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Simple screening method for improving membrane protein thermostability

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Abstract

Biochemical and biophysical analysis on integral membrane proteins often requires monodisperse and stable protein samples. Here we describe a method to characterize protein thermostability by measuring its melting temperature in detergent using analytical size-exclusion chromatography. This quantitative method can be used to screen for compounds and conditions that stabilize the protein. With this technique we were able to assess and improve the thermostability of several membrane proteins. These conditions were in turn used to assist purification, to identify protein ligand and to improve crystal quality.

Keywords

Membrane proteins; Size exclusion chromatography; Protein thermostability

1. Introduction

Integral membrane proteins play an essential role in many diverse cellular processes, including the transport of solutes across the cell membrane and facilitating communication between cells and their environment. As a result, membrane proteins are the targets of many pharmaceuticals, including β -blockers, antidepressants and bronchodilators. While approximately one third of human genes encode membrane proteins [1], high resolution structures of membrane proteins are few compared to their water soluble protein counterparts. Indeed, fewer than 300 independent structures of integral membrane proteins have been determined from all organisms thus far (<http://blanco.biomol.uci.edu/index.shtml>). A better understanding of the structure of membrane proteins would provide invaluable answers to many fundamental questions in physiology, pharmacology and medicine.

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The pursuit of high-resolution structural information on membrane proteins is a challenging task. The major bottlenecks include the expression of sufficient quantities of functional and stable proteins and the optimization of the growth of well-ordered crystals for X-ray analysis. The best crystallization conditions cannot be formulated *a priori*; they must be determined empirically.

The best indicator of the crystallizability of a membrane protein are its monodispersity and thermostability [2; 3; 4; 5; 6; 7; 8; 9; 10; 11]. Typically, a monodisperse sample, a sample in which the protein exists in a unique oligomeric state in solution, displays a single symmetrical peak on size exclusion chromatography (SEC) column [12; 13; 14; 15]. Such a monodisperse sample defines an ideal starting point for membrane protein crystallization [2; 3]. Monitoring monodispersity has been used repeatedly to find the optimum detergent, pH and other stabilizing conditions, which translate into better crystal growth [5]. SEC coupled with fluorescence can successfully monitor monodispersity of a GFP-fusion protein before purification [7].

While monodispersity is often essential for the crystallization of water-soluble and membrane-bound proteins alike [16], thermostability is particularly important for membrane proteins [17], perhaps because they are often unstable in detergent solution to start with. Thermostability of a protein, typically characterized as its melting temperature, is the most reliable indicator of its overall stability [18]. Therefore, membrane protein thermostability is often used as a parameter for choosing better crystallization candidates or improving their crystallizability. Proteins derived from thermophilic organisms have proven to be more amenable to crystallization [19; 20]. In addition, mutagenesis of membrane proteins has resulted in increased thermostability [21]. More stable proteins can be purified in harsher detergents with smaller micellar size and, therefore, form better-ordered crystals [22].

Thermostability of a purified sample can be assessed by several methods, including differential scanning calorimetry [23; 24], circular dichroism [25; 26], gel electrophoresis and fluorescence [25] and absorbance [27; 28]. Such classical methods require large amounts of purified protein. A more sensitive approach assesses thermostability by measuring binding activity using radiolabeled substrate, which can be accomplished without protein purification [22]. More recently, however, a technique capable of doing high-throughput thermostability screens on membrane proteins using a thiol-specific fluorochrome was developed [29]. Most of these techniques quantify the fraction of protein that survives treatments at elevated temperatures.

In this paper we describe a simple method to screen for protein stability in a quantitative manner. The goal is to identify conditions that stabilize a purified sample while maintaining its monodispersity. The method was applied to three examples. Screening using this method led to improved solubility of a membrane bound enzyme in detergent, identification of the permeating ion for a channel and improvement of the X-ray diffraction from crystals of a secondary transporter.

