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Regiospecific Protein Perturbation on F NMR Shifts and Photoisomerization of Fluororhodopsins. An Interpretation Based on Recent Crystal Structures of Rhodopsin

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Based on structural information provided by recently reported crystal structures of rhodopsin, we present rationales for the regiospecific protein perturbation on the previously reported ^{19}F chemical shifts of the vinyl and trifluoromethylrhodopsins and their photoproducts. The crystal structures also suggest that H-bonding is a likely cause for the earlier reported regiospecific photoisomerization of the 10-fluororhodopsins. Photoisomerization was revealed by chemical shift of the photoproducts. Additionally, possible use of 3-bond F,F coupling constants for following photoisomerization of retinal-binding proteins is discussed.

key words: Rhodopsin, photoisomerization, F NMR, protein perturbation

INTRODUCTION

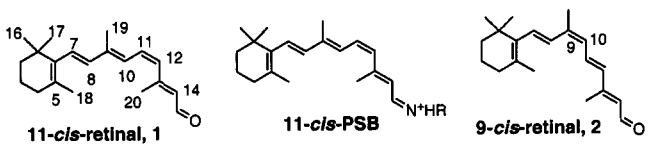
Rhodopsin is a heptahelical membrane protein located in the eye retina responsible for scotopic vision. It is a photoreceptor protein activated by the covalently bonded 11-*cis*-retinal chromophore with Lys-296 forming a protonated Schiff base (PSB). The photoisomerization of the chromophore initiates the vision process [1]. It has long been our desire, along with many others, to understand fully the protein-chromophore interactions in the binding site. This paper presents a comprehensive summary of the F-chemical shifts and photoisomerization data of the fluororhodopsins from this research group, accumulated in the last two decades and analyzed in light of the specific protein-substrate interactions and the currently available 3-dimensional structures of rhodopsin [2, 3].

The F NMR study of visual pigments should be considered complementary to the extensive ^{13}C NMR studies in the literature [4]. The fluorine labels, replacing hydrogen atoms, report information for regions that are one bond away from the carbon atoms. In fact, the locations of the fluorine labels are more likely to yield information directly related to protein-substrate interactions.

DISSCUSSION

^{19}F NMR Studies of Rhodopsins and Photoproducts

The strategy involves replacing a hydrogen atom of the rhodopsin chromophore with fluorine and measuring the change in the NMR chemical shift of the ^{19}F. Fluorine is selected based on its small size and its NMR chemical shift sensitivity [5]. After the synthesis of F-labeled retinal, the natural chromophore of rhodopsin (11-cis retinal, **1**) is substituted with the synthetic fluorinated chromophore and analyzed by ^{19}F NMR spectroscopy. The change in ^{19}F chemical shift of the chromophore (retinylidene PSB) in solution and in the protein is called fluorine opsin shift (FOS). A comparison of the FOS values at different locations in the chromophore will reveal which positions of the chromophore are strongly perturbed by protein binding and subsequently indicate specific protein interactions.



A total of eleven (11) fluorine-monolabeled rhodopsin analogs were prepared and measured by ^{19}F NMR [6]. Likewise, the corresponding chromophore PSB in solution was also prepared and measured (Table 1). In all cases, the chemical shift of the photo-bleached product was identical to that of the all-*trans*-retinylidene PSB indicating the extrusion of the all-*trans* chromophore from the binding site after photoisomerization.

Factors affecting ^{19}F chemical shifts include electron anisotropy (ring current effect), specific interactions (i.e. hydrogen bonding), van der Waals interactions and weak electric effects [7]. More recent work by Oldfield and co-workers [8] showed that weak electric field effects (α $1/r^3$) are the dominant factors, which obviated the need to invoke second order van der Waals interactions (α $1/r^6$). Experimentally, Gribble and co-workers [9] assert that fluorine chemical shift is affected by steric depolarization. Using 1-fluoronaphthalene and its 8-alkyl-substituted derivatives, they demonstrated that

