J Biomol NMR (2011) 50:191–195
DOI 10.1007/s10858-011-9523-3

COMMUNICATION

Electrostatically-driven fast association and perdeuteration allow detection of transferred cross-relaxation for G protein-coupled receptor ligands with equilibrium dissociation constants in the high-to-low nanomolar range

Laurent J. Catoire · Marjorie Damian · Marc Baaden · Éric Guittet · Jean-Louis Banères

Received: 25 March 2011 / Accepted: 25 May 2011 / Published online: 19 June 2011

Abstract The mechanism of signal transduction mediated by G protein-coupled receptors is a subject of intense research in pharmacological and structural biology. Ligand association to the receptor constitutes a critical event in the activation process. Solution-state NMR can be amenable to high-resolution structure determination of agonist molecules in their receptor-bound state by detecting dipolar interactions in a transferred mode, even with equilibrium dissociation constants below the micromolar range. This is possible in the case of an inherent ultra-fast diffusive association of charged ligands onto a highly charged extracellular surface, and by slowing down the ^{1}H–^{1}H cross-relaxation by perdeuterating the receptor. Here, we demonstrate this for two fatty acid molecules in interaction with the leukotriene BLT2 receptor, for which both ligands display a submicromolar affinity.

Keywords Kinetics · Transferred NOE · G protein-coupled receptor · Signal transduction · Structural biology

Electronic supplementary material The online version of this article (doi:10.1007/s10858-011-9523-3) contains supplementary material, which is available to authorized users.

L. J. Catoire (✉)
Laboratoire de Biologie Physico-Chimique des Protéines Membranaires, UMR 7099 CNRS/Université Paris-7, Institut de Biologie Physico-Chimique (FRC 550), 13 rue Pierre et Marie Curie, 75005 Paris, France
e-mail: laurent.catoire@ibpc.fr

M. Damian · J.-L. Banères
Institut des Biomolécules Max Mousseron, UMR 5247 CNRS, Université Montpellier 1 and Université Montpellier 2, 15 Avenue C. Flahault, 34093 Montpellier, France

M. Baaden
Laboratoire de Biochimie Théorique, UPR 9080 CNRS/ Université Paris-7, Institut de Biologie Physico-Chimique (FRC 550), 13 rue Pierre et Marie Curie, 75005 Paris, France

É. Guittet
Centre de Recherche de Gif, Laboratoire de Chimie et Biologie Structurales, ICSN, UPR 2301 CNRS, 91198 Gif-sur-Yvette, France

G protein-coupled receptors (GPCRs) are integral membrane proteins encountered in many eukaryotic tissues (Bockaert and Pin 1999). They represent the predominant mediators of signal transduction between the exterior and the interior of the cells. Understanding the molecular basis of GPCR activation requires a detailed knowledge of the conformational changes occurring on the receptor upon the binding of a ligand (Rosenbaum et al. 2009; Nygaard et al. 2009). In this respect, determining high resolution structures of agonists in their receptor-bound state represents an important achievement. Even though X-ray crystallography is the method of choice to obtain structural information on GPCRs in different sub-states (e.g. Cherezov et al. 2007; Rasmussen et al. 2011; Rosenbaum et al. 2011), NMR spectroscopy is a valuable alternative to study the bound structure of agonists (Inooka et al. 2001; Luca et al. 2003; Kofuku et al. 2009; Catoire et al. 2010). In solution, one of the most powerful approaches is to collect dipolar interactions in a transferred mode (trNOE) (Balaram et al. 1972). However, the inherent association properties of agonists, including equilibrium dissociation constants (K_d) in the nanomolar (nM) range, in addition to the long overall correlation times (τ_c) of GPCR/surfactant complexes, usually make the kinetics of exchange too slow compared to the auto-(ρ) and cross-relaxation (σ) rates (Clore and Gronenborn 1982, 1983; Campbell and Sykes 1993; Williamson

2006). Two factors can extend the range of trNOE measurements: an electrostatically-driven association combined with the perdeuteration of the receptor. This is illustrated here with two fatty acid compounds, namely leukotriene B4 (Borgeat et al. 1976) (LTB4; 5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid) and the heptadecanoid 12-HHT (Hamberg et al. 1974) (12*S*-hydroxyheptadeca-5*Z*,8*E*,10*E*-trienoic acid) (Fig. 1A), in the presence of perdeuterated human BLT2 receptor (e.g. Yokomizo et al. 2000) (u-^{2}H-BLT2) stabilized in solution by amphipols (Tribet et al. 1996; Dahmane et al. 2009; Popot 2010; Popot et al. 2011) (see Material and Methods in Supplementary Material, SM).

