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GPCR stabilization using the bicelle-like architecture of mixed sterol-detergent micelles†

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Abstract

The biophysical characterization of purified membrane proteins typically requires detergent mediated extraction from native lipid membrane environments. In the case of human G protein-coupled receptors (GPCRs), this process has been complicated by their conformational heterogeneity and the general lack of understanding the composition and interactions within the diverse human cellular membrane environment. Several successful GPCR structure determination efforts have shown that the addition of cholesterol analogs is often critical for maintaining protein stability. We have identified sterols that substantially increase the stability of the NOP receptor (ORL-1), a member of the opioid GPCR family, in a mixed micelle environment. Using dynamic light scattering and small-angle X-ray scattering, we have determined that the most thermal stabilizing sterol, cholesteryl hemisuccinate, induces the formation of a bicelle-like micelle architecture when mixed with dodecyl maltoside detergent. Together with mutagenesis studies and recent GPCR structures, our results provide indications that stabilization is attained through a combination of specific sterol binding to GPCRs and modulation of micelle morphology.

Keywords

thermostability; cholesteryl hemisuccinate (CHS); cholesterol; opioid; NOP receptor (ORL-1); GPCR; SAXS

1. Introduction

Recent attempts at characterizing the Human Membrane Proteome identified approximately 6000 human membrane proteins constituting approximately 30% of the human genome [1, 2]. The characterization of membrane proteins typically requires their extraction from the lipid membrane bilayer, an environment that bestows considerable stabilization on these embedded proteins. Protein extraction from the membrane and its subsequent purification therefore represents a major obstacle that requires the use of detergents/surfactants, which assemble into globular micelles that adopt different geometrical shapes in solution [3, 4], and shield the hydrophobic core of membrane proteins from exposure to aqueous medium through formation of a protein-detergent complex (PDC) [5]. Finding suitable detergent(s) that will stabilize the protein's structure and maintain its activity is therefore pivotal.

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G protein-coupled receptors (GPCRs) represent the largest class of human membrane proteins involved in signal transduction (80%) [6], and remain a largely untapped reservoir of pharmaceutical targets as ~40% of all marketed drugs target only a small fraction of the 800+ members in this protein family [7, 8]. GPCRs share a common fold consisting of a seven transmembrane (TM) alpha helical bundle flanked by a N-terminus or a globular extracellular domain that plays a role in ligand recognition and/or binding in some receptors, and an amphipathic helix (helix VIII) followed by an intracellular domain at the C-terminus [9]. Common among the rhodopsin family of GPCRs is a palmitoylation site immediately following helix VIII, which anchors this region to the membrane bilayer and may target these proteins for partitioning into cholesterol rich rafts and caveolae [10, 11]. Analysis of the crystal structures of the β_1 and β_2 adrenergic receptors (β_1 AR and β_2 AR) has led to the identification and characterization of a specific cholesterol binding site at a cleft created by helices II, III, and IV [12–16]. Many residues within this specific binding site have been found to be conserved among the GPCR families, and thus this region has been termed the cholesterol consensus motif (CCM) [13].

The nonionic alkyl glucoside detergents, specifically n-Dodecyl- β -D-Maltoside (DDM), are the most widely used detergents for the isolation and purification of GPCRs. Above the critical micelle concentration (CMC), DDM micelles form oblate ellipsoids where the polar axis is shorter than the equatorial axes [3, 4, 17]. DDM mixtures with various additive detergents containing various acyl chain lengths or different head groups can be used to adjust the micelle's physiochemical properties and dimensions for optimal membrane protein stability. Additionally, the use of detergent soluble cholesterol analogs is becoming increasingly popular, with numerous reports showing that these compounds may help protect GPCRs from thermal inactivation [13, 18–20]. Several successful GPCR structure determination efforts have shown that the addition of cholesterol analogs is often critical for maintaining receptor stability [13–15, 21–25]. However, it is unclear if the protective function of these sterol analogs is achieved through modulation of detergent micelle morphology and physiochemical properties, or through a specific sterol-protein interaction.

This study is focused on understanding the effect of cholesterol analogs on DDM/GPCR PDCs. In a case study, we show that the type of cholesterol analog supplemented to the PDC substantially influenced the thermostability of ORL-1 (Opioid Receptor Like -1; also known as NOP (nociceptin opioid peptide) receptor), a member of the opioid receptor (OR) GPCR family. All four opioid receptors have been shown to partition into lipid rafts, and methyl- β -cyclodextrin (M β CD) mediated depletion of cholesterol has been shown to modulate their downstream signaling [26–33]. Importantly, the ORs contain a tryptophan residue, Trp^{4.50} (superscript corresponds to the Ballesteros-Weinstein numbering [34]), located at the heart of a putative CCM, which is critical for π -stacking interactions with cholesterol's sterol core [13]. The importance of ORL-1's Trp^{4.50} residue has been underscored through mutagenesis studies. In an effort to understand the differences in effects attributed to the different cholesterol analogs, biophysical studies of various sterol/DDM micelle mixtures (free of protein) were conducted to measure differences in the mixed micelle morphologies. Using dynamic light scattering and small-angle X-ray scattering, we have determined that the most thermal stabilizing sterol, cholesteryl hemisuccinate, induces the formation of a bicelle-like architecture when mixed with DDM detergent.

