Impact of purification conditions and history on A_{2A} adenosine receptor activity: The role of CHAPS and lipids

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Abstract

The adenosine A_{2A} receptor (A_{2A}R) is a much-studied class A G protein-coupled receptor (GPCR). For biophysical studies, A_{2A}R is commonly purified in a detergent mixture of dodecylmaltoside (DDM), 3-(3-cholamidopropyl) dimethylammonio propane sulfonate (CHAPS), and cholesteryl hemisuccinate (CHS). Here we studied the effects of CHAPS on the ligand binding activity and stability of wild type, full-length human A_{2A}R. We also tested the cholesterol requirement for maintaining the active conformation of the receptor when solubilized in detergent micelles. To this end, the receptor was purified using DDM, DDM/CHAPS, or the short hydrocarbon chain lipid 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC, di-6:0PC). After solubilization in DDM, DDM/CHAPS, or DHPC micelles, although A_{2A}R was found to retain its native-like fold, its binding ability was significantly compromised compared to DDM or DDM/CHAPS with CHS. It therefore appears that although cholesterol is not needed for A_{2A}R to retain a native-like, α -helical conformation, it may be a critical component for high affinity ligand binding. Further, this result suggests that the conformational differences between the active and inactive protein may be so subtle that commonly used spectroscopic methods are unable to differentiate between the two forms, highlighting the need for activity measurements. The studies presented in this paper also underline the importance of the protein's purification history; *i.e.*, detergents that interact with the protein during purification affect the ligand binding properties of the receptor in an irreversible manner.

Introduction

Membrane proteins are essential biomolecules needed for a vast array of cellular processes including cellular signaling, ion and metabolite transport, adhesion, and migration [1], to name a few. G protein-coupled receptors (GPCRs) are integral membrane proteins consisting of seven α -helical segments that span the plasma membrane and respond to different extracellular stimuli (e.g. peptides, neurotransmitters, and small molecules). Upon binding to a ligand, GPCRs transmit a cellular signal mainly through intracellular G proteins and arrestins [2].

Structural studies typically require milligrams of purified protein [3, 4], and unlike water-soluble proteins, membrane proteins require a membrane mimetic system to stabilize their hydrophobic transmembrane regions. However, obtaining substantial amounts of active GPCRs remains a challenge. This is in part due to their low expression in native tissues, structural flexibility, and instability when in detergent solutions [5]. Because of these challenges, structure-function studies of GPCRs using their native, full-length sequence, are not commonly carried out; modifications to their native sequences have been used to stabilize the receptors, facilitating crystal formation [6, 7].

Detergents with a hydrophobic tail of 6-12 carbon atoms are commonly used to solubilize, stabilize and crystallize GPCRs [8]. In the case of GPCR X-ray crystal structures, the membrane protein was first solubilized in detergent prior to crystallization in detergent micelles, lipidic cubic phases, or bilayered micelles (also referred to as bicelles), without exception.

Even though many GPCRs are stable and retain functionality in detergent micelles, GPCRs and other membrane proteins often unfold and aggregate when solubilized in detergents [9], altering their native structure and eliminating or suppressing their biological function(s). At this point, selecting a detergent that retains membrane protein stability and function is typically a matter of trial and error [10]. In the case of the wild type, full-length human A_{2A} adenosine receptor ($A_{2A}R$), a class A GPCR, purification with the detergent dodecylmaltoside (DDM) requires the presence of a cholesterol analog (cholesteryl hemisuccinate, CHS) in order for the receptor to retain its ligand binding activity [11, 12]. It should be pointed out that three cholesterol interaction sites have been identified from molecular dynamic simulations [13], and cholesterol's presence was observed in one $A_{2A}R$ crystal structure [14]. However, it remains unclear whether cholesterol stabilizes the high affinity conformation of the receptor due to changes to the membrane properties, direct lipid-protein interactions, or a combination of both.