Kinetic experiments (Fig. 1B) indicate that the dissociation rate constants k_{off} for LTB4 and 12-HHT are 50 and 18 s^{-1}, respectively. This translates into an approximately 3.6 times longer bound time for 12-HHT than LTB4. Interestingly, 12-HHT, that has been described as the putative natural ligand for BLT2 (Okuno et al. 2008; Iizuka et al. 2010), displays a higher efficiency in BLT2-mediated calcium mobilization than LTB4 (Okuno et al. 2008). Our data are thus in agreement with recent models that correlate the intrinsic ligand efficacy in signaling with the mean lifetime of the agonist/receptor complexes (Sykes et al. 2009). This would mean that, as previously suggested (e.g. Sykes et al. 2009), the equilibrium binding properties, i.e. how tightly ligands bind to receptors, do not fully account for the signaling efficacy.

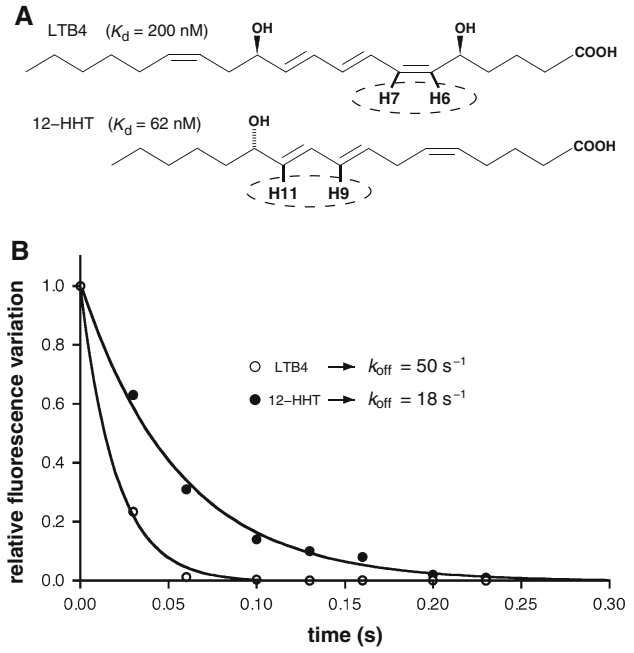

Fig. 1 **A** Chemical structures of LTB4 and 12-HHT with the corresponding *in vitro* affinities for BLT2 (*cf.* SM Fig. S1). The two-spin systems used in Fig. 4 are *circled* with a *dashed line*. **B** Time-dependent decrease in fluorescence signal due to complex formation between LTB4-568 (Sabirsh et al. 2005) and BLT2 in the presence of either LTB4 (*open circles*) or 12-HHT (*closed circles*). The dissociation rates were obtained from these plots as reported in SM § A2

Association rate constants k_{on} of $2.5 \times 10^{8}\ M^{-1} \cdot s^{-1}$ for LTB4 and $2.9 \times 10^{8}\ M^{-1} \cdot s^{-1}$ for 12-HHT can be derived from the experimentally measured K_d values (SM Fig. S1) of 200 and 62 nM. These values indicate that 12-HHT binds onto BLT2 slightly faster than LTB4. Both k_{on} exceed by $\times 3$ the limit usually—but improperly—cited in the literature for biomolecular diffusional associations. These fast-associating k_{on} are not physically unrealistic, however, even for large biomolecules, cases of protein–protein association have been reported with k_{on} values close to or in excess of $10^{9}\ M^{-1} \cdot s^{-1}$ (Schreiber and Fersht 1996; Gabdoulline and Wade 2002). In this case, electrostatic interactions prevail because of their long-range nature, while they do not affect k_{off}, which is governed by short range interactions, including van der Waals and hydrophobic interactions, salt bridges and hydrogen bonds. Indeed, both agonists have a net charge of -1 and interact with the highly positively charged extracellular surface of the receptor (Fig. 2). The electrostatic potential of the latter was calculated for a model of the BLT2 receptor after a 0.5 μs molecular dynamics simulation in a fully hydrated lipid bilayer (SM Fig. S2). This simulation suggests a well-accessible binding pocket situated close to the surface (Fig. 2B), as observed for other class A GPCRs (Nygaard et al. 2009).