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Table 1. ^{19}F NMR Chemical Shifts (ppm) of F-Rhodopsins Compared to the Corresponding Free Chromophores (PSB) in Solution

F-Analog	Rhodopsin	PSB(CD_2Cl_2)	FOS (δΔ)
9-*cis* 8F	-99.3	-105.9	6.6
9F	-96.4	-103.3	6.9
10F	-115.3	-119.7	4.4
11F	-81.0	-87.1	6.1
12F	-114.3	-120.9	6.6
14F	-123.5	-131.4	7.9
10,14-F_2	-115.4; -121.2	-119.1; -128.8	3.7; 7.6
11-*cis* 8F	-103.7	-116.8	13.1
10F	-107.4	-112.2	4.8
11F	-75.9	-78.0	2.1
12F	-94.6	-107.8	13.2
14F	-117.1	-125.5	8.4
8,12F_2	-105.2; -92.9	-117.0; -104.6	11.8; 11.7
10,14F_2	-111.8; -117.7	-115.3; -122.7	3.5; 5.1

steric crowding of the fluorine atom due to the proximity of the alkyl substituents produces a large low-field shift of the ^{19}F signal. Collectively taken, the experimental data and the theoretical results mentioned above agree that ^{19}F chemical shift is influenced by neighboring atom perturbations and is distance dependent. Ring current effect may be positive or negative depending on the orientation of ^{19}F with respect to the plane of the ring, but at interaction distances between the ring and ^{19}F labels, it is likely to be small, about 2 ppm [10]. Hydrogen bonding also causes small changes in the ^{19}F chemical shift, about 1-2 ppm downfield [11].

In analyzing the FOS values, a general trend was observed and that is the downfield shift of the ^{19}F signal in the protein representing the overall change in medium environment from organic solution (methylene chloride) to the hydrophobic protein cavity. To gauge the normal baseline FOS value, we conduct a cursory examination of the FOS of the 9-*cis* chromophore series first. It is well-known that the 9-*cis*-retinal binds with rhodopsin and gives similar absorption and circular dichroic properties with the normal 11-*cis* rhodopsin. Technically, the 9-*cis*-retinal (**2**) occupies the same binding cavity as the 11-*cis*, but most likely without as strong specific protein substrate interactions as in 11-*cis* since the 9-*cis* has much lower quantum yield of photoisomerization and red-shift characteristics. The FOS values of the 9-*cis* analogs showed a relatively narrow range of 4.4 to 7.9 ppm, which is considered the overall medium effect of changing from solution to protein cavity environment and any excessively higher values of FOS are, therefore, indicative of specific protein interactions.

In the 11-cis chromophore series, most of the FOS values measured were <8 ppm with the exception of 12F and 8F, which are in the order of 12-13 ppm. An examination of the recent x-ray crystallographic (2.6 Å) rhodopsin structure [3] (Protein Data Bank, ID 1L9H) reveals that the chromophore in the protein cavity has very close neighboring amino acids:

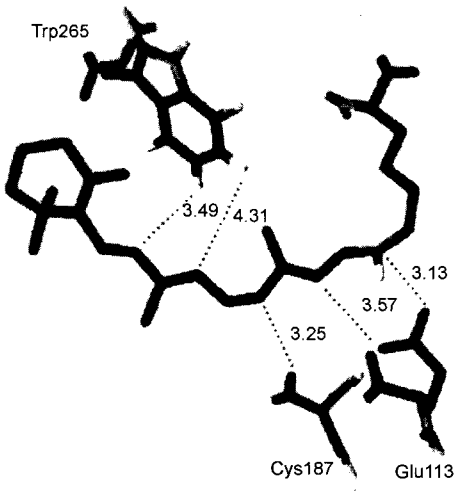


Figure 1. The 11-*cis* chromophore and nearest neighboring amino acid residues in the binding cavity of rhodopsin (1L9H).

