A high-throughput method for membrane protein solubility screening: The ultracentrifugation dispersity sedimentation assay

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Abstract

One key to successful crystallization of membrane proteins is the identification of detergents that maintain the protein in a soluble, monodispersed state. Because of their hydrophobic nature, membrane proteins are particularly prone to forming insoluble aggregates over time. This nonspecific aggregation of the molecules reduces the likelihood of the regular association of the protein molecules essential for crystal lattice formation. Critical buffer components affecting the aggregation of membrane proteins include detergent choice, salt concentration, and presence of glycerol. The optimization of these parameters is often a time- and protein-consuming process. Here we describe a novel ultracentrifugation dispersity sedimentation (UDS) assay in which ultracentrifugation of very small (5 μL) volumes of purified, soluble membrane protein is combined with SDS-PAGE analysis to rapidly assess the degree of protein aggregation. The results from the UDS method correlate very well with established methods like size-exclusion chromatography (SEC), while consuming considerably less protein. In addition, the UDS method allows rapid screening of detergents for membrane protein crystallization in a fraction of the time required by SEC. Here we use the UDS method in the identification of suitable detergents and buffer compositions for the crystallization of three recombinant prokaryotic membrane proteins. The implications of our results for membrane protein crystallization prescreening are discussed.

Keywords: membrane proteins; stability; aggregation; monodispersity; ultracentrifugation; detergent; high throughput

The last 10 years have seen a dramatic increase in the number of high-resolution membrane protein structures (Berman et al. 2000; Loll 2003; Raman et al. 2006). This rapid progress can be attributed in part to the growing number of tools available to structural biologists: crystallization robots, a wide range of commercially available

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Abbreviations: C_8E_4, n-octyltetraoxyethylene; $C_{12}E_8$, olyoxyethylene(8)dodecyl ether; $C_{12}E_9$, polyoxyethylene(9)dodecyl ether; CMC, critical micelle concentration; cymal-5, 5-cyclohexyl-1-pentyl-β-D-maltoside; cymal-6, 6-cyclohexyl-1-hextyl-β-D-maltoside; DDM, n-dodecyl-β-D-maltoside; DDMG, n-dodecyl-N,N-dimethylglycine; DM, n-decyl-β-D-maltoside; DTM, n-decyl-β-D-thiomaltoside; FC-12, n-dodecylphosphocholine; FPLC, fast performance liquid chromatography; MP, membrane protein; NG, n-nonyl-β-D-glucoside; NM, n-nonyl-β-D-maltoside; OG, n-octyl-β-D-glucoside; OM, n-octyl-β-D-maltoside; SEC, size exclusion chromatography; TriDM, n-tridecyl-β-D-maltoside; UDM, n-undecyl-β-D-maltoside; UDS, ultracentrifugation dispersity sedimentation.

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detergents, and protein from recombinant sources (Drew et al. 2006; Wagner et al. 2006). Nevertheless, obtaining high-resolution membrane protein structures is still a considerable challenge. Overexpression, detergent solubilization, and purification of active membrane protein remain major hurdles (Eshaghi et al. 2005). Even if highly pure, active membrane protein can be obtained, crystallization is by no means certain.

Monodispersed protein particles are the building blocks of stable crystal lattices. Because of their hydrophobic nature, membrane proteins in solution tend to nonspecifically aggregate over time. This irregular association of the protein molecules in solution reduces the likelihood of the regular association essential for crystal lattice formation.

One of the key factors in determining the formation of nonspecific aggregates is the choice of detergent. Large micelle-forming detergents such as n-dodecyl-β-D-maltoside (DDM) and polyoxyethylene(9)dodecyl ether ($C_{12}E_9$) are more likely to maintain a membrane protein in solution; however, the large size of the micelle means less of the protein molecule is exposed to form protein–protein interactions essential for crystal lattice formation. In contrast, small micelle detergents, such as n-octyl-β-D-glucoside (OG) and n-octyl-β-D-maltoside (OM), leave more of the protein molecule exposed to form the protein–protein contacts vital for strong crystal-lattice formation but can also cause undesirable aggregation if the hydrophobic regions are not covered completely by the small micelle.