Proton NMR relaxation rates of ^{1}H natural abundance in macromolecules are governed by indirect dipolar pathways. Deuteration of the receptor reduces spin diffusion, and, by doing so, substantially diminishes the rates of relaxation processes (e.g. Markus et al. 1994). As a consequence, dilution of the ^{1}H thermal bath allows the use of a longer NOESY mixing time (τ_m), the detection of longer interdipolar distances, and can shift the limit of trNOE observation towards higher affinities. Moreover, internal motions preceding the dissociation event can also facilitate the observation of trNOE by decreasing the ρ and σ values.

12-HHT in solution in excess with respect to the BLT2 receptor gives rise to trNOEs (Fig. 3). As already observed with LTB4 (Catoire et al. 2010), most of the cross-peaks between olefinic and aliphatic protons appear only in the presence of the wild-type receptor. This is in contrast with those corresponding to intra-olefinic interactions, or between an olefinic proton with an adjacent aliphatic proton, that can be observed with the free ligand in solution, or in the presence of either amphipol only or with a BLT2 mutant that does not bind specifically 12-HHT (see SM § C). In other words, except in rigid parts of the molecule that are located at or close to the unsaturations, both

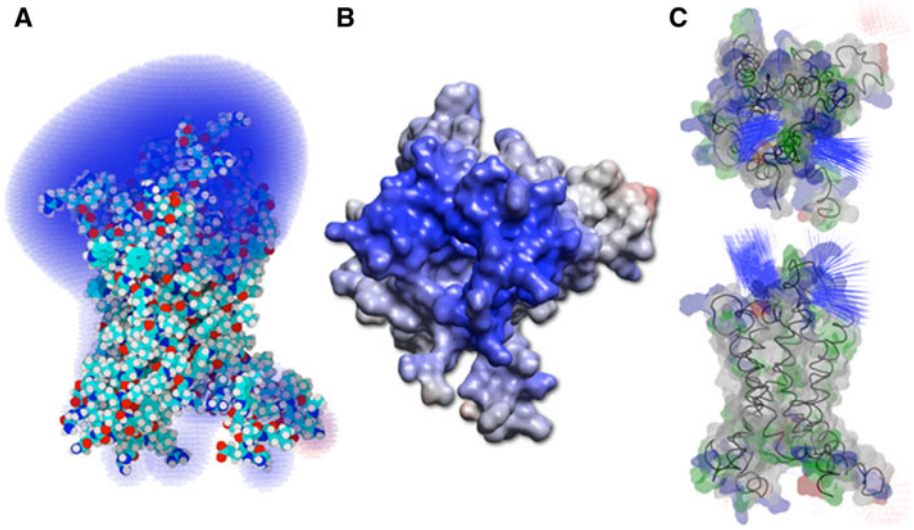

Fig. 2 Electrostatic potential (Ep) of a BLT2 receptor model calculated on a simulation snapshot. The Ep maps are colored from −400 kT/e in *red* to +400 kT/e in *blue*. In **A**, the reach of the receptor's Ep is illustrated by a cloud. In this side view the extracellular ligand binding site is located at the top. **B** shows a top view of the binding site surface colored by Ep. **C** illustrates Ep field lines in a combined top/side view. **A** and **B** were prepared with Yasara (Krieger et al. 2002), and **C** with VMD (Humphrey et al. 1996) (see SM § A3 and the video file available as SM)

