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Fluorine-19 NMR of integral membrane proteins illustrated with studies of GPCRs

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Fluorine-19 is a spin-½ NMR isotope with high sensitivity and large chemical shift dispersion, which makes it attractive for high resolution NMR spectroscopy in solution. For studies of membrane proteins it is further of interest that ^{19}F is rarely found in biological materials, which enables observation of extrinsic ^{19}F labels with minimal interference from background signals. Today, after a period with rather limited use of ^{19}F NMR in structural biology, we witness renewed interest in this technology for studies of complex supramolecular systems. Here we report on recent ^{19}F NMR studies with the G protein-coupled receptor family of membrane proteins.

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Introduction

The favorable nuclear spin properties of fluorine-19 were exploited in ^{19}F NMR experiments with fluorinated organic compounds already early in NMR history. As a result, the principles of ^{19}F NMR have been extensively described in the literature, including serial coverage in the *Annual Reports on NMR Spectroscopy* (starting with [1]) and monographs (e.g. [2]). It was also readily recognized that the absence of a natural ^{19}F background and the high sensitivity of ^{19}F NMR observation favor the use of fluorine-19 labels for studies of proteins. ^{19}F NMR labels incorporated into a protein can provide information on the local environment of the labels, including solvent exposure, and on the conformational states and dynamics of the protein [3–5,6••]. ^{19}F NMR experiments with integral membrane proteins further enable characterization of variations of motional and structural properties in different environments, such as lipid vesicles, organic solvents and detergents [7–17,18••,19•,20•,21•].

As can be seen from Table 1, references to ^{19}F NMR studies of membrane proteins in the 21st century, and specifically during the last two years are scarce, and therefore our reference list includes only a small number of featured articles. The renewed interest that we see today is closely linked with breakthroughs in membrane protein crystal structure determination, which enable formulation of new questions about structure/function correlations that might be investigated with the use of NMR in solution [22,23]. In this review we focus on recent crystal structure-guided applications of ^{19}F NMR for studies of G protein-coupled receptors (GPCRs).

Fluorine-19 NMR in a nutshell

Due to the previously mentioned high popularity during the early history of NMR, fundamental considerations on the use of fluorine-19 NMR in structural biology have been extensively covered in the literature [3–5,6••]. Here we summarize some features of special interest for studies of membrane proteins.

The spin-½ fluorine-19 has high sensitivity for NMR observation, corresponding to 83% of the ^{1}H NMR sensitivity. In spite of this high intrinsic sensitivity, recent ^{19}F NMR applications with membrane proteins were dependent on the availability of state-of-the-art cryoprobes (see Figure 3 for details), due to the fact that membrane proteins must generally be studied in dilute solutions. For many applications it is also attractive that informative ^{19}F NMR data can be collected at field strengths corresponding to ^{1}H resonance frequencies of 600 MHz or lower. Due to the large chemical shift anisotropy (CSA) of some ^{19}F-labels used with proteins (see the next section), experiments at lower fields often yield superior results to those obtained with higher-field instruments [6••].

As with other spin-½ NMR nuclei, multidimensional experiments have been developed, which are in common use in organic chemistry [24]. Heteronuclear and homonuclear two-dimensional and three-dimensional experiments with ^{19}F have been applied also with soluble proteins, but few applications have been described with membrane proteins, probably mainly because of limited sensitivity [20•,25–29].

Table 1

Survey of ^{19}F NMR studies with membrane proteins

Membrane protein	Expression system	Milieu[a]	Year[b] (Ref)
M13 phage coat protein	*E. coli*	Lipid vesicles	1978 [7,8]
Gramicidin A	Synthetic	Lipid vesicles	1979 [9,10]
D-Lactate dehydrogenase	*E. coli*	Triton X-100, Triton X-100/SDS, C12E7, lipid vesicles	1986 [11,12]
Bacteriorhodopsin	*E. coli*	$CH_3OH/CHCl_3$, Triton X-100	1987 [13]
Rhodopsin	HEK293S (mammalian cell)	DM, OG, OG/DMPC	1999 [14,15]
Diacylglycerol kinase	*E. coli*	DPC	2002 [16]
ClC-ec1. Cl^-/H^+ antiporter	*E. coli*	DM	2009 [17]
β_2-Adrenergic receptor	*sf9* (insect cell)	DDM/CHS, MNG	2012 [18••,21•]