2. Principle of the method

Protein stability assures a better starting point for crystallization screening as well as for other biochemical or biophysical characterizations. Our method begins with a purified protein sample that is capable of generating a monodisperse peak in gel filtration (Figure 1). The protein sample is first put under stress conditions with the aim of denaturation or aggregation. These denaturation methods include heating, addition of chaotropic agents or concentrating until precipitation. Once the denaturing conditions are established, the protein is subjected to the same treatment in the presence of putative substrate or other possible stabilizing agents. The goal is to find a condition that can rescue the protein from

denaturation. Quantification of the non-denatured fraction is achieved by analytical size-exclusion chromatography on high pressure liquid chromatography (HPLC).

Temperature can be used to denature a membrane protein in solution. In this case, the samples are incubated for 10 minutes at over a temperature range that includes total denaturation of the protein. The samples are then injected onto an analytical SEC column coupled to an HPLC instrument. The peak height of the SEC profile at each temperature is proportional to the fraction of protein remaining after denaturation. From a series of these data, a melting curve is generated and an apparent melting temperature (T_m) estimated. Here, T_m is defined as the temperature for which the peak height becomes half that of the original unheated sample (4 °C). Addition of putative substrates or suspected stabilizing agents should increase the resistant fraction leading to an increase in the apparent T_m . Thus, the assessment of protein thermostability, and the comparison of different stabilizing factors can be achieved in a quantitative manner.

3. Materials and methods

The membrane protein sample was purified by metal affinity chromatography followed by preparative size exclusion chromatography at 4 °C using Purification Buffer (100 mM NaCl, 5 % glycerol, 50 mM Tris-HCl pH 7.5, and 0.05 % dodecyl- β -D-maltopyranoside (DDM)). Purified samples in aliquots of 20–50 μ g (100 μ l) were incubated at different temperatures for 10 minutes (Figure 1) using a thermocycler (Bio-Rad, Hercules, CA). Following brief centrifugation to remove the aggregates, all the supernatant was injected on a Shodex KW804 analytical SEC column (Thomson, Clear Brook, VA) on HPLC (Shimadzu, Columbia, MA) in buffer containing 200 mM Na₂SO₄, 50 mM Tris pH 8.0, 3 mM NaN₃ and 0.05 % DDM. Melting curve was constructed by measuring the height of the SEC peak for each temperature. The height of the SEC peak for the control (the untreated sample kept at 4 °C) was used as a reference for normalization. The melting curves were fitted with a Boltzmann sigmoidal equation using Prism5 (GraphPad Software, La Jolla, CA), from which T_m was extracted. For screening of stabilizing conditions, aliquots were incubated with the additives for 2 hours at 4 °C, followed by incubation at the particular T_m for 10 minutes. Alternatively, entire melting curves were constructed for the screen conditions.

4. Results

4.1 Improved solubility of a lipid phosphatase

Integral membrane lipid phosphatases (LPs) play an essential role in the biosynthesis and catabolism of various lipid species in many bacteria. For example, in *Escherichia coli* the LPs BacA, LpxT, and PgpB dephosphorylate the essential carrier lipid undecaprenyl phosphate [30] and confer resistance to the antibiotic bacitracin upon overexpression [31]. Additionally, bacterial LPs are structural homologs of glucose-6-phosphatase [32], a key enzyme in human gluconeogenesis. While the catalytic mechanism between LPs is likely to be conserved, details of their three-dimensional structure and substrate recognition are unknown [33].

In order to gain structural insight into the mechanism of bacterial LPs, 20 homologous LPs were expressed and purified in DDM and their monodispersity was characterized by analytical SEC. At protein concentrations below ~1 mg/ml, several LPs were monodisperse as judged by the sharp symmetrical SEC profile exemplified by LP-1 (Figure 2A). However, upon concentration, all of these LP samples precipitated from solution and could not be concentrated further. As a result, a variety of detergents, compounds, and conditions were screened with the goal of increasing the solubility of LPs for preparative SEC and eventual crystallization trials.

The strategy utilized to improve the solubility of the LP-1 protein was to use our stress test to screen for suitable detergents (Figure 1). LP-1 was over expressed in *E. coli*, solubilized in DDM and bound to Talon resin (Clontech, Mountain View, CA). After washing, the Talon resin was resuspended in the Purification Buffer (See Materials), and LP-1 was removed from the beads by treatment with thrombin. LP-1 overexpressed in 6 L culture typically yielded 4 mg of protein. This pool at a concentration of 0.6 mg/ml was concentrated to the apparent solubility limit of ~1.5 mg/ml and incubated with different detergents at 0.5 or 5 times the critical micelle concentrations at 4 °C for 2 days. Judged by analytical SEC, among the variety of detergents tested, both Cymal-6 and Foscholine-10 were found to improve the solubility of LP-1 without having an adverse effect on its monodispersity (Figure 2B).

