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TITLE: Lactobionamide-based fluorinated detergent for functional and structural stabilization of membrane proteins.

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ABSTRACT

Membrane proteins (MPs) are important drug discovery targets for a wide range of diseases. Conventional detergents such as *n*-Dodecyl β -D-maltoside have been used largely and efficiently to solubilize MPs with varying degrees of success concerning MPs functionality and stability. Fluorinated surfactants (FSs) have shown a great potential for the stabilization of various MP. However, so far only a limited number of reports have demonstrated the ability of FSs to solubilize MPs from biological membranes. We report herein the use of a fluorinated lactobionamide-based detergent named FLAC6 for functional and structural stabilization of membrane proteins. We first demonstrated that FLAC6 efficiently solubilized three membrane proteins i.e. the native adenosine receptor A_{2A}R, a G protein-coupled receptor, and two native transporters AcrB and BmrA. The resulting affinity purified MPs were highly pure, homogenous and aggregates free. Furthermore, the functionality of each MP was well maintained. Finally, striking overstabilization features were observed. Indeed, the T_m of native A_{2A}R, AcrB and BmrA could be improved by 7, ~9 and ~23 °C, respectively when FLAC6 was used instead of the reference detergent. This work illustrates that FLAC6 is an efficient tool to maintain structural and functional integrities of different MPs belonging to different classes, providing a new avenue for functional stabilization of highly druggable and challenging membrane proteins involved in unmet medical needs.

INTRODUCTION

Membrane proteins (MPs) make up between 20 and 30% of the total eukaryotic proteome [1]. They are composed of integral membrane proteins which are embedded in the hydrophobic region of the lipid bilayer, as they span across the membrane once (single-pass), or multiple times (multi-pass), and extrinsic or peripheral membrane proteins that weakly associate with the hydrophilic surfaces of the lipid bilayer or intrinsic membrane proteins [2]. Membrane proteins are the gatekeepers of the cell and are therefore essential for its function. They are capable of regulating the flow of molecules and information across the plasma and internal membranes of organelles such as mitochondria, endoplasmic reticulum, golgi and nuclei [3]. They are involved in regulating a large panel of important cellular processes such as cell adhesion, energy transduction, signaling, cell recognition and transport. Membrane proteins (MPs) can therefore function as receptors (e.g G protein-coupled receptors or GPCRs), transporters, channels or anchoring proteins. Because of the importance of those functions, MPs are of high interest for fundamental research and pharmaceutical purposes since more than 60% of therapeutic targets are MPs [4]. Understanding the structure function relationship is key to developing effective drugs against unmet medical needs. In order to gain a true understanding of the molecular basis of membrane protein functions, they must be isolated by physically disrupting the membrane. This is often done using detergents or other non-polar solvents. Unfortunately, MPs are often unstable outside of their membrane environment. Several attempts have been made to improve MP stability by changing the native amino acid sequence through protein mutagenesis, truncation and engineering [5]. MPs have also been stabilized using classical antibodies or nanobodies [6]. These methods are very helpful for structural investigations of MPs, but they require significant efforts of engineering and/or generation of relevant antibodies. In addition, there is an important risk in altering the native conformation of the protein target. An emerging alternative consists of maintaining the native sequence of MPs and looking for suitable solubilization conditions that ensure the conservation of the functional and structural integrity [7, 8]. Solubilization reagents are available in a range of strengths from harsh anionic detergents such as SDS and Sarkosyl, to mild zwitterionic, Fos-Cholines (FC) and CHAPS detergents, to weaker non-ionic *n*-Dodecyl β -D-maltoside (DDM) and octyl glucoside (OG) detergent [9-13]. Detergent

common features consisting on a hydrophobic acyl tail and a hydrophilic head group allow them to remove the lipids surrounding the membrane protein. So far one of the most popular detergents used for MP structural and functional studies is n-Dodecyl β -D-maltoside (DDM). While it has generally good solubilization properties, the stability of MPs is often compromised and requires the use of additional stabilizing reagents such as cholesteryl hemisuccinate [14]. This has motivated a number of recent efforts to develop new polymers [15-17] and mild detergents with enhanced stabilization properties such as MNG (Maltose-Neopentyl Glycol), GNG (Glucose-Neopentyl Glycol) [18-20] and calixarene-based detergents [8, 21].

Fluorinated surfactants (FSs) are known to be less destabilising towards MPs because they hardly compete with protein–protein and protein–lipid/hydrophobic cofactor interactions and various reports have shown the superiority of FSs over hydrogenated detergents in stabilizing MPs [22, 23]. Indeed, the fluorinated chains of FSs exhibit the peculiar property of being both hydrophobic and lipophilic due to the weak van der Waals interactions between fluorocarbons and hydrocarbons, thereby preserving native interactions while avoiding delipidation. Moreover, fluorinated chains are bulkier than hydrogenated chains and thus protrude less easily between protein transmembrane helices. Other biochemical applications of FSs such as *in vitro* synthesis [24], inserting MPs into preformed membranes [25] or MPs refolding [26] have been successfully reported making FSs promising tools for MPs stabilization.

Recently, Keller and colleagues showed that the fluorinated analogue of DDM called F₆OM was able to partition into lipid membranes and to solubilise them almost as efficiently as its hydrogenated counterpart [26]. On the contrary a phosphocholine derivative called F₆OPC failed to show any detergency. More recently we could show that Diglucose fluorinated derivatives exhibited detergency in terms of both, lipid-vesicle solubilization as well as membrane protein extraction [27]. To explore that further, we report herein the evaluation of a lactobionamide-based fluorinated detergent named FLAC6 for the functional and structural stabilization of MPs. The evaluation was performed using three different full length and wild type membrane proteins, namely adenosine receptor A_{2A}R, AcrB and BmrA belonging to 3 classes of MPs (GPCR, pump and transporter) which were expressed in two cell types, insect cells (Sf9) and bacteria (*E.coli*). The theoretical molecular weights of A_{2A}R, AcrB and BmrA are ~45, ~113 and ~65 kDa, respectively (with a migration on SDS-PAGE at

42, 100 and 72 kDa; respectively). Their quaternary structure may correspond to monomers, trimers and dimers for A_{2A}R, AcrB and BmrA, respectively.

