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Beyond Standard Molecular Dynamics: Investigating the Molecular Mechanisms of G Protein-Coupled Receptors with Enhanced Molecular Dynamics Methods

Jennifer M. Johnston and

Department of Structural and Chemical Biology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

Marta Filizola

Department of Structural and Chemical Biology, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, New York, NY 10029, USA

Marta Filizola: marta.filizola@mssm.edu

Abstract

The majority of biological processes mediated by G Protein-Coupled Receptors (GPCRs) take place on timescales that are not conveniently accessible to standard molecular dynamics (MD) approaches, notwithstanding the current availability of specialized parallel computer architectures, and efficient simulation algorithms. Enhanced MD-based methods have started to assume an important role in the study of the rugged energy landscape of GPCRs by providing mechanistic details of complex receptor processes such as ligand recognition, activation, and oligomerization. We provide here an overview of these methods in their most recent application to the field.

Keywords

Enhanced methods; Biased simulations; Metadynamics; Umbrella sampling; Free-energy calculations; Coarse-graining; Ligand recognition; GPCR activation; Molecular mechanisms; Oligomerization; Dimers; GPCR structure; GPCR dynamics; GPCR function

6.1 Introduction

GPCRs are components of complex macromolecular machines with multiple ligand-induced ‘active’ states that can engender different signaling outputs through association with specific accessory proteins. These functionally versatile macromolecular complexes, recently termed GPCR signalosomes (Huber and Sakmar 2011), are suggested to operate through a multitude of dynamic steps and allosteric signaling conduits whose properties may not depend necessarily on their individual elements. Thus, it appears evident that a conceptual framework strongly relying on a combination of high-content experimental platforms and computational approaches is necessary to account for the complexity of these signalosomes

and ultimately understand the relationships between their structure, dynamics, and function (Huber and Sakmar 2011).

One way to interpret the functional versatility of GPCRs is in terms of their structural plasticity, through system representation as energy landscapes (Deupi and Kobilka 2010; Choe et al. 2011; Vaidehi and Kenakin 2010). Building upon a concept put forth in 1991 by Frauenfelder and colleagues (1991), the energy landscape of a protein is described as a hyper-surface in a conformational space of very high dimension, with a very large number of valleys (conformational sub-states) and peaks (energy barriers). The valleys and peaks of such a rugged landscape cannot be classified individually, but must be described by distributions. Specific energy levels are no longer relevant, but are more accurately described in statistical terms. At any given time, an individual protein molecule can jump between conformational sub-states as it navigates its own energy landscape, and this exploration is critically dependent on temperature (Frauenfelder et al. 1991). The available X-ray crystal structures of GPCRs (for a review see Katritch 2012) represent single, static sub-states of these proteins. Sections of missing density in many of the structures (e.g. Wu et al. 2012) highlight the inherent flexibility, even at low temperatures, of some regions of the receptor, particularly parts of the intra and extracellular loops and the N- and C-termini, and remind us that the dynamic and adaptable nature of the GPCR structure is a key part of their functionality.

Ligands with different efficacies can modulate the GPCR energy landscape by shifting the conformational equilibrium towards active or inactive conformations, thus eliciting different physiological responses (Deupi and Kobilka 2010; Vaidehi and Kenakin 2010). Examining the molecular basis of this ligand-induced conformational shift is not an easy task using standard MD approaches due to the limited timescales they can access. Although much has been accomplished by standard MD of atomistically-represented GPCRs using some of the largest multiprocessor computing clusters, running the most efficient MD codes currently available (e.g., see the work recently done to simulate ligand binding to the β 2-adrenergic receptor (B2AR) (Dror et al. 2011)), these hardware/software configurations are presently the prerogative of a small group of investigators. Undoubtedly, atomistic descriptions provide the most comprehensive and complete representations of a GPCR, but it is arguable that multiple, lengthy stochastic simulations of such systems are not best suited to access so-called “rare events” in the lives of GPCRs. Since the number of calculations required per step of a MD simulation of a GPCR system scales with the square of the number of particles, reducing the system size considerably increases the speed with which simulations can be performed. This size reduction can be achieved by eliminating the explicit representation of a component of the system (e.g. the solvent or the membrane, as in Generalized Born models), or by grouping individual atoms into interaction sites (e.g., coarse-grained bead models). Of course, such decisions must be taken with care and are inevitably dependent on the nature of the question that the simulation is designed to answer. In the cases where the fastest degrees of freedom can be neglected, these approaches have helped to smooth the energy landscape of GPCRs and thereby extend the range of accessible time and length scales.

The reduced representation of a GPCR system may, however, still be insufficient to bridge the gap between the timescales accessible to standard MD simulations and average experimental timescales. We will illustrate here how both simplified physical representations and enhanced MD-based methods have recently proven useful in the study of the rugged energy landscape of GPCRs by providing otherwise inaccessible details of important events in the life cycle of these receptors, such as recognition of ligands, ligand-specific conformational changes, and oligomerization. However, we acknowledge that both the rate at which advances are being made in this field, and the space constraints of this chapter do not permit an entirely comprehensive review here, but rather our goal is to provide an overview by way of a few selected examples.

6.2 Ligand Recognition in GPCRs

A thorough understanding of the mechanisms by which GPCRs recognize their ligands is fundamental to successful drug discovery. While multiple long timescale atomistic standard MD simulations (of the order of hundreds of μ s) have recently permitted researchers to visualize, for instance, the binding of different ligands to the B2AR (Dror et al. 2011), biased MD techniques have been successfully employed to enhance the probability of observing such events during shorter simulation timescales. Three studies we discuss in this section focus on the premise that from knowledge of the pathways by which a ligand can exit a receptor, one can infer specific details of the binding mechanism (Gonzalez et al. 2011; Selvam et al. 2012; Wang and Duan 2009). The probability of spontaneous ligand exit from a binding cavity, on the timescale accessible to MD simulations, can be enhanced by addition of an external force imposed upon the ligand, as is the case for steered MD and random acceleration MD. We compare the results of these studies with those obtained by the very long timescale, unbiased simulations from Dror and colleagues (2011). A fourth study we discuss below focuses on a binding event in which the ligand travels along a predetermined pathway from the bulk solvent, towards a bound state within the receptor (Provasi et al. 2009). We also comment on a novel method of altering the orthosteric binding pocket of receptor homology models, to permit successful docking of ligands of differing shape and size to that in the template structure (Kimura et al. 2008).

6.2.1 Exploring the Binding Site in Homology Models

Prior to the application of receptor engineering innovations that have given rise to the recent influx of solved crystal structures of GPCRs, homology modeling was the only tool in the arsenal of the researcher wishing to computationally investigate any receptor other than rhodopsin. However, for drug discovery purposes, a limitation of GPCR homology models has been that the binding pockets are not always big enough to accommodate the variety of ligands that have been demonstrated to bind to a particular receptor through other experimental assays. Indeed, retinal is a small, covalently bound ligand, so ligands of interest that are different in shape or size to retinal, and most importantly, not covalently bound, might not be expected to be a good fit to receptor models generated from a rhodopsin backbone template. Kimura and colleagues (2008) have developed a method for increasing the available space for ligand binding within the orthosteric pocket of a model of a GPCR, which may be readily transferable to other types of binding pockets for other proteins. Once

the binding pocket has been expanded using their algorithm, the endogenous ligand can be docked into the pocket, and induced fit docking methods, sidechain rotamer sampling or MD methods can be used to refine the pocket before virtual screening or docking of novel ligands is performed.

Conceptually, the method proposed by Kimura and colleagues is similar to inflating a balloon inside the pocket. By slowly increasing the pressure on the cavity walls during a MD simulation, the pocket can be expanded. To achieve this, the authors placed several (>20) Lennard-Jones (LJ) beads of small radius ($0.25 \text{ \AA}$) within the cavity, and these particles collided with the cavity walls with velocities consistent with a Maxwell distribution according to the temperature of the simulation. A slow increase in pressure was implemented by increasing the radii of the particles. The particles filled the pocket, and were tethered by weak ($k = 1 \text{ kcal}/(\text{mol } \text{\AA}^2)$) harmonic forces to their four immediate neighbors, but these bonds were reassigned with every increase of the particle radius. The radius was increased in steps of $0.05\text{--}0.1 \text{ \AA}$ every 300–500 ps. The simulations were performed using NAMD, with explicitly represented solvent and palmitoyl-oleoyl-phosphatidyl-choline (POPC) lipid bilayer. The system was minimized and equilibrated for several hundreds of picoseconds prior to the pocket expansion stages. During the expansion stages, the backbone dihedral angles of the protein were weakly restrained ($k = 2.0 \text{ kcal}/(\text{mol } \text{rad}^2)$) to prevent gross deformations and unwinding of the TM helices.

Kimura and colleagues tested their protocol on three receptors; firstly, in their 2008 paper (Kimura et al. 2008), they generated an apo state of the rhodopsin structure (PDB ID: 1LH9 (Okada et al. 2002)). By removing the retinal ligand and simulating the receptor for a period of $\sim 4 \text{ ns}$, the sidechains were rearranged such that the pocket was too small to accommodate retinal without modification. After the pocket expansion simulations, docking studies were performed with GLIDE (Friesner et al. 2004, 2006; Halgren et al. 2004) and a comparison of the crystal structure, the MD relaxed structure and the structure after expansion with the balloon potential indicate that re-docking the retinal is significantly improved between the relaxed structure and the balloon expansion structure, but the crystal structure pose is not recovered exactly, with an RMSD of $7.2 \text{ \AA}$ between the re-docked pose and the crystallographic pose.

Secondly, they built a homology model of the chemokine receptor type 2 (CCR2) based on a rhodopsin template. Three potent antagonists, RS-504393 (MacKerell et al. 1998), TAK-779 (Baba et al. 1999), and a Teijin lead compound (Shiota 1999) were docked into the receptor at different stages of the pocket expansion. At $r = 0.35 \text{ \AA}$, a cluster of poses correlating with the available mutagenesis data was observed. Docking into the pockets formed with $r > 0.35 \text{ \AA}$ yielded many more poses, owing to the increased space available in the pocket, but clustering these poses did not yield any that matched with the known mutagenesis data.

Finally (Krystek et al. 2006), the same authors have built and refined a homology model of B2AR from a rhodopsin template (PDB ID: 1F88 (Okada et al. 2002)) and validated this model according to the paradigm of structure activity relationships (SAR) and mutagenesis data existing at the time (the study was published in 2006, prior to the solution of the B2AR structure (Cherezov et al. 2007)). After pocket expansion, docking of agonists such as

propranolol into the $r = 0.3$ state satisfied available experimental data of agonist ligand binding, although required the re-orientation of some side chains (Strader et al. 1988, 1989). It seems that key advantage provided by the balloon expansion methodology is that it generates an ensemble of models, unbiased by the inherent dependence on the template introduced during the homology modeling process, which may then be evaluated in the context of the available mutagenesis, SAR and small molecule pharmacophoric data. However, these expanded models require further refinement before the results of any docking studies may be considered any more than qualitatively accurate.

