Chapter 3: Study of Glt\textsubscript{Tk} in its membrane environment: comparison of three methods to create larger nanodiscs

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Abstract

Technological developments have opened the way for biochemical and structural characterization of membrane proteins in lipidic environments, which are more native environments than traditionally used detergent micelles. These developments include: methods to reconstitute the detergent purified protein into nanodiscs with the use of a membrane scaffold protein (MSPs), or into a saposin-lipoprotein nanoparticle system (Salipro) and methods to extract the proteins directly from the membrane with the use of maleic acid derivative polymers, including DIBMA and SMA. However, in the procedures for nanodiscs formation using MSPs and membrane protein extraction using polymers, often difficulties are encountered related to obtaining sufficient yields of homogeneously sized supramolecular assemblies. In this chapter, I show attempts to extract the glutamate transporter homologue Glt\textsubscript{Tk} from crude membrane vesicles using DIBMA, which had very low yields, as well as from proteoliposomes, which gave more promising results by implementing an extra step of sonication. I also show a successful attempt to increase to size of an MSP-nanodiscs (with the belt protein MSP2N2) by increasing the amount of lipids used during the nanodisc assembly.
Introduction

In 2020, five structures of GltTk embedded in nanodiscs were reported. The use of different substrates and a series of substrate concentrations revealed an ensemble of conformations of the protomers, depicting the different states in the framework of the elevator mechanism. It also corroborated other works that previously proposed the independent operation (i.e. no cross-talk) of protomers in the glutamate transporters.

Figure 1 | Structural and functional importance of lipids in GltTk. (A) View from the membrane plane of GltTk in nanodiscs solved in sodium-only condition; it shows the densities of MSP2N2 (grey, mesh) and its distortions around the different conformations of the protomers. The lipids between the protomers are shown in black sticks and red mesh (figure adapted from 1). (B) Slice of the trimer from the top view. In grey, the lipid densities found between the scaffold and the transport domains. (C) Sequence of transport steps from the outward-open state (with TBOA, left most panel), outward-occluded state (with L-Asp), outward-occluded state (apo) and inward open state. Close to the gate (magenta) were found the lipid densities represented in red mesh.

The distortions of the belt protein, helped visualizing the deformations that the membrane around the protein may undergo during the transport (Fig. 1A). Moreover, the re-analysis of the density maps that represent the different states in the transport cycle of GltTk, revealed lipid-like densities around the periphery of the protein (Fig. 1A-B) and in proximity of the hairpin loop 2 (Fig. 1C). Similarly to what was suggested for ASCT2 and for
Glt<sub>Ph</sub>, these lipids most likely serve both structural and functional roles. The structural role is obvious as the positions of lipids between the protomers and between the domains within the same protomer are well conserved in all the available structures and likely provide both stability and the preservation of the quaternary structure as well as assist movements of transport domains during the transport cycle (Fig. 1B). Furthermore, at different steps of the transport cycle, the lipid density around the hairpin loop 2 assumes different orientations, hinting to the possibility that during the transport cycle the lipidic environment facilitates the gating (Fig. 1C).

While this work revealed the majority of the states of the protomers during the transport cycle, it failed to capture two expected conformations: the outward-open Na<sup>+</sup>-only state and the inward occluded state. Furthermore, it was puzzling that in the presence of both substrates (sodium and L-aspartate) the protomers adopted an intermediate outward state and only in the presence of the blocker of EAATs, DL-threo-beta-hydroxyaspartate (TBOA) the fully outward state. One of the possible explanations suggested by the authors is that the tightness of the belt protein (MSP2N2) around Glt<sub>Tk</sub> might create artefacts similar to the ones created by crystal contacts. Therefore, it seems reasonable that such artefacts can be reduced if a protein is surrounded by a larger number of lipids.

Apart from the reconstitution of membrane proteins using belt proteins of the family of MSP (Membrane Scaffold Proteins), other strategies have been developed, including the use of peptides instead of scaffold proteins that create the peptidiscs the reconstitution with saposins-lipoproteins. Furthermore there are methods that prevent the use of any detergent for the solubilization of the membrane protein and these include the solubilization with amphipols or the extraction of the protein of interest directly from membranes with the use of different polymers, such as maleic acid derivatives: poly styrene-co-maleic acid (SMA) and poly diisobutylene-alt-maleic acid (DIBMA). Both SMA and DIBMA polymers have been widely employed for both structural and functional studies, since they have provided a useful kit to study membrane proteins in their native environment.

In an attempt to create larger nanodiscs (hence containing more lipid molecules) we adopted three strategies (schematically represented in Fig. 2):

1. The solubilization of Glt<sub>Tk</sub> directly from the crude membrane vesicles preparation with DIBMA;
2. The solubilization of Glt<sub>Tk</sub> from proteoliposomes with DIBMA;
3. Increase of the lipid concentration during the reconstitution of GltTk in nanodiscs using belt protein MSP2N2.

