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NMR as a Tool to Investigate Membrane Protein Structure, Dynamics and Function

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Summary

Membrane protein NMR occupies a unique niche for determining structures, assessing dynamics, examining folding, and studying binding of lipids, ligands and drugs to membrane proteins. However, it also faces special challenges including sample preparation, size limitation, spectral crowding, and sparse data accumulation that are not encountered with soluble proteins. This Perspective gives a snapshot of our view of current achievements, future opportunities, and possible limitations in this rapidly developing field.

Membrane proteins account roughly for a third of all proteins expressed by the genomes of most organisms and carry out some of the most important cellular functions.¹ They generate energy, provide communication between cells and the outside world, transduce signals, are responsible for nutrient and waste transport, and carry electrical currents. Despite half a century of superb biochemical, biophysical, and structural work on membrane proteins, they continue to challenge structural biologists, biochemists and cell physiologists because of their complex behavior in lipid bilayer membranes. For example, of the nearly 120,000 proteins whose structures have been solved (February 2016), less than 2% are membrane proteins and only a handful of these have been solved by NMR spectroscopy.² A common challenge for structure determination of membrane proteins by all methods is sample preparation. Membrane proteins have to be expressed in high yields, they need to be extracted from their native environments by using appropriate detergents that preserve their structures and functions, and ultimately they must be transferred into an environment that is suitable for the chosen method of structure determination.

Solution versus solid-state NMR

Analyzing membrane protein structure and function by NMR bypasses the need for crystallization in a detergent that supports crystal formation, but it poses new challenges. These challenges are different for the two principal methods that have been applied to study the structures and dynamics of membrane proteins, namely solution and solid-state NMR. Solution NMR methods can be applied to macromolecular complexes that undergo fast rotational diffusion with correlation times <100 ns. This restricts the method to proteins embedded in lipid micelles and relatively small bicelles and nanodiscs. On the other hand,

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solid-state NMR (ssNMR) methods have no principal limitations on size because they work on static samples or samples that are mechanically rotated at high speed. This allows one to prepare membrane proteins in lipid bilayers, which more closely mimic their natural environment. For this reason, ssNMR is in principle the preferred method to study membrane proteins, especially if their structures and dynamics depend on the structure and composition of the lipid bilayer. However, ssNMR methods, which have been in development for membrane protein structural biology for more than 30 years, still face technical hurdles that limit the size of structures that can be solved by this method. SsNMR spectra are generally complex, leading to severe spectral crowding of uniformly labeled samples of large proteins, and creative isotope labeling schemes³ are necessary. Therefore, most proteins whose structures have been studied by ssNMR contain sequences of less than 50–100 residues, i.e. their protomer molecular masses are typically less than 10 kDa.⁴ Apart from some notable exceptions^{5–8}, most of these proteins contain one or two transmembrane (TM) spans, which however often form oligomeric structures in lipid bilayers^{9–12}. These limits seem to be mostly due to sample heterogeneity and dynamics, which both lead to increased resonance line widths and thus spectral overlap in crowded ssNMR spectra. Line widths may also be inhomogeneously broadened in highly concentrated, non-crystalline samples that are prone to aggregation in some ssNMR studies. Dynamics and resonance line widths also pose problems to solution NMR studies of integral membrane proteins, but cutoffs for complete backbone resonance assignments currently approach about 40 kDa for solution NMR.^{13–16} In addition, careful attention needs to be paid to the choice of the membrane mimetic in solution NMR to ensure that it supports the native structure and function of the protein.¹⁷ Despite these limitations, NMR spectroscopy has become very popular to study membrane proteins because it is the only method that can provide detailed residue-by-residue dynamic information on these proteins without labeling them with reporter groups that are generally required for EPR or fluorescence spectroscopy of membrane proteins. In addition to solving complete structures of membrane proteins, NMR now also finds wide application when combined with selective isotope labeling to measure interactions with selected ligands and to probe physiologically important conformational changes. Since sample preparation is critical in all solution and ss-NMR applications of membrane proteins, in box 1 we introduce the very different kinds of samples that are commonly used in membrane protein NMR spectroscopy and we discuss each of their advantages and limitations.

