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Design, Synthesis and Properties of Branch-chained Maltoside Detergents for Stabilization and Crystallization of Integral Membrane Proteins: Human Connexin 26

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Abstract

A challenging requirement for structural studies of integral membrane proteins (IMPs) is the use of amphiphiles that replicate the hydrophobic environment of membranes. Progress has been impeded by the limited number of useful detergents and the need for a deeper understanding of their structure-activity relationships. To this end, we designed a family of detergents containing short, branched alkyl chains at the interface between the polar head and apolar tail. This design mimics the second aliphatic chain of lipid molecules and reduces water penetration, thereby increasing the hydrophobicity within the interior of the micelle. To compare with the popular straight-chained maltoside detergents, the branch-chained β -D-maltosides were synthesized efficiently in pure anomeric form. The branch-chained maltosides form smaller micelles by having shorter main chains, while having comparable hydrophobicity to the detergents with only straight chains. Selected branch-chained and straight-chained maltoside detergents were examined for their ability to solubilize, stabilize, and aid the crystallization of human connexin 26, an α -helical IMP that forms hexamers. We showed that the branch-chained maltosides performed as well as straight-chained analogues and enabled crystallization in different space groups.

1. Introduction

Integral membrane proteins (IMPs) are encoded by about a third of the human genome, performing essential functions as receptors, transporters and channels. Owing to their profound physiological significance in numerous human diseases, IMPs comprise more than half of all current drug targets. In spite of remarkable progress made in recent few years,¹ high-resolution structural studies of IMPs lag far behind that of soluble proteins because IMPs have to be solubilized and stabilized by amphiphiles that mimic the hydrophobic environment of membranes. Only a few IMPs such as bacteriorhodopsin,^{2–5} rhodopsin,^{6,7} the acetylcholine receptor⁸ and the sarcoplasmic reticulum Ca^{++} -ATPase⁹ are sufficiently abundant in their

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Supporting Information Available: Characterization data for new detergents. This information is available free of charge via the Internet at <http://pubs.acs.org>.

native membranes to enable high-resolution electron crystallography. Most IMPs are present in low copy numbers within cell membranes, necessitating the use of recombinant methods for expression. Consequently, detergents are required for their extraction and purification.¹⁰ High-resolution structural studies require that the protein in the detergent micelle maintain a native fold, exist as homogeneous, monodisperse protein-detergent complexes (PDCs), and be stable for at least several days, if not weeks. The selection of a suitable detergent is critically important and remains one of the major bottlenecks of successful sample preparation and structural studies.^{11,12}

Although numerous amphiphiles and detergents have been used in membrane biochemistry, a majority of the IMP structures solved by X-ray crystallography have relied primarily on only a few detergents, usually based on sugar, polyethylene oxide and amine oxide head groups.¹³ A serious limitation is that IMP crystals grown in available detergents are often of low quality and difficult to improve. Hundreds of detergent molecules are bound to a membrane protein,¹⁴ reducing the surface area for protein-protein contacts in the crystal lattice, often leaving large channels for detergents and solvent. Theoretically, shrinking the size of the detergent micelle in the PDC should increase hydrophilic, protein-protein interactions and potentially improve crystal packing. This idea is consistent with the observation that detergents with shorter alkyl chains or smaller polar head groups often yield higher resolution protein crystals. In contrast, detergents with longer alkyl chains generally confer higher protein stability. Thus, a balance between the hydrophobicity and hydrophilicity of a detergent molecule¹⁵ will likely affect its ability to solubilize and stabilize IMPs and enable crystallization.

Careful optimization of protein constructs and screening protein homologues are common practices in membrane protein structural biology.^{16,17} Since many of the challenges in this field are associated with the use of amphiphiles,^{17,18} the development of new detergents can be equally important to generate stable PDCs for high-resolution structural studies. Clever ideas exist for designing novel amphiphiles such as protein-based nanodiscs,^{19,20} amphiphilic polymers (amphipols),^{21,22,23} peptide-based amphiphiles,²⁴⁻²⁷ fluorinated detergents^{23,28} and tripod detergents.²⁹⁻³⁰ Some of these novel amphiphiles (e.g. nanodiscs and amphipols) have found broad applications in membrane protein biochemistry and are used for the solubilization and stabilization of IMPs for functional studies. However, the repertoire of successful amphiphiles used for the crystallization of IMPs is still quite limited, and only a few popular head-to-tail detergents have prevailed.¹³ We recently introduced a novel design of steroid-based facial amphiphiles,³¹ and some example molecules in this class have been used in the structure determination of several monotopic P450 enzymes.^{32,33}

As part of our long-term effort to develop custom amphiphiles for better performance in membrane protein structural biology, we describe here a new class of branch-chained maltoside detergents in which short aliphatic branches have been introduced between the hydrophilic and hydrophobic interface. The effect of short branches on the detergent micellar properties was analyzed and compared with the straight-chained analogues, and this information is expected to be helpful to guide detergent selection. We also report the success of this new class of detergents for stabilization and crystallization of human connexins 26 (Cx26).