The efficacy of all these strategies was evaluated through a combination of SDS-gel electrophoresis, size-exclusion chromatography (SEC), dynamic-light scattering (DLS), and transmission electron microscopy (TEM).

**Materials and methods**

**Solubilization with DIBMA from crude membrane vesicles**

The fractions of 1 g/ml membrane vesicles are prepared as described in Chapter 2. Prior to the addition of DIBMA to the fractions, they were diluted three times with the resuspension buffer (50 mM Tris pH 8.0) and DIBMA (Anatrace) was added at the appropriate concentration (1, 2 or 3% DIBMA). Each vial was then incubated at 4°C while agitating for 1, 2, 4, 6 and 20 hours. At the end of the incubation time, each sample was ultracentrifuged at 100K xg for 20 min. Prior the analysis on SDS gel, the pellet was
resuspended in the same volume as the supernatant, in order to make a fair comparison. The soluble fraction was added to His-trap column previously equilibrated with 50 mM Tris pH 8.0, 300 mM KCl and left incubated for 1 hour with a continuous flow of 0.2 ml/min. After the incubation, a washing buffer containing 10 mM imidazole was used and after that the bound proteins were eluted at 50 mM imidazole. The eluted fraction was then applied to a Superdex 200 increase 10/300 column, pre-equilibrated with 50 mM Tris pH 8.0, 100 mM KCl.

**Solubilization with DIBMA from proteoliposomes**

The procedure for both protein purification and preparation of proteoliposomes is described in Chapter 2. The difference between the ones used for uptakes or SSM and the ones used for solubilization is that the latter are sonicated at 70% amplitude for 15 s on and 45 s off for 16 cycles prior addition of the protein. Once the protein is added at the ratios tested (1:5, 1:20 and 1:50), the reconstitution proceeded as in the other protocols. DIBMA was added at 1:1, 1:2, 1:3, 1:4 (w:w) ratio to the proteoliposomes and left incubating for 1, 3, 6 hours or overnight. The solubilized fraction was separated from the insolubilized through ultracentrifugation at 100K xg for 20 min. Prior the analysis on SDS gel, the pellet was resuspended in the same volume as the supernatant.

**Purification of the sample from DIBMA and lipids**

The loading of the nanodiscs solubilized with DIBMA on SDS gel results in smearing of the bands. Proteins can be separated through a precipitation using methanol, chloroform and water. In order to do this, we followed the protocol from Anatrace:

- Measure volume of polymer-containing protein sample
- Add 4x volume of cold methanol to sample and mix thoroughly via vortexing
- Add 1x volume of cold chloroform to sample and mix thoroughly via vortexing
- Add 3x volume of cold water, mix, and centrifuge for 2 min at 4°C and 14,000 g.
- Remove top aqueous layer, add 4x volume of methanol, and mix
- Pellet proteins by centrifuging at 5,000 g for 1 min and 20,000 g for 5 min at 4°C
- Remove supernatant and dry pellet
- Add SDS loading buffer, run SDS-PAGE as normal

Results

**Extraction from membrane vesicles**

There are reports in the literature, describing the extraction of the membrane protein of interest with DIBMA, by simply incubating the membrane vesicles preparations with variable concentrations of the polymer \(^{16,17}\). Protocols for extraction from crude membranes typically report a range from 1-3% (w/v) of DIBMA with 2% being the preferred concentration. We attempted extraction of Glt\(_{Tk}\) by testing three different concentrations of DIBMA on 1.5 g of membrane vesicles that contain around 1 mg of Glt\(_{Tk}\). At 1, 2, 4, 6 and 20 hours from the start of incubation, samples were taken and the soluble fractions were separated from the insoluble fractions through ultracentrifugation. First, a comparison between 1- and 2-hour incubation time was made by comparing the samples on SDS polyacrylamide gels (Fig. 3). A band between 55 and 35 KDa corresponds to the monomer of Glt\(_{Tk}\) of 46.64 KDa. From figure 3 it can be seen that for all the conditions tested, the protein was found in the insoluble fraction and no major differences were visible on SDS gel between the different concentrations tested. For longer incubations, only 3% DIBMA was used and also in the latter cases, the band corresponding to the protein is visible only in the “pellet” lane. These results suggest that no or little protein is solubilized with this approach.

