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Detergent selection for enhanced extraction of membrane proteins

Buenafe T. Arachea [1], Zhen Sun, Nina Potente [2], Radhika Malik [3], Dragan Isailovic, Ronald E. Viola *

Department of Chemistry, University of Toledo, 2801 W. Bancroft St., Toledo, OH 43606, United States

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ABSTRACT

Generating stable conditions for membrane proteins after extraction from their lipid bilayer environment is essential for subsequent characterization. Detergents are the most widely used means to obtain this stable environment; however, different types of membrane proteins have been found to require detergents with varying properties for optimal extraction efficiency and stability after extraction. The extraction profiles of several detergent types have been examined for membranes isolated from bacteria and yeast, and for a set of recombinant target proteins. The extraction efficiencies of these detergents increase at higher concentrations, and were shown to correlate with their respective CMC values. Two alkyl sugar detergents, octyl-β-D-glucoside (OG) and 5-cyclohexyl-1-pentyl-β-D-maltoside (Cymal-5), and a zwitterionic surfactant, N-decylphosphocholine (Fos-choline-10), were generally effective in the extraction of a broad range of membrane proteins. However, certain detergents were more effective than others in the extraction of specific classes of integral membrane proteins, offering guidelines for initial detergent selection. The differences in extraction efficiencies among this small set of detergents supports the value of detergent screening and optimization to increase the yields of targeted membrane proteins.

Introduction

Membrane proteins are the last major frontier for protein structural investigation. Approximately 20–30% of the proteins that are encoded in the human genome are predicted to be integral membrane proteins [1] and another 10–20% of the total proteins are proposed to be membrane-associated [2]. Improved methods can now recognize a wider range of transmembrane topologies [3], but the predicted number of membrane proteins has remained about one-quarter of the human genome [4]. These proteins include the cell surface receptors, signal transducers, metabolite transporters and membrane channels that are the targets for the majority of the drugs in the clinic. The development of these drugs has been accomplished despite the paucity of structural information about their biological targets. The design of improved methods that lower the barriers to membrane protein extraction, stabilization, crystallization and structural determination will accelerate structure-guided approaches to support the production of new drugs.

Extraction of membrane proteins from the phospholipid bilayer environment is a critical first step in their purification and structural characterization. This extraction requires disruption of the bilayer structure to effect protein removal, without also irreversibly disrupting the protein structure. The primary agents used to extract membrane proteins are amphiphilic molecules such as detergents that can substitute for and mimic the stabilizing properties of the natural phospholipids, and ever-widening arrays of molecules with detergent-like properties are being specifically synthesized for this purpose. The properties of several classes of detergents and their interactions with membrane proteins have been examined [5–8], and there are many examples of optimizing the use of detergents to extract and stabilize specific target proteins of interest [9–12]. In most of these studies the range of detergents selected was quite limited, with selection based primarily on previous successes in the extraction of other membrane proteins. More recent studies have examined the properties of new classes of surfactants for membrane protein extraction [13,14], high-throughput methods for detergent screening [15,16], and refined approaches to examine the stabilization [17–19] and the purification [20] of integral membrane proteins. But, other than very broad generalities, there are no guidelines to suggest which detergents will be the most efficient in extraction of a given protein or class of proteins from membranes. In the absence of such guidance most studies of membrane proteins begin with extractions that use the same set of detergents previously shown to be effective with other membrane proteins.

It is unlikely that any single detergent or group of detergents will be uniformly superior for the efficient extraction of the majority of the diverse classes of membrane proteins. However, it is not

* Corresponding author. Fax: +1 419 530 1583.
E-mail address: ron.viola@utoledo.edu (R.E. Viola).
[1] Current address: Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN.
[2] Current address: Caymen Chemical Co., Ann Arbor, MI.
[3] Current address: Department of Structural and Chemical Biology, Mount Sinai School of Medicine, New York, NY.

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clear how the variations in protein properties influence which types of detergents will accomplish their most efficient extraction while also minimizing structural disruption. We have begun a systematic investigation with the overall aim of addressing these issues. Several non-ionic and zwitterionic detergents were used to extract endogenous proteins from the membranes of bacteria and yeast. Trends in the extraction efficiency of surfactants have been correlated with specific protein classes. In addition, several types of integral membrane proteins have been recombinantly expressed in several microbial cell lines, and the extraction efficiencies of different detergents have been compared for these target proteins.

Materials and methods

Reagents and chemicals

All chemicals and reagents were analytical grade and were purchased from commercial sources. Glycerol was obtained from Fisher, isopropyl-β-D-1-thiogalacto-pyranoside (IPTG)[4] from Gold Biotechnology and buffers from USB Corporation. Precast acrylamide gels and protein molecular weight markers were from Invitrogen. Sodium dodecylsulfate (SDS) was purchased from Gold Biotechnology. Bruker Daltonics provided the α-cyano-4-hydroxycinnamic acid matrix as well as the peptide calibration standards kit. The detergents used in this study were provided by Anatrace.

Plasmids and cell growth

Escherichia coli cell lines (C41, C43, BL21(DE3) and XL-1 Blue) and expression vectors were purchased from Novagen. *Pichia pastoris* cells (KM71H) and pPICZ vector were obtained from Invitrogen. *E. coli* cells were transformed with expression plasmids containing the *BtuB, KcsA* or NaDC3 genes under the control of a *lacZ* promoter and grown on plates containing ampicillin. Wild type cells were also transformed with the empty expression vectors to confer antibiotic resistance. Colonies were picked and used to grow overnight cultures, and were subsequently used to inoculate bulk cultures (4 × 1 L LB Medium). Cells were grown in an incubator shaker with 250 rpm shaking speed, induced with IPTG for protein production and then grown to late log phase. *P. pastoris* cells were transformed with a *pPICZ* plasmid [21] under control of the AOX1 promoter. Cells were initially grown in a glycerol-containing medium in the presence of antibiotic zeocin, and then switched to a methanol containing medium for protein expression as previously described [22]. The cell pellets obtained from the different organisms were used as the protein source for the recombinant and constitutive membrane proteins.

