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Application of amphipols for structure-functional analysis of TRP channels

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Abstract

Amphipathic polymers (amphipols), such as A8-35 and SApol, are a new tool for stabilizing integral membrane proteins in detergent-free conditions for structural and functional studies. Transient receptor potential (TRP) ion channels function as tetrameric protein complexes in a diverse range of cellular processes including sensory transduction. Mammalian TRP channels share ~20% sequence similarity and are categorized into six subfamilies: TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPP (polycystin), and TRPML (mucolipin). Due to the inherent difficulties in purifying eukaryotic membrane proteins, structural studies of TRP channels have been limited. Recently, A8-35 was essential in resolving the molecular architecture of the nociceptor TRPA1 and led to the determination of a high resolution structure of the thermosensitive TRPV1 channel by cryo-EM. Newly developed maltose-neopentyl glycol (MNG) detergents have also proven useful in stabilizing TRP channels for structural analysis. In this review, we will discuss the impact of amphipols and MNG detergents on structural studies of TRP channels by cryo-EM. We will compare how A8-35 and MNG detergents interact with the hydrophobic transmembrane (TM) domains of TRP channels. In addition, we will discuss what these cryo-EM studies reveal on the importance of screening different types of surfactants towards determining high resolution structures of TRP channels.

Keywords

cryo-EM; amphipols; detergent; TRPA1; TRPV1; TRPV2

Introduction

Transient receptor potential (TRP) channels are a distinct family of Ca²⁺-permeable non-selective cation channels that have been implicated in a diverse range of cellular processes, including pain and temperature sensation, neuronal development, angiogenesis, cardiac and pulmonary function, and cancer (Venkatachalam & Montell, 2007). Biochemical, biophysical and structural analyses of TRP channels may provide insight into the molecular

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mechanisms by which these channels function and lead to significant advances in structure-based drug design for TRP channel-related diseases.

The mammalian TRP family is subdivided into six branches: TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPP (polycystin), and TRPML (mucolipin) (Venkatachalam & Montell, 2007). TRP channels are integral membrane proteins that form homo- and hetero-tetramers and require a lipid membrane environment for proper function. Each monomer contains six transmembrane helices (TM1-6) with varying sizes of cytoplasmic amino- and carboxy-termini (Venkatachalam & Montell, 2007). Like most integral membrane proteins, studying the structure and function of TRP channels *in vitro* has been difficult due to the challenges in producing pure, functional protein (le Maire, Champeil & Moller, 2000; Moiseenkova-Bell & Wensel, 2011; Moiseenkova-Bell & Wensel, 2009). Purification requires the screening of many detergents to extract and stabilize the protein from the cell membrane. Detergent-solubilized membrane proteins have historically required an addition of lipids into the buffers and/or reconstitution into lipid membranes to maintain protein function and stability (Garavito & Ferguson-Miller, 2001; Gohon & Popot, 2003). Moreover, detergent-solubilized membrane proteins generally have very limited ranges of storage and operating conditions for structural studies (Seddon, Curnow & Booth, 2004). Biophysical and structural characterization of TRP channels presents a formidable challenge due to these inherent difficulties (Moiseenkova-Bell & Wensel, 2011; Moiseenkova-Bell & Wensel, 2009).

To gain an understanding into the molecular topology and function of TRP channels, several laboratories have employed two different approaches for structural analyses. The first strategy was a “divide and conquer” approach, which provided structures of smaller soluble domains at atomic resolution (Fujiwara & Minor, 2008; Inada et al., 2012; Jin, Touhey & Gaudet, 2006; Lau, Procko & Gaudet, 2012; Lishko et al., 2007; Phelps et al., 2008; Phelps et al., 2010; Shi et al., 2013; Yamaguchi et al., 2001). A second strategy utilized electron microscopy (EM), which revealed full-length TRP channel structures (Cao et al., 2013; Cvetkov et al., 2011; Huynh et al., 2014; Liao et al., 2013; Moiseenkova-Bell et al., 2008). The use of amphipathic polymers (amphipols) to stabilize the TM domain of TRP channels after detergent solubilization and purification contributed to the success of TRP channel structure determination by cryo-EM at side-chain resolution (Liao et al., 2013; Popot et al., 2011; Zoonens & Popot, 2014).

