14-Fluorobacteriorhodopsin and Other Fluorinated and 14-Substituted Analogues. An Extra, Unusually Red-Shifted Pigment Formed during Dark Adaptation†

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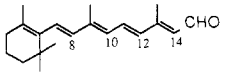
ABSTRACT: Five vinyl-substituted fluororetinal analogues (8-F, 10-F, 12-F, 14-F, and 13,14-F_2) were found to give bacteriorhodopsin analogues with properties similar to those of the parent system. Of these, only 14-fluororetinal was found to give an extra red-shifted BR analogue ($\lambda_{max} \leq 680$ nm) in equilibrium with the normal 587-nm pigment. The 680-nm pigment was enriched upon irradiation. It rearranged to the 587-nm pigment at room temperature ($\Delta E^{\ddagger} = 20.8$ kcal/mol). Chromophore extraction experiments revealed the all-trans geometry for the 680-nm pigment. 14-Chlororetinal gave a similarly red-shifted pigment while 14-methylretinal did not. A scheme for dark adaptation of the 14-halogenated bacteriorhodopsins has been proposed in which the new red-shifted pigment was assigned the all-trans,15-syn geometry.

In addition to the possible use of fluoro substituents as reporting groups in ^{19}F NMR studies of protein–substrate interactions (Sykes & Hull, 1978; Gerig, 1978; Liu et al., 1987), the presence of a highly electronegative fluorine atom could lead to new specific protein–substrate interactions. Thus, for the labeled retinal chromophores, such an interaction has been demonstrated in 10-fluororhodopsin postulated to involve a nearby polar amino acid residue (Liu et al., 1986; Shichida et al., 1987) and in 18,18,18-trifluorobacteriorhodopsin, with the nearby second point charge (Rao et al., 1986). We report here the result of a reconstitution study of 14-fluororetinal (**1**)



1 : 14-F; **4** : 10-F
2 : 14-Cl; **5** : 8-F
3 : 12-F; **7** : 14-Me
6 : 13-deMe, 13,14-F_2

and other fluorinated and 14-substituted retinal analogues with bacterioopsin (BO). New red-shifted intermediates in halogenated bacteriorhodopsin (BR) analogues (14-fluoro- and 14-chlorobacteriorhodopsin) have been detected. Since formation of new pigments appears to be restricted only to 14-halogenated analogues, a regiospecific protein–substrate interaction is also suspected.

Materials and Methods

The syntheses and characterization properties of the halogenated retinal analogues used in this study are in the literature (Asato et al., 1978; Asato & Liu, 1986). All isomers were purified by preparative high-performance chromatography before use. White membrane was isolated from a 20-L culture of JW2N strain of *Halobacterium halobium* according to a published procedure (Oesterhelt, 1982). Procedures for isolation of purple membrane and formation of BR analogues were the same as those reported (Oesterhelt & Stoeckenius, 1974). All absorption spectra were recorded on a Shimadzu UV–vis 2100 recording spectrometer equipped with a temperature control unit, TOC 280 (Kyoto, Japan), or on a Perkin-Elmer λ-5 recording spectrometer.

Reconstitution with 14-Halogenated Retinals. All pigment formation experiments were performed under dim red light at 25 °C by the addition of 1.1 equiv of the retinal analogues, in 1.5–5.5 μL of ethanol, to 600 μL of white membrane stock suspension. The stock suspension of the isolated white membrane is 11.9 μM in 100 mM sodium phosphate buffer, pH 6.0. The visible absorption spectrum of the resulting suspension was monitored at different time intervals. The fluorinated bacteriorhodopsin, 14-F-BR, resulting from the addition of 13-*cis*-14-fluororetinal (**1a**) or *all-trans*-14-fluororetinal (**1b**), was monitored for 1 day, while the chlorinated bacteriorhodopsin, 14-Cl-BR, generated from 13-*cis*-14-chlororetinal (**2a**) and *all-trans*-14-chlororetinal (**2b**), was monitored over a period of 5 days due to the slow rate of pigment regeneration.