Box 1**Sample Preparation for Solution and Solid-State NMR of Membrane Proteins** **Micelles**

The most popular and most simple way to prepare membrane proteins for solution NMR is to disperse them in lipid micelles. Lipids with relatively mild detergent activity are preferred. Dodecylphosphocholine (DPC), which has a phosphocholine headgroup like most phospholipids do and a single twelve carbon aliphatic chain, has been one of the most popular detergents to study membrane proteins by solution NMR.^{15,18,21,24–25,37,55,76–77} Short chain phospholipids such as dihexanoyl-phosphatidylcholine (DHPC)^{29,78–79} or diheptanoylphosphatidylcholine^{20,80} also form micelles and have been popular for solution NMR studies of membrane proteins. Other detergents that have been used occasionally include lyso-phosphatidylglycerol, lauryl-dimethylamine oxide (LDAO), n-dodecyl- β -maltoside (DDM), and octyl- β -glucoside (β -OG).

Bicelles are mixtures of bilayer-forming phospholipids, most commonly dimyristoyl-phosphatidylcholine (DMPC), and non-bilayer forming lipids, most commonly DHPC. The ratio between long- and short-chain lipid is referred to as the q -value. They form ellipsoid structures with sizes that depend on the ratio between the two kinds of lipids. For solution NMR, only relatively small bicelles with excess non-bilayer forming lipids are useful ($q=0.25$ to 0.5).⁸¹ As bicelles get larger with increasing excess of bilayer-forming lipid, they become flatter and eventually connect to form sheets of bilayers with DHPC-lined holes. The larger bicelles ($q=2.5$ and larger) can be oriented in the magnetic field to perform ssNMR experiments.³⁴ Membrane proteins have been incorporated in both kinds of bicelles. Bicelles are more bilayer-like and therefore may be better models than micelles for studying membrane proteins in a quasi-bilayer-like environment. However, the small q “isotropic” bicelles that undergo rotational diffusion that is fast enough to produce well-resolved resonances for solution NMR experiments exhibit a mostly micellar character. The bilayer and non-bilayer forming lipids presumably diffuse

rapidly within these structures and most likely both kinds of lipids exchange rapidly with the surface of the embedded protein.

Nanodiscs are small patches of lipid bilayer surrounded by segments of amphipathic helical proteins that stabilize these patches.⁸² The most commonly used nanodiscs are formed with two copies of the lipoprotein MSPD1 at their perimeter. They contain about 160 lipids per particle leading to a molecular mass of 150 kDa per empty particle. The size of these nanodiscs is at the upper end of still being useful for obtaining highly resolved solution NMR spectra of included membrane proteins. To improve nanodiscs for such studies, shorter versions of MSPD1 have been designed.²⁶ They produce smaller nanodiscs with only 40 to 120 lipids per particle (60 to 120 kDa, without embedded membrane protein). Indeed, higher quality NMR spectra of embedded membrane proteins could be obtained with some of these. These systems are probably the most bilayer-like and therefore the most physiological environments that can be used for solution NMR spectroscopy of membrane proteins. Membrane proteins have also been studied in amphipols⁸³ and lipodiscs⁸⁴.

Oriented bilayers

Stacks of lipid bilayers oriented between glass plates or large bicelles oriented by the external magnetic field have been frequently used in ssNMR experiments of membrane proteins.³⁴ In these approaches, orientations of chemical bonds relative to the magnetic field and, therefore, relative to the lipid bilayers are measured and used as inputs for structure calculations.

Unoriented bilayers

Magic angle spinning (MAS) solid-state NMR works well with microcrystalline, precipitated, or proteoliposome-reconstituted membrane proteins in proteoliposomes that are packed at high density into MAS rotors.^{31–32} In this case, structures are determined mostly from measured inter-nuclear correlations, from which distances between the correlated nuclei are obtained. As in NOE-based approaches in solution NMR, these distances serve as the experimental constraints to calculate the structures of microcrystalline or bilayer-embedded membrane proteins.³²