Since their first use in place of detergent for membrane protein stabilization eighteen years ago (Tribet, Audebert & Popot, 1996), amphipols have gained a reputation as a valuable tool for biophysical studies of membrane proteins (Popot et al., 2011; Zoonens & Popot, 2014). Amphipols have the ability to bind tightly to the TM domain of membrane proteins and to mimic a membrane lipid environment (Etzkorn et al., 2014; Tribet et al., 2009). A8-35 is a commercially available amphipol that has been used for determination of several membrane protein structures by electron microscopy (EM) (Fig. 1a) (Althoff et al., 2011; Catoire et al., 2010; Flotenmeyer et al., 2007; Gohon et al., 2008; Kevany et al., 2013; Popot et al., 2011; Tribet, Audebert & Popot, 1996; Tsybovsky et al., 2013; Wilkens, 2000; Zoonens & Popot, 2014). These studies demonstrated that detergents could be exchanged to A8-35 while preserving the fold and stability of membrane proteins. A8-35 forms a ~10–20 Å belt around

the TM domains that can be detected by EM (Althoff et al., 2011; Gohon et al., 2008; Kevany et al., 2013; Popot et al., 2003; Tsybovsky et al., 2013).

In addition, the newly developed maltose-neopentyl glycol (MNG) class of detergents has been shown to stabilize membrane proteins for structural studies (Rasmussen et al., 2011a; Rasmussen et al., 2011b; Ring et al., 2013; Westfield et al., 2011; Zhang et al., 2012) and was recently employed for structural determination TRPV2 by cryo-EM (Huynh et al., 2014).

In this review, we discuss the utilization of amphipols and detergents in recent structural analyses of TRP channels by EM.

Use of amphipols for structure determination of TRPA1 channel by EM

TRPA1 is a Ca^{2+} -permeable, non-selective cation channel and one of the key pain sensors in mammals (Jordt et al., 2004). TRPA1 is activated by numerous exogenous electrophilic compounds and by endogenous reactive species generated during oxidative stress. Structural analyses of TRPA1 will facilitate novel design of analgesics and anti-inflammatory agents (Andrade, Meotti & Calixto, 2012).

The TRPA1 single-particle EM structure was the first TRP channel structure resolved with the incorporation of amphipols (Cvetkov et al., 2011). TRPA1 was initially purified in Fos-Choline 12 (FC12) detergent and was shown to be functional when reconstituted into small unilamellar vesicles (Cvetkov et al., 2011); however amphipols were needed to stabilize TRPA1 for EM studies. To achieve this, FC12-purified TRPA1 was exchanged to A8-35 amphipol by dialysis in the presence of Bio-Beads SM-2 (Cvetkov et al., 2011). Size-exclusion chromatography (SEC) revealed that A8-35 preserved the tetrameric state of TRPA1 (Fig. 1b) (Cvetkov et al., 2011). The A8-35 solubilized TRPA1 protein gave a homogenous preparation that was used to determine the TRPA1 EM structure at 16 Å resolution (Fig. 1c and d) (Cvetkov et al., 2011). This moderate resolution structure revealed a tetramer with a large cytoplasmic density and a TM domain. The large cytoplasmic density was able to account for the N-terminus with 14 to 17 ankyrin repeats and the C-terminal coiled-coiled domain. A model of the TRPV1 TM domain was fitted into the TRPA1 EM map in the TM region. Similar to other membrane proteins, an uneven belt was apparent around the TM portion of the protein, which likely represents A8-35 densities (Fig 1d and 2b) (Althoff et al., 2011; Cvetkov et al., 2011; Gohon et al., 2008; Kevany et al., 2013; Popot et al., 2003; Tsybovsky et al., 2013).