2. Materials and Methods

All organic reactions were carried out under anhydrous conditions and an argon atmosphere, unless otherwise noted. Reagents were purchased at the highest commercial quality and used without further purification, unless otherwise stated. NMR spectra were recorded using Bruker DRX-500, AMX-500 or AMX-400 instruments, which were calibrated using residual undeuterated solvent as an internal reference. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. Electrospray ionization (ESI) mass spectrometry (MS)

experiments were performed on an API 100 Perkin Elmer SCIEX single quadrupole mass spectrometer at 4000V emitter voltage. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer using MALDI (matrix-assisted laser-desorption ionization) or ESI.

Synthesis of branched alkyl β -D-pyranomaltoside detergents

A mixture of 2-dodecanol (4.95 g, 26.6 mmol), 1-thio-ethyl-hepta-*o*-benzoyl- β -D-maltose (29.7 g, 26.6 mmol) and 4 Å molecular sieves (5.0 g) were stirred in anhydrous dichloromethane (120 mL) for 30 min. The solution was cooled to -15°C , and then *N*-iodosuccinimide (5.99 g, 26.6 mmol) and silver trifluoromethanesulfonate (2.04 g, 7.98 mmol) were added under gaseous N_2 . The reaction mixture was slowly warmed to room temperature and was stirred overnight. The reaction was quenched by addition of a solution of saturated sodium bicarbonate, and the aqueous phase was extracted with dichloromethane. The combined organic layers were washed with saturated sodium thiosulfate solution and brine, dried over sodium sulfate, filtered, and the solvent was evaporated *in vacuo* to afford the alkoxyl glycosylation product as yellow oil. The oil was dissolved in anhydrous methanol (100 mL), to which was added a catalytic amount of sodium methoxide (143 mg, 2.6 mmol) at room temperature. After stirring for 2 hrs, the reaction mixture was neutralized with Dowex-50 (H^+) to pH 6-7. The resulting mixture was filtered, and the filtrate was concentrated *in vacuo*. The crude product was first isolated by column chromatography over silica gel, followed by further purification by Dowex 1(OH) chromatography (methanol). 2-Dodecyl- β -D-maltopyranoside (Mal 11_1) was obtained as a white powder (10.1 g, 74% over 2 steps, > 99% purity), and no β -glycoside anomer was detected using this procedure. ^1H NMR (400 MHz, CD_3OD) δ 5.16 (d, $J = 4.0$ Hz, 1H), 4.34 (d, $J = 7.6$ Hz, 0.5H), 4.34 (d, $J = 8.0$ Hz, 0.5H), 3.88-3.80 (m, 4H), 3.71-3.50 (m, 5H), 3.44 (dd, $J = 3.6, 9.6$ Hz, 1H), 3.38-3.33 (m, 1H), 3.26 (t, $J = 9.6$ Hz, 1H), 3.20 (dd, $J = 7.6, 8.8$ Hz, 1H), 1.65-1.56 (m, 1H), 1.48-1.29 (m, 17H), 1.22 (d, $J = 6.4$ Hz, 1.5H), 1.16 (d, $J = 6.4$ Hz, 1.5H), 0.90 (t, $J = 6.8$ Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 103.9, 102.9 (superimposed signals for two anomer carbons), 102.1, 81.4 (81.45), 81.4 (81.37), 77.9 (77.95), 77.9 (77.91), 77.8, 76.6, 76.5, 75.8, 75.1, 74.9, 74.8, 74.7, 74.2, 71.5, 62.8, 62.3, 38.5, 37.7, 33.1, 31.0, 30.9, 30.8, 30.5, 26.6, 26.4, 23.8, 22.0, 19.8, 14.5. IR (film): $\nu_{\text{max}} = 3374, 2925, 2360, 1075, 774\text{ cm}^{-1}$. HR-MS (ESI) calcd for $\text{C}_{24}\text{H}_{47}\text{O}_{11}$ ($\text{M} + \text{H}$) $^+$ 511.3113, found 511.3114.