6.2.2 Exploring Ligand Egress Pathways with Random Acceleration MD

Random acceleration MD (Wang and Duan 2007, 2009), also called random expulsion MD (Ludemann et al. 2000) uses a randomly directed, externally applied force to encourage unbinding of a ligand from the pocket of a receptor. This ensures thorough exploration of the pocket, and good sampling of possible modes of ligand egress. According to the formulation, the direction of the force applied to the center of mass (COM) of the ligand is chosen at random, and it is maintained for N steps. During these steps, the COM of the ligand is expected to move a minimum distance r_{min} , i.e. the average velocity, $\langle v \rangle$, of the ligand will maintain a threshold value of at least:

$$\langle v \rangle = r_{min} / N \Delta t, \quad (6.1)$$

where Δt is the timestep of the MD simulation.

Upon encountering an unyielding portion of the binding site, the velocity of the ligand falls below the threshold velocity and the trajectory is considered to be complete, thus a new random direction is assigned. For each new trajectory, the direction of the force, and thus the movement of the ligand, is maintained as long as the velocity of the ligand exceeds the threshold value, or until N steps of MD have been completed. By using multiple trajectories, the ligand can thoroughly probe the binding pocket until it finds a suitable exit pathway or no egress, if appropriate. The key feature of this technique, which makes it particularly effective for the purpose of exploring ligand binding pathways, as compared to e.g. steered MD, is that no prior knowledge of an exit pathway is required.

In 2007, Wang and Duan (2007) applied the random acceleration MD technique to the rhodopsin crystal structure, (PDB ID: 1U19 (Okada et al. 2004)), embedded in a POPC bilayer and solvated with explicit water. The endogenous ligand, 11-cis-retinal, is covalently bonded to K296^{7.43} within a deep binding pocket formed by the TM domain of rhodopsin. The superscript refers to the Ballesteros-Weinstein generic residue numbering scheme (Ballesteros and Weinstein 1995), where the first number (e.g., 1 in 1.48) indicates the helix, and the second number (e.g., 48 in 1.48) represents the residue position in that helix relative to the most conserved residue in the helix (numbered 50 by definition). EL2 in rhodopsin forms a beta-hairpin fold that completely blocks access to this binding pocket from the extracellular side. Thirty-eight random acceleration MD simulations were performed on the rhodopsin system and the predominant exit pathways were observed to be towards the extracellular side, through interhelical clefts, either between TM4 and TM5, or between

TM5 and TM6. These exit pathways involved transient breakage of the interhelical interactions, which reformed immediately upon complete exit of the ligand from the binding site.

In 2009, the same authors conducted a similar investigation on the B2AR (Wang and Duan 2009). A total of 100 random acceleration MD trajectories were performed on the B2AR crystal structure (PDB ID: 2RH1) (Cherezov et al. 2007), which was first crystallized in the inactive conformation, with carazolol (an inverse agonist) located in a binding pocket defined by strong interactions with polar residues in TM3, TM5 and TM7. The second extracellular loop (EL2) forms a short helix in the B2AR crystal structure, and is extended outward, rendering the binding pocket slightly open to the extracellular side. Egress from the binding site is, however, restricted by two bulky aromatic residues (F193 on EL2 and Y308^{7,35} on TM7) and a salt bridge between EL2 and TM7, formed by D192-K305^{7,32}.

The random acceleration MD trajectories suggested that the dominant exit pathway of carazolol from the B2AR crystallographic binding pocket was via the extracellular opening of the binding pocket. The salt bridge between EL2 and TM7, (D192-K305^{7,32}), was broken during exit along this pathway. Furthermore, if the salt bridge can be thought of as bisecting the extracellular opening of the receptor, exit via this dominant pathway can be subdivided into two, and egress was found to be approximately equally distributed between both the A1 sub-pathway (toward TM 5, 6 and 7, thick red arrow 1 in Fig. 6.1), and A2 sub-pathway (toward TM 2, 3 and 7, thick red arrow 2 in Fig. 6.1), of the salt bridge. Five additional exit pathways were observed through inter-helical clefts. Of these, the most statistically significant offered an exit through transient breakage of the interhelical interactions between TM4 and TM5. The predominant barrier to ligand egress was presented by the interactions between the ligand and the polar groups within the binding pocket.

During 120 ns of unbiased, standard MD simulation of the receptor in the absence of ligand, the authors found that the D192-K305^{7,32} salt bridge was dynamic in nature, and that a conformational change in F193 caused it to rotate outward toward TM7 and forming a 'hydrophobic cluster' between EL2 and TM7. The authors repeated their random acceleration MD simulations from this state after re-docking the carazolol. Overall, the average ligand egress time was slightly longer from the putative 'ligand-free' conformation than from the crystal structure and this increase was attributed to the barrier formed by the clustering of F193 and the salt bridge connecting TM7 and EL2. Furthermore, the population of the dominant pathway was shifted strongly toward exit via the A2 sub-pathway (arrow 2 in Fig. 6.1), between ECL2 and TM2, 3, and 7. The authors used these results to propose a binding pathway for carazolol. First, the ligand was found to access the receptor via the cleft between TM2, TM3 and TM7 at the extracellular opening. Subsequently, the ligand interacted with F193 as it passed through the TM7-EL2 junction on the way to the bottom of the binding pocket, where it was oriented and stabilized by the polar interactions with TM3, TM5 and TM7. The difference in results between the B2AR and rhodopsin studies described here, alongside speculative reports of different lipid phase ligand entry pathways for e.g. the cannabinoid receptor (Hurst et al. 2010) and for the PAR1 (Zhang et al. 2012) indicate that ligand binding pathways may not be general across receptor types.

6.2.3 Forced Ligand Unbinding by Steered MD

Under experimental conditions, unbinding of a ligand can be monitored by means of Atomic Force Microscopy (AFM), by applying a time-dependent external force to the system through the atomistically fine tip, and measuring the mechanical resistance properties of the biomolecule. Such a process can be mimicked virtually, using steered MD. Steered MD uses the application of an external force to drive the system towards a desired state, be it via a conformational change, or a ligand-unbinding event. Steered MD is a non-equilibrium technique, during which the MD pathway is irreversible. Analysis of the position and interactions of the dissociating ligand, and the evolution of the applied forces during a forced ligand unbinding by steered MD can provide reliable qualitative insights into the irreversible work required for the unbinding process (Isralewitz et al. 1997; Jensen et al. 2002; Fishelovitch et al. 2009; Yang et al. 2009). Such information may enable researchers to determine structural features of these receptorligand complexes that contribute crucially to ligand binding. Unlike random acceleration MD, the direction of the force must be predetermined, though usually this is unknown. The egress pathway can therefore often be arbitrary, and as such the sampling of different possible modes of ligand exit may be less comprehensive than with random acceleration MD.

In steered MD, the elastic force is proportional to the change in the spring extension, relative to its equilibrium length:

$$F(t) = k(vt - \vec{r}(t) - \vec{r}_0) \bullet \vec{n} \quad (6.2)$$

where k is the spring constant; v is the constant velocity of pulling, mimicking the retracting cantilever; r_0 and $r(t)$ are the ligand center of mass position at initial and current time t respectively and n is the direction of the pulling vector.

The potential of mean force (PMF) along the reaction coordinate was calculated by the second-order cumulant expansion of the irreversible work measurements (Park et al. 2003) according to:

$$W(t) = v \int_0^t F(t) dt \quad (6.3)$$

$$PMF = \langle W \rangle - \frac{1}{2k_B T} (\langle W^2 \rangle - \langle W \rangle^2) \quad (6.4)$$

where $\langle W \rangle$ is the mean work averaged from the six trajectories, k_B is Boltzmann's constant and T is the bulk temperature.

Gonzalez and colleagues embedded the crystal structure of the human B2AR (PDB ID: 2RH1) (Cherezov et al. 2007) and a MODELLER (Eswar et al. 2007; Fiser and Sali 2003) generated structure of the human B1AR, based on the turkey B1AR crystal structure (PDB ID: 2VT4) (Warne et al. 2008) into POPC membranes, and then used steered MD to remove the two crystallized ligands, cyanopindolol and carazolol, from the binding sites of B1AR and B2AR respectively, through different channels (Gonzalez et al. 2011). Steered MD simulations were performed at constant velocity of 10 Å/ns and the spring constant was set

to 250 pN/Å. Trajectories were repeated six times, and lasted approximately 3 ns each. The force profile, as a function of simulation time, revealed the ease with which the ligand can be extracted from the receptor along the different pathways (Gonzalez et al. 2011). To address the shortcomings of an arbitrary choice of exit pathway from the receptors, CAVER (Petrek et al. 2006) was used to establish feasible egress pathways connecting the orthosteric binding pocket with the surface of the receptor. This showed two possible pathways, depicted in Fig. 6.1, by red arrows labeled 1 and 2. Two additional, lipid phase exit pathways between TM1/7 and TM5/6 inter-helical clefts were also tested (red arrows 3 and 4 in Fig. 6.1). Though not found by the CAVER exploration, they have been implicated as entry/exit pathways for retinal in rhodopsin (Hildebrand et al. 2009), and an exit pathway between TM5/6 was found to be significant in the random acceleration MD study of rhodopsin, from Wang and Duan (2007) (neither of these pathways was found to be significant in their investigation of B2AR (Wang and Duan 2009)). In Gonzalez's investigation, the initial force peaks for extracting ligands through pathways 3 and 4 were found to be twice that for extraction via pathway 1 or pathway 2, so the lipid phase extraction channels were considered to be unfavorable for the adrenergic receptors. For B2AR, extraction of carazolol via pathway 1 (bounded by TM5, 6 and 7) was determined to have two force peaks, indicating breaking of interactions between ligand and receptor along the exit pathway. The first (and highest) peak in the force occurred early in the simulations and represents extraction from the orthosteric binding pocket, breaking key interactions with D3.32, S5.42 and N7.39, while the second peak occurred later, and represents breaking interactions with D192 and N301 in the extracellular loops EL2 and EL3 respectively. Extraction of cyanopindolol from B1AR through the same channel showed similar behavior, showing not only a second barrier, arising from interactions with D217 in EL2, and D7.32, but also some subsequent barriers, as additional interactions in the extracellular loops were broken. Extractions of the ligands through channel 2 (bounded by TM2, 3 and 7) for both receptors demonstrate two retention sites after the initial orthosteric pocket interactions were broken, before being released into the solvent. The potentials of mean force (PMFs) of these extractions indicate channel 1 is favored for both receptors, but only by a small amount (~1 kcal/mol). The PMFs also show that the difference between the bound and the unbound states is positive, indicating that the bound state is favored in all cases.