Irradiation of 14-Halogenated Bacteriorhodopsin. A light source equipped with an optical fiber light guide (Unisoku, Japan) was used. The light through a KL-56 filter has a λ_{max} of 560 ± 10 nm and an intensity of 159 W/m^2 at a distance of 2 cm from the end of the optical fiber.

Reconstituted membranes derived from white membrane and 13-*cis*-14-fluororetinal or *all-trans*-14-fluororetinal were irradiated at 10 °C with 560 ± 10 nm light. A large buildup of the 680-nm species was observed, and absorption spectra were recorded at different time intervals. The reconstituted membrane from 14-chlororetinals also was irradiated with the same apparatus through a KL-46 interference filter (Toshiba, Japan, 460 ± 10 nm), and absorption spectra, 350–800 nm, were recorded.

Chromophore Extraction. All chromophore extractions were performed as fast as possible in the dark to minimize isomerization of 14-halogenated retinals. An ethanol solution of **1a** (8 μL, 4.45 mM) was added to 3 mL of a white membrane suspension. Aliquots of 200 μL were removed at 1 min, 3 min, 10 min, 1 h, and 1 day. Each aliquot was denatured by the addition of 4 mL of a CH_2Cl_2–CH_3OH solution (1:1 v/v) with sonication for 30 s at 0 °C and partitioned by the addition of 4 mL of hexane followed by centrifugation at 3000 rpm to enhance the phase separation. The upper organic layer

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was dried by filtration through a small column of $MgSO_4$ and evaporated with a gentle stream of nitrogen. The residue was dissolved in 200 μL of CH_2Cl_2. One-fourth of the extracts was injected onto a silica gel column, Develosil SI-60 column (8 mm × 300 mm), eluted at 6 mL/min with ether in hexane (15:85 v/v), and monitored at 360 nm. The ratio of **1a** and **1b** was calculated from the area of each peak.

For studies of the composition of 14-fluororetinals during irradiation, a portion (600 μL) of the reconstituted membrane suspension was irradiated for 5 min at 4 °C. Immediately after irradiation, half (300 μL) of the suspension was extracted, while the remaining half was allowed to stand in the dark at 25 °C for 1 h before extraction. Similar sets of experiments were performed with pigment mixtures derived from **1b**, **2a**, and **2b**.

Kinetics. The 14-F-BR sample was enriched in the 680-nm pigment by irradiation for 5 min with 560-nm light. Decay of the new pigment was monitored at 680 nm at each of the following temperatures: 5, 10, 15, 20, and 25 °C. The natural logarithm of the absorbance at 680 nm was plotted against time for each temperature. The resulting graphs proved to be linear for each temperature, showing that the decay of 680-nm intermediate is first order. Three different measurements for the average lifetime of the 680-nm intermediate were determined.

Other BR Analogues. Preparation of BR analogues with 12-fluoro- (**3**), 10-fluoro- (**4**), 8-fluoro- (**5**), and 13-demethyl-13,14-difluoro- (**6**) retinal involved the use of BO preparation from the purple membrane, according to published procedures (Oesterhelt & Stoeckenius, 1974; Oesterhelt & Schumann, 1974). Retinyloxime was not removed from BO. The ratio of absorbance at 280 and 350 nm for the BO sample was 2.1. The retinal analogue was added in 2-fold excess.

Absorption maximum of protonated retinylidene Schiff base (PSB) of halogenated retinals was determined according to the following general procedure. To 2 mL of methanol in a cuvette were added 10 μL of a ~5 mM solution of a retinal analogue in ethanol and 10 μL of a hexane solution of 0.5 M *n*-butylamine. After 15 min at room temperature, 10 μL of 0.5 M trifluoroacetic acid (or camphorsulfonic acid) in hexane was added and the spectrum recorded.

Procedures for recording absorption spectra of M and O intermediates were the same as those reported (Crouch et al., 1986).

