means of an Amicon ultrafiltration cell fitted with a YM-30 membrane filter (Amicon), followed by a dialysis against several exchanges of 500 mL of buffer A-20 for the complete removal of methyl α-mannoside. The dialyzed preparation was mixed with concanavalin A–Sepharose beads (3 mL of swollen beads/mg of iodopsin) that had been equilibrated with buffer A-20, gently shaken for 2 h, and kept in the dark overnight. Then, it was mixed with NH_2OH (HCl form and pH adjusted to 6.6 with NaOH) at a final concentration of 10 mM. The iodopsin adsorbed to the beads was exposed to an intense orange light (light from a 1-kW tungsten lamp was passed through a Toshiba VO58 cutoff filter, >560 nm) for 5 min to completely bleach it into R-photopsin and *all-trans*-retinal oxime. Subsequent procedures were performed in the light. The beads with R-photopsin were packed in a column (10 × 80 mm, the second concanavalin A–Sepharose column) and washed at a flow rate of 2.5 mL/h with three bed volumes of buffer A-20 containing 10 mM NH_2OH for complete cleavage of the random Schiff-base of *all-trans*-retinal into retinal oxime and with five bed volumes of buffer A-20 without NH_2OH for complete elution of the retinal oxime and free NH_2OH. Finally, R-photopsin was eluted from the column by buffer A-20 supplemented with 100 mM methyl α-mannoside. Such a high concentration of methyl α-mannoside increased the concentration of R-photopsin in the eluate. The yield of R-photopsin from the second column was about 75%. All experiments were carried out by use of this eluate without removal of methyl α-mannoside from it. The eluate was composed of greater than 96% R-photopsin and less than 3% B-photopsin (the opsin moiety of chicken blue-sensitive pigment; absorption maximum = 455 nm), which was estimated spectrophotometrically after regeneration of the pigments by addition of 11-*cis*-retinal. The ratio of absorbances A_{280}/A_{571} (optical purity) was 3.6–3.8.

Preparation of Bovine Scotopsin. Two hundred bovine retinas isolated from freshly dissected eye balls were shaken vigorously by hand for 1 min in 200 mL of ROS buffer supplemented with 40% (w/v) sucrose, followed by centrifugation (20000*g*, 20 min). The pellet was centrifuged again in the same buffer containing 40% (w/v) sucrose. All the floating materials and supernatants obtained in these centrifugations were mixed together with the same volume of ROS buffer and then centrifuged (20000*g*, 30 min). The pellet was washed four times with ROS buffer. The crude rod outer segments (ROS) thus obtained were subjected to sucrose stepwise density gradient centrifugation (Matsumoto et al., 1975). Purified ROS recovered at the interface between the two sucrose solutions [36% and 29% (w/v) in ROS buffer] were collected, sedimented by centrifugation (20000*g*, 20 min), and then suspended in 40 mL of ROS buffer supplemented with 100 mM neutralized NH_2OH. The suspension was exposed to the intense orange light (>560 nm) for 10 min for complete bleaching of rhodopsin into scotopsin and retinal oxime. Subsequent manipulations were carried out in the light. The bleached ROS were washed five times with ROS buffer by centrifugation (20000*g*, 20 min), followed by solubilization of scotopsin with buffer B ([CHAPS] = 2%; [PC] = 1.0 mg/mL). Clear supernatant obtained by centrifugation (74000*g*, 1 h) was mixed with concanavalin A–Sepharose beads (0.3 mL of swollen beads/mg of scotopsin) equilibrated with buffer A-20 in advance, shaken gently for 2 h, and kept overnight at 4 °C. Then, the beads were packed in a column (typically, 10 × 130 mm) and washed at a flow rate of 20 mL/h with buffer A-20 to elute retinal oxime. Finally, the scotopsin was eluted by buffer A-20 supplemented with 100

[2] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PC, phosphatidylcholine from fresh egg yolk; $PhMeSO_2F$, phenylmethanesulfonyl fluoride.