2. Experimental Procedures (Materials and Methods)

2.1 ORL-1 Cloning, Expression and Purification

The wild type (WT) human ORL-1 gene (UniProt accession code P41146) was synthesized by DNA2.0 and then cloned into a modified pFastBac1 vector (Invitrogen) containing an expression cassette with an HA signal sequence followed by a FLAG tag at the N-terminus,

and a PreScission protease site followed by a 10xHis tag at the C-terminus. Thirty amino acids were deleted from the C-terminus, which helped to increase receptor yield (data not shown). Recombinant baculoviruses were generated by using the Bac-to-Bac system (Invitrogen) and were used to infect *Sf9* insect cells at a density of 2×10^6 cells/mL at a multiplicity of infection of 5. Infected cells were grown at 27 °C for 48 h prior to being harvested, and the cell pellets were stored at -80 °C.

Insect cell membranes were disrupted by dounce homogenization of cell pellets in a hypotonic buffer containing 25 mM HEPES [pH 7.5], 10 mM MgCl₂, 20 mM KCl, and protease inhibitor cocktail (Roche). Extensive washing of the raw membranes was performed consecutively by repeated dounce homogenization and centrifugation in the same hypotonic buffer (~two times), followed by high osmotic buffer containing 1.0 M NaCl, 25 mM HEPES [pH 7.5], 10 mM MgCl₂, and 20 mM KCl (~three-four times). Purified membranes were resuspended in 25 mM HEPES [pH 7.5], 20 mM KCl, and 30% (v/v) glycerol, flash-frozen with liquid nitrogen and stored at -80 °C until further use.

Purified membranes were thawed and incubated with 5 μM Compound-24 (C-24; 1-benzyl-N-{3-[spiroisobenzofuran-1(3H),40-piperidin-1-yl]propyl} pyrrolidine-2-carboxamide [35] was provided as a gift from Girolamo Calo, Remo Guerrini and Stephano Molinari at the University of Ferrara, Ferrara, Italy), 50 mM HEPES [pH 7.5], 500 mM NaCl, 20 mM KCl, and 5% glycerol (v/v), and incubated at 4 °C for 1 hour. Iodoacetamide (Sigma) was then added to the membranes at a final concentration of 1 mg/mL for another 15 minutes before solubilization with 10 mM DDM (~0.5% (w/v) n-dodecyl-β-D-maltopyranoside; Anatrace), and 2 mM CHS (~0.1% (w/v) cholesteryl hemisuccinate; Anatrace or Sigma) for three hours at 4 °C. The supernatant was isolated by centrifugation at $160,000 \times g$ for 45 minutes, supplemented with 25 mM imidazole [pH 7.5], and incubated with TALON IMAC resin (Clontech) overnight at 4 °C. Typically, 0.75 mL of resin (slurry) per 1 L of original culture volume was used. After binding, the resin was divided and washed with 20 column volumes of wash buffer 1 (WB1: 50 mM HEPES [pH 7.5], 500 mM NaCl, 20 mM KCl, 10 mM MgCl₂, 1 mM ATP, 10% glycerol (v/v), 25 mM imidazole, 5 μM C-24, 1 mM DDM supplemented with various sterols at the indicated concentration), and 25 column volumes of WB2 (same as WB1, but without ATP and MgCl₂), before protein elution with 4 column volumes of elution buffer (EB: 50 mM HEPES [pH 7.5], 500 mM NaCl, 20 mM KCl, 10% glycerol (v/v), 250 mM imidazole, 5 μM C-24, 1 mM DDM supplemented with various sterols at the indicated concentrations). CHS (cholesteryl hemisuccinate) was purchased from Sigma or Anatrace; Façade (3α-hydroxy-7α,12α-di-((O-β-D-maltosyl)-2-hydroxyethoxy)-cholane) from Avanti; and CHAPS (3-[(3-Cholamidopropyl)-Dimethylammonio]-1-Propane Sulfonate/N,N-Dimethyl-3-Sulfo-N-[3-[[3α,5β,7α,12α]-3,7,12-Trihydroxy-24-Oxocholan-24-yl]Amino]propyl]-1-Propanaminium Hydroxide Inner Salt), CHAPSO (3-[(3-Cholamidopropyl)dimethylammonio]-2-Hydroxy-1-Propanesulfonate), Sodium Cholate (3α,7α,12α-Trihydroxy-5β-Cholan-24-oic Acid Monosodium Salt), Sodium deoxycholate (3α,12α-Dihydroxy-5β-Cholan-24-ic Acid, Monosodium Salt), BigCHAP (N,N'-bis-(3-D-Gluconamidopropyl)Cholamide), BigCHAP deoxy (N,N'-bis-(3-D-Gluconamidopropyl) Deoxycholamide, BigCHAP deoxy), and CHOBIMALT (β-cholesteryl-(maltosyl-β-(1,6)-maltoside)) were purchased from Anatrace. Exchange of the different DDM/sterol mixtures was verified through the correlation of retention time and the measured micelle sizes described below.

