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How do short chain non-ionic detergents destabilize GPCRs?

Sangbae Lee[†], Allen Mao[†], Supriyo Bhattacharya[†], Nathan Robertson[%], Reinhard Grisshammer[‡], Christopher G. Tate^{*§}, and Nagarajan Vaidehi^{*†}

[†]Department of Molecular Immunology, Beckman Research Institute of the City of Hope, 1500 E. Duarte Road, Duarte, California 91010, USA

[%]Heptares Therapeutics Ltd, BioPark, Broadwater Road, Welwyn Garden City, AL7 3AX, UK

[‡]Membrane Protein Structure Function Unit, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Department of Health and Human Services, Rockville, Maryland 20852, USA

[§]MRC Laboratory of Molecular Biology, Cambridge Biomedical Campus, Francis Crick Avenue, Cambridge CB2 0QH, UK

Abstract

Stability of detergent-solubilized GPCRs is crucial for their purification in a biologically relevant state and it is well-known that short chain detergents such as octylglucoside are more denaturing than long chain detergents such as dodecylmaltoside. However, the molecular basis for this phenomenon is poorly understood. To gain insights into the mechanism of detergent destabilization of GPCRs, we used atomistic molecular dynamics simulations of thermostabilized adenosine receptor (A_{2A}R) mutants embedded in either a lipid bilayer or detergent micelles of alkylmaltosides and alkyl-glucosides. A_{2A}R mutants in dodecylmaltoside or phospholipid showed low flexibility and good interhelical packing. In contrast, A_{2A}R mutants in either octylglucoside or nonylglucoside showed decreased α -helicity in transmembrane regions, decreased α -helical packing and the interpenetration of detergent molecules between transmembrane α -helices. This was not observed in octylglucoside containing phospholipid. Cholesteryl hemisuccinate in dodecylmaltoside increased the energetic stability of the receptor by wedging into crevices on the hydrophobic surface of A_{2A}R, increasing packing interactions within the receptor and stiffening the detergent micelle. The data suggest a three-stage process for the initial events in the destabilization of GPCRs by octylglucoside: (i) highly mobile detergent molecules form small micelles around the receptor; (ii) loss of α -helicity and decreased interhelical packing interactions in transmembrane regions through increased receptor thermal motion; (iii) transient separation of transmembrane helices allowed penetration of detergent molecules into the core of the receptor. The relative hydration of the headgroup and alkyl chain correlates with detergent harshness and suggests new avenues to develop milder versions of octylglucoside for receptor crystallization.

*Corresponding Author, NVaidehi@coh.org, cgt@mrc-lmb.cam.ac.uk.

Supporting Information.

Details on data sets for molecular dynamics along with supporting figures (Fig. S1 – Fig. S15), tables (Table S1 and S2), and videos (Online movie file 1 and 2). This material is available free of charge via the Internet at <http://pubs.acs.org>.

The authors declare no competing financial interest.

Graphical abstract

Keywords

G protein–coupled receptors (GPCRs); Molecular dynamics simulations; Thermostability; Adenosine receptor; Detergent micelle; Cholesteryl hemisuccinate; Energetic stability

Introduction

The use of surfactants, also known as detergents, has a long history in the purification and crystallization of membrane proteins.^{1–4} Initially, industrial detergents such as Triton X100 were the only ones available. These were a mixture of different aliphatic chain lengths, including short chain derivatives, and therefore these detergents often resulted in inactivation of membrane proteins and they had limited utility for protein crystallization. With the development of chemically homogeneous detergents of different chemical structures, such as octylglucoside (OG) and lauryl-dimethylamine Noxide (LDAO), it became possible to purify some membrane proteins^{5,6} and, in the case of the bacterial photosynthetic reaction center and porins, to produce well-ordered crystals and determine the first atomic structures of membrane proteins.^{7,8} However, it was appreciated even at this time that OG was a harsh detergent that often inactivated membrane proteins and that milder detergents were essential for working with most other membrane proteins.⁹ The alkylmaltoside series has proven the most successful of these new detergents with the majority of bacterial transporters and ion channels being either purified and/or crystallized in dodecylmaltoside (DDM).¹⁰ However, the shift of emphasis towards structure determination of mammalian membrane proteins highlighted that even DDM may not be mild enough for preserving their integrity upon solubilization and therefore even milder detergents were required.^{11,12} Thus there has been a continued effort to develop novel mild detergents, including bicyclic alkylmaltosides,¹³ bifacial amphiphiles¹⁴ and maltose-neopentylglycols.¹⁵