Structure Determination

Solution NMR

Heteronuclear NMR methods that have been developed for soluble proteins can be readily applied to membrane proteins. For structure determination, the proteins are typically uniformly ¹⁵N- and ¹³C-labeled by expression in minimal media containing ¹⁵N-ammonium sulfate and ¹³C-glucose as nitrogen and carbon sources. To improve spectral resolution protein side-chains are frequently deuterated by using ²H,¹³C-glucose instead of ¹³C-glucose. Cultures are grown in ²H₂O and amide protons are back-exchanged during protein purification and sample preparation. Side-chain deuteration reduces the ¹³C relaxation rates leading to narrower resonances, but the drawback is that much fewer protons are present for measurement of ¹H-NOEs that, apart from chemical shift-derived dihedral angle

information, provide distance constraints for structure determination. Therefore, often only backbone conformations are reported for earlier solution NMR structures of larger membrane proteins. Much progress has been made in the 15 years since the first NMR structure of a larger membrane protein has been reported.¹⁸ For example, protons of selected amino acids¹⁹ have been re-introduced by selective labeling in a perdeuterated background, which has led to better defined structures (Figure 1)^{20–21}. Paramagnetic relaxation enhancement (PRE) and residual dipolar coupling (RDC) have also significantly improved the solution structures of several membrane proteins^{22–25}. So far, only relatively few studies have used bicelles and even fewer have used nanodiscs as bilayer mimicking environments. Importantly, OmpX in nanodiscs²⁶ exhibited only relatively small differences compared to its counterpart in micelles, validating the micelle as a suitable lipid environment. Comparative studies with VDAC-1²⁷, as well as OprH and OprG in nanodiscs²⁸ show that the loops of these proteins are still disordered in this bilayer-like environment as they are in micelles (the structure of OprG solved in micelles²⁹ is shown in Figure 1), whereas more structured loops were observed when these proteins were crystallized and the proteins made crystal contacts through these loops, indicating that the crystal and not the micelle environment is problematic for these proteins.

Solid-state NMR

Although ¹H-detected experiments have recently gained popularity³⁰, heteronuclear detection is more typical in ssNMR because the very strong dipolar couplings of protons produce extremely broad static linewidths (tens of kHz) that require very high MAS frequencies to be narrowed to well-resolved resonances. For uniform labeling, proteins are often expressed in ¹³C-glycerol minimal media for ¹³C-¹³C correlated spectroscopy and additionally in ¹⁵N-ammonium sulfate for ¹³C-¹⁵N correlated spectroscopy. ¹³C-detected experiments are performed to assign spin-systems and heteronuclear experiments are used to sequentially assign neighboring spin systems.^{31–32} As in solution NMR, dihedral angles from the assigned chemical shifts, and more recently, PREs have been used to supplement structure calculations (Figure 1).⁷ Proton driven spin diffusion (PDSF) or dipolar-assisted rotational resonance (DARR) experiments are frequently used to measure internuclear distances to determine MAS ssNMR structures. A single uniformly ¹³C, ¹⁵N-labeled microcrystalline sample was sufficient to determine the structure of the transmembrane domain of the *Yersinia enterocolitica* adhesin A (YadA) (Figure 1).⁵ Rotational or transferred echo double resonance (REDOR/TEDOR) experiments provide distance information and are mostly used with selectively labeled samples to obtain specific long-range distances and to distinguish between different molecular models rather than for complete structure determinations.³³

A completely different ssNMR method uses oriented bilayers that are either deposited on stacks of glass coverslips or oriented in the magnetic field. Since chemical shifts in solid samples are strongly orientation-dependent and since bond vectors in α -helices are circularly repetitive, tilt angles of TM α -helices in membranes can be determined with high precision. Polarization-inversion spin exchange at the magic angle (PISEMA) experiments, which measure orientation-dependent ¹⁵N chemical shifts and ¹⁵N-¹H dipolar couplings, are very popular to obtain these measurements.³⁴ Orientations of selectively deuterated side-

chains are frequently derived from quadrupole splittings of orientated ^2H -NMR spectra and have been used to obtain their conformations.³⁵ All of these methods depend strongly on the quality of bilayer alignment because mosaic spread of the alignment leads to broadened line widths. The structures of several small membrane proteins have been solved by oriented ssNMR.