When purifying $A_{2A}R$, the zwitterionic detergent 3-(3-cholamidopropyl) dimethylammonio propane sulfonate (CHAPS) is often used to solubilize CHS into micelles. In the current studies, we employed the wild type, full-length human $A_{2A}R$ to investigate the effects of CHAPS on the ligand binding activity and stability of this receptor. We also solubilized $A_{2A}R$ in micelles, with and without CHS, to further test the notion that a cholesterol analog is needed to retain the native fold and ligand binding

activity of A_{2A}R. Finally, we tested the efficacy of 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC), a short chain phospholipid that self-assembles into micelles, and which acts as a biologically relevant detergent [9, 15]. DHPC has been shown to minimally perturb membrane proteins [16] and has been studied extensively in conjunction with the long hydrocarbon chain lipid, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, di-14:0PC) [16-19]. This lipid mixture (i.e., DHPC/DMPC) is tunable, and forms a number of different morphologies (e.g. bilayered micelles, unilamellar vesicles, multilamellar vesicles, perforated lamellae, ribbon-meshed lamellae) depending on the total lipid concentration, the molar ratio of DMPC-to-DHPC, net charge of the system, and temperature [20, 21].

Materials and Methods

Expression and purification of A_{2A}R from yeast membrane preparations A_{2A}R was expressed in *Saccharomyces cerevisiae* cells, BJ5464, using the multi-integrating pITy-A_{2A}R-His₁₀ plasmid, as previously described [11]. In order to improve protein purity and reproducibility between purifications, a previous purification protocol [11, 22] was adapted to employ membrane preparations instead of crude cell lysis [23]. Briefly, cell pellets were collected via centrifugation 24 hours post-induction. 600 ml of liquid culture with a total OD₆₀₀ of 22 was separated into 50 mL aliquots, centrifuged at 3,220xg, and cooled to -80°C. Both lipid and detergent purifications started from cell pellets, with cell pellets consistently collected at an OD close to 22, which reduced batch-to-batch variability.

For details regarding the purification protocol refer to [23]. For these studies, additional detergent and lipid mixtures were used as follows. The homogenized membrane preparations were resuspended in 22 ml Buffer A (composed of 10% glycerol, 50 mM sodium phosphate monobasic, and 300 mM sodium chloride at pH 8, supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) and complete EDTA-free protease inhibitor tablets (Roche Applied Science, Indianapolis, IN)), and the appropriate detergent or lipid: 1) 2% (w/v) DDM+1% (w/v) CHAPS+0.2% (w/v) CHS (all from Anatrace, Maumee, OH); 2) 2% (w/v) DDM+0.2% (w/v) CHS; 3) 2% (w/v) DDM+1% (w/v) CHAPS; or 4) 2% (w/v) DDM, and 5) 6.25% (w/v) DHPC (Avanti Polar Lipids Alabaster, AL). 6.25% (w/v) DHPC corresponds to 138 mM, approximately 10 times the critical micellar concentration (CMC) of DHPC (11-16 mM) [15]. The concentrations of DDM and CHAPS used correspond to approximately 200 and 3 times their CMC (i.e., approximately 0.2 mM and 6 mM, respectively) [24].

The elution buffers contained the appropriate detergent or lipid mixtures: 1) 0.1% (w/v) DDM+0.1% (w/v) CHAPS+0.02% (w/v) CHS; 2) 0.1% (w/v) DDM+0.02% (w/v) CHS; 3) 0.1% (w/v) DDM+0.1% (w/v) CHAPS; 4) 0.1% (w/v) DDM; or 5) 0.8% (w/v) DHPC. Purified A_{2A}R samples were stored at 4 °C and used within one week of preparation to ensure maximal ligand binding activity.

Protein purity, concentration and biophysical characterization

Samples were separated via electrophoresis on 12% SDS-PAGE, and protein bands were detected via staining with Sypro Ruby (Life Technologies, Carlsbad, CA). For Western blotting mouse anti-A_{2A} (Santa Cruz Biotechnology, Santa Cruz, CA, catalogue # 32261) primary antibody was used at a 1:5000 dilution. Alexa Fluor 488 goat anti-mouse (Life Technologies, Carlsbad, CA, catalogue # A11029) was used as the secondary antibody at a 1:5000 dilution. Fluorescence was detected using a BioSpectrum Imaging System (UVP, Upland, CA). Protein concentration was determined using UV absorbance at 280 nm as described in [11], and protein purity was quantified from the Sypro Ruby stained gel images using FIJI [25].

For characterizing the protein's secondary structure, circular dichroism (CD) measurements were conducted using a Jasco J-810 spectropolarimeter (Jasco, Easton, MD), as previously described in [11]. Measurements were performed at 25°C, and spectra collected with 1 nm resolution, with at least 3 integrations per spectrum. Reference spectra containing the appropriate buffer were collected and subtracted from their respective spectra. Fluorescence spectra were collected using a PC-1 spectrofluorimeter (ISS, Champaign, IL), as described previously in [11]. To minimize light scattering effects, measurements were collected with the excitation polarizer set to 90° and the emission polarizer set to 0°. Intrinsic fluorescence was measured at 15°C, and excitation was set to 280 nm. CD and fluorescence measurements were taken at a protein concentration of 0.05 – 0.06 mg/ml.

