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Improved Technique for Reconstituting Incredibly High and Soluble Amounts of Tetrameric K^+ Channel in Natural Membranes

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Abstract The reconstitution of large amounts of integral proteins into lipid vesicles is largely prompted by the complexity of most biological membranes and protein stability. We optimized a particular system which maximized the incorporation efficiency of large soluble amounts of KcsA potassium channel in *Escherichia coli* membranes. The effects of two detergents, octylglucoside and 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), on KcsA reconstitution were compared. Reconstitution efficiency was found to be incredibly high for CHAPS-treated proteoliposomes followed by dialysis at room temperature. This approach may allow more accurate investigation of integral membrane proteins in their natural membrane environment via biophysical or biochemical techniques.

Keywords Ion channel expression and reconstitution · Lipid–protein interaction · Membrane assembly · Potassium ion channel

Introduction

Purification from the native membrane and further reincorporation of a purified membrane protein into an artificial or natural membrane are known to be crucial steps in studying the function and structure of these molecules. Protein reconstitution has played a central role in identifying and characterizing the mechanisms of transport function (Jones 1998; Rigaud et al. 1995). Furthermore, the reconstitution of membrane proteins to form two-dimensional crystals confined in a membrane has led to important high-resolution structural information by electron crystallography (Kühlbrandt and Wang 1991; Rigaud et al. 2000).

The ability to investigate membrane proteins has been limited by lack of critical steps in biochemical, biophysical and molecular biological techniques. One of the limiting factors in obtaining structural information is related to the lack of reconstituted systems containing optimal amounts of soluble and stabilized proteins. Extreme efforts are therefore required to make reconstitution of available proteins or ion channels even more favorable for structure–function analysis. Here, we deal with the choice of detergent (octylglucoside [OG] and 3-[(cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate [CHAPS]) and reconstitution conditions that can be used to enhance the protein-to-lipid ratio as well as the stability and solubility of oligomeric integral membrane proteins in proteoliposomes by taking potassium channel KcsA as a suitable model protein.

Materials and Methods

For enhanced incorporation of KcsA into *Escherichia coli* membranes, the protein expression and purification strategies were optimized to increase the protein stability for proteoliposome reconstitution trials. Briefly, 2 l of Luria-Bertani-containing ampicillin (100 μg/ml) was inoculated with overnight-grown culture from *E. coli* strain BL-21 (DE3), which was transformed with pQE60-KcsA vector. KcsA protein was expressed with a C-terminal

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hexahistidine (6× His) tag according to the method described previously (Raja and Vales 2009a). The protein was eluted in elution buffer (25 mM KCl, 50 mM KH_2PO_4, 1 mM DDM, 400 mM imidazole, protease inhibitor [PI], 5% glycerol, pH 7.0). The presence of DDM precluded the use of the Bradford assay; therefore, the protein concentration was assessed by SDS gel, using a standard of bovine serum albumin (BSA) as described previously (Raja et al. 2007). KcsA protein fractions were either used directly for reconstitution or stored at 4°C for not more than 2 days.

E. coli total lipid extract (10 mg) dissolved in chloroform was dried onto the glass surface under a nitrogen stream. One milliliter of reconstitution buffer (150 mM KCl, 10 mM KH_2PO_4, PI, pH 7.0) containing either 5 mM OG or 35 mM CHAPS was directly added to the dried lipid film. Adding CHAPS at this step helps to destabilize multilamellar structures and therefore reduces the sonication time. This mixture was incubated at room temperature (RT) for 1 h. Lipids were briefly sonicated to clarity with a probe sonicator on ice (10 cycles, 30 s/cycle). This is a critical step. If the solution gets clear, the sonication should be stopped before 10 cycles. Liposomes were centrifuged at 5,000 rpm for 3 min to remove titanium particles from the sonication step. For proteoliposome preparation, 100 µl of reconstitution buffer was added to the glass tube, followed by either 5 mM OG or 35 mM CHAPS. Freshly purified DDM-solubilized KcsA protein (to a final 1:100 or 1:50 protein:lipid mol%) was directly added to the mixture, followed by 900 µl of OG- or CHAPS-solubilized liposomes. The detergent-solubilized mixtures were briefly vortexed and subsequently incubated at RT for 30–45 min.