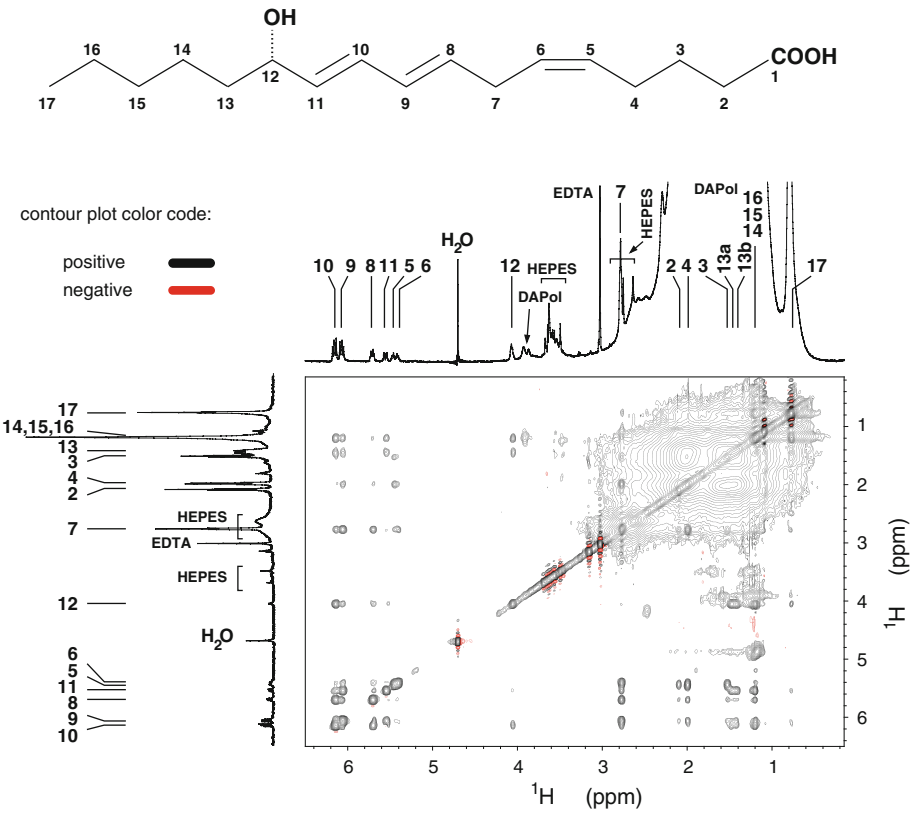

Fig. 3 ^{1}H–^{1}H dipolar interactions in 12-HHT (110 μM) in the presence of u-^{2}H-wild-type BLT2 (12 μM, K_d = 62 nM) associated with partially deuterated amphipols (DAPol) (receptor/DAPol ratio of 1:5 (w/w)) observed in a 2D NOESY spectrum (illustration with τ_m = 0.4 s). The corresponding 1D ^{1}H spectrum is shown above the 2D spectrum. 1D spectrum of free fatty acid molecule in solution is displayed on the left side. Numbers refer to the annotated protons on the corresponding 12-HHT chemical structure above the spectrum. The NMR experiments were carried out at 25°C (see the time-dependent stability of A8-35-folded BLT2 at this temperature in Catoire et al. 2010) and 600 MHz on a Bruker Avance spectrometer equipped with a cryoprobe. The following parameters were used for 2D NOESY experiments: data size = 256(t_1) × 8,192(t_2) complex points, t_{1max} = 32 ms, t_{2max} = 511 ms, 256 acquisitions per increment. ^{1}H chemical shifts are referenced to H_2O (calibrated at 4.7 ppm at 25°C). Chemical shift assignments are based on a COSY spectrum (data not shown). Data processing was performed and analyzed with the TOPSPIN software