Cell membrane preparation

Bacterial cells were resuspended in buffered solution and sonicated with a 50% duty cycle for ten cycles with a total exposure time of 5 min to disrupt the cell membrane, followed by centrifugation for 30 min at 10,000 rpm and 4 °C. The resulting membrane-containing pellets were resuspended in buffer, centrifuged at 10,000 rpm for 20 min, and then resuspended and centrifuged a second time to separate any soluble or loosely associated proteins. The integral proteins from this washed membrane fraction were then extracted as described below. Preparation of the outer membrane (OM) required homogenization of the membrane pellet, then incubation of the supernatant from the cell disruption for 1 h at room temperature in 20 mM Hepes, pH 7.2, containing 5 mM EDTA and 20 mM lauryl sarcosine detergent. This was followed by a high speed centrifugation (2 h at 34,000 rpm) to pellet the OM. Yeast cells were resuspended in 20 mM potassium phosphate, pH 7.2, and lysed by using a Bead Beater cell disruptor. The cell lysate was centrifuged (1 h at 35,000 rpm) and the pellet was resuspended in phosphate buffer for extraction.

Extraction and solubilization of membrane proteins

Different membrane preparations were examined to measure the extraction efficiencies for specific detergents. Stock solutions of each detergent were prepared at 20 times their critical micelle concentration (CMC) values and then diluted as needed for the initial working concentrations. An equal volume of resuspended cell pellet was used for the comparison of detergent extraction efficiencies, with the total amount of extracted protein normalized per gram of cells. Each pellet was suspended in a buffered detergent solution and incubated with rocking at 4 °C for 3 h. The samples were then centrifuged at 40,000 rpm for 1 h to separate and remove the unextracted membranes. A set of representative specialty non-ionic and zwitterionic detergents [5] were initially chosen to determine which types of detergents were most effective for total protein extraction from the various cell types. Control extractions were run with buffer solutions and the extraction efficiencies for these specialty detergents were compared to a commonly used strongly denaturing ionic detergent (SDS), a zwitterionic detergent (LDAO) and a mild, non-ionic surfactant (Tween 20) (Fig. 1). Following the initial extractions, the set of detergents were expanded to include a larger number from the most efficient classes of detergents for each type of membrane as well as for the individual membrane proteins.

Gel electrophoresis

SDS–PAGE gels, run in triplicate with equal protein loading, were used to analyze the extraction efficiency of each detergent for the constitutive and recombinant membrane proteins and then normalized by the concentration factors. Total protein concentrations were determined by measuring the absorbance at 280 nm using a Nanodrop 2000 spectrophotometer (Thermo Scientific). After staining the intensity of each band was quantified by using either the *ImageJ* software package program (http://rsb.info.nih.gov/ij) or the *Un-scan-it* software (Silk Scientific).

Mass spectrometry sample preparation

Bands corresponding to constitutive membrane proteins were examined by MALDI mass spectrometry to identify the individual proteins. Initial trials were performed using the total membranes isolated from XL-1 Blue cells to determine the optimal concentration needed for protein identification in MS analysis. Protein bands were excised from the gels, destained and digested overnight with trypsin following the method of Shevchenko et al. [23].

MALDI-mass spectral analysis

The samples were prepared for mass spectrometric analysis using the dried droplet method. Briefly, 1 μl of each sample was mixed with an equal volume of saturated α-cyano-4-hydroxycinnamic acid dissolved in 1:1 (v/v) acetonitrile:water containing 0.1% TFA. The samples were spotted onto a MTP 384 ground steel target plate along with a peptide standards mixture for mass calibration, prepared using the same method described above. Mass

[4] *Abbreviations used:* CMC, critical micelle concentration; Cymal-5, 5-cyclohexyl-1-pentyl-β-D-maltoside; DM, n-decyl-β-D-maltoside; Fos-choline-10, Decylphosphocholine; IPTG, isopropyl-β-D-1-thiogalacto-pyranoside; LDAO, lauryldimethylamine-N-oxide; MALDI, matrix assisted laser desorption ionization; Mega-9, nonanoyl-N-methylglucamide; OG, n-octyl-β-D-glucoside; SDS, sodium dodecyl sulfate; Tween20, polyoxyethylene (20) sorbitan monolaurate.