3-dodecyl- β -D-maltopyranoside (Mal 10_2): ^1H NMR (400 MHz, CD_3OD) δ 5.16 (d, $J = 4.0$ Hz, 1H), 4.33 (d, $J = 7.6$ Hz, 0.5 H), 4.32 (d, $J = 7.5$ Hz, 0.5 Hz), 3.91-3.76 (m, 3H), 3.72-3.50 (m, 6H), 3.44 (dd, $J = 3.6, 9.6$ Hz, 1H), 3.38-3.32 (m, 1H), 3.26 (t, $J = 9.2$ Hz, 1H), 3.21-3.19 (m, 1H), 1.64-1.46 (m, 4H), 1.44-1.29 (m, 14H), 0.95-0.88 (m, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ 103.6, 103.3, 102.9, 82.1, 81.6, 81.4, 78.0, 76.5, 75.1, 74.9, 74.8, 74.2, 71.6, 62.8, 62.4, 35.3, 34.5, 33.1, 31.1, 31.0, 30.8, 30.5, 28.7, 27.3, 26.4, 26.1, 23.8, 14.5, 10.1, 9.6. IR (film) $\nu_{\text{max}} = 3353, 2923, 2854, 1147, 1073, 1022\text{ cm}^{-1}$. HR-MS (ESI) calcd for $\text{C}_{24}\text{H}_{46}\text{O}_{11}\text{Na}$ ($\text{M} + \text{Na}$) $^+$ 533.2932, found 533.2938.

All other branch-chained detergents shown in Table 1 were synthesized similarly and purified to >99% purity.

Measurement of hydrodynamic radius of detergent micelles by dynamic light scattering

The hydrodynamic radius (R_h) was determined using a DynaPro Titan (Wyatt Technology Corporation, CA) equipped with a plate reader and a laser operating at 830 nm. The scattered light was measured at an angle of 158° relative to the primary beam. Aliquots (50 μL) of various concentrations of detergents in distilled, deionized (di) water were placed in a 384-well plate, which was maintained at 20°C . Samples were repeated in triplicate with 5 acquisitions for each well. All detergent stock solutions were carefully filtered through a 0.2 nm membrane

and diluted with di water, which was also filtered through a 0.02 μm filter. R_h values were determined using the integrated Dynamics software that analyzes the time scale of the scattered light intensity fluctuations by an autocorrelation function. The viscosity value of pure water (1.0 centipoise at 20 °C) was used for all analyses, with the assumption that low concentrations of detergents ($\leq 0.6\%$) do not have a significant effect. The reported value was an average of all valid acquisitions.

Evaluation of detergent hydrophobicity by reverse-phase HPLC

HPLC was performed on an instrument equipped with both a UV detector and an evaporative light scattering detector (ELSD). The detergent sample was analyzed using a C18 column (Waters, Catalogue No. 186002431, 150×4.6 mm) and a mobile phase of 45% $\text{CH}_3\text{CN}/55\%$ H_2O at a flow rate of 1.0 mL/min. The HPLC retention factor (k') was calculated according to the equation: $k' = (t_r - t_0)/t_0$, where t_0 = retention time of the solvent front and t_r = retention time of the detergent molecule.³⁴

Measurement of critical micelle concentration (CMC)

The CMC value of each detergent was determined by monitoring the fluorescence ($\lambda_{\text{ex}} = 388$ nm, $\lambda_{\text{em}} = 477$ nm) of the ammonium salt of 8-anilino-1-naphthalenesulfonic acid (ANS), which becomes highly fluorescent when incorporated into the hydrophobic micellar environment.³⁵ Solutions containing 10 μM ANS and different concentrations of detergents were measured at room temperature on a Cary Eclipse Fluorescence spectrophotometer (Varian). All measurements were performed in triplicate using separately prepared solutions. The CMC was defined as the breakpoint in the plot of fluorescence intensity versus detergent concentration (Figure S1).