Results

14-Fluorobacteriorhodopsin. The progress of pigment reconstitution of the two isomeric 14-fluororetinals with the white membrane is shown in Figure 1. For the 13-cis isomer (Figure 1a), immediate formation of a pigment analogue with an absorption maximum at 587 nm was observed. Upon standing, a slight decrease of the 587-nm peak was accompanied by the appearance of a tail absorption in the red (curve 4, Figure 1a). The difference spectrum (not shown) between the initial and final spectra showed that the tail absorption was due to a new pigment with λ_{max} at ≤680 nm. For the all-trans isomer, a 440-nm prepigment was formed initially, which gave way eventually to the same 587-nm pigment as in the case of the 13-cis isomer. However, the spectrum taken during early stages of conversion of the 440-nm pigment (curve 1 in Figure 1b) revealed initially a preference for the formation of the red-shifted 680-nm pigment component. Only after prolonged standing was a constant isomer composition containing a slight excess of the trans isomer obtained (Table I). Since the absorption maxima remained the same during dark adaptation of samples (from 10 min to 1 h), starting with either isomer

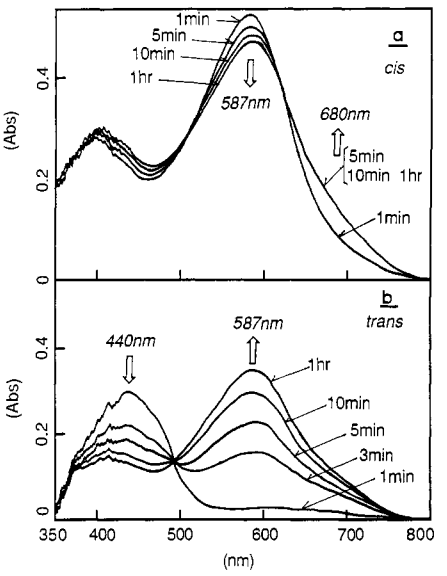


FIGURE 1: (a) Formation of 14-F-BR analogue from the interaction of 13-*cis*-14-F-retinal with white membrane: 1 min, 5 min, 10 min, and 1 h after incubation. (b) Formation of 14-F-BR analogue from the interaction of *all-trans*-14-fluororetinal with white membrane: 1 min, 3 min, 5 min, 10 min, and 1 h after incubation.

Table I: Distribution of Isomers of 14-Fluororetinal Chromophore during Reconstitution and Irradiation by HPLC Analyses of Extracted Retinals

time	13-*cis*-14-F-retinal		*all-trans*-14-F-retinal	
	% cis	% trans	% cis	% trans
1 min	99	trace	trace	>99
3 min	80	20	trace	>99
10 min	70	30	27	73
1 h	45	55	45	55
1 day	47	53	45	55
after irradiation with 560-nm light at 4 °C				
0 min	7	93	5	95
1 h	40	60	52	48

while the isomer composition changes during the same period (Table I), the two isomeric pigments must coincidentally have the same absorption maxima.

Irradiation with 560-nm light led to enrichment of the 680-nm pigment (Figure 2) and complete isomerization to the all-trans isomer as shown by extraction experiments (Table I). The residual 587-nm pigment shown in the UV–vis spectrum (Figure 2) as well as the 680-nm pigment must have the all-trans geometry. On the basis of extraction results of the dark-adapted sample (54 ± 2% trans) and the approximately 10% of the 680-nm pigment in dark-adapted samples at room temperature which was shown by extraction to be all-trans (Figure 1), one would estimate the 587-nm pigment to consist of approximately equal amounts of the two isomeric pigments (44% each).

The photochemically enriched 680-nm pigment was found to be thermally unstable and reverted to the 587-nm pigment upon standing (Figure 2). The process was followed at five different temperatures. The averaged unimolecular rate of decay and calculated averaged lifetimes are listed in Table II. The Arrhenius plot of the rate constants proved to be linear. An activation energy of 20.8 ± 0.3 kcal/mol and activation entropy of 62.5 ± 1 eu were calculated for the decay of the 680-nm species by linear regression analysis.