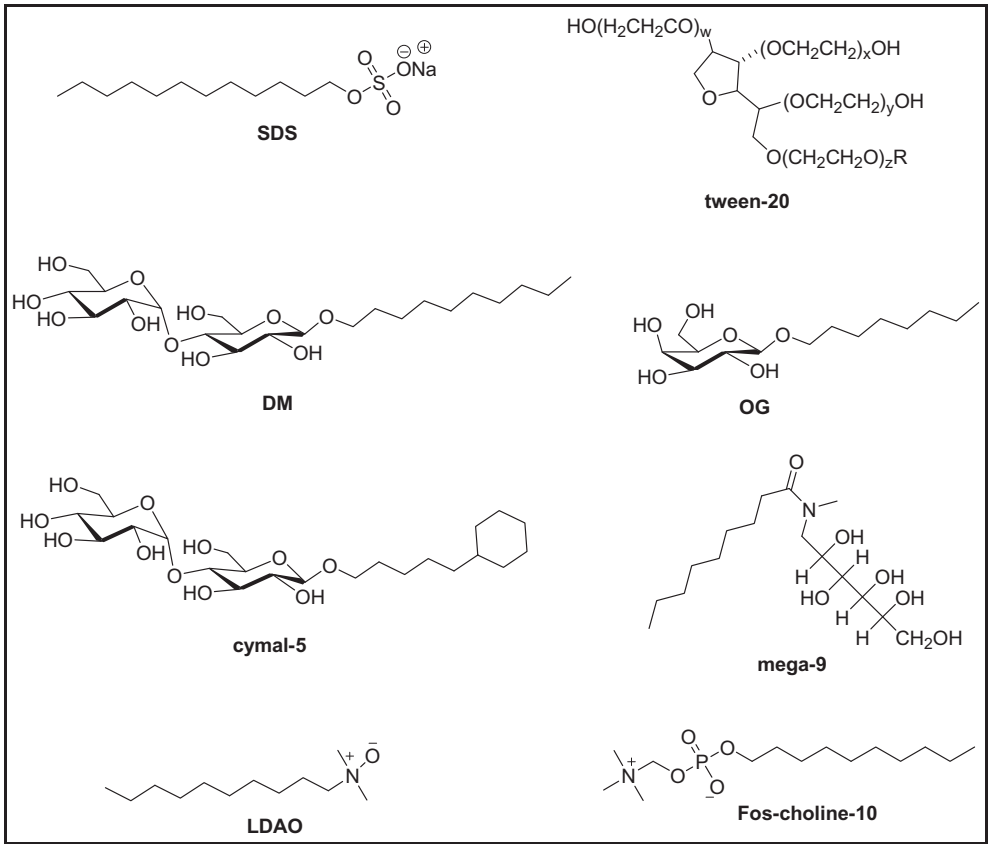

Fig. 1. Structures of the ionic, non-ionic and zwitterionic detergents used for the extraction of membrane proteins. SDS, sodium dodecyl sulfate; Tween20, polyoxyethylene (20) sorbitan monolaurate; DM, *n*-decyl-β-D-maltoside; OG, *n*-octyl-β-D-glucoside; Cymal-5, 5-cyclohexyl-1-pentyl-β-D-maltoside; Mega-9, nonanoyl-N-methylglucamide; LDAO, lauryldimethylamine-N-oxide; Fos-choline-10, decylphosphocholine.

spectra were acquired by an UltrafleXtreme MALDI TOF/TOF instrument (Bruker Daltonics) in positive ion mode using a Smartbeam II laser operating at a repetition rate of 1 kHz. The MS spectra were acquired in the m/z range from 800 to 3500 using reflectron mode and flexControl software (Bruker Daltronics). The signal obtained from a total of 1000 acquisitions was summed for each peptide mass fingerprinting (PMF) search. For the MS/MS analysis, the LIFT TOF–TOF method [24] was used to analyze fragments formed by laser induced dissociation (LID). The MS/MS spectra were collected using 1000–2000 acquisitions for each sample with laser power settings higher than those used for the PMF experiments. Data analysis and PMF and MS/MS searches were carried out using the flexAnalysis (Bruker Daltonics) and Mascot (Matrix Sciences) [25], respectively. Protein identities and classifications were verified by using the UniProt database (www.uniprot.org).

Results

Detergent efficiencies in membrane protein extractions

Different species of microorganisms can have membranes with varied structural organizations and each can contain unique sets of proteins to carry out the specialized functions of that organism. Extractions have been carried out on a representative Gram-negative enterobacteria (XL-1, BL-21 and C41 strains of *E. coli*), and a methylotrophic yeast (*P. pastoris*) to determine how efficiently each type of surfactant will extract the membrane proteins from these organisms. Six members from various structural classes of non-ionic and zwitterionic detergents (Fig. 1) were tested for their capacity to extract proteins from these membranes.

Extractions from *E. coli* membranes were carried out from each of these strains and also from the purified outer membrane (OM) and inner membrane (IM) fractions of C41 cells by incubating equal aliquots of each membrane fraction for defined times with a buffer containing each detergent at twice their CMC levels. In addition to these specialty detergents, extractions were also conducted with two commonly used detergents, SDS and Tween 20 (Fig. 1). SDS is an ionic detergent that effectively solubilizes membrane proteins, but can disrupt protein structural integrity. Tween 20 is a non-ionic, polysorbate surfactant that is less disruptive. Triton X-100 was considered as an additional non-ionic control surfactant, but was eliminated because of strong absorbance that interfered with the protein concentration determinations. The total protein obtained with each surfactant was compared to measure overall extraction efficiency.

Extraction of the washed membrane fractions from *E. coli* strain XL-1 with buffer alone leads to the release of low levels of protein (Table 1). The proteins extracted with the buffer control in the absence of detergents are likely to be weakly associated peripheral membrane proteins and cytosolic proteins that interact with membrane protein partners. SDS was included in the extraction experiments as a positive control and, as expected, was found to extract the largest amount of total protein from each membrane sample. At the other end of the range Tween 20 was only slightly more effective than buffer alone in the extraction of proteins from their membrane environment. Among the specialty detergents N-decylphosphocholine (Fos-choline-10), a zwitterionic, lipid-like surfactant, and octyl-β-D-glucoside (OG), a non-ionic alkyl sugar detergent, were found to be nearly as effective as SDS in the extraction of proteins from the total membrane of *E. coli* XL-1 cells

Table 1
Membrane protein extraction efficiency with different surfactants.

Detergent[b]	Type[c]	[detergent] (mM)	Total protein extracted from different membranes[a]			
			E. coli (XL-1)	*E. coli* (C41 IM)	*E. coli* (C41 OM)	*P. pastoris*
None	–	–	5.2 ± 0.4	7.4 ± 1.5	15.0 ± 2.8	3.1 ± 0.6
SDS	I	20	94.3 ± 3.5	115.3 ± 15.2	89.3 ± 12.4	37.7 ± 4.5
DM	NI	3.2	26.2 ± 2.0	21.5 ± 3.0	29.8 ± 1.3	7.6 ± 1.0
OG	NI	50	78.0 ± 6.8	85.9 ± 6.5	55.1 ± 9.9	18.0 ± 1.0
Cymal-5	NI	10	29.6 ± 2.1	46.5 ± 9.1	42.8 ± 2.5	10.0 ± 1.1
Mega-9	NI	50	33.7 ± 3.5	43.1 ± 1.9	60.9 ± 1.6	8.1 ± 0.7
LDAO	ZI	4	9.7 ± 0.2	12.2 ± 1.5	26.3 ± 3.7	4.0 ± 0.2
Fos-choline-10	ZI	22	89.9 ± 4.0	32.2 ± 4.1	29.2 ± 5.7	16.9 ± 2.3
Tween 20	NI	0.12	9.3 ± 0.7	7.8 ± 1.0	28.3 ± 3.8	3.9 ± 0.2