Carboxylate functional groups confer A8-35 with the ability to solubilize membrane proteins, but these same functional groups make A8-35 sensitive to calcium ions, $\text{pH} < 7$, and salt concentrations above 200–300 mM (Popot et al., 2011; Tribet et al., 1996; Zoonens & Popot, 2014). SAPol, another amphipol (Fig. 1a), contains a sulfonated group and is unaffected by low pH, high ionic strength, or high Ca^{2+} concentrations (Popot et al., 2011). Specifically, SAPol has been proven to be an effective stabilizing agent in Ca^{2+} concentrations up to 30 mM and NaCl concentrations up to 300 mM (Dahmane et al., 2011). We tested the stability of TRPA1 after exchange to SAPol (a kind gift from J.L. Popot) and observed that SAPol increased the stability and solubility of TRPA1 (Fig. 1b). The use of the

higher salt concentrations together with SApol could optimize the yield of TRPA1 protein for structural studies, and compatibility of SApol with Ca^{2+} could be important for TRPA1 ligand binding assays in the future. Based on these results that we obtained for TRPA1, it is clear that use of the A8-35 and SApol could be expanded for stabilizing other TRP channels (Fig. 1).

Use of amphipols for structure determination of TRPV channels by cryo-EM

The TRPV subfamily of ion channels has six members involved in a diverse range of cellular functions (Venkatachalam & Montell, 2007). TRPV1-4 are Ca^{2+} -permeable nonselective cation channels with approximately 50% sequence homology and are involved in nociception and inflammation (Venkatachalam & Montell, 2007). TRPV5 and TRPV6 are important for Ca^{2+} absorption by epithelial cells (Venkatachalam & Montell, 2007). Among the TRPV subfamily members, TRPV1 is essential for pain and temperature sensation (Caterina et al., 1997). Capsaicin, the active component of chili peppers, activates TRPV1 causing a pain sensation, yet it can also be used as an analgesic for treating pain.

The first cryo-EM structure of rat full-length TRPV1 at 19 Å resolution revealed a possible low resolution architecture of the channel; however, the use of decyl- β -D-maltoside detergent (DM) contributed to sample heterogeneity, which likely affected the final reconstruction of the channel (Moiseenkova-Bell et al., 2008). A more recent study employed a truncated TRPV1 construct (minimal TRPV1) in order to achieve a biochemically homogenous sample (Liao et al., 2013). Minimal TRPV1 still displayed predominant protein heterogeneity after purification using dodecyl- β -D-maltoside detergent (DDM) (Liao et al., 2013). Together, our previous biochemical observation and availability of these new results suggest that these detergents are not suitable for structural studies of TRPV1 and that the 19 Å cryo-EM structure of full-length TRPV1 does not represent the overall architecture of the full-length protein.

A major key to stabilizing minimal TRPV1, leading to the high resolution structural analysis of the channel at 3.4 Å by cryo-EM (Liao et al., 2013), was the use of A8-35 amphipols and a previously established protocol for purification of TRPA1 (Cvetkov et al., 2011). Similar to TRPA1, DDM-purified TRPV1 was exchanged to A8-35 amphipol by dialysis in the presence of Bio-Beads SM-2 for further structural studies (Cvetkov et al., 2011; Liao et al., 2013). These results confirmed that TRP channels need a membrane-like support for their stability after extraction from plasma membrane and A8-35 is a favorable substitute for the lipid membrane (Etzkorn et al., 2014; Popot et al., 2011; Zoonens & Popot, 2014). A8-35 allowed for a high resolution analysis of the TM domain of TRPV1 while the N- and C-terminal densities were not fully and clearly resolved in the cryo-EM structure (Henderson, 2013). The TM region, including the channel pore, resembles those of previously determined Na^+ and K^+ channels structures by X-ray crystallography. Moreover, structures of TRPV1 in the presence of activators revealed minimal conformational changes in the TM region of the channel, suggesting that the pore region facilitates gating of the channel upon ligand binding (Cao et al., 2013). The N-terminus of TRPV1 contains six ankyrin repeats; however, the first two ankyrin repeat densities were not observed in the minimal TRPV1 cryo-EM structure possibly due to the flexibility of the ankyrin repeats (Liao et al., 2013).

