Study of the Shape of the Binding Site of Bovine Opsin Using 10-Substituted Retinal Isomers†

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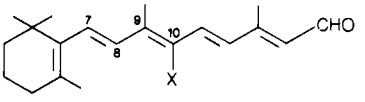
ABSTRACT: The 9-cis, 11-cis, 13-cis, and all-trans isomers of 10-fluoro-, 10-chloro-, 10-methyl-, and 10-ethylretinals have been prepared and characterized. Results of their interaction with bovine opsin are reported. The data have been analyzed in terms of the conformational properties of the retinal isomers and their steric compatibility with the binding site as defined by the two-dimensional map disclosed earlier. The need to expand the active zone and previously undetected restrictions in the third dimension are noted.

Specific protein modification in studies of protein–substrate interactions, known as site-specific mutagenesis, is a relatively new and increasingly popular technique. In contrast, specific substrate modification in studies of protein–substrate interactions is a well-established technique primarily employed by the organic chemist, which carries a less glamorous name of analogue studies. Nevertheless, it should be clear that the goal of both approaches is the same.

For visual pigments, a large number of analogues have been prepared on the basis of variation of the polyene geometry (Wald et al., 1955; Liu et al., 1984a), substituent, or other more extensive structural modifications (Kropf et al., 1973; Balogh-Nair & Nakanishi, 1982a,b). Several review articles are now available on synthetic retinoids (Frickel, 1984; Liu & Asato, 1984), visual pigment analogues (Balogh-Nair & Nakanishi, 1982a,b), and bacteriorhodopsin analogues (Balogh-Nair & Nakanishi, 1982a,b).

Continuing the analogue studies carried out in Hawaii in the last decade (Liu, 1982; Liu et al., 1984b; Crescitelli & Liu, 1985) we have now prepared and characterized a series of isomeric retinals specifically modified with different substituents at the C-10 position. Their interaction with bovine opsin



X=H, F, Cl, Me, Et

together with the implications of the results on the shape of the binding site is the subject of this paper.

Here we report the synthesis and select properties of the retinal analogues and identify spectrally the analogue photopigments that were generated. The substituent groups introduced at the 10-position are F, Cl, CH_3, and C_2H_5. In this paper we will examine the influence of these different substitutions on the regeneration process and on the spectral properties of the analogue photopigments. In the following paper (Liu et al., 1986) we will consider the photosensitivities of the regenerated bovine and gecko pigments. In a preliminary experiment (H. Matsumoto and R. S. H. Liu, unpublished results; Asato et al., 1978), 9-*cis*-10-fluororhodopsin (cattle) was observed to be unusually insensitive to light, and in the following paper this extraordinary property will be explored with all four C-10 retinal analogues.

All the results will be evaluated in terms of possible steric effects of the substituent groups of varying size, in relation to the shape of the opsin binding site, in addition to other protein–substrate secondary interactions. Recently, the binding site of bovine opsin was described in terms of a two-dimensional projection map (Liu et al., 1984a), which served to rationalize the failure of the 13-cis and all-trans isomers of retinal to form stable pigment analogues. Now, it accounts for new selectivities to be seen in the data of this and the subsequent paper and for reactions of two different opsins (cattle and gecko) with different stereoisomers of a series of substituted retinal analogues.

Experimental Procedures

General Procedures for Preparation of Isomers of Retinal Analogues. The synthetic procedures described below afforded mixtures of the all-trans, 13-cis, and 9-cis isomers. The hindered 11-cis and 7-cis isomers were obtained by direct irradiation of the all-trans isomer or the synthetic mixture in acetonitrile according to established procedures (Denny & Liu, 1977; Liu & Asato, 1982). All isomers were isolated by preparative HPLC on a 5-μm silica gel column and characterized by their ^{1}H NMR (Table I) and UV spectra (Table II). All UV absorption spectra were recorded on a Perkin-Elmer λ-5 or Coleman 124 spectrometer and NMR spectra on a Nicolet NM-300 or an IBM NR-80 spectrometer.

10-Fluororetinal. Procedures for the preparation of the compound were disclosed earlier (Asato et al., 1978). Conditions for HPLC separations were as follows: solvent, 8% ether–hexane; relative order of increasing retention time of isomers, 9-cis, 13-cis, 11-cis, all-trans. Spectral data have been reported (Asato et al., 1978).

