^{19}F NMR and UV−Vis Absorption Spectroscopic Studies of Fluorinated Octopus Rhodopsin and Its Photoproducts

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Isomeric octopus pigment analogues from 9-cis and 11-cis 10-fluoro retinal have been prepared and their photochemical as well as other properties examined. While most of the properties of the fluorinated analogues remained similar to those of the parent system, the following differences were noted. Fluorine substitution at the 10-position increased the UV opsin shift (UV OS) values of the pigments and increased the fluorine opsin shift (FOS) compared to that in bovine rhodopsin. For the 9-cis 10-fluoro octopus pigment, a reduction in photosensitivity and in FOS were observed compared to that in bovine rhodopsin. These results suggest that in octopus rhodopsin the environment around the fluorine probe in the 11-cis chromophore is more sterically hindered compared to that in bovine, while the opposite is likely for the 9-cis.

Introduction

Visual pigment consists of 11-*cis*-retinal covalently bound to an apoprotein via a protonated Schiff base (PSB) linkage. The optimum wavelength of sensitivity in retinal proteins, including visual pigments and bacterial pigments, extends widely within the visible region (400−700 nm) from pigment to pigment, although the common chromophore, retinal, absorbs light only around 380 nm. Light isomerizes the 11-cis chromophore to the all-trans form, and the visual pigment proceeds to form a series of transient photointermediates, which initiates an enzymatic cascade of visual excitation.[1−4] Absorption maxima of these photointermediates differ from each other, although they contain the same chromophore and protein.[5,6] It is expected that the absorbance of the visual pigments and their photointermediates must arise from interactions between the opsin and its retinylidene chromophore.

A central unanswered question in visual pigments is how do retinal−opsin interactions regulate wavelength absorption. Retinal−opsin interactions were extensively studied by the modification of either component, specific protein modification as site-directed mutagenesis or specific retinal modification by organic synthesis. Artificial pigments from these modified components were studied by several spectroscopic methods to elucidate the chromophore−protein interactions, such as low-temperature spectrophotometry,[7,8] laser flash photolysis,[9] resonance Raman spectroscopy,[10] ^{13}C NMR,[11] and ^{19}F NMR.[12]

In bovine rhodopsin, the chromophoric PSB is neutralized by a charged counterion, Glu-113.[13,14] In the octopus rhodopsin binding site, it has been recently shown that the equivalent residue (Tyr-112) is protonated and, therefore, is not the charged counterion to the PSB.[15] The same result was earlier determined with Asp-91.[16] Hence, there seem to be no charged species near the chromophoric moiety in the binding pocket. In bovine rhodopsin, a charged counterion Glu-113 is suggested to be close to C-12, and the location of 10-F in the 9-cis configuration should be close to this region.[17]

In a study of chain fluorinated rhodopsin analogues, it was found that their properties are similar to the parent rhodopsin, except that the 9-cis 10-F pigment showed a marked reduction in photosensitivity.[8,18] With red light irradiation at −191 °C, its batho intermediate transformed only to the 11-cis isomer, whereas the batho intermediate of the parent rhodopsin, as well as that of the 11-cis 12-F pigment analogue, converted to a mixture of both 11-cis and 9-cis isomers. Furthermore, at −265 °C, the 9-cis 10-F pigment was practically photoinsensitive. For these altered photochemical properties, a regiospecific interaction between 10-F in the chromophore and the protein was suggested. The two postulated models for the specific fluorine−opsin interaction are hydrogen bonding between F and an acidic H of a neighboring amino acid residue and repulsive interaction between F and an electron dense atom of a proximal amino acid. At this point, it will be interesting to find out if the 9-cis 10-F octopus system will bear photochemical behavior similar to the 9-cis 10-F bovine and gecko 10-F systems.[18] Simultaneously, the F NMR chemical shift measurements will be carried out to detect these environmental changes. Since the fluorine atom is only slightly larger than hydrogen and highly electronegative, it is expected that the fluorine label might act as a sensitive probe for the chromophore−protein interaction.

Octopus rhodopsin has homology close to bovine rhodopsin. A number of residues are conserved including the ring-binding pocket of bovine rhodopsin, characterized by F-261, W-265,[19] and Y-268. The covalent linkage between the other end of the chromophore to the protein through K-296 (K-306 in octopus rhodopsin) is also conserved. Resonance Raman data[20] showed that the C=N bond vibrational mode for both pigments is the

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same. The configuration of the 11-*cis*-retinal chromophore in both systems is 11-cis-12-s-trans and the C=N is anti.[21] However, there are indications that the hydrogen bond between the PSB and counterion in the bovine system is stronger than in the octopus system. Substantial perturbations (compared to the bovine system) around its C11=C12−C13 bond were also detected.[21]

So far, most of the retinal analogue studies have been conducted on a vertebrate system, bovine rhodopsin. In contrast, far fewer studies have been conducted on the invertebrate rhodopsin system. Difficulties involved in the preparation of the pigment from retinal and invertebrate opsin limited the regeneration of invertebrate rhodopsin to octopus[22,23] and squid[24] systems.

In this paper, we report the preparation of 10-fluorinated analogues of octopus rhodopsin and their photochemical properties studied using UV−vis absorption and ^{19}F NMR spectroscopy. Because of difficulties in accumulating a large amount of the octopus apoprotein needed for NMR studies, the current study is limited to the 10-F system, where regiospecific interaction with protein was suspected.

Experimental Section

Retinals. The 9-cis and 11-cis isomers of retinal were isolated by high-performance liquid chromatography (HPLC) (Gulliver, JASCO) on a silica porous column (Finepak SIL-5, JASCO) from the isomeric mixture.[25] Procedures for the preparation and isolation of the 9-cis and 11-cis isomers of the 10-fluorinated retinal (10-F retinal) by HPLC were described in the literature.[26] The HPLC conditions used for isolation were as follows: column, silica porous (Finepak SIL-5, JASCO); solvent, 5% ether−hexane; flow rate, 2 mL/min; monitoring wavelength, 370 nm.

Octopus Microvillar Membrane and Bleached Microvillar Membrane. Octopus microvillar membranes were prepared according to Tsuda.[27] All procedures were done at 4 °C under dim red light. The concentration of rhodopsin was determined by absorption measurements after solubilization with 2% sucrose monolaurate (w/v; SM1200, Dojin) using a molar extinction coefficient at 476 nm (30 000 cm^{-1} M^{-1}).[22,28]

The preparation procedures for bleached microvillar membranes were according to Koutalos et al.[22] with modifications. The microvillar membranes were suspended in a 40% sucrose solution (w/v) in Na−K phosphate buffer (66 mM Na/K phosphate, pH 7.0) with neutralized 200 mM hydroxylamine added to the membrane suspension. The bleaching of rhodopsin was carried out in an ice cold bath using light of wavelengths longer than 480 nm (Toshiba VY-47). With a 150 W halogen projector lamp, bleaching was completed within an hour. The bleached membranes were first washed with 10 times volume of Na−K phosphate buffer by centrifugation (38000*g*, 60 min) and then three times with the same buffer (38000*g*, 30 min) to remove residual hydroxylamine. The bleached membranes were further washed three times with a 2% solution (w/v) of bovine serum albumin (BSA) (Sigma, Fraction V) in 20% sucrose in the same buffer (w/v) to remove retinal oxime from the membranes. The pellet was suspended in 40% sucrose in the same buffer (w/v) used for the washes and stored at −80 °C until use for pigment regeneration.