The solubilization, purification, thermostability, behavior in solution as well as functionality were all evaluated for the three MPs prepared using FLAC6 or a reference detergent, pointing out promising features of this detergent for MP fundamental studies and drug discovery.

MATERIAL & METHODS

Expression and Membrane preparation

Full-length and wild-type human A_{2A}R was cloned into a pOET1 transfer plasmid in frame with a N-terminal hemagglutinin signal sequence, Strep-tag II and 8xHis tag. The baculovirus was produced according to the manufacturer's protocol (flashback ULTRA™ system, *Oxford Expression Technologies*). Sf9 insect cells were infected with recombinant baculovirus and grown at 28 °C for 64 hours in an orbital shaker as described [28]. Cell pellets were collected, washed in Hepes buffer pH 7.4, 200 mM NaCl, 1X protease inhibitor cocktail (*Sigma*) and then stored at -80°C until use. BmrA was over-expressed in *E.coli* as previously described [7, 29]. AcrB was also produced in *E.coli* as previously reported [30, 31]. Frozen cell pellets were thawed, resuspended in Hepes buffer (A_{2A}R), PBS buffer (AcrB) or TBS buffer (BmrA) pH 7.4, 200 mM NaCl, 1X protease inhibitor cocktail. Cell lysis was performed on ice using a BeadBeater homogenizer with 0.1mm diameter glass beads. Membrane fractionation was then carried out at 4°C by sequential centrifugations. For both insect or *E.Coli* cells, two centrifugations were performed: 4000g for 15min and 100000g for 45min. Membrane pellets were washed twice in buffer containing high salt (1M NaCl) to remove membrane associated proteins only for A_{2A}R. Membrane enriched pellets were resuspended in Hepes, PBS or TBS buffer pH 7.4, 200 mM NaCl, 1X protease inhibitor cocktail and glycerol 20%, quantified using the *Pierce Micro BCA Protein Assay Kit (Thermo Scientific)* and then flash-frozen and stored at -80°C until use.

Membrane protein solubilization

Membrane were incubated for 2h at 4°C at a final total protein concentration of 5mg/ml in 50mM Hepes buffer pH 7.4, 200 mM NaCl ($A_{2A}R$), or 50 mM Tris pH8, 100 mM NaCl (BmrA) or 50mM Phosphate buffer pH8, 150mM NaCl (AcrB) 1X protease inhibitor cocktail, and with 10-fold the critical micelle concentration (CMC) of detergent FLAC6 (*CALIXAR*), DDM (*Carl Roth Int*) or SDS. The synthesis FLAC6 will be described in another manuscript. The extraction with SDS served as a “positive” control. After solubilization, samples were centrifuged at 100000g for 45min at 4°C and an aliquot of the pellet and the supernatant from each solubilization condition were analyzed by SDS-PAGE and/ or western-blot.

SDS-PAGE and Western-blot

$A_{2A}R$ samples were denatured with 5x Laemmli buffer and incubated at RT for 20 min prior to analysis without heating to avoid the formation of aggregates. Proteins were separated by SDS-PAGE on a 4-15% acrylamide gel (4–15% Mini-PROTEAN® TGX Stain-Free™ Gel, *Bio-Rad*) and subsequently immobilized by electro-transfer to a PVDF membrane. The immunodetection of $A_{2A}R$ with an anti- $A_{2A}R$ monoclonal antibody HRP (7F6-G5-A2, Santa cruz Biotechnology), and of BmrA with an anti-His HRP antibody (*Sigma*), were performed by using the SNAP i.d. system (*Millipore*). A SDS-PAGE stain free gel was used to analyze the fractions of AcrB.

Thermostability assay

Membranes of $A_{2A}R$, AcrB or BmrA (4mg/ml total protein) were solubilized under different conditions (see solubilization method above) for 2 hours at 4°C. Solubilized fractions were obtained after ultracentrifugation (100,000g) for 1h at 4°C. The solubilized fraction serves to make 50µl aliquots to be submitted to one temperature each as part of a gradient of temperature ranging from 25 to 85°C using PCR thermal cycler (*PeqSTAR 2x gradient, Peqlab*). Samples were subsequently centrifuged 40min

(at 20000g) and supernatants were analyzed by SDS-PAGE and western-blot using an anti- A_{2A}R antibody (7F6-G5-A2) and an anti-His antibody. The relative intensity of the target protein at each temperature was quantified by Western-blot using the Image Lab software 4.1 from *Bio-rad*. Each condition was performed in duplicate. The intensity was plotted as a function of the temperature, normalized and fit to the Boltzmann equation with the least square method using the Solver Add-in of the Excel software. This method was previously described [28, 32].

Clear Native-PAGE (CN-PAGE) and Western-blot

Non-denatured proteins were separated by native-PAGE on a 4-15% acrylamide gel (4–15% Mini-PROTEAN[®] TGX Stain-Free[™] Gel, *Bio-Rad*) using 25 mM imidazole as anode buffer and 7.5 mM imidazole, 0.05% deoxycholate, 0.01% DDM as cathode buffer. Clear Native PAGE gels were migrated for 90min at 200V and 4°C. Proteins were then immobilized by electro-transfer to a PVDF membrane. The immunodetection of A_{2A}R, AcrB or BmrA was performed using the SNAP i.d. system (*Millipore*) with an anti-A_{2A}R (7F6-G5-A2) and an anti-His antibodies (AcrB and BmrA).

