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

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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 1H–1H 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

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 submicromolar (nM) range, make the kinetics of exchange too slow compared to the auto-($\rho$) and cross-relaxation ($\sigma$) rates (Clore and Gronenborn 1982, 1983; Campbell and Sykes 1993; Williamson...
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: 5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid) and the heptadecanoid 12-HHT (Hamberg et al. 1974) (12S-hydroxyheptadeca-5Z,8E,10E-trienoic acid) (Fig. 1A), in the presence of perdeuterated human BLT2 receptor (e.g. Yokomizo et al. 2000) (u-2H-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 \( k_{	ext{off}} \) 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.

Association rate constants \( k_{	ext{on}} \) of 2.5 \( \times 10^8 \) M\(^{-1}\).s\(^{-1}\) for LTB4 and 2.9 \( \times 10^8 \) M\(^{-1}\).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 \( k_{	ext{on}} \) exceed by \( \times 3 \) the limit usually—but improperly—cited in the literature for biomolecular diffusional associations. These fast-associating \( k_{	ext{on}} \) are not physically unrealistic, however, even for large biomolecules, cases of protein–protein association have been reported with \( k_{	ext{on}} \) values close to or in excess of \( 10^9 \) M\(^{-1}\).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 \( k_{	ext{off}} \), 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 \( \mu \)s 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 \( (\tau_{\text{m}}) \), 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 \( \rho \) and \( \sigma \) values.

12-HHT in solution in excess with respect to the BLT2 receptor gives rise to trNOE (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 $\tau_m = 0.4$ 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 $= 256(t_1) \times 8,192(t_2)$ complex points, $t_{1\text{max}} = 32$ ms, $t_{2\text{max}} = 511$ ms, 256 acquisitions per increment. $^1$H chemical shifts are referenced to H2O (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.
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 ~10⁸ M⁻¹·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.

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References


SUPPLEMENTARY MATERIAL

Electrostatically–Driven Fast Association and Receptor Perdeuteration Allow Detection of Transferred Cross–Relaxation for G Protein–Coupled Receptor Ligands with Equilibrium Dissociation Constants in the High–to–Low Nanomolar Range

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A. Material and Methods

A1. The Unsaturated Fatty Acid Preparation, Synthesis of Polymeric Surfactant DAPol, NMR Sample Preparation and NMR Spectroscopy are similar to the procedures described in Catoire et al., 2010.

A2. Kinetic Measurements by Fluorescence

Transient kinetic experiments were performed using a Cary Eclipse fluorimeter equipped with a rapid mix accessory from Applied Photophysics in which equal volumes (0.1 ml) of reactants were mixed with a dead time of about 5 ms. LTB4 labeled with Alexa Fluor–568 (LTB4–568; Sabirsh et al., 2005) was used as a fluorescent tracer. The mixture containing BLT2 and the non–fluorescent ligand was mixed with either 10 or 20 µM LTB4–568, and the time–dependent changes in LTB4–568 fluorescence were monitored by exciting at 578 nm and detecting the emission intensity at 605 nM. The dependence of fluorescence on time was fitted to a single exponential rate equation. Since the BLT2:ligand complex is mixed with a high and excessive concentration of LTB4–568, the binding of the fluorescent ligand completely prevents the rebinding of the nonfluorescent one. The process of binding of LTB4–568 to the receptor is therefore controlled by the rate of dissociation of the unlabeled compound. The first–order rate constant describing the fluorescence change upon binding of LTB4–568 to BLT2 is thus the dissociation rate constant, \( k_{\text{off}} \), of the unlabeled molecule from the receptor (Bednar et al., 1997; Singh et al., 2011). This rate constant remains unaffected when the concentration of LTB4–568 is increased twofold, indicating that the inferred rate constant indeed serves as the measure of the dissociation rate of 12–HHT and LTB4 from the BLT2 site.

A3. Molecular Modeling of BLT2 Receptor

A BLT2 homology model was built using the Yasara software (www.yasara.org; Krieger et al., 2002) based on chain A of the turkey \( \beta_1 \) adrenergic receptor (PDB–id 2VT4) as template. Yasara homology modeling parameters were as follows: speed = slow, PsiBLASTs = 6, Evalue Max = 0.5, Templates Total = 5, Templates SameSeq = 3, OligoState = 1, Templates SameSeq = 3, LoopSamples = 50, TermExtension = 10. Subsequently, pKa calculations were performed with the Yasara software (Krieger et al., 2006) at neutral pH suggesting a protonation state where all histidines except His181 are neutral. All other ionisable residues are in their standard protonation states. A molecular dynamics (MD) simulation was set up with the Yasara program (Krieger et al., 2004), using the AMBER03 forcefield (Duan et al., 2003) and Yasara’s membrane protein plugin for insertion in a phosphatidyl–ethanolamine bilayer (Figure S2). A snapshot was extracted from the end of the 0.5 µs production run. The protein configuration from this snapshot was used for analysis of its electrostatic potential depicted in Figure 2.
B. Simultaneous Graphical Estimation of Auto- and Cross-Relaxation Rate Constants in the Bound State from 2D NOESY Experiments in the Presence of Chemical Exchange