First, OG- or CHAPS-treated samples were subjected to detergent removal by Bio-Beads SM-2 at room temperature. Bio-Beads are convenient for rapid screening of reconstitution trials and are used for efficient removal of all kinds of detergents (Rigaud et al. 1998). Prewashed Bio-Beads were resuspended in reconstitution buffer and added directly to both detergent-solubilized samples, at a Bio-Bead-to-detergent ratio of 10 (w/w), and stirred for 1 h. A second portion of beads was then added for an additional 1 h of incubation, followed by a third addition for about 2 h to ensure complete detergent removal. Since CHAPS is a less hydrophobic detergent, a fourth addition of beads was performed because of the lower adsorptive capacity of this detergent (Highsmith 1990).

Proteoliposomes were obtained by ultracentrifugation at 100,000×*g* for 1 h at 4°C, and the resultant pellet was resuspended in 1 ml reconstitution buffer. For protein determination by SDS-gel electrophoresis, proteoliposomes were directly treated and mixed with 6× SDS buffer containing 1 M Tris (pH 6.8), 80% glycerin, 10% SDS, 10% β-mercaptoethanol and 0.01% bromophenol blue. Samples were directly loaded on 12% acrylamide SDS-gel. Gels were stained by Coomassie brilliant blue dye.

Results and Discussion

It is well established that KcsA forms an extremely stable homotetramer (~68 kDa) even in the presence of detergent, like SDS (Raja and Vales 2009a; Raja et al. 2007). Figure 1a (left panel) shows a representative SDS gel of KcsA proteoliposomes (1:100 protein: lipid mol%) after removal of OG or CHAPS by Bio-Beads. After ultracentrifugation of proteoliposomes, the supernatant (S-KcsA) contained significant amounts of unincorporated KcsA tetramer (T). Surprisingly, the proteoliposomes (PL-KcsA) contained either protein or protein–lipid aggregates, indicating that these reconstitution conditions drastically impaired the reconstitution efficiency and protein stability.

Detergents with high critical micelle concentrations such as OG and CHAPS can be easily removed by dialysis in 1–2 days (Allen et al. 1980). As a next step, we carried out experiments in which dialysis was performed at either RT or 4°C. Detergent-treated mixtures were filled into a dialysis tube (Dialysis Membrane; SpectraPor, Rancho Dominguez, CA) prewashed with double-distilled water. The dialysis tube was closed tightly to ensure that no air bubbles formed in the tube. Samples were dialyzed against 1 l of dialysis buffer (150 mM KCl, 10 mM KH_2PO_4, 1 mM sodium azide, PI, pH 7.0) for 48 h. The dialysis buffer was replaced four times (after every 12 h) in 48 h. The dialysis bag was inverted up and down several times to ensure thorough mixing of samples at least six to eight times throughout dialysis. Avoiding this step resulted in clearly visible protein–lipid aggregation.

Figure 1a (right panel) represents two different reconstitution conditions for OG-treated mixtures at either 4°C (PL-4°C) or RT (PL-RT). At 4°C, the efficiency of KcsA reconstitution was drastically decreased for both preparations. Dialyzing samples at RT resulted in improved reconstitution efficiency in proteoliposomes (1:50 P:L mol%) containing higher amounts of KcsA tetramer (T). However, protein degradation was drastically increased, resulting in tetramer dissociation into monomeric (M) subunits (~18 kDa). Qualitatively similar results were obtained for proteoliposomes treated with dodecylmaltoside (DDM), indicating protein aggregation during reconstitution (not shown).