Regenerated Pigments. Retinal and 10-F retinal isomers used were highly purified by HPLC. The content of octopus opsin in the bleached microvillar membranes was estimated from the maximal absorbance of octopus rhodopsin regenerated in the presence of a molar excess of 11-*cis*-retinal. In the case of 10-F analogue pigments, an equimolar amount of 10-F retinal was estimated by titration. The same amount of bleached microvillar membrane was mixed with 9-cis or 11-cis 10-F retinal. The regeneration was complete within 3 h at 15 °C. After hydroxylamine was added to the mixture (final concentration; 1 mM), the preparation was incubated for 1 h at 4 °C to convert the free retinal and the random Schiff base to retinal oxime. The regenerated membranes were washed twice with the buffer, followed by BSA-wash by centrifugation (75000*g*, 20 min) as described in the preparation of the bleached microvillar membrane. Thus, the final precipitate was suspended with the buffer as a regenerated pigment membrane. Immediately after the regenerated pigment membrane was solubilized with detergent, the 10-F pigment degraded partially. The extent of degradation was estimated to be ∼50%, and additional degradation was not observed in the dark at 15 °C for a day. Thus, the content of the pigment and free 10-F retinal (∼50%) was constant during the NMR measurements at 15 °C in the dark.

For NMR measurements solubilized 10-F pigments were concentrated (Centriprep 30, Amicon) to 0.4 mM (500 μL) and D_2O was added to the sample (final concentration 10%). The photoproducts of 11-cis or 9-cis 10-F pigments were prepared by irradiation with blue light from a 150 W halogen projector lamp (Color Cabin III, Cabin, coupled with a Toshiba V-40 filter) or in the cases of 9-cis 10-F pigments further irradiation with orange light (Toshiba O-58). All samples (two regenerated pigments and three photoproducts) were kept at −80 °C in the dark until use.

UV−Vis Spectoroscopic Measurements. Absorption spectra were measured with a Shimadzu MPS-2000 recording spectrophotometer. For the correction of scattering of the membrane suspension, opal glasses were placed in both the sample and reference side. The reference cuvette contained the same opsin solution as the sample cuvette for regeneration spectra. The sample was irradiated with light from a side illumination system (Shimadzu, 50 W halogen lamp) that passed through a glass cutoff filter (orange light, Toshiba O-58, $\lambda >$ 582 nm) or a band-pass filter (blue light, Toshiba V-40, peak wavelength, 400 nm, half-bandwidth, 100 nm). The temperature of the sample cuvette was kept constant at 15 °C by a water circulator (RMS, LAUDA).

For determination of the wavelength of the absorption maximum (λ_{max}) of regenerated pigments, the sample containing 10 mM hydroxylamine was irradiated with panchromatic light to convert the pigment into opsin and its corresponding retinal oxime. The wavelength of the absorption maximum of the sample was estimated from the difference of the absorption spectrum before and after the irradiation.

To compare the photosensitivity of regenerated pigments, each regenerated pigment was irradiated successively with weak blue light to convert the pigment into metarhodopsin. After no further spectral change was observed, the absorbance change at 530 nm was calculated using the following equation and plotted against the successive integrated irradiation time (Figure 4).

$$\Delta A = \log\frac{A_t - A_\infty}{A_0 - A_\infty}$$

where A_0, A_t, and A_∞ represent absorbance at 530 nm measured at time 0 (starting point), t, and ∞ (end of the photoreaction), respectively. The back photoreaction from metarhodopsin (trans

