ELSEVIER

Biophysical Chemistry 56 (1995) 31–39

Biophysical Chemistry

Effect of modification of the chromophore in retinochrome

Noriko Sekiya, Kazuo Yoshihara *

Suntory Institute for Bioorganic Research, 1-1, Wakayamadai, Shimamotocho, Osaka 618, Japan

Abstract

14-Methyl-, 14-chloro-, 14-fluoro-retinals have been incorporated into apo-retinochrome to investigate the effect of the electronegativities of C-14 substituents on the protonation of the Schiff base. The extent of protonation decreased in going from 14-methyl- to 14-chloro- to 14-fluoro-retinal. There was usually no protonation in the case of the 14-fluoro analogue. The chromophore–protein interactions are also discussed.

Keywords: Retinochrome analogues; 14-Substituted retinals; Non-protonated Schiff base; Counterion; Electronegativity; Rhodopsin; Bacteriorhodopsin

1. Introduction

Retinochrome was found by Hara and Hara [1] in 1965 as the second photopigment in the retina of the squid, *Ommastrephes sloani pacificus*. Numerous biological and physiological studies [2,3] on this protein have been reported mainly by Hara and coworkers. Retinochrome in membranes of myeloid bodies is mostly located in the inner segments of the visual cells [4], while another retinal protein, cephalopod rhodopsin [5,6], is located in rabdomal membranes of the outer segments. Retinochrome has an all-*trans*-retinal Schiff base chromophore linked to Lys-275 of the protein [2,8,9]. Although the Schiff base is protonated by supposedly an acidic protein residue as in other retinal proteins, the counterion of the Schiff base has as yet not been determined. The all-*trans* chromophore of retinochrome is photochemically isomerized to the 11-*cis*-isomer [7] in the photoproduct, meta-retinochrome (Fig. 1). Similar to vertebrate rhodopsins, cephalopod rhodopsin [4,6] has an 11-*cis*-retinal chromophore which is photoisomerized to the all-*trans*-isomer [7] in meta-rhodopsin. These complementary isomerizations of the chromophores in rhodopsin and retinochrome (Fig. 1), together with the existence of a retinal binding protein [10] which is capable of transporting retinal between these proteins, indicate that the physiological function of retinochrome is to isomerize all-*trans*-retinal to 11-*cis*-retinal, which can then be used as the chromophore in rhodopsin [11].

Of the all-*trans*-retinal analogues studied so far, retinochrome appears to isomerize all analogues to the 11-*cis*-isomer [12]. Structural studies of retinochrome and rhodopsin are helpful for understanding the mechanism by which these retinal proteins achieve such a high degree of selectivity in the all-*trans* to 11-*cis* or 11-*cis* to all-*trans* isomerizations.

In the low temperature studies of the photolytic process of retinochrome (λ_{max} 496 nm), the intermediates prelumiretinochrome (λ_{max} 465 nm) [13], lu-

* Corresponding author.

SSDI 0301-4622(95)00012-7

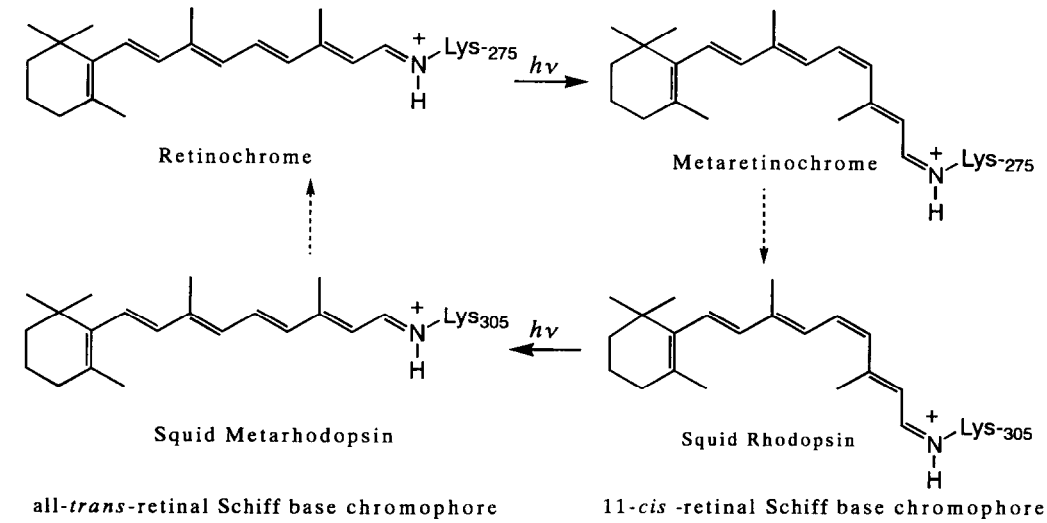

Fig. 1. Photoisomerization of retinal chromophores in retinochrome and squid rhodopsin.

miretinochrome (λ_{max} 475 nm) [14], and meta-retinochrome (λ_{max} 470 nm) [14] were trapped at 25, 83 and 273 K, respectively. The prelumi- and lumiretinochrome were also detected in pico- and millisecond time-resolved measurements, respectively [15]. FT-IR difference spectroscopic studies [16,17] of retinochrome supported the 11-*cis* geometry of the chromophore in the photointermediates. The spectra also showed hydrophobic to hydrophilic change in the environment of an unidentified carboxylic acid residue in the protein during the thermal relaxation process, retinochrome via lumi to meta.