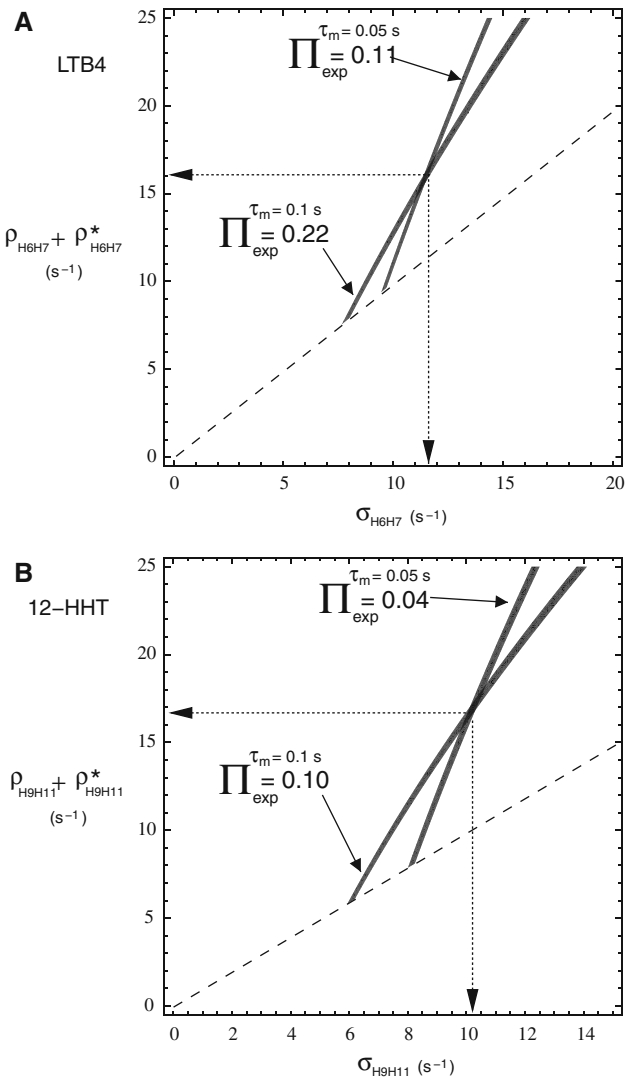

Fig. 4 Simultaneous graphical estimation of auto (ρ) and cross (σ) relaxation rate constants of LTB4 and 12-HHT in their BLT2-bound states in the presence of chemical exchange (see respective k_{off} values in Fig. 1B). **A** and **B** correspond to superimposed projections along the cross to diagonal NOESY peak volume ratio, Π, axis of contour plots of theoretical Π taken at experimental Π values. NOESY volumes are measured at two mixing times (τ_m) (see corresponding LTB4/BLT2 and 12-HHT/BLT2 NOESY spectra in Catoire et al. 2010 and SM § C, respectively). Illustration with the dipolar interactions between nuclei H6 and H7 of LTB4 (**A**) and H9 and H11 of 12-HHT (**B**) in the presence of ~9-fold excess of ligand over u-^{2}H-BLT2. ρ* represents other non-dipolar relaxation contributions and/or a contribution from some other spins of the lattice. Contour lines are drawn for $\rho + \rho*$ greater than or equal to σ (above the *dashed line*) (see SM § B for any details on this graphical method)

extremities of the unsaturated fatty acid molecule remain unstructured in the absence of the wild-type receptor. A positive control using LTB4 as competing agonist confirms the specificity of the detected interaction (SM § C).

To demonstrate that trNOE can be observed with tight-binding ligands, ρ and σ in the bound state can be estimated from 2D NOESY experiments in the presence of chemical exchange (Ni 1992). One convenient way is to use a graphical approach where both ρ and σ can be estimated simultaneously from the experimental ratio of cross to diagonal peak volumes, Π_{exp}, knowing the k_{off}, the relative population of ligand *vs.* receptor, and ρ in the free state (see SM § B). Fig. 4 illustrates two examples in the case of strong dipolar interactions, i.e. corresponding to short inter-proton distances in a rigid part of both molecules. In particular, Fig. 4 indicates values of σ close to 10 s^{-1}, i.e. below the respective dissociation rates of LTB4 and 12-HHT (Fig. 1), fulfilling one of the most stringent criteria to observe trNOE when τ_c of large complexes become very long (Clore and Gronenborn 1982, 1983; Campbell and Sykes 1993; Williamson 2006). Hence, BLT2/amphipol complexes in solution, which display a τ_c of ~55 ns (SM Fig. S3 and S4), are compatible with the observation of trNOE in the case of slow-intermediate (Levitt 2001) chemical exchange.