Preparation and crystallization of connexin 26

Wild-type human connexin 26 (Cx26) and various single-amino acid mutated forms with a C-terminal hexahistidine tag were expressed in Tn5 insect cells using a baculovirus vector. Protein was solubilized by incubating Tn5 membranes with 2% (wt/vol) of the maltoside detergent. The C-terminal hexahistidine tag enabled one-step purification by cobalt affinity chromatography. Purified Cx26 was eluted in a buffer (20 mM Tris-HCl pH 7.5, 1.0 M NaCl, 200 mM imidazole) containing $\approx 10 \times$ CMC concentration of the maltoside detergent. A PD10 desalting column was used to perform buffer exchange into 20 mM MES, 1.0 M NaCl, protease inhibitors at pH 6.0 and the same detergent ($10 \times$ CMC). The protein was concentrated by centrifugal ultrafiltration to ~ 6 mg/mL, and crystallization screens were prepared using an Innovadyne Screenmaker 96+8 robot. Crystals of Cx26 in various maltoside detergents were grown by vapour diffusion at 20 °C with a well solution containing 0.1 M Tris-HCl pH 8.5, 0.2-0.3 M LiSO_4 or 0.2 M malonate and 25-30% polyethylene glycol 400.

Size-exclusion chromatography

Purified samples of Cx26 in maltoside buffers were subjected to analytical SEC using a SepaxTM nanofilm SEC-250 column (Sepax Technologies, Newark, DE) connected to a DionexTM Ultimate 3000 HPLC. The absorbance at 280 nm was recorded versus the elution volume.

Electron microscopy

An aliquot (~ 5 μL) of the purified Cx26 in maltoside buffer was applied to EM grids that had been rendered hydrophilic by glow discharge. After ~ 90 sec, the grid was rinsed with di water, a drop of 2% uranyl acetate was applied for ~ 60 sec, and the grid was blotted with filter paper and air dried. Grids were examined using a Tecnai F20 transmission electron microscope (Philips/FEI) operating at 120 kV in low electron-dose mode.

Thermal stability assay

The thermal stability of Cx26 was measured as described previously.³⁶ Upon thermal denaturation, C202 in the fourth transmembrane α -helix becomes accessible for labeling by CPM [7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin], which upon binding undergoes a large increase in quantum yield. For these experiments, an aliquot (1 μ g) of concentrated protein was diluted into 20 mM Hepes buffer (pH 7.5), 150 mM NaCl, 2x, 5x, 10x CMC of detergent in the presence of CPM (5 μ M). Samples were heated and maintained at 37 °C, and fluorescence was monitored using excitation and emission wavelengths of 405 and 480 nm, respectively, in a Tecan Genios Pro plate reader. Four measurements were made with and without protein for each detergent type and concentration. The baseline was subtracted, and quadruplet measurements were averaged. Data shown in Figure 5 are for the 2x CMC detergent concentration. We attributed the variation of fluorescence maximum of the denaturation curves to several possible factors including the error in measuring the protein concentration ($\pm 10\%$), possible protein precipitation upon denaturation, and unknown fluorescence intensity variations of the CPM-protein conjugate in the presence of different detergents.

3. Results and Discussion

Design and synthesis of branch-chained detergents

Our custom detergents were designed with a short aliphatic branch at the interface between the hydrophobic tail and the hydrophilic head to mimic the second aliphatic chain of lipid molecules (Figures 1a and 1b). We reasoned that the short branch could be accommodated near the polar-apolar boundary of the micelle without significantly affecting the overall packing. As discussed by C. Tanford,³⁷ some water-hydrocarbon contacts at the interface between the hydrophobic core and the polar mantle of the detergent micelles are inevitable. We surmise that adding a short alkyl branch at the polar-apolar interface will reduce the water penetration. The associated reduction in the water content would increase the hydrophobicity within the interior of the detergent micelles, as illustrated in Figure 1a. By this approach, the branch-chained detergents may form smaller micelles by having shorter main chains, while having comparable hydrophobicity to detergents with only straight chains.

Nearly 50% of the currently available high-resolution X-ray structures of α -helical IMPs have been solved using maltoside detergents.¹³ The maltoside detergents are appealing because they have a sufficient detergent effect to solubilize membranes, and yet they are sufficiently mild to not denature most IMPs. Thus, demonstration of the above design principles was exemplified for the popular maltoside detergents by appending short branches (1-3 carbons) on the hydrophobic tails. The molecules shown in Figure 1b have a total of 12 carbons in the alkyl chain. Of note, some known detergents also contain aliphatic branches at various positions, such as the Triton detergents and some short-chained phosphocholines (e.g. dihexanoylphosphatidylcholine and the Fos-choline-ISO detergents developed by Anatrace, Inc.), but none of these molecules embodies the same design principles that we describe. In our previous work,³⁸ we observed that phosphocholine detergents containing long branched chains were not as efficient as single straight-chained detergents in refolding β -barrel membrane proteins. Interestingly, some bromo-branched maltoside detergents have been used in the crystallization of a pentameric ligand-gated ion channel.³⁹

The published method to synthesize straight-chained β -D-maltoside detergents uses acetobromomaltose as the glycosyl donor, which is activated by reaction with a stoichiometric amount of silver salt (Ag_2CO_3) and a trace amount of iodine.^{40,41} However, the reaction products are often contaminated with undesired α -anomer byproducts. As a result, the reaction requires a large excess of alkyl alcohol (10 equivalents) to suppress formation of the byproduct.