The positive results from the solubility screen were subsequently implemented on a preparative scale with the goal of obtaining an LP-1 sample of sufficient quantity for crystallization trials. Replacement of DDM with Cymal-6 in the Purification Buffer permitted the concentration of LP-1 to ~4 mg/ml without precipitation. Supplementation of the Cymal-6 buffers with Foscholine-10 further improved the solubility of LP-1 to ~12 mg/ml. Preparative SEC of LP-1 in this detergent mixture lead to a 10-fold increase of the yield relative to DDM (Figure 2C). Therefore, this strategy successfully identified conditions for improved solubility of a purified membrane bound enzyme, and crystallization trials of LP-1 in the Cymal-6/Foscholine-10 mixture are currently being pursued.

4.2 Substrate identification of a novel FNT family member

Members of the Formate Nitrite Transport family (FNT) function as channels that conduct various polyatomic anions across the plasma membranes of bacteria as well as those of the malaria parasite and pathogenic fungi [34]. The nitrite channel NirC and the formate channel FocA, both found in gram-negative bacteria, are two of the best studied representatives of this family. Structural studies of FocA from *Vibrio cholerae* have elucidated its molecular basis of ion selectivity and gating mechanism [34].

We have characterized another member of the FNT family, FNT3, from the pathogenic anaerobic soil bacterium *Clostridium difficile*. Genetic linkage analysis suggests that the channel plays a role in sulfite transport (BKC and DNW, manuscript in preparation). Specifically, FNT3 is encoded in an operon with three genes sharing sequence homology with a multisubunit sulfite reductase. Indeed, members of the FNT family are often co-transcribed with metabolic enzymes in order to provide substrates for their coupled enzymes or to rapidly remove their toxic byproducts from the cytoplasm [35; 36]. Consequently, we hypothesized that sulfite or the product of sulfite reduction, sulfide, could be the physiological permeating ion of the FNT3 channel from *C. difficile*.

The specific interaction between the selectivity filter of a channel with its permeating ion often stabilizes the ion structure [37; 38], thereby increasing its thermostability. As the physiological permeating ion of FNT3 is unknown, we hypothesized that such ions would display a stabilizing effect of FNT3 denaturation. Thus we determined the apparent T_m of the purified channel protein in the presence of high concentrations of various possible permeating anions. Purified FNT3 samples were incubated in the presence of several candidate anions over a wide temperature range, with the pH of the solutions maintained in a narrow range of 8.5 to 9.0, followed by SEC analysis on HPLC.

The melting temperature for FNT3 was measured as $53.3 \pm 0.1^\circ\text{C}$ in the absence of test compounds (Figure 3). Among the compounds tested, sodium bisulfide (HS^-), the product of the sulfite reductase enzyme, was found to have the greatest stabilizing effect on the protein, increasing the apparent T_m by 8.5°C . In contrast, the substrate of the reductase,

sodium sulfite (SO_3^{-2}), had little effect on the T_m of the channel protein. As FNT channels may function as molecular “release valves” for the products of their linked enzymes [39], this data suggested that the bisulfide ion is the permeating anion for FNT3 *in vivo*. Indeed, competitive transport assays in proteoliposomes and X-ray crystal structures of the channel are consistent with this conclusion (BKC and DNW, manuscript in preparation).

4.3 Improvement of crystal quality of a transporter protein

Membrane transport proteins undergo cycles of conformational change to translocate solutes across the membrane [40; 41]. For example, the leucine transporter, LeuT, adopts outward-facing, occluded, and inward-facing conformations to catalyze leucine uptake [42]. In the absence of stabilizing factors such as substrates or inhibitors, the transporter may dynamically sample these conformations. As a result, a pure and monodisperse transporter preparation may resist crystallization due to this conformational heterogeneity. Presumably, identifying agents or substrates that favor a conformationally homogeneous protein population would lead to an improvement in crystal quality.