These pathways suggested by random acceleration MD and steered MD can be directly compared with the recent entry pathways observed for similar ligands (antagonists alprenolol, propranolol and dihydroalprenolol) binding to B1AR and B2AR, by Dror and colleagues, during 82 standard MD simulations, ranging from 1 to 19 μ s in length (Dror et al. 2011). During their 82 simulations, Dror and colleagues observe 21 binding events in total. Out of 12 binding events for alprenolol to B2AR, in 6 cases alprenolol replicates the crystallographic pose. The authors note that entry is almost exclusively through the cleft between ECL2 and TM5, 6 and 7 (11 out of 12 binding events) i.e. pathway 1 in Fig. 6.1. In the remaining event, entry was through pathway 2. Similar results were observed for binding dihydroalprenolol to B1AR. These results are largely in agreement with the preference noted by Gonzalez and colleagues (2011), but opposite to that of Wang and Duan (2009). Also, similarly to Gonzalez and colleagues, but again unlike Wang and Duan, Dror and colleagues observed two energetic barriers to ligand binding through pathway 1 for B2AR (Dror et al.

2011). The main barrier is that presented by ligand dewetting and orientation within the binding pocket, and is comparable to that observed during ligand extraction from the orthosteric pocket for both of the biased MD studies. The other barrier observed by Dror and colleagues occurred prior to ligand entry into the binding pocket and was not observed during the random acceleration MD simulations (Wang and Duan 2009), but according to Dror and colleagues, is at least as large, if not larger than the barrier to binding in the pocket (Dror et al. 2011). This barrier is comparable to the second barrier in the force profile observed during Gonzalez and colleagues' steered MD simulations (Gonzalez et al. 2011) and was attributed to dewetting of both the ligand and the binding pocket by Dror and colleagues, noting that alprenolol loses 80 % of its hydration upon binding, and 63 % of this is at the point of entering the pre-binding vestibule (Dror et al. 2011). Gonzalez and colleagues present data showing that the number of water molecules within 3 Å of the ligand in their simulations increases dramatically, from ~ 10 to ~30 molecules, at the point of encountering this second barrier to ligand egress (Gonzalez et al. 2011).

The biased MD simulations are largely able to reproduce the characteristics of binding to B2AR, i.e. dominant exit through the extracellular opening of B2AR, no or limited exit through interhelical pathways (Dror and colleagues observe that alprenolol partitions into the bilayer but never enters the receptor through an interhelical pathway). The steered MD is also able to replicate the existence of two barriers to ligand binding at stages along the entry/egress pathway comparable to those observed in the unbiased simulations. Furthermore, many of the detailed features of the dynamics of the ligand binding events can be captured by both the random acceleration MD and steered MD, including conformational changes in F218/193 and F/Y7.35 in B1AR/B2AR respectively; changes in ligand hydration and the dynamic nature of the salt bridge between K305 and D192. The key advantage of the biased events is the much shorter simulations that are required to obtain these details.

Following the simulations described above, Selvam and colleagues (2012) have used a similar steered MD methodology to Gonzalez and colleagues, to demonstrate that the PMF of extracting ligands from the B1AR and the B2AR can be used to differentiate selective ligands from their non-selective counterparts. The work performed to extract a B1AR selective antagonist (Esmolol) from the B1AR was greater than that required to extract ICI-118,551 (B2AR selective) and the reverse is also true: more work was required to extract ICI-118,551 from B2AR than Esmolol.

6.2.4 Exploring Ligand Binding Pathways Using Well-Tempered Metadynamics

Well-tempered metadynamics (Barducci et al. 2008) is an enhanced sampling technique that enables more efficient exploration of the multidimensional free energy surfaces of biological systems by adding Gaussian bias to a standard MD simulation (Laio and Parrinello 2002; Leone et al. 2010). The dynamics is biased by a non-Markovian (history-dependent) potential, constructed as a sum of Gaussian "hills" localized along the trajectory in the direction of the reaction coordinate (or collective variable, CV) of interest. The accumulation of this biasing potential enables the simulated system to overcome high energy barriers in order to explore efficiently its free energy landscape as defined by the CVs. The success of a metadynamics study is predicated on a careful *a priori* choice of a set of CVs to

provide a satisfactory description of the process of interest. In well-tempered metadynamics, the height of the added Gaussian hills depends both on a temperature scaling factor and the underlying bias, and decreases to zero once a given energy threshold is reached. As a result, convergence of the algorithm to the correct free energy profile can be proven rigorously, and exploration of physically relevant regions of the conformational space is ensured for complex systems.

In 2009, we performed well-tempered metadynamics to elucidate the mechanistic details of flexible-ligand, flexible-protein docking of naloxone (NLX), a non-selective antagonist for opioid receptors, from the bulk water environment into the orthosteric binding pocket of a B2AR-based homology model of δ opioid (DOP) receptor (Provasi et al. 2009). Using the multiple walker method (Raiteri et al. 2006), several well-tempered metadynamics simulations can be simultaneously performed, each contributing to the same history-dependent bias potential; this is a key advantage of the method, enabling the most efficient use of cluster computing resources. Thus, the DOP receptor was simulated for 500 ns, in a hydrated DPPC-cholesterol lipid bilayer, using ten walkers. All simulations were performed using GROMACS 4.0.5 (Van der Spoel et al. 2005) with PLUMED (Bonomi et al. 2009).

To represent the binding event, two CVs were chosen to describe (1) the distance between the center of mass of the heavy atoms of NLX and the center of mass of the heavy atoms of the alkaloid-binding pocket of opioid receptor and (2) the distance between the center of mass of the DOP receptor alkaloid binding pocket and the center of mass of the heavy atoms of the middle residues of the EL2. This second CV was selected to enable enhanced conformational sampling of the EL2 region of the DOP receptor, given its uncertain predicted structure (this work was performed before the crystal structure of the DOP receptor became available (Granier et al. 2012)). Using these two CVs, we reconstructed the free-energy surface of the NLX binding event, determining that the non-selective antagonist NLX exhibits a molecular recognition site on the DOP receptor surface at a cleft formed by EL2 and EL3, and ends in a preferred orientation into the receptor alkaloid binding pocket, after visiting some less stable states in between. The most stable NLX-bound state of DOP corresponded to a conformation in which NLX interacted directly with D128^{3.32} via a salt bridge, and with Y308^{7.43}, W274^{6.48}, H278^{6.52}, in broad agreement with experimental data from mutagenesis and competition binding assays (Befort et al. 1996a, b; Li et al. 1999; Mansour et al. 1997; Bot et al. 1998; Spivak et al. 1997; Surratt et al. 1994). The ligand was stabilized in a specific orientation through interactions with a number of residues on TM3, TM5, TM6 and TM7, in particular: M132^{3.35} as well as F218^{5.43} and F222^{5.47}. The recent crystal structure of the DOP bound to naltrindole confirmed some key interactions in the bound state, in particular, the interactions with Y308^{7.43}, H274^{6.52}, M132^{3.36} and D128^{3.32} were observed. On the pathway to this final binding mode, NLX visited a number of different less stable alternative pockets and in particular, two metastable states characterized by different degrees of opening of EL2. The existence of these metastable states before binding to the orthosteric pocket is reminiscent of the pathways observed for the B2AR using steered MD (Gonzalez et al. 2011) and unbiased MD (Dror et al. 2011), though the intermediate poses visited by NLX along the binding pathway are, as expected, not the same as those observed for ligand binding to B2AR. Some residue positions are, however,

implicated to interact along the entry pathway for both receptors, in particular Y/L7.35, H/W6.58 and D300/D290 in the B2AR and DOP receptor respectively.

Sampling of the NLX ligand in the bulk solvent was corrected for, using a methodology put forward by the Roux lab (Roux 1999; Allen et al. 2004). Using the free energy surface from the metadynamics simulation, with the collective variables described above, we obtained a restrained free-energy profile, following the protocol and equations described in (Provasi et al. 2009). Our calculated equilibrium constant, of $K_{eq} = 80 \pm 13$ nM, for the final bound state of NLX at DOP was remarkably close to the majority of reported experimental values (e.g. Toll et al. 1998), and thus, this methodology offers great potential for describing, quantitatively, the binding events of other GPCRs.

6.3 Activation Mechanisms in GPCRs

Activation processes of GPCRs are known to occur on timescales that are inaccessible to current simulations using standard MD. From recent crystal structures, the most pronounced, common conformational rearrangements that mark the activation of a receptor include: breaking of the so-called “ionic lock” between the E/DRY motif in TM3 and acidic residues in TM6, upon a large (i.e. ~ 11 Å in the case of B2AR (Rasmussen et al. 2011a)) outward movement and slight rotation of the intracellular end of TM6; smaller (i.e. ~ 6 Å for B2AR (Rasmussen et al. 2011a)) outward movement of TM5; and some smaller slightly inward movements of TM3 and TM7. Here we describe activated GPCR models obtained using reduced system representations and steered MD, as well as combinations of adiabatic biased MD and metadynamics techniques. We also discuss ligand-specific conformations obtained depending on the physiological response of the bound ligand. In particular, we discuss applications to two GPCRs, the B2AR and the 5HT_{2A} serotonin receptor, and their perceived success as judged by virtual ligand screening and, in the case of B2AR, when compared with the recently published crystal structures.

6.3.1 Using Guided MD to Build Activated GPCRs

There are very few currently available X-ray crystal structures of GPCRs displaying the characteristics of an active state, and these are: the ligand free opsin crystal structures at either low pH (PDB ID: 3CAP (Park et al. 2008)) or in complex with a synthetic peptide derived from carboxy terminus of the alpha-subunit of the heterotrimeric G protein (PDB ID: 3DQB (Scheerer et al. 2008)); the crystal structure of the proposed active state of the A2A adenosine receptor (PDB ID: 3QAK, 2YDO (Xu et al. 2011; Lebon et al. 2011)); the crystal structures of metarhodopsin II (PDB ID: 3PXO (Choe et al. 2011)) and a constitutively active metarhodopsin II (PDB ID: 4A4M (Deupi et al. 2012)); and finally the crystal structures of B2AR in complex with the Gs protein (PDB ID: 3SN6 (Rasmussen et al. 2011b)), and a camelid nanobody (PDB ID: 3P0G (Rasmussen et al. 2011a)).

In 2011, two groups combined experimentally derived restraints and ligand binding data from extensive literature searches with steered MD to guide the generation of the active states of the B2AR (Simpson et al. 2011) and the 5HT_{2A} receptor (Isberg et al. 2011), and have assessed the success of their approaches by virtual screening of test sets of known agonists, antagonists and inverse agonists, diluted among non-binders.

To build an initial model for the active state of the B2AR, Simpson and colleagues have combined the intracellular part of the opsin crystal PDB ID: 3DQB (Scheerer et al. 2008) with the extracellular portion of the inactive B2AR crystal structure at 2.1 Å (PDB ID: 2RH1 (Cherezov et al. 2007)) to generate a template, and used an alignment based on common motifs in the transmembrane regions as input for MODELLER version 9v1 (Fiser and Sali 2003). Two hundred initial models were narrowed down to a single model that scored well according to the MODELLER objective function (measuring spatial restraint violation) and had a low backbone RMSD from the template.