2.2 Thermostability Data Collection

Thermal stability data was collected using a modified procedure incorporating a thiol-reactive N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM) fluorophore, which undergoes an increase in the fluorescence emission following interaction with Cysteine residues [36]. Based on amino acid threading onto the β₂AR structure (PDB

code 2RH1), ORL-1 contains seven buried Cys residues (out of thirteen found on this construct) in the TM core that become exposed and available for reaction with the CPM dye upon thermal unfolding/denaturation. Reactions contained 5 μ M CPM and ~5 μ g purified ORL-1 in a buffer (EB, but without imidazole) containing 50 mM HEPES [pH 7.5], 500 mM NaCl, 20 mM KCl, 10% glycerol (v/v), 5 μ M C-24, 1 mM DDM and sterols at the indicated concentrations. Fluorescence of the CPM dye was measured on a Cary Eclipse Fluorescence Spectrophotometer using 387 nm excitation filter with a 2.5 nm bandpass and a 463 nm emission filter with a 10 nm bandpass. Measurements were made in 1 $^{\circ}$ C intervals from 20 – 90 $^{\circ}$ C with a ramp rate of 2 $^{\circ}$ C/min, and the background fluorescence of buffer in the absence of protein was subtracted. Midpoints of the thermal transitions (T_m) were obtained using a least squares non-linear regression analysis (GraphPad Prism) of fluorescence signal versus T plots to the following equation [37]:

$$y = \frac{([y_f + m_f T] + [y_u + m_u T] \exp[(H^* \{(T - T_m)/T_m\})/(R^* T)])}{(1 + \exp[(H^* \{(T - T_m)/T_m\})/(R^* T)])}$$

where y is the observed fluorescence signal, y_f and y_u are the intercepts, m_f and m_u are the slopes of the pre- and post-transition baselines, T_m is the midpoint of the transition curve, T is the temperature in Kelvin, and R is the gas constant, and H is the enthalpy change for unfolding at T_m .

2.3 Light Scattering Measurements

Dynamic light scattering (DLS) of the detergent samples dissolved in 150 mM NaCl, 50mM TRIS [pH 8.0] with a DDM concentration of 5 mM and supplemented with various sterols was measured in a DynaPro (Wyatt, Santa Barbara, CA) instrument to obtain the apparent hydrodynamic radius of micelles, R_h . Experiments were performed in quartz cuvettes at 20 $^{\circ}$ C with 5 second acquisition times, and 50 acquisitions were collected to complete one measurement. The DLS data were analyzed using Dynamics 6.0 software using an assumed Rayleigh sphere model for the analysis.

2.4 SAXS Data Collection

Small angle X-ray scattering (SAXS) data were collected at the Bio-SAXS beamline BL4-2 of the Stanford Synchrotron Radiation Lightsource (SSRL, Stanford, CA). The beam size was slit-collimated to 0.2×0.2 mm, and the X-ray energy was calibrated to 11 KeV, which corresponds to a wavelength of $\lambda = 1.1271$ Å. A Rayonix Mx225-He CCD detector was used at a sample to detector distance of 2 m, and the sample temperature was maintained at 20 $^{\circ}$ C. Testing of various CHS/DDM mixed detergent concentrations (5 mM to 200 mM DDM) revealed no change in micelle size (via Guinier analysis); therefore, to minimize inter-micelle scattering effects, the DDM concentration was a constant 5 mM (and supplemented with various sterols) for all reported data. All detergent samples were dissolved in 150 mM NaCl, 50 mM TRIS [pH 8.0], and were injected into a capillary for collection using automated liquid handling equipment. Twenty frames per sample with 1 second exposure times were collected and integrated radially, and the data from each sample were averaged, and subtracted from the background buffer in the absence of detergent. Guinier analysis was performed using Primus software (EMBL) [38], and MatLab software (MathWorks) was used for analysis of the two shell ellipsoid fitting by the methods described by Lipfert et al [4].