Despite all the work to develop new detergents, the fundamental mechanism how non-ionic detergents denature membrane proteins is unknown. The stability of any given membrane protein has evolved in the context of the biological membrane, where the protein has to be sufficiently stable to function and to confer a phenotype on the whole organism. Membrane proteins have not evolved to be stable in detergent micelles. This is not a fundamental limitation of the membrane protein, because many membrane proteins have been engineered through site-directed mutagenesis to be highly stable in detergent solution,¹⁶ even in highly

denaturing detergents such as sodium dodecyl sulfate.¹⁷ Many reasons have been put forward for why detergents destabilize membrane proteins compared to lipids, although there is currently no accepted consensus.¹⁸ Additionally, it is difficult to obtain molecular level insights into the mechanism of destabilization of membrane proteins in detergent micelles through experimentation.¹⁹

We have chosen to study the stability of G protein-coupled receptors (GPCRs), a superfamily of seven helical transmembrane proteins, in detergent micelles using atomistic molecular dynamics (MD) simulations and thermostability measurements, and have compared the results of the detergent simulations with those in a phosphatidylcholine (POPC) bilayer.

The properties of many different detergent micelles have been studied extensively by MD²⁰⁻²⁷ and, more recently, simulations of ion channels and porins in detergent micelles have also been performed.²⁸⁻³¹ However, MD simulations have not been used to study the stability of GPCRs embedded in detergent micelles. The receptor we have used for this study, the human adenosine A_{2A} receptor (A_{2A}R), was chosen because its stability in lipid bilayers has already been thoroughly studied by MD simulations, both for the wild type receptor and engineered thermostable mutant in different conformations.³²⁻³⁴ In addition, there is a wealth of biochemical data on A_{2A}R stability, in particular in different detergent solutions.³⁵⁻³⁷ A_{2A}R has been crystallized by a number of groups using three different strategies (thermostabilization^{38,39}, T4 lysozyme fusion^{40,41} and antibody binding⁴²) to facilitate the formation of well-ordered crystals. Structures have also been determined bound to a variety of different antagonists and agonists. The structures bound to an antagonist represent the inactive state of the receptor, whereas the agonist-bound structures represent an active-intermediate that has undergone substantial conformation changes.^{38,39} Here we have studied the dynamics of A_{2A}R in both the inactive and active-intermediate states. The microsecond time scale available for these studies is insufficient to observe the full denaturation process of a receptor, but we found this was sufficient to observe the substantial differences between the effects of long chain and short chain detergents on the structure of A_{2A}R mutants.

Results

Before studying the A_{2A}R mutants in detergent micelles, we used MD simulations to assemble the micelles of four detergents, n-dodecyl- β -D-maltoside (DDM), n-decyl- β -D-maltoside (DM), n-nonyl- β -D-glucoside (NG) and n-octyl- β -D-glucoside (OG) in the absence of protein to test the validity of our simulations (see Methods). Following an aggregated 500 ns MD simulation on each micelle, the micelles of all the detergents assembled into an oblate spheroid, as previously observed for DDM.^{20,43,44} The radius of gyration and the eccentricity of each micelle (Supplemental Table S1) were found to compare well with Small Angle X-ray Scattering (SAXS) measurements for these micelles.⁴⁵ The agreement between the MD simulations and the experimental data for the shape and size of the micelle particles shows that the simulations recapitulated the experimental properties of the detergent micelles.

Two structures of A_{2A}R were chosen for the MD simulations in detergent, namely A_{2A}R-StaR2³⁹ with the antagonist ZM241385 bound (PDB code 3PWH) and A_{2A}R-GL31³⁸ with the agonist adenosine bound (PDB code 2YDO). Both of the A_{2A}R mutants contain thermostabilizing mutations (see Methods) that stabilize the inactive R state for A_{2A}R-StaR2³⁵ and the active-intermediate R' state for A_{2A}R-GL31.³⁶ Each of the receptors was first assembled into a detergent micelle containing either 192 molecules of DDM, 144 molecules of DM, 155 molecules of NG or 123 molecules of OG. These values are larger than the aggregation number for the pure detergent and reflect the total number of detergent molecules specifically bound to membrane proteins determined from biochemical assays.^{46,50} As expected, the radius of gyration and the eccentricity of each protein-detergent micelle are larger than for the pure detergent (Supplementary Table S1). After assembly, each receptor embedded in detergent was subjected to a further 5 simulations, 100ns each and a representative structure of the most populated conformation ensemble determined (Fig. 1). After completion of the simulations, a series of analyses were performed on both the detergent and receptor for each of the detergents studied.