[a] *Abbreviations*: SDS, sodium dodecyl sulfate; DM, n-*decyl*-β-D-maltopyranoside; OG, n-*octyl*-β-D-glucopyranoside; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPC, n-dodecylphosphocholine; DDM, n-*dodecyl*-β-D-maltopyranoside; CHS, cholesteryl hemisuccinate; MNG, maltose neopentyl glycol.
[b] The year of the first publication is shown. The references include only the first and the last publications.

Fluorine-19 chemical shifts are outstandingly sensitive to the local environment characterized by van der Waals interactions, local electrostatic fields, and similar effects. ^{19}F-labels are therefore highly sensitive reporters of changes in protein conformation and solvent environment. Direct applications are measurements of the immersion depth of membrane proteins into lipophilic environments by observation of isotope effects between H_2O and D_2O [30,31], or of effects from O_2 as a paramagnetic relaxation agent [16,32]. In general, however, interpretation of ^{19}F chemical shifts in terms of protein structure, or in terms of the nature of conformational changes is rather limited [33].

Introducing ^{19}F NMR labels into membrane proteins

Since ^{19}F does not occur naturally in proteins, its use in structural biology typically relies on incorporation of extrinsic ^{19}F-labels at structurally and/or functionally interesting locations of the target protein. Three methods of fluorine labeling are described here: chemical conjugation of fluorine-containing small molecules with reactive amino acids, biosynthetic introduction of fluorinated amino acid analogs which are compatible with a natural aminoacyl-tRNA synthetase, and site-specific incorporation of fluorinated amino acids which do not have a naturally compatible aminoacyl-tRNA synthetase.

Chemical modification of amino acid side chains

Fluorine can be posttranslationally introduced by chemical conjugation of small ^{19}F-containing molecules with reactive –SH or reactive $–NH_n$ moieties in proteins. It is an important advantage of the chemical modification approach that it can be applied with otherwise unlabeled proteins, which may either be obtained from natural sources or as recombinant proteins from cells grown in an optimized medium. This can be especially useful for membrane proteins, which are often poorly expressed even in eukaryotic cell lines. Two preferred reagents for membrane protein studies are 2,2,2-trifluoroethanethiol (TET) [14,15,18••,20•] and 3-bromo-1,1,1-trifluoroacetone (BTFA) [16,19•,21•], and 4-(perfluoro-tert-butyl)-phenyliodocetamide (PFP) [34] and S-ethyl-trifluorothioacetate (SETFA) [35] have also been used. TET and BTFA are commercially available, exhibit high labeling efficiency, and cause minimal structural perturbations. BTFA–protein conjugation is a one-step process that results in a stable thioester bond. Conjugation with TET starts with activation of cysteine–SH groups by 4,4-dithiodipyridine (4-DPS), and in a second step a disulfide linkage is formed. Therefore, TET-labeling may be reversible, especially at higher temperatures [18••,20•].

Trifluoromethyl probes have higher sensitivity for NMR detection and usually smaller CSA than monofluorinated probes, and are therefore the best option for studies of membrane proteins. Whereas, for example, NMR spectra of 3-fluorotyrosine-labeled alkaline phosphatase showed strong deteriorating effects due to CSA-induced line broadening at higher magnetic fields [36], trifluoromethyl groups may provide narrow signals even at high fields.