10-Chlororetinal. This analogue was prepared by a slight modification of the procedure according to (Köbrich et al. (1967). Thus, the C_{14}-chlorotriene prepared from β-ionone

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Table I: Characteristic ^1H NMR Signals of Isomers of 10-Substituted Retinals[a]

compds and isomers	chemical shift (δ, ppm)[b]									J (Hz)	
	CH_3-5	CH_3-9	CH_3-13	H_7	H_8	H_{11}	H_{12}	H_{14}	H_{15}	7,8	11,12
10-chloro											
all-trans	1.75	2.01	2.33	6.23	6.66	7.31	6.37	5.91	10.06	16.0	15.8
13-cis	1.75	2.03	2.17	*c*	*c*	7.24	6.82	5.79	10.05	*c*	14.7
11-cis	1.73	1.90	2.27	6.30	6.72	6.21	6.31	5.89	10.03	15.9	12.0
9-cis	1.75	2.20	2.34	6.38	6.61	7.29	6.75	6.00	10.08	15.9	14.8
7-cis	1.44	2.00	2.34	6.16	6.64	7.12	6.78	5.99	10.05	12.8	14.8
11-cis,13-cis	1.78	1.69	2.20	6.13	6.38	6.09	6.32	5.73	9.95	16.0	12.5
10-Me											
all-trans	1.73	1.99, 2.06[d]	2.36	6.33	6.58	7.32	6.46	5.90	10.03	15.8	15.5
13-cis	1.73	2.01, 2.06	2.16	6.31	6.63	7.24	7.42	5.75	10.14	15.9	15.6
11-cis	1.71	1.82, 1.90	2.22	6.13	6.42	6.39	6.02	5.82	9.98	16.0	12.1
9-cis	1.74	1.98, 2.02	2.33	6.21	6.67	7.39	6.36	5.89	10.03	15.9	15.6
7-cis	1.38	1.89, 1.90	2.33	6.03	6.45	7.21	6.39	5.89	10.03	12.6	15.6
9-cis,13-cis	1.73	2.04	2.14	6.22	6.66	7.29	7.35	5.75	10.12	15.9	15.5
10-Et[e]											
all-trans	1.71	2.04	2.35	6.32	6.54	7.21	6.43	5.91	10.03	15.9	15.7
13-cis	1.71	2.15	1.93	6.31	6.55	7.11	7.44	5.76	10.11	16.0	15.7
11-cis	1.8	2.14	2.30	6.24	6.50	6.22	6.43	5.96	10.08	16.1	12.1
9-cis	1.74	2.01	2.33	6.22	6.63	7.27	6.36	5.90	10.03	15.9	15.8
7-cis	1.41	1.88	2.33	6.04	6.47	7.11	6.40	5.90	10.03	12.2	15.7
9-cis,13-cis	1.74	2.03	2.15	6.22	6.62	7.19	7.38	5.77	10.11	15.7	15.7

[a] Nicolet NM-300 spectrometer, 20% acetone-d_6 in CCl_4. [b] Acetone-d_5 as internal standard, δ = 2.04 ppm. [c] Not resolved, overlapping with signals of 11-cis,13-cis present in the sample. [d] CH_3-9 and CH_3-10. [e] Signals for the ethyl group fall in the region 1.04–1.07 and 1.15 ppm (for 11-cis) for the CH_3 and 2.42–2.50 ppm for the CH_2.

Table II: UV–Vis Absorption Maxima (nm) for 10-Substituted Retinals[a]

isomers	10-F	10-Cl	10-Me	10-Et
all-trans	373	280, 366	374	376
13-cis	284, 355	*b*	364	371
11-cis	283, 356	269, 335 (sh)[c]	270, 340	250, 335 (sh)
9-cis	358	281, 360	280, 362	280, 365
7-cis	284, 360	303, 352	356	280, 259
9-cis,13-cis	359	*b*	*b*	288, 359

[a] In hexane. [b] Isomerically pure sample unavailable. [c] sh = shoulder.