![Figure 3](image)

**Figure 3 | Trials of solubilization of Glt\(_{Tk}\) from crude membrane vesicles with DIBMA.** The samples resulting from the incubation with 1,2 and 3% DIBMA for 1, 2, 4, 6 and 20 hours were loaded on SDS gel and the comparison was made between the sample before centrifugation, the supernatant and the pellet.
The separated fractions (obtained with 3% DIBMA and 2 hours solubilization) were also loaded on a His-trap Ni\(^{2+}\)-resin for 1 hour and after a wash with a low concentration of imidazole, the elution was followed at 280 nm using a buffer containing 50 mM imidazole (Fig. 4A). From the SDS-PAGE analysis (Fig. 4C) we can observe that all the fractions eluted contain contaminants. Nevertheless, the fraction corresponding to the peak of the absorbance was loaded on a size-exclusion column in order to separate the proteins by size (Fig. 4B). After SEC, the fractions eluted early corresponding to bigger sizes (namely S1 and S2), contained little or no protein, meaning that they likely membrane patches and nanodiscs with other proteins. The other fractions at later elution volumes (S3 and S4) showed multiple bands. Moreover, the His-tag western blots on these gels showed multiple false positives (data not shown).

This experiment was repeated with different Ni\(^{2+}\)-resin incubation times and different concentrations of imidazole and resulted in similar results. In order to have a better understanding of the efficiency of this method, one of the fractions from SEC was also analyzed by electron microscopy after negative staining. Apart from some particle that resemble nanodiscs of the expected size, the samples presented heterogeneity of sizes and contaminants (Fig. 4D). These results all together show that using DIBMA directly on crude membrane vesicle preparations can yield broad ranges of sizes of nanodiscs, unspecific binding of other proteins to the Ni\(^{2+}\)-resin, together with a much weaker binding of the protein of interest to the same column (where perhaps the His-tag is hindered) compared to the detergent solubilized protein or the protein reconstituted into nanodiscs.
Figure 4 | From purification to negative staining. (A) Elution from His-trap column followed at 280 nm; (B) Elution on SEC of sample H3 from His-trap; (C) SDS-gel of the samples highlighted on the chromatograms of (A) and (B); (D) Negative staining of the sample resulting from solubilization of crude membrane vesicles and purification steps on IMAC and SEC.

**Extraction from proteoliposomes**

In order to avoid contaminations from other proteins, other approaches for the extraction of the protein with DIBMA include the purification with detergent and reconstitution of the protein of interest in proteoliposomes prior the incubation with DIBMA \(^{18-22}\). Different lipid:protein ratios used in the reconstitution as well as different DIBMA:proteoliposomes (w/w) ratios used for extraction yield to a broad range of different sizes \(^{20}\). In a first attempt to extract the protein directly from the proteoliposomes, three reconstitution ratios, namely 1:5, 1:20 and 1:50 (w/w), were tested. From \(^{20}\) we know that lipid:DIBMA ratios equal or above 1:1 can yield smaller nanodiscs. Therefore, starting from the smaller lipid to protein ratio, we tested from 1:1 up to 1:4 lipid:DIBMA ratios (Suppl Fig. 1). While 1:1 and 1:2 ratios do not seem to yield to any solubilization even after overnight incubation, 1:3 and 1:4 yield to a better solubilization and the two ratios show similar results (Suppl. Fig. 1). Also in this case, the purification on Ni\(^{2+}\)-resin was necessary to separate the nanodiscs containing the proteins from the empty ones. After IMAC, the SEC was performed and it did not allow for
the complete separation between different sizes as a single peak is visible and it corresponds to the void volume of the column. In fact, analyzing this fraction with electron microscopy, we observed a broad variety of liposomes sizes and some particles that could resemble nanodiscs (Suppl. Fig. 1). A homogeneity in proteoliposomes sizes may be achievable with the use of sonication prior addition of the protein, during the preparation of the vesicles. We tested this on all three reconstitution ratios and after the incubation with DIBMA and the nanodiscs separation, we were able to observe that the 1:5 reconstitution ratio prevented the formation of aggregates and yielded nanodiscs that eluted later compared to the ones formed with the other ratios (Fig. 5A). A further confirmation of this difference comes from DLS which, by using a regularization fitting, showed particles with an average hydrodynamics radius ($R_h$) of 18 nm for the 1:5 reconstitution and 41 nm for both 1:20 and 1:50. For all the reconstitution ratios used, the samples were monomodal and a high polydispersity index was observed ($=> 30 \, %$Pd).

![Figure 5](image-url)  

**Figure 5 | Comparison of sizes from both SEC and DLS of proteoliposomes solubilized with DIBMA.** (A) SEC chromatogram of the fractions eluted from IMAC for the three ratios; (B) Regularization fitting of the same samples analyzed on DLS.

