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Single-Particle Cryo-EM of Calcium Release Channels: Structural Validation

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Abstract

Few tools are available to determine the structure of large integral membrane proteins such as intracellular Ca^{2+} release channels, RyRs and IP₃Rs. Single particle cryo-EM can readily determine the structure of such channels to intermediate resolution, and can be used to quantitatively assess conformational variability. However, due to the, often low, image contrast of these cryospecimens, methods for validation are critical to insure the accuracy of such structures, and to put limits on their interpretability. The low-resolution structure of RyR has been well established for some time, but high-resolution has been slow to emerge. The structure of IP₃R channel by cryo-EM had a number of false-starts, but improved validation methods have recently lead to a demonstrably accurate reconstruction.

Introduction

Cell function depends on the regulated flow of specific ions across the biological membrane in and out the cell as well as between intracellular compartments. Ion channels form a class of integral membrane proteins permitting regulated but passive transfer of ions across membranes. Understanding the structural basis for ion transport is one of the most important frontiers in structural biology. Integral membrane proteins are the most difficult targets for structural analysis due to their tight association with lipid membranes *in vivo* and their intrinsic dynamic nature. While X-ray crystallography has begun making some strides in this area, ion channels remain one of the most complex and structurally least understood of the cellular proteins. It is estimated that membrane proteins represent 20–30% of the genome [1], and yet of the 82,224 structures currently listed in the PDB, only ~5800 (7%) are reported as membrane proteins, and the PDBTM, a subset of the PDB containing only structures believed to contain transmembrane domains, currently has only 1827 entries (2%). Large sections of ion channels such as the Ca^{2+} release channels have virtually no sequence homology with any known structures. Moreover, some membrane proteins form unusually large macromolecular assemblies, whose enormous size makes X-ray crystallography or NMR difficult to apply.

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At present, single-particle electron cryomicroscopy (cryo-EM) has emerged as one of the most effective and straightforward techniques for the study of large membrane proteins and their interactions. Among these are the intracellular Ca^{2+} release channels also known as ryanodine and inositol-1,4,5-trisphosphate receptors (RyRs and IP₃Rs) that form exceptionally large integral membrane protein complexes of over 2.3 and 1.2 MDa, respectively. These channels mobilize Ca^{2+} from intracellular stores such as endoplasmic/sarcoplasmic reticulum (ER/SR) into cytoplasm and play important roles in generation of Ca^{2+} signals regulating a wide array of cellular processes, including muscle contraction and brain function.

To date, the best 3D structures of complete tetrameric RyR and IP₃R channels have been solved by single particle cryo-EM at 10–17 Å resolution [2–5], and high-resolution crystal structures of both channel proteins are currently limited to soluble cytoplasmic fragments representing only ~10–15 % of the entire protein [6–13]. While attempts have been made to dock these crystal structures into existing cryo-EM maps [9,12,13], it is important to note that the accuracy of these efforts depends on the resolution of the cryo-EM map, and assumes that the fragment structures retain an identical conformation as in the full assembly [14]. The reliability of docking improves dramatically when the cryo-EM maps have achieved sufficient resolution to unambiguously localize α -helices (~7–8 Å), explaining why further resolution improvement in cryo-EM studies of entire channels is such a critical goal.

Cryo-EM of soluble proteins has recently achieved resolutions approaching those provided by X-ray crystallography (3–5 Å) [15], but the apparent quality of these structures varies. This general perception has prompted the cryo-EM community to more aggressively develop tools for the critical assessment of maps at all resolution ranges [16]. At the high resolution extreme, existing methods developed over decades for x-ray crystallography are being adapted to cryo-EM. However, in the low-resolution regime (worse than 5–7 Å), completely different validation and assessment tools are required. As membrane protein structures reported to date fall almost entirely in this resolution range, in this manuscript we survey some of the methods that have been applied to controversial structures in the field, and discuss the growing consensus that, as cryo-EM is maturing as a field, these validation methods should be required of all new structures [16]. This review briefly summarizes our current knowledge about the 3D structure of Ca^{2+} release channels and outlines the emerging ideas on structural validation of cryo-EM density maps.

From discovery to structure of Ca^{2+} Release Channels

The story of Ca^{2+} release channels begins with discovery that physiological responses, such as striated muscle contraction, are triggered by release of Ca^{2+} from ER/SR [17,18]. Subsequent studies established that specific proteins tightly bound to SR/ER membranes function as Ca^{2+} -permeable ion channels, which are activated by selective ligands, inositol-1, 4, 5-trisphosphate or ryanodine [19–22]. Cloning of receptor proteins established that both RyR and IP₃R are unusually large membrane proteins, comprising four subunits of ~5,000 amino acids for RyR [23] or ~2,700 amino acids for IP₃R [24,25]. Furthermore, mammalian genes have been identified encoding three homologous isoforms (types 1–3) of each receptor.