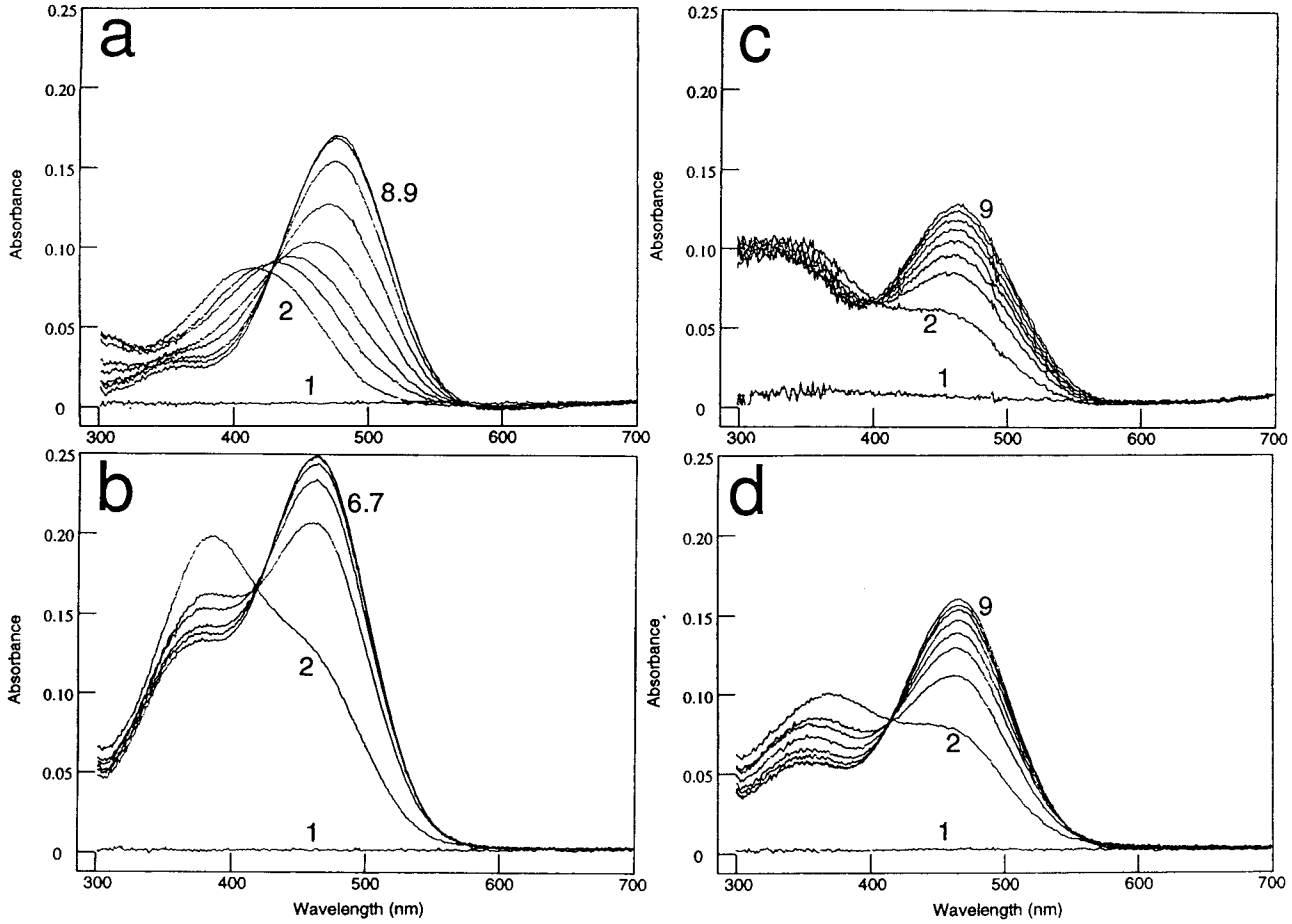

Figure 1. Formation of octopus natural and artificial pigments. Bleached membranes (6.4 nmol octopus opsin) and an equimolar amount of 11-cis, 9-cis, 11-cis 10-F, and 9-cis 10-F retinal (**a**−**d**, respectively) were mixed and incubated at 15 °C. The equimolar amount of each retinal was determined by titration experiments described in the Experimental Section. The absorption spectra were measured before (curve 1) and just after (approximately 45 s, curve 2) the addition of retinal. Then, the absorption spectra were measured successively at 3, 6, 12, 24, 48, 96, and 192 min after the addition (curves 3−9, respectively).

pigment) was also investigated in the manner similar to the regenerated pigments except that orange light was used for irradiation.

The photochemical reaction pathways between 9-cis and 11-cis pigments were analyzed based on the following model, and the reaction kinetics were analyzed with IGOR Pro (Wave Metrics, Inc.) on Power Macintosh 7200 (Apple).

$$\text{9-cis pigment} \xrightarrow{k_1} \text{trans pigment} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \text{11-cis pigment}$$

^{19}F NMR Measurements. ^{19}F NMR spectra were recorded on a Bruker AMX600 spectrometer with a 6 ms pulse and 6 s delay between pulses at 15 °C. A total of 8000 to 16 000 scans were accumulated and processed with a line broadening of 200 Hz. The spectral shape was monitored every 4000 scans. FELIX (Biosym Inc.) was used for data processing and signal assignment on a Silicon Graphics workstation. Trifluorotrichloroethane in CD_2Cl_2 was used as an external standard at −82.2 ppm.

Results

Pigment Formation. The spectral changes during the formation of octopus pigments of retinal and F-retinal are shown in Figure 1. When a given amount of bleached microvillar membranes was mixed with equimolar amounts of retinal or F-retinal, absorbances around 480 nm increased with time, indicating that a pigment was formed.

The 9-*cis*-retinal bound to octopus opsin faster than 11-*cis*-retinal. The half-life time of pigment formation was ca. 2 min for 9-*cis*- and 18 min for 11-*cis*-retinal pigment. The pigment for 11-cis 10-F retinal formed faster than natural 11-*cis*-retinal, with a half-life time of 2.7 min. The substitution of fluorine atom in 11-*cis*-retinal enhanced the rate of pigment formation, but in the 9-*cis*-retinal the F atom did not seem to have an influence on formation of pigments.

The wavelengths of maximum absorption of pigments are shown in Table 1. The absorption maximum of each PSB is also given.[12,29] The UV opsin shifts (UV OS; the difference in wavenumbers between the λ_{max} of the PSB and that of the corresponding pigment) of octopus pigments are smaller than that of corresponding bovine pigment, 0.61 and 0.62 times that of bovine pigment (isorhodopsin and rhodopsin, respectively). In the bovine system, the substitution of a fluorine atom in retinal caused an increase in the UV OS value (1.27 and 1.33 times; rhodopsin and isorhodopsin, respectively). In the case of octopus 11-cis 10-F pigment, the increase in UV OS was observed at a magnitude (1.18 times larger) similar to that observed in the bovine system. The increase in UV OS of 9-cis 10-F octopus pigment (1.54 times) was, however, larger than the expected value. This difference in UV OS value between 11-cis and 9-cis forms resulted in similar λ_{max} values of the synthetic pigments (466 and 465 nm, respectively).