Characteristics of the detergent micelle around A_{2A}R-StaR2

The distribution of detergent molecules in the micelles around A_{2A}R-StaR2 differed dependent upon the detergent type. For DDM and DM, there was a higher probability of the hydrophobic aliphatic 'tail' being closer to the receptor than the hydrophilic maltoside 'head group', as shown by the radial distribution function (Fig. 2). Thus the number density of tail groups of DDM and DM around A_{2A}R-StaR2, peaks at 18Å and 20Å from the receptor core, respectively, while the distance of their head groups peaks at 27Å and 32Å, respectively. In contrast, there was no significant difference between the distribution of the tail and headgroups of the short chain detergents (Fig. 2). This substantiates visual inspection of representative A_{2A}R-StaR2-detergent micelle structures where the micellar surface composed of DDM and DM has a greater density of hydrophilic groups on the outer surface of the micelles compared to the micelles of NG or OG (Fig. 1 and Fig. S1). Additionally, the rate of movement of detergent molecules within the micelle showed substantial difference between the long chain and short chain detergent micelles. For DDM or DM, individual detergent molecules diffuse relatively slowly, and thus the position of a given molecule varies little with time (Fig. 2 and Fig. S2). In contrast, molecules of NG and OG diffuse rapidly, often with the detergent tumbling within the micelle, allowing it to sometimes sample the whole space of the micelle within the length of the simulation. Another difference between the long chain and short chain detergents is that on a number of occasions OG molecules were observed to penetrate into the receptor core (Supplementary movie; discussed further below). Tumbling or penetration of DDM or DM into A_{2A}R-StaR2 was not observed.

The structure and stability of receptor-detergent complexes in different detergents

To validate the MD simulations of the receptors, we compared experimentally-determined apparent T_m determinations³⁵ with the average potential energy of the receptor conformations calculated over the entire MD trajectory (Fig. 3). The good correlation between the experimental data and the computational data suggests that MD simulations are good models for understanding the stability of GPCRs in both detergents and membranes.

Interestingly, the highest stability for A_{2A}R-StaR2 was observed for the receptor solubilized in DDM supplemented with CHS, followed by receptor embedded in membranes. DDM was the best detergent to stabilize A_{2A}R-StaR2, closely followed by DM, but both NG and OG destabilized the receptor significantly. The average potential energy is the sum of the internal energy of the receptor and the interaction energy of the receptor with the detergent or palmitoylphosphatidylcholine (POPC), cholesterol (wherever applicable) and water molecules. The difference in the stability of A_{2A}R-StaR2 in various detergents comes predominantly from the receptor detergent interaction energies and the non-bond energy of the receptor (Fig. S3). This could arise due to the more frequent interaction of the hydrophobic portions of DDM and DM with the receptor with respect to their head groups, compared to NG and OG where there is an equal probability of head groups or tails interacting with the hydrophobic portions of the receptor. In addition, the larger micelle size of DDM and DM more effectively shields the hydrophobic portions of the receptor compared to NG and OG. This is reflected in the higher hydrophobic solvent accessible surface area (SASA) of A_{2A}R in OG compared to DDM (Fig. S4).

What are the consequences on the receptor structure when the receptor is inserted into a detergent micelle that is destabilizing?

Variations in the structure of A_{2A}R-StaR2 observed in the MD trajectories were clustered based on two properties that measured the structural deviation from the crystal structure, namely, the root mean square deviation (RMSD) in the coordinates of the backbone atoms and the percentage α -helicity of residues in the transmembrane (TM) helices (Fig. 4). Both DDM and POPC maintain A_{2A}R-StaR2 in a state similar to the crystal structure, although there is greater flexibility of the receptor in the lipid bilayer compared to in a DDM micelle (Fig. 4). However, it is noticeable that the harsher the detergent, the greater the deviation from the crystal structure and the lower the α -helicity of the transmembrane regions (Fig. S5). This is particularly apparent for A_{2A}R-StaR2 in either OG or NG where there are significant perturbations in the middle of TM7, the cytoplasmic end of TM6 and in the loop regions. Each of the transmembrane regions in A_{2A}R-StaR2 maintains a stable α -helix in DDM and DM simulations, but TM5, TM6, and TM7 show relatively low helicity (50 to 70%) in OG.