Glu-113, Cys-187 and Trp-265. In Figure 1, the distances between the carbon atoms (C-8, C-10, C-12, C-14 and C-15) and the nearest amino acid to the ^{19}F probe are indicated. It shows the oxygen of Cys-187 is 3.25 Å to C-12 and the hydrogen atom of Trp-265 is 3.49 Å to C-8. The actual distances between the ^{19}F atom and the amino acid residues are expected to be shorter inasmuch as the fluorine atom extends one bond away from the carbon framework (C-F bond about 1.41 Å [12]) of the chromophore in the direction of the amino acid. The amino acid residues in close proximity perturb the electron density of the ^{19}F probe to a more downfield chemical shift (steric depolarization). A comparison of the FOS with the distance values shows an inverse relationship. F-12 having the closest distance to the protein exhibits the highest FOS. This is followed by C-8, C-14 and C-10.

Although the F-8 is relatively distant to Trp-265, its experimental FOS is the same as that of C-12 (13 ppm). We attribute the large FOS value of F-8 partly to the change in the dihedral angle, C5-C6-C7-C8 ($\varphi_{6\text{-}7}$), of the chromophore upon binding as suggested by calculated results using model structures [6] showing that F chemical shielding is more sensitive to variations in $\varphi_{6\text{-}7}$ compared to $\varphi_{12\text{-}13}$.

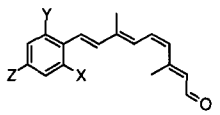
Experimentally, the question about the $\varphi_{6\text{-}7}$ angle as a binding requirement was addressed through the use of fluorophenylretinal analogs as chromophores. Several phenyl substituted retinal analogs were prepared and tested for binding. Their ring-chain torsional angles were calculated and the results reported in Table 2.

The binding of 11-*cis*-*o*,*o*-bistrifluoromethyl, 11-*cis*-*o*,*o*,*p*-tristrifluoromethyl- and *o*-fluoro-*o*-trifluoromethylphenyl retinal (**3-5**) are expected in that the calculated $\varphi_{6\text{-}7}$ (58.2, 56.6 and 51.5 degrees, respectively) is similar to the torsional angle of the normal chromophore (calcd., 54.4°). On the other hand, the non-binding of 11-*cis*-*o*-fluoro-, 11-*cis*-*o*,*o*-difluoro-, and

Table 2. Calculated Torsional Angles and ^{19}F NMR Chemical Shifts of Triluoromethylated Rhodopsins Compared to the Corresponding Free Chromophores (PSB) in Solution

Rhodopsin Analog	Calculated $\varphi_{6\text{-}7}$	Rh	Chemical shift (ppm) PSB (CD_2Cl_2)	FOS ($\delta\Delta$)
11-*cis*-*o*,*o*-bis-CF_3-phenyl	58.2°	-55.5	-58.8	3.6
		-60.7	-58.8	-1.9
11-*cis*-*o*,*o*,*p*-tris-CF_3-phenyl	56.6°	-56.8	-59.6	2.8
		-61.2	-59.6	-1.6
		-64.0	-61.7	2.3
11-*cis*-*o*-F-*o*-CF_3-phenyl	51.5°	-61.6	-60.2	-1.4
		-114.5	-112.4	-2.1
11-*cis*-9-CF_3		-53.9	-58.2	4.3
9-*cis*-9-CF_3		-60.2	-65.1	4.9
9-*cis*-13-CF_3		-57.8	-61.4	3.6
normal, cyclohexenyl	54.4°			
11-*cis*-*o*-F-	24.6° (F in the 5-Me position)			
11-*cis*-*o*,*o*-di-F-	29.7°			
11-*cis*-*o*,*p*-bis-CF_3-	46.63° (CF_3 in the 5-Me position)			

11-*cis*-*o*,*p*-bistrifluoromethylphenylretinal (**6-8**) is at first somewhat perplexing due to the fact that although the calculated $\varphi_{6\text{-}7}$ in the free chromophore (24.6°, 29.7° and 46.63°, respectively) is less than the normal chromophore, theoretically speaking, it can be altered by free rotation to fit the cavity just like the bistrifluoromethyl analog or the normal chromophore. Its inability to bind with the protein implies the absence of sufficient amounts of the twisted conformation for these planar analogs in equilibrium, and there is a requisite interaction between protein and the ring portion of the chromophore to hold it within the cavity.