Next, we performed similar experiments for CHAPS-treated samples. Figure 1b (left panel) illustrates the reconstitution profile of KcsA tetramer after dialysis at 4°C. Neither enhanced incorporation nor protein aggregation/degradation was detected, indicating that a significant amount of protein was not reconstituted. Interestingly,

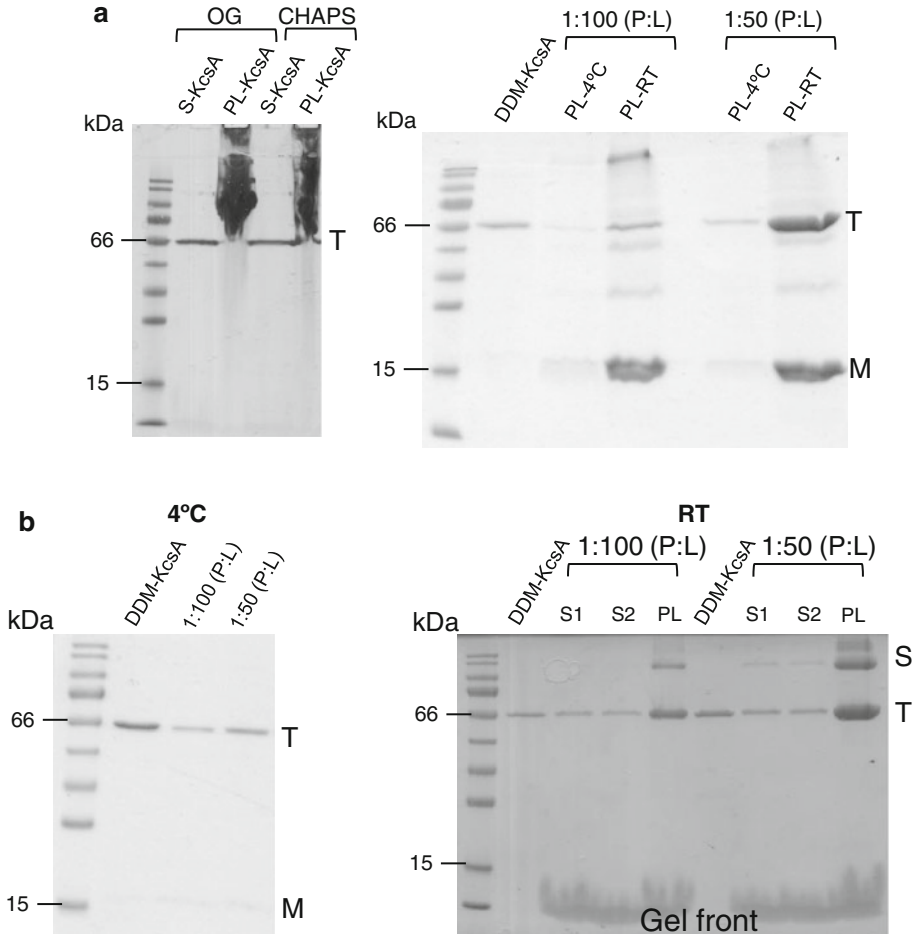

Fig. 1 **a** Representative SDS gels of KcsA reconstitution into proteoliposomes. *Left* OG- or CHAPS-treated samples were subjected to detergent removal by Bio-Beads at RT. S-KcsA and PL-KcsA correspond to supernatant and proteoliposome fractions, respectively. *Right* KcsA reconstitution (at two different P:L ratios) into proteoliposomes after removal of OG by dialysis at two different temperatures. **b** Proteoliposome reconstitution upon removal of CHAPS by dialysis at 4°C (*left panel*) or at RT (*right panel*). *S1* and *S2* correspond to duplicate fractions of supernatants. The supramolecular KcsA is denoted by *S*, and tetramer and monomer fractions are indicated by *T* and *M*, respectively. The protein molecular weight marker (kDa) is shown on the left of each gel