Amphipols likely stabilized the TM domain of TRPV1 allowing for high resolution structure determination of this region of the protein (Popot et al., 2011).

The interaction between A8-35 and TM domain of the minimal TRPV1 is unexpectedly different from that previously observed in TRPA1 (Cvetkov et al., 2011) and other membrane protein structures (Althoff et al., 2011; Etzkorn et al., 2014; Gohon et al., 2008; Kevany et al., 2013; Popot et al., 2003; Tsybovsky et al., 2013). It has been suggested that A8-35 is strongly attached to the hydrophobic TM domain of membrane proteins and forms an unevenly distributed belt along the edge of the TM region as seen in the structures of the mitochondrial supercomplex B ($I_1III_2IV_1$) (Fig. 2a), TRPA1 (Fig. 2b) and other membrane proteins (Althoff et al., 2011; Cvetkov et al., 2011; Etzkorn et al., 2014; Gohon et al., 2008; Kevany et al., 2013; Popot et al., 2011; Popot et al., 2003; Tsybovsky et al., 2013). These structures are determined by cryo- and negative stain EM at low resolution varying between 16–19 Å, and only give us an overall understanding of how A8-35 interacts with the hydrophobic core of the membrane proteins.

In the recent TRPV1 reconstructions at 8.8 Å (Fig. 2c, TRPV1 map was generously provided by Yifan Cheng) (Liao et al., 2013) and at 3.4 Å (Fig. 2d) by cryo-EM (Liao et al., 2013), we can, for the first time, observe how A8-35 possibly interacts with a tetrameric membrane protein at high resolution. The A8-35 belt is clearly present in these two TRPV1 cryo-EM structures when displayed from the top view at low iso-surface threshold as suggested by the authors (Fig. 2c and d) (Liao et al., 2013) and are similar to the top views for the previously reported low resolution structures of the mitochondrial supercomplex B ($I_1III_2IV_1$) (Fig. 2a) (Althoff et al., 2011) and TRPA1 (Fig. 2b) (Cvetkov et al., 2011).

Intriguingly, cross-sectional views at the same low iso-surface threshold show the predicted densities corresponding to A8-35 display minimal interaction with the TM regions of the channel. In the 8.8 Å resolution structure, A8-35 forms an uneven “cloud of densities” with minimal contact to the TM region of the channel (Fig. 2c). In the 3.4 Å resolution structure, the putative A8-35 density forms a uniform belt around the TM domain of the protein (Fig. 2d). While this may be due to the imposed four-fold symmetry during EM reconstruction and refinement (Liao et al., 2014), the belt shows minimal direct interaction with TM domain of the channel (Fig. 2d). At this resolution, it is possible that the polar head groups of A8-35 were detected while the hydrophobic hydrocarbon chains of A8-35 are not apparent due to their lower electron density; however the interaction of TRPV1 with small hydrophobic ligands in the TM domain was detected (Cao et al., 2013) suggesting that A8-35 adsorbed to the TM region of TRPV1 would be detectable. Another high resolution structure with and without imposed symmetry of a different membrane protein by cryo-EM is needed to further understand the interaction of A8-35 with the TM domain of membrane proteins.