3: X = Y = CF_3, Z = H
4: X = Y = Z = CF_3
5: X = F, Y = CF_3, Z = H
6: X = F, Y = Z = H
7: X = Y = F, Z = H
8: X = Z = CF_3, Y = H

An examination of the rhodopsin structure shows that the ring-chain torsional angle is caused by the orientation of Trp-265 and Ala-269 in relation to the other amino acids that form the walls of the ring pocket. This torsional twist works synchronously with the protein interactions at the ring pocket acting as a retaining mechanism to lock the chromophore in place. Hence the torsional twist and the bulky substituents are both needed to hinder the rotation of the ring and its subsequent expulsion. This situation is observed in the ^{19}F NMR of the bistrifluoromethyl analog.

The binding of bis-trifluoromethylphenylretinal in rhodopsin was studied by ^{19}F NMR spectroscopy (Figure 2) [13]. In solution, the two CF_3 groups are equivalent due to rapid ring-

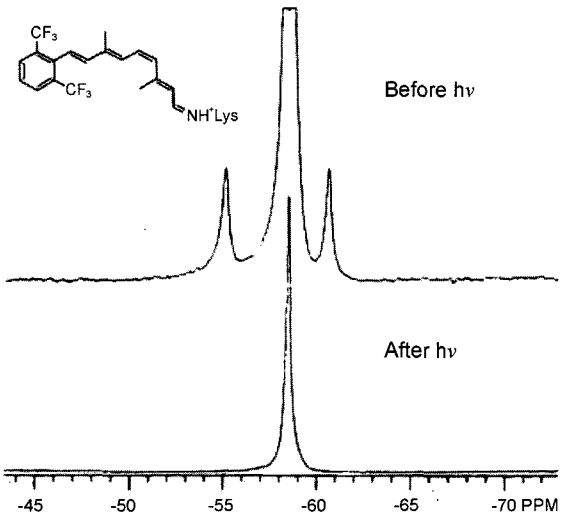


Figure 2. The ^{19}F NMR spectrum of the visual pigment (approximately 10^{-4} M in CHAPS) from the *o*,*o*-bis(trifluoromethyl)phenyl retinal analog with excess fluororetinal (upper spectrum). The chemical shifts for the three peaks are -55.2 (LW=88 Hz), -58.5 (50 Hz, free excess fluororetinal), and -60.7 (72 Hz) ppm with CF_3CCl_3 as an external standard. Spectrum of the same sample after photobleaching with orange light (>460 nm) (lower spectrum).

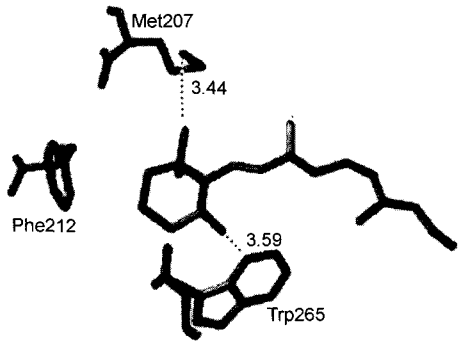


Figure 3. The nearest amino acid residues to the methyl-5 and methyl-1 positions of the 11-*cis* chromophore in the binding cavity of rhodopsin (1L9H).

chain conformation equilibration, but in the protein cavity, this is restricted causing the appearance of two CF_3 signals of equal intensity. One signal occurs downfield of the solution chemical shift, an expected phenomenon. However, the other signal shows upfield of the solution signal. An examination of the ring binding pocket reveals that several residues are closely interacting with the ring portion serving as its initial recognition site and most of these are aromatic amino acids that fan out like walls of the ring pocket. The closest amino acid to C-16 is Met-207 with the C of S-CH_3 being 3.4 Å away (Figure 3). Incorporating the C-H and C-F bonds in this distance will make a very crowded accommodation for the CF_3 group, explaining the downfield shift of 3.6 ppm. For the C-18 counterpart, the CF_3 group will project toward Gly-121 but on its side, it is exposed (3.59 Å) to the aromatic ring of