His-tag affinity chromatography

The soluble protein fractions were loaded onto a TALON column equilibrated with 50mM Hepes buffer pH 7.4, 200 mM NaCl (A_{2A}R), 50 mM Tris pH8, 100 mM NaCl (BmrA) or 50mM Phosphate buffer pH8, 150mM NaCl (AcrB), 2CMC of DDM or FLAC6. After a 2h incubation at 4°C, the resin was washed with 12 Column Volumes (CV) of Wash Buffer containing Hepes, phosphate or Tris buffer, 2CMC of DDM or FLAC6 and 20mM Imidazole. Target proteins were eluted with 4 CV of washing buffer with 150mM Imidazole. Samples of each fraction T, S, FT, W and E (corresponding to Total, Solubilized, Flow through, Wash and Elution, respectively) were analyzed by SDS-PAGE and/ or western-blot.

Size exclusion chromatography

Affinity purified A_{2A}R, AcrB or BmrA were concentrated using Centriprep contractors with a 50K cut-off and loaded to a superdex 200 Increase 5/150 GL(GE-Healthcare) at 0.1 ml/min. The running buffer was 50mM Hepes buffer pH 7.4, 200mM NaCl (A_{2A}R), 50 mM Tris pH8, 100 mM NaCl (BmrA) or 50mM Phosphate buffer pH8, 150mM NaCl (AcrB), all in presence of 1CMC of DDM.

Functional assays

To assess the functionality of A_{2A}R, radioligand binding assays were performed at 4°C in triplicate using 96 well plates. Protein at a final concentration of 24 µg/ml (1µg - 50µL per well) was incubated at 4°C in the presence of 3.6µM ³H-CGS21680 (0.6µM final, 10µL per well) (*Perkin-Elmer*) +/- 1mM of cold ligand (0.17mM final) in binding buffer (50mM Tris-HCl pH 7.4, 10mM MgCl₂, 0.5mM EDTA) or CGS21680. After 2h of incubation, 60µL of 0,1% γ-globulin (prepared in wash buffer) and 120µl of 25% PEG6000 (prepared in wash buffer) were added per well, mixed and further incubated for 15mins at RT. Samples were then filtered using PEI-pre-coated GF/B plates (*Perkin-Elmer*). After 4 washes with ice-cold wash buffer (50mM Tris-HCl pH 7.4), 25µl of scintillation reagent was added per well, and the samples were incubated for 1 hour. CPM detection was performed using the Microbeta2 equipment (*Perkin Elmer*), applying 5 min counting per well.

To test the functionality of AcrB, fluorescent polarization binding assays using rhodamine G6 as previously described [33] were performed. Therefore, the polarization values of Rhodamine 6G (ranging from 4µM to 1mM) in the presence or absence of AcrB were measured using 530nm and 560nm as excitation and emission wavelengths, respectively. ATPase assays were used to assess BmrA activity as previously reported [21].

RESULTS & DISCUSSION

Solubilization using FLAC6

Figure 1 displays the chemical structure of FLAC6. This molecule consists of a lactobionamide polar head group attached through an amide bond to a C₉ alkyl tail, containing six perfluorinated terminal carbon atoms. Two structurally related lactobionamide-based fluorinated surfactants showed improved stabilizing properties for the fragile *b_{6f}* complex when compared to DDM [34]. However, their ability to both solubilize and stabilize MPs has not previously been reported. We conducted a comparative study of this newly developed FLAC6 versus DDM and have evaluated their ability to solubilize and stabilize of wild type and full-length membrane proteins. We chose three full length and unmutated targets for which DDM has already been used for solubilization and purification. GPCR, pump channel and transporter proteins, namely adenosine receptor A_{2A}R, AcrB and BmrA, respectively [29, 31, 35]. The three targets were expressed in two different expression systems *E.coli* and insect cells, which provide a sufficient diversity in terms of lipid composition of the biological membrane as well as the nature of MPs. For each MP, solubilization trials were carried out using FLAC6 and SDS being a “positive” control for solubilization. **Figure 2** shows that SDS allowed an almost total solubilization of all three membrane proteins A_{2A}R, AcrB and BmrA. This was expected given that this detergent is extremely harsh. Moreover, better solubilization was obtained with FLAC6 when compared to DDM (**Figure 2**, compare lanes corresponding to soluble and pellet fractions for each condition). This clearly demonstrates that the novel fluorinated detergent FLAC6 exhibits efficient and broad solubilization properties of membrane proteins belonging to different families and expressed in different expression systems (bacterial and insect cells).

Purification of membrane proteins using FLAC6

To evaluate the impact of this novel solubilizing agent on membrane protein purification, his-tag affinity purifications were performed for A_{2A}R, AcrB and BmrA solubilized in FLAC6. **Figure 3** shows that the majority of solubilized BmrA could bind to the Talon affinity column. All solubilized AcrB bound to the column and

eluted from the resin. The efficiency of binding of A_{2A}R was difficult to evaluate due to the non-specific signal of the antibody used for western blot. SDS-PAGE stain free gels showed the specificity of the purification (**Figure 3A, 3B** and **3C** for A_{2A}R, AcrB and BmrA, respectively). Side by side gel panels representing the three purified proteins displayed in **Figure 3D**, show a high degree of purity for all preparations (>95%), except of a degradation product observed for A_{2A}R, which is consistent with a previous report [28]. This clearly demonstrates that FLAC6 efficiently solubilizes the three proteins A_{2A}R, AcrB and BmrA and thereby allows an efficient purification.

