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Analysis of the oligomeric state of mycobacterial membrane protein large 3 and its interaction with SQ109 with native cell membrane nanoparticles system

Weihua Qiu *, Youzhong Guo

Department of Medicinal Chemistry, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298, USA
Institute for Structural Biology, Drug Discovery, and Development, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23219, USA

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ABSTRACT

Mycobacterial membrane protein large 3 (Mmpl3) as a trehalose monomycolate lipid transporter contributes to cell wall biosynthesis. Inhibition of Mmpl3 can suppress cell growth and lead to mycobacterial death. SQ109 is a hydrophobic inhibitor of Mmpl3. We have devised a detergent-free strategy to characterize the SQ109/Mmpl3 interaction using the Native Cell Membrane Nanoparticles (NCMN) system, a new method for extracting membrane proteins that better retains native lipids. The homogeneity of the Mmpl3 NCMN particles was confirmed with electron microscopy. The hydrophobic protein-ligand interaction analysis shown for Mmpl3 using the NCMN system may broadly apply to other membrane proteins.

Membrane proteins are essential for many physiological processes and involved in numerous pathological conditions, thus serving as the primary targets for multiple prescribed drugs on the market. However, the traditionally detergent-based approaches for membrane protein research have significant drawbacks. Thus, the current detergent-free systems such as SMALP (Styrene Maleic Acid-Lipid Particles), the NCMN (Native Cell Membrane Nanoparticles) system, and others have emerged as alternatives for membrane protein research [1].

The NCMN system has been developed mainly for high-resolution structure determination of membrane proteins. However, protein samples prepared using this detergent-free system maybe also ideal for many biophysical and biochemical analyses [1]. Protein-ligand interaction analysis is an essential theme in membrane protein research and related drug discovery and development. The structure and function of membrane proteins can be modulated through protein-ligand interactions. Traditionally, characterization of membrane protein-ligand interactions is conducted *in vitro* using a detergent-solubilized membrane protein sample. If the ligands are hydrophobic, detergents are also often used for their solubilization in these characterizations. In contrast, the NCMN system is entirely detergent-free. It is designed so that no detergent is involved in solubilizing hydrophobic ligands. However, unlike detergents, membrane-active polymers cannot solvate hydrophobic ligands, especially those that are small. Here, we have reported a strategy to analyze protein-ligand interactions in an entirely detergent-free system by taking advantage of the unique properties of organic solvents, such as DMSO, using the NCMN system. This system may have broad applications for analyzing interactions between hydrophilic or hydrophobic ligands and membrane proteins.

To demonstrate how this system can be used, we tested the binding of mycobacterial membrane protein large 3 (Mmpl3) NCMN-encapsulated particles to its hydrophobic ligand SQ109 [2]. Mmpl3 is an emerging drug target for Tuberculosis (TB) treatment, one of the top 10 diseases in the world. The World Health Organization (WHO) End TB strategy provides a blueprint to eliminate TB gradually, with a target of fewer than 100 cases per million people by 2035 [3]. The unique feature of mycobacteria is that it possesses a particular cell wall containing mycolic acid, which prevents attacks from the human immune defense system. Although many efforts and financial support have been put forth to develop efficient drugs to eradicate TB, multidrug resistance (MDR) and extensive-drug resistance (XDR) TB variants remain highly challenging. So, new anti-TB drugs with less treatment duration, lower dosing frequency, and fewer pill burdens are in high demand [4].

Mycobacteria are slow-growing and acid-fast aerobic microbes. Mycobacteria are classified into three groups: 1) *mycobacterium tuberculosis* (Mtb) complex, which causes severe and contagious tuberculosis in humans; 2) nontuberculous causing mycobacteria, such as

* Corresponding author at: Department of Medicinal Chemistry, Institute for Structural Biology, Drug Discovery, and Development, Virginia Commonwealth University, Richmond, VA 23298-0540, USA.
E-mail address: wqiu@vcu.edu (W. Qiu).