There are a number of methods for screening the aggregation status of membrane proteins. The method used by most laboratories is size exclusion chromatography (SEC) (Scott et al. 2001; Lemieux et al. 2002, 2003; Kawate and Gouaux 2006). By separating the particles in a pure protein sample based on their size, it is possible to assess the extent of aggregation. The advantage to this method is that most laboratories have SEC columns and equipment as standard. However, SEC is low throughput, with typically only 2–3 samples analyzed per day, and requires large volumes of protein and detergent-containing buffers. In addition, the protein sample is usually applied to the column at a much lower (in our hands usually 10-fold lower) concentration than that used for crystallization trials. Since aggregation is often concentration dependent, the results may not be indicative of the state of the protein sample prior to crystallization setup. One alternative method is negative stain electron microscopy, which was used successfully to screen for monodispersity of the membrane protein succinate quinone oxidoreductase prior to crystallization. However, this method is lower throughput than SEC and comparatively demanding in terms of specialized equipment and expertise. Much time must be spent both optimizing the concentration of the protein sample loaded into the microscope (Harris 1996; Ruprecht and Nield 2001) and analyzing the micrographs.

Another method frequently employed to assess aggregation of soluble proteins is light scattering (Wilson 2003). Recently a high-throughput solubility assay for recombinant protein immunogens has been reported (Stenvall et al. 2005). So far, neither method has been routinely used for analysis of membrane proteins.

Here we describe a novel alternative, high-throughput ultracentrifugation dispersity sedimentation (UDS) assay for assessing the aggregation status of pure, concentrated membrane protein prior to crystallization. In this method, protein aggregates in small volumes of detergent-solubilized membrane proteins are removed by ultracentrifugation. Subsequently, the amount of remaining soluble protein in the samples is quantified by SDS-PAGE analysis. We have applied this assay to several membrane proteins currently undergoing crystallization trials in our laboratory. Results are shown for three recombinantly expressed prokaryotic transport proteins referred to as MP (membrane protein)-A (belonging to the ATP-binding cassette Superfamily) and MP-B and MP-C (belonging to the Major Facilitator Superfamily). Based on the results of our analyses to date, we suggest a list of detergents for initial screening of all new membrane protein samples.

Results and Discussion

The UDS method (see Fig. 1 for a summary of the methodology) allows rapid determination of the dispersity of pure membrane protein samples. The assay is based on the assumption that protein aggregates are orders of magnitude heavier than dispersed protein particles and can therefore be removed by sedimentation at high *g* forces. Prior to UDS, the membrane protein of interest is purified in a high ionic strength buffer containing 300 mM NaCl. This serves to suppress heavy aggregation if the primary detergent is not optimal. The primary detergent is usually DDM since this has been shown to maintain many membrane proteins in a stable state over the prolonged periods of time (2–4 d) required for sample preparation (data not shown).

The first step of the UDS assay (Fig. 1) involves the removal by ultracentrifugation (100,000*g*; 45 min; 4°C) of any aggregates that may have accumulated during purification. The concentration of the protein is then determined using a typical method such as the bicinchoninic acid assay. The sample is then diluted at least fivefold into test buffer, usually to a concentration of about 0.1 mg/mL. At this concentration, membrane protein aggregation is usually low even in suboptimal detergents.

For detergent/buffer exchange the membrane protein is immediately applied to a Vivaspin 500 concentrator with the appropriate molecular weight cutoff filter. The detergent is exchanged using three successive concentration-dilution steps. In the case of the proteins described here,

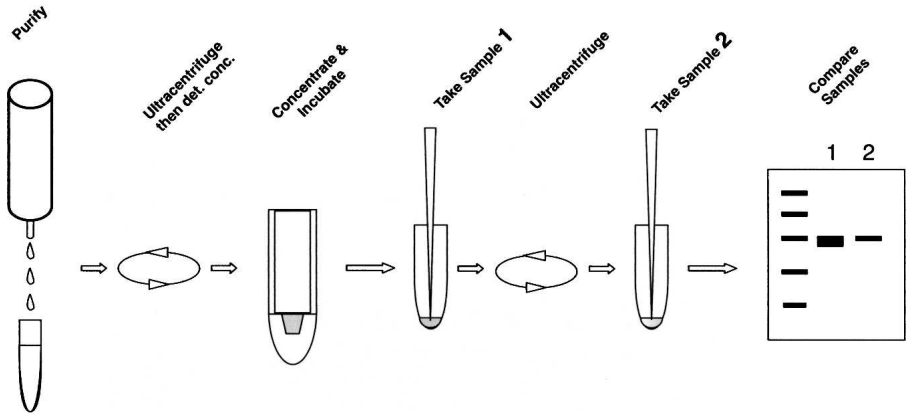

Figure 1. Schematic workflow of UDS is shown for a membrane protein where no previous information on behavior in different detergents is available.