B1. Theory

Auto and cross-relaxation rate constants in the bound state, \( B \), respectively \( \rho^B \) and \( \sigma^B \), can be in principle extracted from 2D NOESY experiments in the presence of chemical exchange following the theoretical treatment described by Feng Ni in Ni, 1992. The exchange can be written as Eq. (1), where \( P, l \) and \( Pl \) represent respectively the concentrations of protein, ligand and ligand-protein complex in solution, \( k_{on} \) and \( k_{off} \) correspond to the association and dissociation rate kinetic constants:

\[
P + l \xleftrightarrow{k_{on}}_{k_{off}} Pl
\]

The time evolution of the deviation from the equilibrium of the longitudinal magnetization \( M \) of a homonuclear \( n \)-spin system can be described by a set of coupled first-order differential equations (Solomon, 1955; McConnell, 1958):

\[
\frac{dM}{dt} = -\Gamma M
\]

where \( \Gamma \) represents an extended relaxation matrix including the chemical exchange:

\[
\Gamma = R + K
\]

\( R \) and \( K \) represent respectively the relaxation-rate and kinetic matrices. A formal solution of Eq. (2) is given by:

\[
M = \exp (-\Gamma t)M_0
\]

where \( M_0 \) is a diagonal matrix composed of the initial magnetizations of all spins.

Introducing the exchange in Eq. (3) renders \( \Gamma \) non-symmetric. However, under certain circumstances, i.e. fast exchange, a perturbation-like calculation can help to solve Eq. (2) (Landy & Rao, 1989). Otherwise, \( \Gamma \) can be made symmetric by the following transformation:

\[
S^{-1} \Gamma S = \Gamma'
\]

where \( S \) is a diagonal matrix (Olejniczak, 1989; Ni, 1992):

\[
S = \begin{bmatrix}
\sqrt{N} & 0 \\
0 & \sqrt{q \times N}
\end{bmatrix}
\]

\( N \) is a diagonal submatrix composed by the number of equivalent spins in both free- and bound-state \( F \) and \( B \). \( q \) is the ratio of ligand in the bound \( (p^B) \) and free \( (1-p^B) \) states:

\[
q = \frac{p^B}{1-p^B}
\]
\( \Gamma' \) can be diagonalized by an orthogonal transformation, such that:

\[
\Lambda = L^T \Gamma' L \tag{8}
\]

\( L \) corresponding to the matrix of eigenvectors of \( \Gamma' \) and \( \Lambda \) is a diagonal matrix composed by the eigenvalues \( \lambda_i \) of \( \Gamma' \).

Combining Eq. (4) with Eq. (5) yields:

\[
M = \exp \left( -S \Gamma' S^{-1} t \right) M_0 \\
= \exp \left( -S L A L^T S^{-1} t \right) M_0 \\
= SL \exp \left( -\Lambda t \right) L^T S^{-1} M_0 \tag{9}
\]

As indicated in Ni, 1992, at equilibrium, the initial magnetization \( M_0 \), representing all the initial magnetizations of the spins of the ligand, can be written using the submatrix \( N \):

\[
M_0 = \begin{bmatrix}
M^F_0 & 0 \\
0 & M^B_0
\end{bmatrix} \\
= c \begin{bmatrix}
(1 - p_B)N & 0 \\
0 & p_B N
\end{bmatrix} \\
= c(1 - p_B)SS \tag{10}
\]

where \( c \) is the equilibrium magnetization of a single spin in arbitrary units. Eq. (9) then becomes:

\[
M = c(1 - p_B)SL \exp \left( -\Lambda t \right) L^T S^{-1} SS \\
= c(1 - p_B)SL \exp \left( -\Lambda t \right) L^T S \tag{11}
\]

The magnetization of the free ligand can be obtained from the above equation using the subdivision of \( S \) that corresponds to the spins of the free ligand. Eq. (11) can be rewritten as follows:

\[
M^F = c(1 - p_B)\sqrt{NQ^F} \sqrt{N} \tag{12}
\]

where \( Q^F \) is the upper diagonal block extracted from \( L \exp(-\Lambda t)L^T \):

\[
L \exp(-\Lambda t)L^T = \begin{bmatrix}
Q^F & E \\
E & Q^B
\end{bmatrix} \tag{13}
\]
B2. Application: an isolated two spin-system

Considering two spins of the ligand, I and S, in the free (F) and in the bound (B) states, such that:

\[
M_0 = \begin{bmatrix}
M^F_{I0} & 0 & 0 & 0 \\
0 & M^F_{S0} & 0 & 0 \\
0 & 0 & M^B_{I0} & 0 \\
0 & 0 & 0 & M^B_{S0}
\end{bmatrix}
\] (14)

\[
R = \begin{bmatrix}
\rho^F_I & 0 & 0 & 0 \\
0 & \rho^F_S & 0 & 0 \\
0 & 0 & \rho^B_I + \rho^* & \sigma_{IS}^B \\
0 & 0 & \sigma_{IS}^B & \rho^B_S
\end{bmatrix}, \quad K = \begin{bmatrix}
q \times k_{off} & 0 & -k_{off} & 0 \\
0 & q \times k_{off} & 0 & -k_{off} \\
-q \times k_{off} & 0 & k_{off} & 0 \\
0 & -q \times k_{off} & 0 & k_{off}
\end{bmatrix}
\] (15)

where,

\[
\rho^F_i \text{ and } \rho^B_i \text{ are respectively the total or auto spin-lattice relaxation rates of spin } i \text{ in the free and bound states:}
\]

\[
\rho_i = \sum_{j \neq i} \rho_{ij} + \rho^*_i
\] (16)

\[
\rho_{ij} \text{ represents the spin-lattice relaxation rate constant of the spin } i \text{ by the spin } j \text{ in the absence of interference effects, and } \rho^*_i \text{ represents other non-dipolar relaxation contributions and/or a contribution from some other spins of the lattice. We make the following approximations:}
\]

\[
\rho^F_I \simeq \rho^F_S = \rho^F, \quad \rho^B_I \simeq \rho^B_S = \rho^B
\] (17)