The 14-F-BR pigment exhibited proton pumping activity and possessed a photocycle with intermediates M, λ_{max} =

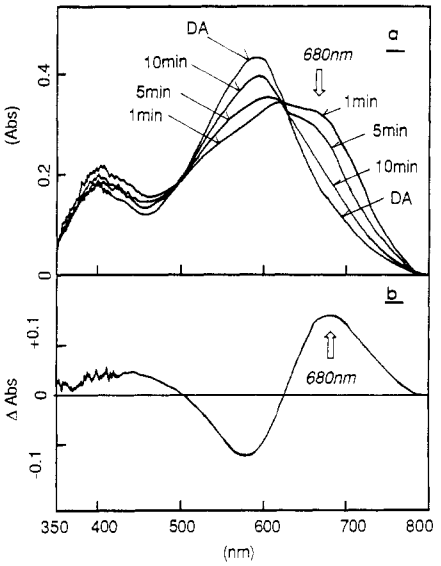


FIGURE 2: Decay of the 680-nm intermediate after irradiation of 14-F-BR with 560-nm light for 5 min at 10 °C. (a) Absorption curves of the dark-adapted (DA) sample before irradiation, and 1, 5, and 10 min after irradiation. (b) Difference between spectra of 14-F-BR at 1 min after irradiation and DA.

Table II: Decay Rates of the 680-nm Intermediate Determined at Various Temperatures

temp (°C)	av rate of decay $k \times 10^3$ (s^{-1})	av lifetime (s)
5	2.13 ± 0.094[a]	469 ± 44
10	3.90 ± 0.29	256 ± 19
15	7.63 ± 0.28	131 ± 4.8
20	14.06 ± 0.8	71 ± 4.0
25	26.40 ± 1.2	38 ± 1.7

[a] Uncorrected standard deviation from three measurements.

410–420 nm, $T_{1/2}$ = 30 ms, and O, λ_{max} = 690–700 nm, $T_{1/2}$ = 100 ms (Table III). Therefore, the 680-nm pigment obtained during steady-state irradiation could not be due to a long-lived O intermediate but rather a stable new pigment in equilibrium with the dominant isomeric pigments.

Other Halogenated Analogues. We have also prepared the BR analogues of 8-, 10-, 12-fluoro-, and 13-demethyl-13,14-difluororetinals (Table III). In contrast to the 14-fluoro isomers, no extra, red-shifted pigments were detected in these systems. The opsin data for the fluorinated analogues have been obtained and are shown in Table V. In addition, we have

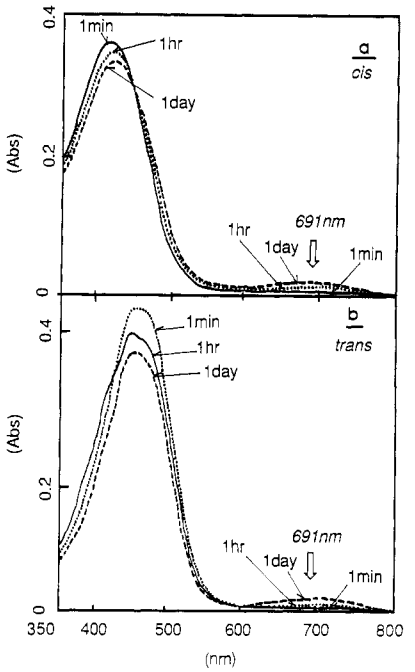


FIGURE 3: (a) Formation of 14-Cl-BR analogue from the interaction of 13-*cis*-14-chlororetinal with white membrane: 1 min, 1 h, and 1 day after mixing. (b) Formation of 14-Cl-BR analogue from the interaction of *all-trans*-14-chlororetinal with white membrane: 1 min, 1 h, and 1 day after mixing.