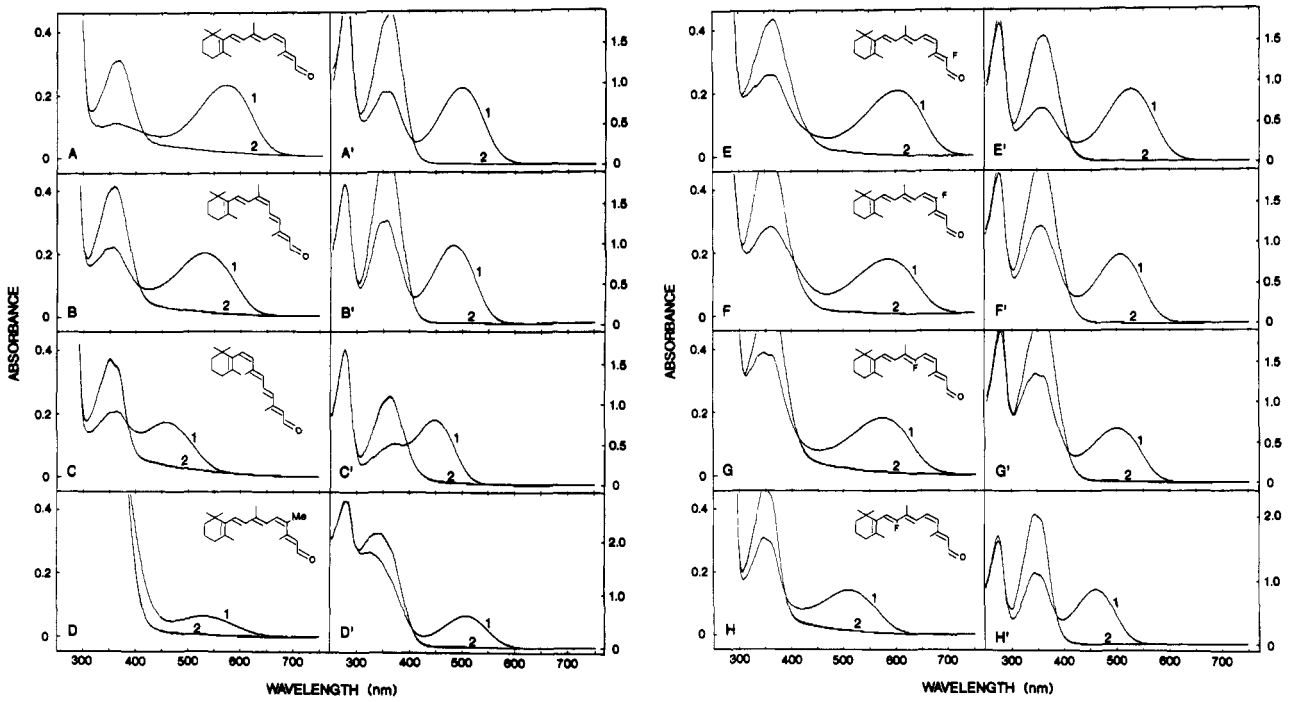

FIGURE 1: Absorption spectra of iodopsin, rhodopsin, and their isomers and analogues before and after complete bleaching in the presence of NH_2OH: (A) 11-*cis*-iodopsin; (A′) 11-*cis*-rhodopsin; (B) 9-*cis*-iodopsin; (B′) 9-*cis*-rhodopsin; (C) 7-*cis*-iodopsin; (C′) 7-*cis*-rhodopsin; (D) 12-methyl-11-*cis*-iodopsin; (D′) 12-methyl-11-*cis*-rhodopsin; (E) 14-fluoro-11-*cis*-iodopsin; (E′) 14-fluoro-11-*cis*-rhodopsin; (F) 12-fluoro-11-*cis*-iodopsin; (F′) 12-fluoro-11-*cis*-rhodopsin; (G) 10-fluoro-11-*cis*-iodopsin; (G′) 10-fluoro-11-*cis*-rhodopsin; (H) 8-fluoro-11-*cis*-iodopsin; (H′) 8-fluoro-11-*cis*-rhodopsin. All the absorption spectra were measured at 4 °C (panels A, C, D, D′, and F) or 15 °C (all the others). After completion of each pigment formation, the sample was incubated with 1 mM NH_2OH (final concentration) at 4 °C for 20 min (D) or with 10 mM NH_2OH at 4 °C for 30 min (A and D′), 20 min (F), or 30 s (C) or at 15 °C for 20 min (H) or 30 min (all the others), followed by measurement of the absorption spectrum (curve 1 in each panel). Then the sample was irradiated with the orange light (>520 nm) at 4 °C for 15 min (curve 2) except for panels C and D. In the case of 7-*cis*-iodopsin (C), the spectral changes in the dark were monitored at 4 °C, and the final spectrum after the incubation for 45 min is shown (curve 2; see also Figure 2). 12-Methyliodopsin (D) was additionally supplemented with NH_2OH at a final concentration of 10 mM and then irradiated with the orange light (>520 nm) at 4 °C for 15 min (curve 2). In every case, it was confirmed that an additional irradiation (15 min) induced no spectral change.

mM methyl α-mannoside. When rhodopsin was regenerated by addition of equimolar 11-*cis*-retinal to the scotopsin thus purified, the optical purity (A_{280}/A_{500}) was 1.8–2.3.

Spectrophotometric Analyses. All the absorption spectra were recorded on a Hitachi (Model 330) or a Perkin-Elmer (Model λ-5) spectrophotometer. The sample cell was held at constant temperature (4 or 15 °C), the surface of which was kept dry with a stream of nitrogen gas.

Isomers and analogues of retinal were synthesized by the methods described previously (Liu & Asato, 1982; Asato et al., 1978; Liu et al., 1984). Prior to the binding experiments, they were purified again by high-performance liquid chromatography when necessary, reanalyzed by ^1H NMR spectroscopy, and finally dissolved in ethanol. The content of each isolated opsin was estimated from the maximal absorbance of iodopsin or rhodopsin regenerated in the presence of molar excess of 11-*cis*-retinal. In the following binding experiments with an isomer or analogue of 11-*cis*-retinal, a small molar excess of each isomer or analogue (1–4 μL of ethanol solution) was added to the R-photopsin or scotopsin solution (0.4 mL). The mixture was kept at 15 °C in the dark, and then the absorption spectra were recorded at an appropriate time interval until two successive spectra were superimposable (completion of pigment formation). In most case, 4 μL of 1 M NH_2OH solution in buffer A-20 (pH 6.6) was added to the mixture (10 mM final concentration). Again, the absorption spectra were recorded successively at 15 °C in order to confirm that the free retinal or the random Schiff-base was completely converted to retinal oxime (Figure 1, curves 1). It usually took 20–30 min. Then, the sample was irradiated with an orange light (>520 nm) to convert the pigment into opsin and its corresponding retinal oxime (Figure 1, curves 2). The absorption maximum of each pigment was calculated from the difference spectrum before (curve 1) and after (curve 2) the irradiation. In some cases, samples were cooled to 4 °C before the addition of NH_2OH, and the subsequent procedures were performed at 4 °C (see also the legend to Figure 1).