Furthermore, with only two spins:

\[
\rho^B = \rho^B_{IS} + \rho^*
\] (18)

with the assumption \( \rho^B_{IS} \simeq \rho^B_S = \rho^* \)

\( \sigma_{IS}^B \) is the cross-relaxation rate in the bound state only. Based on experimental measurements, \( \sigma \) in the free state is negligible for both ligands in this study.

To simplify the writing, \( k_{off} = k \) and \( \sigma_{IS} = \sigma \). Hence, \( R \) and \( K \) become:

\[
R = \begin{bmatrix}
\rho^F & 0 & 0 & 0 \\
0 & \rho^F & 0 & 0 \\
0 & 0 & \rho^B_{IS} + \rho^* & \sigma \\
0 & 0 & \sigma & \rho^B_{IS} + \rho^*
\end{bmatrix}, \quad K = \begin{bmatrix}
q \times k & 0 & -k & 0 \\
0 & q \times k & 0 & -k \\
-q \times k & 0 & k & 0 \\
0 & -q \times k & 0 & k
\end{bmatrix}
\] (19)

The matrix \( S \) used in the transformation of Eq. (5) is:

\[
S = \begin{bmatrix}
n_I & 0 & 0 & 0 \\
0 & n_S & 0 & 0 \\
0 & 0 & \sqrt{n_I \times q} & 0 \\
0 & 0 & 0 & \sqrt{n_S \times q}
\end{bmatrix}
\] (20)
with \( n_i \) representing the number of equivalent spins \( i \) for each resolved resonance. In cases treated in this study (see § B4), \( n_i = 1, \forall i \). Hence, the transformation represented by Eq. (5) gives rise to a symmetric \( \Gamma' \) matrix:

\[
\Gamma' = \begin{bmatrix}
qk + \rho^F & 0 & -k\sqrt{q} & 0 \\
0 & qk + \rho^F & 0 & -k\sqrt{q} \\
-k\sqrt{q} & 0 & k + \rho^B_{IS} + \rho^* & \sigma \\
0 & -k\sqrt{q} & \sigma & k + \rho^B_{IS} + \rho^*
\end{bmatrix}
\]  

(21)

\( \Gamma' \) is diagonalized into \( \Lambda \) by an orthogonal transformation (Eq. (8)), leading to the following eigenvalues:

\[
\Lambda = \begin{bmatrix}
\lambda_1 & 0 & 0 & 0 \\
0 & \lambda_2 & 0 & 0 \\
0 & 0 & \lambda_3 & 0 \\
0 & 0 & 0 & \lambda_4
\end{bmatrix}
\]  

(22)

with

\[
\lambda_1 = \frac{1}{2}(k + kq + \rho^* + \rho^B_{IS} + \rho^F + \sigma - \\
\sqrt{(k + kq + \rho^* + \rho^B_{IS} + \rho^F + \sigma)^2 - 4(\rho^F(\rho^* + \rho^B_{IS} + \sigma) + k(\rho^F + q(\rho^* + \rho^B_{IS} + \sigma)))})
\]  

(23)

\[
\lambda_2 = \frac{1}{2}(k + kq + \rho^* + \rho^B_{IS} + \rho^F + \sigma + \\
\sqrt{(k + kq + \rho^* + \rho^B_{IS} + \rho^F + \sigma)^2 - 4(\rho^F(\rho^* + \rho^B_{IS} + \sigma) + k(\rho^F + q(\rho^* + \rho^B_{IS} + \sigma)))})
\]  

(24)

\[
\lambda_3 = \frac{1}{2}(k + kq + \rho^* + \rho^B_{IS} + \rho^F - \\
\sqrt{-4(k(\rho^F + q(\rho^* + \rho^B_{IS} - \sigma)) + \rho^F(\rho^* + \rho^B_{IS} - \sigma)) + (kq + \rho^* + \rho^B_{IS} + \rho^F - \sigma)^2 - \sigma})
\]  

(25)

\[
\lambda_4 = \frac{1}{2}(k + kq + \rho^* + \rho^B_{IS} + \rho^F + \\
\sqrt{-4(k(\rho^F + q(\rho^* + \rho^B_{IS} - \sigma)) + \rho^F(\rho^* + \rho^B_{IS} - \sigma)) + (kq + \rho^* + \rho^B_{IS} + \rho^F - \sigma)^2 - \sigma})
\]  

(26)

For a two-spin system, the \( Q^F \) matrix block in Eq. (13) can be written as follows:

\[
Q^F = \begin{bmatrix}
Q^F_{11} & Q^F_{12} \\
Q^F_{21} & Q^F_{22}
\end{bmatrix}
\]  