filters were used with a cutoff of 50 kDa. After the buffer has been diluted 1000-fold, the protein is concentrated to a 5-μL volume at 5–20 mg/mL and stored at 4°C for 16 h. This preincubation step allows time for any aggregation to develop.

Prior to ultracentrifugation, a 1-μL control sample (1) of the protein is removed, mixed with SDS-PAGE loading buffer (LDS, Invitrogen), and stored (Fig. 1). The remaining protein solution is ultracentrifuged at 350,000*g* for 45 min at 4°C. Thereafter, a second 1-μL sample (2) is taken, and the amount of protein is compared to that in sample (1) by comparing protein densities on Coomassie Brilliant Blue-stained SDS-PAGE. In terms of the time, detergent, and amount of protein required, UDS is far superior to SEC. In our hands, only about 50–100 μg of protein is required to screen a single detergent, while up to 14 samples can be run in parallel using a standard ultracentrifugation rotor such as the TLA-120 (Beckman-Coulter). Centrifugation is often used to remove aggregates from protein samples (D'Arcy 1994) and SDS-PAGE has been used to assess the recovery of membrane protein following solubilization (Columbus et al. 2006). However, this method is unique in combining both techniques to screen for optimal buffer conditions for membrane proteins prior to crystallization screens.

Application of the UDS assay to the identification of optimum buffer conditions

Many biochemical assays that are used to investigate membrane protein function are fully compatible with elevated concentrations of glycerol and salt. These agents are widely applied to stabilize membrane proteins in solution. Glycerol generates a more native environment for the membrane proteins by reducing the concentration of water and increasing the hydrophobicity (Iwata 2003; Byrne and Jormakka 2006), and salt ions reduce the energetic cost of accommodating hydrophobic surfaces in aqueous solution (Vogel et al. 2001). However, high salt and glycerol concentrations can interfere with crystallization (Iwata 2003). Hence, crystallization of membrane proteins necessitates a different approach to protein preparation than biochemical assays.

Since solubilized MP-A had previously been biochemically characterized in the presence of 10% glycerol and 100 mM NaCl, we performed initial SEC under these conditions. MP-A showed heavy aggregation in n-decyl-β-D-maltoside (DM), DDM, OG (Fig. 2A), and $C_{12}E_9$ (data shown for DDM and OG only). To facilitate the screening of detergents, we made use of the UDS assay. The results obtained agreed with SEC data and enabled higher-throughput testing (Fig. 2B). In the first round we assessed the aggregation status of MP-A in 12 different detergents. All the samples were maintained in buffer containing 100 mM NaCl and 10% glycerol. Three detergents, polyoxyethylene-(8)dodecyl ether ($C_{12}E_8$), n-dodecylphosphocholine (FC-12), and n-tridecyl-β-D-maltoside (TriDM), maintained MP-A in a stable, monodispersed state under these conditions (Fig. 2B). SEC analysis of MP-A in $C_{12}E_8$ confirmed the absence of aggregation in this sample (Fig. 2D).

The UDS assay was repeated in the presence of FC-12, $C_{12}E_8$, or TriDM in a range of reduced salt (0–50 mM) and no glycerol buffers (Fig. 2B). In the case of $C_{12}E_8$ and TriDM, salt proved to be essential for stability. In contrast to TriDM, MP-A was stable in as little as 50 mM NaCl in the presence of $C_{12}E_8$. Therefore, $C_{12}E_8$ conveyed a higher degree of stability on MP-A than TriDM did. Only FC-12 maintained MP-A soluble and monodispersed in the absence of both salt and glycerol (Fig. 2C).

Crystallization trials were performed using MP-A purified in FC-12 and $C_{12}E_8$. Preliminary crystals were obtained using FC-12 (Fig. 2E). Here the UDS assay was applied to the incremental optimization of the sample buffer using only a few milligrams of protein.