[a] Total protein was determined by measuring the absorbance at 280 nm, averaged for three independent extractions and expressed as mg protein per gram of cell pellet.
[b] Detergents were used at twice their CMC values: SDS, sodium dodecyl sulfate; DM, *n*-decyl-β-D-maltoside; OG, *n*-octyl-β-D-glucoside; Cymal-5, 5-cyclohexyl-1-pentyl-β-D-maltoside; Mega-9, nonanoyl-N-methylglucamide; LDAO, lauryldimethylamine-N-oxide; Fos-choline-10, Decylphosphocholine; Tween 20, polyoxyethylene (20) sorbitan monolaurate.
[c] I = ionic, NI = non-ionic, ZI = zwitterionic.

(Table 1). In contrast, decyl-β-D-maltoside (DM), another alkyl sugar detergent, and the other non-ionic specialty detergents extracted less than half of these total protein levels, while dodecyldimethylamine-N-oxide (LDAO), a commonly used zwitterionic detergent, was only slightly more effective than the buffer control (Table 1).

A similar pattern was observed when the bacterial membrane of C41 *E. coli* cells was fractionated into the inner and outer membrane components. Again, SDS was the most efficient at extracting proteins from both the IM and OM of *E. coli*, while OG was among the most effective of the non-ionic detergents (Table 1). However, two other non-ionic detergents, 5-cyclohexyl-1-pentyl-β-D-maltoside (Cymal-5) and nonanoyl-N-methyl-glucosamide (Mega-9), were found to function more efficiently than the zwitterionic Fos-choline-10 in total protein extraction from both the IM and OM fractions of this C41 strain. So, even with this small set of detergents, differences were observed in extraction efficiencies between the membranes of these two *E. coli* strains.

Extracting membrane proteins from a yeast species proved to be more challenging, with only about one-third of the extracted protein amounts achieved, on average, compared to the levels obtained from bacterial membrane extractions (Table 1). Once again, a similar pattern was observed with SDS as the most effective surfactant, followed by OG and then Fos-choline-10. But, in this case, half of the detergents tested were found to be quite ineffective, with the total extracted protein obtained from treatment with these detergents within a factor of two of the protein levels measured for the buffer control (Table 1). In an attempt to identify surfactants that are more effective in extracting proteins from the yeast membrane the study with *P. pastoris* was expanded to include a greater diversity of surfactants, including a wider range of alkyl glucosides, maltosides and thiomaltosides, along with some alkyl amine-N-oxides and alkyl polyoxyethylenes. However, each of the surfactants examined in this expanded set also performed only slightly better than the buffer control, with OG and Fos-choline-10 still showing the best extraction efficiency among the non-ionic and zwitterionic detergents tested.

Effects of increasing surfactant levels

Surfactants have typically been examined at or above their CMC values for effective membrane protein solubilization [26]. For example, the binding of dodecyl-β-D-maltoside (DDM) to membrane and liposome samples was found to decrease at lower detergent concentrations, while the achievement of binding equilibrium was significantly delayed at detergent levels below the CMC value [27]. For our extraction studies each surfactant level was typically set at twice their CMC values, but the effect of varying surfactant levels was also examined to determine if higher concentrations might lead to enhanced extraction efficiency. As expected, when the concentration of detergents such as OG, DM, LDAO and Fos-choline-10 were decreased below their respective CMC values their efficiency in extracting membrane proteins from the BL-21 *E. coli* strain decreased by a factor of two or greater (Fig. 2). With increasing concentrations the extraction efficiencies of the more effective surfactants increased nearly linearly at concentrations up to four times their CMC values. When DM and Fos-choline-10 were examined at higher concentrations the increase in extraction efficiencies began to plateau, while the amount of extracted protein continued to increase at higher levels of OG (Fig. 2). LDAO remained relatively ineffective even at the highest detergent levels, with the amount of protein extracted at 20 times CMC only slightly greater than that obtained at 2 times CMC. At their maximum efficiency values DM and Fos-choline-10 extracted about 4-fold more total protein than LDAO, while OG extracted 10-fold more protein than was achieved with LDAO (Fig. 2).

A similar trend was seen when the extraction of *P. pastoris* membrane proteins was examined at detergent levels ranging from half CMC to 20 times their CMC values by selecting an effective detergent (Fos-choline-10) and a detergent that were much

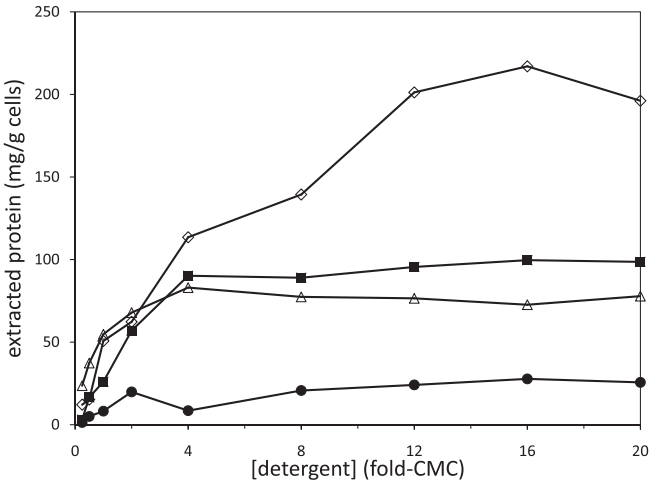

Fig. 2. Total extracted BL-21 *E. coli* membrane proteins with increasing detergent levels. Extracted protein (mg protein per g cell pellet) *vs.* fold-CMC of different detergents, with each data point representing the average of two different extraction experiments. (▲) DM (CMC = 1.8 mM); (◇) OG (CMC = 6.5 mM); (■) Fos-choline (CMC = 11 mM); (●) LDAO (CMC = 0.15 mM).

less effective (Cymal-5). LDAO remained ineffective at extracting yeast membrane proteins even when tested at 10–20 times its CMC value, and also began to cause protein precipitation and denaturation at these higher levels. The efficiency of Cymal-5 increased at higher concentrations, extracting up to 10-fold more total protein at the highest detergent levels (data not shown). However precipitation, which is indicative of protein denaturation, was observed beginning at 8 times CMC levels and above with this detergent. Fos-choline-10 remained the most effective specialty detergent at extracting yeast membrane proteins, with a nearly 10-fold increase in total extracted protein at the highest surfactant levels. But, this detergent also began to cause protein denaturation in the 4–8 times CMC range.