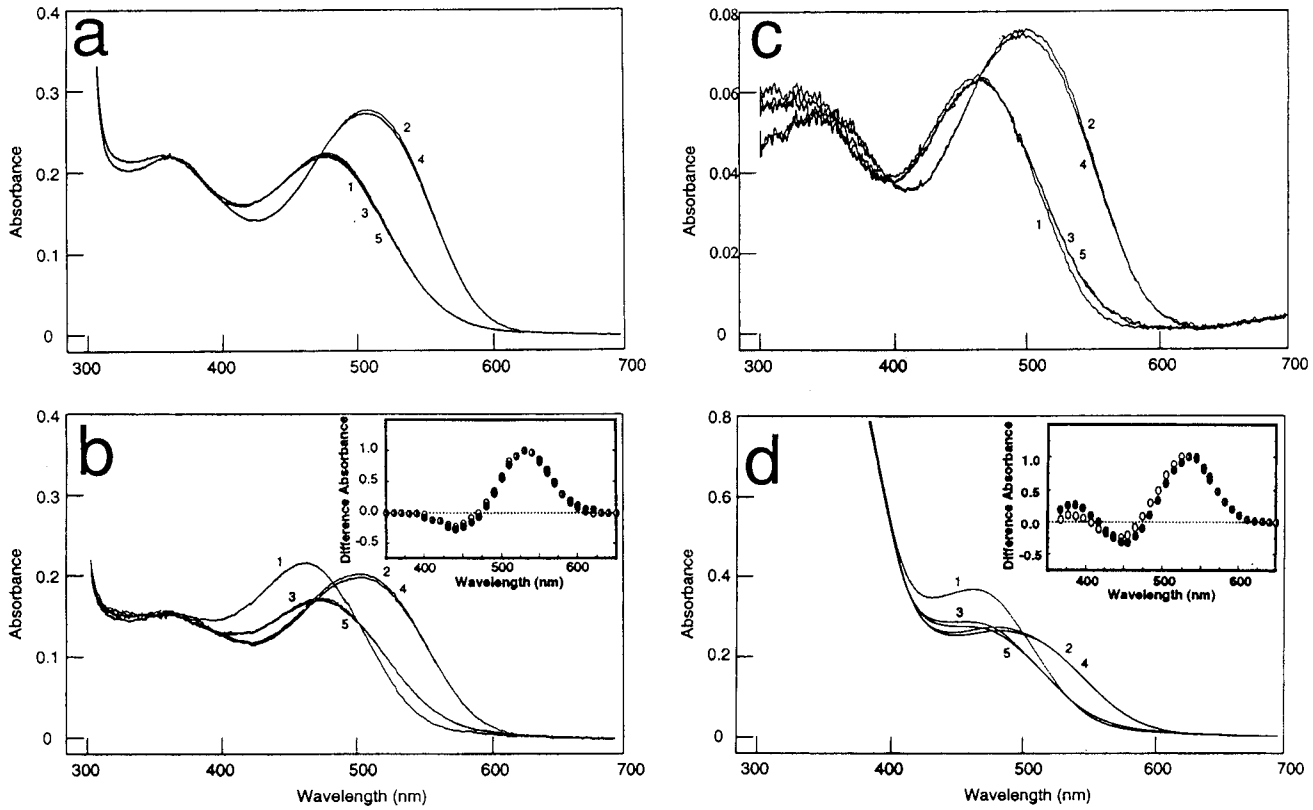

Figure 2. Photoconversion of octopus natural and artificial pigments at 15 °C. (a−d) The regenerated pigments (11-cis, 9-cis, 11-cis 10-F, and 9-cis 10-F pigment; curve 1 in a−d, respectively) were irradiated with blue light (Toshiba, V-40) to convert the pigments into corresponding photoproducts (curve 2). The photo-steady-state mixture (curve 2) was then irradiated with orange light (Toshiba O-58) (curve 3). The irradiation with blue light was repeated (curve 4) and followed with orange light irradiation (curve 5). Inset in b: Difference absorption spectra of the photoconversion from natural retinal pigment. The difference spectra were normalized at their difference maxima as 1.0. (○) Difference spectrum between curve 2 and 1 in a. (●) Difference spectrum between curve 2 and 3 in b. Inset in d: Difference absorption spectra of the photoconversion from 10-F retinal pigment. The difference spectra were normalized at their difference maxima as 1.0. (○) Difference spectrum between curve 2 and 1 in c. (●) Difference spectrum between curve 2 and 3 in d.

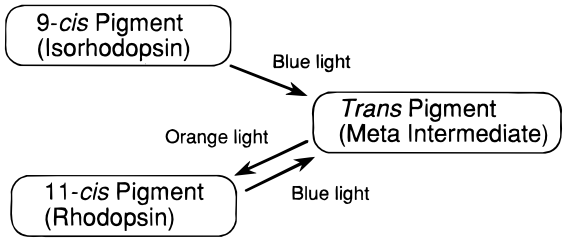

Figure 3. Schematic representation of the photoconversions of regenerated octopus pigments. The 9-cis pigment regenerated with retinal or the 10-F retinal was converted into the trans pigment (metarhodopsin) with initial blue light irradiation. The irradiation with orange light to the metarhodopsin caused an isomerization of the chromophore from all trans to 11-cis (rhodopsin or 11-cis 10-F rhodopsin). Successive irradiation with blue light and orange light caused a photoreversible reaction between 11-cis pigment and trans pigment. The pigment regenerated with 11-cis isomers of retinal and 10-F retinal showed photoreversible reaction between 11-cis pigment and trans pigment.

The regenerated pigments, both with natural retinal and 10-F retinal, were stable at 15 °C in the membrane in the dark. Upon solubilization of pigments formed with 10-F retinal in sucrose monolaurate, absorbance around 370 nm increased possibly due to partial dissociation of 10-F retinal from opsin. Several other detergents (digitonin, CHAPS, Tween 20, and dodecylmaltoside) were tested but found to cause pigment denaturation.

Photoconversion of the Pigments. Figure 2a shows the photoconversion of regenerated pigment with 11-*cis*-retinal (octopus rhodopsin). When the pigment (curve 1) was irradiated with blue light for 5 min at pH 6.5, the absorption maximum shifted to a longer wavelength by 20 nm and the absorbance increased at the wavelength longer than 470 nm (curve 2). This blue light photoproduct was photoreversible to the 11-cis pigment by orange light irradiation. The difference spectrum of the photoreaction (open circles in inset of Figure 2b) was the same as that of native rhodopsin.

Irradiation of 9-cis pigment (curve 1 in Figure 2b) with blue light induced the shift of maximum wavelength to a longer wavelength and the absorbance decreased. Irradiation of the blue light photoproduct with orange light resulted in a hypsochromic shift of the absorption spectrum. The absorption spectrum of the final photoproducts (curve 3 in Figure 2b), however, was different from that of the 9-cis pigment, λ_{max} being about 476 nm and the extinction coefficient being smaller. Further irradiation of the photoproduct (curve 3 in Figure 2b) with blue light caused a bathochromic shift, and the resulting absorption spectrum (curve 4 in Figure 2b) is identical to that of curve 2 in Figure 2b. The latter two processes are reversible (curves 2−5 in Figure 2b). The difference spectrum between curves 3 and 2 in Figure 2b (filled circles in inset of Figure 2b) is the same as that between octopus rhodopsin and metarhodopsin (open circles in inset of Figure 2b), indicating that the photoproduct of orange light irradiation (curve 3 in Figure 2b) must be the 11-cis pigment (rhodopsin). These photoprocesses originating from the 9-cis pigment at 15 °C are summarized in Figure 3.

The photoconversion of the 11-cis 10-F pigment was essentially the same as that of the 11-cis parent pigment (Figure