(27)

with the following \( Q^F_{ij} \) literal expressions:
\[ Q_{11}^F = Q_{22}^F = \frac{1}{4} \left\{ \begin{array}{c}
- 4 + e^{-\frac{1}{2}(k + q + \rho^* + \rho_{IS}^B + \rho^F) - \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F - \sigma) + (\rho^* + \rho_{IS}^B - \rho^F - \sigma)^2} t \times \\
\frac{k - kq + \rho^* + \rho_{IS}^B - \rho^F + \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F - \sigma) + (\rho^* + \rho_{IS}^B - \rho^F - \sigma)^2}}{\sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F - \sigma) + (\rho^* + \rho_{IS}^B - \rho^F - \sigma)^2}} e^{-\frac{1}{2}(k + q + \rho^* + \rho_{IS}^B + \rho^F + \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F + \sigma) + (\rho^* + \rho_{IS}^B - \rho^F + \sigma)^2}} t \times \\
\frac{k(-1 + q) - \rho^* - \rho_{IS}^B + \rho^F + \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F - \sigma) + (\rho^* + \rho_{IS}^B - \rho^F - \sigma)^2 + \sigma}}{\sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F - \sigma) + (\rho^* + \rho_{IS}^B - \rho^F - \sigma)^2}} e^{-\frac{1}{2}(k + q + \rho^* + \rho_{IS}^B + \rho^F + \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F + \sigma) + (\rho^* + \rho_{IS}^B - \rho^F + \sigma)^2}} t \times \\
\frac{k(-1 + q) - \rho^* - \rho_{IS}^B + \rho^F - \sigma + \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F + \sigma) + (\rho^* + \rho_{IS}^B - \rho^F + \sigma)^2}}{\sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F + \sigma) + (\rho^* + \rho_{IS}^B - \rho^F + \sigma)^2}} e^{-\frac{1}{2}(k + q + \rho^* + \rho_{IS}^B + \rho^F + \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F + \sigma) + (\rho^* + \rho_{IS}^B - \rho^F + \sigma)^2}} t \times \\
\frac{k - kq + \rho^* + \rho_{IS}^B - \rho^F + \sigma + \sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F + \sigma) + (\rho^* + \rho_{IS}^B - \rho^F + \sigma)^2}}{\sqrt{k^2(1 + q)^2 - 2k(-1 + q)(\rho^* + \rho_{IS}^B - \rho^F + \sigma) + (\rho^* + \rho_{IS}^B - \rho^F + \sigma)^2}} \right\} \]
\[ Q_{12}^F = Q_{21}^F = \frac{1}{4} \left\{ \right. \\
- e^{-\frac{1}{2} (k + kq + \rho^* + \rho_{IS}^B + \rho_F - \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F - \sigma)^2 - (\rho^* + \rho_{IS}^B - \rho_F - \sigma)^2 - \sigma})t} \times \\
\left[ \frac{k - kq + \rho^* + \rho_{IS}^B - \rho_F + \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F - \sigma) + (\rho^* + \rho_{IS}^B - \rho_F - \sigma)^2 - \sigma}}{\sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F - \sigma) + (\rho^* + \rho_{IS}^B - \rho_F - \sigma)^2}} \right] \\
- e^{-\frac{1}{2} (k + kq + \rho^* + \rho_{IS}^B + \rho_F + \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F - \sigma) + (\rho^* + \rho_{IS}^B - \rho_F - \sigma)^2 + \sigma})t} \times \\
\left[ \frac{k(-1+q) - \rho^* - \rho_{IS}^B + \rho_F + \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F - \sigma) + (\rho^* + \rho_{IS}^B - \rho_F - \sigma)^2 + \sigma}}{\sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F - \sigma) + (\rho^* + \rho_{IS}^B - \rho_F - \sigma)^2}} \right] \\
+ e^{-\frac{1}{2} (k + kq + \rho^* + \rho_{IS}^B + \rho_F + \sigma + \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F + \sigma) + (\rho^* + \rho_{IS}^B - \rho_F + \sigma)^2})t} \times \\
\left[ \frac{k(-1+q) - \rho^* - \rho_{IS}^B + \rho_F + \sigma + \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F + \sigma) + (\rho^* + \rho_{IS}^B - \rho_F + \sigma)^2}}{\sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F + \sigma) + (\rho^* + \rho_{IS}^B - \rho_F + \sigma)^2}} \right] \\
+ e^{-\frac{1}{2} (k + kq + \rho^* + \rho_{IS}^B + \rho_F + \sigma - \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F + \sigma) + (\rho^* + \rho_{IS}^B - \rho_F + \sigma)^2})t} \times \\
\left[ \frac{k - kq + \rho^* + \rho_{IS}^B - \rho_F + \sigma + \sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F + \sigma) + (\rho^* + \rho_{IS}^B - \rho_F + \sigma)^2}}{\sqrt{k^2(1+q)^2 - 2k(-1+q)(\rho^* + \rho_{IS}^B - \rho_F + \sigma) + (\rho^* + \rho_{IS}^B - \rho_F + \sigma)^2}} \right] \\
\left. \right\} \\
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B3. Principle of the graphical estimation

Based on the Solomon equation (Eq. (2)) and the subsequent theoretical treatment described in § B1 applied to a two-spin system (§ B2), one can figure out the evolution of the longitudinal magnetization with time during the NOESY mixing period in the presence of a large excess of ligand over the receptor. In the case of 2D transferred NOE, cross and diagonal peaks observed correspond to the magnetization of the free ligand, $M^F$. The evolution of the latter is described by Eq. (12) and can be subdivided as follows:

$$M^F = \begin{bmatrix} M^F_{11} & M^F_{12} \\ M^F_{21} & M^F_{22} \end{bmatrix}$$  \hspace{1cm} (28)

where $M^F_{11}$ and $M^F_{22}$ represent the volumes of the diagonal peaks of spins 1 and 2, respectively, and $M^F_{12}$ and $M^F_{21}$ the volumes of cross peaks.