Selectivity of target membrane protein extractions

An examination of the efficiency in total membrane protein extraction is useful to establish general guidelines for the selection of the initial surfactants to test with new protein targets. However, since many proteins of interest are overexpressed before subsequent extraction and purification, three integral membrane proteins with different functions were selected for examination under these higher expression conditions with a set of non-ionic surfactants. KcsA is a voltage-gated ion channel that assembles its α-helical subunit into a tetramer in the membrane and controls the passage of potassium ions across cell membranes [28]. BtuB is a β-barrel cobalamin transporter located in the outer membrane that is classified as a TonB-dependent cofactor transporter [29]. NaDC3 is a member of the sodium-dicarboxylate antiporter family and is involved in shuttling the important brain metabolite, N-acetylaspartate from its site of synthesis to oligodendrocyte cells for hydrolysis [30]. The structures of KcsA and BtuB have been solved to high resolution and serve as test proteins for both extraction and subsequent structural studies. The structure of NaDC3 has not been determined and this protein will establish the effectiveness of our proposed experimental approaches for the extraction of new membrane proteins for subsequent characterization.

Each of these membrane proteins was cloned and expressed in host organisms and the efficiency of extraction was examined with different classes of detergents by using SDS–PAGE. Fos-choline-10 was found to be highly effective in extracting the potassium ion channel protein KcsA from the *E. coli* host membrane, along with Cymal-5, OG and DM (Table 2). A similar general extraction pattern was seen when KcsA was expressed in *R. rubrum*, a photosynthetic bacterium strain that has been adapted for high membrane protein expression [31]. OG was now the most effective detergent in extracting the greatest amount of KcsA from the more extensive membrane of this organism, followed by Cymal-5 and Fos-choline-10. OG was also clearly the most effective detergent in extracting BtuB from the OM of *E. coli* cells. However, in contrast to its effectiveness with KcsA, DM was particularly ineffective in the solubilization of BtuB (Table 2).

The full length NaDC3 transporter was also effectively extracted with OG and Fos-choline-10, but tended to precipitate after extraction into these detergents. An analysis of the amino acid sequence suggested that this protein is likely organized into two domains [32], each of which was predicted to form stable folded structures [33]. Based on these predictions the N-terminal (a.a. 1–303) and C-terminal (a.a. 326–586) domains were separately cloned and expressed. Extraction of each domain lead to significantly different detergent extraction patterns compared with the full length protein. Mega-9, a glucamide with a 9-carbon chain, was most effective at extracting the more hydrophobic N-terminal domain of NaDC3 (Table 2). For the more hydrophilic C-terminal domain OG was more effective, similar to what was observed with full length NaCD3, but the zwitterionic Fos-choline-10 was particularly ineffective in extracting the separate domains of this transporter, in contrast to its efficiency with full length NaDC3 (Table 2). In contrast to the instability of the full length extracted protein, the individual domains were found to be stable in their optimal extraction detergents and did not show any precipitation after storage for 2–3 days at 4 °C.

To extend the initial results with these representative classes of detergents, the extraction of these target proteins was then examined with a wider range of surfactants to begin correlating surfactant properties with the properties of each type of target protein. For KcsA, Fos-choline-10 remained a highly effective solubilizing agent along with the alkyl dimethylamine oxides. An extended family of eleven alkyl maltosides, glucosides, thiomaltosides and thioglucosides were found to be no more effective than DM and, in most cases, somewhat less effective in the extraction of KcsA. The full length NaDC3 transporter was extracted by Fos-choline-10, OG and also by methyl-6-(N-heptylcarbamoyl)-α-D-glucoside (Anameg7) with high efficiency, but stability issues in these detergents continued to hamper subsequent studies. The C-terminal domain of NaDC3 was also examined against an extended series of detergents and decyl-β-D-thioglucoside (DTG) was identified as the most effective surfactant, followed by two other glucosides, 3-cyclohexyl-1-pentyl-β-D-glucoside (Cyglu3) and Anameg7. Anameg7 was most effective for extraction of the N-terminal domain of NaDC3, along with two thioglucosides (OTG and DTG) and decanoyl-N-hydroxyethyl–glucamide (Hega-10) (data not shown).

Selectivity in the extraction of constitutive membrane proteins

Comparing the extraction with specific detergents for specific target proteins provides a useful view of detergent efficiencies for these proteins, but cannot provide the broad range of information needed to correlate detergent properties with different classes of membrane proteins. This wider view of detergent efficiencies can be obtained by examining the extraction of a variety of consti-

Table 2
Detergent extraction of specific target proteins.

Detergent[a]	Relative levels of extracted protein					
	KcsA[b] (*E. coli* XL1)	KcsA[b] (*R. rubrum*)	BtuB[b] (*E. coli* OM)	NaDC3[c] Full	NaDC3[c] N-term	NaDC3[c] C-term
None	560	640	150	490	530	160
DM	3070	3110	60	450	1020	460
OG	2790	5640	3000	1780	1120	650
Cymal-5	3130	4740	530	490	1240	160
Mega-9	630	2880	1300	460	1790	110
LDAO	880	440	870	490	560	–
Fos-choline-10	3280	3710	1340	1650	540	190

[a] See footnote b in Table 1 for detergent abbreviations.
[b] Relative levels of extracted proteins determined by using the *ImageJ* software package to calculate the density of each band (http://rsb.info.nih.gov/ij).
[c] Relative levels of extracted proteins determined by using the *Un-scan-it* software (Silk Scientific) to measure band pixel counts.