examined two other 14-substituted BR analogues. *all-trans*-14-Methylretinal was reported to give a 425-nm (unprotonated) pigment (Schiffmiller et al., 1985). The present work (Table III) shows that the 13-cis isomer also yielded only a blue-shifted 420-nm pigment. Nor was there any long wavelength absorbing pigment detected upon irradiation (460 nm) of the 420–430-nm pigments. A different situation existed for the 14-chlororetinal system. For the all-trans isomer, a 440–475-nm pigment was formed after 1 h of incubation and a 691-nm species was observed after 1 day (Figure 3). For the 13-cis isomer, the formation of a 430-nm pigment was accompanied by a red-shifted 691-nm pigment. The latter remained as a minor component (~5%) in the equilibrium mixture (after 5 days). Extraction experiments showed that equilibration of the isomers proceeded very slowly. After 5 days, the mixture of the two isomers approached a common equilibrium composition of approximately 40% 13-cis and 60%

Table III: Properties of Fluorinated and 14-Substituted BR Analogues

analogue		pigments	intermediates M	intermediates O	λH^+[a]
retinal	13-cis	400–410, 554[b]	412[c]	640[c]	+
	all-trans	420–430, 568[a]			
14-F (**1**)	13-cis	587	410–415	690–700	+
	all-trans	440, 587, 680			
14-Cl (**2**)	13-cis	440–475, 691			
	all-trans	430, 691			
12-F (**3**)	all-trans	566,[d] 591			
10-F (**4**)	13-cis	557	410	650	+
	all-trans	431, 565			
8-F (**5**)	all-trans	530			
13,14-F_2 (**6**)	13-cis	600	420		+
	all-trans	450, 600			
14-methyl (**7**)	13-cis	420			
	all-trans	425			
13-demethyl-14,20-ethano (**8**)	13-cis	600 547[e]			
13-demethyl-12,14-ethano (**9**)	576[e]				

[a] Proton pumping activity. [b] Scherrer et al. (1989). [c] Stoeckenius et al. (1979). [d] For dark-adapted sample. The 13-cis isomer was unavailable. [e] Fang et al. (1983); Chang et al. (1985).

Table IV: Distribution of Isomers of 14-Chlororetinyl Chromophore during Reconstitution by HPLC Analyses of Extracted Chromophores

	isomer distribution (%)			
	all-trans-14-Cl + BO		13-cis-14-Cl + BO	
time of extraction	13-cis	all-trans	13-cis	all-trans
1 min			100	0
10 min	6	94	99	1
	6	94		
30 min	6	94	95	5
1 h	6	94	93	7
4 h	14	86		
5 days	39	61	45	55
after irradiation with 460-nm light at 4 °C				
0 min	61	39	68	32
	57	43	60	40
30 min	53	47	65	35
	58	42	63	37
dark adpated	43	57	47	53
	37	63	46	54

Table V: Opsin Shift Data for Fluorinated BR Analogues

retinal	BR (nm)	PSB[a] (nm)	opsin shift[b] (cm^{-1})
all-trans[c]	567	443	4940
14-F, all-trans	587	455	4940
	680	455	7270
12-F, all-trans	591	447	5450
10-F, all-trans	562	442	4830
8-F, all-trans	530	427	4550
13,14-F_2, all-trans	600	466	4790

[a] *n*-Butyl protonated Schiff base in methanol. [b] Difference of columns 2 and 3 expressed in cm^{-1}. [c] Data of Balogh-Nair et al. (1981).

all-trans (Table IV). Irradiation of either sample (Figure 4) increased slightly the amount of the 691-nm pigment, which peaked at 30 min of irradiation. Therefore, the 14-chloro system appears to exhibit the same general characteristics as the 14-fluoro system, albeit reacting at a much slower rate.

Discussion

The most unusual feature among all the halogenated BR analogues examined in this study is the additional red-shifted pigments present in both the 14-F and 14-Cl systems during formation of the BR analogues. The 14-chloro pigment, while exhibiting the same characteristics as the 14-fluoro, was formed at a very slow rate; hence, the 14-F system has been studied and is discussed below in more detail.