RESULTS

Rate of Pigment Formation. In all experiments, either R-photopsin or scotopsin was mixed with a small molar excess of the retinal isomer or analogue (see Experimental Procedures). Since the molar extinction coefficients of some retinal isomers or analogues and their corresponding pigments were not known, the rate constants of the pigment formation, which should follow second-order kinetics, could not be determined. Therefore, apparent half-lives ($T_{1/2}$) have been determined (Table I), which are inversely proportional to the rates of the binding reactions.

Like scotopsin, R-photopsin failed to form a pigment with 13-*cis*- or *all-trans*-retinal. The other isomers and all the analogues of retinal tested here bound with R-photopsin as well as with scotopsin. The binding reactions with R-photopsin, however, were considerably faster than those with scotopsin.

As shown in Table I, the rates of formation of 9-*cis*-, 14-fluoro-, 12-fluoro-, and 10-fluoroiodopsins were comparable to that of 11-*cis*-iodopsin (iodopsin) and were almost complete (>90%) within only the first scan (ca. 0.5 min) of the spectrum ($T_{1/2} < 0.25$ min). Accordingly, the rates could not be compared with each other. 7-*cis*- or 8-fluoroiodopsin was formed more slowly, while the formation of 12-methyliodopsin was

Table I: Half-Life of Pigment Formation and Relative Absorbance at the Maximum

	iodopsin (chicken)		rhodopsin (bovine)	
	$T_{1/2}$ (min)[a]	A_{max}[b,c]	$T_{1/2}$ (min)[a]	A_{max}[b,d]
11-cis	<0.25	100	<0.85	100
9-cis	<0.25	99	<0.85	104
7-cis	0.40	76	204	84
14-F	<0.25	103	1.0	95
12-F	<0.25	86	<0.4	92
10-F	<0.25	84	<0.6	71
8-F	<0.60	83	12.5	92
12-Me	3.0	37	166	72

[a] Time required for formation of 50% of a pigment. [b] Absorbance at the maximum when the formation of a pigment was saturated. [c] Normalized against the absorbance (0.215) of 11-*cis*-iodopsin at the maximum (571 nm). [d] Normalized against the absorbance (0.950) of 11-*cis*-rhodopsin at the maximum (500 nm).

the slowest among the pigments from R-photopsin.

The pigments from scotopsin were formed even more slowly (Table I). It should be noted that the series of $T_{1/2}$ values of rhodopsin isomers and analogues is roughly equal in sequence of magnitude to that of iodopsin ones.

Amounts of Pigments Formed. The maximal absorbance of a pigment after formation completion was normalized to that of 11-*cis*-iodopsin (0.215 at 571 nm) or 11-*cis*-rhodopsin (0.950 at 500 nm) (A_{max} in Table I). Owing to the absorption of free retinal (or random Schiff-base) remaining in the sample and to the instability of the pigment to NH_2OH (see below), these A_{max} values can not be used for estimating relative molar extinction coefficients of pigments even if we assume that none of the opsins denature during the incubation. The lower A_{max} value of 12-methyliodopsin (37%) compared to those of the others (>70%) should be ascribed to the thermal instability of the pigment or R-photopsin at 15 °C rather than to the low value of the molar extinction coefficient.

Stabilities of Pigments against NH_2OH. All the pigments derived from scotopsin except for 12-methylrhodopsin were stable at 15 °C for 30 min in the presence of 10 mM NH_2OH; i.e., the decrease of the absorbance at the maximum of each pigment was negligible during this period. About 50% of 12-methylrhodopsin degraded within 10 min at 15 °C, but the degradation was suppressed to 15% at 4 °C during 30 min of incubation.

On the other hand, all the isomers and analogues of iodopsin were unstable against 10 mM NH_2OH at 15 °C. However, the amounts of degradation (5–25%) of 11-*cis*-, 9-*cis*-, 14-fluoro-, 10-fluoro-, and 8-fluoroiodopsins during incubation for 30 min in the presence of 10 mM NH_2OH at 15 °C were remarkably reduced at 4 °C. More evidently, less than 12% of 12-fluoroiodopsin was decomposed during incubation for 20 min at 4 °C, while at 15 °C it decomposed completely within 10 min.

7-*cis*-Iodopsin was, however, almost completely bleached even at 4 °C within 30 min (Figure 2A). Plots of absorbance change at 463 nm (absorption maximum of this pigment; see below) against time of incubation in the presence of 10 mM NH_2OH (Figure 2A, inset) demonstrated that the bleaching process displayed a single-exponential decay (to R-photopsin and 7-*cis*-retinal oxime), except for the initial period of the incubation which should be mainly due to the conversion of free 7-*cis*-retinal or the random Schiff-base into retinal oxime.