Functionality of membrane proteins produced using FLAC6

The next step was to verify whether MP preparations were still functional. We therefore analyzed the function of each solubilized and purified membrane protein. For each of them, we compared FLAC6 to the most stabilizing conditions previously reported (DDM/CHS for A_{2A}R and DDM for BmrA and AcrB) [36] and named, reference conditions. **Figure 4A** depicts significant differences between radioligand binding of ³H-CGS-21680 to A_{2A}R produced in DDM/CHS (reference) and the FLAC6 condition. This difference may be due to the presence of inactive A_{2A}R or to a possible interference of FLAC6 with binding. To address this, we replaced FLAC6 by DDM/CHS after solubilization and purification during the final steps of the purification. Interestingly, ligand binding activity could be recovered suggesting that the protein produced in FLAC6 was functional and that FLAC6 may displace the binding of ³H-CGS-21680 to A_{2A}R. Similar type of interference was observed with a calixarene-based detergent and melatonin (data not shown). However, we cannot exclude that FLAC6 unfolds A_{2A}R and that we recover the activity in a reversible manner after buffer exchange.

The ATPase activity of BmrA prepared in FLAC6 was 2.5 higher when compared to the reference condition DDM (**Figure 4B**). Similarly, an increase of ligand binding activity (3-fold) was observed for AcrB in FLAC6 instead of DDM (K_d of 9.46μM vs 27.99μM, respectively) (**Figure 4C**). Thus, FLAC6 based solubilizations and purifications allow to maintain and improve ligand binding activities of AcrB and A_{2A}R as well as the ATPase activity of BmrA.

Behavior in solution of membrane proteins produced using FLAC6

To ensure the absence of aggregates in our membrane protein preparations, we evaluated their behavior in solution using Native PAGE Western blot analysis (**Figures 5A, 5C and 5E**). Whereas small traces of aggregates were observed in the gel loading wells, the majority of A_{2A}R, AcrB or BmrA were non-aggregated and could therefore migrate into the gel. To confirm these results, we applied analytical size exclusion chromatography (SEC) on affinity purified proteins solubilized and purified using FLAC6. Figures **5B, 5D** and **5F** show that most of the proteins displayed similar and typical non-aggregated protein profiles, with the proteins eluting after the void volume, which is consistent with the Native-PAGE result. Thus, Native PAGE and SEC confirmed that AcrB, BmrA and A_{2A}R were aggregates free when FLAC6 was used for the solubilization and the purification.

Thermostability of solubilized membrane proteins

The ability of FLAC6 to stabilize A_{2A}R, AcrB and BmrA was further assessed using a previously established western blot-based thermal shift assay [28]. This assay relies on the assumption that unstable heated proteins are prone to aggregate and therefore after ultracentrifugation and western blot the band intensity corresponding to the protein will decay proportionally to its instability. By measuring the amount of MPs that remains soluble at increasing temperatures the denaturing point (T_m), corresponding to 50% of soluble proteins, can be estimated. A_{2A}R solubilized using DDM displays a T_m of 36.4 °C (**Figure 6A** and **table 6D**), which is in good agreement with what has been previously reported using the same method [28, 37]. Replacing DDM by FLAC6 at the solubilization step resulted in a T_m of 43.5 °C. A similar thermostability improvement was also observed for AcrB which gained 8.6°C in presence of FLAC6 in comparison to DDM (**Figure 6B** and **6D**). The same tendency but significantly amplified was noticed for BmrA which was over-stabilized to a T_m of 58.6°C when FLAC6 was used instead of 36°C for the DDM condition (~23 °C improvement). This suggests that this novel fluorinated detergent maintains the structural and functional integrities of MPs by increasing their thermostability.

Recently, we reported the development of a series of glucose-based surfactants with a fluorinated chain [27]. These surfactants had the ability to solubilize lipid vesicles and extract a broad range of proteins from *E.Coli*. However, none of the three proteins studied in this work could be efficiently extracted (Data not shown). Interestingly, we demonstrated herein that FLAC6 was efficient on solubilization, functional stabilization and thermo-stabilization. The superiority of FLAC6 may arise from two parameters. On the one hand, the sugar-based lactobionamide polar head group brings good water solubility of the detergent which is packed in a rather compact volume due to the linear arrangement of the two sugar moieties compared to the bulky branched arrangement of the Diglu series [27, 38]. Therefore, the lactobionamide polar head group can be considered as a close analog to the maltoside polar head group with a slightly more aggressive character as previously demonstrated with the hydrogenated analog H₁₂-Lac [34]. On the other hand, the 9-carbon alkyl chain adds sufficient hydrophobicity and mild detergency due to the 6 perfluorinated carbon atoms. Other Lactobionamide-based fluorinated detergents were also studied in the stabilization of the fragile cytochrome *b₆f* complex and showed a stabilization of the dimeric form while DDM resulted in the dissociation of inactive monomers [34]. This work highlights the advantage of FLAC6 in both solubilizing and stabilizing functional and non-aggregated MPs similar to what was observed with stabilizing lipids or ligands. This novel fluorinated lactobionamide-based detergent is therefore of high interest for enabling structure-function studies and drug discovery of challenging unstable MP targets of high medical relevance.

CONCLUSION

Here we report the use of a fluorinated detergent FLAC6 which consists of a lactobionamide polar head group attached through an amide bond to a C₉ alkyl tail, with six perfluorinated terminal carbon atoms. This detergent shows a great potential for remarkable membrane protein solubilization and stabilization. We first demonstrate that FLAC6 was efficient in solubilizing three different membrane proteins of very different structure and function (GPCR, pump channel and transporter namely A_{2A}R, AcrB and BmrA, respectively). Then, solubilized MPs could successfully be affinity purified to high purity, resulting in pure, homogenous, aggregate-free and functional preparations. Finally, striking overstabilization features were observed with improvements of the T_m of native A_{2A}R, AcrB and BmrA by 7, ~9 and ~23 °C, respectively when FLAC6 was used instead of the reference detergent. This work demonstrates that FLAC6 is an efficient tool to maintain structural and functional integrities of different classes of MPs which represents the main prerequisite for their functional and structural investigations. Taken together, these findings support the relevance of this new fluorinated detergent as promising solubilizing and stabilizing agent, representing therefore an efficient and valuable addition to the growing membrane protein toolbox.