The alterations in secondary structure are also reflected in the decrease in the average number of interhelical hydrogen bonds and van der Waals interactions (Fig. S6). A_{2A}R-StaR2 in DDM has the highest number of interhelical interactions (61 contacts) with the lowest number of interhelical interactions in OG (48 contacts). The greater flexibility of A_{2A}R-StaR2 in OG has a secondary consequence, because as the transmembrane helices move apart, then it is possible for a detergent molecule to intercalate between them. Out of five 100 ns simulations of A_{2A}R-StaR2 in OG, significant penetration of the α -helical bundle was observed on three occasions (Fig. 5; Online movie file 1). The remaining two simulations that did not show penetration of OG within 100ns simulation time showed penetration when the simulations were extended to 200ns each. In all these simulations the OG molecule entered the receptor through TM6 and TM7 (see Fig. S7). In one example, the antagonist ZM241385 is also displaced from its normal binding position in the orthosteric binding pocket, suggesting that OG could inhibit antagonist binding. However, it is also

likely that further ingressions of OG into the core of the receptor could lead to further unfolding and ultimately to complete denaturation, which is the outcome of trying to solubilize and purify A_{2A}R-StaR2 in OG (which is on a significantly longer time scale than the simulations). To eliminate the possibility that the tumbling and penetration of the OG molecules are not arising due to smaller micelle size (123 molecules) compared to DDM, we performed the OG micelle simulations starting with 192 OG molecules similar to the number of detergents in the DDM micelle. The OG molecules in these simulations showed tumbling in the micelle and penetration into the receptor that confirmed that this phenomenon is characteristic of the short chain detergents (see Fig. S8).

Stabilizing membrane proteins with either ligands, cholesteryl hemisuccinate or lipids

In experimental systems, the stability of a GPCR is crucial for subsequent success in its purification and crystallization. The first step of the process is to perform high-throughput detergent screening to identify those detergents that maintain the protein in a monodisperse and functional state.^{51,52} It is well known that adding either ligands⁵³ or lipids⁵⁴ can improve the stability of a membrane protein in detergent. So for example, ZM241385 binding to A_{2A}R improves its stability,^{37,39} as does the binding of agonists to A_{2A}R-GL31.^{37,38} Simulations suggested that in both cases, adding a ligand improved the helicity and decreased the flexibility for both A_{2A}R-StaR2 and A_{2A}R-GL31 (Fig. S9A-B), which correlates with the increase in number of interhelical packing interactions (Fig. S9C). Cholesteryl hemisuccinate (CHS) is another factor that is known to improve the stability of A_{2A}R in DDM.^{37,55} Indeed, it is not possible to purify A_{2A}R in DDM unless CHS is added to each step of the procedure,⁵⁵ although once the protein has been thermostabilized in the inactive state (A_{2A}R-StaR2) CHS is no longer required to maintain functionality during purification.³⁵ Simulations of A_{2A}R-StaR2 in DDM supplemented with CHS did not show any significant difference in its flexibility compared to the receptor in DDM alone (Fig. 6). In contrast, simulations of A_{2A}R-GL31 in DDM supplemented with CHS showed an appreciable increase in α -helicity and a decrease in RMSD compared to the crystallized receptor after MD simulations (Fig. 6A-D); this effect was also observed in simulations of the receptors in a POPC lipid bilayer with cholesterol (Fig. S10A). The basis for the stabilizing effect of CHS appears to be its interaction with A_{2A}R-GL31 with TM4. In addition, interactions between CHS and residues N181^{5,42}, A184^{5,45} on TM5 and H230^{6,32}, K233^{6,35} on TM6 stabilizes these helices (Fig. 6E and F). This is the same site where CHS resides in some of the crystal structures of the β_1 -adrenergic receptor⁵⁶ and the β_2 -adrenergic receptor,^{57,58} although all the residue contacts in the crystal structures are not captured in the MD simulations.

The presence of phospholipids in a detergent micelle is another factor that is known from experimentation to improve the stability of many membrane proteins.⁵⁹ This can be specific to a particular lipid or it can be a more general effect that can be affected by many different lipids. To observe the effects of lipids on the stability of A_{2A}R-StaR2, simulations were performed where an annular layer of POPC lipids surrounded the receptor in an OG micelle.

In contrast to the receptor in pure OG micelles, the A_{2A}R-StaR2 is less flexible in the OG micelle when surrounded by a POPC annular layer, and OG shows no penetration into the

receptor (Online movie file 2). The OG molecules tumble and displace the POPC molecules from the vicinity of the receptor (Fig. S11). However, the alkyl chains of the lipid POPC molecules point to the hydrophobic regions of the TM helices of the receptor as shown by the decrease in the hydrophobic solvent accessible surface area (Table S2), and increase in the stability of the receptor compared to just the OG micelle (see Fig. S3).