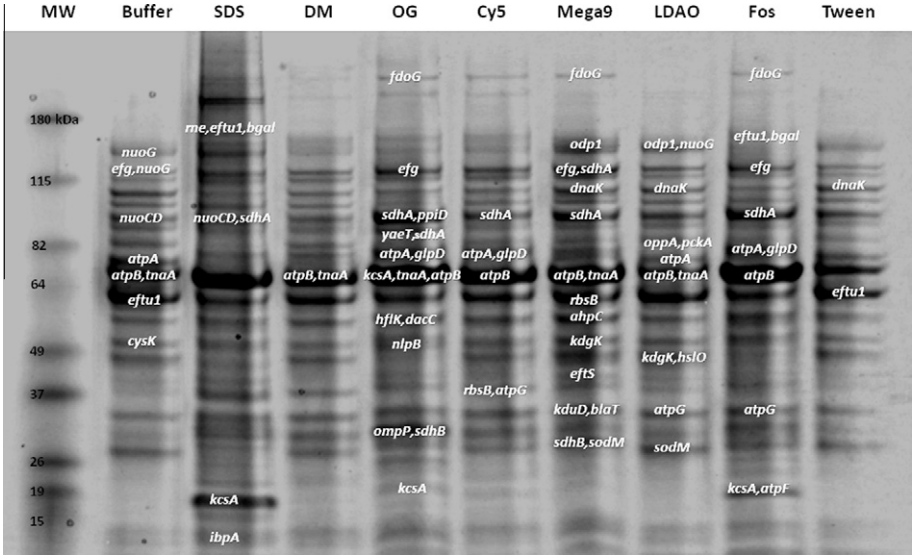

Fig. 3. Constitutive membrane proteins extracted by various detergents from the total cell membrane of *E. coli* XL-1 cells. Each detergent extract was concentrated to load an equal amount of protein (~50 μg) in each gel lane. The annotated proteins were each identified by peptide mass fingerprinting and, in some cases, further confirmed by MS/MS fragmentation.

tutive membrane proteins. Membrane extracts from *E. coli* were prepared by using the same set of specialty detergents, with the total protein extracted by each detergent measured, and the number and amount of the various extracted proteins quantitated by SDS–PAGE and identified by mass spectrometry. Extracts from each treatment were either concentrated or diluted to achieve the same amount of total protein loaded and separated by gel electrophoresis as an aid for the quantitation and identification of the individual proteins.

Relatively low levels of membrane and cytosolic proteins were extracted with buffer treatment alone, while the inclusion of SDS extracted the greatest amount of total protein and Tween 20 was only slightly more effective than the buffer control (Fig. 3). The number of distinct protein bands observed by 1-D gel electrophoresis ranged from as few as 20 to as many as 35 depending on the particular detergent used. As expected, mass spectral analysis identified mixtures containing two or more proteins in many of these bands (Fig. 3). Among the specialty detergents Fos-choline-10 was nearly as effective as SDS in extracting the greatest number of membrane proteins, followed by OG. Mega-9, Cymal-5 and DM were less effective in total protein extracted, with the zwitterionic detergent LDAO performing only slightly better than the buffer control (Fig. 3).

A total of 35 unique constitutive proteins were identified from the detergent extracts of the *E. coli* total membrane by using peptide mass fingerprinting and MS/MS analysis, with 14 assigned as membrane proteins, 3 periplasmic proteins and 18 classified as cytosolic or proteins with undetermined localization (Fig. 3). Fractionation of the outer membrane of the *E. coli* C41 strain followed by detergent extractions yielded 13 OM proteins, included one previously identified from total membrane extractions, 8 IM proteins, including 4 not observed from the total membrane, 1 additional periplasmic protein, 2 ribosomal proteins and 5 additional soluble/cytosolic proteins (Fig. 4). Thus, the different detergent extrac-

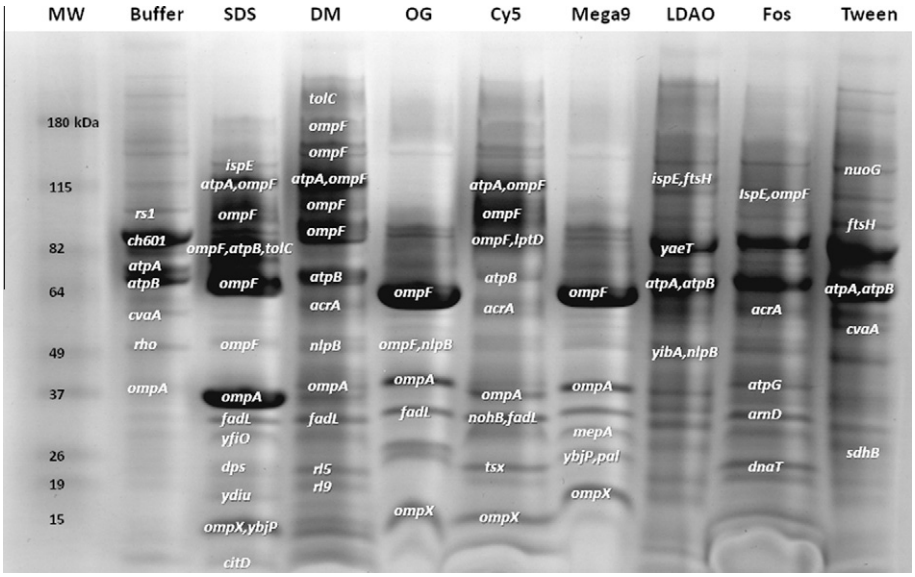

Fig. 4. Constitutive membrane proteins extracted by various detergents from the outer cell membrane of *E. coli* XL-1 cells. Each detergent extract was concentrated to load an equal amount of protein (~50 μg) in each gel lane. The annotated proteins were each identified by peptide mass fingerprinting and, in some cases, further confirmed by MS/MS fragmentation.

tions from both *E. coli* membrane fractions yielded a total of 59 unique proteins, including 36 integral, periplasmic and ribosomal proteins identified from these bacterial membranes.