Isberg and colleagues generated a single homology model of the 5HT_{2A} based on the B2AR inactive structure, PDB ID: 2RH1 (Cherezov et al. 2007). They modified this structure, to match the active characteristics found in the opsin structure, PDB ID: 3DQB (Scheerer et al. 2008). Specifically, (1) the lower parts of TM5 and TM6 were tilted outwards, manually; (2) the missing density of IL3 in the B2AR structure was replaced by the opsin IL3 structure, and (3) the Gα_i peptide was inserted into the structure and mutated to Gα_s and then subsequently to the Gα_q subtype. Finally, (4), the rotamer of R131^{3,50} was set to their G protein interacting conformation, and W286^{6,48} was set to its presumed active conformation, as predicted by some earlier investigations (see e.g. Holst et al. 2010; Shi et al. 2002, among others), but not yet observed in any active receptor crystals to date (see supplementary figure 7a of Taddese et al. 2012). This modified structure was then used as the input to MODELLER version 9v6 (Eswar et al. 2007).

In agreement with subsequent observations of the activated B2AR during long timescale MD simulations (Rosenbaum et al. 2011), which revealed that interaction with either the G protein or a nano-body G protein surrogate is essential to maintaining an activated receptor conformation, both groups have included interactions with the G protein to complete their activated conformations. The 3DQB opsin structure represents the apo-receptor, stabilized by a peptide derived from the main binding site of the heterotrimeric G protein, the C-terminus of the α subunit (GαCT). Isberg and colleagues included the G protein peptide, mutated to Gⁱq while Simpson and colleagues modeled the whole heterotrimeric assembly, using structural details from a number of different crystallographic structures (namely PDB IDs: 1AZS (Tesmer et al. 1997), 1GOT (Lambright et al. 1996) and 1GP2 (Wall et al. 1995)).

The initial model of the B2AR from Simpson and colleagues was energy minimized using the CHARMM (Brooks et al. 1983) force field and an implicitly represented membrane/solvent environment described by the Generalized Born algorithm with the simple switching function (GBSW) (Im et al. 2004). In the GBSW model, the influence of the membrane is included as a solvent-inaccessible, infinite planar slab of low dielectric constant. A simple smoothing function is included to approximate the dielectric boundary between the “bulk water” and the “membrane” (Im et al. 2003). For this study, the membrane thickness was set at 35 Å with a smoothing length of 5 Å. The use of the implicit membrane can minimize problems associated with complete sampling of phase space during short standard MD simulations, since the number of explicit particles for which pair-wise interactions are required to be calculated is dramatically reduced. In particular for this application, this meant that the entire G protein assembly could be included without the prohibitive

contribution to the number of particles from explicitly represented solvent. The simulation length total was approximately 175 ns. The choice to use an implicit solvent model could be controversial, given previous reports of a structural and functional role for H-bonding interactions of waters entering the rhodopsin structure upon activation (Grossfield et al. 2008), and the internal crystallographic waters noted in many X-ray derived structures of GPCRs. Simpson and colleagues have indirectly incorporated the effects of such solvation, through specific experimentally derived restraints that would open out parts of the structure sufficient to permit entry of water molecules upon activation under explicit solvation conditions.

Isberg and colleagues embedded their model of the 5HT_{2A} receptor in an explicitly represented DPPC membrane, with the TIP4P explicit water model and counterions under the OPLS-AA 2001 (Kaminski et al. 2001) forcefield. Their simulations were carried out using the Schrödinger implementation of the DESMOND software package, version 2.4 originally developed by DE Shaw and associates (Chow et al. 2006). Perhaps reflecting the increased number of degrees of freedom, these simulations are shorter in duration, four stages of 5 ns simulations with restraints for a total of 20 ns. The choice of DPPC may not be the best lipid for activation studies of GPCRs, since Vogel and colleagues presented evidence that, at least for rhodopsin, the fully saturated chains of DPPC can inhibit the activation transition to the receptor activated meta II state (Vogel et al. 2004). Furthermore, the melting temperature of DPPC is 41 °C, and so, under physiological conditions, the membrane might be expected to be gel-like, casting some doubt on the choice of this lipid as a valid membrane mimetic. Nevertheless, Isberg and colleagues ensured a fluid membrane environment for their 5HT_{2A} model, by maintaining their simulation conditions at T = 325 K (~51 °C).

Both groups derived sets of restraints from previously published experimental information to define their activated states, and in both cases, during the earliest parts of the simulations, harmonic restraints were applied to the backbone of transmembrane regions of the receptor, to prevent distortions of the helical structure. Isberg and colleagues applied pairwise harmonic distance constraints, collated from mutagenesis and X-ray crystallographic data, to inter-helical, ligandreceptor and G protein-receptor distances. These constraints were used to drive the conformation of the 5HT_{2A} towards a presumed activated structure, achieved once they are satisfied. Simpson and colleagues applied six sets of harmonic distance restraints to the B2AR receptor model during MD simulations of 150 ns in production length, to drive the conformation towards an activated state. The basis of these restraints was from experimental evidence sourced from the available literature on both B2AR and other class A GPCRs (in particular, the muscarinic receptor and rhodopsin). The sets of restraints were derived from site-directed spin labeling, disulfide cross-linking, engineered zinc binding and site-directed mutagenesis experiments. The restraints were introduced slowly, as the system was heated to simulation temperature, and all but the toggle switch restraints were maintained throughout the 150 ns production simulation.

The success of the models, in both cases, was probed by virtual screening during which it was demonstrated that the predicted active models successfully discriminated known receptor-selective agonists from antagonists. Simpson and colleagues refined their active

structure by flexibly docking full agonists epinephrine, isoproterenol, TA-2005 and salmeterol into the binding pocket, and screened these refined receptors using GLIDE (Friesner et al. 2004, 2006; Halgren et al. 2004). They were able to check all binding poses, because they used a fairly small set of ligands, comprising 29 full and partial B1/B2AR agonists, 38 B1/B2AR antagonists and inverse agonists, and 56 non-peptide ligands selective for opioid, muscarinic, neurokinin, neurotensin, bradykinin and serotonin receptors. Docking scores demonstrated that the active model showed a preference for B1/B2AR agonists over antagonists, inverse agonists and other ligands. Active enrichment curves showed that the enrichment over the first 10 % of ligands was significantly greater than expected at random. Furthermore, enrichment for B2AR-selective agonists was higher than for B1AR-selective agonists, and enrichment for antagonists was only observed once all the agonists had been found. Isberg and colleagues performed a larger scale virtual screen, also using GLIDE. They selected 182 known 5HT_{2A} ligands, including 139 agonists (112 phenylethylamine agonists and 27 others), 39 antagonists and 4 inverse agonists. These were diluted with 9257 diverse compounds from the ZINC database (Irwin et al. 2012). GLIDE docking scores demonstrated that the 29 highest ranked hits were phenylethylamine agonists, and these were the best scoring class of molecules by a large margin, with a mean G-score 1.5 lower than other agonists. Phenylethylamine antagonists also scored better than other agonists, other antagonists, and other ligands. The virtual screenings seem to demonstrate the success of modeling the active state of both receptors, using restraints derived from experimental data. However, the subsequent solution of the active state crystal structures of B2AR (Rasmussen et al. 2011a, b; Rosenbaum et al. 2011) has enabled direct comparison between the B2AR crystal and the active model, and Taddese and colleagues (2012) have discussed this comparison in a brief paper in 2012. Overall, they report an average RMSD of 1.3 Å in the invariant TM region (i.e. residues that have the same environment when compared over a number of structures) between the active model from Simpson and colleagues (2011) and the active B2AR structure. The overall RMSD is perhaps less explanatory than the residue-by-residue variation between the B2AR model and the B2AR crystal structure, which ranges from 1.0 Å in the TM region, to 7.2 Å in the loops. The larger value of RMSD for the loop regions is not unexpected owing to their high mobility. The active B2AR model was based on an active, G protein bound state of opsin, and the difference between the opsin template and the active B2AR crystal structure is 1.7 Å over the invariant TM region. The active model shows good agreement with the overall position of the helices in the structure PDB ID: 3SN6 for TM1, TM2 and TM5, but seems more like the opsin template for TM3, TM4, TM6 and TM7. The orthosteric binding pocket appears to have been successfully modeled, with an RMSD of 1.3 Å between the residues in the active model and the crystal structure, and this may be the reason for the strong enrichment results for the virtual screening. The key receptor-G protein interactions were correctly predicted from the opsin-G protein complex template, with 27/38 residues on the receptor and 20/34 residues on the G protein surface correctly predicted. Overall the modeling moderately successfully reproduced the nature of the activated characteristics observed in the crystal structure, but the atomic resolution specificity of the interactions seen in the single conformation of the X-ray structure was not reproduced. This can be thought of as a direct consequence of the simplification or averaging over interactions collated from different structural/biophysical sources introduced by the homology modeling process, but

can also be attributed to the static nature of a crystallographic structure versus the dynamic manner in which the model was constructed.

6.3.2 Combined Adiabatic Biased MD with Metadynamics to Explore Activation Pathways

As mentioned earlier in this chapter, and as observed in experimental studies, the value of GPCRs as pharmaceutical targets is enhanced by the concept of “functional selectivity”, or “biased agonism”, a concept wherein a single ligand might display different efficacies at a single receptor isoform depending on the effector pathway coupled to that receptor (Rives et al. 2012). A simple mechanistic explanation for this phenomenon can be derived from the dynamic equilibrium of GPCRs between different conformational sub-states. Different ligands or changes in protein-protein interactions (such as may be observed in receptor dimerization) can shift the equilibrium population of these sub-states toward conformations of the receptor that are kinetically and structurally distinct. Evidence of different sub-states can be inferred from crystallographic structures: not all of the attributes usually associated with an ‘activestate’ receptor are present for active-state crystal structures solved in the presence of different agonists (Provasi et al. 2011). We have recently developed a general computational strategy that combines adiabatic biased MD with path CV metadynamics, which we have used to characterize possible metastable active states of rhodopsin (Provasi and Filizola 2010); to discern the effects of ligands with variable efficacies on the conformation of the B2AR receptor (Provasi et al. 2011) and, finally, to offer a mechanistic explanation of allosteric effects of ligand binding in a GPCR dimer of the 5HT_{2A} serotonin receptor and the glutamate receptor (mGluR2) observed by our experimental colleagues (Fribourg et al. 2011).