and (chloromethyl)triphenylphosphorane was converted to a mixture of 9-cis and all-trans 10-chlorotriene C_{15}-aldehyde. Thereafter, a standard $C_{15} + C_5$ chain extension afforded the desired compound. Pure isomers were isolated by HPLC separation of the synthetic or the irradiated mixture: 10% ether in hexane with isomers appearing in the following order, 13-cis, all-trans, 11-cis, 9-cis, 7-cis. Spectral data are listed in the tables. Generally, the chemical shift and coupling constant data are sufficient for assignment of the polyene geometry (Liu & Asato, 1982, 1984). However the introduction of the 10-chloro substituent made the assignment of the geometry at the 9,10-double bond more difficult. The all-trans and the 9-cis isomers were distinguished on the basis of their reactivity toward opsin (9-cis being reactive and all-trans unreactive). Results of a study of difference proton nuclear Overhauser effect (NOE) by irradiating the methyl groups of the major synthetic isomer revealed the following results, which were consistent with the assignment of the all-trans geometry (irradiate {observed enhancement}): 5-CH_3 {H-8}; 9-CH_3 {H_7, H_{11}, H_{15}}.

10-Methylretinal. We were unable to repeat the reported method for preparation of the title compound (Tanis et al., 1978). Instead, the introduction of the 10-alkyl substituent was effected by the Peterson reactions of β-ionone with ethyl 2-(trimethylsilyl)propanoate. Subsequent standard chain-extension reactions (Liu & Asato, 1982) yielded the desired retinal. HPLC conditions were as follows: solvent, 6% ether in hexane; order of appearance, 13-cis, 11-cis, 9-cis, 7-cis, all-trans. Spectral data are listed in the tables. Reasons for the assignment of the configuration about the 9,10 double bond were the same as those outlined for 10-chlororetinal.

10-Ethylretinal. A similar procedure as described above for the preparation of 10-methylretinal was employed for the synthesis of the title compound. The 10-ethyl substituent was introduced by the Peterson reaction of β-ionone with 2-(trimethylsilyl)butanoate. Order of HPLC elusion was 11-cis, 9-cis,13-cis, 13-cis, 9-cis, 7-cis, and all-trans.

Bovine Pigment Analogues. The procedure for pigment regeneration described previously has been followed (Matsumoto et al., 1978; Liu et al., 1984b). Two detergents were used to solubilize the pigment: digitonin or 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate (CHAPS) (Kropf, 1982). Yields and difference absorption spectra of the pigment analogues, listed in Table IV, were determined in the same manner as described previously (Matsumoto et al., 1980a; Liu et al., 1984b). Briefly, opsin (2–10 μM) solubilized in digitonin or CHAPS and a 2–5-fold excess of the retinal analogue were incubated at room temperature for 1–10 h. Upon complete pigment formation as indicated by a constant reading in the visible region of the absorption spectrum, hydroxylamine was added to a final concentration of 40 mM. After the absorption spectrum was recorded, the sample was bleached with yellow light. The difference spectrum between those before and after photobleaching was then computed.

The extinction coefficient of 10-fluororhodopsin was determined according to the following procedure. A stock solution of 2×10^{-5} M opsin solubilized in 1% digitonin was used for titration against stock solutions of 11-*cis*-retinal and 10-fluororetinal. Into two sets of cuvettes was introduced 0.5 mL of diluted stock opsin solution with concentration ranging from 2×10^{-6} to 1×10^{-5} M. To these cuvettes was added either an excess amount of 11-*cis*-retinal (to a final concentration of 9.0×10^{-5} M) or 10-fluoro-11-*cis*-retinal (5.8×10^{-5} M). After incubation at 30 °C for 3.5 h to allow complete regeneration of pigments, the absorbance at λ_{max} for each sample was recorded. Data for the five pairs of samples (Table III) showed that the ratio of pigment formation was independent of opsin concentration, thus suggesting that 10-fluororhodopsin, as well as rhodopsin, was formed in quantitative yield under the reaction conditions. The difference in absorbance at λ_{max}

Table III: Relative Yields of 10-Fluororhodopsin to Rhodopsin at Varying Concentration of Opsin[a]

[opsin] (M)	absorbance at λ_{max}[b] rhodopsin	10-F-Rhod	ratio of abs (rhod/ 10-F-Rhod)
2×10^{-6}	0.072	0.053	1.36
4×10^{-6}	0.15	0.11	1.36
6×10^{-6}	0.23	0.17	1.35
8×10^{-6}	0.30	0.22	1.36
1×10^{-5}	0.38	0.28	1.36

[a] In digitonin. [b] Determined after 3.5 h of incubation at 30 °C.