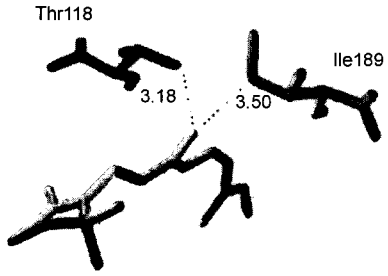


Figure 4. The binding cavity of rhodopsin (1L9H) around the methyl-9 position of the 11-*cis* chromophore.

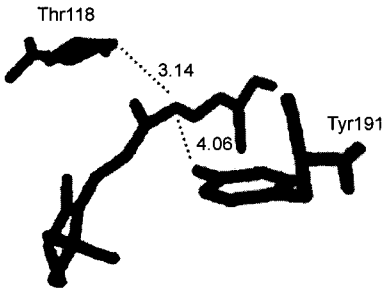


Figure 5. The binding cavity structure of rhodopsin (IF88) in the C-10 position of the 11-*cis* chromophore.

Trp-265 (see Figure 4) which induces ring current effect. It is reported that this shielding effect can produce about 2 ppm upfield [14]. Its neighboring amino acids such as Gly-121 (3.64 Å) and Glu-122 (4.03 Å) are relatively distant compared to those of CF_3-16 that include Met-207, Ala-269, Phe-212 and Phe-208. Hence, the upfield signal of -60.7 ppm in Figure 3 is assigned to the CF_3 counterpart of CH_3-5 and the downfield signal at -55.2 ppm belongs to the CF_3 counterpart of CH_3-1.

This interpretation is consistent with the result of the *o*-fluoro-*o*-trifluoromethylphenyl analog, **5**. The upfield shift of the CF_3 signal at -61.6 ppm indicates the position CH_3-5, instead of CH_3-1, is assumed by the CF_3. The upfield F-signal indicates that it is occupying a location of relatively empty space. Reversing the assignment of the CF_3 to methyl-1 contradicts the torsional $\varphi_{6\text{-}7}$ requirement for binding [13].

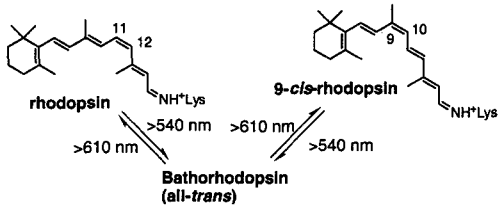
It is to be noted that the FOS of the CF_3 signal is not as large as those of the F signal. This is reflected in the F-shift data of *o,o*-bistrifluoromethylated chromophore, and for 9-CF_3 and 11-CF_3 analogs (FOS~3-5 ppm) (Table 2).

An examination of the binding cavity of the 11-*cis* chromophore shows the 9-CF_3 group is extending to the region bound by Thr-118 (3.18 Å to C19) and Ile-189 (3.50 Å to C19). See Figure 4. In fact, in a limited variable temperature study (determined by sample stability and viscosity) lowering of temperature to 5°C resulted in a broadening of the signal suggesting possible restricted rotation of the 9-CF_3 group.

Another location where a small FOS was observed is the F-11 position (2.1 ppm). The 9-*cis* isomers of the 11F analog gave normal FOS values, but the 11-cis isomer gave low FOS in both mono- and di-fluoro analogs (Table 1). Examination of the C-11 location in the rhodopsin structure (1L9H) showed that there are no close lying amino acid residues in the C-F trajectory. This is in agreement with the earlier assertion that a small FOS implies an area of free space (fewer distant neighbors than in solution).