Relatively recently, rotationally aligned ssNMR, which combines the advantages of MAS- and oriented-sample NMR, was developed to solve the backbone structure of the chemokine receptor CXCR1, a seven-helix G-protein coupled receptor (GPCR).⁶ It is noteworthy that molecular fragment replacement (MFR) with CS-Rosetta³⁶ was an important component of this structure determination. RDC-based MFR in addition to PREs has also been successfully applied to solve the solution NMR structure of the mitochondrial uncoupling protein 2.³⁷ A hybrid solution and solid-state NMR approach was employed to solve the structure of pentameric phospholamban in lipid bilayers.³⁸

Dynamics

NMR is the pre-eminent method to obtain residue-specific dynamic information on macromolecules including membrane proteins. NMR provides dynamic information ranging from ps to s down to atomic resolution.³⁹ Essential biological functions, such as conformational exchange, ligand or inhibitor binding, folding and unfolding, and allosteric regulations in membrane proteins can be detected by employing a wide range of NMR techniques. Traditional NMR dynamics studies probe site-specific motions by measurement of longitudinal (R_1) and transverse (R_2) relaxation rates and heteronuclear NOEs. These experiments measure motions in the ps-ns time range and provide valuable information on whether the protein is well-structured, flexible, or completely disordered. In addition, the model-free formalism can be employed to separate internal from global motions in a residue specific manner, and, in favorable cases, extract slow ms exchange processes.⁴⁰ An extended model-free analysis of the β -barrel membrane protein OmpA revealed a rotation of the protein within the micelle. This analysis also found backbone segmental motions that increased the further the residues were from the mid-plane of the membrane.⁴¹

Carr-Purcell-Meiboom-Gill (CPMG) and $R_{1\rho}$ relaxation dispersion experiments are designed to capture μs -ms conformational exchange processes amidst a collection of modulated relaxation rate constants upon the change of the “spin-echo” or effective “spin-locked” radio frequency field strength, respectively.³⁹ A comprehensive CPMG relaxation dispersion study revealed that slow conformational exchanges in the ADP/ATP carrier (AAC) were asymmetrically distributed, with large exchanges concentrated in domain I despite its unique three-fold quasi-symmetry.⁴² The exchange rate of the TM region was increased or decreased substantially when a substrate or inhibitor bound, respectively. Surprisingly, the relative populations of the bound and unbound states did not change significantly, suggesting that ligand binding changed the energy barrier of conversion by stabilizing (substrate) or destabilizing (inhibitor) the transition state.

ZZ-exchange is a powerful technique to measure ms-s conformational exchange rates, i.e., rates that are slow on the NMR timescale. Morrison et al showed that the homodimeric small

multidrug resistance transporter EmrE adopts an antiparallel topology.⁴³ Using TROSY ZZ-exchange experiments, they further demonstrated that the tetraphenylphosphonium (TPP⁺)-bound form of EmrE interconverted with equal rates between the inward and outward facing conformations. The interconversion rates changed with different substrates by almost 3 orders of magnitude, i.e., a property that may be unique for multidrug recognition and transport.⁴⁴ Combined solution and ssNMR dynamics measurements were used to determine the rates of conformational exchange of apo- and TPP⁺-bound forms of EmrE, with that of apo-EmrE exchanging 50-fold faster than that of TPP⁺-EmrE.⁸ Interestingly and importantly, the conformational exchange of EmrE did not depend on whether the experiments were performed in small isotropic $q=0.33$ bicelles, larger $q=3.2$ bicelles, or in DMPC lipid bilayers. A generic model of functional and dynamical aspects of a membrane transporter that can be studied by NMR is shown in Figure 2a.

Fast (ps-ns) and slow (μ s-s) time-scale dynamics were connected in a study on the bacterial outer membrane enzyme PagP.⁴⁵ The fast dynamics revealed that a long extracellular loop had an elevated mobility when the protein was solvated in DPC, which presumably permitted a DPC molecule to enter and block the active site. However, in the detergent CYFOS-7 that cannot enter the active site in the lumen of the β -barrel structure, PagP exchanged between at least two conformations in order to catalyze lipid synthesis.⁴⁶ ZZ-exchange and CPMG relaxation dispersion experiments revealed significant conformational exchange occurring in the mobile loop and adjacent catalytic residues of PagP. A palmitate may enter the active site in the more flexible conformation, followed by catalysis in the more rigid conformation.

Intermediate time-scale protein dynamics in the ns- μ s range can be detected by PRE or RDC measurements using ensemble approaches. Extensive PRE measurements on OmpG porin were analyzed by fitting the data to multiple structural ensembles.⁴⁷ This analysis revealed that some extracellular loops adopt three different exchanging conformations in lipid bicelles. The three conformations likely represent the open, closed, and an intermediate state of the pH-gated pore of OmpG.