The major pigment from either the all-trans isomer, or the 13-cis isomer, of 14-fluororetinal has an absorption maximum at 587 nm. This corresponds to an opsin shift identical with that of the *trans*-BR of the parent system (Table V). The new red-shifted pigment ($\lambda_{max} \leq 680$ nm[1]), with additional absorption near 440 nm (Figure 2b), apparently in equilibrium as a minor component with the "normal" 587-nm pigments, has an unusually large opsin shift value, a point to be discussed later. Its formation appears to be associated with the electronic properties of the 14-fluoro substituent, and the effect is regiospecific. These conclusions are based on the observations that similar red-shifted pigments were not detected in analogues with the fluoro substituent located at other positions of the polyene chain or in the 14-methyl analogue (a methyl substituent is about the same size as a chlorine atom) (Pauling, 1960) (Table III). The presence of the new pigment seems to have no effect on the photocycle or on the proton pumping ability. Hence, M intermediates have been detected for all three fluorinated analogues that have been examined so far (Table III).

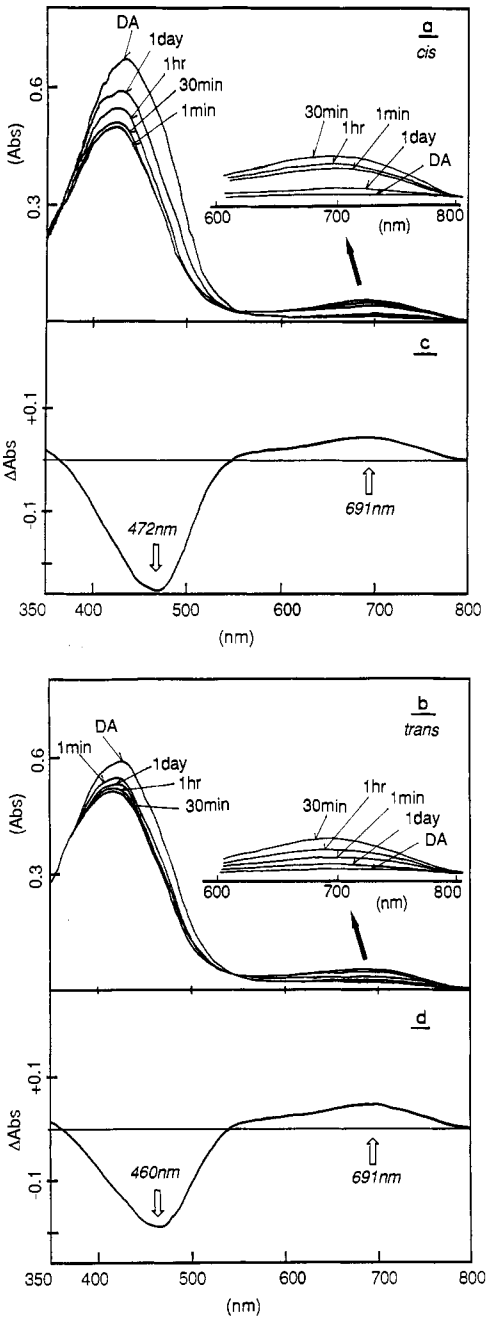
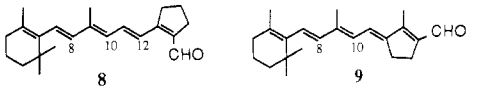


FIGURE 4: (a) Spectra of 14-Cl-BR derived from 13-*cis*-14-chlororetinal and white membrane dark adapted (DA) before irradiation and 1 min, 30 min, 1 h, and 1 day after irradiation with 460-nm light for 5 min at 25 °C. The region around 600–800 nm is expanded in the inset to show the formation of the 691-nm intermediate. The difference between the spectra at 30 min after irradiation and DA is shown in (c). (b) Spectra of 14-Cl-BR derived from *all-trans*-chlororetinal and white membrane before irradiation and 1 min, 30 min, 1 h, and 1 day after irradiation with 460-nm light for 5 min at 25 °C. The region around 600–800 nm is expanded to show the formation of the 691-nm intermediate. The difference between the spectra at 30 min after irradiation and before irradiation is shown in (d).