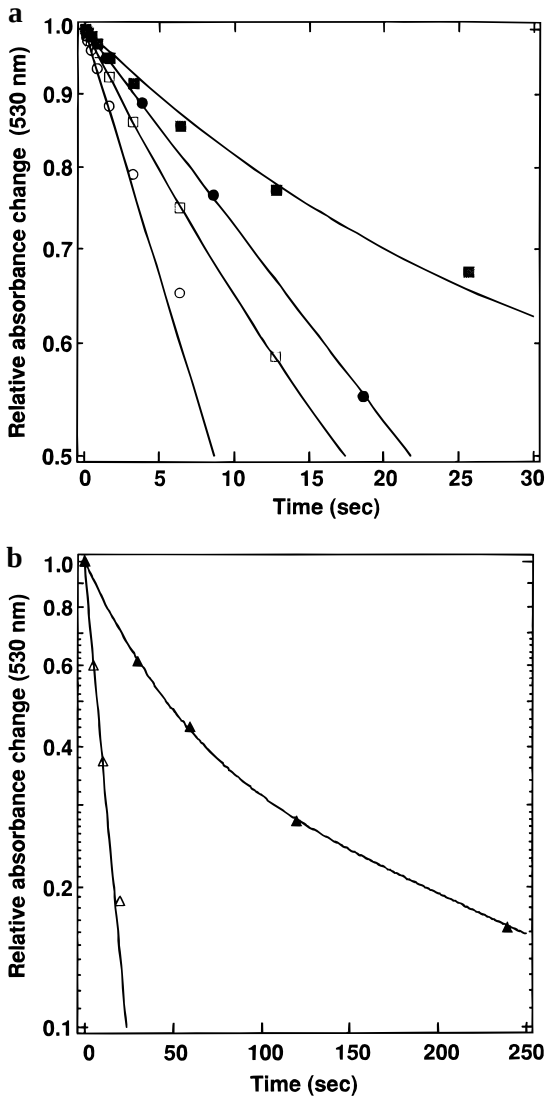

Figure 4. Kinetics of photoreactions of octopus natural and 10-F retinal pigments. (a) Regenerated pigments irradiated with blue light as described in the Experimental Section. The relative absorbance change at 530 nm was calculated and plotted against irradiation time. (○) 11-cis retinal pigment. (□) 9-cis retinal pigment. (●) 11-cis 10-F pigment formed by irradiation of 9-cis 10-F pigment. (■) 9-cis 10-F pigment. The curves represent the fitted curves based on the model for photoreaction pathways of 9-cis pigment (see Figure 3 and in the text). (b) Metarhodopsin of natural (△) and 10-F retinal pigments (▲) irradiated with orange light. The relative absorbance change at 530 nm was calculated and plotted against the irradiation time. The curves represent the fitted curves.

2c). The 9-cis 10-F pigment represented the same course of photoconversion as that of the 9-cis parent pigment (Figure 2d). The difference absorption spectrum between curves 2 and 3 in Figure 2d is the same as that between curves 1 and 2 in Figure 2c (filled and open circles in inset of Figure 2d, respectively). The results indicate that the photoconversion pathway is not affected by fluorine substitution at the 10-position of retinal.

Relative Photosensitivities of the Pigments. Although the overall photoconversion pathway was the same for both native and 10-F pigments, photosensitivities of the latter were lower. Therefore we investigated the kinetics of photoreactions of 11-cis and 9-cis pigments (Figure 4a) and that of metarhodopsin (trans pigments) (Figure 4b). To compare the photosensitivities of the pigments quantitatively, the photochemical reaction pathways between 9-cis and 11-cis pigments were analyzed

TABLE 1: Absorption Maxima of Pigments and Their Opsin Shift

		octopus[a]		bovine	
	PSB[b] (nm)	pigment[c] (nm)	OPS[d] (cm^{-1})	pigment (nm)	OPS[d] (cm^{-1})
11-cis	442[e,f]	476	1616	500[f]	2624[f]
9-cis	433[e,f]	463	1496	484[f]	2434[f]
11-cis 10-F	428[g,h]	466	1905	499[h]	3324
9-cis 10-F	420[g,h]	465	2304	486[h]	3233

[a] Determined in the present study. [b] Absorption maxima of protonated *n*-butylamine Schiff base. [c] In 2% SM1200. [d] The difference in wavenumbers between the λ_{max} of the PSB and that of the corresponding pigment. [e] In methanol. [f] Data from Fukada et al.[29] [g] In ethanol. [h] Data from Colmenares et al.[12]

based on the following model:

$$\text{9-cis pigment} \xrightarrow{k_1} \text{trans pigment} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \text{11-cis pigment}$$

The rate constant (k) of the photoreaction is represented as follows.

$$k = I\epsilon\Phi$$

where I represents irradiation light intensity, ϵ the molar extinction coefficient of the pigment at irradiation wavelength, and Φ the quantum efficiency of the pigment, respectively.

The change in relative absorbance of each of the photoreactions shown in Figure 4a,b can be represented by an exponential function of each rate constant: The photoreaction from the trans pigment to the 11-cis pigment can be represented by a single-exponential curve of k_2, $\exp(-k_2t)$, while that from the 11-cis pigment to the trans pigment by another single-exponential curve of $(k_3 + k_2)$, $\exp(-(k_3 + k_2)t)$, and that from the 9-cis pigment by a sum of two exponentials, $\exp(-k_1t) + \exp(-(k_3 + k_2)t)$. From an analysis of the photoreaction kinetics of the natural and 10-F pigments, the rate constants can be obtained. The ratio of the rate constants is equal to that of the sensitivities of the pigments ($\epsilon\Phi$) when the same irradiation light is used.

The rate constants, k_1, k_2, and k_3 for native pigments and $k_{1,F}$, $k_{2,F}$, and $k_{3,F}$ for fluorinated pigments, respectively, were obtained as described below.

1. The photoreaction of native trans pigment to 11-cis pigment (open triangles in Figure 4b) was fitted to a single-exponential curve, and k_2 was obtained as $(1.85 \pm 0.06) \times 10^{-2}$ s^{-1}.

2. The photoreactions from native 11-cis pigment to trans pigment (open circles in Figure 4a) was successively fitted with a single-exponential curve. The reaction kinetic constants thus obtained [$(7.92 \pm 0.18) \times 10^{-2}$ s^{-1}] are a sum of the rate constants of the forward and back reactions ($k_2 + k_3$).

3. We analyzed the photoreaction kinetics from the 9-cis pigment based on the model shown above and found that the reaction kinetics shown in Figure 4a (open squares) can be represented by a sum of two exponentials; one is $\exp(-k_1t)$ and another is $\exp[-(k_2 + k_3)t]$. The plots were safely fitted with a sum of two exponentials, $\exp(-8.26 \times 10^{-2}t)$ and $\exp(-1.85 \times 10^{-2}t)$. We assigned the former one to $k_2 + k_3$ and the latter one to k_1, because the former value [$(8.26 \pm 0.30) \times 10^{-2}$ s^{-1}] was similar to that obtained from the data of the 11-cis pigment [$(7.92 \pm 0.18) \times 10^{-2}$ s^{-1}] within experimental error. These results support our model for the photoreaction pathway of the 9-cis pigment (Figure 3).

4. It is possible to deduce the ratio of rate constants of forward and back reactions of native pigments (k_2/k_3) from the

ratio of the amount of both pigments in the photo-steady-state mixture of 11-cis and trans pigments because the ratio of the amount of both pigments equals the ratio of the rate constants. According to the results of Tsuda et al.,[28] we can estimate the ratio of the rate constants of forward and back reactions (k_3/k_2) as 2.41± 0.24.