Trials of other peracetylated glycosyl donors including thiomaltoside and trichloroacetimidate gave similar results as using acetobromomaltose. Since the purity of a detergent is critical for crystallizing membrane proteins,^{42,43} our synthesis adopted a different, highly stereoselective β -glycosylation methodology by the use of perbenzoylated ethylthiomaltoside as the glycosyl, which is highly stable and easily synthesized in large scale. Thus, an equal equivalent of a secondary alcohol was reacted with perbenzoylated ethylthiomaltoside that was activated by reaction with *N*-iodosuccinimide (NIS) and a catalytic amount of silver trifluoromethanesulfonate (Figure 1c).⁴⁴ The reaction usually reached completion within 2 hours. Removal of the benzoyl protective groups used catalytic amounts of sodium methoxide in methanol. This two-step method yielded the branched β -D-maltoside as the single anomer at a yield of 69-78% in multi-gram scale syntheses.

Physical properties of branch-chained detergents

In this study, we used the shorthand Mal *x*_{*y*} notation for naming the β -D-maltosides, where *x* and *y* represent the total number of carbon atoms in the primary alkyl chain and the secondary branch, respectively, and *y* is simply omitted for the straight-chained maltosides (*y* = 0). For the branch-chained maltosides and their straight-chained analogues, we compared the following measurements: solubility, hydrophobicity, critical micelle concentration (CMC), and size of the micelles.

As expected, increasing the length of either the primary alkyl chain or the branch conferred enhanced hydrophobicity, estimated by *k'*, the detergent's normalized HPLC retention factor.³⁴ As shown in Table 1, *k'* is roughly related inversely to the CMC value. We observed that adding two carbons on the branch had a similar effect on the CMC as extending one carbon on the straight chain (Figure 2). The CMC of a methyl-branched maltoside (Mal *x*₁) fell between Mal *x* and Mal (*x*+1). It has been demonstrated previously that adding an aliphatic branch in detergents has a much smaller effect on the CMC values compared to elongating the main alkyl chain with the same number of carbon atoms.^{45,46} In terms of the effect on the CMC, our approximation that each carbon increment on the short branch (≤ 3 in our studies) is equivalent to a half-carbon increment on the main chain is in good agreement with previous studies.^{45,46} (We did not study maltoside detergents with a total of >14 carbon-chain because of their low solubility.)

The micelle size was estimated by the hydrodynamic radius (*R_h*) over a range of detergent concentrations (0.10-0.60% wt/vol). As shown in Figure 3, the micelle size remained approximately constant over the concentration range investigated for each straight-chained maltoside from nonyl (Mal 9) to tridecyl (Mal 13) and for the maltosides with a single methyl branch (Mal *x*₁), as well as for Mal 9₂. Small angle X-ray and neutron scattering studies have also shown that the micelle sizes of Mal 8 (OM) and Mal 12 (DDM) are monodisperse and not sensitive to the detergent concentration.⁴⁷⁻⁴⁹ The mean values of *R_h* over the detergent concentration range shown in Figure 3 are 2.6 ± 0.14 nm for Mal 9 and 2.4 ± 0.13 nm for Mal 9₁; 2.9 ± 0.12 nm for Mal 10 and 2.7 ± 0.10 nm for Mal 10₁; 3.1 ± 0.07 nm for Mal 11 and 3.0 ± 0.07 nm for Mal 11₁; and 3.4 ± 0.09 nm for Mal 12 (DDM) and 3.4 ± 0.10 nm for Mal 12₁. Of note, the micelle sizes are slightly smaller upon addition of a methyl branch on Mal 9 (NM), Mal 10 (DM) and Mal 11 (UDM) (Inset of Figure 3). Overall, for the class of β -D-maltoside detergents, our data indicate that very short branches have a minimal effect on the micelle size compared to micelles formed by straight-chained molecules. However, incorporation of longer branches (C3) or short branches onto longer primary alkyl chains produced larger micelles with increased detergent concentration, and this effect was more dramatic for the longer-chained maltosides. The structural changes of maltoside detergents (e.g. having a longer branch in Mal 10₃) can lead to the alteration of their molecular shapes that govern their self-assembly properties^{50,51} and may contribute to the above results. We