Biosynthetic incorporation of fluorinated amino acid analogs

Biosynthetic incorporation of fluorinated aliphatic or aromatic amino acid analogs has been popular in early ^{19}F NMR studies of proteins [7,9,11]. For membrane proteins, fluorinated aromatic amino acids were primarily used, presumably because the relative scarcity of aromatic amino acids was expected to simplify the assignment process. Auxotrophic bacterial strains are available, and fluorinated aromatic amino acid analogs, such as *m*-monofluorotyrosine, 4-monofluorotryptophan, 5-monofluorotryptophan and 6-monofluorotryptophan, and o-monofluorophenylalanine, m-monofluorophenylalanine and p-monofluorophenylalanine are commercial reagents, which makes the labeling process quite efficient [37]. ^{13}C-enriched and ^{15}N-enriched fluorinated amino acid analogs have been used to reduce spectral overlap in ^{19}F-detected multidimensional heteronuclear correlation NMR experiments, and to obtain NMR-based assignments of the ^{19}F signals

[28,29,38]. Detailed biosynthetic labeling protocols can be found in earlier reviews [3,37].

Sequence-specific incorporation of fluorinated amino acid analogs

A potentially highly exciting approach is sequence-specific incorporation of ^{19}F-labels into genetically encoded positions, which entails the use of an extrinsic orthogonal tRNA/aminoacyl-tRNA synthetase pair to incorporate ^{19}F-labeled amino acids at positions defined by a TAG amber codon or a frameshift codon. Phenylalanine analogs such as p-OCF_3-Phe and p-CF_3-Phe have been incorporated into soluble proteins [39–42] and at least one membrane protein [43]. Genetically encoded labeling was also used for in-cell ^{19}F NMR measurements [39,44].

Three-dimensional protein structures guide ^{19}F labeling

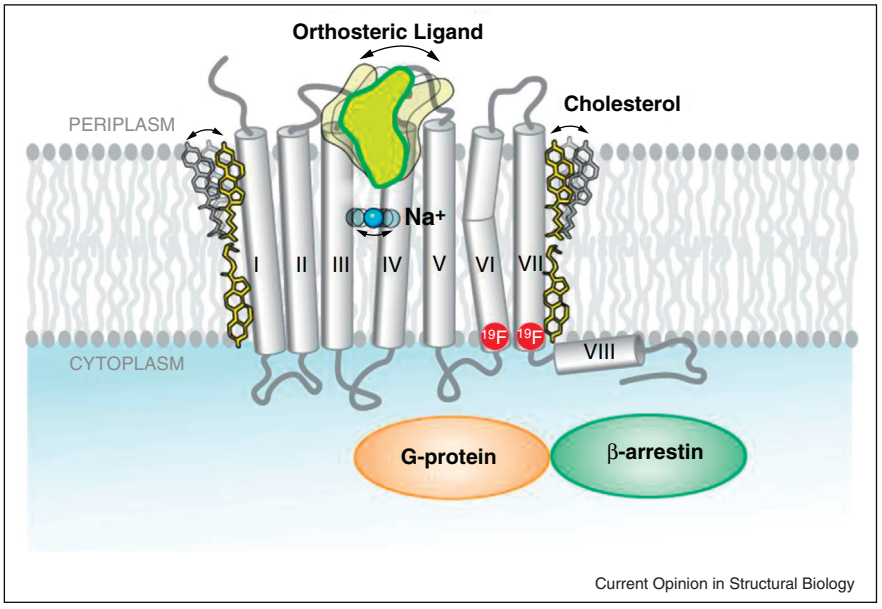
In principle, strategies for the introduction of ^{19}F-labels into polypeptide chains can be developed based on the amino acid sequence, by taking the positions of reactive amino acids into account, such as cysteines and lysines, and by identifying interesting sequence locations for mutagenesis. However, reference to three-dimensional structures often enables more meaningful strategies. Examples may include the identification of sites near active centers or near binding sites for allosteric effectors. In principle, strategies based on three-dimensional structures can lead to triangulation of function-related interaction networks in larger systems, as will be illustrated below with studies of GPCRs.