Discussion

The stability of membrane proteins in detergent is a key parameter that dictates the probability of success in their purification, crystallization and structure determination.^{12,60} Despite this, there is little understanding of how detergents destabilize membrane proteins or how adding lipids can stabilize them. Here we show that MD simulations can perhaps offer some insights into some of the initial molecular events that could destabilize different conformations of A_{2A}R. The simulations of A_{2A}R in the harsh detergent OG showed that the detergent was highly mobile and formed a micelle around the receptor with no discernible difference in hydrophobicity between the region around the hydrophobic core of the receptor and its surface. Addition of POPC annular lipid layer stabilized the receptor in the OG micelle by covering the receptor hydrophobic region of the TM helices.

In contrast, simulations of A_{2A}R in the mild detergent DDM showed that the detergent was not very mobile and formed a micelle around the receptor that showed high hydrophobicity in its interior and low hydrophobicity on the exterior. Thus DDM mimics the hydrophobic environment of the membrane better than OG, and the corresponding interhelical contacts within A_{2A}R are better preserved in DDM than in OG. It is well known that the addition of CHS and/or ligands can stabilize membrane proteins upon detergent solubilization. MD simulations suggest that ligands stabilize interhelical interactions within A_{2A}R, whilst CHS interacts directly with the receptor, reducing the flexibility in the transmembrane helices and thereby improving the stability of the receptor. The simulations offer only a hint of some of the processes that may occur in a receptor-detergent micelle, because the timescale of the all-atom MD simulations (100 ns) are several orders of magnitude smaller than the timescale for the purification of a membrane protein (hours to days). Thus the ultimate result of adding a harsh detergent to an unstable receptor, which is receptor aggregation and precipitation, was not observed. However, in three out of five simulations it was observed that OG molecules inter-penetrated between transmembrane domains and could even displace the bound antagonist ZM241385, which binds with nanomolar affinity to A_{2A}R.⁶¹ This appears to be a consequence of the significantly increased dynamics of A_{2A}R in OG compared to DDM, because OG ingress into the receptor always followed the separation of two adjacent α -helices. In DDM, where A_{2A}R is far less dynamic, no interpenetration of detergent into the receptor was observed, regardless of the conformation of the receptor. This hints at the possibility that breaking interhelical interactions and the partitioning of the detergent into the receptor could be initial steps in the inactivation process, which leads ultimately to irreversible denaturation.

Both OG and NG appear to have a significant advantage for membrane protein crystallography, because the size of the micelle they form around a small membrane protein is less likely to occlude hydrophilic loops essential for the formation of crystal contacts.^{62,63}

It is note-worthy that so far, GPCRs whose structures have been determined in the absence of either antibodies, nanobodies or T4 lysozyme fusions have either been crystallized in NG, OG, hega-10 or in the lipidic cubic phase.^{38,56,64} No structures have been determined from receptors crystallized in either DDM, DM or lauryl maltose neopentyl glycol (LMNG), presumably due to the large size of the detergent occluding hydrophilic surfaces and preventing close associations necessary for crystal lattice formation. Of course, the significant disadvantage of OG and NG is that they are so harsh that they rapidly denature most mammalian membrane proteins. This raises the interesting question of whether the MD simulations offer any insight into routes to design new detergents that have the size of OG or NG, but the mildness of DM or DDM. The major difference observed in the behavior of DDM and OG during the MD simulations was the rate of tumbling and diffusion within the micelle. Intuitively, this could be a reflection on the relative hydrophobicity of the alkyl chain and the sugar head group, previously referred to as the hydrophile-lipophile balance (HLB).⁶⁵ Here we have calculated a more pertinent parameter for alkyl maltosides and glucosides that takes into account the degree of hydration of the headgroup compared to the hydration of the alkyl chain during the MD simulations. This ratio of hydration numbers (RHN; hydration number for the tail group divided by the hydration number for the head group) gives large values for the alkyl glucosides (OG, NG, DDG: harsh detergents) and small numbers for the alkyl maltosides (OM, DM, DDM, LMNG: mild detergents) (Fig. 7). The prediction is therefore that decreasing the RHN ratio of OG by increasing the hydrophilicity of the headgroup of OG would result in a milder detergent, as is observed for OM (the interhelical packing interactions of A_{2A}R-StaR2 in OM detergent is close to that of DM detergent) (Fig. S12). MD simulations of novel detergents will offer an attractive route to testing these *in silico* before embarking on their synthesis, assaying their biophysical properties and finally seeing whether they do, indeed, maintain membrane proteins in a biologically relevant conformation for a sufficient length of time to allow crystallization and structure determination.