3. RESULTS

3.1 Cholesterol derivatives and ORL-1 thermostability

ORL-1 was expressed in *Sf9* insect cells, and a thorough search for optimal detergents identified DDM as the best detergent for extraction and purification of this receptor (data not shown). To date, little is known about the thermostabilizing effect of sterol addition to OR PDCs. Because cholesterol is insoluble in the absence of lipids (unpublished observations), we tested the effect of cholesterol analogs mixed with DDM on their ability to promote the thermal stabilization of ORL-1 using a thiol-reactive fluorescent probe (CPM assay) [36]. The tested cholesterol analogs are soluble in detergent solutions by virtue of polar prosthetic groups attached to different portions of the tetracyclic sterol core (Figure 1). The sterol additives were dissolved in DDM at a molar ratio of 0.2:1 (sterol:DDM), and experimental determination of the CMC using a modified 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence based assay [39] indicated that the sterol/DDM mixtures had a similar CMC to DDM alone (data not shown). Each sterol/DDM mixture was then exchanged at $\sim 5 \times$ the CMC on IMAC resin loaded with ORL-1, followed by measurement of the thermal stability of the different ORL-1/C-24/sterol/DDM complexes using the CPM assay. Thermostability of ORL-1 in the presence of most sterol/DDM mixtures was similar to DDM alone (Figure 2). However, in the presence of CHOBIMALT and CHS, the thermostability of ORL-1 was significantly improved by 5 and 15 °C, respectively (Figure 2).

To better understand the influence of CHS on the thermostability of ORL-1, we measured its T_m at various CHS:DDM ratios (Figure 2). In the presence of CHS, all of the transitions were shifted to higher temperatures compared to ORL-1 PDCs containing only DDM. A maximal increase in thermal stability was observed at a CHS:DDM ratio of ~ 0.1 ; further increasing the CHS:DDM ratio resulted in a negligible increase in the transition temperature.

To determine if the CHS-dependent increase in thermostability is the result of a specific interaction with a cholesterol binding site on the receptor, we mutated Trp175^{4.50}, located at the heart of the CCM, to an alanine residue. Fluorophore labeled antibody (α FLAG tag) based flow cytometry on *Sf9* cells expressing either ORL-1 W175^{4.50}A or WT-CCM ORL-1 suggested that expression of the receptor was not significantly affected by the mutation [40]. However, extracting the W175A protein from the membranes yielded low quality receptor as observed by analytical size exclusion chromatography, and the lack of a prominent thermal transition in the CPM assay suggested that many of the buried cysteines were exposed prior to heating the sample (data not shown).

3.2 Hydrodynamic radii of Sterol/DDM mixed micelles

To test whether the addition of cholesterol analogs to DDM would impact the physical characteristics (e.g. size) of the detergent micelle, we used Dynamic Light Scattering (DLS) to determine the hydrodynamic radius (R_h) of the different sterol/DDM mixtures. For DDM detergent micelles at 5 mM concentration the measured $R_h = 35$ Å, which is in agreement with the published radius of gyration value of 35 ± 1 Å [4]. A typical globular protein displaying a $R_h = 35$ Å would correspond to an apparent MW of 63 kDa (where $MW_{\text{apparent}} \text{ (kDa)} = [0.168 \cdot R_h]^{2.3398}$; DynaPro manual), a value which is close to literature values for the mass of DDM micelles (70–72 kDa) [41, 42]. With the exception of CHS and CHOBIMALT, all sterol/DDM mixtures resulted in a decrease of the R_h values by 2–4 Å compared to DDM alone (Table 1). While the addition of CHOBIMALT to DDM had no effect on the R_h value, titration of CHS to DDM at increasing ratios resulted in an increase of the R_h ; with CHS:DDM ratios of 0.1 and 0.2, the R_h increases by 3 and 6 Å, respectively, when compared to DDM micelles.

3.3 Micelle radius of gyration measured by SAXS

To further analyze the impact of sterols on DDM micelle size, we used SAXS to obtain the radii of gyration (R_g) of the different micelles using Guinier analysis (Figure 3). The calculated R_g for DDM in 150 mM NaCl was 33.2 ± 0.2 Å (Table 1), similar to previously published results [4]. The measured R_g for DDM determined by SAXS was about ~ 2 Å less than the R_h determined by DLS, and the same trend was also observed for most of the mixed micelles (Table 1). With the exception of CHS, the R_g measurements indicated that sterol mixed micelles are smaller than DDM alone. In contrast, the addition of CHS to DDM resulted in larger micelle dimensions confirming the results of the DLS experiments. To better understand the effect that CHS has on DDM micelles, we fit the full SAXS scattering profile to a two shell ellipsoid form factor model [4].