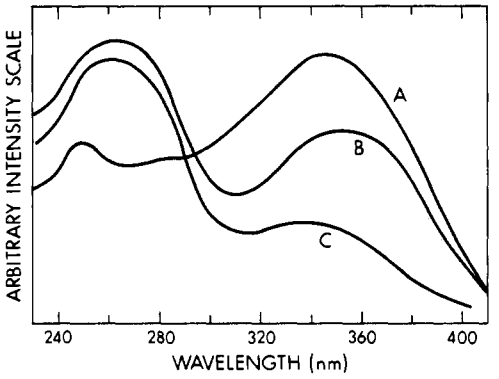


FIGURE 1: UV–vis absorption spectra of 10-fluoro- (A), 10-methyl- (B), and 10-ethyl-11-*cis*-retinal (C) in hexane. The intensity scale is of arbitrary absorbance unit. No attempt is made to correlate relative absorbance values.

was therefore attributed to the difference in the extinction coefficients for the two pigments. The calculated ϵ_{max} for 10-fluororetinal was found to be 42 000/1.36 = 30 600.

RESULTS

The UV–vis absorption spectra of the 10-substituted retinals are characterized by a decrease in intensity of the 360-nm band in the 11-cis isomer, which is accompanied by an increase in intensity of a band below 300 nm. This change appears to be qualitatively proportional to the size of the 10-substituent. Selected spectra are shown in Figure 1.

The visual pigment analogues exhibit properties generally similar to those derived from isomers of the parent retinal (Liu et al., 1984b). However, the employment of a computerized UV–vis absorption spectrometer in the current study has significantly improved the sensitivity, making possible the detection of those pigments formed in less than 5% yield. In Figure 2, the case 10-ethyl-11-*cis*-retinal is shown as a representative example.

All pigment absorption maxima listed in Table IV are those from the difference absorption spectra. The yields were calculated on the basis of the following assumptions for the extinction coefficients of the pigment analogues. Since the λ_{max} of 10-fluororhodopsin was shown to be smaller than that of rhodopsin (30 600 vs. 42 000), the extinction coefficients for all other 10-substituted 11-cis analogues were assumed to have a similar small value of 30 000. For all other geometric isomers, the extinction coefficients were assumed to have a more normal value of 40 000. The complete data for bovine pigment analogues are shown in Table IV.

The minimized conformations of the isomers of retinal analogues have been obtained by molecular mechanics calculations. The individual isomers of retinal and the 10-substituted analogues of retinal were constructed in CHEMGRAF (Chemical Design Ltd., Oxford), using as a starting point the geometry suggested by the X-ray structure of 11-*cis*-retinal (Gilardi et al., 1972). For this study, the 12-s-trans conformation has been assumed. Complete energy minimizations were calculated for each analogue of the 11-cis isomer with MMP1, a molecular mechanics program for π systems (Allinger & Yuh, 1983), to give stable conformations presumed to be close to the global minimum and close to those suggested by the molecular models. Selected data from such calculations on two isomers of retinal and the 11-cis isomers of 10-substituted retinals are listed in Table V.

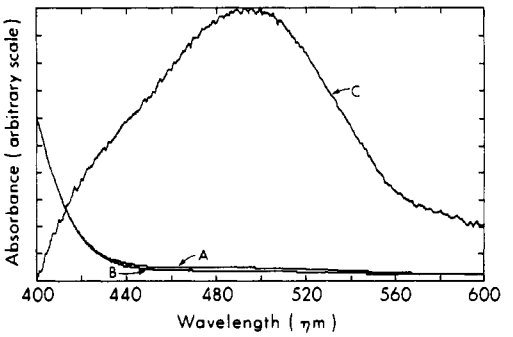


FIGURE 2: Representative difference absorption spectrum for a low-yield (~5%) pigment analogue (curve A) absorption spectrum of 10-ethyl-9-*cis*,13-*cis*-rhodopsin after addition of an excess of hydroxylamine; (curve B) absorption spectrum of the same sample after irradiation with light >450 nm; (curve C) difference spectrum between curves B and A and plotted on an expanded scale.