Functional Studies

pH titration

Determinations of pKa's by NMR provide unique residue-specific information on the ionization states of amino acid residues, which frequently modulate protein function. In homodimeric EmrE, coupled deprotonation of two equivalent glutamates from each monomer were thought to be prerequisite for substrate binding. However, a recent pH-titration NMR study showed that the two glutamates are not equivalent and have distinct pKa values.⁴⁸ Interestingly, apo-EmrE switches conformations in an only partially protonated state, questioning the established single-site alternating access model. This latter observation first made by solution NMR has been confirmed in a more recent combined solution/ss-NMR study.⁴⁹ The results revealed that previously unknown intermediate states may exist and thus offer new mechanistic details on transport and possible additional functions of EmrE in the membrane.

Small ligand screening

Saturation transfer difference (STD) or saturation transfer double difference (STDD) NMR, which works best for weakly bound ligands, is a sensitive routine method for drug screening of many soluble proteins. However, although membrane proteins constitute more than half of all drug targets⁵⁰, STD so far has enjoyed only limited use for membrane proteins, probably due to the complicating presence of the membrane mimetic. Recent STD-NMR studies employed membrane proteins purified from eukaryotic cell membranes.^{51–52} Binding was only weakly dependent on the ligand⁵² or mutant⁵¹ that was used. However, new developments may be needed to expand the use of this method in the future. When the ligand undergoes fast exchange between bound and free states, transferred NOE spectroscopy can be applied to determine the structure of the bound state,⁵³ which is important for drug design. For tightly-bound ligands ($K_d \sim \text{nM}$), inter-molecular NOEs have been employed to determine protein-drug complexes at high resolution.^{21,24,54–55} ^2H -NMR is an excellent method to measure the orientation and dynamics of bound ligands in the solid state. The binding site and orientation of the anti-influenza drug amantadine when bound to the influenza M2 proton channel in a lipid bilayer has been determined by this method.¹⁰

Protein-protein interactions

In principle very similar to STD experiments, cross-saturation (CS) and transferred cross-saturation (TCS) can detect the interface of protein-protein complexes. In these solution NMR experiments, unlabeled membrane proteins can be stabilized in micelles, bicelles, nanodiscs, or even in liposomes, whereas the directly detected protein, usually soluble, is deuterated for optimal detection.⁵⁶ This method has been applied to identify the precise binding interface between a chemokine and the GPCR CXCR4 or plastocyanin and photosynthetic membrane proteins and provides mechanistic insight into these respective interactions.^{57–58} More traditional NMR chemical shift perturbation and resonance line-broadening approaches have been employed to detect the interaction of arrestin with rhodopsin.⁵⁹ Since ssNMR methods have no principle limitations on size, both protein partners can be directly observed by exploring creative isotope labeling strategies in combination with specific ssNMR pulse schemes.⁶⁰ SsNMR experiments also revealed that alternating interactions of phospholamban with the sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) regulate the Ca^{2+} pump activity, which depends on the equilibria between transient conformational states of phospholamban.⁶¹

Protein-ligand interactions

GPCRs contain multiple binding sites for agonists and antagonists and some of these work by allosteric mechanisms. Solution^{62–63} and solid-state⁶⁴ NMR chemical shift perturbations and lineshape analyses have been employed to reveal mechanisms of activation and inactivation and to probe the dynamics of GPCRs. Selective isotopic labeling during protein biosynthesis^{63,65–66} and chemical conjugation with isotope-labeled compounds, either dimethylation of lysines^{62,67} or alkylation of cysteines^{68–70}, have been used to gather site-specific information and to avoid presently intractable spectral overlap of a uniformly labeled sample. Biosynthetic labeling schemes potentially access any labeling sites, even those in the hydrophobic environment of the membrane protein, whereas chemical labeling

is limited by solvent accessibility and the efficiency of the chemical reactions. Despite this limitation, chemical labeling has the advantage that highly sensitive nuclei, such as fluorine-19, can be incorporated, leading to excellent signal/noise. Liu et al employed ^{19}F -NMR to observe conformational changes of two cytoplasmic residues upon binding of a diverse array of ligands on the extracellular side of the β 2-adrenergic receptor (AR).⁶⁸ These results revealed that long-range structural plasticity is critical for signal transduction and that different pharmacological ligands shift populations and affect the selection of preexisting conformations.⁷¹ Biosynthetic methionine labeling enabled the direct observation of a conformational change of the TM regions upon activation of the AR.^{63,65} Interestingly, the rate of changing the AR into the active state was slower and the population of the active state was higher when the receptor was solubilized in nanodiscs instead of micelles.⁷² A generic model of ligand (agonist/antagonist) interactions with GPCRs and possibilities of allosteric regulation that can be studied by NMR is shown in Figure 2b. The conformational and dynamic heterogeneity observed in these and other recent NMR studies provides novel insights into membrane protein function that would be impossible to obtain by crystallography alone.