Another approach for structural investigation on retinal proteins is the replacement of the native chromophore with artificial retinals. A series of dihydroretinal chromophores in rhodopsin and bacteriorhodopsin revealed that the positive charge of the Schiff base is delocalized over the chromophores, and is stabilized by electrostatic interactions with charged and/or uncharged residues of the proteins [18,19]. Another series of alkylated and halogenated analogues were used to estimate the shape and size of the chromophore cavity in rhodopsin [20].

Our incorporation studies of all-*trans*-10-, 12-, and 14-fluorinated (F-) retinals (Figs. 2 (2–4) into apo-retinochrome [21] revealed that only the 14-fluorinated (14-F-) chromophore gave a pigment with spectroscopic properties differing from those of the

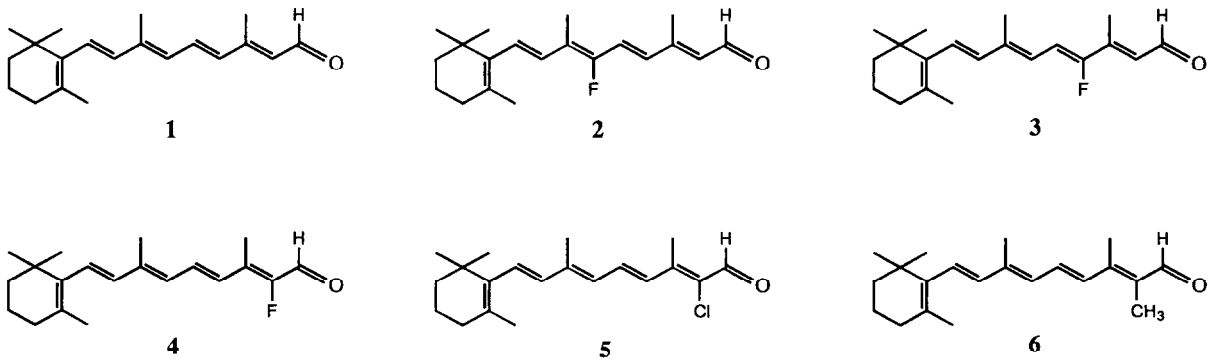

Fig. 2. (1) All-*trans*-retinal and the analogues incorporated into apo-retinochrome: (2) 10-F-, (3) 12-F-, (4) 14-F-, (5) 14-Cl-, and (6) 14-Me-retinals.

native pigment. The result seemed to suggest that this 14-F-retinochrome was apparently an equilibrium mixture between two species: a major species with a non-protonated Schiff base and a minor retinochrome-like species with a protonated Schiff base. A possible explanation for the formation of the non-protonated species is that an electrostatic interaction of the fluorine atom with a protein residue produces an environment less favorable for protonation from an acidic residue within the binding site.

This paper describes results of incorporation of 14-fluoro-, 14-chloro (Cl)- and 14-methyl- (Me) retinals (5 and 6) in apo-retinochrome to investigate the effect of 14-substitution.

2. Materials and methods

All experiments were carried out under dim red light unless otherwise described. UV and visible absorption spectra were obtained using microcuvetts (1 cm path length) and a Shimadzu 2200 spectrometer (Kyoto, Japan). Aporetinochrome was used as reference. Circular dichroism (CD) was measured with a JASCO J-600 spectropolarimeter (Tokyo, Japan).

2.1. Materials

All-*trans*-retinal (1) was purchased from Sigma. All-*trans*-14-Cl- and Me-retinals (5 and 6) were prepared by the Honer–Emmons reaction of a C-18 ketone (8-(2,6,6-trimethyl-1-cyclohexenyl)-3,5,7-octatrien-2-one) with triethylphosphonochloroacetate and triethylphosphono-2-propionate, respectively, followed by diisobutylaluminum hydride reduction and MnO_2 oxidation. Structures of the synthetic compounds were confirmed in comparison of the ^{1}H-NMR data with the published data. Mixtures of