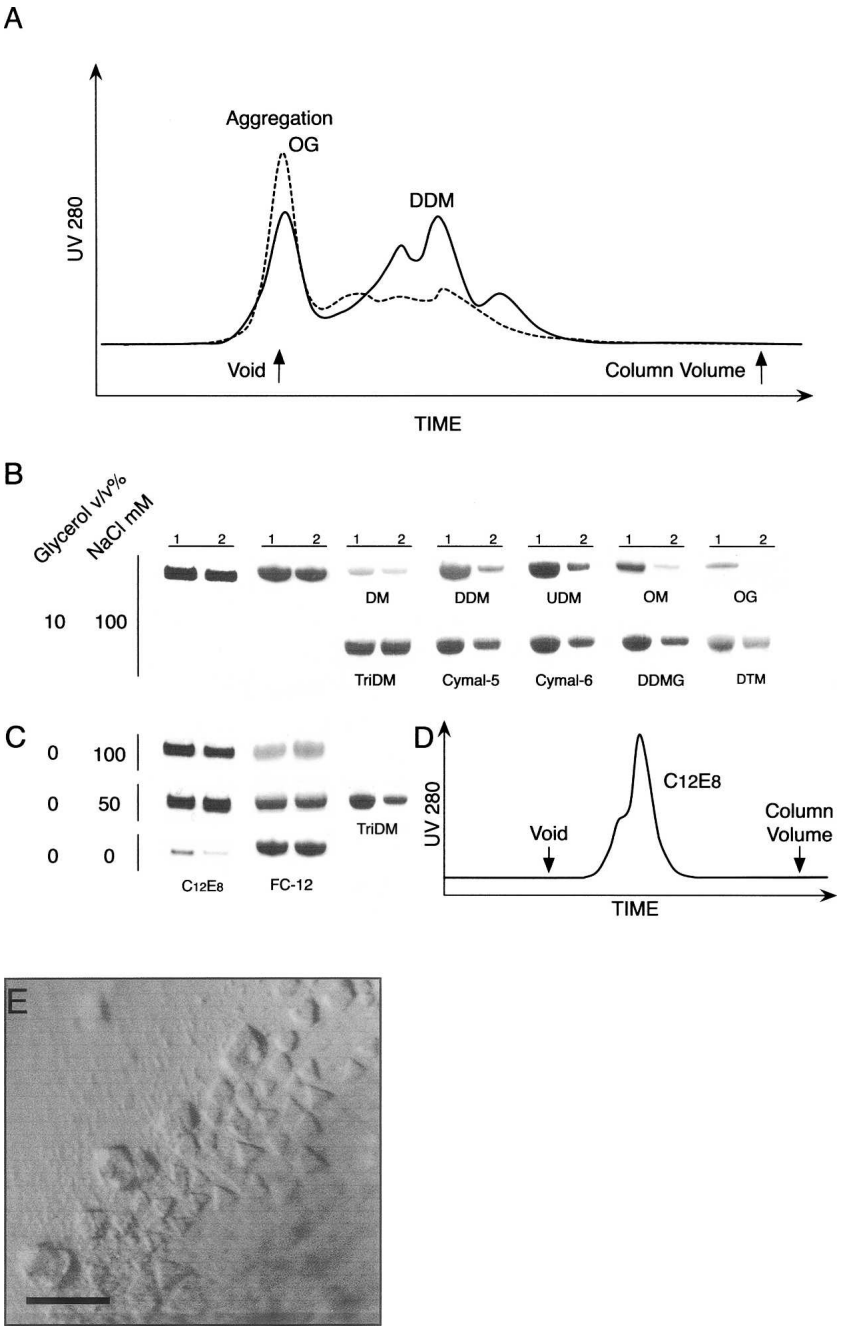

Figure 2. (*A*) SECs for MP-A maintained in OG (broken line) and DDM (solid line). (*B*) UDS of MP-A in a range of different detergents in the presence of 10% glycerol and 100 mM NaCl. (*C*) UDS of MP-A in $C_{12}E_8$, FC-12, and TriDM in 0% glycerol and decreasing concentrations of NaCl. (*D*) SEC of MP-A in buffer containing 0.05% $C_{12}E_8$ and 100 mM NaCl. (*E*) Crystals of MP-A obtained in 0.1% FC-12. Bar, 50 μm.