However, steric effects do play a significant role in determining rates of pigment formation as well as absorption characteristics of the resultant pigment analogues. Hence, both

[1] As pointed out by a reviewer, the 680-nm value obtained from Figure 2b for λ_{max} of the pigment could be slightly too high because of its partial overlap with the 587-nm pigment.

the all-trans and the 13-cis isomers of 14-methylretinal (**7**) gave only "prepigments" absorbing in the 420–430-nm range, which was shown earlier to contain an unprotonated imine linkage (Schiffmiller, 1985). And the five-membered-ring-containing analogues (**8** and **9**) (Fang et al., 1983; Chang et



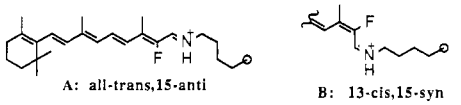
8 **9**

al., 1985) gave pigments with λ_{max} = 576 and 547 nm, respectively, at extremely sluggish rates (150 min for full pigment formation from **8** and 15 days for **9**). In contrast, for the less crowded 13-demethylretinal pigment formation (λ_{max} = 600 nm) is unaffected (Gartner et al., 1983). Steric effects were also evident in the halogenated series with the bulkier chlorine substituent greatly inhibiting pigment formation.

On the basis of current knowledge of structures of the parent BR isomers and chemical information on the 14-fluoro system, it is possible to speculate on a possible structure of the red-shifted pigment. From resonance Raman and NMR studies, it has been determined that the configuration of the chromophore in *trans*-BR and *cis*-BR is all-trans,15-anti and 13-cis,15-syn, respectively (Harbison et al., 1984; Smith et al., 1984). Interconversion of these two structures is achieved (Mathies et al., 1987) by a simple volume-conserving bicycle-pedal process (Warshel, 1976; Warshel & Barboy, 1982).

For the stable isomeric 587-nm pigments in the 14-fluoro system, it seems reasonable to assume that they also have the all-trans,15-anti and 13-cis,15-syn structures. Knowing that the 680-nm pigment contains the all-trans chromophore, it seems reasonable to propose the structure all-trans,15-syn for the red-shifted chromophore. Below we suggest a possible role of such a structure in the observed dark adaptation of the 14-fluoro pigments.

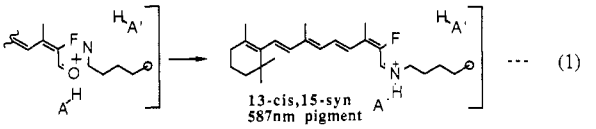
Structure A contains the currently accepted all-trans,6-s-trans geometry for *trans*-BR (Lugtenburg et al., 1988). A skewed (shortened) butyl group is appended to the chromophore so that the longer, tethered all-trans chromophore fits within the common binding cavity shared by the shorter 13-cis,15-cis chromophore (structure B) with the common points



A: all-trans,15-anti B: 13-cis,15-syn

of attachment (circled C_α and the ring). It further allows its conversion to a shorter 13-cis isomer during the rapid photochemical process (Liu et al., 1985). Furthermore, failure of BO to give pigment analogues with other mono-cis retinal isomers (Stoeckenius et al., 1979) suggests that the binding site nearby the polyene chain must fit snugly within a rigidly defined binding site.[2]