3.4 Ellipsoid Fit of SAXS data

The I versus q ($\text{\AA}^{-1}$) SAXS profiles of DDM and the mixed sterol/DDM micelles indicated the presence of a two-component ellipsoid model with the DDM maltoside head group likely constituting the outer shell and the hydrophobic alkyl detergent tail making up the inner shell (Figure 4) [4]. Previous studies showed that DDM micelles form oblate ellipsoids where the polar axis of rotation is smaller than the two equatorial axes [3, 4, 17]. We therefore fit the full SAXS profile to a two-shell ellipsoid model in order to determine the vector lengths of the two ellipsoid shells where a single minor a_1 and two major b_1 axes describe the inner shell, and a_2 and b_2 (parallel with the a_1 and b_1 vectors, respectively) represent the outer shell thickness (Figure 3). The fit of DDM micelle SAXS data revealed an a_1 and b_1 of 15.9 and 29.2 Å, respectively, and an a_2 and b_2 both equal to 5.0 Å. These results are in agreement with previously published parameters of DDM micelles [3, 4, 17]. Consistent with the R_g values, the hydrophobic core dimensions (a_1 and b_1) of micelles containing sterols with polar tails are smaller in magnitude, particularly along the b_1 vector (Table 1). The outer shell dimensions (a_2 and b_2), likely composed of maltoside head groups and polar moieties from the cholesterol analogs, show differences that can be attributed to the sterol added. Addition of sterols with relatively large polar groups, such as BigCHAP, BigCHAPdeoxy, and Façade, to DDM revealed a modest increase in the a_2 and b_2 vector (note that CHOBIMALT was not analyzed by SAXS). The parameters obtained for the mixed CHS/DDM micelle were the most interesting. The addition of CHS to DDM induced a significant increase in the b_1 parameter in a CHS concentration dependent manner (increase of 11 Å at a CHS:DDM ratio = 0.2), while a_1 displayed only a modest increase in length by ~ 1 Å (at a ratio of 0.1 and 0.2, the CHS:DDM $a_1 = 16.5$ Å). On the other hand, the outer shell b_2 dimension did not change significantly in contrast with a_2 which displayed a relatively large increase in thickness by ~ 3.5 Å. A model rationalizing these features and possible effects on the protein stability are discussed in the following section.

4. DISCUSSION

Having evolved under pressure of natural selection, cholesterol is the primary sterol found in higher eukaryotes [43]. In the case of plants and fungi, cholesterol is replaced by phytosterols and ergosterol, respectively. The unique rigid planar structure of sterols imparts special properties on membranes by modulating its fluidity [44], thickness [45], curvature [46], and permeability [47]. Many of these “effects” are due to the increase in ordering of lipid acyl tails when packed against the rigid sterol tetracyclic backbone. Differential membrane physicochemical parameters have been implicated in the regulation of integral membrane protein function [48–50], and it is known that cholesterol modulates the function and activity of GPCRs in membrane bilayers [51]. Activation of rhodopsin induces a series of conformational intermediates that involve an expansion of the protein in the lipid bilayer, an event that is inhibited in membranes containing high cholesterol content due to an

increase in lateral pressure [52, 53]. This finding is confirmed by studies on the oxytocin receptor that shows a more compact structure in the presence of cholesterol [54], suggesting that this stabilizing effect may be common. Lateral compression and hydrophobic matching between the lipid bilayer and a protein's transmembrane core, parameters that are affected by cholesterol content, are critical for maintaining the structural integrity of embedded membrane proteins [55–57] (reviewed in [58]).

We examined the thermal stabilization of ORL-1 in DDM micelles supplemented with the different sterols shown in Figure 1. Our results indicated that the stability of ORL-1 is strongly dependent on the sterol with which it is reconstituted. Compared to DDM alone, CHOBIMALT increased the T_m by ~5 °C. However, in the presence of CHS we observed a much larger increase in the thermal transition temperature by almost ~15 °C compared to DDM alone (Figure 2). Although a previous study showed equal thermostabilization of κ OR by CHOBIMALT and CHS [19], this could possibly be explained by the presence of CHOBIMALT's large headgroup (discussed below) or from differences in the assay for thermal stability. We found the thermostabilizing effect of CHS to be dependent on its ratio with DDM; however, increasing amounts of CHS exceeding a CHS:DDM ratio of 0.1:1 did not result in any further stabilization of the receptor (Figure 2C & 2D). Previous studies have shown that CHS increases the thermostability and half-life of β_2 AR in detergent micelles [13, 59], and cholesterol has been shown to increase the melting temperature of β_2 AR in a membrane bilayer mimetic known as lipidic cubic phase (LCP) [60]. Similar results have been observed for other rhodopsin-like family members; cholesterol enhances the thermal stability of the oxytocin receptor [18], CHS addition to DDM increases the stability of the A_{2A} adenosine receptor [20], and CHS and CHOBIMALT protect κ OR from thermal inactivation [19]. Although ligand binding to proteins has a stabilizing effect that typically leads to an increase in thermostability [61, 62], the mechanism in which cholesterol and CHS are able to mediate GPCR thermostabilization is unclear.