Economical screening of detergents

In contrast with MP-A, many membrane proteins are stable in a range of detergent and buffer conditions. Nevertheless, crystallization is often facilitated if membrane proteins can be maintained in small micelle detergents such as OG, OM, or n-octyltetraoxyethylene (C_8E_4), as demonstrated by the relatively large number of membrane protein structures solved in these detergents (Raman et al. 2006). Because of their small micelle size, such detergents are thought to be far less likely to interfere with the formation of stable crystal lattices.

Two other membrane proteins currently undergoing crystallization trials in our laboratory, MP-B and MP-C, were both successfully crystallized in DDM and data was collected to 4.5 Å (Fig. 3A,B) and 6.5 Å, (Fig. 3C,D), respectively. To improve chances of obtaining crystals diffracting to higher resolutions, both membrane proteins were analyzed by UDS over a range of detergents with a low or high critical micelle concentration (CMC). Both proteins proved to be very stable under these conditions (Fig. 4A,C). Available SEC data for MP-B and MP-C (Fig. 4B,D) correlated extremely well with the UDS results, which demonstrates the general applicability of this method to membrane proteins other than MP-A. The data showed that both proteins were stable in the small micelle-forming detergent OM (Fig. 4A,C). The use of OG, however, led to strong aggregation for both proteins. Therefore, OM is a possible alternative detergent or additive in the next stages of MP-A and MP-C crystallization.

UDS as a prescreening method for high-throughput membrane protein crystallization

The data presented here clearly show that the results obtained using UDS were comparable with those using SEC. This indicates that the difference in protein concentration used for the two methods was not critical, at least for the test proteins. We believe that this novel approach is an ideal method for identifying detergents and other buffer conditions, which maintain target membrane proteins in a state suitable for crystallization trials. Importantly, the UDS is an inexpensive way to screen high CMC detergents such as OG, n-nonyl-β-D-glucoside (NG), n-nonyl-β-D-maltoside (NM), 5-cyclohexyl-1-pentyl-β-D-maltoside (CYMAL-5), 6-cyclohexyl-1-hexyl-β-D-maltoside (CYMAL-6), and others. For SEC, a buffer volume of ~100 mL was used, whereas a few hundred microliters of detergent-containing buffer is sufficient for one UDS experiment. Therefore, in terms of the amount of detergent required, SEC is at least 100 times more expensive than the UDS.

Homolog screening of membrane proteins is an increasingly used strategy to obtain membrane protein structures by both individual laboratories (Chang et al. 1998; Locher et al. 2002; Dawson and Locher 2006) and structural genomics consortia. This approach entails the cloning and expression of homologous proteins in parallel and crystallization thereafter. The rationale behind this approach is that although given membrane protein homologs from different organisms often have the same biological function, expression and stability in identical