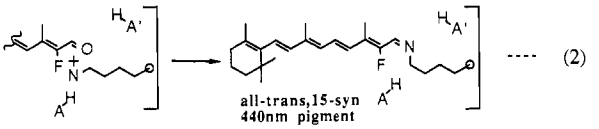
To account for the observed rapid rate of formation of the 13-cis pigment and the intermediacy of unprotonated pigments for the all-trans isomer, also suggested by data of the parent BR system (Schreckenbach & Oesterhelt, 1977), we found the need to postulate possible involvement of two proton sources (HA and HA′) nearby the imino bond. Such a possibility has recently been proposed in a model for the photocycle of BR (Fodor et al., 1989; Lugtenburg et al., 1988). Alignment in the transition state of the aminobutyl group of Lys-216 and the 13-cis chromophore shown in step 1 could



(1)

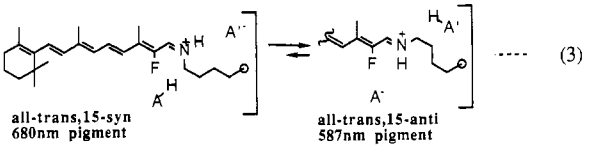
lead directly to the 13-cis,15-syn chromophore containing a relaxed butyl side chain of the lysine residue, with the imino nitrogen readily protonated by the neighboring H–A group.

On the other hand, interaction of the all-trans chromophore with the similarly positioned lysine group (i.e., equal in distance from the C_α of lysine to the hydrophobic pocket) would lead to a 15-syn chromophore with the lone-pair electrons of the imino nitrogen oriented away from H–A (step 2). Its pro-



(2)

tonation could take place only through interaction with a probably more remotely located second proton source (H–A′). A longer lived unprotonated prepigment would result. Protonation of the imine by H–A′ should give the corresponding all-trans,15-syn pigment. Conversion to the more stable all-trans,15-anti structure could then be achieved by an extended bicycle-pedal process (step 3). Interchange of protons, as a



(3)

result of reorientation of the imino nitrogen, would lead to the all-trans,15-anti structure, believed to be that of the 587-nm pigment. The all-trans,15-syn precursor could then correspond to that of the 680-nm pigment.

We note with interest that a recent study of retinal isomer ratio in dark-adapted samples of BR seems to suggest the presence of a pigment near 650 nm at higher temperatures (Scherrer et al., 1989). That the red-shifted pigment is formed more favorably in the 14-F system could be due to electron repulsive interaction between A^- and 14-F in the all-trans,15-anti structure. The more remote location of A'^{-1} could then account for the red-shift absorption characteristic of the new pigment, in agreement with suggestions made by Blatz and Mohler (1972) for more weakly hydrogen bonded retinyl chromophores. The scheme also implies that from the all-trans isomer formation of the 680-nm pigment should precede that of the normal 587-nm pigment. This is in agreement with what was observed during early stages of reconstitution as shown in Figure 1b.

The explanation proposed above for the unusual red-shifted pigment is regiospecific. It is consistent with results of other monofluorinated (8-F, 10-F, 12-F) analogues for absence of similar pigments. The opsin shift data for the fluorinated BRs are shown in Table V. In general the values are within 10% of that of *trans*-BR (perhaps with the exception of the 12-F), indicating an absence of unusual protein–fluorine interactions. The large value for the minor 680-nm pigment clearly stands out. A normal opsin shift value was also observed for the 13,14-difluoro system. The absence of a red-shifted pigment there is probably due to the replacement of the 13-methyl group by a smaller fluorine atom, making the system more similar to 13-demethylretinal. The latter is known to exhibit different stereoselectivity toward the binding site of BO (Gartner et al., 1983).

[2] These generalizations are probably not applicable to other retinal analogues (Gartner et al., 1983) or pigments formed under acidic medium where stereoselectivity of the binding site appears to be quite different.

In summary, we have shown that formation of the unusually red-shifted pigments from two 14-haloretinals is regiospecific and is associated with the electronic properties of the 14-substituent. Their presence and the different rates of formation of isomeric pigments can be rationalized by a simple but structurally detailed mechanistic scheme. While the scheme is consistent with all known chemical information available so far, it remains speculative. Independent corroborative evidence to verify the features proposed in the scheme and to identify the exact role of the halogen substituent in promoting specific protein–substrate interactions is obviously desirable.

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