FIGURES LEGEND

Figure 1- Chemical structures of *n*-dodecyl β -D-maltoside (DDM) and of the new fluorinated detergent FLAC6.

Figure 2- Membrane proteins Solubilization using FLAC6.

Membranes were incubated for 2 hours in the presence of FLAC6, DDM or SDS at 4°C before being subjected to ultracentrifugation. The soluble fraction was analyzed by Western-Blot (**A** and **C**) or by stain free gel (**B**) to evaluate the solubilization efficiency. The work was performed on (**A**) native human adenosine receptor ($A_{2A}R$), (**B**) bacterial multidrug efflux pump AcrB and (**C**) the multidrug resistance ABC transporter BmrA. Each protein of interest is indicated by an arrow. SDS and DDM were used as “positive” controls and as “reference” for solubilization, respectively. S and P correspond to the soluble and the pellet fractions, respectively. * indicates potential degradation products.

Figure 3- Membrane protein purification using FLAC6.

SDS-PAGE analysis of a His-tag affinity purification using FLAC6 as a detergent starting from biological membranes containing (**A**) $A_{2A}R$, (**B**) AcrB or BmrA (**C**). Signal on stain free gel for each protein target is indicated by an arrow. T, P, S, FT, W and E correspond to Total, Pellet, Soluble, Flow through, Wash and Elution fractions, respectively. An Asterix indicates a possible degradation product. Side by side SDS-PAGE stain free gels illustrate the degree of purity for $A_{2A}R$, AcrB and BmrA purifications (**D**).

Figure 4- Evaluation of the functionality of AcrB, BmrA and $A_{2A}R$ after solubilization and purification using FLAC6.

$A_{2A}R$ was either solubilized and purified using DDM/CHS (labelled “DDM/CHS”), FLAC6 (labelled “FLAC6”) or solubilized with FLAC6 and then further purified with DDM/CHS (labelled “FLAC6+DDM/CHS”). Purified $A_{2A}R$ was assessed for its functionality in a radioligand binding assay using 3H -CGS-21680 (**A**). Total (protein

sample), non-specific (detergent buffer) and specific signals (total – non-specific) are indicated. BmrA purified in DDM or in FLAC6 were functionally evaluated by an ATPase activity assay (B). Ligand binding assay using Rhodamine G fluorescence polarization for the evaluation of the activity of FLAC6 or DDM based solubilization & purification of AcrB (C).

Figure 5- Behavior in solution of purified membrane proteins.

Native PAGE and western blot analysis were performed to assess the behavior of A_{2A}R (A), AcrB (C) and BmrA (E) in solution. Analytical size exclusion chromatography was used to confirm the non-aggregated state of purified A_{2A}R (B), AcrB (D) and BmrA (F). The column void volume V₀ is indicated by an arrow on each chromatogram. The elution volume of each of the four protein standards is indicated according to the manufacturer in the absence of any detergent (ferritin, aldolase, ovalbumine and ribonuclease A, corresponding to, 440, 158, 43 and 13,7 kDa, respectively). * indicates possible protein aggregates.

Figure 6- Thermostability of A_{2A}R, AcrB and BmrA.

The thermostability was measured by the percent of soluble membrane proteins present after applying a heating range from 25 to 75°C, as previously reported [28, 37]. A- Thermostability curves of A_{2A}R solubilized with DDM or FLAC6. B- Thermostability curves of AcrB solubilized with DDM or FLAC6. C- Thermostability curves of BmrA solubilized with DDM or FLAC6 The T_m was calculated as the temperature at which 50% of the protein remained soluble. Dose (temperature) vs normalised response curve fitted for all graphs. Table D summarizes the mean and the variation between FLAC6, and reference conditions ~~duplicates~~ for each membrane protein (A_{2A}R, AcrB and BmrA).

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AUTHORS CONTRIBUTION

CF, SI & RB performed the expression, solubilization, purification, thermal-shift and functional assays. GD designed FLAC6 and DC synthesized and characterized it. DC and GD designed and synthesized the FLAC6 compound. AJ designed the experiments and managed the project. AJ wrote the paper with the help of SI and GD.

CONFLICT OF INTEREST

The detergent reported in this study, FLAC6 is commercially available at calixar.com

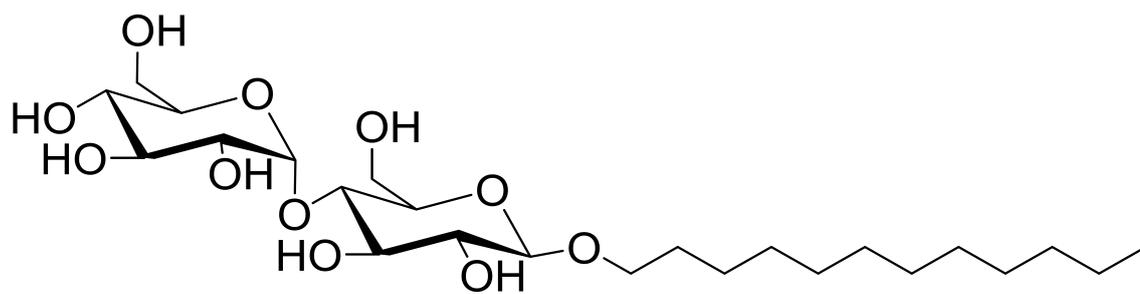
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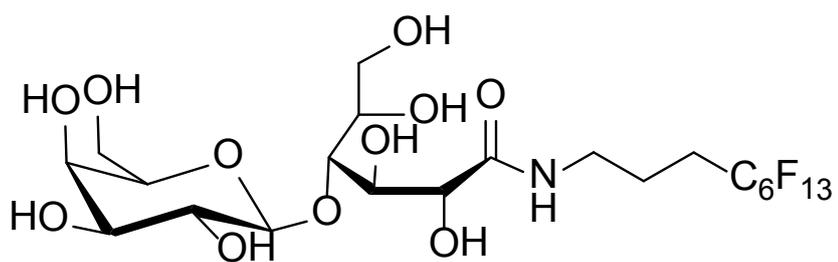
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Figure 1