Several GPCR crystal structures show cholesterol or CHS bound in a cleft formed by helices II, III, and IV [12–16]. Within this CCM cleft are several residues that favor cholesterol binding, the most notable of which is Trp^{4.50} [13]. Trp^{4.50} is 94% conserved among all GPCRs in the rhodopsin family and has been shown to interact with cholesterol rings through π -stacking interactions. All ORs contain the key Trp^{4.50} residue at the heart of the CCM, suggesting that cholesterol and appropriate analogs thereof potentially modulate the receptors through direct protein-sterol interactions. To explore if this effect stems from a specific interaction at the CCM, we mutated Trp¹⁷⁵^{4.50} in ORL-1 to an alanine and attempted to measure the correlation between T_m at different CHS:DDM ratios. Despite flow cytometry data showing adequate expression of the W175A mutant ORL-1 in Sf9 cells [40], the protein extracted from the washed membranes displayed no thermal transitions and a poor SEC profile suggesting the protein was partially unfolded (data not shown). Albeit indirect, this finding suggested that the tryptophan within the CCM is critical for the integrity of the receptor. This could explain the observations that the four opioid receptors preferentially associate with cholesterol rich lipid rafts, and that M β CD-mediated cholesterol depletion has significant effects on signaling [26–33]. It has been shown that the δ OR and μ OR require cholesterol for proper agonist binding activity [26, 63, 64], and CHS is able to modulate agonist binding and efficacy to μ OR, although purportedly through an increase in CHS:detergent microviscosity [48].

To understand the effects of sterols on the thermostability of ORL-1, we measured the size and shape of sterol-mixed micelles in the absence of protein using DLS and SAXS. Analysis of PDCs is not reported since the excess free micelle leads to sample heterogeneity, and the presence of protein invalidates the simplified two shell ellipsoid model used in the SAXS analysis. We found that the overall size of the micelles, as approximated by their

hydrodynamic radius (R_h) or their radius of gyration (R_g), was dependent on the type of sterol mixed with DDM (Table 1). Whereas most studied sterols resulted in a smaller micelle size, CHOBIMALT had no apparent effect on the micelle size, which is consistent with the NMR based observations by Howell *et al.* [19]. In contrast, the properties of CHS were unique, since its addition to DDM resulted in an increase in the R_h and R_g in a concentration-dependent manner (Table 1).

More detailed information about the shape of the micelles was obtained from fitting the full SAXS profiles using a two-component scattering ellipsoid model with hydrophilic detergent head groups constituting an outer shell that surrounds an inner shell composed of the hydrophobic alkyl chains. The parameters obtained for the mixed CHS:DDM micelle indicated a swelling of the micelle; a substantial increase in the b_1 radius of ~34% was observed, as was a modest but potentially significant increase in the length of a_1 by ~4%. Comparison of the outer shell dimensions indicate that CHS promotes a significant increase in the a_2 radius by ~70%, but does not change b_2 .

To explain these observations we propose the following model for mixed CHS/DDM micelles (Figure 4). CHS, like cholesterol and appropriate analogs [65], avoids highly curved regions and more readily inserts into the flatter parts of the micelle. The CHS sterol core intercalates in between the DDM hydrocarbon tails, thereby reducing the micelle curvature through the expansion of b_1 due to favorable stacking interactions. The sterol mediated ordering effect on DDM hydrocarbon tails results in an increase in thickness of the hydrocarbon layer (parameter a_1), allowing the central core of the micelle to resemble a membrane-like bilayer. The thickness of the outer micelle shell (parameter a_2) is most likely increased as a consequence of two interacting forces. The hydrophobic-based condensing of DDM alkyl tails by CHS may cause DDM head groups to alter their orientation into a more extended configuration, and favorable electrostatic interactions between the DDM and CHS head groups likely contribute to the micelle packing and increase in a_2 thickness. The bulkier head group of CHOBIMALT probably prevents condensing effects and electrostatic interactions with the maltoside head groups of DDM, providing an explanation for the smaller R_h value observed with this sterol/detergent mixture. NMR spectroscopy has demonstrated that the OH group of cholesterol participates in hydrogen bonding interactions with phospholipids [66], suggesting that the head groups of cholesterol analogs are important for similar interactions. In fact, studies with dipalmitoylphosphatidylcholine (DPPC) liposomes reconstituted with cholesterol or CHS indicate that the head groups of CHS and DPPC form both electrostatic and hydrogen bonding interactions causing greater membrane stabilization [67]. Thus, this model suggests that the arrangement of a CHS/DDM micelle resembles the architecture of a bicelle with DDM and CHS molecules forming a membrane-like bilayer disc flanked by a belt of DDM molecules.