In the first stage of the method, a monodimensional pathway defining activation of the receptor was derived. Frames depicting the receptor in intermediate states between ‘inactive’ and ‘active’ X-ray-derived crystal structures (or models, in the absence of crystal structures) defined the pathway. The frames were derived from multiple (e.g. 5 or 10) statistically independent adiabatic biased MD simulations (Marchi and Ballone 1999). Like targeted MD (Schlitter et al. 1994), adiabatic biased MD drives the system towards the desired final state, by enforcing a reduction in RMSD between the initial and target conformations, to enable observations of a conformational transition during a single trajectory. In contrast to TMD, the adiabatic biased MD algorithm (Marchi and Ballone 1999) ensures exploration of low-energy pathways by keeping the total potential energy of the system constant during the MD run through application of a time-dependent biasing potential. The harmonic biasing potential is only applied when the RMSD is bigger than the minimum value previously achieved during the trajectory. The adiabatic biased MD trajectories were short (e.g. 1–5 ns), with small elastic constants (0.01 kJ/nm²) and independently drawn Maxwellian initial velocities. All of the independent adiabatic biased MD trajectories were then pooled, and all of the sampled conformations clustered based on the RMSD of the TM domain of the receptor. Representative structures from these clusters were then chosen as reference frames to define the path CVs for further metadynamics simulations. The number of clusters, and hence frames, was between 4 and 10, depending on the nature of application. See Fig. 6.2 for an example of this.

To obtain information about the relative stability of the states populated by the receptor during transition from inactive to active conformations, metadynamics simulations with path collective variables (Branduardi et al. 2007) can be performed. The CVs defining the phase space are the position (s) along and the distance (z) from the pre-determined activation pathway. The reference frames, k , define the position of the progression of the system from inactive ($s \sim 0$) to active ($s \sim 1$) during the pre-determined transition (CV 1), and its distance (z) from it (CV 2), according to the following equation:

$$s(R(t)) = Z^{-1} \sum_{i=1}^k \frac{i-1}{k-1} e^{-\lambda d_{TM}(R(t), R^{(i)})} \quad (6.5)$$

where $\delta = \langle d_{TM}(R^{(i)}, R^{(i+1)}) \rangle$ is the average distance between two adjacent frames in the transition pathway, the constant exponent, λ , must be chosen in such a way that $\lambda \times \delta \cong 1$, and Z is a normalization factor defined by:

$$Z \sum_{i=1}^k e^{-\lambda d_{TM}(R(t), R^{(i)})} \quad (6.6)$$

while

$$z(R(t)) = -\lambda^{-1} \log Z \quad (6.7)$$

The free-energy of the receptor as a function of s and z can then be reconstructed from converged well-tempered metadynamics simulations of varying length (~80–300 ns) (see Provasi et al. 2011; Provasi and Filizola 2010; Fribourg et al. 2011 for details of the simulation protocols). Convergence of the reconstructed free-energy can be assessed in two ways: firstly, by ensuring minimal variation in the free-energy difference between the minima around the experimental endpoints with time, and secondly, in a converged simulation, exploration of the FES represented by the CVs faces no further energy barriers, therefore one can expect frequent re-crossing of the values of the CVs after convergence is attained.

All simulations in each of the applications of this method to date have been carried out using an explicit atomistic representation of all components, and the receptors were embedded in solvated POPC/10 % cholesterol membranes. The versatility of the method lies in the implementation using Plumed plugin (Bonomi et al. 2009), meaning it has been performed using GROMACS (Van der Spoel et al. 2005) and the OPLS-AA force-field (Kaminski et al. 2001) for rhodopsin and B2AR (Provasi et al. 2011; Provasi and Filizola 2010) and NAMD 2.7 (Phillips et al. 2005) with the Charmm27 force-field (MacKerell et al. 1998) for the 5HT_{2A}, mGluR2 monomers and dimer (Fribourg et al. 2011).

This combinatorial approach was initially validated on rhodopsin (Provasi and Filizola 2010), where the activation pathway was defined between the crystal structure of a photoactivated, deprotonated intermediate of rhodopsin (PDB ID: 2I37) and the low pH crystal structure of opsin (PDB ID: 3CAP), the only crystal structure displaying characteristics of an active state available at the time of this study. Simulations were

performed for wild-type rhodopsin, embedded in a POPC membrane, with either a charged or an uncharged residue E134^{3,49} within the E(D)RY motif, to simulate proton uptake from the bulk occurring in the late stage of rhodopsin activation.

Reconstruction of the rhodopsin free energy landscape indicated three common metastable states between 2I37 and 3CAP along the adiabatic biased MD pre-calculated transition path. Two of the identified minima are comparable to active intermediates of bovine rhodopsin, characterized by different amplitude of the outward movement of TM6. This was revealed by comparing intra-molecular distance analysis of these states with results from double electron-electron resonance spectroscopy (Altenbach et al. 2008). The largest predicted separation between TM3 and TM6 was in line with data obtained for Meta IIb from spectroscopy (Knierim et al. 2007), and for opsin from crystallography (Park et al. 2008).

Key residues, thought to influence the activation pathway, were mutated and additional simulations were carried out on these receptors for comparison to the WT. An interaction between K231^{5,66} and E247^{6,30} appeared to stabilize the predicted active conformation with the largest separation between TM3 and TM6, as also seen in the opsin crystal structure (Park et al. 2008). Furthermore, we carried out metadynamics simulations of the K231A^{5,66} mutant rhodopsin with either a charged or an uncharged residue E134^{3,49} within the E(D)RY motif. We hypothesized that removal of a polar interaction between K231^{5,66} and E247^{6,30} by replacing K231^{5,66} with alanine would switch the equilibrium toward the predicted conformation with a smaller separation between TM3 and TM6. Our simulations showed that the putative active conformation of rhodopsin with the largest separation between TM3 and TM6 was destabilized in the presence of a neutral E134^{3,49} mimicking the activation-dependent proton uptake from the bulk.

In the most comprehensive study using this methodology (Provasi et al. 2011), we were able to present compelling evidence for the concept that ligands of differing efficacy access structurally distinct conformations of the B2AR. Path CV metadynamics simulations were conducted for six ligands, representing a full agonist (epinephrine), weak partial agonists (dopamine and catechol) inverse agonists (carazolol and ICI-118,551) and a neutral antagonist (alprenolol). These different ligands were also compared to the unliganded receptor. Parameters for the ligands were obtained manually, by analogy with existing molecules in the OPLS-AA force-field, while Coulomb point charges were obtained from quantum chemical geometry optimization using Gaussian 03 and restricted Hartree-Fock calculations with the 6-31G* basis set. The ligands were either docked into the receptor using Autodock 4.0 (Morris et al. 1996) (for dopamine, catechol and epinephrine) or positioned according to their crystallographically determined binding modes. The activation pathway was homogeneously defined by ten frames, between the inactive state of the B2AR, represented by PDB ID: 2RH1 (Cherezov et al. 2007) and the active state, represented by the nanobody stabilized structure, PDB ID: 3P0G (Rasmussen et al. 2011a). Metadynamics simulations for each receptor/ligand complex were run for approximately 300 ns until the difference between the free energies of the metastable states observed was converged. The activation of the receptor was also measured in terms of other structurally pertinent variables, by reweighting the free energy surface. These variables were: the distance between the sidechains of the R131^{3,50} and E268^{6,30} residues of the ionic lock; the dihedral

angle of the sidechain of W286^{6,48}, i.e. the rotamer toggle switch; and the outward displacement of TM6. Comparison of the free energy surfaces of the receptor activation when complexed with different ligands demonstrated selective inactive or active conformations depending on their known elicited functional responses.

In the absence of ligand, the receptor was found to have two low energy conformations, one with $s \sim 0.2$, i.e. close to the inactive state, and a second, closer to an active state, with $s \sim 0.6$, separated by a low energy barrier. This corresponds well with the known basal activity of the B2AR, and the inherent conformational flexibility of the unliganded B2AR may help to explain the difficulty in obtaining crystallographic structures of the B2AR in the absence of ligand.

The free energy surface for B2AR complexed with alprenolol, ostensibly a neutral antagonist, i.e. a ligand that competitively binds in the orthosteric binding pocket of a receptor and blocks the binding of (and thus cellular responses to) agonists or inverse agonists, also displayed two stable states. One, close to the inactive, $s \sim 0.2$, was marginally (~ 1 kcal/mol) more stable than the second state, at $s \sim 0.6$. This type of free energy profile illustrates possible reasons for some studies having found alprenolol to behave in different ways in different assays: most notably, as an inverse agonist (Elster et al. 2007; Hopkinson et al. 2000) or as a partial agonist (Callaerts-Vegh et al. 2004).

The profiles for the inverse agonists (ligands that preferentially stabilize a receptor in an inactive conformational state), ICI-118,551 and carazolol both displayed single conformational states with low values of s ($s < 0.2$), in deep free energy wells. The free energy minimum, in each case, was approximately 4 kcal/mol lower in energy than the next lowest minima. This corresponds with the solution of many crystal structures in inverse agonist bound conformations.

Results for the agonists were less clear-cut. The free energy profiles for both partial (catechol and dopamine) and full (epinephrine) agonists each displayed a deepest free energy minimum at the expected value of s , i.e. $s \sim 0.6$ for the partial agonists, and $s > 0.9$ for the full agonist. However, other minima, only marginally less stable than these were found in all cases, at lower values of s . A feasible explanation for this arises from the absence of G protein: as demonstrated by Rosenbaum and colleagues (2011), during very long timescale MD simulations, an active conformation of the B2AR will quickly lose many of the active characteristics in the absence of the G protein or a stabilizing nanobody surrogate.

The results from this study provide an atomic resolution view of structurally distinct receptor conformations stabilized by different ligands, and demonstrate that ligands with varying efficacies might be used to control population shifts toward desirable GPCR conformations. Such knowledge might be useful to aid rational drug design: biased ligands may represent novel, more efficacious therapeutics.

Finally, in conjunction with tissue and animal behavioral studies, we have recently applied this methodology to determine some of the mechanistic details driving the antipsychotic properties of drugs targeting a heteromeric arrangement of the serotonin 5HT_{2A} and mGluR2 (Fribourg et al. 2011), in particular, why some 5HT_{2A} inhibitors (e.g. clozapine)

may exhibit neuropsychological effects while others (e.g. methysergide) do not. Oligomeric receptor complexes have been shown to have distinct signaling properties when compared to their monomeric components, and these receptors have been shown to form a specific heterooligomeric complex in mammalian brain tissue that is implicated in schizophrenia. The complex integrates the responses from the Gq-coupled mGluR2 and that of the Gi-coupled 5HT_{2A} to modulate signal transduction and influence downstream signaling events. To investigate this computationally, and to provide a structural context for the experimental observations, we built homology models of both receptors: mGluR2 was rhodopsin based, using PDB ID: 1U19 for the inactive state and 3DQB for the active-like state, while 5HT_{2A} was based on B2AR, using 2RH1 as the inactive template and 3P0G as the active-like template. To these models, we applied the methodology as in the previous cases, comparing the free energy profiles of activation for 5HT_{2A} bound to a strong agonist, DOI, a neutral antagonist, methysergide, and an inverse agonist, clozapine. For the monomeric 5HT_{2A}, clozapine and methysergide stabilized two different inactive-like conformations, while DOI stabilized an active like conformation. These experiments were repeated for dimeric arrangements of the receptors, with the lowest energy ligandstabilized conformations of 5HT_{2A} complexed with a ligand-free mGluR2. When complexed with the clozapine-stabilized 5HT_{2A} conformation, the mGluR2 conformation is shifted toward an activated state. In contrast, when bound to either the DOI-stabilized active-like 5HT_{2A}, or the methysergide-stabilized inactive-like 5HT_{2A} conformation, the conformational equilibrium of mGluR2 is shifted toward an inactive-like state. These results (in combination with the tissue and animal studies) suggest that the formation of the heteromeric complex enables modulation of the mGluR2 response to its endogenous ligand by binding of different ligands to the 5HT_{2A}. Therefore, the strong 5HT_{2A} agonist, DOI, can greatly stimulate Gq signaling, while decreasing Gi signaling, but the inverse agonist, clozapine, abolishes Gq signaling while simultaneously stimulating Gi signaling. These results go some way to explaining the complexities surrounding the disparity between the neuropsychological effects of 5HT_{2A} inhibitors, some of which demonstrate antipsychotic effects, while others do not. A drug bound to one receptor of the heteromer was shown to influence the signaling response of the partner to its endogenous ligand and such mechanistic insights are invaluable to therapeutic strategy design for disorders in which oligomeric arrangements of receptors are implicated.