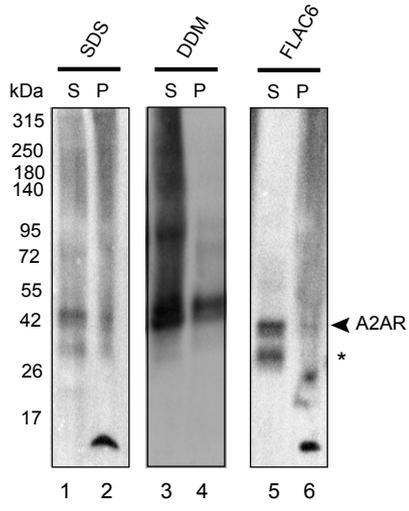
n-Dodecyl β-D-maltoside



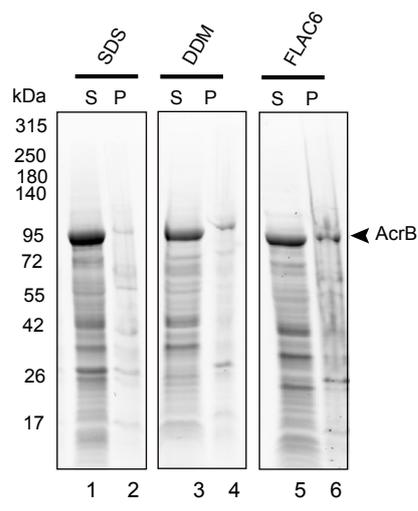
FLAC6

Figure 2

A



B



C

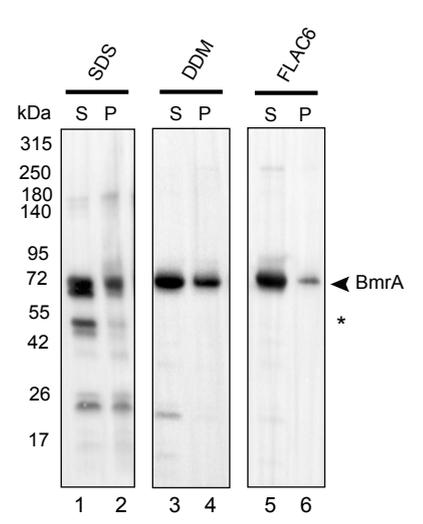
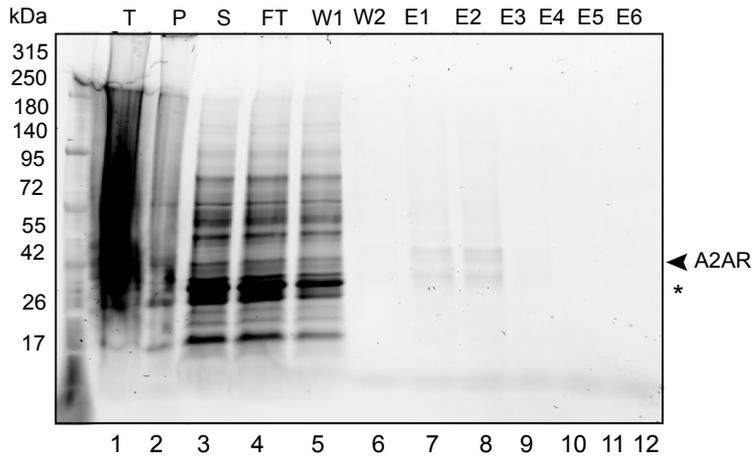
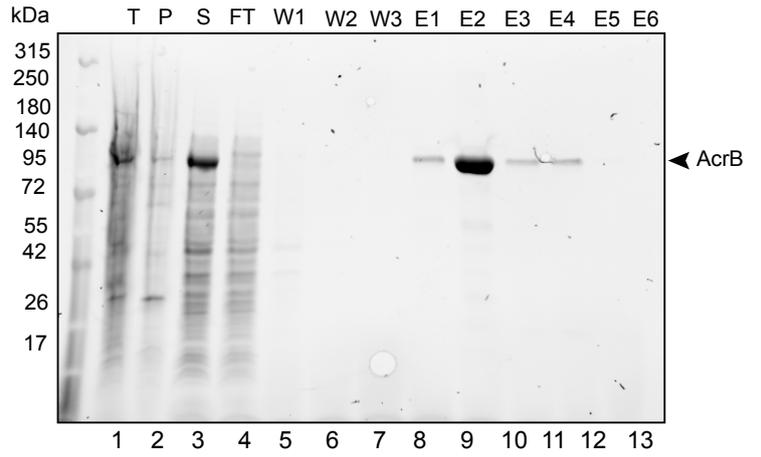