Many of the identified IM proteins belong to bioenergetic protein complexes (Table 3), including subunits of ATP synthase, succinate dehydrogenase and NADH-quinone reductase complexes [34]. However, each of these protein subunits is optimally extracted by different types of detergents. Fos-choline-10 effectively extracted several ATP synthase subunits, while OG and Mega-9 also gave the best extractions of the succinate dehydrogenase and LDAO was most efficient with an NADH-quinone reductase subunit. Four of the eight ATP synthase subunits were extracted and identified, including the α-, β- and γ-subunits of the F_1 catalytic core (atpA, atpB and atpG) and the b subunit from the F_0 proton channel (atpF) (Table 3). Two of the four succinate dehydrogenase subunits (sdhA and sdhB) were extracted and identified, as was the nuoG subunit of NADH-quinone reductase. For other IM proteins OG and Fos-choline-10 are the most effective at extracting the proteins involved in membrane biogenesis and protein folding, while Tween20 effectively extracted a metalloprotease involved in protein degradation (ftsH), and Cymal-5 was the only detergent to extract the cytochrome bd ubiquinol oxidase (yhcB) [34].

Cymal-5 and Fos-choline-10 were generally effective in extraction of the periplasmic proteins, while LDAO was effective for extraction of the transport proteins but the least effective for a periplasmic peptidase and a respiratory complex protein (Table 3). The outer membrane porins were effectively extracted with the alkyl sugar detergents, but LDAO was generally ineffective for this class of membrane proteins. Cymal-5 and DM were quite effective in extracting OM transport proteins, while the OM proteins involved in membrane assembly and integrity were each extracted most efficiently by different types of detergents (Table 3).

Discussion

The extraction of integral membrane proteins from their lipid bilayer environment requires a delicate balance through the choice of surfactants with properties that are sufficient to disrupt the stabilizing forces between the proteins and the surrounding lipids without also irreversibly disrupting the internal stabilizing forces in the protein. Because of the wide range in the nature and extent of these stabilizing forces among different types of membrane proteins it is unlikely that a single set of ideal surfactant properties will be found that can achieve an optimal balance for every type of membrane protein. There are, however, some general trends that have been observed in surfactant properties. Detergents with small, charged head groups and relatively short alkyl chains tend to be much more disruptive to membrane protein structures than detergents with larger, neutral head groups and longer alkyl chains [35]. As a consequence ionic detergents such as SDS, while effective in the extraction of membrane proteins, tend to bind to these proteins with high affinity and cause significant protein denaturation. Even commonly used non-ionic and zwitterionic detergents, such as OG and LDAO, can cause denaturation of extracted membrane proteins [36].

Increasingly, milder and less denaturing non-ionic detergents have been chosen for the extraction, stabilization and crystallization of integral membrane proteins. In general, these classes of surfactants are less efficient than ionic surfactants in extracting proteins from their membrane environment. While the detailed mechanisms of detergent solubilization of membrane proteins still have not been fully delineated [37–39], it is clear that increasing the concentration of these detergents above their CMC values leads to higher levels of extracted proteins. However, this higher extraction efficiency must be balanced with an increasing tendency to cause protein denaturation even among these milder, non-ionic detergents. Given the 100-fold difference in CMC values for the detergents that were examined (Fig. 2) it is clear that the extraction efficiency is much more closely correlated with the CMC values of the detergents than with their absolute concentrations. The detergent levels that cause protein denaturation also varies with the type of detergent used and will likely also depend on the type of membrane protein being extracted.

A relatively large number of soluble, cytosolic proteins remained associated with the membrane fractions even after extensive washings before detergent extraction. Examination of the cytosolic proteins that were identified from the detergent extracts shows that some of these proteins have functional partners or proposed binding interactions with membrane proteins [40]. For example, dnaK is a heat shock chaperone (Hsp70) that functions together with a membrane anchored co-chaperone (djlA) [41]. The heat shock protein ibpA was found to copurify with an OM export protein (yfcU) by using an IMAC pull-down assay [42]. Glycerol-3-phosphate dehydrogenase (glpD) has multiple membrane associated functional partners, including a glycerol transporter (glpF) [43], an IM protein (plsY) and a periplasmic phospho-diesterase (glpQ) [44]. There are likely other protein interactions, either with specific functional partners or through non-selective binding, that have not yet been identified which would allow many of these identified cytosolic proteins to remain associated with the bacterial membrane during fractionation and washing.

While the extraction of many individual membrane proteins followed the same general trend that was observed for total protein extraction, there were a number of significant differences in extraction efficiencies for specific types of proteins. OG, Fos-choline-10 and Cymal-5 are each quite effective at extracting a wide range of membrane proteins. However, there are also a number of cases where there same detergents are among the least efficient at extracting a particular type of membrane protein. Based on the proteins that have been examined OG would not be the best detergent to choose for the extraction of bioenergetic complex proteins or drug efflux proteins, and Fos-choline is not particularly effective in the extraction of outer membrane porins (Table 3). In contrast, detergents such as LDAO and Tween20 which were generally ineffective in membrane protein extraction were efficient in the extraction of periplasmic transport proteins and an inner membrane metalloprotease (Table 3).

Despite the high efficiency with which many of these specialty detergents are able to extract specific membrane proteins, there were also a group of proteins that were only observed when the extraction was conducted with SDS. These included a cell wall processing protein (ygaU), an IM NADH reductase subunit (nuoCD) and a 50S ribosomal protein (rl28). A wider range of non-denaturing detergents must be examined to find suitable extraction conditions for these, and for other unextracted membrane proteins. There were also a group of proteins, primarily cytosolic in location, that were only observed in the buffer extraction. These included an Hsp60 chaperone protein (ch601), elongation (efts) and termination (rho) factors, and a cytoplasmic enzyme (cysK). It is likely that the wide range in the nature and extent of binding interactions between different proteins and their membrane environments will require a range of properties in the surfactants used to optimally extract each type of protein without causing extensive denaturation. A two-stage extraction protocol in which milder detergents are used for an initial extraction, followed by treatment with harsher detergents, could lead to the enrichment of proteins of interest into one of these extracts.