Without a significant coulombic contribution to the interaction, i.e. with k_{on} of $\sim 10^8\ M^{-1} \cdot s^{-1}$, and/or unhindered access of the ligand to the binding site, perdeuteration would not be sufficient. Fortunately, this accelerated diffusive association does not seem to be specific of BLT2. For instance, the β_2 adrenergic receptor, which is also characterized by a highly positively charged extracellular surface (SM Fig. S5), has diffusive agonists that associate with k_{on} close to or higher than those measured here (Hegener et al. 2004). On the nuclear longitudinal relaxation timescale, this provides the opportunity to study structures of tight-binding ligands, i.e. with K_d of a few tens of nM, bridging the gap between pharmacology and NMR.

Acknowledgments We are extremely grateful to Fabrice Giusti (UMR 7099) for the gift of deuterated surfactant amphipols. We thank Monica Zoppè (Scientific Visualization Unit, Inst. of Clinical Physiol., Pisa, Italy) for access to innovative visualization methods to illustrate the strong electrostatic potential gradient of BLT2. Particular thanks are due to Jean-Luc Popot (UMR 7099) and Jacky Marie (UMR 5247) for helpful comments on this manuscript. This work was supported by the Centre National de la Recherche Scientifique (CNRS), Paris-7 University, and by grants from the E.U. (Specific Targeted Research Project LSHG-CT-2005-513770 IMPS Innovative tools for membrane protein structural proteomics) and from the French Ministry of Research (ANR-06-BLAN-0087 and ANR BLAN07-1_191475). L.J.C. is a 2009 recipient of Projets Exploratoires/ Premier Soutien (PEPS, *Leukomotive* project) from the CNRS.

References

Balaram P, Bothner-By AA, Dadok J (1972) Negative nuclear Overhauser effects as probes of macromolecular structure. J Am Chem Soc 94:4015–4017

Bockaert J, Pin JP (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. EMBO J 18:1723–1729

Borgeat P, Hamberg M, Samuelsson B (1976) Transformation of arachidonic acid and homo-gamma-linolenic acid by rabbit polymorphonuclear leukocytes. Monohydroxy acids from novel lipoxygenases. J Biol Chem 251:7816–7820

Campbell P, Sykes BD (1993) The two-dimensional transferred nuclear Overhauser effect: theory and practice. Annu Rev Biophys Biomol Struct 22:99–122

Catoire LJ, Damian M, Giusti F, Martin A, van Heijenoort C, Popot JL, Guittet E, Banères JL (2010) Structure of a GPCR ligand in its receptor-bound state: leukotriene B4 adopts a highly constrained conformation when associated to human BLT2. J Am Chem Soc 132:9049–9057

Cherezov V, Rosenbaum DM, Hanson MA et al (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. Science 318:1258–1265

Clore GM, Gronenborn AM (1982) Theory and applications of the transferred nuclear Overhauser effect to the study of the conformations of small ligands bound to proteins. J Magn Res 48:402–417

Clore GM, Gronenborn AM (1983) Theory of the time dependent transferred nuclear Overhauser effect: applications to structural analysis of ligand–protein complexes in solution. J Magn Res 53:423–442

Dahmane T, Damian M, Mary S, Popot JL, Banères JL (2009) Amphipol-assisted in vitro folding of G protein-coupled receptors. Biochemistry 48:6516–6521

Gabdoulline RR, Wade RC (2002) Biomolecular diffusional association. Curr Opin Struct Biol 12:204–213

Hamberg M, Svensson J, Samuelsson B (1974) Prostaglandin endoperoxides. A new concept concerning the mode of action and release of prostaglandins. Proc Natl Acad Sci USA 71:3824–3828

Hegener O, Prenner L, Runkel F, Baader SL, Kappler J, Häberlein H (2004) Dynamics of beta2-adrenergic receptor-ligand complexes on living cells. Biochemistry 43:6190–6199

Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. J Mol Graph 14:33–38

Iizuka Y, Okuno T, Saeki K et al (2010) Protective role of the leukotriene B4 receptor BLT2 in murine inflammatory colitis. FASEB J 24:4678–4690

Inooka H, Ohtaki T, Kitahara O, Ikegami T, Endo S, Kitada C, Ogi K, Onda H, Fujino M, Shirakawa M (2001) Conformation of a peptide ligand bound to its G-protein coupled receptor. Nat Struct Biol 8:161–165

Kofuku Y, Yoshiura C, Ueda T et al (2009) Structural basis of the interaction between chemokine stromal cell-derived factor-1/CXCL12 and its G-protein-coupled receptor CXCR4. J Biol Chem 284:35240–35250

Krieger E, Koraimann G, Vriend G (2002) Increasing the precision of comparative models with YASARA NOVA—a self-parameterizing force field. Proteins 47:393–402

Levitt M (2001) In spin dynamics. Wiley, Chichester, pp 490–493

Luca S, White JF, Sohal AK, Filippov DV, van Boom JH, Grisshammer R, Baldus M (2003) The conformation of neurotensin bound to its G protein-coupled receptor. Proc Natl Acad Sci USA 100:10706–10711

Markus MA, Dayie KT, Matsudaira P, Wagner G (1994) Effect of deuteration on the amide proton relaxation rates in proteins. Heteronuclear NMR experiments on villin 14T. J Magn Reson B105:192–195

Ni F (1992) Complete relaxation matrix analysis of transferred nuclear Overhauser effects. J Magn Reson 96:651–656

Nygaard R, Frimurer TM, Holst B, Rosenkilde MM, Schwartz TW (2009) Ligand binding and micro-switches in 7TM receptor structures. Trends Pharmacol Sci 30:249–259

Okuno T, Iizuka Y, Okazaki H et al (2008) 12(S)-Hydroxyheptadeca-5Z, 8E, 10E-trienoic acid is a natural ligand for leukotriene B4 receptor 2. J Exp Med 205:759–766

Popot JL (2010) Amphipols, nanodiscs, and fluorinated surfactants: three nonconventional approaches to studying membrane proteins in aqueous solutions. Annu Rev Biochem 79:737–775

Popot JL, Althoff T, Bagnard D et al (2011) Amphipols from A to Z. Ann Rev Biophys 40:379–408

Rasmussen SG, Choi HJ, Fung JJ et al (2011) Structure of a nanobody—stabilized active state of the $\beta(2)$ adrenoceptor. Nature 469:175–180

Rosenbaum DM, Rasmussen SG, Kobilka BK (2009) The structure and function of G-protein-coupled receptors. Nature 459:356–363

Rosenbaum DM, Zhang C, Lyons JA et al (2011) Structure and function of an irreversible agonist-$\beta(2)$ adrenoceptor complex. Nature 469:236–240

Sabirsh A, Wetterholm A, Bristulf J, Leffler H, Haeggström JZ, Owman C (2005) Fluorescent leukotriene B4: potential applications. J Lipid Res 46:1339–1346

Schreiber G, Fersht AR (1996) Rapid, electrostatically assisted association of proteins. Nat Struct Biol 3:427–431

Sykes DA, Dowling MR, Charlton S (2009) Exploring the mechanism of agonist efficacy: a relationship between efficacy and agonist dissociation rate at the muscarinic M3 receptor. J Mol Pharmacol 76:543–551

Tribet C, Audebert R, Popot JL (1996) Amphipols: polymers that keep membrane proteins soluble in aqueous solutions. Proc Natl Acad Sci USA 93:15047–15050

Williamson MP (2006) In: Craik D (eds) Modern magnetic resonance. Kluwer, The Netherlands, pp 1339–1344

Yokomizo T, Kato K, Terawaki K, Izumi T, Shimizu T (2000) A second leukotriene B(4) receptor, BLT2. A new therapeutic target in inflammation and immunological disorders. J Exp Med 192:421–432