To estimate particle sizes, Dynamic Light Scattering (DLS) measurements were taken using a Brookhaven Instruments 90Plus Particle Analyzer (Brookhaven Instruments, Holtsville, NY). A 50 µL micelle solution was loaded into Eppendorf UVette small volume cuvettes (Fisher Scientific, Pittsburgh, PA) and mounted on the instrument's small-volume cuvette adapter pedestal.

Radioactive ligand binding

Ligand binding of purified receptors was carried out as described previously [11], with minor modifications. Briefly, approximately 1 ml of purified receptor was incubated with a Ni-NTA Superflow resin (approximately 15 µl of settled resin) overnight. **If necessary, samples were centrifuged briefly to allow the resin to settle, the elution buffer volume was removed, and the sample volume adjusted to achieve a target concentration of 55 µg/ml. 180 µl of the protein/resin mix was added per well, to enable loading of 10 µg of A_{2A}R-His₁₀ per well in a 96-well plate (glass fiber type B filters, Millipore, Billerica, MA) for saturation and point measurement ligand binding analysis.** For saturation binding experiments, protein samples were incubated with increasing amounts of [³H] CGS 21680 (Perkin Elmer, Waltham, MA) for 4 hours. Non-specific binding was determined using 10 µM of the cold ligand, CGS 21680, which was added to the samples at each value of titrated ligand. The counts obtained from these controls were subtracted from the sample data. For ligand binding activity point measurements, 100 nM of the titrated ligand was added to each A_{2A}R sample, and 10 µM of cold ligand was added to measure non-specific binding. Binding was measured using a Wallac 1450 Trilux MicroBeta Jet (Perkin Elmer). **Error bars reflect effects of variability in both technical and**

biological replicates. Saturation binding data were fit to a single-site binding model using the Curve Fitting toolbox in Matlab 7.10 (R2010a), and point measurement data were compared to data from A_{2A}R purified and solubilized in DDM/CHAPS/CHS.

Protein thermal denaturation

A thiol-reactive fluorescent probe, 7-(Diethylamino)-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) (Invitrogen, Carlsbad, CA) was used to characterize the thermal stability of A_{2A}R in various membrane-mimetic environments. CPM stocks were protected from light and stored at -80 °C as described in [22]. 5.6 μM CPM was added to the protein sample while A_{2A}R was bound to Ni-NTA Superflow resin prior to elution (CPM was allowed to react with accessible free thiols, protected from light, on an end-over-end mixer at 4 °C overnight). The following day the protein was eluted from the Ni-NTA Superflow resin with 500 mM imidazole as described in [23].

Fluorescence measurements were performed on a PC-1 (ISS) spectrofluorimeter, as described in [22] with some modifications. Measurements were taken with the excitation set to 280 nm (intrinsic absorbance of the purified receptor) and the emission at 460 nm, close to the emission maximum of CPM bound to the protein sample. Thus, any fluorescence from CPM close to the emission peak is expected to occur due to tryptophan excitation and energy transfer to the CPM in its vicinity; **further details are included in the Supplemental Information.** The fluorescence signal is expected to change as the protein unfolds. CPM fluorescence was normalized to the maximal and minimal intensity values.

Results and Discussion

The full-length, wild type human A_{2A} receptor (A_{2A}R) was expressed in yeast BJ5464 cells, and membrane preparations were carried out as described in [23] and the Materials and Methods section. Samples of A_{2A}R were extracted and purified from cellular membranes using different detergent and lipid mixtures: DDM, CHAPS, CHS, and DHPC. To solubilize CHS in DDM in the absence of CHAPS, we used a sonication protocol described by Stevens and the JCIMPT at Scripps Research Institute (jcimpt.usc.edu/protocols/JCIMPT_PreparationofCHSStock.pdf). Samples were separated via electrophoresis, and protein bands were detected using Sypro Ruby staining and Western analysis using anti-A_{2A}R antibodies (Figure 1).