Like cholesterol, only CHS and CHOBIMALT contain a double bond between C5 and C6 of the B- ring, which results in a more planar steroid core, enhanced packing against detergent acyl tails and putative specific binding sites on membrane proteins. In addition, all other studied sterols have hydrophilic groups attached either to the tetracyclic ring system or to the tail, thereby preventing their insertion into DDM micelles in the same orientation as CHS or CHOBIMALT, and thus reducing their potential for direct interactions with ORL-1 and other GPCRs. These factors likely combine to yield a more disordered DDM micelle that is smaller in size as evident from DLS and SAXS data, which is sufficient to destabilize GPCRs.

All DLS and SAXS measurements of the micelles were performed in the absence of embedded protein. However, superimposition of an oblate ellipsoid representing the inner hydrophobic shell of a CHS/DDM micelle (ratio = 0.2) with the high resolution β_2AR

crystal structure (PDB code 2RH1), clearly illustrates the spatial relationship of a GPCR's PDC (Figure 5). Modeling of cholesterol against the outer bilayer half of the CCM illustrates a possible configuration that the sterol rings may adopt in the micelle (green cholesterol molecule in Figure 5).

Consequently, our data and the model based on these data suggest that the stabilizing effect of CHS on ORL-1 embedded in mixed CHS/DDM micelles is likely two-fold. First, the bicelle-like structure of CHS/DDM micelles provides a more membrane-like environment, and second, CHS molecules insert in the hydrocarbon slab of the micelle in a similar orientation as cholesterol molecules insert in the membrane, which makes it possible for CHS to directly interact with cholesterol binding sites of ORL-1. We expect that these beneficial attributes should apply to most GPCRs and other membrane-embedded proteins. Taken together, these results suggest that unstable membrane proteins preferring DDM for extraction and purification, even those without CCMs, could benefit from the bicelle-like micelle properties provided by supplementation of CHS to this detergent.

5. Conclusion

A number of different sterol/DDM mixtures were tested for their ability to stabilize the opioid receptor ORL-1 for structural studies. Out of nine sterol-like detergents studied, only CHS and CHOBIMALT showed substantial thermostabilization of ORL-1. These two cholesterol analogs are chemically similar, each having a cholesterol-like tetracyclic ring system and non-polar tails, thereby permitting their penetration in the micelle core. The other seven sterols tested contained polar groups attached either to the ring system or to the hydrophobic tail and were likely incorporated within the micelle in configurations preventing direct sterol-ORL-1 interactions. CHS, the sterol that conferred the highest thermostability upon ORL-1, displayed the unique ability to increase the size of DDM micelles. Based on experimental data, we propose a model in which CHS molecules induce a bicelle-like architecture in the mixed CHS/DDM micelles. The membrane-like core of CHS/DDM micelles provides a more native stabilizing environment for membrane-embedded proteins and allows CHS molecules to directly interact with cholesterol binding sites on ORL-1. These attributes should extend to other GPCRs and proteins which are naturally embedded in a membrane bilayer environment. We therefore conclude that cholesterol analogs with planar steroid cores, hydrophobic tails, and small polar head groups attached to the hydroxyl group at the C3 position of ring-A should be the most effective in stabilizing membrane proteins in mixed micelles.

The abbreviations used are

DPH	1,6-diphenyl-1,3,5-hexatriene
Compound-24	1-benzyl-N-{3-[spiroisobenzofuran-1(3H),40-piperidin-1-yl]propyl}pyrrolidine-2-carboxamide
β₁AR	β ₁ adrenergic receptor
β₂AR	β ₂ adrenergic receptor
CCM	cholesterol consensus motif
CHS	cholesteryl hemisuccinate
CPM	coumarin maleimide, N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide
CMC	Critical micelle concentration

DPPC	dipalmitoylphosphatidylcholine
DLS	dynamic light scattering
GPCR	G protein-coupled receptor
R_h	hydrodynamic radius
IMAC	immobilized metal ion affinity chromatography
LCP	lipidic cubic phase
MβCD	methyl- β -cyclodextrin
DDM	n-Dodecyl β -D-maltoside
ORL-1	nociceptin opioid peptide receptor
OR	opioid receptor
PDC	protein-detergent complex
R_g	radius of gyration
SAXS	small-angle X-ray scattering
Sf9	Spodoptera frugiperda cell line 9
T_m	thermal transition temperature
TM	transmembrane
WT	wild type

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Figure 1. Schematic diagram of cholesterol, cholesterol analogs and DDM

Chemical structures of: cholesterol, with its four rings labeled A–D, DDM, the detergent used throughout these studies, and various cholesterol analogs. Notice that only CHS and CHOBIMALT have cholesterol like iso-octyl tails and planar steroid cores (by virtue of a double bond between C5 and C6 of ring B).