Figure 3

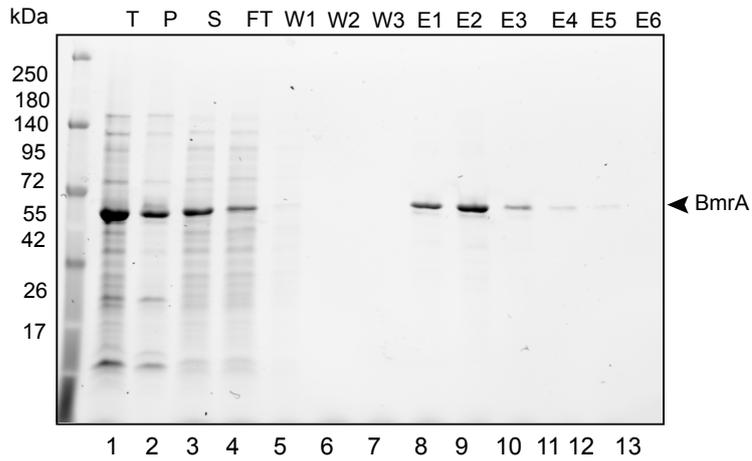
A



B



C



D

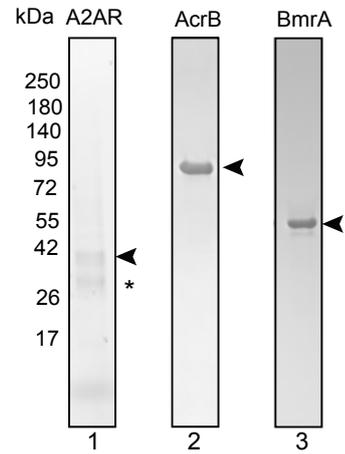
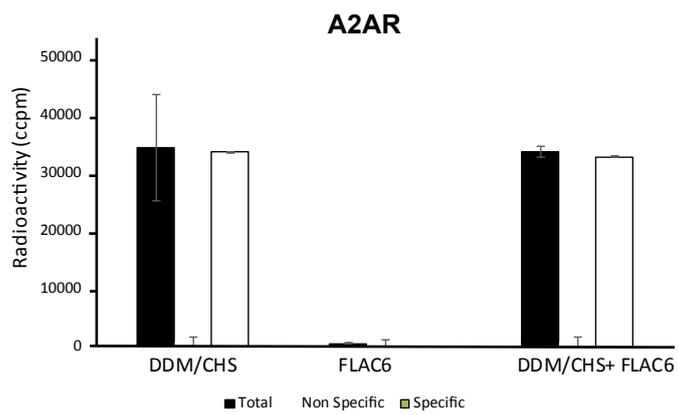
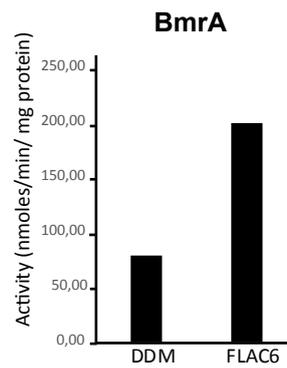