5. Now we can estimate the rate constants (k_1, k_2, and k_3).

$$k_3/k_2 = 2.41 \pm 0.24$$
$$\therefore k_3 = (2.41 \pm 0.24)k_2$$

$$k_3 + k_2 = (3.41 \pm 0.24)k_2 = (8.09 \pm 0.17) \times 10^{-2}\ s^{-1}$$
$$\therefore k_2 = (2.37 \pm 0.29) \times 10^{-2}\ s^{-1} \quad (1)$$

$$k_3 = (5.72 \pm 0.34) \times 10^{-2}\ s^{-1} \quad (2)$$

$$k_1 = (1.85 \pm 0.06) \times 10^{-2}\ s^{-1} \quad (3)$$

6. The photoreaction from the fluorinated trans pigment to the fluorinated 11-cis pigment was composed of two exponentials (filled triangles in Figure 4b). We designated the faster component as the photoreaction of the pigment and the slower one as a degradation process of fluorinated pigments during the photoreaction, the latter to account for the small decrease of absorbance around 470 nm (curves 2−5, Figure 2d) observed during irradiation. Thus,

$$k_{2,F} = (2.88 \pm 0.14) \times 10^{-2}\ s^{-1} \quad (4)$$

7. The photoreaction from the 11-cis 10-F pigment to the trans 10-F pigment takes place faster than the reverse one. Thus, the degradation of the fluorinated pigments was not prominent during this photoreaction. This allowed fitting of the kinetic data with a single-exponential curve (filled squares in Figure 4a). Thus,

$$k_{2,F} + k_{3,F} = (3.18 \pm 0.02) \times 10^{-2}\ s^{-1} \quad (5)$$

8. The photoreaction kinetics from the 9-cis 10-F pigment (filled squares in Figure 4a) can be represented by a sum of two exponentials: the data best fitted by the sum of $\exp(-5.20 \times 10^{-2}t)$ and $\exp(-2.28 \times 10^{-3}t)$. We assigned the former one to $k_{2,F} + k_{3,F}$ and the latter one to $k_{1,F}$, because the former value [$(5.20 \pm 0.39) \times 10^{-2}\ s^{-1}$] was close to that obtained from the data of the 11-cis pigment [$(3.18 \pm 0.02) \times 10^{-2}\ s^{-1}$]. We used the average of the both values for $k_{2,F} + k_{3,F}$. Thus,

$$k_{2,F} + k_{3,F} = (4.19 \pm 0.39) \times 10^{-2}\ s^{-1} \quad (6)$$

and,

$$k_{1,F} = (2.28 \pm 0.13) \times 10^{-3}\ s^{-1} \quad (7)$$

9. From the ratio of the rate constant of native (k_2) and fluorinated trans pigments ($k_{2,F}$), the following relationship was obtained.

$$k_{2,F} = (0.302 \pm 0.021)k_2 = (0.125 \pm 0.019)k_3 \quad (8)$$

TABLE 2: Relative Photosensitivity of Octopus and Bovine Pigments

	octopus pigment[a]			bovine pigment[b]	
	11-cis	9-*cis*	trans	11-*cis*	9-*cis*
retinal	100	32.3 ± 2.2	41.4 ± 5.6	100	24
10-F retinal	60.7 ± 7.7	3.98 ± 0.61	12.5 ± 1.9	73	13

[a] Determined in the present study. [b] Data from Liu et al.[18]

10. From the comparison of photoreaction kinetics of 11-cis pigments (circles in Figure 4a), we can estimate $k_{3,F}$ as follows:

$$(k_{2,F} + k_{3,F})/(k_2 + k_3) = [(0.125 \pm 0.019)k_3 + k_{3,F}]/1.41k_3$$
$$= [(4.19 \pm 0.39) \times 10^{-2}]/(8.09 \pm 0.17) \times 10^{-2} = 0.518 \pm 0.049$$

Thus,

$$k_{3,F} = (0.607 \pm 0.077)k_3 \quad (9)$$

11. From $k_{1,F}$ and $k_{2,F} + k_{3,F}$, we can estimate $k_{1,F}$ as follows:

$$k_{1,F}/(k_{2,F} + k_{3,F}) = k_{1,F}/[(0.125 \pm 0.019) + (0.607 \pm 0.077)]k_3$$
$$= (2.28 \pm 0.13) \times 10^{-3}/(4.19 \pm 0.39) \times 10^{-2} = 0.0544 \pm 0.0059$$

Thus,

$$k_{1,F} = (0.0398 \pm 0.0061)k_3 \quad (10)$$

12. From eqs 1, 2, 3, 8, 9, and 10, the relative photosensitivities of the pigments were obtained and summarized in Table 2.

^{19}F NMR Spectra of 10-F Pigments and Their Photoproducts. ^{19}F NMR spectra of 10-F pigments (11-cis and 9-cis form) and their photoproducts were recorded as shown in Figure 5. Relatively sharp peaks are due to retinals released from the chromophore-binding pocket of the opsin moiety during solubilization, judging from their difference in line widths. In Figure 5a, the two resonance peaks were assigned to *trans*-retinal (−123.5 ppm) and 9-*cis*-retinal (−118.4 ppm) based on their chemical shifts. A rather broad peak at −105.5 ppm was assigned to the 11-cis 10-F pigment because upon irradiation with blue light, its signal intensity had decreased and a new signal appeared at −119.9 ppm (Figure 5b). The latter should be due to metarhodopsin (trans pigment). The peaks due to free retinal did not change upon blue light irradiation.

For the spectrum of the 9-cis 10-F pigment (Figure 5c), two strong peaks corresponding to unbound 9-cis and all-trans 10-F retinals, from denatured protein, at −118.5 and −123.6 ppm (as in Figure 5a) predominate. The signal that corresponds to the 9-cis 10-F pigment is the broad shoulder at −116.0 ppm, determined from periodic monitoring of spectral changes during the entire acquisition period of the NMR run. The processing of these various data blocks using different line-broadening parameters showed the peak was consistently fixed at −116.0 ppm. This agreed with earlier trials using different spectral NMR parameters and spectrometers. Additionally, the chemical shift is consistent with that of the 9-cis 10-F model PSB (−119.7 ppm) presented in Table 3. By irradiation with blue light, a sharp peak due to metarhodopsin appeared at −120.2 ppm (Figure 5d). This peak position (−120.2 ppm) is almost the same as that observed in Figure 5b (−119.9 ppm). Further