^{19}F NMR applications with GPCRs

GPCRs constitute the largest membrane protein family in the human genome, with over 800 unique sequences [45,46]. They are also important drug targets, since over 30% of all prescription drugs on the market act via GPCRs [47–49]. GPCRs have a seven-transmembrane-helix topology and contain multiple binding sites for drugs and allosteric effectors (Figure 1). They recognize a diverse array of orthosteric ligands, including small organic compounds, peptides and small proteins. Upon ligand binding a signal is transmitted over a distance of about 30 Å across the cell membrane to intracellular partner proteins, such as a G protein or β-arrestin (Figure 1). Other compounds, such as cholesterol or sodium, may act as allosteric effectors which modulate GPCR activity [50,51]. Depending on the chemical structure, different ligands have variable signaling efficacies [18••,52]. More than 20 GPCR crystal structures have been reported during the last seven years, which include structures of inactive and active states of some proteins, and a G protein-bound state of the β_2-adrenergic receptor (β_2AR) [53].

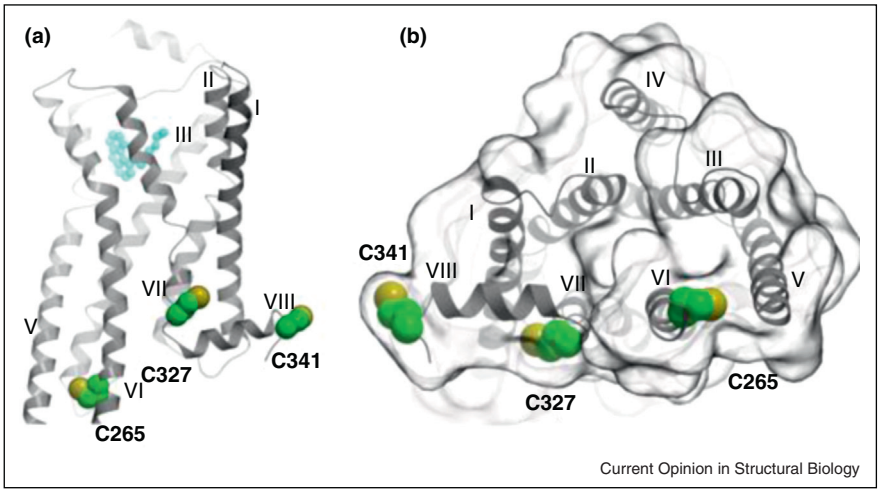
GPCRs have so far almost exclusively been expressed in eukaryotic systems, such as yeast, insect or mammalian

Figure 1



GPCR structure and function. GPCR structures are characterized by seven transmembrane helices, which stretch across the lipid bilayer cell membrane. On the periplasmic surface there is an 'orthosteric' ligand binding site (the arrow indicates mobility in the ligand binding site). Allosteric binding sites are located along the periphery of the seven-helix bundle (cholesterol) and in the center of the bundle (Na^+). Binding of drug molecules to the orthosteric site elicits signaling to partner proteins in the cytoplasm, for example, G protein and β-arrestin. The two red circles indicate locations for fluorine-19 labels that might be used for ^{19}F NMR observation of conformational changes associated with transmembrane signaling between the orthosteric site and the cytoplasmic protein surface, which bridges a distance of about 30 Å.

Figure 2



Locations of ^{19}F-labels that were recently used for β_2AR functional studies [18••,19•,20•,21•]. The green and yellow spheres indicate three TET-labeled cysteine residues in positions 265, 327 and 341. **(A)** Side view of β_2AR. An agonist, BI-167107, in the orthosteric binding site is drawn in cyan. **(B)** View of the cytoplasmic surface of β_2AR.

cells, which are not readily amenable to ^{15}N and ^{13}C labeling for NMR studies. Chemical conjugation of ^{19}F-labels into GPCRs for ^{19}F NMR experiments is therefore an attractive approach for studies in non-crystalline milieus.