**Excess of lipids for reconstitution of the protein into nanodiscs with MSP2N2**

Another way to obtain larger nanodiscs is by changing the ratio between the membrane protein, the protein belt and the lipids during the reconstitution. Many ratios have been tested in the past for Glt$_{1\kappa}$ to obtain nanodiscs of homogeneous sizes (Suppl. Fig. 2) and the one that was selected to solve the structures of the transport cycle ensemble was the one that yielded nanodiscs of 11 nm diameter, namely the 3:5:100 Glt$_{1\kappa}$:MSP2N2:lipid molar
In these experiments, the largest quantity of lipids that has been used was 400 per trimer of GltTk and it yielded a large fraction of aggregates. As reported in 23, a low lipid:MSP2N2 ratio could result in underloaded nanodiscs, with only few lipid molecules around the membrane protein. As recently reported 24, the use of a high concentration of lipids helped with obtaining nanodiscs of 18 nm and little heterogeneity in the sample. We tested if such a higher concentration of lipids also yielded a larger size for GltTk’s nanodiscs. Therefore, instead of 3:5:100, we used 3:5:725 GltTk:MSP2N2:lipid ratio. After the usual purification to eliminate empty nanodiscs, the SEC revealed a single peak eluting around 1 ml earlier than the one with nanodiscs obtained with the previous ratio, hinting to the possibility to have obtained larger homogenous nanodiscs. The DLS measurements agree with the size increment, with an average $R_h$ difference of approximately 3 nm between the particles from the different ratios (6 nm for 3:5:100 and 9 nm for 3:5:725). The samples also presented a monomodal characteristic with a polydispersity around 20% in all of the conditions.

Discussion

There is a steadily increasing number of functional and structural studies on membrane proteins thanks to the development of methods to encapsulate them in lipid nanodiscs or to extract them from their native environment 25–28. There are many advantages for using nanodiscs to study membrane proteins, as they offer the ability to gather information about movement of the protein complexes. In fact, due to the new technological advances and
rapid development of cryo-EM, it became possible to solve the structures of membrane proteins without need to crystalize them, hence allowing also to reveal states which, in principle, would not be possible to capture with crystal structures. For example it was possible to confirm the hypothesis about the independence of each monomer during the transport cycle of Glt$_{T_k}$¹ that was based on some individual structures, MD simulations, cross-linking studies and other biochemical experiments. With the work presented here, we aimed to check the effect of nanodisc geometry on the conformational ensemble by trying to obtain larger nanodiscs, in order to avoid bias given by the tight constraints of the belt protein. We considered using the method based on the extraction of the protein and the membrane around it, using the DIBMA copolymer. DIBMA, compared to SMA, has been reported to not only form more stable nanodiscs, but also to bear aliphatic rather than aromatic groups that make them more suitable for UV spectroscopy ²⁹. Solubilization of the protein of interest directly on membrane preparations should work at a concentration of ~2% DIBMA (w/v) according to the authors that tested the compound. In our experiments, we could not solubilize Glt$_{T_k}$ in a quantity that was large enough to purify it from the rest of the solubilized fraction. Moreover, it was difficult to obtain homogeneity of the sample as shown on the SEC and electron microscopy micrographs. One strategy that could be adopted in the future is sonication of the crude membrane vesicles after the homogenization step, as sonication was a crucial step that helped obtaining homogenous proteoliposomes in the second strategy that we tested in this work. In accordance with the publication from ²⁰, we noticed that the 1:5 protein:lipid ratio (w/w) as well as the 1:3 proteoliposome:DIBMA (w/w) ratio helped obtaining particles that eluted earlier than the retention volume of the SEC column and gave an estimated size of around 18 nm diameter on DLS. A microscopical analysis would help defining whether the homogeneity of the sample is enough to proceed with the structure determination. Finally, a combination of SEC analysis and DLS confirm that the use of 7-fold larger quantity of lipids than the one that was used in the past can be beneficial. All the approaches presented in this chapter have a potential to be further optimized in the future to obtain larger nanodiscs which might be useful for both biochemical and structural studies.
Supplementary figures

**Supplementary figure 1 | Solubilization of the proteoliposomes reconstituted at 1:50 protein:l lipid ratio and purification of the nanodiscs.** (A) SDS gel of the solubilization trials on the proteoliposomes with different lipid:DIBMA ratios; (B) SEC chromatogram resulting from the purification on IMAC; (C) Micrograph of cryo-EM data collection of the purified sample.
Supplementary figure 2 | Different reconstitution ratios trials. Different GlkT:MSP2N2:lipids ratios yield to different SEC chromatograms. The higher the lipids content, the higher the chances to have aggregates (8-9 ml) and free belt protein or empty nanodiscs (12-14 ml), as shown in the gel.
References


20. Voskoboynikova, N. et al. Evaluation of DIBMA nanoparticles of variable size and