The most labile pigment among those tested was 12-methyliodopsin, which was attacked by 10 mM NH_2OH more quickly than 7-*cis*-iodopsin at 4 °C: The decomposition was too fast to record the spectral change as was done for 7-*cis*-iodopsin.

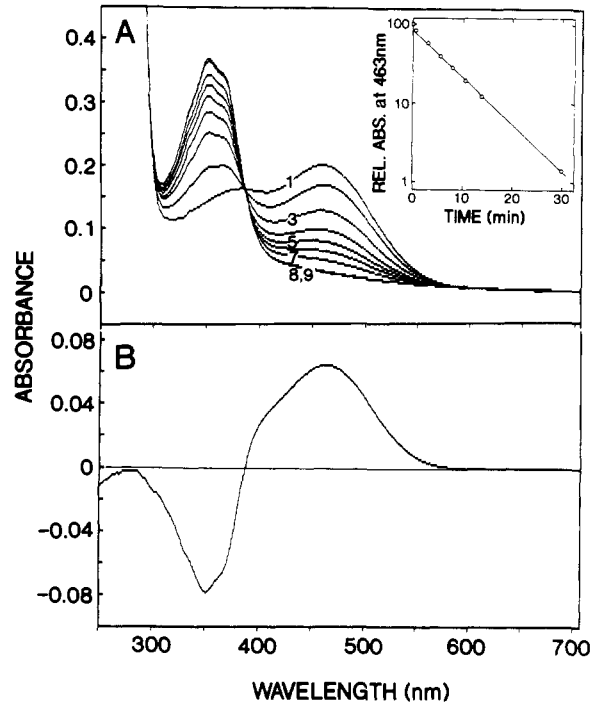

FIGURE 2: Decomposition of 7-*cis*-iodopsin in the presence of 10 mM NH_2OH in the dark. (A) The spectral change in the presence of NH_2OH. After complete formation of 7-*cis*-iodopsin, the sample was cooled to 4 °C (curve 1) and then mixed with NH_2OH at a final concentration of 10 mM. A spectral change due to the dilution of the sample by the addition of 1 M NH_2OH was negligible. The absorption spectra of the sample kept in the dark at 4 °C were successively recorded at 0.5, 3, 5.5, 8, 10.5, 14, 30, and 45 min after addition of NH_2OH (curves 2–9, respectively). Inset: The kinetics of thermal bleaching of 7-*cis*-iodopsin in the presence of NH_2OH. The change in absorbance at 463 nm was plotted semilogarithmically against incubation time. (B) The difference absorption spectrum showing the conversion of 7-*cis*-iodopsin into R-photopsin and 7-*cis*-retinal oxime. The spectrum was calculated from the difference between curves 4 and 8 in panel A. The maximum was located at 463 nm.

Absorption Maxima of Pigments. Absorption maxima of all the pigments except for 7-*cis*-iodopsin were estimated from the difference spectra before (Figure 1, curves 1) and after (curves 2) irradiation of the pigments in the presence of NH_2OH (Table II).

Owing to the instability of 7-*cis*-iodopsin in the presence of NH_2OH, the absorption maximum of 7-*cis*-iodopsin (463 nm; Figure 2B) was estimated by calculating the difference between two curves in the later phase (curves 4 and 8 in Figure 2A) of the incubation of 7-*cis*-iodopsin with 10 mM NH_2OH (Figure 2A, inset).

12-Methyliodopsin was incubated with 1 mM NH_2OH at 4 °C for 20 min for conversion of free 12-methylretinal or the random Schiff-base into retinal oxime, though about 20% of the pigment was decomposed during the incubation. Then, NH_2OH was additionally supplemented at a final concentration of 10 mM. Immediately, the absorption spectrum of 12-methyliodopsin was recorded, and the maximum was estimated from difference spectra before (Figure 1, panel D, curve 1) and after (curve 2) bleaching.

Discussion

The current study is made possible by application of the improved method for purification of iodopsin (Fukada et al., 1989; Okano et al., 1989), in which the CHAPS–PC (phosphatidylcholine) system was used as the solubilizer. Since the protein–chromophore interaction in visual pigments has been

Table II: Absorption Maxima of Pigments and Their Differences in Wavenumber

	iodopsin	rhodopsin		PSB[b]	difference in wavenumber (cm^{-1})		
	CHAPS–PC (nm)[a]	CHAPS–PC (nm)[a]	digitonin (nm)	MeOH (nm)[a]	PSB – Iod[c]	PSB – Rh[d]	Rh – Iod[e]
11-cis	571	500	498[f]	442	5111	2624	2487
9-cis	534	484	483[f]	433	4368	2434	1935
7-cis	463	451	450[f]	432	1550	975	574
14-F	607	527	527[f]	455	5504	3003	2501
12-F	586	507	506[f]	446	5357	2698	2659
10-F	574	499	498[g]	428	5943	3324	2619
8-F	514	463	463[f]	410	4935	2792	2143
12-Me	534	507	489[h]	440	4001	3003	997

[a] Determined in the present study. [b] Absorption maximum of a protonated retinylidene Schiff-base (PSB) in methanol. To 2 mL of methanol in an optical cuvette were added 10 μL of an approximately 5 mM solution of 11-*cis*-retinal (or its analogue) in ethanol and 10 μL of a hexane solution of 0.5 M *n*-butylamine. The mixture was allowed to stand at room temperature for at least 15 min and then monitored by the absorption spectra. To this solution was added 10 μL of 0.5 M trifluoroacetic acid in hexane, and then the spectrum was recorded. [c] For each isomer and analogue of retinal, the difference in absorption maximum between PSB and the pigment with R-photopsin was calculated on a scale of wavenumber. [d] Difference between PSB and the pigment with scotopsin. [e] Difference between the pigments with scotopsin and with R-photopsin. [f] Data from Liu et al. (1988). [g] Data from Shichida et al. (1987). [h] Data from Liu et al. (1984).

studied mainly with bovine rhodopsin, the data on iodopsin should be compared with those on bovine rhodopsin rather than on chicken rhodopsin.