6.4 Oligomerization Mechanisms in GPCRs

In the previous section, we have mentioned the distinct properties of oligomeric arrangements of some GPCRs as compared to those of their monomeric components (Milligan 2009). However, the ability of GPCRs to assemble into stable, heteromeric or homomeric, oligomeric arrangements remains a subject of much debate (Fonseca and Lambert 2009; Lambert 2010). From a computational perspective, understanding the thermodynamics and kinetics of GPCR oligomerization is not a trivial problem owing to (1) the large size of the complex system, and (2) the stochastic nature of the process. In this section we survey the enhanced MD-based methods that have recently been used in combination with reduced representations of the GPCR systems to study the dynamic behavior of dimeric/oligomeric arrangements of these receptors.

6.4.1 Spontaneous Self-Assembly of Coarse-Grained Representations of GPCRs

The number of calculations required by a biological molecule scales as the square of the number of particles included in its representation, and consequently, a lengthy simulation of a large, explicitly represented GPCR, particularly in a dimeric or oligomeric state, becomes particularly difficult at atomistic resolution. This issue has prompted several recent efforts focused on reducing the number of particles included in a simulation of a protein and its environment, and a number of strategies have been proposed. We focus here on the MARTINI coarse-grained (CG) model (Marrink et al. 2008), one of several “bead models” that have been devised to coarsely represent biomolecules (see Tozzini 2005, 2010 for recent reviews).

The MARTINI forcefield has proven to be a successful means of studying oligomerization for GPCRs and has been used in extensive studies of oligomerization for rhodopsin (Periole et al. 2007, 2012) opioid receptors (Johnston et al. 2011; Provasi et al. 2010) and the B1AR and B2AR (Johnston et al. 2012). Developed by Marrink and co-workers, the MARTINI force-field (Marrink et al. 2004, 2007, 2008; Monticelli et al. 2008; Lopez et al. 2009) offers coarse-grained (CG) representations of proteins, several lipid types and cholesterol. Close comparability to both experimental and atomistic MD approaches in residue-level detail is ensured by the extensive calibration of a large library of chemical building blocks against thermodynamic data (in particular, oil/water partition coefficients). These versatile building blocks permit the force field to flexibly represent a large range of biomolecules without the need to re-parameterize the model in each case. The MARTINI force field is compatible with GROMACS (Van der Spoel et al. 2005) and its CG mapping of heavy atoms (including water molecules) to beads is a 4:1 ratio, except in the case of molecules containing rings, where a mapping of 2:1 is used. There are four main types of beads, P = polar, N = non-polar, C = apolar and charged D Q, with subtypes depending on hydrogen bonding capacity and polarity. Each CG bead has a mass of 72 atomic mass units (amu), equivalent to the mass of four water molecules, except for ring structure beads, which have a mass of 45 amu. This improves efficiency and enables a simulation timestep of 20–40 fs, approximately ten times that possible with an all-atom GROMACS simulation. Based on comparison of diffusion rates for atomistic and MARTINI CG models, the CG model affords a scaling factor of 4 for the effective time sampled, though there has been some recent debate surrounding the best time step (Winger et al. 2009; Marrink et al. 2009).

The earliest application of the MARTINI CG model to GPCRs was the self-aggregation of rhodopsin monomers in four different explicit, CG, lipid membrane environments (Periole et al. 2007). Systems with 16 CG rhodopsin monomers (based on the 1L9H structure (Okada et al. 2002)) at a protein:lipid ratio of 1:100, were simulated for up to 8 μ s. Such time scales would be very difficult to reach in an atomistic representation. The results of these simulations indicate a localized membrane adaptation to the presence of the receptor, supposedly driven by the motivation to overcome the hydrophobic mismatch between the length of the hydrophobic part of the monomeric receptor and the equilibrium hydrophobic bilayer thickness. Hydrophobic mismatch has been shown to promote self-assembly of rhodopsin reconstituted in membrane bilayers (Kusumi and Hyde 1982; Ryba and Marsh 1992). The details of such phenomena could not be modeled in a simulation using an

implicit representation of a membrane, like the generalized Born method (Im et al. 2004) described earlier in this chapter.

Periole and colleagues simulated rhodopsin in different phosphocholine (PC) lipid environments under non-biased conditions, and they observed a clear dependence on chain length. Bilayer adaptation was manifest as local thickening near helices TM2, TM4, and TM7 in (C12:0)2PC, (C16:1)2PC, and (C20:1)2PC bilayers and as local thinning near helices TM1/H8 and TM5/TM6 in (C20:1)2PC and (C20:0)2PC. The results indicated a higher propensity for protein:protein contact interactions in (C16:1)2PC. Clear reorganization and increase in scope of the rhodopsin dimer interfaces was observed during the simulations, indicating a search for a shape complementarity that maximized the buried accessible surface area. The number of contact interfaces was higher in (C12:0)2PC and (C16:1)2PC, where strong forces were introduced by the hydrophobic mismatch, than in (C20:1)2PC and (C20:0)2PC, where the forces were more balanced. Three contact interfaces were clearly visible on the 6–8- μ s time scale in (C20:1)2PC; these included previously suggested homo- and heterodimerization interfaces in rhodopsin and other GPCRs, involving the exposed surfaces of the helices TM1/TM2/H8, TM4/TM5, and TM6/TM7. The authors concluded that hydrophobic mismatch drives self-assembly of rhodopsin into liquidlike structures with short-range order, and that the interactions may not be dictated by specific residue-residue contacts at the contact surfaces.

6.4.2 Combining CG Representation and Biased MD to Investigate GPCR Dimerization

By combining the MARTINI reduced representation of the system with biased MD techniques, it has been possible to make predictions about the relative strength of dimers formed at different interfaces in an explicit membrane environment.

We pioneered the use of a free energy approach to characterize oligomerization of GPCRs. Two studies of dimerization of the DOP receptor were performed using established methodologies, firstly umbrella sampling, from which we derived the PMF of a dimerization event (Provasi et al. 2010), and secondly, a metadynamics study in which we established the most favorable orientation of the individual protomers involved in different dimeric arrangements (Johnston et al. 2011), i.e. comprised of different contact interfaces, of the DOP receptor. The computational results of this study compared favorably with inferences from cysteine crosslinking experiments, supporting the role of specific residues at the interfaces.

To characterize dimerization for the DOP receptor, we performed biased MD simulations of a CG representation of a homo-dimeric arrangement of this receptor in an explicitly CG represented, POPC:10 % cholesterol-water environment. Since these studies were conducted prior to the crystallographic solution of the opioid receptor structures in 2012 (Wu et al. 2012; Granier et al. 2012; Thompson et al. 2012; Manglik et al. 2012), an all-atom structural model of the DOP receptor protomer from *Mus musculus*, was generated by homology modeling, using the crystal structure of the B2AR at 2.4 Å resolution (PDB ID: 2RH1) (Cherezov et al. 2007) in MODELLER 9v3 (Eswar et al. 2007), using the same strategy we recently reported in the literature for the human DOP receptor (Provasi et al. 2009). The loop regions were built *ab initio* using ROSETTA 2.2 (Wang et al. 2007). A pair of the

resultant DOP receptor models was placed facing one another at a putative symmetrical interface, involving residue 4.58, inferred from cysteine cross-linking data on this and other GPCRs (see e.g. Guo et al. 2005, 2008).

In an effort to improve the stability of the secondary structure of the CG representation of the receptor, we combined an elastic network model (ENM) with the MARTINI CG representation, using a method developed and termed ELNEDIN by Periole and colleagues (2009). ENMs are ideally suited to preserve the secondary or tertiary structure of biomolecules, since they are structure derived, and therefore introduce an intrinsic bias toward the structure upon which they are established. In a novel extension to the ELNEDIN method, we applied a secondary-structure-dependent construct to models of the DOP receptor dimer (Provasi et al. 2010). The strength of the force constant for the harmonic restraint, K_{SPRING} , was determined by the secondary structure of each of the residues. If the residue was determined to have a defined secondary structure (by DSSP (Kabsch and Sander 1983)), e.g. α -helix as in the case of the TM regions of the DOP receptor, then a force constant of $K_{\text{SPRING}} = 1,000 \text{ kJ/mol/nm}^2$ was applied. For a sequence of >2 residues with undefined secondary structure (e.g. coil, bend, or turn), a force constant of $K_{\text{SPRING}} = 250 \text{ kJ/mol/nm}^2$ was applied. A comparison of the RMSF for the Ca beads for the CG representation with an equivalent atomistic simulation indicated a qualitative agreement, permitting the secondary structure of the helices to be maintained, without compromising the flexibility of the loop regions.

We used the CVs illustrated in Fig. 6.3a to describe the relative position of interacting DOP receptor protomers *A* and *B* during the simulations: CV1 represented the distance r between the COM of protomers *A* (C_A) and *B* (C_B); CV2 described the rotational angle θ_A , defined by the projection on to the plane of the membrane of the COM of the TM4 of *A*, the C_A , and the C_B , and CV3 corresponded to the equivalent rotational angle θ_B . To allow exploration of an experimentally-supported TM4 interface of DOP receptor homodimers involving position 4.58 in a reasonable timescale, we limited the sampling of the two rotational angles θ_A and θ_B to a $\sim 25^\circ$ interval, using steep repulsive potentials.

The system setup was the same for both of the studies (Johnston et al. 2011; Provasi et al. 2010). All simulations were performed using GROMACS (Van der Spoel et al. 2005) incorporating the Plumed plugin (Bonomi et al. 2009). In the first of these two, we used umbrella sampling to define the PMF of dimerization as a function of the separation between the centers of mass of the two protomers.