Hence, from Eq. (12) and (27), providing $k$, $q$ and $\rho^F$ are known, at a fixed NOESY mixing time $\tau_m$, the ratio $M^F_{21}/M^F_{11}$ (that corresponds to $\Pi_{exp}$ in the article) can be plotted as a function of $\rho^B (\equiv \rho_{IS}^B + \rho^* \text{ in § B2})$ and $\sigma^B (\equiv \sigma \text{ in § B2}).$

---

Legend: (A) 3D plot of the ratio of cross vs. diagonal peak volumes ($M^F_{21}/M^F_{11}$) as a function of the auto- and cross-relaxation rate constants in the bound state at a fixed mixing time. (B) Projection along the $M^F_{21}/M^F_{11}$ axis of the contour plot corresponding to the experimental value of $M^F_{21}/M^F_{11}$ ($\equiv \Pi_{exp}$ in the article). The contour line is drawn for $\rho^B$ greater than or equal to $\sigma^B$ (above the dashed line) as $\rho$ is always $\geq \sigma$ (for long rotational correlation times $\rho^B$ and $\sigma^B$ are almost equal in the limits of an exclusive dipolar relaxation, i.e. $\lim \inf_{\tau_c \to \infty} \sigma = \rho$, with $\rho^B \simeq W_0+2W_1$ and $\sigma^B \simeq W_0$, where $W_i$ represent transition probabilities in a two-spin system; e.g. Levitt, 2001a). Illustration at $\tau_m = 100 \text{ ms}$ with a 9-fold excess of 12-HHT over BLT2 receptor in the presence of chemical exchange ($k = 18 \text{ s}^{-1}; \rho^F = 0.09 \text{ s}^{-1}; q = 0.06$) (see also § B4).
and \( q \) are respectively deduced from kinetic and UV measurements, and \( \rho^F \) can be measured by NMR (e.g. Ernst et al., 1987) or roughly estimated for a two-spin system \( IS \) using model-free spectral density functions (Lipari & Szabo, 1982).

Intrinsic physical relaxation properties of the spin-system, like those represented by \( \rho \) and \( \sigma \) rate constants, are independent of experimental variables like the NOESY mixing time \( \tau_m \), in contrast to the \( M_{21}^F / M_{11}^F \) ratio. Superimposing several projections along the \( M_{21}^F / M_{11}^F \) axis of contourplots of experimental \( M_{21}^F / M_{11}^F \), measured from NOESY experiments performed at different \( \tau_m \), as a function of \( \rho \) and \( \sigma \), gives rise in theory to one single crossing point corresponding to one couple of \( \rho^B \) and \( \sigma^B \) values. In practice, the crossing point shifts slightly with \( \tau_m \) towards smaller \( \rho \) and \( \sigma \) values because of the two-spin system approximation (see next Figure). Hence, this graphical method yields slightly underestimated values of both \( \rho \) and \( \sigma \) and becomes more imprecise at larger \( \tau_m \).

![Contourplot diagram](image_url)

Legend: projections of three contourplots along the \( M_{21}^F / M_{11}^F \) axis representing the ratio of NOESY cross vs. diagonal peak volumes measured at 3 mixing times \( (\tau_{m1} = 50 \text{ ms}, \tau_{m2} = 100 \text{ ms}, \tau_{m3} = 200 \text{ ms}) \) (ligand 12-HHT, see § B4). Dotted black lines roughly defines the accuracy of the graphical determination for \( \rho_B \) and \( \sigma_B \).

In the above Figure, the dashed vertical blue line represents the limit of \( M_{21}^F / M_{11}^F \) when \( \tau_m \to 0 \), as, at very short mixing time, \( M_{21}^F / M_{11}^F \) becomes independent of the auto-relaxation rate in the bound state as illustrated in the next Figure for an unrealistic very short mixing time \( (\tau_m = 100 \mu\text{s}) \):
Legend: A and B represent two views of a 3D plot of cross vs. diagonal peak volumes ratio ($\frac{M_{2,1}^F}{M_{1,1}^F}$) as a function of the auto- and cross-relaxation rate constants in the bound state at a short $\tau_m$. Illustration with a $k_{off}$ of 18 s$^{-1}$, $q = 0.06$, $\rho^F = 0.09$ s$^{-1}$ and $\tau_m = 100$ µs.
B4. Numerical applications

Numerical simulations are shown for two spins that are close in space, where contributions from additional neighboring spins are only included in a leakage term in the total longitudinal rate $\rho$ of each spin.