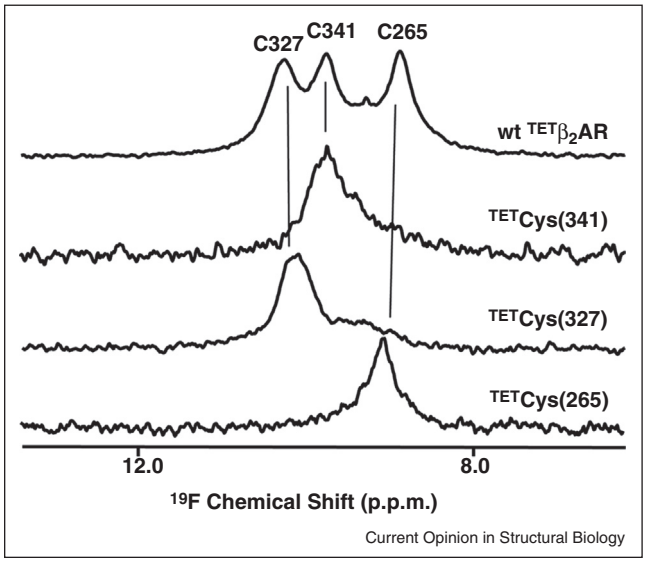
Questions for ^{19}F NMR with GPCRs

A key problem in GPCR research is to rationalize how the receptors transduce information encoded in the chemical structures of orthosteric ligands across the cell membrane to appropriate intracellular partners, such as the G protein and β-arrestin (Figure 1). While crystal structures describe the molecular architecture in great detail and may identify structural differences between activated and inactive forms of a given GPCR, ^{19}F NMR can provide additional information on local conformational polymorphisms and the rate of associated conformational exchange processes.

^{19}F labeling and sequence-specific NMR assignments of β_2AR

^{19}F NMR studies of the β_2AR have recently been reported by two different groups [18••,19•,20•,21•]. β_2AR contains three native cysteines in positions 265, 327 and 341, which are located near the cytoplasmic protein surface (Figure 2). One project made use of the accessibility of these cysteines for covalent labeling with TET [18••,20•]. The resulting 1D ^{19}F NMR spectrum consists of three peaks (Figure 3, top trace). Site-specific mutagenesis was employed to obtain single-residue labeled β_2AR variants. Comparison of the four spectra in Figure 3 then yielded sequence-specific assignments of the three ^{19}F NMR signals, and in functional studies the single-residue TET-labeled proteins enabled detailed line shape analyses of the individual signals [18••,20•]. In the second project the residue Cys265 was selectively labeled with BTFA, and the observed ^{19}F NMR signal was assigned to this residue [19•,21•].

Figure 3



Mutagenesis-based sequence-specific ^{19}F NMR assignments in β_2AR. 564 MHz 1D ^{19}F NMR spectra at 298 K are shown of the carazolol complexes with the TET-labeled wild-type protein, β_2AR (^{TET}C265, ^{TET}C327, ^{TET}C341), and for three single-residue TET-labeled variants, β_2AR (^{TET}C265, C327S, C341A), β_2AR (C265A, ^{TET}C327, C341A) and β_2AR (C265A, C327S, ^{TET}C341). The resonance assignments derived from comparison of the four spectra are indicated at the top. The spectra were recorded with a Bruker Avance 600 spectrometer (Bruker Biospin, Billerica, MA) equipped with a 5 mm ^{1}H/^{19}F, ^{13}C/^{15}N QCI cryoprobe. Reproduced from [18••].