Shape of Retinal Binding Site of R-Photopsin. The current data on R-photopsin and scotopsin [for the latter, see also Liu et al. (1984)] show that the binding sites in the two proteins are similar in chromophore specificity; i.e., they can interact with neither 13-*cis*- nor *all-trans*-retinal but readily accommodate the other mono-cis isomers and all the analogues of 11-*cis*-retinal. Thus, the shape of the retinal binding site of R-photopsin must resemble that of scotopsin. However, the rate of binding of each retinal with R-photopsin was much greater than that with scotopsin. It seems likely that the difference in rate of pigment formation between scotopsin and R-photopsin would be due to the difference in location, not in shape, of the binding site between the two proteins: The binding site of R-photopsin is located near the molecular surface, while that of scotopsin is buried inside. This idea has been suggested on the basis of the accessibility of the chromophore of iodopsin to various reagents (Matsumoto et al., 1975).

The slow formation of 7-*cis*-rhodopsin would be due to some conformational readjustment of scotopsin required for accommodation of 7-*cis*-retinal, which has a highly distorted ring–chain conformation (DeGrip et al., 1976). It is likely that R-photopsin would undergo a similar distortion for accommodation of 7-*cis*-retinal.

It should be noted that the $T_{1/2}$ value of 12-methylrhodopsin (166 min) was much larger than those of all other 11-*cis*-retinal analogues. On the basis of the low yield of formation of 12-methylrhodopsin, Liu et al. (1984) proposed that an area near the hydrogen atom at C_{12} of retinal in the retinal binding site of scotopsin might be occupied by some amino acid residue. The present observation of the slow formation of 12-methylrhodopsin may support the proposal. Some conformational readjustment would take place on accommodation of the analogue. The exact yield of 12-methylrhodopsin cannot be estimated because its molar extinction coefficient is not known. However, the apparent yield observed in this study (A_{max} = 73%; Table I) is much larger than the previous value (2% in digitonin or 5% in CHAPS; Liu et al., 1984). This can be ascribed, at least partly, to a protective effect of glycerol and/or phosphatidylcholine (PC) on a specific conformation of the retinal binding site (Okano et al., 1989).

In contrast, the apparent yield of 12-methyliodopsin (A_{max} = 37%) was much lower than those of all other pigments (above 70%; Table I). The formation of 12-methyliodopsin ($T_{1/2}$ = 3.0 min) was also the slowest among the pigments formed with R-photopsin (Table I). In addition, only this pigment decomposed quickly in the presence of 10 mM NH_2OH even at 4 °C. These observations taken together also suggest the possible presence of a blocking group of some amino acid residue in R-photopsin interfering with the methyl group at the C_{12} position of this compound as proposed in the case of scotopsin (Liu et al., 1984). The failure of 13-*cis*-retinal to form an iodopsin isomer is consistent with this idea because in 13-*cis*-retinal the methyl group at the C_{13} position protrudes into the area occupied by the blocking group (Liu & Mirzadegan, 1988). The apparent yield of 12-methyliodopsin (37%) being lower than that of 12-methylrhodopsin (72%) suggests that the area in the retinal binding site of R-photopsin would be restricted more rigorously than that of scotopsin. A more distorted conformation of R-photopsin on accommodation of 12-methylretinal would be responsible for the lowest stability of 12-methyliodopsin among all the pigments examined here.

As discussed in the previous paper (Okano et al., 1989), the absorption maximum of iodopsin was influenced by detergents; the native spectral property of iodopsin was better preserved in the CHAPS–PC system than in digitonin. On the other hand, the absorption maxima of pigments from scotopsin in the CHAPS–PC system were in fairly good agreement (within 2 nm) with those in digitonin except for 12-methylrhodopsin (Table II). Such a contrast between iodopsin and rhodopsin can be well explained by the notion that the chromophore of iodopsin located near the molecular surface may be more easily affected by environmental effects than that of rhodopsin. The reason for the difference in 12-methylrhodopsin between absorption maxima obtained in the CHAPS–PC system and in digitonin is not clear, but the value in digitonin (Liu et al., 1984) would be less reliable in view of the very low yield of the pigment formed (2%).

Electrostatic Interaction between R-Photopsin and the Chromophore. The location of the counterion to the Schiff-base (Blatz et al., 1972) and the presence of a second point charge near C_{13} (Koutalos et al., 1989) have been discussed in connection with the absorption maximum of rhodopsin. Similar charges should be considered in iodopsin for the analysis of the present set of data.