Methods

Receptor Preparation

All the MD simulations were performed using the GROMACS package⁶⁶ with the GROMOS force field.⁶⁷ The initial coordinates of A_{2A}R-GL31 and A_{2A}R-StaR2, including the coordinates of the agonist adenosine and antagonist ZM241385, were taken from the PDB file with the PDB code 2YDO³⁸ and 3PWH,³⁹ respectively. The antagonist ZM241385 bound A_{2A}R-StaR2 thermostable mutant (PDB code 3PWH) in the inactive state contains eight mutations at positions A54L^{2.52}, T88A^{3.36}, R107A^{3.55}, K122A^{4.43}, L202A^{5.63}, L235A^{6.37}, V239A^{6.41}, and S277A^{7.42}.³⁷ The Ballesteros-Weinstein amino acid numbering system used for class A GPCRs are shown as superscripts.⁶⁸ The agonist adenosine bound active-like state mutant A_{2A}R-GL31 (PDB code 2YDO) has four mutations L48A^{2.46}, A54L^{2.52}, T65A^{2.63}, and Q89A^{3.37}.³⁸ We performed MD simulations for both A_{2A}R-StaR2 and A_{2A}R-GL31 with and without their respective antagonist and agonist ligands bound.

Building and Optimizing Micelle structures

The number of detergent molecules per micelle was chosen to be larger than the aggregation number of each detergent studied here. We used 192 DDM molecules, 144 DM, 155 NG and 123 OG molecules to build their respective micelles. The aggregation numbers for these detergents are given in Table S1. The hydrophobic region of the GPCR, which is cylindrical in shape, was estimated to be 60Å, from the range of the minimum and maximum of the Z coordinates of the heavy atoms (see Fig. S13). The radius of the GPCR is estimated to be 30Å as shown in Fig. S13. Based on this estimated size, the hydrophobic tail of the detergents would reside in 20Å ring like sections around the hydrophobic middle of the GPCR cylinder. We divided the micelle into six ring sections spaced 3.3Å apart. Also the 6 rings would be able to accommodate 32 methyl groups found at the end of the detergent tail. For example, this geometrical constructs resulted in accommodating 192 DDM molecules close to the aggregation number of DDM (~200). The first step was to create 4 pseudo-atom points that would represent the ideal spacing and packing for each detergent molecule. For example, for the DDM molecule, the pseudo-atom point 1 was chosen at the end of the hydrophobic tail of DDM, point 2 was the in the middle of the hydrophobic tail, point 3 and point 4 were in the head group (see Fig. S13). These points were used to place all the DDM molecules in the same orientation. For the 192 DDM molecules, x, y, and z coordinates for all points were calculated to maximize spacing between each detergent molecule. A transformation matrix between the original coordinates of the detergent molecules and the new array using the four pseudo-atom points were calculated for each of the 192 DDM molecules, and applied to generate new coordinate files for the detergent molecules. The internal conformations of the detergent molecules were taken from the protein structure database. The same procedure was repeated for DM, NG and OG with the same 6 rings sections, and spacing change based on the number of molecules 144, 150 and 120, respectively. The chemical structures of the detergents used in this study are shown in Fig. S14 and the starting micelle particle are shown in Fig. S13B.

Building of the Receptor-Micelle Complexes

The A_{2A}R-GL31/StaR2 receptor was inserted into each of the four detergent micelles built as described above. The receptor-detergent complex was constructed by inserting the receptor into hollow micelles. The detergent molecules were placed randomly with the terminal alkyl (methyl) groups of their tails at minimum distance of 4Å from the receptor exterior to avoid steric clashes. Each detergent head group was placed approximately equidistant from its nearest neighbors. After energy minimization of this system, the receptor-micelle system was solvated with waters⁶⁹ by superimposing a box of water followed by removal of water molecules that were too close to the receptor (within 4Å of receptor). We used the SPC forcefield for describing the waters.⁶⁹ Chloride ions were added to the systems as the counter ions for neutralizing the whole system. In the DDM simulation with CHS, the DDM molecules were randomly replaced with 25 CHS molecules in keeping with the concentration ratio (w/w) of CHS to DDM used in experiments namely, 0.1% DDM to 0.01% CHS.^{70,71} The receptor-micelle complexes were equilibrated using MD simulations as described in the next section. The resulting receptor-detergent micelle complexes had 6,003 atoms in OG to 11,115 atoms in DDM for simulations.