Photochemistry of the fluororhodopsins

F-substituents exert a minor regioselective effect on photoisomerization of retinals. For example, in the case of photoisomerization of all-*trans*-retinal and the fluorinated analogs in non-polar hexane, where isomerization takes place exclusively at the tri-substituted double bonds, the same two product isomers (13-*cis* and 9-*cis*) were obtained. Their ratio changes upon fluorine substitution and depending on its location. Thus, the initial ratio of 4.0:1 of 13-*cis* to 9-*cis* for the parent system was changed to 3.1:1 and 2.3:1 for respectively 10F-retinal and 14F-retinal [15]. More spectacular was the regiospecific perturbation of the F-substituents on photoisomerization of the retinal chromophore in fluororhodopsins. The results are summarized below.



Irradiation of rhodopsin (λ_{max}=498 nm) is known to produce first the stable photoproduct bathorhodopsin (λ_{max}=546 nm). Upon further irradiation with longer wavelength light, it was shown that bathorhodopsin, which is believed to contain the retinal chromophore in the all-*trans* configuration, undergoes reversibly secondary photoisomerization to the 11-*cis* rhodopsin and additionally to the isomeric 9-*cis* pigment in an approximate ratio of 3:1 [16]. In fact, retention of such photochemical reactivities together with stability of batho and later dark intermediates (lumi and meta's) are considered good indications of the absence of any specific interaction of protein residue with the electronegative fluorine atom. This is clearly demonstrated, for example, in the case of 12-fluororhodopsin [17].

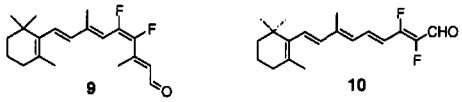
However, in the case of 10-fluororhodopsin, the corresponding bathorhodopsin was found to isomerize regiospecifically to the 11-*cis* with no detectable amount of the 9-*cis* isomer [18] (i.e., the 11-*cis*/*trans* photoisomerization reaction being completely reversible). At the same time the 9-*cis*-10-F-rhodopsin pigment was found to be photostable at liquid nitrogen temperature [18], or exhibit a considerably reduced quantum yield of photobleaching at room temperature [19].

The loss of reactivity of photoisomerization was shown to be regiospecific at the 9,10-bond because bathorhodopsins with the F-label located at other positions of the polyene

chain isomerized in the same manner as the parent system [17,20]. It was postulated that regiospecific isomerization at the 11,12-position is due to supramolecular control by the binding pocket with isomerization at the 9,10-bond hindered by specific protein perturbation. This was envisioned to be either in the form of H,F hydrogen bonding attractive interaction between the fluorine label and a nearby acidic protein residue [19] or repulsive interactions of the F-label with a nearby electron rich residue [18]. We have now examined the protein structure with the intention to identify the nature of perturbation on the 10-F substituent.

Examination of the binding cavity of modeled 9-*cis*-rhodopsin [21] (based on rhodopsin crystal structure [2] (DBID: 1F88) and an energy minimized 9-*cis* retinyl chromophore) around the C-10 shows that it is close to Thr-118 whose O is measured to be 3.14 Å away. Since the O projects in the direction of C-F, the actual F,H distance will be shorter after taking into consideration O-H and C-F bond lengths. On the other side of the chromophore is the hydrogen-bonding network of Glu-181, Tyr-268 and Tyr-191. Most likely, hydrogen bonding impedes the initial photoisomerization resulting in the loss of photosensitivity of 9-*cis*-10-F rhodopsin. We might add that the regiospecific interaction of hydrogen bonding is not expected to cause a large ^{19}F NMR shift for ready detection (estimated to be about 1-2 ppm downfield shift only, see above).

On a different front, through the use of vicinally bis-labeled fluoro-analogs (11,12-difluororetinals, **9**), we have successfully demonstrated possible use of three-bond F,F coupling constants as a reliable means for monitoring the primary photoisomerization process in rhodopsin [22]. And, repulsive interactions would lead to a large steric depolarization (down field shift). The three-bond F,F coupling in cis and trans isomers are 0-30 Hz and >100 Hz, respectively [23], a difference that is sufficiently large for differentiation of configuration even for the broad band protein samples. Hence, the change from a broad singlet to a doublet of >100 Hz coupled constant upon photoirradiation of the 11,12-difluororhodopsin (Figure 6) reinforces the notion of 11-*cis* to 11-*trans* photoisomerization of the retinyl chromophore being the primary process of vision. A 13,14-difluoro-bacteriorhodopsin was also prepared from 13-demethyl-13,14-difluororetinal, **10** [24]. However, difficulty in obtaining NMR spectra of solubilized bacteriorhodopsin analogs [25] (unstable in the presence of detergents), precluded application of this method for following the 13-*trans* to 13-*cis* photoisomerization of a bacteriorhodopsin analog.