We tried to crystallize a bacterial membrane transporter for which the substrate is unknown but a number of substrate candidates have been suggested. The protein was purified in high yields and showed monodisperse behavior in the standard Purification Buffer. In such conditions, the protein produced crystals that diffracted X-rays to a resolution of 6 Å (Figure 4C). As the quality of these crystals failed to improve by standard optimizations, we reasoned that binding by the substrate could lock the protein into a homogenous conformation, which would improve the crystal resolution. Thus, we screened a number of candidate compounds for their ability to stabilize the protein. The apparent T_m of the protein was determined as 44 °C (Figure 4A). Addition of various putative substrates and other salts improved the thermostability of the protein to different degrees (Figure 4B). In particular, compound F showed a clear effect. Strikingly, addition of this compound completely rescued the transporter protein from thermal denaturation. The melting curve of the transporter in the presence of the compound revealed an increase in T_m of 6 °C (Figure 4A). Subsequently, the inclusion of this compound during purification and crystallization improved the crystal quality from 6 Å to 2.8 Å (Figures 4C and 4D).

5. Discussion

In order to study membrane proteins *in vitro*, it is fundamental to maintain the sample's stability. This is particularly true for crystallization experiments, for which the thermostability, along with monodispersity, is essential. Stable and monodisperse membrane proteins, like aquaporins or outer membrane porins, readily crystallize and their crystals tend to diffract to high resolution [43; 44; 45].

The stability of a protein can be improved by finding the appropriate conditions, which in turn helps crystallization. In several cases, the use of additives [46; 47; 48; 49; 50], detergent mixtures [51; 52], or lipids [53; 54; 55] has been important for successful structure determination of membrane proteins. The key is to find a compound that stabilizes the protein in solution. If the additive turns out to be a specific substrate or ligand of the protein, it may lock the protein into a preferential conformation, conferring stability, homogeneity and structural rigidity. However, to search for such stabilization compounds/conditions can be very tedious, and a simple and quantitative method is desired.

While various techniques can monitor the thermostability of a protein and methods like ultracentrifugation [56] and blue native gel [57] can analyze its monodispersity, analytical size-exclusion chromatography can characterize both simultaneously [15]. In this work we have used analytical SEC on HPLC to measure the melting temperature of membrane

protein samples directly. The method can be used to screen various compounds and conditions for their ability to maintain the protein's thermostability. We have used this method to address several questions for three different types of membrane proteins.

Although applicable only to purified protein samples, the assay presented in this paper has several advantages. It is simple, flexible and quantitative. And most importantly, it reports on monodispersity and stability of the protein simultaneously. Only 30–50 μg of sample are required for each injection, and the run can be completed in less than 30 minutes. Also, the method is easy to implement. Moreover, when the HPLC is equipped with an autosampler, 30 to 40 samples can be analyzed within a day and the method becomes medium-throughput. The method is flexible since both sample manipulation and detection of the signals can be modified to suit for different needs. The technique can be easily adapted to resist many extreme conditions and to screen for substrate, inhibitors, pH, detergent, lipids, and so on. At the same time, the SEC profile can be monitored either by UV absorbance or by fluorescence if the protein contains a high enough number of tryptophans. In the later case the amounts of sample required can be reduced to as low as 10 μg . The quantitative nature of our method, in the form of the determination of apparent melting temperature, allows straightforward comparison of compounds or experimental conditions tested. The fact that this method reports on monodispersity and stability simultaneously makes it particularly suitable for screening for crystallization conditions.

As another general method that requires purified protein, microscale fluorescent thermal stability assay has been used to characterize thermostability of membrane proteins in a high-throughput manner [29]. This method needs a specific fluorescence dye that can bind accessible cysteines in the target protein. The method uses N-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM), which gives a fluorescence signal when bound to a cysteine residue. Since most of the cysteine residues are buried in membrane proteins, there is almost no fluorescence for a folded protein; when the sample is heated, the protein starts to unfold and the cysteines become accessible to CPM, yielding a fluorescence signal. This method can be carried out in parallel in a 96 well plate and requires only 30–50 μg of purified protein for each condition. Several G-protein coupled receptor proteins have been characterized using this method, which helped to produce high-quality crystals [58].