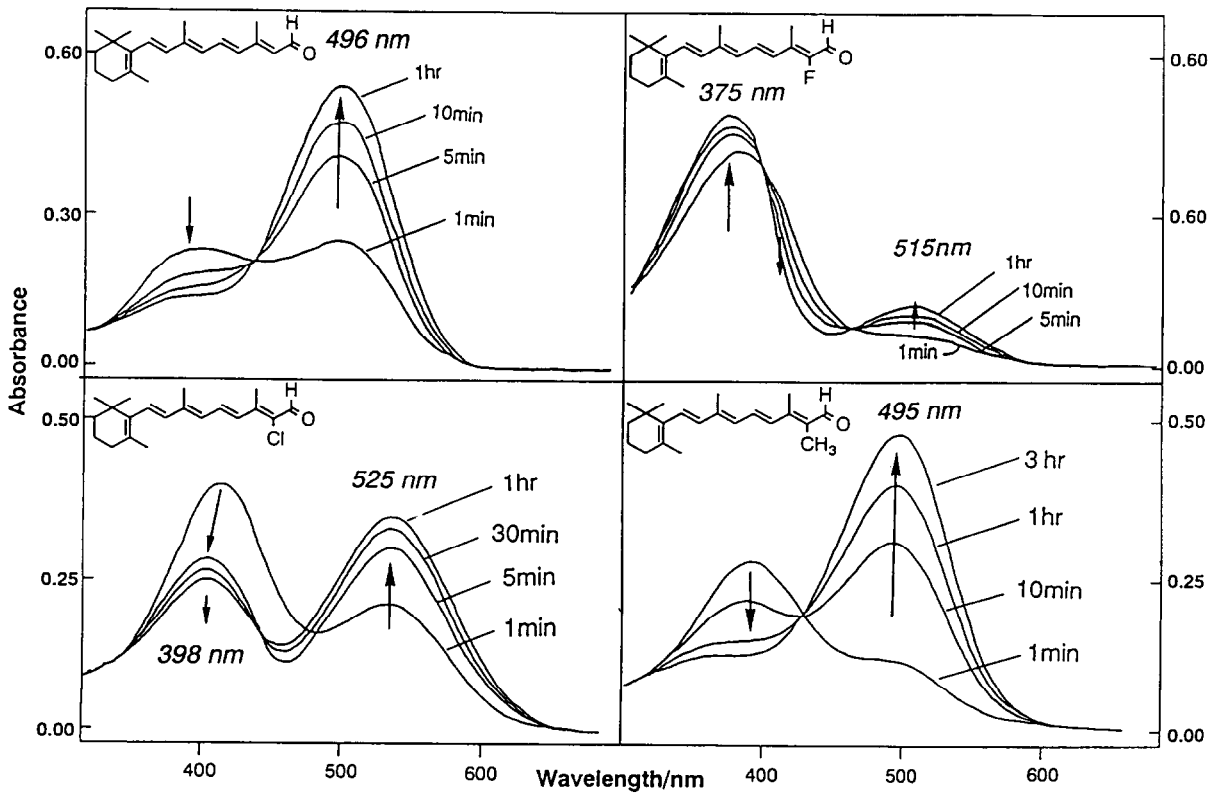

Fig. 3. Formation of retinochrome, 14-F-, 14-Cl- and 14-Me-retinochrome at pH 6.5.

the geometrical isomers of retinal and of the analogues for HPLC analysis of the chromophores were prepared by photoisomerization of the all-*trans* aldehydes in CH_3CN. All-*trans*-retinal and the analogues were purified on preparative SiO_2 HPLC (column: Cosmosil Si-60, ID 10 mm × 300 mm, solvent: 10% diethyl ether in hexane, flow rate: 6 ml/min) just before the incorporation experiments.

Membrane suspensions containing retinochrome and apo-retinochrome, and their digitonin solutions (pH 6.5, 67 mM phosphate buffer) were prepared from the retina of squid, *Todarodes pacificus*, according to the reported method [10,21]. The final concentration of the retinochrome was determined to be ca. 8.3 μM (calculated from its O.D. ca. 0.5 at 498 nm and the ϵ_{max} 6,0700 [3].

2.2. Regeneration experiments of retinochrome and the 14-substituted retinochrome analogues.

Ethanol solutions (2 μl) of all-*trans*-retinal and the all-*trans*-retinal analogues (2 mM) were added to apo-protein solutions (0.5 ml) or to the apo-membrane suspensions (0.5 ml) in the cuvettes at 25° C, and were mixed thoroughly. The formation of retinochrome and analogue pigments was monitored by visible absorption spectra. Although regeneration experiments with apo-retinochrome in solution and in membrane suspension yielded essentially the same results, only the spectra of the solution samples are shown because of the better quality. Retinochrome regenerated from all-*trans*-retinal showed virtually identical properties with native retinochrome.