Table IV: Absorption Maxima (nm) and Yields (%) of Bovine Pigment Analogues from Isomers of 10-Substituted Retinals

compd	isomer[a]	detergent[b]	λ_{max}	yield[c,d]
10-fluoro	11-cis	D	502	100 (6)[e]
		C	500	90
	9-cis	D	486	100 (2)
		C	486	68
	7-cis	D	484	22 (3)
	9-cis,13-cis	D	484[f]	50 (2)
	7-cis,9-cis	D	464[f]	31
	11-cis,13-cis	C	461	61
10-chloro	11-cis	D	480	79
	9-cis	D	474	30 (2)
	7-cis	D		
10-methyl	11-cis	D	508	83
	9-cis	D	498	94
	7-cis	D	505	12
	9-cis,13-cis	D		
10-ethyl	11-cis	D	480	2–3
	9-cis	D	494	8
	7-cis	D	480–490	~1
	9-cis,13-cis	D	493	6.0 (2)
10-H[g]	11-cis	D	498	100
	9-cis	D	483	>90
	7-cis	D	450	40
	9-cis,13-cis	D	485	90

[a] No pigment formed for all 13-cis and all-trans isomers. [b] In digitonin (D) or CHAPS (C). [c] One run unless otherwise indicated in parentheses. Yields based on ϵ = 40 000 unless otherwise specified. [d] All pigments stable, i.e., no noticeable decrease of absorption 10 min after the addition of 0.04 M NH_2OH. [e] Based on ϵ = 30 400; see text. [f] Data recorded on a Perkin-Elmer spectrometer, all others on a Perkin-Elmer λ-5 spectrometer. [g] Based on data in Liu et al. (1984b) and references cited therein.

DISCUSSION

Retinal Isomers. The unhindered isomers of 10-substituted retinals exhibit UV–vis absorption characteristics similar to those of the parent isomers. However, because of increased steric crowding in the 11-cis isomers, their UV–vis absorption spectra show a substantial change with the 360-nm main band decreasing in intensity and a concurrent increase in intensity of a band near 250–280 nm (see Figure 1). This observation is reminiscent of that reported for 9-*cis*,11-*cis*,13-*cis*-retinal

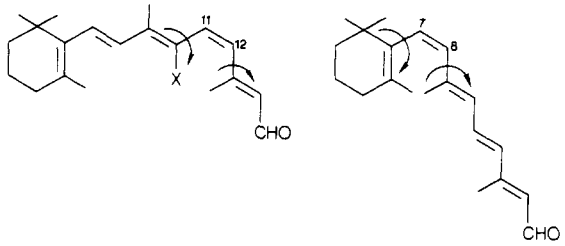
Table V: Calculated Dihedral Angles (deg) in 11-*cis*-Retinal and Its 10-Substituted Analogues[a]

	single-bond twist[b]					double-bond twist				
compd	6,7	8,9	10,11	12,13	14,15	5,6	7,8	9,10	11,12	13,14
11-*cis*-Ret	54.8	−160.8	−173.0	−147.4	177.9	−173.6	178.2	−175.8	12.2	−179.5
10-F-11-*cis*-Ret	55.5	164.1	−166.3	−148.0	178.1	−173.6	179.1	−176.2	14.0	−178.9
10-Cl-11-*cis*-Ret	55.3	160.4	−152.4	−150.9	178.5	−173.5	178.6	−176.8	17.3	−177.8
10-Me-11-*cis*-Ret	54.9	157.5	−152.9	−153.8	179.7	−173.4	178.3	−174.0	17.2	−177.6
10-Et-11-*cis*-Ret	58.0	167.5	−141.9	−155.0	178.8	−173.2	176.1	−173.5	15.6	−179.5
7-*cis*-Ret	53.6	−152.6	177.7	162.5	−174.6	8.6	−179.7	−179.2	−174.2	−174.0

[a] Data based on MMP1 calculations and structural and data manipulation via the CHEMGRAF program. [b] For this study, only structures with 12-s-trans conformation were calculated.

(Knudsen et al., 1980; Chandraratna et al., 1984).