**Case I : LTB4, dipolar interaction between spins H6 and H7**

![Schematic Diagram](image)

<table>
<thead>
<tr>
<th>$\tau_m$ (s)</th>
<th>$M_{2,1}/M_{1,1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
<td>0.112</td>
</tr>
<tr>
<td>0.10</td>
<td>0.217</td>
</tr>
<tr>
<td>0.20</td>
<td>0.318</td>
</tr>
<tr>
<td>0.50</td>
<td>0.410</td>
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</tbody>
</table>

Legend: primary structure of LTB4, with squared in red the localization of the two-spin system chosen, and experimental ratios of cross vs. diagonal peak volumes ($^1$H Larmor frequency = 600 MHz).

![Graph A](image)

![Graph B](image)

Legend: superimposed projections along the $M_{21}^F/M_{11}^F$ axis of contourplots representing the ratio of cross vs. diagonal peak volumes measured from NOESY experiments performed at (A) four or two (B) mixing times. Variables: $k = 50 \text{ s}^{-1}$, $n_6 = n_7 = 1$, $c = 1$ (cf. Eq. 10), $p^B = 0.06 \rightarrow q = 0.064$, $\rho^F = 0.23 \text{ s}^{-1}$. 
**Case II: 12-HHT, dipolar interaction between spins H9 and H11**

<table>
<thead>
<tr>
<th>$\tau_m$ (s)</th>
<th>$M_{2,1}/M_{1,1}$</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.043</td>
</tr>
<tr>
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<tr>
<td>0.20</td>
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<tr>
<td>0.40</td>
<td>0.259</td>
</tr>
<tr>
<td>0.80</td>
<td>0.335</td>
</tr>
</tbody>
</table>

Legend: primary structure of 12-HHT, with squared in red the localization of the two-spin system chosen, and experimental ratios of cross versus diagonal peak volumes ($^1$H Larmor frequency = 600 MHz).

Legend: superimposed projections along the $M_{21}^F/M_{11}^F$ axis of contourplots representing the ratio of cross versus diagonal peak volumes measured from NOESY experiments performed at (A) five or two (B) mixing times. Variables: $k = 18$ s$^{-1}$, $n_9 = n_{11} = 1$, $c = 1$ (cf. Eq. 10), $p_B = 0.054 \rightarrow q = 0.058$, $\rho_F = 0.09$ s$^{-1}$. 
Four parameters determine the contourplot of $M_{21}^F/M_{11}^F$ (II$_{exp}$ in the article) vs. $\rho_B$ and $\sigma_B$: $M_{21}^F/M_{11}^F($exp$)$, $k_{off}$ (≡ $k$ in the simulations), $\rho^F$ and $p_B^B$. Among them, in the two cases treated in this study, $k_{off}$ has a major influence, i.e. 10% variation in $k_{off}$ (typically the experimental standard deviation observed with the method described in § A2) results in a variation of $\sim$1 s$^{-1}$ in both $\rho_B$ and $\sigma_B$ values (see next Figure).

Legend: Impact of experimental errors on the estimation of $\rho_B$ and $\sigma_B$ with the graphical method. Illustration with LTB4/BLT2 sample: $k_{off} = 50$ s$^{-1}$, $n_6 = n_7 = 1$, $c = 1$ (cf. Eq. 10), $p_B^B = 0.06$ $\rightarrow$ $q = 0.064$, $\rho^F = 0.23$ s$^{-1}$. (A) Theoretical $M_{21}^F/M_{11}^F$ ratio taken at experimental value (i.e. 0.22 at $\tau_m = 0.1$ s). The thickness of the line represents the experimental error in the $M_{21}^F/M_{11}^F$ ratio (here ±5%). (B) Impact of the experimental $k_{off}$ uncertainty (±10%) coupled to the experimental error in $M_{21}^F/M_{11}^F$. (C) Impact of the experimental $\rho^F$ uncertainty (±10%) coupled to the experimental error in $M_{21}^F/M_{11}^F$. At the ligand/receptor ratio used here, variations of ±10% in $p_B^B$ have no noticeable incidence on the line thickness in A.
C. Experimental NOEs and study of the specificity of the interaction observed by NMR

Experimental NMR data and structure calculation for LTB4 are described in *Catoire et al. 2010.* The specificity of 12–HHT/BLT2 dipolar interactions observed is based on the same strategy exposed in the study of the LTB4, *i.e.* by performing control experiments in the presence of amphipols only in solution or in the presence of a BLT2 mutant that does not specifically bind 12–HHT, and also by using a competitor agonist (here LTB4) (*vide infra* Figures).