Details of MD simulations

We used the GROMACS v4.6 to perform all the MD simulations in this study. The MD simulations were performed at 300 K. Solvent and solute (receptor-detergent system) were independently coupled to a temperature bath with a relaxation time of 0.1 ps.⁷² The pressure was calculated using a molecular virial and held constant by weak coupling to a pressure bath with a relaxation time of 0.5 ps. For all the equilibration simulations, the receptor was positionally restrained and the simulations were performed at constant volume (NVT). Thus no pressure coupling was applied in these cases. Bond lengths and the geometry of the water molecules were constrained using the SHAKE algorithm.⁷³ The equations of motion were integrated using the leap-frog algorithm and a time step of 2 fs. Center of mass motion was removed every 20 fs. The short-range van der Waals and electrostatic interactions were evaluated at every time step by using a charge-group pair list with cutoff radius of 8 Å between the centers of geometry of the charge groups. Longer-range van der Waals and electrostatic interactions, between pairs at a distance longer than 8 Å and shorter than a long-range cut-off of 14 Å, were evaluated every fifth time step, at which point the pair list was also updated, and were kept unchanged between these updates. For equilibration of the receptor-detergent complexes, the atoms of the protein were positionally restrained using a harmonic restraining force with a force constant of 1,000 kJ/mol/nm during the 200 ps of equilibration. In this step the water molecules and detergent were movable to optimize their packing around the receptor. The system was further equilibrated using NPT ensemble, while the force constant of the restraining force was set to 5 kcal/mol and reduced to zero step-wise at each 2 ns. At this point the pressure coupling was switched on. This results in a total equilibration time of 10 ns. We performed an additional 5 ns of simulations without restraints before the production runs. Five independent simulations each to 100 ns were performed with different starting velocities. The analyses of the convergence of the five simulation runs, along with the energy analysis are discussed in the Supplementary Information. The average representative structures from MD simulations were calculated as follows. We performed conformation clustering using RMSD cutoff of 1.2 Å on the concatenated MD simulation trajectories from the five simulations for any given system. The most representative structure of the most populated cluster was calculated as the frame that has the smallest RSMD to the center of this cluster of conformations.

Building of the receptor-bilayer complexes and MD simulations in a bilayer

The starting conformations of A_{2A}R were taken from the crystal structures of GL31 and StaR2 (PDB codes 2YDO for GL31 and 3PWH for StaR2). The structures were solvated in SPC water and explicit lipid (palmitoylcholinephosphatidylcholine, POPC) using the *inflatagro* package in GROMACS.⁶⁶ We performed the MD simulations on A_{2A}R in a POPC bilayer lipid using PBC (periodic boundary conditions) with GROMOS96 force field.⁷⁴ We used SETTLE⁷⁵ and LINCS⁷⁶ algorithm for the bond and angle for water and all other bonds with a 2 fs time step. A 12 Å cutoff distance was used for non-bonded interactions and the coordinates were saved every 2 ps for analysis. In addition, we used the PME (particle mesh Ewald) method^{77,78} for long-range van der Waals interaction. Each system was equilibrated for 200 ps at 310K using a NVT ensemble followed by 10 ns of MD under constant pressure, NPT condition with a pressure of 1 bar. In this stage, receptor and ligand were kept in place using position restraints. After NPT equilibration, we performed a total of

10 production runs for a total of 100 ns with different velocities using the constant volume, NVT ensemble. Additional details are provided in the SI (supporting information) text.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Representative structures from the most populated MD conformation ensemble of A_{2A}R-StaR2 in the detergents, DDM (A and C) and OG (B and D). Atoms in detergent are depicted as spheres (hydrophilic atoms as red; hydrophobic atoms as cyan) and the receptor is shown in cartoon representation (dark blue). The A_{2A}R-StaR2-detergent micelle complexes are shown in the two different views, equivalent to either parallel to the membrane plane (side view; A and B) and viewed from the intracellular (IC) surface (C and D). See Supplementary Information (Fig. S1) for data on simulations in DM and NG.

Figure 2.

OG is highly mobile in the A_{2A}R-StaR2–detergent complexes compared to DDM. The Radial Distribution Function (RDF) gives the density for either the head-group or tail-group of the detergents as a function of distance from the center of mass of A_{2A}R-StaR2 in DDM (A) and OG (C); tail group, black curve; head group, red curve. B and D, Spatial distribution plots of two representative detergent molecules for DDM (B) and OG (D) within 6 $\text{\AA}$ of A_{2A}R-StaR2 from the most populated ensemble. The initial position of the detergent molecules is shown in stick representation and the resultant spatial distributions are shown as dots; green, DDM and red, OG.). See Supplementary Information (Fig. S2) for data on simulations in DM and NG.

Figure 3.