It is tempting to interpret the result in terms of increased twisting of the 12,13 bond in these hindered 11-cis isomers from that already known to be present in the corresponding parent isomer (Gilardi et al., 1972; Honig & Karplus, 1971). However, MMP1 calculations (Table V) suggest a possible alternative explanation. It appears that the more hindered isomers (10-alkyl and 10-Cl) prefer to assume a conformation by twisting at both the 10,11 and the 12,13 single bonds. Such



a doubly twisted structure is consistent with the notion of a minimization of steric crowding and at the same time a maximization of resonance stabilization. This interpretation in fact parallels the conformation of the severely crowded 7-cis isomers where, in addition to twisting of the 6,7 bond, a substantial twist of the 8,9 bond was detected (Matsumoto et al., 1980b; Liu et al., 1983). The calculated conformation for the 7-*cis*-retinal (Table V) enhances that interpretation. Therefore, it appears the two-bond twist may be a general phenomenon for the more hindered isomers of retinal.[1]

Pigment Analogues. In contrast to the free retinal isomers, the main band of the analogues remains in the usual 460–500-nm region. There is however a possible decrease of the extinction coefficients of all analogues. For the only case (10-fluororhodopsin) where ϵ has been determined, the extinction coefficient for this analogue with a relatively small substituent (X = F) is 30400, approximately 75% of that found in rhodopsin (X = H). Hence, the yield data calculated on the basis of the assumption that the extinction coefficients for the pigments have a value of 30000 (hindered isomer) or 40000 (unhindered) (Table IV) should be considered as qualitative. Nevertheless, some trends are noteworthy.

With the exception of the more sterically crowded 11-*cis*-10-ethylretinal, the yields of pigment analogues from the isomers of the 10-substituted retinals generally follow the trend of those of the parent retinal (Liu et al., 1984b). Therefore, in the 10-fluoro series the 11-cis and the 9-cis isomers gave the highest pigment yields while those containing the 7-cis geometry gave a lower pigment yield.

Interestingly the 11-cis,13-cis isomer of 10-fluororetinal is sufficiently stable at room temperature for binding studies.[2]

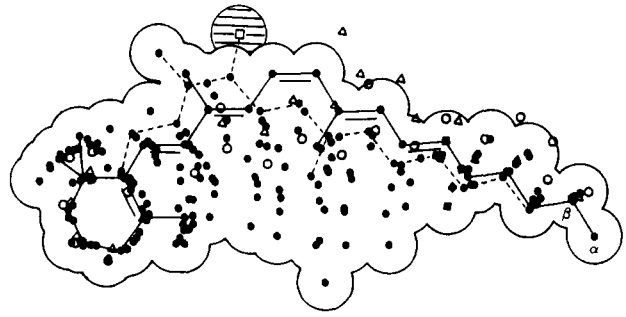


FIGURE 3: Two-dimensional projection map of the binding site of rhodopsin modified from that of Liu et al. (1984a). The α-carbon has now been rigidly fixed in order to minimize the pivotal motion of the β-atom. The dots correspond to the position of the carbon atoms in all active isomers of anchored retinyl–butyl Schiff bases. Those for the 11-cis and 9-cis isomers are connected respectively by the dashed and solid lines. The triangles and circles correspond to the atoms for the unreactive 13-cis and all-trans isomers. The solid squares are for the nitrogen atoms, and the empty square is the carbon atom for the 10-substituent, in, e.g., 10-methyl-9-*cis*-retinal. The shaded area is the area corresponding to the van der Waal radii of the added atom. It is also the proposed expanded area of the binding site.

That a pigment was formed with an absorption maximum different from all other isomers safely indicates the formation of a new pigment analogue. This result suggests that the pigment obtained in low yield during interaction of the parent 11-*cis*,13-*cis*-retinal isomer with bovine opsin (Hubbard & Wald, 1952–1953) could also be due to a unique isomeric pigment analogue. (See a parallel case in 9-*cis*,13-*cis*-retinal; Crouch et al., 1975.)[3]

For the 10-ethyl series, while the pigment yields were found to be generally low, there is a slight preference for the 9-cis rather than the 11-cis geometry. Possible implications will be discussed below.

Binding Site Map. The two-dimensional binding site map disclosed earlier (Liu et al., 1984a) and reproduced in Figure 3 was constructed on the basis of the assumption that all 10 isomeric rhodopsins (Liu et al., 1984b) share the common hydrophobic pocket in opsin in addition to their attachment to Lys-296 through a protonated Schiff-base linkage (Liu & Asato, 1985). These limitations represent an extended and modified view of the existence of a longitudinal restriction for the visual chromophore (Matsumoto & Yoshizawa, 1978; Matsumoto et al., 1980b). After further pivotal freedom of motion of the trimethylcyclohexenyl ring within the hydrophobic pocket was allowed, all isomeric rhodopsin analogues were constructed. The summation of the positions of the carbon atoms in these isomers defines the two-dimensional

[1] Attempts are under way to generalize the current MMP1 calculation to include all other conformations. The relative importance of the 12-s-trans and 12-s-cis conformations could be of interest for discussion of properties of free molecules.