note that the micelle size of many commercial detergents increases by concentration (data not shown). Detergents that form small micelles are usually favored for crystallizing membrane proteins in order to increase the likelihood of protein-protein interactions; hence, the ability to customize the micelle size will be helpful to guide detergent selection and concentration.

It is noteworthy that each of the branched detergents is a mixture of two diastereomers at the site of branching. To probe the effect of chirality, two optically pure compounds related to Mal 11_1 were prepared (Figure 4). To assess the effect of the position of the branch in the primary aliphatic chain, we also synthesized undecyl chains with a branch in the middle (compound **1**) and at the terminus (compound **2**) (Figure 4). The CMC and the size pattern of both pure isomers of Mal 11_1 were nearly identical to the mixed form (data not shown). Interestingly, compound **1** gave different micelle sizes at different concentrations, while the micelle size of compound **2** was constant (3.1 ± 0.1 nm) and almost identical to that of UDM (3.1 ± 0.07 nm) and slightly larger than Mal 11_1 (3.0 ± 0.07 nm) (Figure 5). From the aspect of micellar size homogeneity, variants of compound **2** with branches at the alkyl terminus comprise interesting structures to be explored in future studies.

Solubilization, stabilization and crystallization of Cx26 using branch- and straight-Chained maltosides.

The branch-chained maltosides have been tested in the solubilization, stabilization and crystallization of a polytopic, α -helical IMP, the human gap junction channel protein Cx26. Mutations in this particular connexin isoform are the most common cause of nonsyndromic deafness.⁵² Gap junction channels are formed by the end-to-end docking of two hemi-channels, each comprised of 6 connexin subunits. Each subunit contains 4 transmembrane domains, and electron crystallography has revealed that gap junction hemichannels are formed by an annular bundle of 24 transmembrane α -helices.⁵²⁻⁵⁴

Screening of more than twenty commercial detergents to generate stable protein samples and crystals of Cx26 showed that DDM (Mal 12) was the best performer, followed by UDM (Mal 11). Cx26 was not sufficiently soluble in shorter straight-chained detergents such as DM (Mal 10) at the higher protein concentrations required for crystallization. Mal 10_2 and Mal 11_1, with a total of 12 aliphatic carbons but shorter primary alkyl chains than DDM, were selected for further evaluation.

Both Mal 10_2 and Mal 11_1 were as efficient as DDM in solubilizing and extracting Cx26 from membranes, and the purified protein was concentrated to ~6 mg/mL for crystallization studies. Electron micrographs of Cx26 in the maltoside detergents showed that the protein samples were homogenous with donut-shaped particles, characteristic of “end-on” views (Figure 6b).⁵⁵ SEC of purified Cx26 in Mal 11_1 and DDM revealed a main peak with an apparent molecular weight consistent with dodecamers and a right shoulder consistent with hexamers (Figure 6a). Cx26 was clearly more homogeneous with a larger fraction of dodecamers in Mal 11_1 than in DDM. It is interesting that the recently reported X-ray structure of Cx26 in UDM crystallized as a dodecamer;⁵⁶ thus, it is unclear how the crystallization conditions affected the oligomerization state of Cx26.

The isothermal stability of Cx26 was measured by the accessibility of a cysteine residue (C202) in the fourth transmembrane domain of Cx26 for labeling by the thiol-reactive fluorescence dye CPM.³⁶ The fluorescence intensity for all protein samples incubated at 37 °C reached a maximum within 100 minutes. Incubation for longer times led to a decrease of the fluorescence intensity as a result of protein precipitation. Amongst the few maltoside detergents in which Cx26 could be solubilized and purified, DDM afforded the highest thermal stability followed by Mal 11_1, Mal 10_2 and UDM (Figure 7). The solubility of concentrated Cx26 stored for

days at 4°C in optimized pH and salt conditions was similar to the isothermal stability screen performed over minutes at 37°C (data not shown).