**Legend of the figure on next page:** Olefinic $^1$H NMR spectra of 12–HHT (~110 µM) in different environments. *(A)* 12–HHT chemical structure and a zoom on the olefinic $^1$H region of the 1D NMR spectrum of 12–HHT free in an aqueous solution (in *black*). $^1$H assignments are indicated above the spectrum. *(B)* 12–HHT in the presence of free amphipol A8–35 partially deuterated (DAPol). Schematic view (left) of the two coexisting states I (free) or II (associated to particles of DAPol), black arrows symbolizing the chemical exchange. On the right, zoom on the olefinic $^1$H region of the 1D NMR spectrum of 12–HHT free in aqueous solution (in *black* superimposed to the spectrum of 12–HHT in the presence of DAPol in *blue*). Resonances of protons H10 and H11 are enlarged on the right. *(C)* 12–HHT in the presence of perdeuterated wild–type BLT2 receptor ($u$–$^2$H–wtBLT2) complexed by DAPol. The scheme on the left describes four putative states of 12–HHT. In addition to the states shown in *(B)*, states III and IV represent, respectively, 12–HHT interacting with the high affinity site of the receptor and with either a low affinity site and/or the belt of DAPol. Dotted arrows symbolize a lower probability of exchange, compared to solid arrows. On the right, spectrum of 12–HHT in the presence of wild–type receptor (in *green*), compared to the black spectrum from *(B)*. *(D)* Same as *(C)*, but in the presence of the 12–HHT competitor LTB4. Spectra in *red* and *orange* correspond respectively to a LTB4/12–HHT molar ratio $r$ of 10 and 50. *(E)* Same as *(C)* but with a mutant receptor ($u$–$^2$H–mBLT2) (spectrum in *magenta*). See also the legend at the bottom of the figure.
A

B

C

D

E

[Chemical structures and spectra analyses]
In contrast with LTB4 (see Catoire et al. 2010), 12–HHT free in solution gives rise to detectable NOEs (see figure on next page). Indeed, because 12–HHT is slightly smaller than LTB4, the overall correlation time $\tau_c$ of 12–HHT is below the critical correlation time $\tau_{c_{\text{crit}}}$ at which the cross-relaxation vanishes (see Eq. 16.18 in Levitt 2001b), giving rise to NOE cross-peaks with an opposite sign to the diagonal peaks. The pattern of cross-peaks observed, that contains also COSY-type correlations, corresponds exactly to the pattern obtained in the presence of amphipols only in solution or in the presence of a mutant BLT2 receptor that does not bind specifically 12–HHT (vide infra).

**Legend of the figure on next page:** NOESY experiment of 12–HHT free in solution ([12–HHT] $\simeq$ 110 $\mu$M, HEPES/NaOH buffer, pH 8, 25$^\circ$C, $\tau_m = 0.8$ s, $^1$H Larmor frequency = 400 MHz). (A) 12–HHT chemical structure. (B) NOESY spectrum. Positive and negative contour plots are respectively represented in black and red. The corresponding 1D $^1$H spectrum is displayed on top of the 2D spectrum, with numbers referring to the chemical structure in (A). Additional weak COSY-type cross-peaks are also identified with their anti-phase shapes. (C) One row extracted at the H7 proton chemical shift indicated by a dotted line in the 2D spectrum in B.
As observed with LTB4 (see Catoire et al. 2010), in the presence of either amphipols only in solution or a BLT2 mutant that does not bind specifically the ligand, only cross-peaks between olefinic protons or between an olefinic proton with an adjacent aliphatic proton appear in the NOESY spectrum (see spectra in the Figure on next page). In other words, this pattern of trNOEs involves spin located in rigid parts of the molecule, corresponding to the pattern observed with 12–HHT free in solution (vide supra). Compared to spectra obtained in the presence of the wild–type receptor, most of the cross-peaks have volumes 75 to 80% lower and no additional NOEs appear when increasing the mixing time, in contrast with spectra obtained with the wild–type receptor. Structure calculations conducted with either the blue or red set of NOE cross-peaks (see next figure) collected at several mixing times do not lead to an ensemble of converged low energy structures (data not shown).

**Legend of the figure on next page:** Positive contours of 2D NOESY spectra of 12–HHT (∼110 µM) in the presence of u–2H–wild–type BLT2/DAPol complexes (12 µM) (in black), DAPol only (in blue), or u–2H-mutant BLT2/DAPol (12 µM) complexes (in red) (mixing time = 0.4 s, ν_H = 600 MHz, 25°C). Corresponding 1D ¹H spectrum of 12–HHT (∼110 µM) in the presence of the wild–type receptor 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 protons annotated on the corresponding 12–HHT chemical structure above the spectrum. I, II, and III represent enlarged views of three regions indicated by a dotted frame in the global 2D NOESY superimposed spectra.
A second control experiment to assess the specificity of the LTB4–BLT2 interaction was carried out by performing a competition assay by LTB4 in the presence of BLT2 at several LTB4/12–HHT molar ratios, namely 10:1 and 50:1. Both unsaturated fatty acid molecules present enough differences to give rise to specific signals with distinct $^1$H chemical shifts, in particular in the olefinic region (see Figure A on next page). At both LTB4/12–HHT molar ratios tested, intense intra-LTB4 NOE cross-peaks are observed in 2D NOESY spectra. Conversely, NOE cross-peak volumes of 12–HHT decrease when the LTB4/12–HHT molar ratio increases (see Figures B and C on next page), indicating that the BLT2–specific agonist LTB4 displaces 12–HHT from its binding site on BLT2/DAPol complexes.