[2] The same isomer for the parent retinal was reported to rearrange slowly to 13-*cis*-retinal (Wald et al., 1955).

[3] The 9-cis,11-cis,13-cis isomer of 13-demethylretinal is also stable and forms a pigment analogue with a λ_{max} at 485 nm (M. Denny and R. S. H. Liu, unpublished results).

projection of the binding site, and the van der Waal radii of the outermost carbons in this collection of atoms define the perimeter of this area.

Considering conformation properties of molecules, we believe that it is unlikely a cavity shown in Figure 3 exists in opsin at any instant. Rather, the area reflects the maximum extent that the side chains of the amino acid residues comprising the binding site can yield through conformational reorganization during its interaction with the retinal isomers. It is also important to realize that binding interaction is a slow process. Unlike the rapid primary photochemical process, the protein backbone should, in principle, be capable of reorganization. However, on the basis of the abundant results of analogue studies demonstrating selectivity of the binding site [see, e.g., Balogh-Nair and Nakanishi (1982)] and the fact that isomeric rhodopsin has similar photobleaching intermediates as rhodopsin (Kawamura et al., 1980), we believe that the protein backbone in the pigment analogues cannot deviate to a significant extent from its conformation in rhodopsin.

In Figure 3, in addition to the carbon skeleton of the butyl Schiff base of 11-*cis*-retinal,[4] that of the 10-substituted 9-cis Schiff base is also shown. It is interesting to note that while the 10-substituent of the 11-cis isomer remains in the active zone, that of the 9-cis isomer (e.g., 10-methyl) lies beyond the perimeter of the previously defined binding site. On the other hand, the data in Table IV show that the 9-cis isomers are equally reactive as the 11-cis isomers. The reason for this apparent discrepancy lies in the fact that the binding site perimeter determined by this method was limited by the carbon atoms available in the active isomers of retinal. Hence, while it is suggestive of the shape of the binding site, it does not necessarily exclude local regions not populated by any atoms in the retinal isomers. Therefore, the data from the 10-substituted series suggest that the perimeter near carbon 10 should in fact be expanded by including the shaded area as shown. It will be of interest to follow the current analyses to examine whether the perimeter should be expanded in other regions of the map.

It is interesting to compare the current results with those reported earlier for the 12-substituted retinals (Liu et al., 1984a). In the latter case, incubation studies showed low activities of the 11-cis isomers and normal activities for the 9-cis isomers. This trend was considered to be consistent with the presence of a relatively rigid protein wall (more specifically, the counterion) near carbon 12 (Liu et al., 1984a), which rejects any substituent at this position. The 11-cis isomer of 10-ethylretinal gave negligible amount of pigments while the yield for the 9-cis isomer is noticeably higher. The result cannot be accounted for by the location of the counterion. In fact, it is not predicted by the two-dimensional projection map. We suspect the negative result indicates possible restrictions of the binding site in the third dimension, which probably cannot readily accommodate a highly twisted (whether singly or doubly) chromophore into the cavity defined by the side chains of the amino acid residues surrounding the middle portion of the polyene chromophore. The 9-cis isomers with a planar chromophore due to the substituent orienting itself away from the polyene chain will not have such difficulties. That a small amount of pigment was formed for the 9-cis isomer of 10-ethylretinal, in turn, suggests a cavity even slightly more flexible than that implied by the shaded area in Figure 3.

In the same vein, the reported high pigment yield of 14-methyl-11-*cis*-retinal (Chan et al., 1974) at first does not seem to be compatible with the binding site map. However, the twisted conformation of the 12,13 bond in an 11-cis isomer coupled with the fact that the carboxylate counteranion should orient itself perpendicularly to the polyene chromophore (Hargrave et al., 1984) possibly allows the extra 14-methyl group to fit in a space underneath the carboxyl carbon. Such an arrangement unfortunately is not revealed in a two-dimensional map.

The restrictions in the third dimension of the binding site as discussed above are not immediately obvious from a recently postulated three-dimensional model of opsin (Hargrave et al., 1984). It is our hope that a combination of experiments and theory (such as molecular modeling) will eventually lead to a more specific three-dimensional shape of the binding site of opsin.