In addition to these observed differences in extraction efficiencies, detergents have been seen to alter both the gel migration rates and the oligomeric state of certain membrane proteins.

Table 3
Specialty detergent extraction efficiency for different types of membrane proteins.

Protein types	Most efficient	Least efficient
Inner membrane proteins	OG, Fos, Cymal	DM, Mega
Bioenergetic complexes		
ATP synthase		
atpA	DM, Cymal, LDAO, Tween, *Fos*	OG
atpB	All detergents	
atpG	Fos, LDAO, *Cymal, OG*[a]	
atpF	Fos	DM, Mega, LDAO
Succinate dehydrogenase		
sdhA	Fos, OG, Mega, Cymal, *LDAO*	
sdhB	Mega, OG, *Fos, DM*	
NADH-quinone reductase		
nuoG	DM, LDAO, Tween, *Fos*	Mega, OG
Other functions		
Membrane biogenesis (*dacC,hflK*)	OG, *Cymal, DM, Fos*	
Folding of OM proteins (*ppiD*)	OG	LDAO, Tween
Drug efflux (*acrA*)	DM, Cymal, Fos, *LDAO*	OG, Mega
Metalloprotease (*ftsH*)	Tween	All others
Ubiquinol oxidase (*yhcB*)	Cymal	Mega, OG
Periplasmic proteins	Fos, Cymal, OG	
Respiratory complex *(fdoG)*	OG, Mega, Fos, *Cymal*[a]	LDAO, Twee
Transport proteins		
Ribose transport (*rbsB*)	OG, *Fos, DM, Cymal, LDAO*	
Peptide uptake *(oppA)*	LDAO	
Outer membrane proteins	Cymal, OG, DM	LDAO
Porins		
ompA	OG, Mega, Cymal, DM, LDAO	Fos, Tween
ompF (dimer)	OG, Mega	DM, Cymal, LDAO, Fos
ompF (oligomers)	DM, Cymal	OG, Mega, LDAO
ompX	OG, Cymal, Mega, *Fos*[a]	LDAO
Transport proteins		
tolC	DM, *Cymal, LDAO*	OG, Mega, Fos
tsx	DM, Cymal, OG, Fos	Mega, LDAO
fadL	OG, DM, Cymal, Tween, *Mega*	
Membrane assembly and integrity		
nlpB	DM, OG, LDAO, *Fos,Mega, Cymal*	
yaeT	OG, DM	Cymal, Mega
yfiO	DM	OG
lptD	DM, Cymal, Mega, *OG*	LDAO, Fos
pal, ybjP	DM, Mega	Cymal, LDAO
Protease (*ompP*)	OG, *Cymal, Fos, DM*	

[a] Protein extractions by the detergents in *italics* have been suggested by similar gel band positions, but not confirmed by mass spectrometry.

SDS–PAGE is a rapid and reliable method to assess the purity of a protein and to verify its identity based on its relative molecular mass. But, for membrane proteins, this reliability can be compromised by anomalous shifts in band positions caused by the properties of the various detergents used for extraction. For example, ompA is an outer membrane transport protein with a calculated mass of 37.2 kDa. This protein migrates near the 37 kDa standard protein in the buffer control, but this band position varies significantly depending on the extraction detergent, with the migration in SDS showing a lower molecular mass and the band position in OG corresponding to an anomalously higher mass (Fig. 4). Similar shifts in band positions are seen for other membrane proteins and are likely a consequence of differences in the number of detergent molecules that are associated in each protein complex, leading to changes in the effective size for individual protein-detergent complexes.

Changes in the detergent properties can also lead to changes in the oligomeric structure of the extracted membrane proteins. The α-subunit of ATP synthase (atpA) was extracted in a dimeric form with some of the detergents (DM and Cymal-5), while this dimer dissociated when extracted by LDAO and Tween (Fig. 4). The ribose transport protein (rbsB) is extracted as a monomer by Cymal-5 (Fig. 3), but appears at a molecular weight corresponding to a dimer when extracted by Mega-9. The functional trimer of the transport protein tolC is extracted by DM, but extraction with SDS leads to a band that migrates closest to the position expected for the tolC monomer (Fig. 4). The OM porin ompF (37 kDa monomer) is a functional trimer to allow the passage of low molecular weight neutral molecules. ompF is highly abundant in the bacterial OM and is observed to migrate as a dimer in the glucoside detergents OG and Mega-9 (Fig. 4). However, with structurally similar maltosides ompF is observed as a trimer in Cymal-5 and as a trimer and higher oligomers in DM. Variations in the nature of the interactions between related alkyl sugar detergents and individual membrane proteins can not only affect the efficiency of protein extractions, but also the likelihood of subunit dissociation and protein denaturation.

Conclusions

The extraction efficiency of expressed membrane proteins can vary dramatically with different detergents. Because these efficiencies have been shown to differ by an order of magnitude or more, the trends that have been identified show the value of screening detergents from different structural classes to identify the best match for a particular target protein. Identifying detergents that have increased efficiency for the extraction of a protein of interest, especially those detergents that are generally less effective at total protein extraction, will lead to enrichment of that protein in the membrane extracts. Increasing detergent concentrations can lead to improved extraction efficiency, but this enhanced efficiency must be balanced with the increased tendency to cause protein denaturation even with the relatively mild, non-ionic detergents. The optimal extraction conditions may not be the best conditions for long term stability and characterization. However, given the time and effort required to stabilize and purify membrane proteins, a small investment of time and material can provide significantly higher levels of a target protein with which to begin subsequent characterization studies.

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