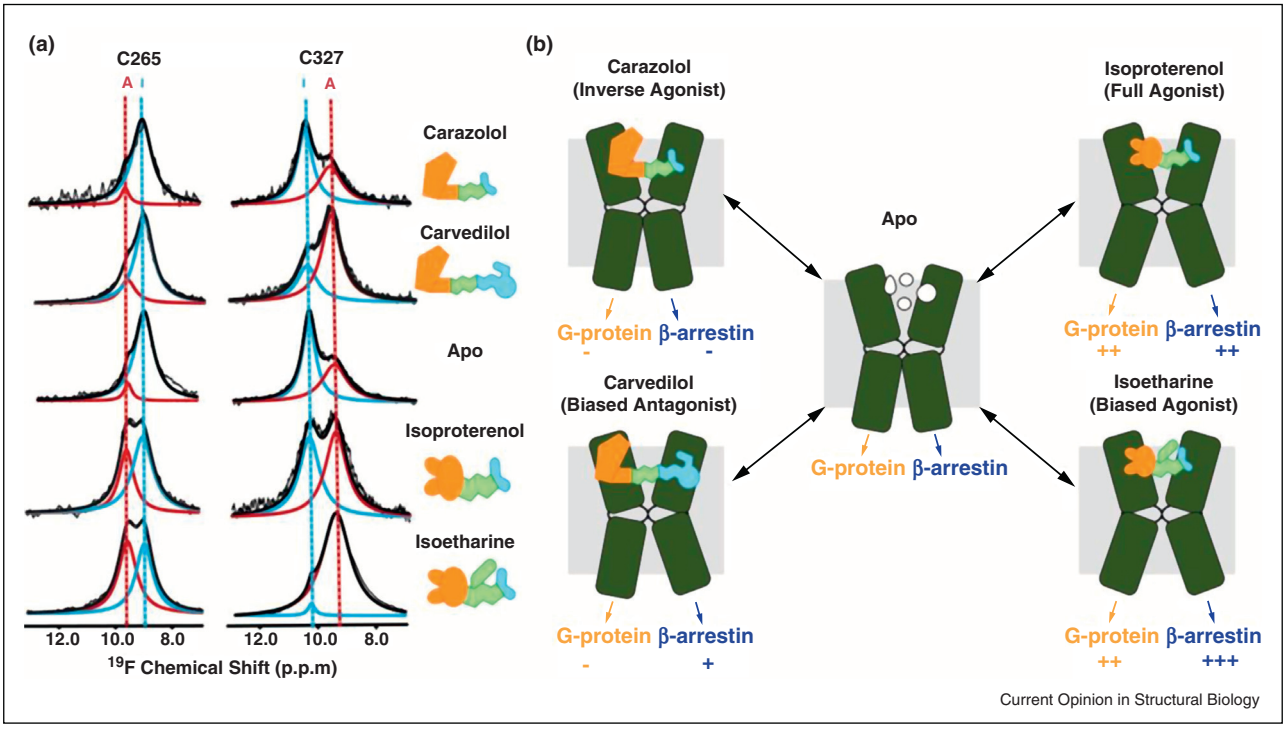
β_2AR is a favorable molecule for the use of ^{19}F NMR, since reactive cysteine residues near the cytoplasmic surface are accessible for ^{19}F-labeling. In the early work with rhodopsin [14,15] and in ongoing experiments with several human GPCRs [unpublished], cysteine residues had to be engineered into informative sequence positions, which then also requires special care to ascertain that such modifications preserve the biological activity.

^{19}F NMR studies of GPCR activation

^{19}F NMR studies of rhodopsin were started prior to the availability of high-resolution GPCR crystal structures [14,15]. In these seminal experiments, Khorana and co-workers introduced TET-labels to endogenous as well as engineered cysteine residues. ^{19}F NMR spectra of various mutant rhodopsins containing only a single ^{19}F-label were recorded in the dark and under light exposure. Chemical shift changes specific to receptor activation were thus observed, which elegantly demonstrated that ^{19}F NMR experiments can be used to monitor local conformational changes related to GPCR activation. In additional studies, close contacts in the three-dimensional structure were quantified by collecting ^{19}F–^{19}F nuclear Overhauser effects (NOEs) between pairs of TET-labels, which had been incorporated by site-specific introduction of TET-accessible cysteines to obtain two-residue TET-labeled rhodopsin [15].