[4] In the figure, a 12-s-trans conformation is assumed even though the 12-s-cis conformation is known to be present in the crystal structure of 11-*cis*-retinal (Gilardi et al., 1972). This is based on recent spectroscopic evidence (Callender et al., 1976) and chemical evidence (Matsumoto et al., 1980b) that the 12-s-trans conformer is favored in rhodopsin.

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Registry No. $ClCH{=}PPh_3$, 29949-92-6; $MeCH(TMS)CO_2Et$, 13950-55-5; $EtCH(TMS)CO_2Et$, 14782-43-5; C_{14}-chlorotriene, 17081-79-7; 9-*cis*-10-chlorotriene C_{15}-aldehyde, 104423-90-7; *all-trans*-10-chlorotriene C_{15}-aldehyde, 104423-91-8; *all-trans*-10-chlororetinal, 18549-13-8; 13-*cis*-10-chlororetinal, 104486-37-5; 11-*cis*-10-chlororetinal, 104486-38-6; 9-*cis*-10-chlororetinal, 17169-64-1; 7-*cis*-10-chlororetinal, 104486-39-7; 11-*cis*,13-*cis*-10-chlororetinal, 104486-40-0; *all-trans*-10-methylretinal, 72648-51-2; 13-*cis*-10-methylretinal, 104527-19-7; 11-*cis*-10-methylretinal, 104527-20-0; 9-*cis*-10-methylretinal, 89576-05-6; 7-*cis*-10-methylretinal, 104527-21-1; 9-*cis*,13-*cis*-10-methylretinal, 104527-22-2; *all-trans*-10-ethylretinal, 104527-27-7; 13-*cis*-10-ethylretinal, 104527-28-8; 11-*cis*-10-ethylretinal, 104423-89-4; 9-*cis*-10-ethylretinal, 104486-41-1; 7-*cis*-10-ethylretinal, 104486-42-2; 9-*cis*,13-*cis*-10-ethylretinal, 104486-43-3; *all-trans*-10-fluororetinal, 68178-28-9; 13-*cis*-10-fluororetinal, 68200-18-0; 11-*cis*-10-fluororetinal, 68200-16-8; 9-*cis*-10-fluororetinal, 68200-15-7; 7-*cis*-10-fluororetinal, 68200-14-6; 9-*cis*,13-*cis*-10-fluororetinal, 68200-19-1; 7-*cis*,9-*cis*-10-fluororetinal, 68200-17-9; 11-*cis*,13-*cis*-10-fluororetinal, 104486-44-4; β-ionone, 79-77-6.

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Photosensitivity of 10-Substituted Visual Pigment Analogues: Detection of a Specific Secondary Opsin-Retinal Interaction†

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ABSTRACT: The photosensitivities of the bovine rhodopsin and gecko pigment 521 analogues regenerated from C-10-substituted analogues of 11-*cis*- and 9-*cis*-retinals were determined by two different methods. A similar reactivity trend was noted for both pigment systems as revealed in the photosensitivity of the gecko pigments and relative quantum yields of the bovine analogues. The 10-fluoro-11-cis photopigments had a photosensitivity less than, but approaching, that of the native (11-cis) visual pigment while the 10-fluoro-9-cis photopigments had a much lower photosensitivity than the parent 9-cis regenerated pigment. The results are interpreted in terms of recently described models of rhodopsin architecture and of the primary molecular reaction of visual pigments to light. The unusually low photoreactivity of the 10-fluoro-9-cis pigment molecule is viewed as the result of a regiospecific hydrogen-bonding interaction of the electronegative fluorine atom to the opsin.

In the preceding paper (Asato et al., 1986) we described the preparation and spectroscopic properties of a series of 10-substituted bovine rhodopsin analogues. Because of the observation, in a preliminary experiment, of the unusually inefficient photobleaching process of 10-fluoro-9-*cis*-rhodopsin, we decided to carry out a more quantitative study of such processes in this and related analogues. Furthermore, in order to test for the generality of such an observation, a parallel study with the gecko pigment analogues has been carried out. The results will be analyzed in light of the current knowledge of the tertiary structure of rhodopsin (Hargrave et al., 1984) and the postulated models for the primary process of vision.

Experimental Procedures

The photosensitivity of the regenerated photopigments was measured by two different procedures. At UCLA, the chromophore sensitivity of the gecko pigments regenerated with the opsin of pigment 521 was determined by the method of

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