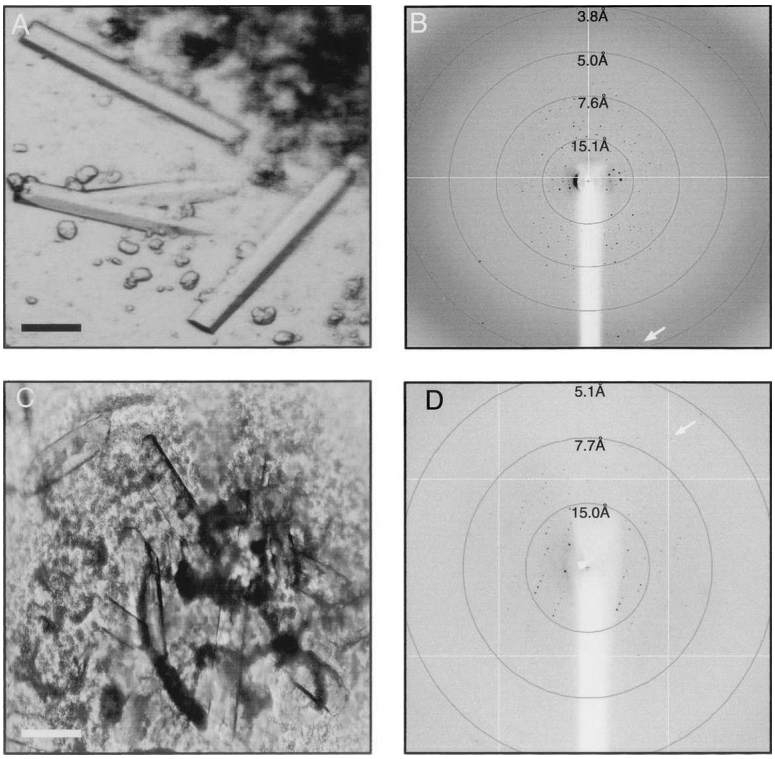

Figure 3. (*A*) Crystals of MP-B grown in the presence of DDM. Bar, 200 μm. (*B*) MP-B diffracted X-rays to up to 4.0 Å. (*C*) Crystals of MP-C grown in buffer containing DDM; the crystals were photographed under a polarizer for clarity. Bar, 200 μm. (*D*) MP-C crystals diffracted anisotropically with some reflections to 6.5 Å.

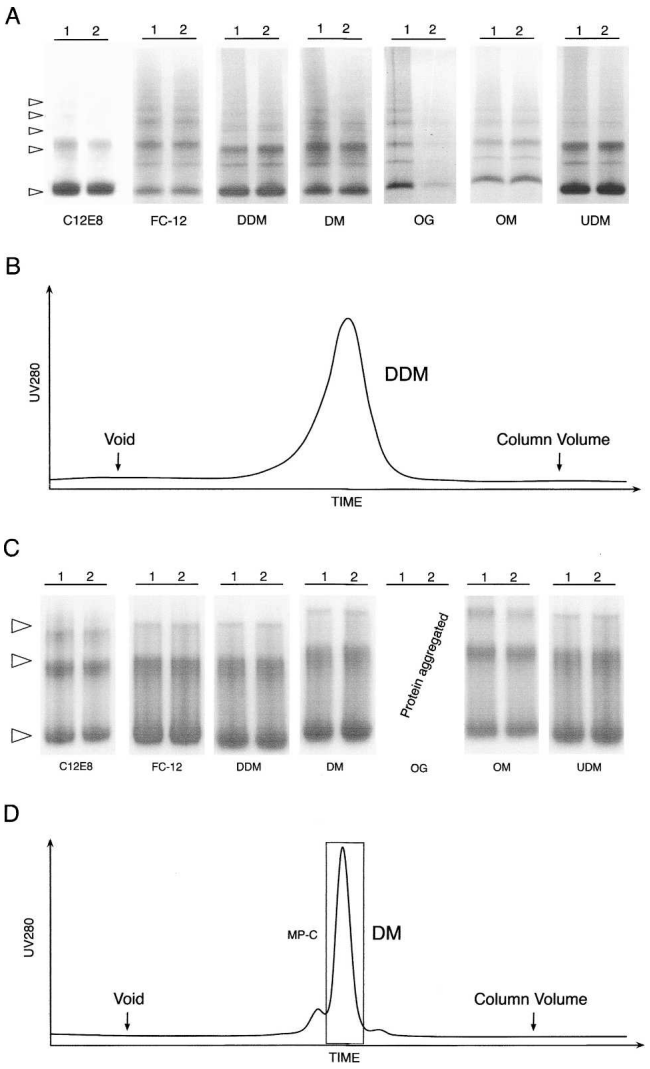

Figure 4. UDS of MP-B (*A*) and MP-C (*C*) in a range of different detergents. Arrows indicate protein bands. SEC analysis of MP-B in DDM (*B*) and MP-C (*D*) in DM.

detergents vary widely (Lundstrom 2006). SEC is difficult to incorporate into a structural genomics pipeline. UDS is highly parallel, uses small amounts of protein, and is comparatively cheap, making it an ideal method for rapid analysis of large numbers of membrane proteins.

An initial detergent screen

The UDS approach allows quick screening for stability of membrane proteins in parallel using relatively small amounts of sample; ~50–100 μg of protein are sufficient to obtain well-interpretable results. Initially, it is not necessary to screen a large number of detergents. A good approach is to test detergents with a range of different chain lengths (C_n): TriDM (C_{13}), DDM (C_{12}), DM (C_{10}), and OM (C_8). We also recommend testing detergent analogs of similar chain lengths but alternative head groups: FC-12 (C_{12}), $C_{12}E_8$ (C_{12}), and OG (C_8).