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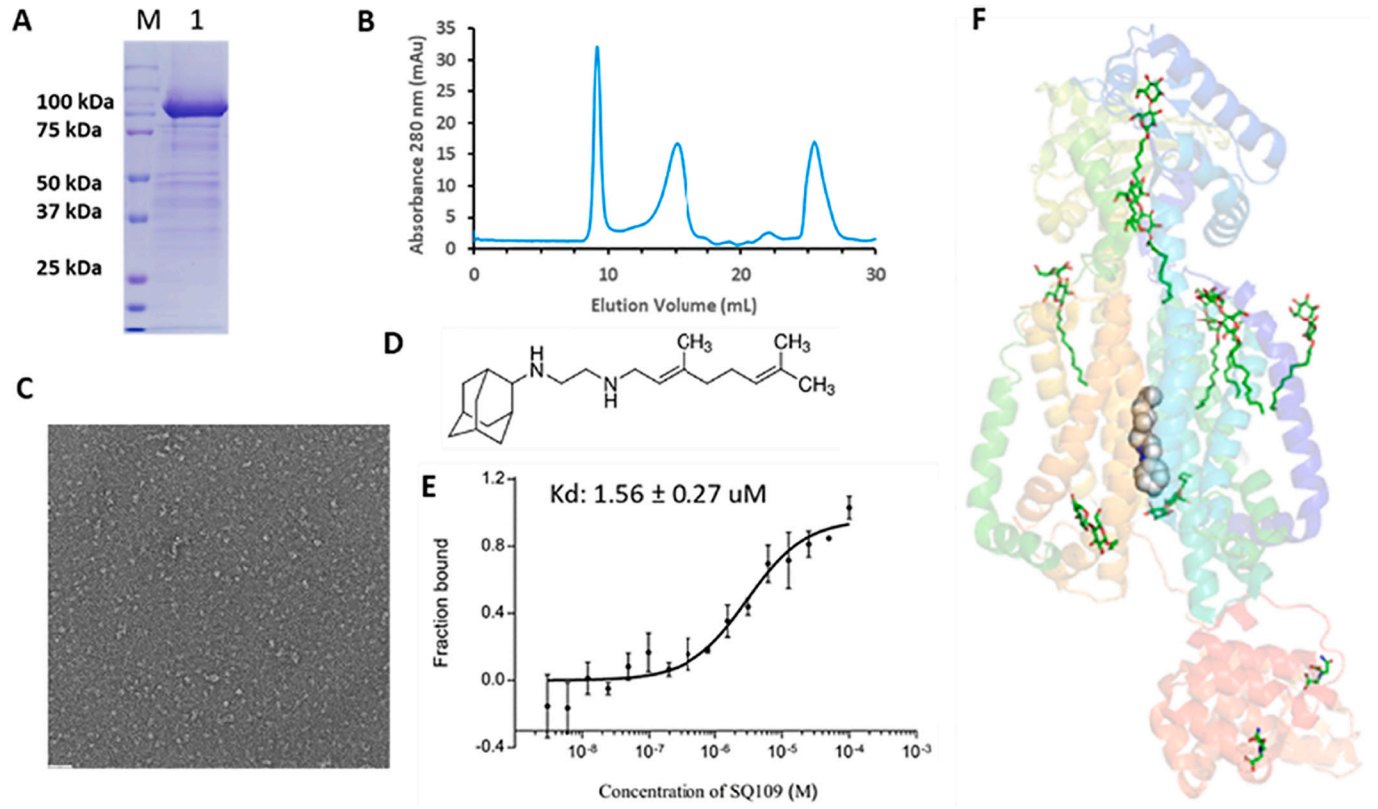

Fig. 1. Biochemical characterization of Mmpl3. (A) The purity of Mmpl3 in the form of NCMN particles was assessed on 12.5% SDS-PAGE gel. (B) The purification profile from superose 6 Increase 10/300 GL shows the Mmpl3 peak around 15 mL. (C) Negative stain EM micrograph shows the homogeneity of the Mmpl3 NCMN particles. (D) Chemical structure of SQ109. (E) Binding affinity analysis of SQ109 to Mmpl3. The assay was performed using MicroScale Thermopherosis Technology (MST). The error bars indicated the mean ± SEM was based on the three individual experiments. (F) Crystal structure of a full-length Mmpl3 complexed with SQ109 missing native cell membrane lipids. SQ109 is shown in the grey colored sphere (PDB ID: 6AJG).