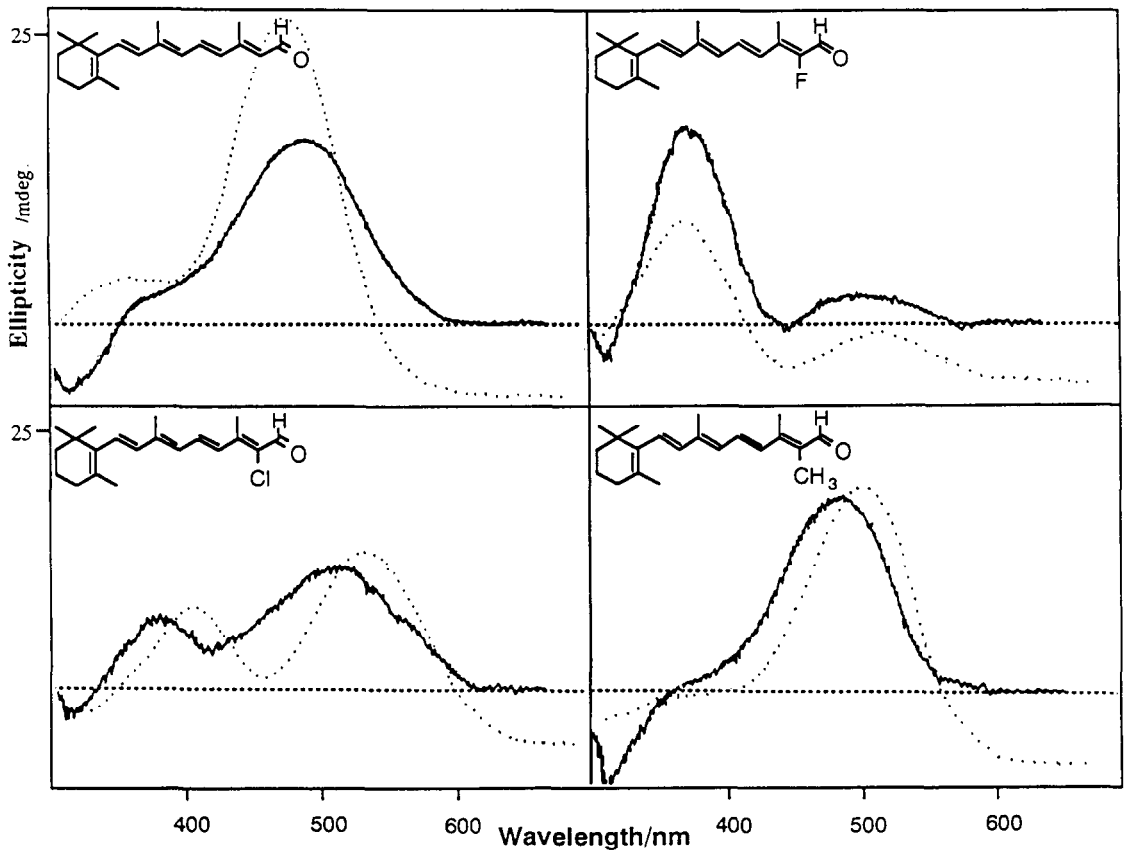

Fig. 4. Circular dichroism (solid lines) and absorption spectra (dotted lines) of retinochrome and the apparent steady state mixtures of 14-F-, 14-Cl- and 14-Me-retinochrome.

Photoreaction of the retinochrome analogues. The regenerated analogues in the cuvettes were irradiated for 3 min by light from a 250 W halogen light source (Unisoku, Japan) passed through a combination of an interference (550 nm for 14-Me- and 570 nm (for 14-Cl-analogues) and a 480 nm high pass filter (KL550, KL570, and Y48, respectively, Toshiba).

2.3. HPLC analysis of chromophores of the analogue proteins.

Methanol (0.5 ml), CH_2Cl_2 (1 ml), and hexane (5 ml) were added successively under vortex mixing to retinochrome and its analogues in the solution or suspension before and after irradiation. After passing the organic layer through an anhydrous Na_2SO_4 column and the removal of solvents, the re-dissolved samples (1 ml hexane) were analyzed by the HPLC mentioned above.

3. Results and discussion

3.1. Regeneration of the retinochrome analogues

In Fig. 3, spectra of regeneration process of retinochrome and the analogue pigments from all-*trans*-retinal, all-*trans*-14-Cl-, and 14-Me-retinals in apo-retinochrome, together with those of all-*trans*-14-F-retinal are shown. During the incorporation process of 14-Cl-retinal into apo-retinochrome, although a retinochrome-like pigment (λ_{max} at 525 nm) was formed after 1 min of mixing, a blue-shift from a 410 nm band (after 1 min of mixing) to a 398 nm band (after 5 min of mixing) was observed. Then a partial conversion of the 398 nm band to the 525 nm band was observed in the following process. 14-Cl-retinal in ethanol has λ_{max} at 398 nm. The 410 nm band in the spectrum after 1 min of mixing seems to be a mixture of the blue-shifted 398 nm band and the band of the chromophore which has no Schiff base linkage with the intact lysine residue in the sample. The CD spectrum of the steady state mixture (Fig. 4) shows positive bands corresponding to the 398 and 525 nm bands.

Incorporation of 14-F-retinal into apo-retinochrome [21] mainly gave a pigment with a blue-shifted λ_{max} at 375 nm (Fig. 3). The 375 nm 14-F-retinochrome was attributed to a species having non-protonated Schiff base linkage for the following reasons: (1) The stability of the band to bovine serum albumin [22] or hexane treatment suggests a covalent bond of the chromophore to the protein, (2) non-protonated Schiff base formation of retinal and the analogues results in a blue-shift of the λ_{max}s, and (3) a positive CD band in the region similar to that for native retinochrome (Fig. 4) suggests that the chromophore in the 375 nm species is linked to the protein in a manner similar to that in retinochrome, thus excluding the possibility of random Schiff base formation in the protein.