Our crystallization experiments were conducted at 20 °C using robotic screens. Interestingly, the two branch-chained detergents and DDM yielded Cx26 crystals under similar conditions, but with different symmetries (Figure 8). Crystals obtained with DDM are of the monoclinic space group C2 with unit cell dimensions of $x = 171.7 \text{ \AA}$, $y = 114.8 \text{ \AA}$, $z = 153.5 \text{ \AA}$, $\alpha = 90^\circ$, $\beta = 112^\circ$, $\gamma = 90^\circ$, while crystals obtained from Mal 11_1 are of the hexagonal space group P3 or higher with unit cell dimensions $x = y = 102.5 \text{ \AA}$, $z = 151.4 \text{ \AA}$, $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$. In both cases, crystals grow to beyond 100 μm size over the course of about 1 week. Crystals grown using Mal 10_2 have a hexagonal morphology but are relatively small ($< 50 \mu\text{m}$). Monoclinic crystals were also obtained from UDM alone, while hexagonal crystals could be generated from UDM by supplementing the crystallization drop with a shorter chained detergent (octyl- β -D-glucoside or nonyl- β -D-glucoside). However, in either case, crystals grown in UDM displayed weaker diffraction than for DDM. These observations suggest that the small branch might have a function akin to small amphiphile additives that are known to partition at the polar-apolar interface of mixed micelles, and are often employed to optimize the crystallization conditions of membrane proteins.^{57,58}

Unfortunately, the crystals of Cx26 grown in Mal 10_2, Mal 11_1 and DDM displayed anisotropic diffraction. In the weak direction, the diffraction of crystals grown in DDM and Mal 11_1 was limited to $\sim 7.5 \text{ \AA}$. However, Mal 11_1 improved the diffraction in the strongest direction ($3.5 \text{ \AA}$ vs. $4.5 \text{ \AA}$ using DDM). The crystals grown in Mal 10_2 diffracted about the same as Mal 11_1, except that their size was smaller. It is notable that Mal 11_1 consistently gave increased protein crystallizability and crystal reproducibility compared to DDM; hence current effort is focused on the evaluation of Mal 11_1. Dehydration of Cx26 crystals grown in branch-chained maltosides may further improve the resolution as indicated in the previous studies.^{55,56}

In the recent X-ray structure at $3.5 \text{ \AA}$ resolution,⁵⁶ wild type Cx26 lacking a His tag was expressed, solubilized in DDM and partially exchanged to UDM by SEC. Monoclinic (C2) crystals were grown by vapor diffusion at 4 °C, and dehydration was essential to improve the resolution limit from $7 \text{ \AA}$ to $3.5 \text{ \AA}$.⁵⁵ A notable disagreement between the X-ray structure⁵⁶ and previously published cryoEM-based models of native, intact channels^{53,54} is the assignment of the transmembrane helices and the structure of the extracellular loops; hence, further high-resolution structural studies on this particular isoform and other important members in the connexin protein family are warranted.

4. Conclusions

The choice of detergent is a critical parameter for successful crystallization of integral membrane proteins. We and others have often observed that the alkyl-chain length of a detergent is crucial for stabilizing and crystallizing IMPs—even one-carbon change can have dramatic effects. Our data using branch-chained maltosides for solubilization, stabilization and crystallization of Cx26 imply that a shorter chained detergent with optimized hydrophobicity can perform as well or better than the longer chained analogues.

The monoclinic crystals of Cx26 generated in this study using straight-chained maltosides (DDM and UDM) are similar to those described by Maeda et al.⁵⁶ It is noteworthy that the use of branched maltosides (Mal 11_1 and Mal 10_2) generated a new hexagonal crystal form that was not seen from the extensive crystallization screening of commercial detergents. It is likely that the smaller but more compact micelle of the branched maltoside detergents allowed for a different crystal packing compared to DDM. The availability of multiple space groups and thus

different packing arrangements may be helpful in differentiating naturally occurring structural features from packing-induced artifacts.

Small micelle forming detergents are usually favored for crystallizing membrane proteins in order to increase the likelihood of protein-protein interactions. We designed, synthesized and characterized a new class of branch-chained maltoside detergents to customize the micellar properties such as the size and hydrophobicity, for better performance in the stabilization and crystallization of IMPs. The ability to customize the micelle size will be helpful to guide detergent selection and concentration. In addition, the branch-chained maltosides can be easily synthesized and are well-behaved chemically. Thus, they can be readily tested by the community for the crystallization of other IMP targets.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Schematic diagram illustrating the design of the branch-chained detergents in which short alkyl branches appended at the polar-apolar interface exclude water penetration and pack more hydrophobicity into the micelle. (b) Example molecules used for crystallization of connexin 26. Although the total number of alkyl chain carbon atoms is 12, as in DDM, the branch-chained maltosides have a shorter primary chain and favor formation of smaller micelles. (c) Synthesis of β -D-maltoside detergents using perbenzoylated ethylthiomaltoside as the glycosyl donor. No undesired α -anomer byproduct was detected.