Figure 2. ORL-1 is thermally stabilized by the cholesterol analogs CHS and CHOBIMALT
(A) (B) Thermal stability data for ORL-1/C-24 in the presence of various sterol:DDM mixtures at a ratio of 0.2:1. (C) (D) Thermal stability data for ORL-1/C-24 in the presence of different CHS:DDM ratios. ¹Thermal transition temperatures (T_m) and standard errors are calculated from Equation 1 [47].

Figure 3. SAXS fitting reveals increasing micelle geometrical parameters in the presence of CHS (A) Cross-section of a two-shell oblate ellipsoid defining the geometrical parameters used for the two shell ellipsoid fits. Full SAXS scattering data (black spheres) fit to a two shell ellipsoid (red line) by the method described by Lipfert et al. [6] for micelles containing (B) DDM, (C) CHS/DDM, 0.1:1, (D) CHS/DDM, 0.2:1. Guinier representation ($\ln(I)$ versus q^2 ($\text{\AA}^{-2}$)) of the low angle data (black squares) and linear fit (red line) is plotted in the inset for the three datasets. Only the DDM and CHS/DDM data is included as a representative of all fits.

Figure 4. Models for DDM versus CHS:DDM micelles

Addition of CHS to DDM micelles (**A**) results in altered mixed micelle morphologies (**B**). CHS inserts mostly in less curved regions of DDM micelles inducing a pseudo-bicelle architecture with the core forming a membrane-like bilayer disk consisting of DDM and CHS molecules flanked with a belt of DDM molecules. Rigid CHS molecules order DDM alkyl tails, resulting in a slight increase in the thickness of the hydrophobic slab, and decrease the area per DDM molecule, which results in reorientation of DDM maltoside head groups (yellow ovals), increasing the thickness of the hydrophilic layer.

Figure 5. Overlay of β_2 AR structure and the hydrophobic shell of a DDM and CHS:DDM micelle

Superposition of β_2 AR structure (colored blue; PDB ID 2RH1) and the inner hydrophobic shell (colored gray) of a (A) (B) DDM micelle and a (C) (D) CHS:DDM micelle determined by SAXS (at a CHS:DDM ratio of 0.2:1). The palmitoyl acyl modification is colored cyan, three cholesterol molecules are colored magenta, Trp158 is colored yellow, and a modeled cholesterol molecule is colored green.

Detergent Micelle Measurements

Table 1

Detergent	DLS R_h (Å) ¹	SAXS R_g (Å) ²	a_1 (Å) ³	b_1 (Å) ³	a_2 (Å) ³	b_2 (Å) ³
DDM	35	33.2 ± 0.2	15.9	29.2	5.0	5.0
CHS:DDM = 0.1	38	37.3 ± 0.1	16.5	36.0	8.4	5.1
CHS:DDM = 0.2	41	44.8 ± 0.2	16.5	40.2	8.7	5.0
CHAPS:DDM = 0.2	32	30.3 ± 0.1	15.5	26.5	5.1	5.1
CHAPSO:DDM = 0.2	32	29.7 ± 0.2	15.4	26.5	5.1	5.1
Na Cholate:DDM = 0.2	32	30.7 ± 0.2	15.3	26.6	4.9	4.9
BigCHAP:DDM = 0.2	33	30.6 ± 0.2	15.4	26.5	5.3	5.3
BigCHAP:deoxy:DDM = 0.2	32	31.9 ± 0.2	15.3	26.5	5.4	5.4
Deoxycholate:DDM = 0.2	33	32.4 ± 0.3	15.0	28.1	5.0	5.0
Façade:DDM = 0.2	31	29.6 ± 0.2	14.9	25.7	5.9	5.8
CHOBIMALT:DDM=0.2	36	N/A	N/A	N/A	N/A	N/A

¹ Hydrodynamic radius values measured via dynamic light scattering (DynaPro, Wyatt).

² Radius of gyration values measured via SAXS Guinier analysis (Figure 3).

³ Parameters fit to a two shell ellipsoid model (Figure 3; [4]).