M. smegmatis; and 3) *M. leprae*, which causes leprosy [5]. Fourteen Mmpls participate in diverse activities from various regulations to antibiotic resistance development [6]. Uncovering the mechanisms of individual Mmpls will help us understand how mycobacteria survive from multidrug resistance. Notably, Mmpl3 actively exports trehalose monomycolate (TMM) as the precursor of mycolic acid for cell wall biosynthesis [7,8]. The dysfunction of Mmpl3 leads to cell growth arrest and ultimately cell death. Strikingly, the Mtb Mmpl3 can rescue the *M. smegmatis* Mmpl3 null mutant [9]. This evidence suggests that these two Mmpl3s perform the same function and can be substituted with each other [10]. Therefore, recently, Mmpl3 has emerged as an anti-TB target. Especially, SQ109 is a hydrophobic small-molecule inhibitor of Mmpl3 in a clinical trial (Phase 2a-3 clinical study) [2].

Mmpl3 plays an essential role in the mechanism of mycobacterial survival. Although two crystal structures of Mmpl3 have been reported [10,11], the crystallographic data showed that the Mmpl3 transporter exists as a monomer without native cell membrane lipids, which may be essential for its transport function. The monomeric state of Mmpl3 is inconsistent with other bacterial multidrug resistance superfamily members, such as AcrB [12], CmeB [13], which all function as trimers. In particular, the Mmpl3 ortholog, CmpL1 from corynebacterium (Ncgl2769; 42% identity; 62% similarity to MmpL3 on a 765 amino acid overlap) has been confirmed as a trimer [14]. In our view, the detergent approach might result in the dissociation of the native oligomerization status of Mmpl3. Analysis of Mmpl3 using a detergent-free system is required to understand the oligomeric state and the interaction between Mmpl3 and SQ109.

It is crucial to confirm the biological activity of a protein before using it for structural analysis. Sometimes, the determined structures might not be biologically relevant, which is especially true for membrane proteins. We cloned the wild type of Mmpl3 transporter (1014 amino acids) into the pMYC vector with a 10× His tag at the C-terminus of Mmpl3. We used *Mycobacterium smegmatis* as the natural host to produce Mmpl3 protein required by the NCMN system [1]. The purity of the Mmpl3 NCMN particles from the one-step NiNTA affinity column was examined using SDS-PAGE (Fig. 1A). The homogeneity of further purified Mmpl3 NCMN particles using a size exclusion column was evaluated with negative stain EM imaging (Fig. 1B and C). The molecular weight of Mmpl3 was about 112 kDa, as shown on the SDS-PAGE image. The Mmpl3 NCMN particles are relatively homogenous after the sizing exclusion column, as shown on the negative stain EM micrograph (Fig. 1C). The Superpose 6 Increase 10/300 GL size exclusion profile shows the NCMN particles eluted at the peak of 15 mL (Fig. 1B), corresponding to a molecular weight of about 440 kDa. Considering the monomer molecular weight is 112 kDa, and NCMN particles contain native cell membrane lipids and membrane-active polymers, Mmpl3 proteins may exist as native trimers in the NCMN particles. This result is also consistent with our previous trimer and monomer analysis of AcrB, another member of the RND superfamily, using the NCMN system [15]. An illustration of the chemical structure of SQ109 is in Fig. 1D. The binding affinity was characterized using MicroScale Thermophoresis Technology by fixed concentration of Mmpl3 NCMN particles and titration with log scale increased concentration of SQ109 in the presence of 5% DMSO (Fig. 1E). Fig. 1F shows the crystal structure of a full-length Mmpl3 complexed with SQ109 displayed in sphere rendering (PDB ID: 6AJG). In the detergent-solubilized Mmpl3, the native lipids associated with the transmembrane domain were washed away. A few detergent molecules seem to occupy the locations where native lipids might naturally locate. The transmembrane domain appears to have many hydrophobic cavities where native lipids were likely lost in the course of