Another attractive technique for analyzing thermostability of proteins that does not require protein purification was recently developed [22; 59; 60]. This method screens for thermostabilizing mutations based on their ability to bind a radiolabeled ligand at elevated temperatures. Combination of alanine/leucine scanning mutagenesis and radiolabeled agonist/antagonist binding was used to define conformationally locked protein constructs with increased thermostability. This procedure can also be extended to test for resistance to any other extreme conditions besides temperature. This method requires availability of a known radiolabeled substrate and it does not provide information about the protein's monodispersity. Warne and his colleagues were able to identify six mutations in a G-protein coupled receptor and their combination resulted in a dramatic increase of 23 $^{\circ}\text{C}$ in its melting temperature [22; 61; 62]. This thermostabilized protein construct was purified in small and harsh detergents and crystallized to high resolution [62].

Despite recent progress in the structural studies of membrane proteins, their crystallization remains challenging. This is particularly so when investigators start attacking proteins from mammalian species [63]. Given the challenges, a variety of techniques are needed to increase their expression levels and to improve their stability. Our simple method, hopefully, will help investigators to overcome the challenge.

6. Conclusion

We developed a method that assesses thermostability for membrane proteins by determining their melting temperature, using analytical size exclusion chromatography. This method can be used to screen for compounds or conditions that stabilize the protein in solution. It can also be used for identification of unknown ligands of the protein. As it monitors protein monodispersity and thermostability simultaneously, its applications have shown that the method is particularly suitable for identifying conditions to improve membrane protein crystal quality.

Monodispersity and Thermostability are known parameters for protein crystalizability. We determine T_m of membrane proteins from size exclusion chromatography profiles. This method can be used to search for crystallization conditions, additives and putative substrates.

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Figure 1. Flow chart of methodology

The method requires a purified protein sample capable of showing a peak on SEC. The screening is accomplished by first adding suspected stabilizing agents to equal aliquots of the sample. Then, all samples are subjected to the same stress treatment. Finally, the samples are individually injected onto a SEC column coupled to an HPLC. The SEC profile allows one to quantify the fraction of the sample that remains monodisperse after the stress treatment.

Figure 2. Identification of detergents for improved solubility of an integral membrane lipid phosphatase

(A) Analytical SEC trace of LP-1 purified in DDM. Inset: commassie stained SDS-PAGE gel. Lanes: 1) Benchmark protein marker, 2) Flow-through, 3) Wash 1, 4) Wash 2, 5–7) Elutions. (B) SEC profiles of LP-1 in DDM supplemented with other detergents. Cymal-6 and Foscholine-10 improved the recovery of LP-1 as evidenced by the increased peak heights. (C) Preparative SEC profiles of LP-1 purified in DDM or the Cymal-6/Foscholine-10 mixture.

Figure 3. Effect of putative permeating ions on channel stability

Melting curves for the FNT3 protein were measured with various candidate compounds of permeating ions. The curve in the absence of any added compound was measured in the Purification Buffer (see Methods). Various sodium salts were added to purified protein at a final concentration of 50 mM. Samples were incubated at the indicated temperatures for 10 minutes, centrifuged and injected on SEC HPLC. The fraction remaining is a direct ratio between peak heights against the 4 °C control of each salt.

Figure 4. Effect of salts and putative substrates on transporter thermostability and crystal diffraction

(A) Melting curves for transporter protein. “Control” curve was measured in Purification Buffer and “+ compound F” curve was determined in the same buffer with the addition of 50 mM of compound F. Samples were heated for 20 minutes at the depicted temperatures, centrifuged and injected onto SEC HPLC. The fraction remaining is a direct ratio between peak heights against the 4 °C control. (B) Fractional peak height upon addition of salts (compounds A to C) and putative substrates (compounds D to G). Screening was performed at 44 °C in the Purification Buffer by adding 50 mM of desired salt or substrate. (C) Diffraction pattern of crystals obtained in the absence of substrate. (D) Diffraction pattern of crystals obtained in the presence of 50 mM of compound F.