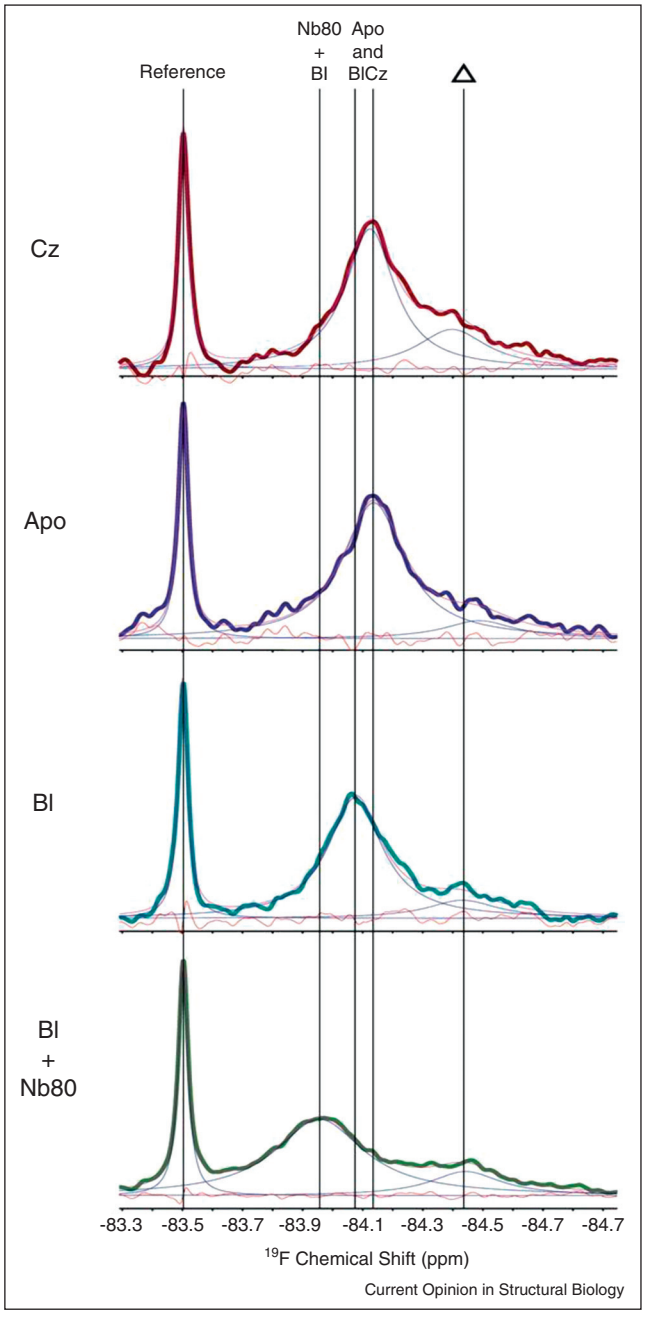
Unlike rhodopsin, β_2AR is activated by binding to diffusible ligands, which exhibit a wide range of different efficacies in their receptor interactions, depending on their chemical structures. In the experiments of Figure 4a, ^{19}F NMR spectra of β_2AR mutants containing single TET-labeled cysteine residues were recorded for complexes with different pharmacological ligands. Two largely independent equilibria were thus discovered by observation of Cys265 in helix VI, and Cys327 in helix VII, respectively. Exchange between the different conformations is slow on the chemical shift frequency scale, with $k < 10\ s^{-1}$ [20•], so that each of the two equilibria is manifested by two partially overlapped signals which have variable relative intensities in complexes with different ligands [18••]. Novel insights into correlations between the chemical structure of different orthosteric

Figure 4



^{19}F NMR observation of conformational equilibria and signaling pathways in β_2AR. **(a)** ^{19}F NMR signals of ^{TET}C265 and ^{TET}C327 in the apo-form and four drug complexes of single-residue TET-labeled β_2AR recorded at 280 K with the same equipment as the data in Figure 3. The experimental spectra (thin black line showing noise) have been deconvoluted into signals of an activated state (A, red) and an inactive state (I, blue) of β_2AR. The thick black line represents the sum of the signals I and A. **(b)** Two β_2AR signaling pathways suggested by the ^{19}F NMR experiments. The helices VI and VII of β_2AR are shown as green kinked cylinders. The structures of the four bound ligands are schematically drawn and their functionalities are indicated. The arrows at the lower end of the cylinders indicate the signaling to the downstream effectors, with plus and minus signs indicating the signaling levels relative to the basal state
Adapted from [18••].