The blue-shifted 398 nm band for the 14-Cl analogue (Fig. 3), which was also stable to the same treatments and showed positive CD bands, may be attributed to a species having a non-protonated Schiff base chromophore. An isosbestic point at 430 nm in the spectra may indicate protonation of the Schiff base in the following process; however, the 398 nm species was not completely converted to the 525 nm species in the steady state spectrum, suggesting an equilibrium between those two species. The rate

Table 1
Schiff base form and physical properties for the 14-substituents

14-Substituents	F	Cl	CH_3	H [a]
Schiff bases [b]	NSB ≫ PSB	NSB < PSB	PSB	PSB
Electronegativity [c]	4.0	3.0	2.5	2.1
r_v [d]/C–X [e] (Å^{-1})	1.35/1.317	1.77/1.776	-/-	1.21/1.09

[a] 14-H for native retinochrome.
[b] NSB: non-protonated Schiff base; PSB: protonated Schiff base.
[c] Cited from [30].
[d] van der Waals radii.
[e] Bond distances to carbon atoms.

determining step in this case may be the protonation reaction, while that in the regeneration of 14-F-retinochrome seems to be the Schiff base formation. The incorporation result suggests that chlorine substitution at the C-14 position also gives rise to the formation of a species with a non-protonated Schiff base. However the ratio of non-protonated species to protonated species is smaller than that in the case of fluorine substitution.

Although incubation of 14-Me-retinal with apo-retinochrome yielded a retinochrome-like pigment (λ_{max} at 495 nm), the regeneration of this pigment proceeded slower than others. In this case, no blue-shifted band in the near UV region was observed. Methyl substitution at the C-14 position did not prevent protonation of the Schiff base, thus resulting in the formation of the protonated Schiff base species.

In Table 1, forms of the Schiff base in the analogue pigments, electronegativities, van der Waals radii, and bond distances with a carbon atom for the C-14 substituents are shown. The most electronegative fluorine atom at the C-14 position produced mainly non-protonated species. The less electronegative chlorine atom produced more protonated species, whereas the methyl group and hydrogen atom (native retinochrome) produced only protonated species.

These results indicate that electronegativity of the C-14 substituents is closely related to the protonation/non-protonation of the Schiff base function. The involvement of steric factors can be excluded because the chlorine and the methyl group which are bulkier than the fluorine atom produce more and fully protonated species, respectively. The slow formation of 14-Me-retinochrome may reflect a rather tight protein environment for the bulky methyl group.

Halogen substitution in organic molecules electrically influences the periphery in two manners: a through-bond electron withdrawing effect and a through-space electrostatic effect. A typical example is the change in pK_a value of acetic acid. Chloro- and fluoroacetic acids (pK_a 2.85 and 2.66, respectively) are more acidic than acetic acid (pK_a 4.8). In retinal analogues, a change of the Schiff base pK_a value (ΔpK_a) caused by introduction of three fluorine atoms to C-20 position has been reported [23]. The pK_a values of 20,20,20-trifluororetinal Schiff bases are much lower than those of retinal Schiff bases in both model Schiff base with n-butylamine (pK_a 1.8 vs. 7.4) and in bacteriorhodopsin (8.0 vs. 13.3). Such ΔpK_a's were explained by a destabilization of the positive charge on the Schiff base due to the electron withdrawing character of the substituents. The pK_a change in the model Schiff base (-5.6) was similar to that in the protein (-5.3). In either case the mixed mode of the through-bond and the through-space effects appears to be contributing to the changes in the pK_a values.

Photoreactions of rhodopsin analogues containing 11-*cis*-10-, 12-, and 14-F-retinal chromophores [20] at $-191°$ C gave bathorhodopsin (all-*trans*-chromophore) like native rhodopsin. Similar to native bathorhodopsin, photoreactions of 12- and 14-F-bathorhodopsin led to mixtures of F-rhodopsin and 9-*cis*-F-rhodopsin. In contrast, 10-F-bathorhodopsin was not converted to 9-*cis*-10-F-rhodopsin but instead reverted to 10-F-rhodopsin. This different photochemical behavior of 10-F-rhodopsin was accounted for by electrostatic interaction between the highly electronegative fluorine atom and an ionic group of the protein. In the incorporation studies of retinal analogues into bacterio-opsin, [24] an unusually red-shifted species (λ_{max} 680 nm) with all-*trans*-chromophore was detected during both the regeneration process of 14-F-bacteriorhodopsin and its photoreaction process. Incorporation of 14-Cl-retinal led to the formation of an apparent equilibrium mixture of a major fraction of 440 nm pigment and a small fraction of red-shifted 691 nm pigment. The formation of the red-shifted species was explained by inhibition of protonation from the native counterion due to the electrostatic interaction between the halogen atoms and protein moiety, possibly the native counterion; this then leads to protonation from another proton source located at a site remote from the fluorine substituent.

In retinochrome, two possibilities for the action of the halogen substituents on the non-protonation of the Schiff bases can be pointed out: a decrease of the Schiff base pK_a due to the mixed mode of the through-space and through-bond effect of the 14-substituents, and furthermore, the through-space effect of the halogen atoms on the protein moiety.