Legend of the figure on next page: Competitive displacement of 12–HHT by LTB4 observed by NMR. (A) Positive contours of a 2D NOESY region of 12–HHT/u-$^2$H-wtBLT2/DAPol in the absence (in red) and presence (in black) of LTB4 (molar ratio LTB4/12–HHT = 10:1; NOESY mixing time = 0.4 s, $\nu_H = 600$ MHz, 25°C). LTB4 assignments are mentioned above the 2D spectrum. (B,C) Two extracted columns from 2D NOESY spectra at the H12 (B) and H7 (C) resonance frequencies (corresponding to the dotted lines in A). In black, the columns are extracted from a 2D NOESY spectrum acquired in the absence of the competitor agonist, in red and blue from 2D NOESY spectra acquired at a 10:1 and 50:1 LTB4/12–HHT molar ratio. The squared frames show the three superimposed columns normalized with respect to the diagonal peak, i.e. at the H12 (B) and H7 (C) resonance frequencies.
Five sets of NOE data were collected with 12-HHT/BLT2 sample. In the Figure below, two enlarged views of two regions of four 2D NOESY superimposed spectra of 12-HHT in the presence of u-2H-wtBLT2/DAPol complexes acquired with the following mixing times: 0.1 s (in green), 0.2 s (in blue), 0.4 s (in red), and 0.8 s (in black). For clarity, the fifth NOESY experiment, acquired at 0.05 s mixing time, is not displayed.
NOE data collected with 12-HHT/BLT2 sample give rise to the following set of experimental NOE-based distance restraints represented by dotted lines on a regular seventeen-sided polygon symbolizing the heptadecanoid (for LTB4, see Catoire et al. 2010):
**Fig. S1.** Binding of LTB4 (*open circles*) or 12-HHT (*solid circles*) to wild-type BLT2 (wtBLT2) receptor. Fluorescence anisotropy-monitored competition experiments were carried out using LTB4-568 as a fluorescent tracer (*Sabirsh et al., 2005*). Data are presented as fluorescence anisotropy (% of maximum, defined in the absence of displacing ligand) as a function of ligand concentration. These experiments indicate equilibrium dissociation constants $K_d$ of 201 and 62 nM for respectively LTB4 and 12-HHT.
Fig. S2. snapshot extracted from a molecular dynamics simulation of 0.5 μs of BLT2 inserted in a solvated phosphatidyl-ethanolamine bilayer in the presence of sodium (blue spheres) and chloride (cyan spheres) ions.
Fig. S3. Size exclusion chromatography of wild-type BLT2 receptor associated to amphipol A8-35 surfactant indicates a protein essentially in a monomeric form. In the pioneer work of amphipol–assisted folding of GPCRs (Dahmane et al. 2009), BLT2 associated with amphipol A8–35 was found to be essentially dimeric. We impute this difference to the neutral pH used in Dahmane et al., that corresponds to the limit of solubility of polyacrylate–based amphipol A8–35. The chromatogram was obtained with a Superose 200 10-300 GL column (Tris-HCl 20 mM, NaCl 100 mM, pH 8 at 20°C). Arrows indicate the void ($V_0$) and total ($V_t$) volumes.
Fig. S4. Overall correlation time $\tau_c$ of $[u-^2H,^{13}C,^{15}N]BLT2/Amphipol A8-35$ complexes (see also Figure S3) estimated by a 1D $[^{15}N,^1H]$-TROSY rotational correlation time (TRACT) experiment (Lee et al., 2006). (A) $^{15}N(\alpha$-spin state)-- (in blue) and $^{15}N(\beta$-spin state)--linked (in black) indole and amino protons 1D spectra of wild-type BLT2 (in 50 mM Hepes /NaOH buffer, 100 mM NaCl, 1 mM EDTA, 0.02% NaN$_3$, 10% D$_2$O, pH 8, 25°C). (B) $I_0$ and $I_i$ are integrations of the $^1H^N$ 1D spectrum region between 7 and 9.5 ppm at $t = 0$ and $t = \Delta_i$, respectively. The blue and black curves correspond to the slow ($\alpha$-spin state of $^{15}N$) and fast ($\beta$-spin state of $^{15}N$) transverse relaxation decays. The exponential fits yield the $R_\alpha$ and $R_\beta$ values indicated, from which an estimate of $\tau_c$ can be derived as described in Lee et al., 2006. At pH 8, amide protons exposed to the solvent, in particular in the extra–or intra–cellular loops, should not contribute significantly to the measurement. But still, as one cannot exclude contributions from non-solvent exposed $^1H^N$ in relatively mobile regions of the protein, the $\tau_c$ estimation obtained with this method is slightly underestimated.
**Fig. S5.** Electrostatic potential (Ep) of the extracellular ligand acceptor site for two GPCR models. Ep is shown for the BLT2 (A) and the β2-adrenergic (B) receptors seen from the extracellular side, looking onto the membrane surface. The insets focus on the ligand acceptor site, both accessible and exhibiting positive potential. The Ep maps are colored from –400 kT/e in red to +400 kT/e in blue. This figure was prepared with Yasara (Krieger et al., 2002).

**E. ADDITIONAL ANIMATIONS**

An animation of the electrostatic field around BLT2 was generated using SpiderMol (Callieri et al. 2010), providing an interactive view of proteins with animated particles and rendered surface. The animation can be viewed on the following website, with a recent WebGL-capable browser (Firefox 4, Google Chrome):


Alternatively, a movie of the animation was generated and will be linked from the above website. It is made available on the following YouTube channel:

http://www.youtube.com/marcbaaden
F. References


Levitt, M. In Spin Dynamics; Wiley: Chichester, UK, 2001a; p528

Levitt, M. In Spin Dynamics; Wiley: Chichester, UK, 2001b; Eq. 16.18 p535


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