The concurrent determination of the structure of the highly homologous TRPV2 by cryo-EM in the newly developed MNG class of detergents (Huynh et al., 2014) allowed for a comparison of the behavior of TRPV channels in A8-35 and MNG detergent. TRPV2 shares nearly 50% sequence identity with TRPV1 (Caterina et al., 1999), suggesting the overall architecture of these channels would be similar. MNG detergents have been shown to

stabilize membrane proteins in a milder manner than commonly used detergents such as DM and DDM (Chae et al., 2010). Like TRPV1, extraction and purification of TRPV2 in DDM resulted in oligomeric heterogeneity of the protein as determined by SEC, blue-native gel and EM imaging (Fig. 3a), suggesting that DDM does not provide a stable environment for TRPV channels. Exchange of TRPV2 from DDM to A8-35 stabilized TRPV2 in its tetrameric state (Fig. 3b), while exchange to SAPol promoted aggregation of TRPV2 into higher oligomeric structures (Fig. 3c). This observation differs from the results that were obtained for TRPA1, which displays a more homogenous behavior in SAPol. This is likely due to the fact that TRPA1 shares only ~15% sequence similarity to TRPV channels, suggesting that screening stabilizing surfactants for each individual TRP channel will be essential for their structural studies. Purification of TRPV2 in MNG detergent led to the most homogenous preparation as determined by SEC, blue-native gel and EM (Fig. 3d). Therefore, MNG-purified TRPV2 was used for structure determination (Huynh et al., 2014).

The structure of TRPV2 in MNG detergent was solved by cryo-EM (Huynh et al., 2014). 2D class averages of 8.8 Å TRPV1 and 13.6 Å TRPV2 display similar features when collected under similar conditions (Fig. 4a, b) (Huynh et al., 2014; Liao et al., 2013). Both show four-fold symmetry and clearly resolved TM and cytoplasmic regions. The crystal structure of a homologous K⁺ channel MlotiK1 fit well into the TM portion of the TRPV2 EM map revealing an extra, uneven density around the TM domain. This likely accounts for the MNG detergent belt around the TM portion of the channel protein (Fig. 4d). This is similar to the size and distribution of MNG detergent belts of other membrane proteins determined by EM (Vahedi-Faridi et al., 2013; Westfield et al., 2011) and similar to the A8-35 belt observed around the TM domain of TRPV1 at 3.4 Å (Fig. 4d, f) (Liao et al., 2013). In contrast, the amphipol belt in the 8.8 Å minimal TRPV1 structure displays an uneven “cloud of densities” (Fig. 4e). This is surprising as the 2D class averages display very similar densities around the TM region of the protein (Fig. 4a, b).

Based on these observations, the conclusion can be made that A8-35 amphipols and MNG detergents share similar stabilizing properties and interaction mechanisms for the TM region of TRP channels. Further characterization of the interaction of amphipols and MNG detergents with the TM domains of ion channels is needed to fully explore this possibility. It is important to understand the exact mechanism of the interactions of A8-35 and MNG detergents with membrane proteins, as this could reveal the strengths and utility of A8-35 and other amphipols in structural biology. While MNG detergents have been used extensively and successfully in X-ray crystallography to solve the structures of several membrane proteins (Rasmussen et al., 2011a; Rasmussen et al., 2011b; Ring et al., 2013; Westfield et al., 2011; Zhang et al., 2012), A8-35 and other amphipols have yet to be used for X-ray crystallography (Popot et al., 2011). Recent structures of TRPV1 and TRPV2 showed that the behavior of these two compounds are very similar and A8-35 has promising potential for use in X-ray crystallography (Huynh et al., 2014; Liao et al., 2013). Nevertheless, A8-35 has already been employed to yield a structure of a TRP channel at side-chain resolution without the use of X-ray crystallography (Liao et al., 2013), and hopefully will allow us to produce more high resolution TRP channel structures.

Conclusion

The TRP superfamily consists of the twenty-eight ion channels with diverse functions. Structural analysis of these channels at atomic resolution is just in its infancy. Amphipols are unique molecules that recently have been used for the stabilization and structure determination of two TRP channels. These results displayed a high potential for the utilization of these amphipolic molecules for the stabilization and structural analysis of the other twenty-six different TRP channels. Moreover, a new class of MNG detergents has also provided the ability to stabilize TRP channels and showed similar structural properties when compared to amphipols. Overall, amphipols have become a promising tool for studying the structure of membrane proteins.