Figure 1. Wild type, full-length human A_{2A}R bands detected using Sypro Ruby staining (A) and Western blotting (B). Samples were treated with a reducing agent (10 μ M TCEP) and separated via 12 % SDS-PAGE, stained with Sypro Ruby (A) and blotted onto a nitrocellulose membrane for Western blot detection with an anti-A_{2A} primary antibody (B). Lanes contain MagicMark protein standards, with molecular weights indicated on the left, and A_{2A}R purified from membrane preparations using various detergents mixtures and DHPC, as shown. Arrows indicate the A_{2A}R monomer and oligomers, and asterisks indicate the main impurities. Results are typical of multiple independent purifications. **The contrast and brightness of images in Figure 1A were adjusted in order to highlight the impurities present in each purification; results shown were not used to quantify protein concentration.** C) Intrinsic fluorescence spectra of A_{2A}R purified using detergents and lipids following excitation at 280 nm. The data represent the average from at least two independent purifications. Lines represent the fine lowess curve fit to the average in GraphPad Prism. DDM/CHAPS/CHS, solid green; DDM/CHS, dashed green; DDM/CHAPS, solid red; DDM, dashed red; DHPC, solid blue. D) CD spectra of A_{2A}R in different micelle environments. Lines represent the fine lowess curve fit to data in GraphPad Prism. DDM/CHAPS/CHS, solid green; DDM/CHS, dashed green; DDM/CHAPS, solid red; DDM, dashed red; DHPC, solid blue. Intrinsic fluorescence and CD measurements were taken at a protein concentration of 0.05 – 0.06 mg/ml.

Differences between the protein obtained from the detergent and lipid purification protocols are highlighted in Figure 1. Sypro Ruby staining (Figure 1A) revealed a pronounced band at approximately 40 kDa, which corresponds to A_{2A}R monomers, and whose identity was confirmed by Western analysis (Figure 1B) and mass spectrometry of protein isolated from gels (results not shown). A final protein concentration of 0.05 – 0.09 mg/ml in 3.5 ml (corresponding to 3.5 – 6.2 mg/L of culture) was determined for the A_{2A}R purifications using UV absorbance at 280 nm (Table 1). As seen in Figure 1A and Table 1, the detergent and lipid purifications yielded varying degrees of receptor purity, as confirmed by Sypro Ruby stain and densitometry analysis.

Despite the differences in purity, A_{2A}R isolated using these different detergent conditions did not show significant differences in their secondary and tertiary structures, as determined by intrinsic fluorescence and circular dichroism (CD) spectroscopy (Figure 1C and 1D). The intrinsic fluorescence emission spectra of A_{2A}R in the different membrane-mimetic environments had a center of mass ranging from 326–328 nm (Table 1). This result implies that the receptor is in a hydrophobic environment, and has a compact and native-like fold (Figure 1C and Table 1). CD analysis was used as a semi-quantitative measure to confirm that the purified receptor was predominantly α -helical (Figure 1D). We have previously reported that A_{2A}R lost its α -helical content when purified in DDM [26]; we believe that the difference here – i.e., the observation of α -helical structure – may result from the improved purity we obtained using our current membrane preparation purification protocol.

The small shift in the intrinsic fluorescence maximum and CD spectra of the protein in these different detergents (DDM/CHAPS/CHS) and lipid (DHPC) could imply minor differences in the structural conformation of the receptor and its local environment. However, it is unclear whether these subtle changes in secondary and tertiary structure correlate to changes in receptor function.

Table 1. Protein purity, concentration and intrinsic fluorescence center of mass ($n \geq 2$ independent purifications). **Total cell density in the lysate was kept constant as described in the Materials and Methods.**

	Purity $\pm$ s.d. (%)	Concentration $\mu\text{g/ml} \pm$ s.d. (mg per L of culture)	Center of mass $\pm$ s.d. (nm)
DDM/CHAPS/CHS	90.1 $\pm$ 3.2	63.0 $\pm$ 7.6 (4.4)	327.9 $\pm$ 0.8
DDM/CHS	96.2 $\pm$ 2.1	55 $\pm$ 8.0 (3.9)	326.2 $\pm$ 0.8
DDM/CHAPS	64.0 $\pm$ 2.1	50 $\pm$ 2.0 (3.5)	327.8 $\pm$ 1.6
DDM	91.7 $\pm$ 2.0	55.0 $\pm$ 7.4 (3.9)	327.0 $\pm$ 0.0
DHPC	73.4 $\pm$ 8.6	88 $\pm$ 46.2 (6.2)	327.7 $\pm$ 1.3

To determine whether A_{2A}R in these different micelle environments was able to bind ligand, we conducted ligand binding assays using 100 nM of the tritiated agonist CGS 21680 (Figure 2). Results were compared to A_{2A}R in DDM/CHAPS/CHS, whose activity we previously characterized in detail [11, 27, 28].