Comparison of the calculated potential energy of A_{2A}R-StaR2 in different environments with apparent T_m values determined experimentally. The apparent T_m of A_{2A}R-StaR2 was determined using a radioligand-based thermostability assay (31) and plotted against the potential energies calculated from the MD simulations. Experimental T_m for comparisons to detergent simulations were determined from purified receptors in the detergents stated. For the comparison with the simulations performed in POPC+cholesterol, the receptors were assayed in insect cell membranes. Magenta, DDM+CHS; red, POPC+CHOL; black, DDM; blue, DM; green, NG; orange, OG. The best fit line was determined by linear regression and error represents standard deviation (s.d.).

Figure 4.

Structural heterogeneity of A_{2A}R-StaR2 in various detergents and in a POPC. (A-E) Population distributions of A_{2A}R-StaR2 are depicted in relation to helical content and RMSD in coordinates from the respective crystal structures. Simulations of A_{2A}R-StaR2 (PDB code 3PWH) were performed in the following environments; A, DDM; B, DM; C, NG; D, OG; E, POPC. The red-dotted lines in the figure are the α-helical content of the crystal structure of A_{2A}R-StaR2. Representative structures extracted from the most populated ensemble of A_{2A}R-StaR2 in the following detergents or lipid are shown: F, DDM; G, DM; H, NG; I, OG; J, POPC. The color scheme ranges from blue to red, with red indicating the lowest helicity in the TM regions. Loop regions are not included in this analysis and are colored dark grey. The percentages of snapshots represented by these structures within the most populated cluster indicated by the red regions are 45% (DDM), 36% (DM), 24% (NG), 29% (OG), and 48% (POPC). Inclusion of MD structures from the yellow regions increases the population to 68% (POPC), 69% (DDM), 40% (NG) and 43% (OG).

Figure 5.

Loss of structural integrity of A_{2A}R-StaR2 in OG. Panels show a series of snap shots of A_{2A}R-StaR2 in OG using the same view of the receptor during a 100 ns simulation. *A.* Starting state; Before ingress, detergents move freely, diffuse rapidly with tumbling within micelle. *B.* Separation of adjacent α -helices (38 ns); Helices move, no ingress. TM6 and TM7 start unraveling. *C.* Intermediate state in the penetration process (64 ns); Start of ingress. Detergent is starting penetration between TM6 and TM7. *D.* Final state; Detergents interpenetrate and inhibit antagonist binding, so ZM241385 (stick model) is displaced off from normal binding site.

Figure 6.

Selective stabilization of A_{2A}R-GL31 compared to A_{2A}R-StaR2 by CHS. The effect of CHS on the distributions of the conformational ensembles is depicted with respect to helicity and RMSD from the crystal structure of the respective receptors. (*A* and *B*) A_{2A}R-StaR2 (antagonist conformation); (*C* and *D*), A_{2A}R-GL31 (active-intermediate conformation). (*E* and *F*) CHS interaction sites identified from the MD simulations of A_{2A}R-GL31. *E*, Representative snapshots from the MD simulations showing the residues in TM4 that make close contact with CHS. Two residues 4.46 and 4.50 have also shown to be important in cholesterol binding in the crystal structure of β_2 -adrenergic receptor (PDB code 3D4S). The hydrogen bonds between head group of CHS and the residues are shown as red-dotted lines and hydrophobic interactions of the tail of CHS and the residues are shown as blue arrows. *F*, The head group of CHS makes strong hydrogen bonds with residues H230^{6.32} and K233^{6.35}. The effect of cholesterol on the conformational ensemble in the POPC bilayer is depicted in the Supplementary information (Fig. S10).

Figure 7.

Relative hydration of detergents in solution. *A.* Hydration numbers for the sugar head groups and alkyl chains of detergents. The hydration number is the average number of waters near each organic moiety in the detergent during the MD simulations. In each case these have been determined by radial distribution functions between each carbon atom in the alkyl chain and the center-of-mass of sugar rings in the head group, to the oxygen atoms of water molecules within 5.0 Å of the detergent. Hydration number profiles for DDM, DM, NG, OG, OM, and dodecyl β-D-glucopyranoside (DDG) are colored in black, blue, green, orange, red, and purple, respectively. *B.* Nomenclature for panel a shown on a model of DDM. *C.* The ratio of hydration numbers (RHN) for each detergent was calculated from the ratio of the summation of average hydration number for the alkyl chain ($\bar{C}_i$ is the average hydration number for carbon in the alkyl chain) to the summation of average hydration number of the head-group ($\bar{S}_i$), as follows.

$$RHN = \left(\frac{\sum_{i=1}^j \bar{C}_i}{\sum_{i=1}^j \bar{S}_i} \right)$$