Umbrella sampling was introduced in 1977 by Torrie and Valleau (1974), and has been considered the prototypic biased MD technique for improving sampling of a PMF as defined by the Kirkwood equation (Kirkwood 1935) (6.8), from the average distribution, $\langle \rho(\zeta) \rangle$, along a predefined reaction coordinate, ζ :

$$W(\zeta) = W(\zeta^*) - k_B T \ln \left[\frac{\langle \rho(\zeta) \rangle}{\langle \rho(\zeta^*) \rangle} \right] \quad (6.8)$$

where $k_B T$ is the Boltzmann factor, and ζ^* and $W(\zeta^*)$ are arbitrary constants. In this method, the reaction coordinate is divided into windows, and a biasing potential ($w(\zeta)$) is introduced

to tether the system to the centre of each window. The biasing potential acts to restrict the variations of the variable in order to ensure enhanced configurational sampling within each of the independent windows, and as a result, along the entirety of the reaction coordinate when all of the windows are combined. The biased simulations have potential energy $[U(\mathbf{R}) + w_i(\zeta)]$, (where $\mathbf{R}$ is the system coordinates) and $w_i(\zeta)$ is a harmonic potential of the form:

$$w_i(\zeta) = \frac{1}{2} K (\zeta - \zeta_i)^2 \quad (6.9)$$

centered on the successive values of ζ_i for each window. K represents the force constant of the potential. The advantage of umbrella sampling, considered to be key from a computational perspective, is that the relative independence of the windows from one another (though this should be considered and confirmed when recombining the windows) allows the biased simulations along the length of ζ to be conducted in parallel on large computer clusters.

To obtain the PMF, $W(\zeta)$ for the range of ζ , that is of interest, the simulations from each of the window must be unbiased and combined. Thus, it is crucial that the histogrammed variations in the measured variable obtained from each of the windows overlap sufficiently such that the whole of the coordinate space is covered.

Roux offers a thorough description of the derivation in (Roux 1995), but briefly, the biased distribution function, $\langle \rho(\zeta) \rangle_{(i)}$ as obtained from the i th biased ensemble, is:

$$\langle \rho(\zeta) \rangle_{(i)} = e^{-w_i(\zeta)/k_B T} \langle \rho(\zeta) \rangle e^{-w_i(\zeta)/k_B T}^{-1} \quad (6.10)$$

where $k_B T$ is the Boltzmann factor and the unbiased PMF from the i th biased ensemble is:

$$W_i(\zeta) = W(\zeta^*) - k_B T \ln \left[\frac{\langle \rho(\zeta) \rangle_{(i)}}{\langle \rho(\zeta^*) \rangle} \right] - w_i(\zeta) + F_i \quad (6.11)$$

ζ^* and $W(\zeta^*)$ are arbitrary constants. F_i is an undetermined constant representing the free energy associated with introducing the biasing potential:

$$e^{-F_i/K_B T} = \langle e^{-w_i(\zeta)/k_B T} \rangle \quad (6.12)$$

Most commonly, the Weighted Histogram Analysis Method, or WHAM (Kumar et al. 1992, 1995) is used to derive the PMF from the output of the simulations. This probably reflects the fact that the Grossfield lab (University of Rochester, NY) has created a very user-friendly and freely distributed implementation of WHAM, but other unbiasing techniques e.g. MBAR (Shirts and Chodera 2008) have proven to be equally useful. WHAM focuses on optimizing the estimate of the coordinate-dependent unbiased distribution function as a weighted sum over all of the data from the biased simulations, and determining the functional form of the weight factors that minimize the statistical error. The derivation of the WHAM equations can be found in (Kumar et al. 1992) but here, in short, the key expressions can be distilled down to:

$$\langle \rho(\zeta) \rangle = \frac{\sum_{i=1}^{N_w} n_i \langle \rho(\zeta) \rangle_i}{\sum_{j=1}^{N_w} n_j e^{-[w_j(\zeta) - F_j]/k_B T}} \quad (6.13)$$

$$F_j = -k_B T \ln \left(\int \langle \rho(\zeta) \rangle e^{-w_j(\zeta)/k_B T} d\zeta \right) \quad (6.14)$$

where N_w is the number of windows; n_i is the number of independent data points, used to construct the biased distribution function, in the i th window at specific value of ζ ; $\langle \rho(\zeta) \rangle_i$ is the biased distribution function in the i th window; $w_j(\zeta)$ is the window potential of the j th window, at specific ζ ; F_j is the (unknown and to be determined) free energy constant. The unbiased distribution function, $\langle \rho(\zeta) \rangle$ is dependent on $\{F_j\}$, and thus the simultaneous Eqs. (6.13) and (6.14) must be solved iteratively, until a consistent solution is obtained for both of them. This unbiased distribution function may then be substituted into the Kirkwood equation (Kirkwood 1935), (6.8), to obtain the PMF along the coordinate ζ .

For our first umbrella sampling simulations of GPCRs (Provasi et al. 2010), 43 independent windows with values of the reaction coordinate in question, i.e. protomer separation, r , between $r_1 = 3.0$ nm and $r_{43} = 4.90$ nm were simulated for 300 ns, using harmonic restraints on the distance. The distribution $p(r)$ of the separation was harvested for the last 250 ns, and the resulting probability distributions were combined using WHAM to derive the free energy as a function of the separation between the protomers.

The free energy surface identified two different, yet energetically very close, homodimeric states of the DOP receptor (D1 and D2) involving the TM4 interface, energetically stabilized relative to the monomeric state, which were separated from each other by a transition state at $r_{TS1} = 3.28$ nm, and from the monomeric state at large values of the separation ($r = 4.90$ nm), by a transition state at $r_{TS2} = 3.75$ nm. In D1, the structure indicated that TM4 from each protomer inserts into a groove on the surface of the opposite protomer, formed by helices TM2, the C-terminal half of TM3, and TM4. As expected from the energetic similarity, the D2 structure was similar to D1 in the overall orientation of the protomers, but corresponded to a slightly less compact ($r_{D2} = 3.40$ nm), and slightly asymmetric arrangement of the protomers.

Using the derived free-energy surface of DOP receptor homodimers, and the formalism described by Roux and co-workers in Allen et al. (2004) and Roux (1999), we were able to calculate several observable quantities from our simulations. The dimerization constant for the identified lowest-energy DOP homodimer was $K_D = 1.02 \mu\text{m}^2$ (see details of the derivation in Johnston et al. 2012). From this calculated K_D , and in combination with a diffusion coefficient D_T value of $0.08 \text{ mm}^2/\text{s}$ determined experimentally for the mu-opioid (MOP) receptor (Sauliere-Nzeh et al. 2010), and also in line with a value of $0.1 \text{ mm}^2/\text{s}$ obtained for several other GPCRs (Barak et al. 1997; Hegener et al. 2004; Henis et al. 1982; Poo and Cone 1973), we obtained an estimate of a few seconds for the half-time of DOP receptor dimers at a contact interface comprised of TM4 in a lipid bilayer mimetic. We

repeated these calculations in a second simulation (Johnston et al. 2011), for a contact interface simultaneously involving contact at both TM4 and TM5 of each protomer. The comparison revealed that the TM4 interface was marginally more stable than the interface involving both TM4 and TM5, indicating that the stability of the dimer pair is dependent on the region of contact between the protomers. These calculated lifetimes of DOP receptor homodimers are consistent with the transient, sub-second to millisecond association inferred by recent single-molecule studies of GPCRs (Hern et al. 2010; Kasai et al. 2011). Whether such a lifetime for DOP receptor homodimers and other GPCRs within the membrane has implications on the functional role of receptor complexes and/or the specificity of their interactions requires more in-depth investigation.

In a second study (Johnston et al. 2011), using this same system setup, we performed a well-tempered metadynamics study, to better explore the TM4-TM4 contact interface of the protomers in this same dimeric arrangement, and compare it with the interface comprising contact at both TM4 and TM5 of each protomer. The well-tempered metadynamics implementation is identical to that described earlier in this chapter. We restricted exploration of CV1, r , to $r = 3.80$ nm, i.e. the value of the transition state observed in the umbrella sampling simulations (Provasi et al. 2010), using a steep repulsive potential, and applied a Gaussian biasing potential to ensure thorough exploration of CV2 and CV3, angles θ_A and θ_B , within a $\sim 25^\circ$ interval for the contact interface involving TM4 only, and a $\sim 17^\circ$ interval for the more symmetric interface involving TM4 and TM5 simultaneously. The FES as a function of angles θ_A and θ_B , for each of the two contact interfaces (TM4, and TM4/5) was reconstructed from the history of the applied bias. We extracted a structure from the minima of each of the resulting 2-dimensional free energy surfaces, and proceeded to characterize the residues representing symmetric contacts between the two protomers in the minimum energy dimeric structures. The results of the calculated relative stability of the two interfaces were in line with inferences from cysteine cross-linking studies. The key residue defining the TM4 interface was V181^{4,58}, and was found to have a C|3-C|3 separation of <7 Å after conversion to an atomistic resolution representation in the OPLS-AA forcefield (Kaminski et al. 2001; Jorgensen et al. 1996), minimization and short equilibration (1 ns) simulation in GROMACS (Van der Spoel et al. 2005). For the TM4/TM5 interface, the cysteine cross-linked residue, T213⁵³⁸, was found within a C|3-C|3 distance of 11 Å between the opposing protomers.

6.4.3 Relative Stability of Dimer Interfaces in GPCRs

In 2012, two papers more comprehensively addressing dimerization of rhodopsin (Periole et al. 2012) and the B1/B2ARs (Johnston et al. 2012) were published almost simultaneously. We have recently combined the two techniques that we have used in the past to investigate the DOP receptor, i.e. umbrella sampling and metadynamics, into a single method to comprehensively characterize dimerization of the B1AR and B2AR, at contact interfaces previously documented to have physiological relevance for a number of GPCRs by experimental techniques. We have focused on contact interfaces involving transmembrane helices TM1/H8 and TM4, and have used umbrella sampling to explore the FES as a function of protomer:protomer separation as in (Provasi et al. 2010), while simultaneously employing metadynamics as in (Johnston et al. 2011) to ensure complete sampling of the

angle space available to the protomers at each interface. The missing segments of the crystal structure of the B2AR (PDB ID: 2RH1 (Cherezov et al. 2007)) were generated using Rosetta (Wang et al. 2007), and a homology model of the human B1AR was built using the turkey B1AR crystal structure (PDB ID: 2VT4 (Warne et al. 2008)). The system for simulation was constructed in an identical fashion to that for the DOP receptor simulations, with both the protein and the environment represented in the CG MARTINI forcefield.

Periole and colleagues (2012) extended their long-timescale simulations of rhodopsin in (C20:1)2PC bilayer (Periole et al. 2007), to multiple repetitions on the 100's of μ s timescale, and with a larger bilayer, encompassing 64 rhodopsin receptors, rather than the original 16 used in the previous study. The dimerization events were clustered and suggested that the most commonly occurring events involved contact interfaces also comprised of TM4, TM4/5 and TM1/H8.