Figure 2. Specific activity measurements for $A_{2A}R$ solubilized in different micelle environments. 100 nM of the titrated ligand was added to each $A_{2A}R$ sample, and 10 μ M of cold ligand was added to measure non-specific binding. The resulting signal (measured in counts per minute) was compared to the signal obtained for $A_{2A}R$ solubilized in DDM/CHAPS/CHS. Results for the sample labeled “DDM reconstituted in DDM/CHAPS/CHS”, correspond to those of the receptor initially purified in DDM only, and subsequently reconstituted in DDM/CHAPS/CHS. Error bars represent the standard deviation from the average of at least two independent purifications, with measurements conducted in triplicate (n=6).

We previously reported ligand binding activity for $A_{2A}R$ in DDM/CHAPS/CHS, but no detectable ligand binding activity was observed for $A_{2A}R$ in DDM [11, 27, 28]. Here, we expand this analysis and report activity for $A_{2A}R$ in DDM/CHS, but almost undetectable activity in DDM/CHAPS (1.8%; Figure 2). Interestingly, we detected ligand binding activity of $A_{2A}R$ purified using the short chain lipid DHPC (4.0%); however the receptor bound with significantly reduced affinity (refer to Fig. S1 in the Supporting Material). It is worth highlighting that if the receptor is first purified in DDM and then reconstituted in DDM/CHAPS/CHS within one day, the receptor does not recover its native ligand binding activity (sample labeled “DDM reconstituted in DDM/CHAPS/CHS” in Figure 2). This result implies that **once the receptor is purified in a detergent that does not promote ligand-binding activity (in this case DDM), changing the detergent mixture to one that promotes ligand-binding activity (such as DDM/CHAPS/CHS) does not recover the receptor’s ligand binding ability.** The inability to restore ligand binding implies that this system exhibits a detergent related hysteresis, which prevents it from regaining its full activity. Similar results have been reported for the cannabinoid receptor CB_2 , where in the absence of CHS the receptor did not show any measurable activity. Importantly, in the absence of CHS even a short exposure of the receptor to micelles leads to a rapid and irreversible loss of function [29]. These results have important implications for nanodisc studies, where receptors are often reconstituted in DDM prior to lipid reconstitution.

Dynamic light scattering (DLS) was used to characterize micelle size (Table 2), and to determine if the lack of ligand binding activity correlated to micelle size/protein aggregation. A_{2A}R completely loses its ligand binding activity when solubilized in DDM/CHAPS or DDM, and has low affinity for CGS2180 when solubilized in DHPC. Large micelles were observed when A_{2A}R was solubilized in DDM/CHAPS or DHPC (47 – 145 nm), while A_{2A}R in DDM had a similar size to A_{2A}R in DDM/CHAPS/CHS and DDM/CHS (3.5 – 10 nm), indicating that ligand binding affinity did not correlate with micelle size (p=0.24). The effect of CHAPS was surprising, in contrast to previous reports of the serotonin receptor and other membrane proteins, which reported a decrease in protein association with this detergent [30-33]; this result further emphasizes that this class of membrane proteins have unique detergent interaction properties that are not easy to predict.

Table 2. Micelle size determined using DLS. Data were collected from at least three independent purifications (n= number of independent purifications and measurements). **The size range shown corresponds to ≥ 98% of the particles measured.**

	Diameter range (nm)	n
DDM/CHAPS/CHS	3.9–9.7	5
DDM/CHS	3.5–7.6	3
DDM/CHAPS	46.8–110.9	3
DDM	4.0–9.7	3
DHPC	84.4–144.5	4

Specific ligand binding point measurements (Figure 2) indicated that A_{2A}R retained high ligand binding affinity only when solubilized in DDM/CHAPS/CHS and DDM/CHS. Even though high affinity ligand binding was lost when A_{2A}R was solubilized in DDM, DDM/CHAPS or DHPC, the receptor retained a native-like fold. These data are strong evidence that cholesterol is not needed to attain an α -helical conformation and native-like fold for A_{2A}R, but may be critical for high affinity ligand binding. Using intrinsic fluorescence and CD we were unable to differentiate between an active and inactive receptor, highlighting the importance of complementary biophysical measurements and functional assays. This result also indicates that the differences between the high affinity state of the receptor and its inactive state are subtle. For example, using atomic force microscopy-based single-molecule force spectroscopy, Zocher *et al.* found that CHS stabilizes structural segments of the β_2 -adrenergic receptor. They also concluded that CHS stabilizes the same structural segments in the absence of CHS [34]. This implies that cholesterol enhances the interactions that stabilize the receptor, but cholesterol does not necessarily alter the receptor's conformation.