Figure 2.

Semi-logarithmic plot of CMC values ($\log[\text{CMC}]$, mM unit) of β -D-maltoside detergents as a function of the alkyl chain length. The $\log[\text{CMC}]$ values of straight-chained maltosides (red circle, CMCs shown in Table 1) were fitted to a straight line generated by least squares regression ($r^2 = 0.997$). The $\log[\text{CMC}]$ values of the branched maltosides (Table 1) were labeled (Mal 9_y, open diamond; Mal 10_y, blue bar; Mal 11_y, green square; Mal 12_y, purple triangle; and Mal 13_y, open circle), and the alkyl lengths for Mal x_y were calculated by the formula $(x + 0.5 * y)$.

Figure 3.

Size of β -D-maltoside detergent micelles estimated by the hydrodynamic radius, R_h , measured by dynamic light scattering. We used a nomenclature in which the first number corresponds to the length of the primary aliphatic chain, ranging from 9-13, and the second number is the length of the branch, from 0-3 (see examples in Figure 1). Maltosides with a total of >14 carbon atoms in the aliphatic chain were not studied because of their low solubility. Bar code indicates detergent concentration. The data were an average of triplicate measurements. The enlarged inset shows error bars for the unbranched maltosides and those with a methyl branch (Mal x_1) that formed homogenous micelles (± 0.1 nm) within the tested concentration range.

Figure 4.

Optically pure (*S*)- and (*R*)-Mal 11_1 (left) and maltosides branching in the middle (**1**) and at the terminus (**2**) of the alkyl chain (right).

Figure 5. Micelle size (R_h) of Mal 11 with a branching methyl group at various positions of the alkyl chain. Structures of compounds **1** and **2** are shown in Fig. 4. Bar code indicates detergent concentration. The data were an average of triplicate measurements with error bars.

Figure 6.

(a) Size-exclusion chromatography of Cx26 in DDM and Mal 11_1. The shoulder in the DDM profile indicates that the sample is more homogeneous in Mal 11_1. (b) Negative-stain electron micrograph of Cx26 in Mal 11_1 showing that the purified protein is homogenous with minimal aggregation. The stain penetrates the lumen of the channel giving rise to the characteristic donut-like appearance in top views.

Figure 7. Isothermal denaturation of Cx26 at 37 °C measured by the CPM fluorescence assay. The differences in stability were consistent for detergent concentrations ranging from 2x to 10x CMC. The data shown at 2x CMC are an average of 4 measurements.

Figure 8.
3D crystals of Cx26 grown in DDM are monoclinic, whereas crystals grown in Mal 11-1 and Mal10-2 are hexagonal.

Table 1

CMC and hydrophobicity assessment of β -D-maltoside detergents

detergent	CMC mM (% wt/vol) ^a	(k _i) ^b	detergent	CMC mM (% wt/vol) ^a	(k _i) ^b
Mal 9	6.0 (0.28) ^c	2.00	Mal 11_1	0.37 (0.019)	6.21
Mal 9_1	3.1 (0.15)	2.36	Mal 11_2	0.11 (0.0060)	9.17
Mal 9_2	2.2 (0.11)	2.96	Mal 11_3	0.093 (0.0050)	14.04
Mal 9_3	1.1 (0.055)	5.52	Mal 12	0.17 (0.0087) ^c	8.22
Mal 10	1.8 (0.087) ^c	2.82	Mal 12_1	0.076 (0.0040)	9.89
Mal 10_1	1.2 (0.058)	3.88	Mal 12_2	0.022 (0.0012)	14.80
Mal 10_2	0.63 (0.032)	5.68	Mal 13	0.033 (0.0017) ^c	13.40
Mal 10_3	0.31 (0.016)	8.15	Mal 13_1	0.020 (0.0011)	16.82
Mal 11	0.59 (0.029) ^c	4.08			

^a CMC in H₂O.^b k_i, normalized HPLC retention factor (C18 column, 45% CH₃CN/55% H₂O).^c Data from the catalogue of Anatrace Inc.