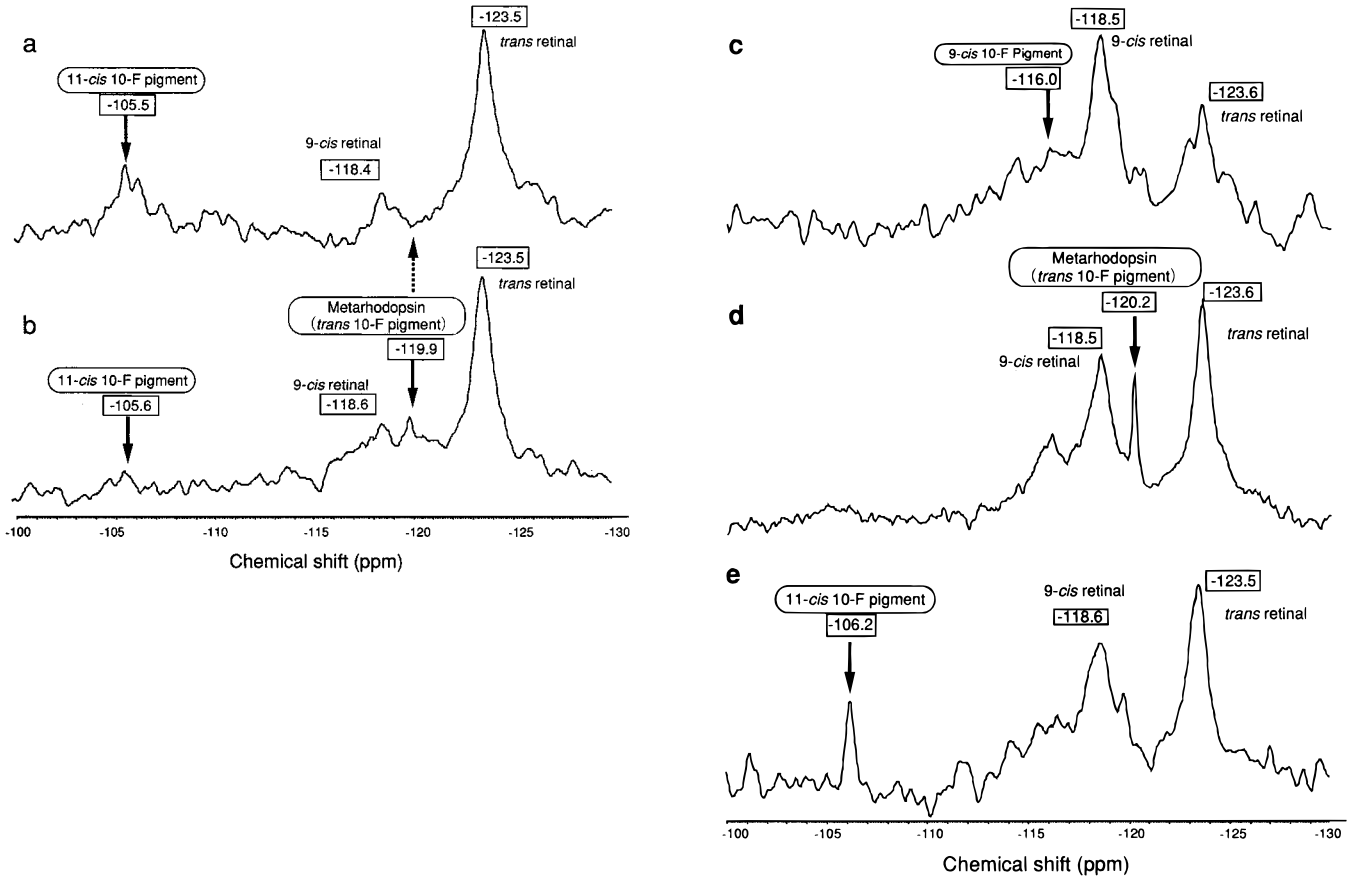

Figure 5. Fluorine NMR spectra of 10-F retinal pigment and its photoproducts. 10-F pigment was solubilized in 2% SM1200 and irradiated, when necessary, as described in the Experimental Section. (a) 11-cis 10-F pigment. (b) Pigment after blue light irradiation of an identical sample as that in a. (c) 9-cis 10-F pigment. (d) Photoproduct of 9-cis 10-F pigment after blue light irradiation of a sample identical to that in c. (e) Photoproduct of d after successive blue and orange light irradiation of an identical sample of c. The assignment of the signal and the value of the chemical shift are given.

TABLE 3: ^{19}F NMR Chemical Shifts and FOS of Retinylidene PSB and Pigments

		octopus[a]		bovine	
	PSB[b,c] (ppm)	pigment (ppm)	FOS[d] (ppm)	pigment (ppm)	FOS[d] (ppm)
9-cis	−119.7[e]	−116.0	3.7	−115.3[e]	4.4
11-cis	−112.2[e]	−105.8	6.4	−107.4[e]	4.8
all-trans	−124.5[e]	−120.0	4.5	ND[f]	ND[f]

[a] Determined in the present study. [b] The protonated *n*-butylamine Schiff base. [c] In $CDCl_3$. [d] The fluorine opsin shift; the difference between the values of PSB and pigment. [e] Data from Colmenares et al.[12] [f] Not determined, because bovine opsin did not form a pigment with *all-trans*-retinal.

irradiation with orange light caused displacement of the peak at −120.2 ppm to −106.2 ppm (Figure 5e). This peak at −106.2 ppm is the same as that of the 11-cis 10-F pigment in Figure 5a within experimental error. The signal assignment was consistent with the scheme of the photoconversion of the 9-cis 10-F pigment (Figure 3).

The ^{19}F chemical shifts of pigments (11-cis and 9-cis form) and their photoproducts (trans form) are listed in Table 3 together with the corresponding PSBs of 10-F retinals. For comparison, the corresponding data set in the bovine system[12] is also given in Table 3.

Discussion

Chromophore Binding Site of Octopus Opsin. Although a lot of information was accumulated on the retinal binding site of vertebrate opsin,[30] little was known about the chromophore binding site of invertebrate opsin. This may be partly due to instability of invertebrate opsin and failure to regenerate the pigment with modified retinal. Improved methods of preparation of octopus opsin enabled us to prepare two types of analogue pigments of octopus, i.e., the dihydro retinals[22] and 14-F retinal.[23] Using an artificial pigment containing 14-F retinal, the pK_a of the Schiff base (an indicator of proton-binding affinity) of both bovine rhodopsin[31] and octopus rhodopsin[23] was studied. The pK_a values of the Schiff base of bovine and octopus rhodopsin were above 16 and 10.6, respectively. These results suggest that the interaction between the protonated Schiff base and counterion is stronger in bovine rhodopsin than in octopus rhodopsin. The 11-cis and trans isomers of the 3,4-dihydro to 11,12-dihydro retinal series formed octopus rhodopsin pigment analogues.[22] On the basis of their UV OS values, it was postulated that a counterion of PSB and another negative charge resides near the middle of the polyene chain and is mainly similar to that of bovine rhodopsin. The interaction between this counterion and chromophore is suggested to be weaker than in bovine rhodopsin, since the UV OS value is smaller in the octopus system.

In the present study, regeneration of octopus opsin with 9-cis and 11-cis retinals and the corresponding isomers of 10-F retinal was carried out. The reconstitution experiment shows that the high electronegativity of the fluorine atom does not perturb the pigment formation. The rate of binding, however, was affected by fluorine substitution in that the 11-cis 10-F showed an accelerated rate, 9 times compared to the 11-cis parent pigment.