Conclusions and future directions

NMR of membrane proteins has come a long way since the first applications to membrane-bound peptides in the late 1970s. Structures of pore forming peptides, peptide hormones and antibiotics, and membrane-inserted phage coat peptides were studied since these early days by solution and solid-state NMR methods. Like for soluble protein NMR, the advent of heteronuclear multidimensional methods have revolutionized membrane protein solution and ss-NMR in the last 15 years and permitted structural and dynamical studies to be extended to membrane proteins in the 10–40 kDa range. This range occupies a unique niche for membrane proteins that are difficult to crystallize and that are too small to be suitable for current electron cryo-microscopy, which also has seen a revolution in the last few years. As for all structural techniques, sample preparation continues to be a challenge with membrane proteins.¹⁷ Functional and other independent experiments are necessary to validate structures and to ascertain that crystallization conditions, lipid micelles, or low hydration and high protein concentrations in lipid bilayers do not distort the structures. Membrane protein NMR also plays a unique role to examine conformational transitions of partially folded membrane proteins such as those involved in intracellular and viral membrane fusion.^{73–75} Hybrid structural techniques are becoming increasingly popular and the combination of NMR with electron cryo-microscopy, x-ray crystallography, EPR and fluorescence spectroscopy, and computation will likely find increased use in the future. Particularly promising areas are NMR studies of conformational changes of membrane proteins in response to small molecule and protein ligands and the changing lipid environment at different physiological states of cellular membranes. Indeed, exploration of the structural and mechanistic biology of membrane proteins by NMR has a bright future and will undoubtedly bring many new exciting discoveries and perhaps even surprises in our current understanding of this important, ubiquitous, and highly druggable class of proteins.

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Figure 1.

A selection of recent *de novo* membrane protein structures determined by solution or solid-state NMR. Protein abbreviations (PDB accession codes) and their organisms of origin are: OprG (2N6L): outer membrane protein G from *Pseudomonas aeruginosa*, a transporter for small amino acids²⁹; YadA (2LME): the transmembrane domain of the adhesin A from *Yersinia enterocolitica*, an outer membrane autotransporter⁵; pSRII (2KSY): phototaxis receptor sensory rhodopsin II from *Natronomonas pharaonis*²⁰; TSPO (2MGY): mitochondrial translocator protein from *mouse*, a cholesterol and porphyrin importer into mitochondria²¹; ASR (2M3G): sensory rhodopsin from cyanobacterium *Anabaena*⁷. β -sheet (top row) or α -helical (bottom row) transmembrane segments are rainbow-color coded from red to pink in the order of their amino acid sequences (N- to C-termini), with additional α -helical (top row) or β -sheet (bottom row) structures colored in cyan. β -barrel proteins (top row) are positioned with the extracellular sides on top and α -helical proteins (bottom row) are positioned with cytosolic or intra-mitochondrial space on the bottom.

Figure 2.

Membrane protein function and dynamics as elucidated by NMR. (a) Dynamics of a transporter as modulated by the addition of a ligand. Conformational exchange between the global outward-facing and inward-facing states may be faster in the un-liganded apo-form than in the ligand-bound form. Conformational exchange dynamics may be further modified by the chemical nature of different ligands. (b) G-protein coupled receptors undergo conformational exchange in the un-liganded apo-state. The motions of different domains may be connected, but not strictly coupled. Different agonists and antagonists bound to one domain may differently affect the equilibrium of activated and inactivated states of another domain. This simplified cartoon shows only a two-state exchange of the two transmembrane helices of the seven-helix receptor that participate in ligand binding, but each state may in reality consist of multiple local conformational sub-states and, in addition, also involves coupled conformational changes of adjacent helices.