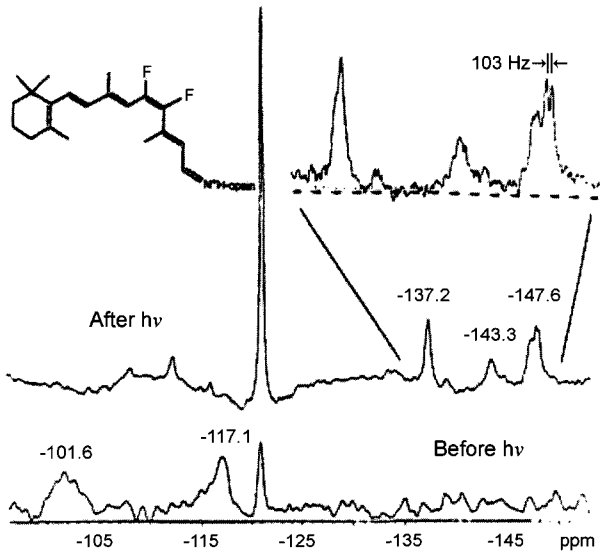


Figure 6. F NMR spectra (376.4 MHz) of 11,12-F_2-rhodopsin and photobleaching products. Lower: spectrum of a concentrated sample of pigment (0.5 mL, ~10^{-4} M) before photoirradiation (spectrometer parameters: 23,216 scans; LB, 200 Hz; pulse delay, 2s). Upper: after photobleaching. Upfield signals are expanded (insert).

Concluding remarks

In this paper we have summarized the F NMR data of labeled rhodopsins, their photoproducts and altered photochemical reactivity of several series of fluororhodopsins prepared at the University of Hawaii. The combined results demonstrate that F NMR can yield information beyond those provided by ^{13}C labels.

The data were interpreted based on the crystal structures of rhodopsin that became available recently [2,3]. While our effort appears to be qualitatively successful, the current interpretations should be considered preliminary because the protein structures used in this study were limited to either the crystal structure of rhodopsin for the 11-*cis*-fluoro-analogs or a modeled 9-*cis*-rhodopsin structure [21] for 9-*cis* fluoro-analogs. Low resolution of the crystal structures (3.5 and 2.8 Å) [2,3] caused by partial decomposition of rhodopsin under the X-ray beam introduces another uncertainty factor.

Also, it should be clear that the replacement of hydrogen by an electronegative fluorine atom and the longer C-F bond length (in particular when considering a trifluoromethyl group) could introduce new protein-substrate interactions causing local realignment of atoms. We hope that in a more comprehensive effort in the future, these issues can be addressed in a more exact manner. When coupled with computer modeling in such an extended effort, one might be able to examine other more perturbed systems. This includes looking for a possible cause of the unique upfield F-shift reported for 9,11-di*cis*-12-fluororhodopsin [26]. Of equal interest will be an examination of fluorine perturbation on the dynamics of photoisomerization through computer simulation.

The Hawaii group has focussed its studies on analogs containing fluorine labels on the retinyl chromophores. Introduction of fluorine labels on the protein residues and a corresponding F NMR study have been reported by others

[27]. Clearly it will be of interest to combine the two approaches. Furthermore, the advent of solid state NMR [4] could allow the use of fluorine, in conjunction with other NMR active labels for distance measurements between strategically separated labels. In fact, the synthesis of a $^{13}C,^{19}F$ doubly labeled retinal analog for such a purpose is in the literature [28].

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