Despite the long timescale and the multiple receptors, Periole and colleagues found that the number of individual dimerization events was insufficient to provide reliable statistics from which to calculate free energy estimates for the dimerization of rhodopsin. Accordingly, they included umbrella sampling simulations to establish five PMFs for this dimerization event at different interfaces observed from the self-assembly simulations, using the virtual bond algorithm (VBA) defined in Fig. 6.3b. Their VBA defined 8 restraints, but in general, the ones found to be most useful were dihedral angles ϕ_1 and ϕ_3 and distance, d . Periole and colleagues use these CVs to investigate PMFs for dimerization at symmetric interfaces involving TM4, TM5, TM4 and TM5, and TM1 and H8. They also considered an asymmetric interface composed of TM4 interacting with TM6 on the adjacent protomer. We only investigated symmetric interfaces, comprised of TM4 and TM1/H8 that have been routinely suggested to have physiological relevance through biophysical experiments. It must also be noted that the arrangement of the helices in TM1/H8 is similar but not identical between the two studies.

The combination of simulation length per window and the number of windows was different between the two studies. Periole and colleagues used a small number of windows, with a weak force constant ($500\text{--}5,000\text{ kJ mol}^{-1}\text{ nm}^{-2}$ on r , and $300\text{ kJ mol}^{-1}\text{ nm}^{-2}$ on ϕ_1 and ϕ_3), and a long simulation length, reaching up to $20\text{ }\mu\text{s}$ (effective timescale) in some cases. In our work, we used many shorter windows, centered at intervals of r of only 0.05 nm . We also used a large force constant, $k = 10,000\text{ kJ mol}^{-1}\text{ nm}^{-2}$ for the harmonic restraint to tether the measured value of the distance to the center of each window. The angles θ_A and θ_B as defined in Fig. 6.3a were restricted by steep harmonic potentials, as described in previous studies (Johnston et al. 2011; Provasi et al. 2010), but were explored using the metadynamics techniques as described in (Johnston et al. 2011). The simulation length of each window was $1\text{ }\mu\text{s}$, which was longer than in the previous umbrella sampling study (in which it was only 300 ns) (Provasi et al. 2010), in order to permit a thorough exploration of the angle range using the Gaussian bias. This novel combination methodology was validated by favorable comparison with the individual methods, on a simple two-dimensional toy system (Johnston et al. 2012).

The similarities between the results of these two independent studies are striking. Most notably, both studies find that the most stable of the interfaces investigated, by a significant margin, was that involving TM1 and H8. It is difficult to compare the exact nature of the interfaces, since firstly, the protomeric arrangement at this interface is not identical between the two investigations, and secondly, the measurement of the separation between the protomers is defined by the interfacial distance in the study of rhodopsin, while it is between the centers of mass of the helical bundles in the study of the B1/B2ARs. Nevertheless, the similarity of the result between the two independent investigations is compelling, particularly when the small contact surface area between the protomers is considered. The TM4 interface was found to be the “weakest” or least stable for both rhodopsin and the B1/B2ARs (no PMF was presented for TM4/5 for the B1/B2ARs). The PMF in the rhodopsin simulation (Periole et al. 2012) shows remarkable qualitative similarity to that found for the same interface in our first umbrella sampling study of the DOP receptor (Provasi et al. 2010). Both PMFs show two minima, separated by a small transition state, with the minimum at larger separation being slightly shallower than that in which the protomers are tightly bound together. Periole and colleagues suggest that the shallower minimum corresponds to a protomeric arrangement in which a small number of lipids “lubricate” the contact surface between the two protomers. Both PMFs show a small barrier for dimerization, between the monomeric state and the shallower minimum for the TM4 interface for both rhodopsin and DOP receptor. No such barrier is noted for TM1/H8 for rhodopsin or the B1/B2ARs, (no TM1/H8 interface has been investigated yet for the DOP receptor). Periole and colleagues suggest this barrier may be related to difficulties in delipidation of the surfaces between the protomers. A significant difference between the studies from these two labs is the choice of lipid membrane mimetic for the environment of the proteins. This is particularly pertinent because Periole and colleagues found that delipidation of some of the contact interfaces was very challenging, even on the (effective) time scale of 20 μ s. Following on from their earlier studies (Periole et al. 2007), Periole and colleagues chose the longer-tailed lipid mimetic of those tested, i.e. C(20:1)2 PC, since this was found to influence dimerization the least. Atomistically, this would represent 1,2, di(D-cis-eicosanoyl)-sn-glycero-3-phosphocholine. In contrast, we used a shorter-tailed lipid membrane mimetic, representing a POPC lipid, with a 10 % cholesterol component. While it is acknowledged that the presence of cholesterol might reduce the mobility of lipids and proteins in the membrane particularly in terms of exchange at the contact interface, plots depicting the residency time at the interface (in terms of percentage of the trajectory spent within 15 Å of the interface helices) of all lipids and cholesterol in every simulation we ran indicated that 75 % of the cholesterol spent less than 60 % of the trajectory within 15 Å of the interface and 75 % of lipids spent less than 20 % of their time at the interface, while the median time at the interface for both lipids and cholesterol was much lower than 10 % of the trajectory. These statistics suggested adequate lipid exchange.

In both studies, the contact interface between the two protomers at TM4 is more extensive than for the TM1/H8 interface, and Periole and colleagues suggest that this means that the strength of the interaction is not dependent on the buried surface area between the protomers for these receptors, as is the case for soluble proteins.

Several post-translational modifications are known to occur for rhodopsin, and in particular, palmitoylation at cysteine residues 322 and 323, has been predicted to play a role in anchoring the receptor to its native membrane environment and specific interactions between the palmitoylate chain and the protein may have a functional relevance for the dark state of rhodopsin (Olausson et al. 2012). The location of these palmitoylated residues, towards the end of the amphipathic helix 8, means that they could feasibly form part of a contact interface between two protomers involving TM1 and H8. Periole and colleagues have accounted for this by performing their umbrella sampling experiments for this interface both with and without palmitoylation. They found that there was no significant difference between the PMF profiles calculated for both cases and thus speculate that the palmitoylation does not contribute to the strength of the contact interface between TM1 and H8 on adjacent protomers.

Both works offer putative physiological explanations for the striking difference in relative strength of interfaces involving different transmembrane regions. We have proposed that the strength of the TM1/H8 interface, and its subsequently calculated “long” lifetime (of the order of minutes), might be reconciled with the sub-second lifetimes reported for the muscarinic receptor (Hern et al. 2010) and the N-formyl peptide receptor (Kasai et al. 2011), through a model wherein a stable dimer is formed at a contact interface involving TM1/H8, and this diffuses through the membrane interacting with other stable TM1/H8 dimers at other, weaker, interfaces. The interactions at the other weaker interfaces are shorter lived, and these may account for the observations of the single molecule experiments. A dimer/tetramer model of oligomerization would also be comparable to the inferences the Scarlata lab in collaboration with us derived (Golebiewska et al. 2011) from the diffusion rates and number and brightness measurements from fluorescence imaging of tagged MOP and DOP receptors in live cells after chronic morphine treatment.

Periole and colleagues compare their results to 2-dimensional rows of dimers of rhodopsin, as suggested by AFM studies of rhodopsin in native disk membranes (Fotiadis et al. 2003). To further test this hypothesis, they built and simulated a system of rows of TM1/H8 dimers interacting in a lipid-mediated or “lubricated” manner with adjacent rows of receptors, matching the structural information derived from the AFM study (Fotiadis et al. 2003). Their system remained stable after 16 μ s of simulation (Periole et al. 2012).

Other information from structural studies supports the TM1/H8 interaction as a contact interface with putative relevance. Two-dimensional electron crystallography of rhodopsin (Schertler and Hargrave 1995) and of metarhodopsin I (Ruprecht et al. 2004) and X-ray crystallography of rhodopsin (Salom et al. 2006), opsin (Park and Schulten 2004) and metarhodopsin II (Choe et al. 2011) have hinted at this interaction, as have the most recent KOP (Wu et al. 2012) and MOP (Manglik et al. 2012) receptor X-ray crystal structures, although the physiological relevance of these arrangements remain to be demonstrated, in spite of recent experimental studies (Knepp et al. 2012) leaning in the same direction.

6.5 Conclusions

Throughout this chapter, we have discussed the ways in which biased MD methods are increasingly becoming invaluable tools to investigate, at the detailed molecular level, key events in the lifetime of GPCRs that take place on timescales in nature that are not routinely accessible to standard approaches. As these techniques become more theoretically comprehensive and computational power becomes greater, the combination of these two factors will progressively reveal important mechanistic details of events in the lives of GPCR signalosomes.

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Fig. 6.1. Exit/entry pathways (red transparent arrows, labeled 1–4) for the B1AR (left) and the B2AR (right). Top (top panels) and side (bottom panels) views. Receptor is shown in cartoon representation, colored blue (TM1) to red (H8). The crystallized antagonists cyanopindolol (in B1AR) and carazolol (in B2AR) are shown in stick representation

Fig. 6.2.

(a) Multidimensional scaling representation of multiple adiabatic biased MD trajectories clustered according to RMSD. Representative structures selected from each cluster (shown in (b): side view and (c): intracellular view) to define a homogenous pathway between the inactive and active states. The receptor is shown in *grey cartoon* representation, in (b) and (c), except for TM5 and TM6, which show the progress from inactive to active, in colors corresponding to the clusters in (a)

Fig. 6.3.

(a) Definition of collective variables (CVs) used in studies of dimers from the Filizola lab (Johnston et al. 2011, 2012; Provasi et al. 2010). CV1 is the distance r between the COMs of the protomers (C_A) and (C_B); CV2 is the rotational angle θ_A defined by the projection on to the plane of the membrane of the COM of the TM_i of A, the C_A , and the C_B , and CV3 corresponded to the equivalent rotational angle θ_B . Shown for the B2AR at a TM1/H8 interface, TM1 and H8 are colored *black*. The CVs are defined according to the interface of interest. (b) Definitions of the virtual bond algorithm used by Periole and colleagues in their

recent publication (Periole et al. 2012). The axes (*black dotted lines* in Fig. 6.3b) are anchored by the backbone CG beads of Cys 187, Gly 121 and Gly51 (shown as *red spheres*) on each protomer, thus the variables are consistent across different interface arrangements. They describe the distance between the receptors, d ; the tilt of long axis of each receptor relative to the receptor-receptor direction θ_1 and θ_2 ; the rotation of the receptors around their long axis (parallel to the membrane normal), ϕ_1 , ϕ_3 or θ_1' and θ_2' , the relative orientation of the receptor's long axis, ϕ_2 . d' is the interfacial receptor distance and is defined as the distance, d , between the receptors from which the distance at the minimum of the PMFs was subtracted, see supplementary material from (Periole et al. 2012). Once again, TM1 and H8 are colored *black*