dialyzing these samples at RT significantly improved the protein reconstitution efficiency (Fig. 1b, right panel). Also, the amount of free protein was found to be remarkably less in supernatants (S1 and S2) after ultracentrifugation. Some bands running at higher molecular weight that correspond to supramolecular (S) KcsA complexes (Raja and Vales 2009b) were detected. Furthermore, the protein gels were scanned and the KcsA tetramer intensity was measured using image processing and analysis in Java (ImageJ; NIH, Bethesda, MD) program. The DDM-solubilized KcsA tetramer band intensity as a total amount of tetramer present before reconstitution was set to 100% in order to calculate the percent tetramer recovered after proteoliposome reconstitution. The quantification of tetramer is shown in Fig. 2 in which the tetramer fraction is plotted against variable conditions for OG- or CHAPS-treated samples. It is obvious that dialyzing CHAPS-treated samples yielded remarkably high incorporation efficiency of KcsA tetramer, with a protein recovery of up to 90%. Although CHAPS has been used as a suitable detergent for KcsA reconstitution into artificial lipid bilayers (Heginbotham et al. 1999), it can also serve to increase the membrane incorporation efficiency of integral membrane

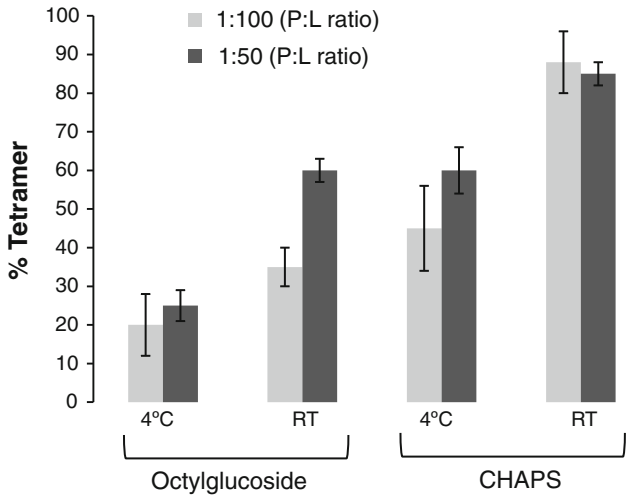

Fig. 2 Quantification of Coomassie blue–stained SDS gels. The percent KcsA tetramer was calculated from OG- or CHAPS-treated proteoliposomes as mentioned in the text. All data points correspond to the average ± SD of two or three experiments

proteins like KcsA channel without affecting the protein solubility and stability. We also determined the biological activity of reconstituted KcsA and found that the presence

of 50–100 mM Na^+ was required for efficient membrane fusion and transfer of incredibly high numbers of active channels into a planar lipid bilayer (not shown), similar to what has been shown previously (Raja and Vales 2009b).

Conclusion

We learned that not all detergents serve equally well for the reconstitution of tetrameric KcsA potassium channel, and the experimental approach must be kept as broad as possible. The optimal detergent that allows high protein incorporation, while avoiding its degradation, must be found experimentally. Despite the efficiency of OG or DDM in terms of protein incorporation, these detergents can be toxic and that may induce protein aggregation. Furthermore, fast detergent removal using Bio-Beads can drastically affect the stability of oligomeric proteins. However, other parameters, such as lipid composition, temperature and buffer composition, should be optimized. Although, there are limitations in reconstitution efficiency at high protein-to-lipid ratios due to protein aggregation at high protein concentrations, our experiments demonstrate the best way to determine the stability of multimeric proteins after proteoliposome reconstitution as well as suitable conditions for incorporating relatively high amounts of oligomeric membrane proteins like KcsA. Although there are many examples in the literature of membrane protein reconstitution into phospholipid vesicles using detergent dialysis, the protein solubility and/or aggregation after reconstitution has not been described by many groups working with KcsA channels. The apparent success of the current approach to the reconstitution of extremely high and soluble amounts of KcsA may therefore be considered quite useful for analyzing complex ion channel behavior in the native environment.

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