Retinal and 14-F-retinal Schiff bases with *N*-Boc-*O*-methyllysin in 50% aqueous ethanol showed apparent pK_as at 5.0 and 4.2, respectively, the difference due to 14-F ($\Delta pK_{a\ 14F}$) being 0.8. However,

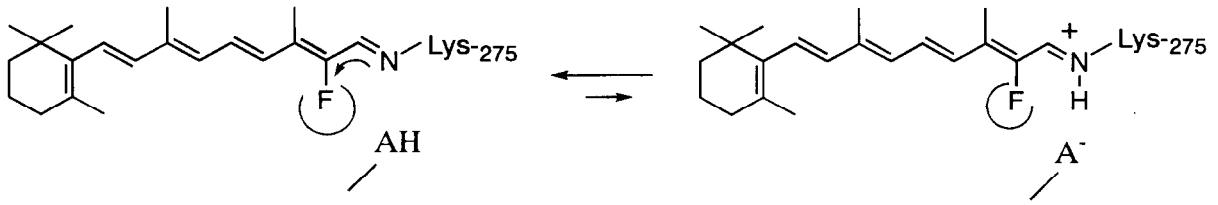

Fig. 5. A possible explanation for equilibrium between non-protonated and protonated Schiff bases. The C-14 fluorine atom exhibits a through-bond electron-withdrawing effect and a through-space electrostatic effect.

$\Delta pK_{a\ 14F}$ in a model Schiff base with n-butylamine in methanol appeared to be 2.4 in a recent report [25]. This difference can be ascribed to a difference in the bases and the solvents. The pK_a of the retinochrome Schiff base can be estimated to be ca. 10 from the absorption spectrum for retinochrome at pH 10.1 [4,5]. If similar effects on pK_a changes both in the model Schiff base and in the proteins, like in the trifluorobacteriorhodopsin analogues, can be expected, even the $\Delta pK_{a\ 14F}$ 2.4, as such, does not produce non-protonated Schiff base species in retinochrome at pH 6.5. The through-space effect to the protein moiety must contribute to the non-protonation in the retinochrome analogues. For the interaction, the 14-substituents and the counterion necessarily have to be closely located; the counterion is located in the same side of C14–X bond as shown in Fig. 5.

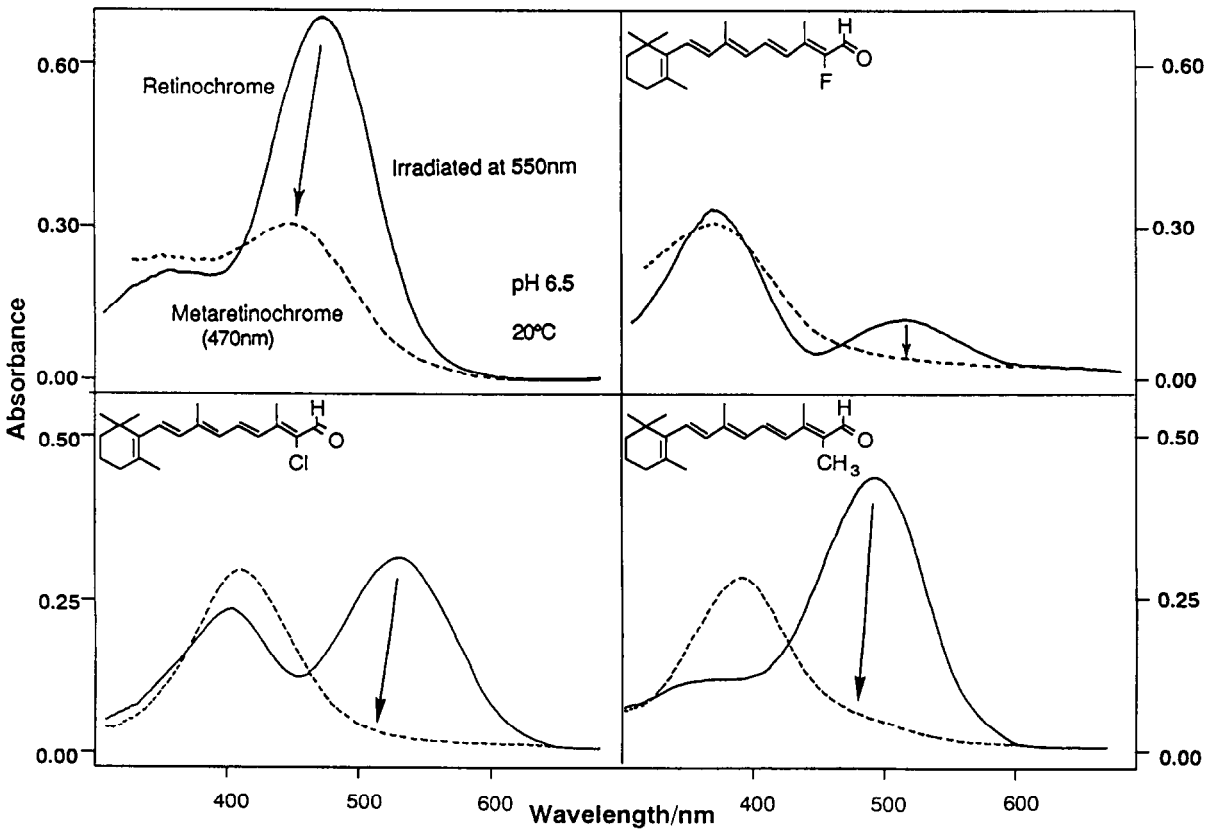

Fig. 6. Spectra of retinochrome and retinochrome analogues before (solid lines) and after (dotted lines) the photoreactions.