Also, it was observed that the absolute magnitude of the UV OS in the octopus system is smaller than in the bovine system (Table 1), as reported previously,[22] about 0.6 of that in bovine. However, fluorine substitution resulted in an increase of the UV OS value. In bovine rhodopsin, the UV OS value is about 1.3 times larger in the 10-F pigments[12] for both the 9-cis and 11-cis isomers. In octopus 10-F pigments, it is about 1.2 times larger for the 11-cis isomer (essentially the same as in bovine), but 1.5 times larger in the 9-cis isomer. The results suggest a specific interaction between 9-cis 10-F retinal and octopus opsin. The effect of the 10-F substituent on enhanced rates of 11-cis pigment formation and the UV OS value is an increased twisting of the 12,13 bond in octopus pigment analogues. Since the conformation around the 12,13 bond is expected to be very sensitive to steric crowding, the free 10-F chromophore is likely to be more twisted than that of the parent 11-cis retinal, possibly making it more compatible with the shape of the binding cavity. In agreement is the more blue-shifted absorption of the octopus pigments. While we are unable to offer an explanation for the fast rate of pigment formation for 9-cis retinal, it is clear that the addition of the 10-F substituent will have no effect on the polyene conformation, hence no additional rate acceleration.

The measured photosensitivities of the octopus pigments (Figure 4a,b) are summarized in Table 2. The photosensitivities of bovine pigments are also given for comparison.[18] In the bovine pigments, the photosensitivity of the 9-cis pigment (isorhodopsin) is much smaller than that of the 11-cis pigment (rhodopsin). Fluorine substitution caused a decrease in photosensitivity of pigments of different magnitudes; in the 11-cis 10-F pigment, it decreased to 73% of rhodopsin, while in the 9-cis 10-F pigment (10-F isorhodopsin) it decreased to 54% of isorhodopsin, resulting in a much lower photosensitivity compared to rhodopsin (13%). The previous work on the 10-F bovine pigment suggested a regiospecific interaction between chromophore and opsin.

In octopus rhodopsin, the photosensitivity of the 9-cis pigment is about 32% of the 11-cis pigment. Fluorine substitution resulted in a decrease in the photosensitivity of the pigment as observed in bovine rhodopsin. However, the magnitude of decrease in photosensitivity is larger in octopus; for the 11-cis 10-F pigment it became 60% of rhodopsin, and for the 9-cis 10-F pigment, it was only 12% (3.98/32.3) of the 9-cis octopus pigment.

The photosensitivity listed in Table 2 is a function of the molar extinction coefficient and the quantum efficiency of the pigment ($\epsilon\Phi$). Although the exact value of the molar extinction coefficient of each regenerated pigment was not obtained, the absorbance of the 10-F pigment was determined to be about 60−70% of the parent pigment (Figure 1). This cannot explain the large decrease in the photosensitivity of the 9-cis 10-F pigment. Thus, the decrease in photosensitivity is larger in octopus pigments than in bovine rhodopsin, indicating a difference in chromophoric structure and environment in the two pigments. It is notable that the decrease in photosensitivity of the octopus 10-F pigments is larger in the 9-cis pigment than in the 11-cis pigment.

Similar to the previous work on the bovine system,[8,18] the results indicate a regiospecific interaction between retinal and the opsin moiety in octopus rhodopsin. The fact that the hydrogen bond between the iminium nitrogen and counterion of octopus rhodopsin is now known to be weaker than that between Glu-113 of bovine rhodopsin and N^+,[21] the F-substituent of the latter is likely to be more basic than that in rhodopsin, which could lead to a stronger H···F hydrogen bonding, hence the observed reduced photosensitivity. However, we also cannot rule out a possible change in dipolar repulsive interaction between the electron-rich F atom and a nearby amino acid residue (e.g., its lone pair electrons) that led to the altered photosensitivity. It should be noted that the change is relatively small at room temperature, magnified only at very low temperatures in the case of bovine pigments.[8]

Fluorine Chemical Shift. The signal of each pigment in ^{19}F NMR spectra was assigned by comparing the chemical shifts with those of the corresponding PSB[12] and by carrying out the photoreaction of the pigments (Figure 2a−d). The appearance and disappearance of the ^{19}F NMR signal were in agreement with results of the UV−vis spectroscopic study (Figure 3). In the spectra, despite efforts to remove unreacted retinal in preparing the NMR samples (see the Experimental Section), a noticeable amount of "free" retinals was observed, clearly due to the pigment's instability upon detergent solubilization.

The FOS (fluorine opsin shift) values of the two isomeric octopus 10-F (Table 3), calculated by taking the difference between the chemical shift of the pigment and that of the PSB, are 3.7 and 6.4 ppm, slightly outside the range of values observed for bovine rhodopsins (4.4−4.8 ppm). Specifically, the FOS value of octopus 9-cis pigment decreased by 0.7 ppm and that of octopus 11-cis pigment increased by 1.6 ppm, compared to the corresponding bovine pigment. On the other hand, the FOS value of metarhodopsin (trans pigment) (4.5 ppm) is between the FOS values of 9-cis and 11-cis pigments.

Two detailed rhodopsin models have recently been disclosed. Orientation of the 10-substituent in such models could suggest source(s) of perturbations in the current set of data, albeit for the octopus rhodopsin. Inspection of the model of Shieh et al.[32] shows that the amino acid residues proximate to H(F)-10 are Ala-117 and Glu-113. In the octopus system, these residues are replaced by Gly and Tyr, respectively. These changes could be the cause of the 1.6 ppm FOS increase in the octopus rhodopsin. In the Pogozheva et al. bovine rhodopsin model,[33] the H(F)-10 is in the vicinity of Pro-291 and Ala-295. These residues are, however, conserved in the octopus system. In the latter model, it is the CH_3-13 that has incurred changes in proximate amino acid residues that can affect the chromophore conformation and, subsequently, the F-10 chemical shift.

For the 9-cis pigment, both the Shieh et al. and Pogozheva et al. bovine rhodopsin models[32,33] suggest that the H(F)-10 is in the vicinity of Ala-117, which is replaced by Gly in the octopus system. This implies less steric bulk in its environment, leading to the observed small FOS, which is 0.7 ppm less than that of the bovine system.

In the *trans*-retinal chromophore, the fact that the metarhodopsin is sufficiently stable at room temperature for NMR and other spectroscopic measurements indicate that the binding pocket of octopus rhodopsin must be less restrictive than that of bovine rhodopsin. This is reflected in the possible formation of the 13-cis analog[22] and a normal FOS value despite the lengthened all-trans chromophore in metarhodopsin.

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