Since A_{2A}R retained high ligand binding affinity when solubilized in DDM/CHAPS/CHS and DDM/CHS, these samples were chosen for saturation ligand binding and temperature unfolding experiments to study differences in ligand binding affinity and

structural stability when the receptor is solubilized into these different micelle environments (Figure 3).

Figure 3. Ligand binding affinity and temperature unfolding studies. A) Saturation ligand binding of ³H-CGS 21680 to A_{2A}R in DDM/CHAPS/CHS (filled circles; solid line) and DDM/CHS micelles (open circles; dashed line). The data points show the average of two different purifications run in triplicate (n=6). A_{2A}R in DDM/CHAPS/CHS had a K_D of 67 nM; we previously reported a K_D of 66 ± 4 nM [11]. A_{2A}R in DDM/CHS had a K_D of 96 nM with a 95% confidence interval of 77 – 114 nM. Data were fit to a single-site binding model (line). Error bars are denoted as the standard deviation. B) Temperature unfolding of A_{2A}R-CPM solubilized in DDM/CHAPS/CHS (filled squares; solid line) and DDM/CHS micelles (open circles; dashed line) via the fluorescence intensity (at 463 nm) of the thiol-reactive probe CPM. Data points were collected from at least three independent experiments and all replicates are shown. Lines connect the median value of the data points.

Saturation binding data were fit to a single-site binding model exhibiting a typical saturation binding curve with an equilibrium dissociation constant (K_D) of 67 nM for A_{2A}R in DDM/CHAPS/CHS, and 96 nM for A_{2A}R in DDM/CHS (Figure 3A). Previously, we have used the thiol-reactive fluorescent probe 7-(Diethylamino)-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM) to determine changes in thermal unfolding of A_{2A}R in the presence of different ligands [35]. As CPM binds irreversibly, the unfolding is not reversible. Here, we removed unbound CPM prior to the temperature denaturation experiments and use bound CPM fluorescence to determine whether the solubilization conditions would impact receptor thermal unfolding behavior, with only minor

differences observed (Figure 3B). The data could not be fit to a single-transition folding model, as there was only a gradual transition from the folded to the unfolded state as temperature increased.

A_{2A}R exhibited high ligand binding affinity when solubilized in DDM/CHAPS/CHS and DDM/CHS micelles. Although there were only subtle differences between the secondary and tertiary structure—based on intrinsic fluorescence and CD data of similar size micelles—it appears that A_{2A}R is slightly more active when solubilized in DDM/CHAPS/CHS micelles.

Conclusion

These results highlight the importance of ligand binding activity screening, as intrinsic fluorescence, CD, and DLS studies were not capable of differentiating between an inactive and an active native-like protein fold. Furthermore, it may be that the conformational differences between an active and inactive protein are so subtle that spectroscopic methods are unable to differentiate between them, or improved methods need to be developed – such as site-specific labeling – in order to determine the subtle differences in protein conformation.

A_{2A}R was minimally active when solubilized in DDM or DDM/CHAPS micelles lacking CHS, and exhibited low affinity when solubilized in DHPC (Figure 2). This low affinity indicates that cholesterol is not needed for the protein to retain its α -helical content and ligand-binding activity, but may be crucial for high affinity binding.

Furthermore, it is important to note that the high affinity conformation was not recovered after purification of A_{2A}R in the absence of cholesterol and its subsequent solubilization in DDM/CHAPS/CHS, indicating that the detergent-protein interaction history is crucial, and alters the ligand binding properties of the receptor in an irreversible matter.

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Author Contributions

ASR, ANN, and JK designed research; ANN and PMM performed the research; ASR, ANN, PMM, and JK analyzed data; ASR, ANN, PMM, and JK wrote and edited the manuscript. ASR oversaw the research.

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FIGURE 1

FIGURE 2

FIGURE 3