The data presented here for MP-B and MP-C demonstrated the importance of the detergent head group, both being unstable in OG but stable in OM. DDM and DM have good track records in membrane protein solubilization, purification, and crystallization (Iwata 2003). In addition to the results presented here for MP-A, FC-12 has been shown to maintain the GABA transporter in a stable, monodispersed, and active state (Li et al. 2001). Other larger micelle-forming detergents, $C_{12}E_8$, shown to be suitable for certain eukaryotic membrane proteins such as SERCA1a and Band3 (Toyoshima et al. 2000; Lemieux et al. 2002), and TriDM, have also been included.

Conclusion

This paper describes a novel method for rapid assessment of the aggregation status of membrane protein using small amounts of pure material. This method could easily be integrated into the workflow of a structural genomics pipeline. The assay can be used for fine-tuning sample buffers, determining detergents suited for crystallization, and clearing the pipeline of proteins that are unlikely to crystallize, and hence, should not be submitted to crystallization trials.

Materials and Methods

Chemicals

All detergents were purchased from Anatrace at the highest purity grade available.

X-ray data collection

Diffraction data for all crystals were collected at the European Synchrotron Radiation Facility (ESRF). Diffraction data for MP-B and MP-C were collected at ID14–2 at 100K using an ADSC Q4 CCD detector and at ID29–2 at 100K using an ADSC Q210 2D CCD detector, respectively.

Expression and purification of the membrane proteins

All three membrane proteins were cloned from genomic DNA into standard expression vectors. Expression was performed in prokaryotic cells using standard protocols. The cells were harvested at 3000*g* and resuspended in lysis buffer containing protease inhibitor tablets (Roche). After lysis using a cell disruptor (Constant Cell Disruption Systems), unbroken cells were removed by centrifugation at 8000*g* for 15 min. Membranes were harvested from the resulting supernatant by ultracentrifugation for 1 h at 100,000*g*. For each of the target proteins, the membranes were solubilized in 1% DDM. Insoluble material was then removed by ultracentrifugation at

100,000*g* for 1 h. All steps following cell harvest were carried out at 4°C; all purification buffers contained 300 mM NaCl. The solubilized proteins were individually purified by one-step Ni^{2+}-NTA (Qiagen) affinity chromatography. The fractions containing the purified target proteins, as assessed by SDS-PAGE, were pooled prior to UDS. The concentration of the protein was confirmed using the BCA assay (Pierce).

UDS

All ultracentrifugation steps were performed on a Beckman-Coulter Optima Max centrifuge at 4°C. All samples were centrifuged at 350,000*g* for 45 min in a TLA-120 (Beckman-Coulter) rotor. Detergents were used at 3× their CMC unless stated otherwise. Buffers for MP-A and MP-B contained 20 mM HEPES at pH 7.0, whereas MP-C buffers contained 100 mM Tris-HCl in addition to the detergent. The first stage centrifugation at 100,000*g* utilizes a TLA-55 rotor. The second small-volume ultracentrifugation is performed with a TLA-120 rotor and 200-μL polycarbonate tubes (Beckman-Coulter). Vivaspin 500 concentrators used for buffer exchange were obtained from Sartorius. The samples were run on 4%–12% Bis-Tris NuPage SDS-PAGE gels from Invitrogen. Protein bands were visualized using Imperial Stain from Pierce.

SEC

For MP-A, all SEC runs were carried out at 4°C on a Superose 6 10/300 (GE Healthcare) column with buffer containing 50 mM HEPES at pH 7.0, 100 mM NaCl supplemented with either 0.05% DDM or 0.05% $C_{12}E_8$. If the protein was maintained in OG (1%), then the buffer was modified slightly to contain 50 mM H at pH 7.0 and 180 mM NaCl. MP-B was analyzed on a Superose 6 10/300 column. The buffer contained 20 mM Tris at pH 8.0, 150 mM NaCl, and 0.03% DDM. The SEC of MP-C in DM (0.2%) with buffer containing 50 mM Tris at pH 7.5, 150 mM NaCl was performed on a Superdex 200 10/300 (GE Healthcare) column.

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