3.2. Photoreactions

Photoreaction of 14-Cl- and 14-Me-retinochromes did not give meta-products, thus giving rise to complete photobleaching of the pigments. Irradiation of 10-F-retinochrome formed the meta-product, while 12- and 14-F analogues were photobleached. HPLC analysis of the chromophores showed 11-*cis* geometry in all photoproducts.

The specific double bond isomerization suggests that the Schiff base cleavage occurred after isomerization. The bond cleavage is facilitated by 12-F, 14-F, 14-Cl, and 14-Me groups in the 11-*cis*-chromophores. The bond cleavage in 12-F-, 14-F- and 14-Cl-retinochrome was considered to be a result of destabilization of the Schiff base bonding, which might be caused by a distortion due to the electrostatic interaction between the halogen substituents and the charged protein residues. In case of the 14-Me analogue, electrostatic interaction should not play the role. The photobleaching might be due to the bulky or hydrophobic property of the methyl group.

In summary, 14-F-retinal has been incorporated into the three retinal proteins, opsin, bacterioopsin, and apo-retinochrome. The rhodopsin analogue with 11-*cis*-14-F-chromophore exhibited virtually the same properties as the native pigment, while the other two pigment analogues having all-*trans*-chromophores, 14-F-bacteriorhodopsin and 14-F-retinochrome, exhibited characteristics different from the native pigments. These differences seem to be derived from the electrostatic effect of the fluorine atoms, suggesting a close location of the 14-fluorine atom and the counterion. This presumably reflects the relative location of the chromophores and the counterions in these pigments.

Site-directed mutation experiments suggested that the counterions of the Schiff bases in rhodopsin and bacteriorhodopsin were Glu-113 [26] and Asp-85 [27], respectively. Those are located in the extracellular side in the membranes. The conformation of the bacteriorhodopsin molecules including the chromophore has mostly been determined by neutron diffraction studies [28] and resonance Raman spectroscopy [29] (Fig. 7). The olefinic 19-methyl group

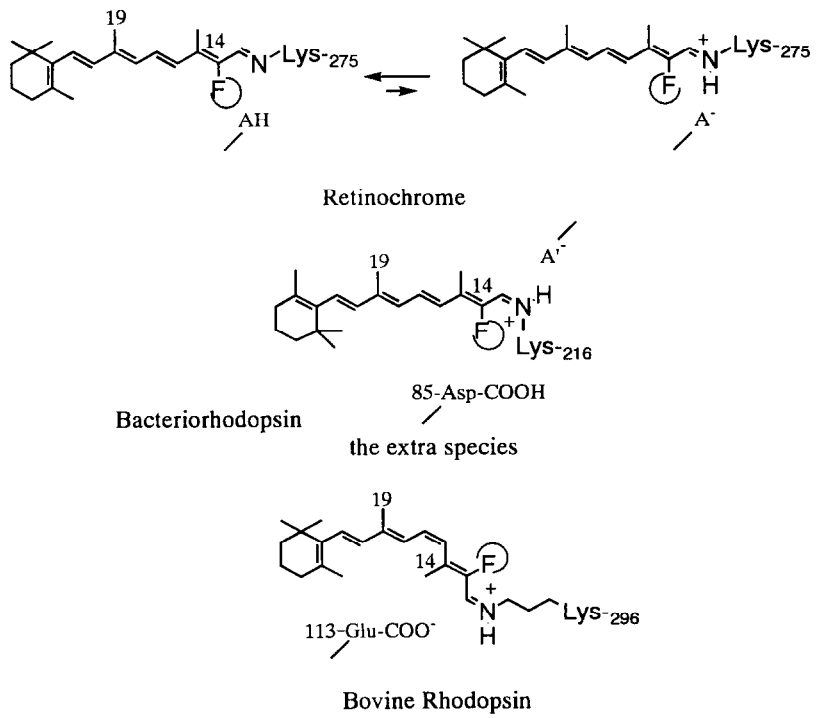

Fig. 7. A speculative view of the relation between the chromophores and the counterions in three retinal proteins.

on the side chain is considered to be directed to the cytoplasmic side of the membrane (direction of C-terminal). However, that in rhodopsin is still unclear and in retinochrome is completely unknown. If those in both proteins have the same orientation as in bacteriorhodopsin (Fig. 7), it is likely that (1) the counterions in these three proteins are located on the opposite (extracellular) side of the 19-methyls, and (2) the orientation of the 14-F's in the all-*trans* chromophores in bacteriorhodopsin and retinochrome with respect to the counterions is such that through-space interaction is feasible. In contrast, the chromophoric 14-F in rhodopsin is directed to the opposite side of the counterion and hence does not interact.

Acknowledgements

We are grateful to professor Koji Nakanishi for useful discussions and advices in preparation of the manuscript.