We evaluated the electrostatic protein–chromophore interaction by calculating the opsin shift [difference in wavenumber between a pigment and a protonated retinylidene Schiff-base (PSB) formed from each retinal isomer or analogue and butylamine]. To distinguish between the opsin shift for

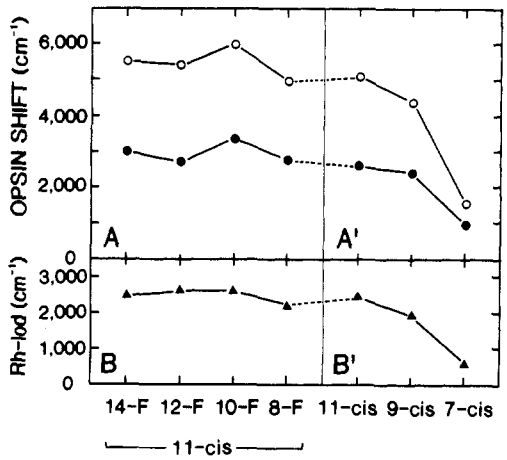

FIGURE 3: Opsin shifts of pigment analogues and isomers. Scotopsin shifts (●) or R-photopsin shifts (○) from Table II were plotted against respective fluorinated pigments (A) and geometric isomers (A'). Differences between scotopsin and R-photopsin shifts (▲) were similarly plotted (B and B').

rhodopsin and that for iodopsin, we wish to coin the terms scotopsin shift (PSB – Rh in Table II) and R-photopsin shift (PSB – Iod in Table II), respectively. From these data one notices that both scotopsin and R-photopsin shifts of 10-fluororetinal are meaningfully larger than those of other fluorinated retinals and the parent 11-*cis*-retinal. This trend becomes more evident in plots of opsin shifts for all fluorinated analogues as shown in Figure 3A.

As the set of fluorinated analogues are in the 11-cis geometry, one may safely assume that the chromophore occupies the same space within the binding site as that of native 11-*cis*-retinal. The compact fluorine atom is not expected to alter sterically the protein–chromophore interaction in any significant manner. Accordingly, the detected unusual specific perturbation by the fluorine substituent at the C_{10} position could only be ascribed to an electrostatic interaction between the electron-rich 10-fluoro substituent and a polar group of the protein, the latter of which has been postulated to be Glu^{122} (Shichida et al., 1987) in helix 3 in a seven α-helix model of rhodopsin (Hargrave et al., 1984). Because of its location it appears that the polar group serves as the second point charge (or an oriented dipole) proposed in rhodopsin, which was first postulated to be near C_{12}–C_{14} (Motto et al., 1980) and more recently near the C_{13} position (Koutalos et al., 1989). Recently, the electrostatic interaction in these regions has also been implicated by ^{13}C NMR studies (Mollevanger et al., 1987; Smith et al., 1988).

It should be emphasized that the profile of scotopsin shifts of the fluorinated retinals (Figure 3A, closed circles) fairly parallels that of the R-photopsin shifts (open circles). Indeed, the differences between both opsin shifts (Rh – Iod values; Table II) are quite constant (Figure 3B), strongly suggesting the presence of a putative second point charge in iodopsin at a position similar to that in rhodopsin. More importantly, these Rh – Iod values coincide well with the value of native 11-cis pigments (Figure 3B', 11-cis). These observations led us to conclude that the remarkable red-shift in the absorption maximum of iodopsin should not be attributed to a difference in the location of the second point charge between iodopsin and rhodopsin.

In contrast, substitution of a sterically bulky methyl group for a hydrogen atom at the C_{12} position induced a large blue-shift of the absorption spectrum of iodopsin or a small red-shift of rhodopsin. This made the Rh – Iod vaue of 12-methyl pigments considerably smaller than that of the native

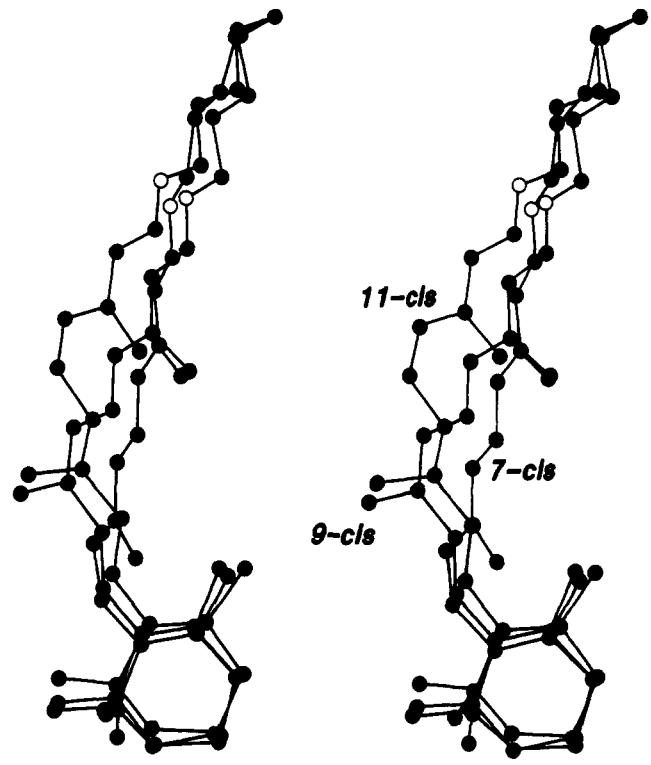

FIGURE 4: Stereoview of 7-*cis*-, 9-*cis*-, and 11-*cis*-rhodopsins anchored rigidly at the α-carbon of the lysine residue and loosely at the center of the cyclohexenyl ring [the hydrophobic pocket: Matsumoto and Yoshizawa (1975)] according to Liu and Mirzadegan (1988). The distances between the imino nitrogens (open circles) for the three isomers are 1.13 (9-cis and 11-cis), 0.78 (7-cis and 11-cis), and 0.92 Å (7-cis and 9-cis).