Figure 5



564 MHz 1D ^{19}F NMR spectra of β_2AR (C77V, C275S, ^{BTFA}C265, C378A, C406A) at 298 K. Cz, complex with the inverse agonist carazolol; Apo, apo-β_2AR; Bl, complex with the full agonist Bl-167107; Bl + Nb80, complex with Bl and a G-protein-mimicking nanobody. The sharp peak at −83.5 is from a small-molecule reference, and Δ indicates a background signal from partially ^{19}F-labeled cysteines in other parts of the β_2AR structure. The vertical lines indicate peak positions of ^{BTFA}C265 assigned to the complexes of β_2AR with carazolol, Bl-167107, and Bl-167107/Nb80, as indicated at the top.
Reproduced from [19•].

ligands and the resulting downstream signaling could thus be obtained. Specifically, ligands that shift the conformational equilibrium manifested in the ^{19}F NMR signal of ^{TET}Cys265 to the activated state are likely to signal through the canonical G protein pathway, whereas ligands that lead to activation observed on ^{TET}Cys327 were found to signal primarily through the β-arrestin pathway (Figure 4). Interestingly, although there is no residual signal intensity after deconvolution with the two resonances I and A in Figure 4a, which would indicate the presence of additional states, the observation of two uncoupled conformational equilibria at the sequence positions 265 and 327 shows that there are at least three (assuming that there is a single inactive state) or four locally different states in β_2AR, indicating that the function of this GPCR is related to an intricate network of local dynamic structural polymorphisms.

With the aforementioned single-residue ^{BTFA}Cys265 variant of β_2AR, a similar strategy to the experiments in Figure 4a was used. When the protein was solubilized in DDM micelles, evidence was obtained for the presence of two or multiple states of the protein in fast exchange on the chemical shift frequency scale [19•]. As a consequence, a single ^{19}F NMR signal was observed, which showed different chemical shifts when different ligands, and in one experiment a ligand and a nanobody were bound to β_2AR (Figure 5). When the BTFA-labeled β_2AR was solubilized with the detergent maltose-neopentyl-glycol (MNG-3), the authors concluded that the exchange between different states was slowed down, and that the ^{19}F NMR signals manifested at least three different states of β_2AR in slow exchange on the chemical shift frequency scale.

From a techniques viewpoint it is of interest that the aforementioned two studies with chemically different ^{19}F-labels attached to the same residue, Cys265, provided complementary information. This comes rather as unexpected, since numerous earlier studies with a variety of different proteins indicated that replacement of natural amino acids with fluorinated analogs caused at most minor perturbations of the protein structures [54,55,56], and the introduction of TET-labels or a BTFA-label in GPCRs did not abolish their functions in the studies reported so far [14,15,18••,19•,20•,21•]. There is thus an indication that more comprehensive information may be obtained from repeating ^{19}F NMR experiments with chemically different ^{19}F-containing groups even if they are attached to the same amino acids in membrane proteins.

Outlook

Due to recent advances in instrumentation, which ensured high sensitivity at moderate field strength, ^{19}F NMR is being recognized as an attractive technique for structural biology of complex systems, in particular membrane proteins. ^{19}F NMR can be used as a 'probe method'

to investigate conformational equilibria and associated rate processes, and can serve as a lead for the use of stable isotope labeling-based NMR studies of structural details associated with the plasticity of the system. With future improvements in membrane protein biochemistry, multi-dimensional NMR experiments with ^{19}F NMR may open additional new avenues for studies of membrane proteins.

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