References

[1] T. Hara and R. Hara, Nature, 206 (1965) 1331.
[2] T. Hara and R. Hara, Biochemical properties of retinochrome, in H. Langer (Editor), Biochemistry and Physiology of Visual Pigments, Springer, Berlin, Heidelberg, New York, 1973, p.180.
[3] T. Hara and R. Hara, Methods Enzymol., 81 (1982) 827.
[4] T. Hara and R. Hara, Nature, 219 (1968) 450.
[5] T. Hara, R. Hara and J. Takeuchi, Nature, 214 (1967) 572.
[6] T. Hara and R. Hara, J. Gen. Physiol., 67 (1976) 791.
[7] T. Hara and R. Hara, Cephalopod retinochrome, in H.J.A. Dartnall (Editor), The Handbook of Sensory Physiology, Vol. VII/I, Springer, New York, 1972, p. 720.
[8] I. Hara-Nishimura, T. Matsumoto, H. Mori, M. Nishimura, R. Hara, and T. Hara, FEBS Lett., 271 (1990) 1.
[9] J. Uematsu, I. Hara-Nishimura, K. Wada, H. Matsubara and T. Hara, Photochem. Photophysiol., 13 (1986) 197.
[10] T. Terakita, R. Hara and T. Hara, Vision Res., 29 (1989) 639.
[11] T. Seki, J. Gen. Physiol., 84 (1984) 49.
[12] (a) K. Tsujimoto, K. Ohmura, M. Ohashi, R. Hara, T. Hara, K. Ozaki and M. Watanabe, Chem. Lett., (1986) 2005; (b) K. Tsujimoto, M. Aoki and M. Ohashi, J. Photochem. Photobiol., A 65 (1992) 73.
[13] F. Tokunaga, T. Watanabe, J. Uematsu, R. Hara and T. Hara, FEBS Lett., 262 (1990) 266.
[14] T. Hara, R. Hara, F. Tokunaga and T. Yoshizawa, Photochem. Photobiol., 33 (1981) 883.
[15] T. Kobayashi, K. Ogasawara, S. Koshihara, K. Ichimura and R. Hara, Photochemistry of retinochrome studied by nanosecond and picosecond spectroscopy, in T. Kobayashi (Editor), Primary Processes in Photobiology, Springer-Verlag, Berlin, 1986, p. 125.
[16] N. Sekiya, A. Kishigami, F. Tokunaga and K. Yoshihara, FEBS Lett., 280 (1991) 107.
[17] A. Kishigami, N. Sekiya, K. Yoshihara, R. Hara, T. Hara and F. Tokunaga, Photochem. Photobiol., 54 (1991) 993.
[18] Y. Koutalos, T.G. Ebrey, M. Tsuda, K. Odashima, T. Lien, M.H. Park, N. Shimizu, F. Derguini, K. Nakanishi, H.R. Gilson and B. Honig, Biochemistry, 28 (1989) 2732.
[19] J.L. Spudich, D.A. McCain, K. Nakanishi, M. Okabe, N. Shimizu, H. Rodman, B. Honig and R.A. Bogomolni, Biophys. J., 49 (1986) 479.
[20] (a) R.S.H. Liu and A.E. Asato, The binding site of opsin based on analog studies with isomeric, fluorinated, alkylated, and other modified retinals, in M.I. Dawson and W.H. Okamura (Editors), Chemistry and Biology of Synthetic Retinoids, CRC Press, New York, 1990, p 51; (b) A.E. Asato, H. Matsumoto, M. Denny and R.S.H. Liu, J. Am. Chem. Soc., 100 (1978) 5957; (c) R.S.H. Liu, H. Matsumoto, A.E. Asato, M. Denny, Y. Shichida, T. Yoshizawa and F.W. Dahlquist, J. Am. Chem. Soc., 103 (1981) 7195; (d) F. Crescitelli and R.S.H. Liu, Proc. R. Soc. Lond. B, Biol. Sci., 233 (1988) 55; (e) Y. Shichida, T. Ono, T. Yoshizawa, H. Matsumoto, A.E. Asato, J.P. Zingoni and R.S.H. Liu, Biochemistry, 75 (1987) 4422.
[21] N. Sekiya, A. Kishigami, F. Tokunaga and K. Yoshihara, Photochem. Photobiol., 56 (1992) 909.
[22] K. Hiraki, T. Hamanaka, K. Yoshihara, Y. Kito, Biochim. Biophys. Acta, 811 (1987) 177.
[23] M. Sheves, A. Albeck, N. Friedman and M. Ottolenghi, Proc. Natl. Acad. Sci. USA, 83 (1986) 3262.
[24] , M.E. Tierno, D. Mead, A.E. Asato, R.S.H. Liu, N. Sekiya, K. Yoshihara, C.-W. Chang, K. Nakanishi, R. Govindjee and T.G. Ebrey, Biochemistry, 29 (1990) 5948.
[25] G. Steinberg, M. Ottolenghi and M. Sheves, Biophys. J., 64 (1993) 1499.
[26] T.P. Sakmar, R.R. Franke and H.G. Khorana, Proc. Natl. Acad. Sci. USA, 86 (1989) 8309.
[27] S. Subramanian, T. Marti and G.H. Khorana, Proc. Natl. Acad. Sci. USA, 87 (1990) 1018.
[28] R. Henderson, J.M. Baldwin, T.A. Ceska, F. Zemlin, E. Beckmann and K.H. Downing, J. Mol. Biol., 213 (1990) 899.
[29] S.O. Smith, J. Lugtenburg and R.A.J. Mathies, Membrane Biol., 85 (1985) 95.
[30] R.D. Chambers, Fluorine in Organic Chemistry, John Wiley & Sons, 1973, p. 5.