detergent solubilization (Fig. S1). Such lost lipids may be essential for supporting the natural structure of Mmpl3 and the fulfillment of its transport function. In the future, a cryo-EM structure of Mmpl3 in NCMN particles will tell us if significant structural differences exist between structures obtained with detergent-free and detergent-based approaches.

The specific binding affinity of SQ109 and Mmpl3 with the detergent method was about 1.65 μM [11]. Our NCMN system obtained the dissociation constant K_d (1.56 μM). This suggests the Mmpl3 purified with detergent and Mmpl3 within the NCMN particles might have identical binding configurations at the binding site of SQ109. However, whether the oligomeric state and tertiary structure of Mmpl3 in the detergent-free and detergent-based systems are also similar remains unknown. However, our current size exclusion purification profile does suggest Mmpl3 exists as trimers. Accurate structural information of membrane proteins is essential in understanding the molecular mechanisms of their activity and related downstream structure-based drug discovery and development. The previous crystal structures of Mmpl3 obtained with a detergent-based approach have provided the initial architecture; however, structural information in multiple conformational states, especially in native cell membrane environments, is still missing. To understand the active mechanism of TMM transport, detergent-free systems such as SMALP, NCMN, and others have recently emerged as alternatives for membrane protein structural biology. Our NCMN system has successfully been applied to determine the high-resolution cryo-EM structure of AcrB together with the naturally associated lipid bilayer patch. These lipids are essential for structural and functional integrity [12]. Here, we demonstrated that we could purify membrane protein Mmpl3 from mycobacteria using the NCMN system. To our knowledge, this is the first study of mycobacterial membrane protein in a detergent-free system.

To date, all crystal structures of Mmpl3 in detergent solutions have shown that it exists as a monomer. However, the detergent itself might be a potential factor that dissociates the oligomers into monomers. The previous comparative crosslinking study with CmpL1 from corynebacterium suggests that Mmpl3 may be a trimer on the cell membrane [14]. Also, some RND family proteins exist as a trimer, but monomeric and dimeric RND protein structures have also been reported [11–13]. It is important to note, however, that all of these structures were determined using detergents. We have demonstrated that the oligomeric state of membrane protein in a native cell membrane environment can be analyzed using the NCMN system. We can successfully extract and purify Mmpl3 in homogenous particles, and this Mmpl3 has specific binding to SQ109 with a binding affinity of around 1.5 μM. The Mmpl3 NCMN particles purity is reasonable based on one-step affinity chromatography. The additional size-exclusive chromatography produced more homogenous high-quality particles for high-resolution single-particle cryo-EM structure determination, currently underway in our lab.

In conclusion, we have purified Mmpl3 with the NCMN system and characterized the binding affinity of Mmpl3 to SQ109. Mmpl3 in the form of NCMN particles may exist as trimers. Using 5% DMSO instead of detergent helps the solubilization of hydrophobic ligands and is compatible with the NCMN system. Ultimately detergent-free systems may have broad applications in illustrating the interactions between hydrophobic ligands and their membrane protein targets.

Notes

None.

CRediT authorship contribution statement

WQ conceived the project, designed and performed the experiments. YG provided the lab space and facility for this project and the NCMN system. YG participated in data analysis. WQ wrote the manuscript. YG participated in manuscript revision.

Declaration of competing interest

Youzhong Guo is the inventor of the NCMN system and he has filed patents together with Weihua Qiu and Thi Kim Hoang Trinh for NCMN polymers and corresponding protocols.

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Appendix A. Supplementary data

The Supporting information containing experimental procedure, additional figures, and results are free of charge at http://pubs.acs.org. Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamem.2021.183793.

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