Figure 4

A



B



C

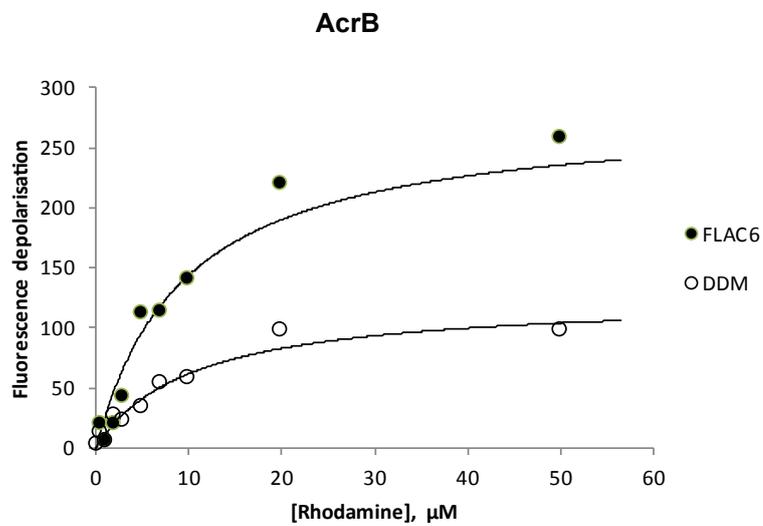


Figure 5

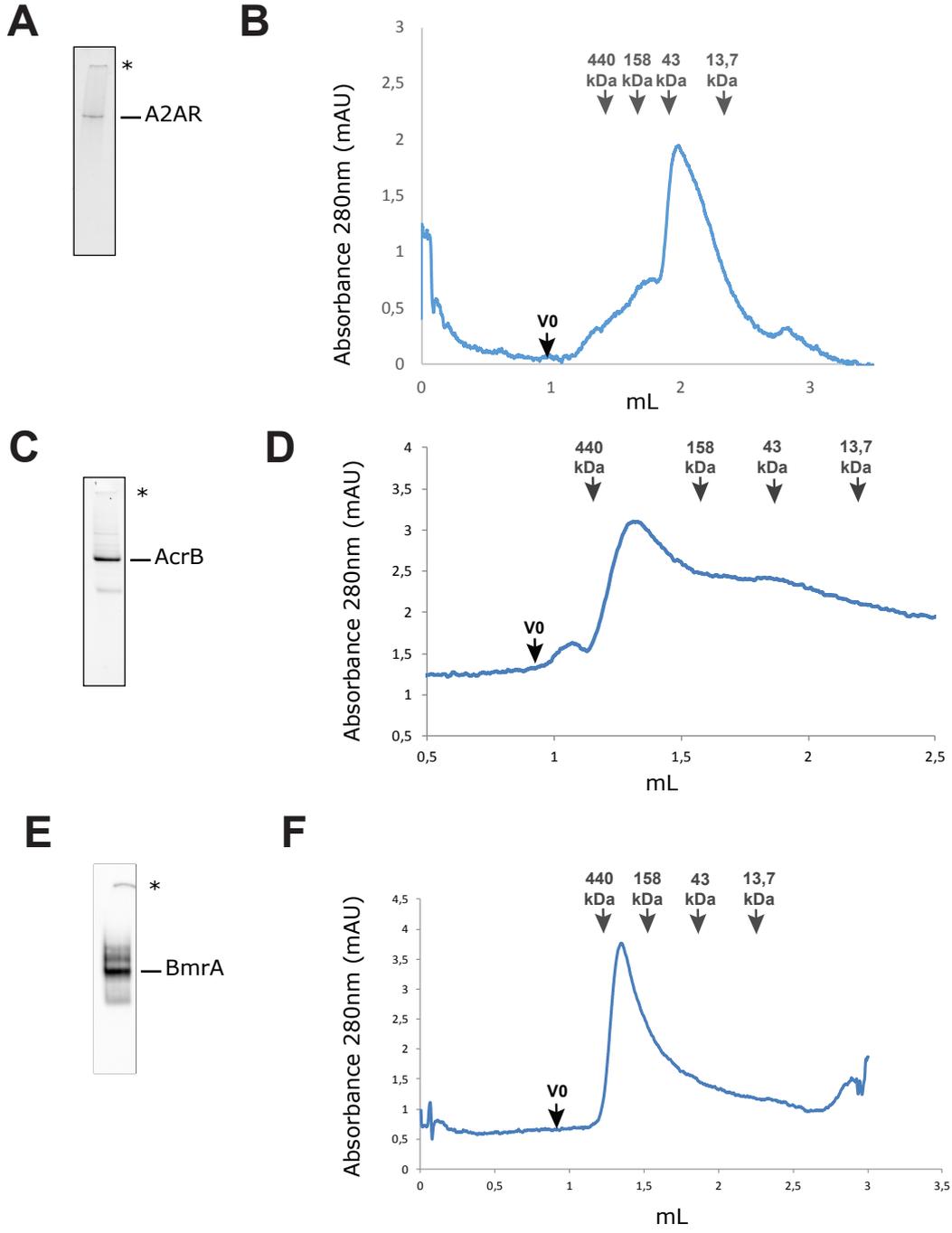
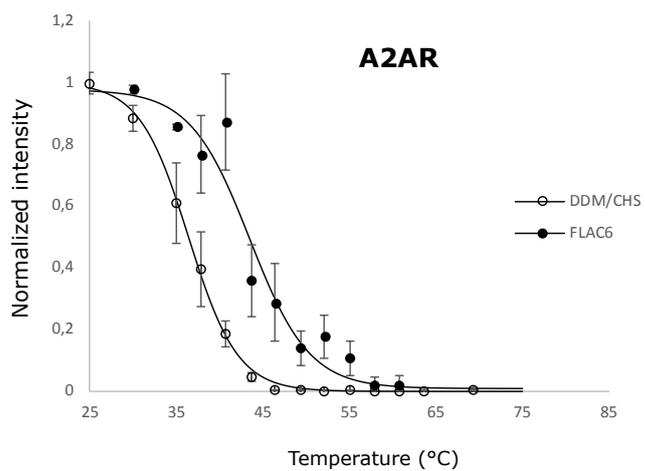
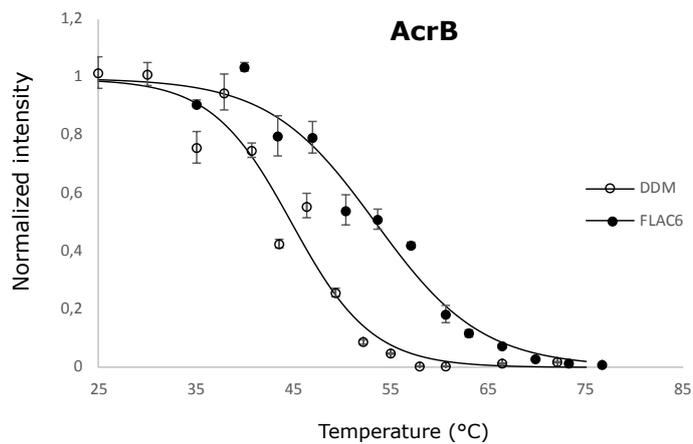


Figure 6

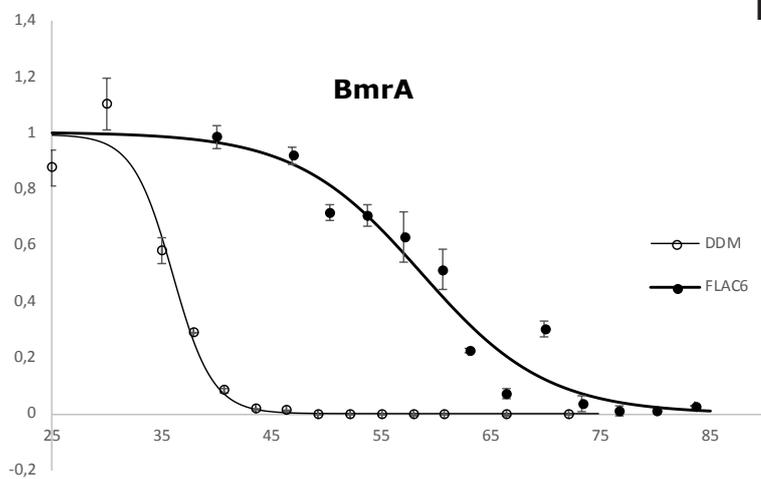
A



B



C



D

	Membrane proteins					
	A2AR		AcrB		BmrA	
	n=2		n=2		n=2	
	Tm	ΔTm	Tm	ΔTm	Tm	ΔTm
ref detergent	36,4	-	44,8	-	36	-
FLAC6 10 CMC	43,5	7,1	53,4	8,6	58,6	22,6