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Abbreviations

A8-35	a poly(sodium acrylate)-based amphipol comprising 35% of free carboxylates, 25% of octyl chains, 40% of isopropyl groups
SApol	sulfonated amphipol
DM	decyl- β -D-maltoside
DDM	dodecyl- β -D-maltoside
MNG	maltose-neopentyl glycol
2D	2-dimensional
TM	transmembrane
TRPA	transient receptor potential ankryin
TRPV	transient receptor potential vanilloid
EM	electron microscopy
SEC	size-exclusion chromatography

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Fig. 1.

Amphipols and single-particle EM of purified TRPA1. **a** Chemical structures of A8-35 and SApol (Popot et al., 2011). **b** SEC of purified TRPA1 in FC12 before and after exchange to either A8-35 or SApol (Cvetkov et al., 2011). **c** Representative negative stained micrograph of purified TRPA1 in A8-35. Scale bar, 70 nm (Cvetkov et al., 2011). **d** Three-dimensional reconstruction of TRPA1 in A8-35. The predicted structure of the TRPV1 TM domain (purple) was docked into the predicted TM domain of the TRPA1 EM structure. Models of the TRPA1 N-terminus (green) and C-terminus (orange) were generated using I-TASSER and fitted into the putative cytosolic density of the TRPA1 EM map (Cvetkov et al., 2011). Scale bar, 50 Å.

Fig. 2.

A8-35 belt in the EM structures of membrane proteins. **a** 19 Å cryo-EM structure of the mitochondrial supercomplex B ($I_1III_2IV_1$) viewed from the matrix side and a cross-sectional view in the TM domain. The fitted supercomplex B crystal structure (PDB: 2YBB) is represented in green ribbons (Althoff et al., 2011). Scale bar, 50 Å. **b** 16 Å negative stain EM structure of full-length TRPA1 shown from the top view and a cross-sectional view in the TM domain. The predicted structure of the TRPV1 TM domain is represented in purple ribbons (Cvetkov et al., 2011). Scale bar, 50 Å. **c** 8.8 Å cryo-EM structure of minimal TRPV1 at low iso-surface threshold shown from the top view and a cross-sectional view of the transmembrane domain. Density corresponding to TRPV1 protein is represented in blue ribbons (PDB: 3J5P) Scale bar, 50 Å. **d** 3.4 Å cryo-EM structure of minimal TRPV1 shown from the top view and a cross-sectional view of the TM domain at low iso-surface threshold. Density corresponding to TRPV1 protein is represented in blue (PDB: 3J5P) (Liao et al., 2013). Scale bar, 50 Å.

Fig. 3. TRPV2 purification screen using different amphipols. SEC, native-gel electrophoresis and negative stained EM micrograph of purified TRPV2 solubilized in **a** dodecyl- β -D-maltoside (DDM), **b** A8-35, **c** SAPol and **d** maltose neopentyl glycol (MNG) detergents. Scale bar, 100 nm.

Fig. 4.

Comparison of the TRPV2 structure in MNG detergent and the TRPV1 structure in A8-35 amphipols. Representative 2D class averages of **a** MNG detergent purified full-length TRPV2 collected with an FEI Tecnai TF20 microscope with a CMOS camera (Huynh et al., 2014). **b** detergent exchanged A8-35 purified minimal TRPV1 at 8.8 Å resolution collected using an FEI Tecnai TF20 microscope with a CMOS camera. **c** 3.4 Å resolution collected using an FEI Polara TF30 microscope with a direct detector camera (Liao et al., 2013). **d** TM domain cross-sections of cryo-EM structure of TRPV2 docked with MlotiK TM domain (red ribbon, PDB: 3BEH) (Huynh et al., 2014). **e** TM domain cross-sections of cryo-EM structure of TRPV1 at 8.8 Å resolution and **f** at 3.4 Å resolution docked with modeled TRPV1 TM domain (blue ribbon, PDB: 3J5P). TRPV1 structure is displayed at low iso-surface thresholds (Liao et al., 2013).