pigments (Table II). The large blue-shift of the maximum of iodopsin would be ascribed to a twist around some single bond(s) of the 12-methyl chromophore due to the strong steric interaction between the 12-methyl group and an amino acid residue in the binding site as discussed above. Alternatively, the 12-methyl group might force the second charge to move away from the C_{13} position, while the small red-shift of 12-methylrhodopsin might reflect a twist around some double bond(s) in addition to some single bond(s) of the chromophore.

The two types of opsin shifts of the geometric isomers displayed a similar trend of changes with each other as depicted in Figure 3A'. As compared with 11-*cis*-rhodopsin, a small scotopsin shift of 9-*cis*-rhodopsin was explained by the longer distance between the second point charge and the polyene side chain (Koutalos et al., 1989). This interpretation would be also applicable to the smallest scotopsin shift of 7-*cis*-rhodopsin. In fact, molecular modeling analyses of these three isomeric chromophores anchored near the center of the cyclohexenyl ring and at the α-carbon of the lysine side chain (Liu & Mirzadegan, 1988) indicate that the orientations of the polyene chains of the three chromophores within the binding site are quite different (Figure 4). The similar trend of changes in the two types of opsin shifts of the geometric isomers also supports the existence of the second point charge at a similar position in both opsins. It is noticeable that the Rh – Iod values decreased dramatically from 11-cis to 7-cis pigments (Figure 3B'). This fact indicates that the polar interaction specific for iodopsin (i.e., the origin of the significant red-shift of the absorption spectrum of iodopsin) is considerably weakened when R-photopsin accepts 7-*cis*-retinal as its chromophore. As depicted in Figure 4, the difference in Rh – Iod values among the three isomeric pigments (Figure 3B') should be attributed to the differences in both orientation

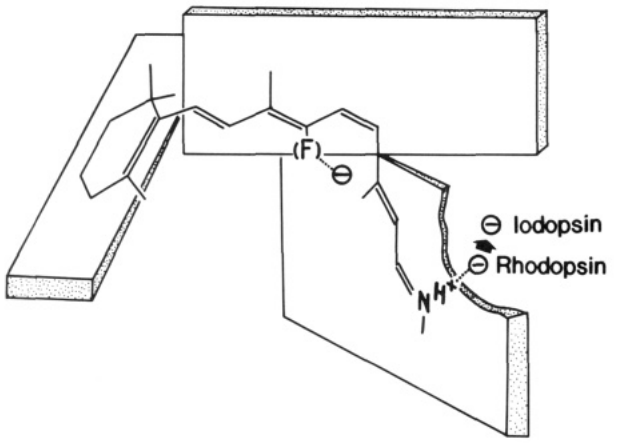

FIGURE 5: Model of the chromophore in iodopsin. The negative counterion of the protonated Schiff-base nitrogen in iodopsin is placed slightly further away than that in rhodopsin. The second negative charge in iodopsin is placed at a position similar to that in rhodopsin near the C_{13} position so as to interact with the fluorine atom of 10-fluororetinal.

of the side chain and location of the iminium nitrogen within the binding site. We speculate that the latter would be a predominant cause of the difference in absorption maxima among pigment isomers and that the difference in the position of the counterion in iodopsin from that in rhodopsin would, in principle, be responsible for the remarkable red-shift of the absorption maximum because the protein–chromophore interaction near the retinal side chain appears to be similar between iodopsin and rhodopsin as discussed above. Thus, our simplest model is that iodopsin would have a Schiff-base weakly hydrogen bonded to the counterion and the second point charge at a position similar to that in rhodopsin (Figure 5).

During the course of preparing the manuscript, we encountered a paper of Chen et al. (1989) in which they proposed, on the basis of absorption maxima of dehydro- and dihydroiodopsin analogues, that the second point charge in iodopsin is located near the β-ionone ring. This is at variance with our conclusion of the second point charge being near the C_{13} position. In reviewing the data, we believe that the disagreement might be at least in part attributable to the diverged values of the absorption maxima of analogue pigments which the two groups estimated by different procedures. First, Chen et al. (1989) used digitonin as a solubilizer while we used the CHAPS–PC system. Conformations of R-photopsin in the two types of detergent may be significantly different as discussed above. Second, we estimated the absorption maxima of pigments from difference spectra between the pigments and their retinal oximes plus opsins in the presence of NH_2OH. On the other hand, they estimated the absorption maxima from difference between spectra before and after partial bleaching in the absence of NH_2OH or before and after pigment formation. We added NH_2OH to every pigment to avoid possible distortion of spectral base lines caused by absorption of long-lived intermediates of pigments or random Schiff-bases expected to exist after irradiation of a pigment in the absence of NH_2OH, thus minimizing any unexpected shift of the estimated absorption maxima of pigments. For example, there is a large difference in the absorption maximum of 9-*cis*-iodopsin,[3] which is a pigment common to the two studies: 500 nm (estimated from difference spectra before and after the pigment formation; Chen et al., 1989) and 534 nm (estimated in this study). Such a large variance of pigment absorption maximum might have led the two groups to different conclusions. However, their hypothesis that iodopsin might have 6-s-trans chromophore to account partly for the significant red-shift of the absorption maximum of iodopsin is quite interesting. The very small R-photopsin shift of 7-*cis*-iodopsin, whose chromophore is expected to be highly twisted at the C_6–C_7 single bond, might support the hypothesis, though it should be tested in further experiments.

[3] The absorption maximum of 9-*cis*-iodopsin in digitonin was estimated to be 510 nm by Wald et al. (1955) or 520 nm by T. Yoshizawa (unpublished observation).

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Activation Parameters for the Halorhodopsin Photocycle: A Phase Lifetime Spectroscopic Study of the 520- and 640-Nanometer Intermediates†

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ABSTRACT: Phase lifetime spectroscopy is used to investigate the kinetics of the 520- and 640-nm intermediates in the halorhodopsin photocycle. These intermediates decay on the millisecond time scale and are strongly implicated in the chloride transport steps. The temperature dependence of the 520 and 640 relaxations was measured for chloride and nitrate buffers at pH 6, 7, and 8 and for iodide buffer at pH 6. The 640 relaxations have small activation energies but large entropy barriers. The two relaxation times observed for the 640 intermediate were interpreted by using a mechanism in which two 640 species exist in equilibrium. The second 640 species is not along the main decay path for the photocycle. A quantitative analysis of the data allowed rate constants and activation parameters to be calculated for the elementary steps of this isomerization process. These parameters are similar for both chloride and nitrate buffers but differ somewhat in iodide. The derived calculated rate constants were consistent with the relaxation times observed for the 520 intermediate. These results indicate that the 520 and two 640 intermediates have very similar free energies as well as similar free energies of activation for the various interconversion processes.

Halorhodopsin (hR),[1] a membrane-bound, retinal protein of *Halobacterium halobium*, functions as a light-driven, inward-directed chloride pump [for a review, see Lanyi (1986a)]. Decay of the initial photoproduct through a series of photocycle intermediates is accompanied by translocation of chloride across the cell membrane. Spectroscopic studies have revealed similarities in the photointermediate absorption maxima and kinetics of the photocycles of hR and bacteriorhodopsin (bR), the light-driven proton pump of *H. halobium* (Zimányi et al., 1989). There is also evidence of structural similarity between the two proteins, which have 28% homology in their amino acid sequences (Blanck & Oesterhelt, 1987). One notable difference in the hR and bR photocycles involves the M_{412} intermediate which corresponds to a deprotonated state of the Schiff base linking retinal to the protein. In bR, the M_{412} intermediate has been implicated in proton transport [cf. Lewis et al. (1974)] while in hR the M-like state appears to be a nonproductive side branch of the main photocycle (Hegemann et al., 1986; Lanyi & Schobert, 1983). These similar proteins which transport very different ions provide an opportunity to identify common features of light-driven ion transport.

The general features of the hR photocycle are outlined in Figure 1. This scheme was mainly derived from previous work (Tittor et al., 1987; Lanyi & Vodyanoy, 1986) but incorporates results from the present study as well. The hR ground state consists of a chloride-dependent equilibrium mixture of the hR_{565} and hR_{578} species. The chloride dissociation constant is estimated to be 10 mM (Tittor et al., 1987). Excitation of the ground state either in the absence of chloride or under saturating chloride results in the formation of the 600-nm absorbing state. The 600-nm intermediate is probably two different species dependent on the path from which it is formed. For it to be one species, chloride must dissociate from hR_{578} in the 5 ps required for formation of hR_{600} (Polland et al., 1985). This would require a dissociation rate constant of 2×10^{11} s^{-1}, which is faster than a diffusion-controlled dissociation (Eigen, 1954; Hemmes et al., 1974). The respective 600 species decay either into the chloride-bound 520 species or into the 640 species. The dissociation constant for chloride in the 520 → 640 equilibrium is estimated at 100 mM (Tittor et al., 1987). This step presumably is the release of the transported ion. Full characterization of these processes is hampered by the lack of sensitive spectroscopic indicators for chloride. The kinetics associated with the 640 species are complex as demonstrated by the observation of several relaxation processes which are very wavelength dependent in the 600–670-nm region (Tittor et al., 1987). In this work, a second 640-nm intermediate is identified as shown in Figure 1. The functional significance of this intermediate remains unclear.

In the present work, phase lifetime spectroscopy is used to measure the relaxation kinetics of the hR_{520} and hR_{640} photointermediates. In previous applications to bR's proton uptake and release kinetics, it was shown that while phase lifetime spectroscopy will give relaxation times similar to flash spectroscopy often the amplitudes of the relaxation processes differ (Sinton & Dewey, 1988). This is a result of the phase mea-

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[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; DNase, deoxyribonuclease; OG, *n*-octyl β-D-glucopyranoside (octyl glucoside); bR, bacteriorhodopsin; hR, halorhodopsin.

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