Structure determination of Ca^{2+} release was not possible until RyR and IP₃R proteins had been purified in detergent-solubilized form [26–29]. Structural studies have been focused primarily on type 1 RyR (RyR1) and type 1 IP₃R (IP₃R1), which are the best functionally characterized isoforms and are abundant in skeletal muscle and cerebellum, respectively. Our current knowledge of the quaternary structure of Ca^{2+} release channels is based solely

on TEM studies [30,31]. Both channels have a common architectural arrangement: the four subunits form a single central ion conduction pathway with an overall characteristic mushroom shape (Figure 1) [2–4]. The cytoplasmic (CY) domains are targets of multiple interactions with large array of intracellular regulatory molecules including proteins that are linked to the channel gating activity. Through low-resolution cryo-EM analysis, some important sites for protein-protein interactions and disease-related “hotspot” amino acids in the protein sequence have been mapped to 3D structure of the tetrameric RyR. Furthermore, conformational changes associated with channel activation have been identified in both the transmembrane (TM) and CY regions of RyR [30,31]. Cryo-EM structural data in combination with recent crystallographic studies of small soluble domains [6–13] has provided important structural insights into our understanding of how Ca^{2+} release channels work at molecular level. However, the lack of atomic details for the entire channel molecule limits the conclusions that we can currently draw thus leaving significant functional and structural ambiguities. Clearly, the field faces challenges related with interpretation and validation of currently available structural data.

Validation of EM structure

The first 3D structure of RyR1 channel was determined from a random conical tilt series of images of negatively stained channel particles [32]. This study provided an initial platform for future EM studies of RyR1 in a frozen-hydrated state [31]. The fact that two EM groups using different approaches (random conical tilt vs. angular reconstitution) achieved essentially the same 3D structure of the channel is a strong indication of accuracy within the resolution limits of these early studies (~ 35 Å). Subsequent cryo-EM studies at higher resolution continued to elucidate structural features thus expanding our mechanistic understanding of RyR channel (Figure 2a). It is worth noting, that RyR1 was among first non-icosahedral structures solved by single particle cryo-EM [33,34]. In contrast to RyR1, early structures of IP₃R1 did not agree [30], and it took more than 15 years to produce a reliable 3D reconstruction of this difficult channel [4,5].

This raises the more general question of how to assess the overall reliability of a cryo-EM reconstruction. Over a decade ago, a method was proposed for validating structures at low resolution through varying the data collection geometry [35]. Specifically, a small set of single particle data is collected wherein each set of particles is imaged in two different orientations, with a known rotation relating them. These tilt-pairs of particle images are then compared to a putative 3-D reconstruction to insure that the same relative rotation is produced computationally as experimentally (Figure 3a). If the orientations are substantially inconsistent, then the structure is incorrect (Figure 3b–d). Due to the requirement for additional experiments, this technique has been slow to gain acceptance, but in recent years it has emerged as a standard [36,37].

Once the accuracy of the quaternary structure has been established, it is important to establish the limiting resolution for reliable interpretation of features in the map. Resolution is generally assessed by splitting the raw particle image data into two halves, then refining the two halves independently, and assessing resolution based on the similarity of the two independent results at each length scale. Historically, however, the definition of “independent” was interpreted different ways by different groups. A more rigorous standard for resolution assessment (the so-called “gold standard” method), which defines what is required to claim such independence, has recently been proposed [38], and reviewers in the cryo-EM community are beginning to enforce this standard in new publications. After participating in designing this standard, we recently showed that the resolution we believed to be ~ 10 Å [4] for our cryo-EM structure of IP₃R1 channel, is actually, closer to 17 Å, due to intrinsic flexibility of the assembly, though it was possible to achieve ~ 14 Å by

classifying the data [5]. When the same test was performed for RyR1, its estimated resolution falls only from ~10 Å to ~12 Å, again, due to flexibility rather than refinement bias.

Very often flexibility is an integral part of the biological function of the molecules themselves, and rigidly locking them into a single conformation would actually distort the true picture of how they function. There are a variety of methods for quantitatively assessing such structural variability [39], including variance maps for localizing regions of variation [40], simultaneous multi-model refinement [41,42], maximum likelihood [43] and biochemical manipulation of the specimen such as binding a specific ligand to the molecule of interest would restrict dynamic behavior of ligand-binding domains.

Furthermore, at low resolution, it can also be useful to refine the same data in multiple software packages (Figure 2). In the case of IP₃R, it was shown that 5 different software packages all produced the same basic quaternary structure (Figure 2b), and that the cross-resolution among these structures was comparable to the “gold standard” resolution determined for the original map [5]. This self-consistency is reassuring, and small visible differences observed among the structures can help give guidance when trying to avoid over-interpreting visual features of a map.

Conclusions and perspectives

Understanding molecular machinery of ion channels at the atomic level remains a major challenge. The current cryo-EM structures of Ca²⁺ release channels have been unambiguously demonstrated to be accurate to intermediate resolution. Debate continues over the architecture of the ion conduction pathway. Structural homology among tetrameric cation ion channels suggests a common architecture for the ion-conduction pathway. The TM region of Ca²⁺ release channels and that of certain K⁺ channels [2–4,44] bear a striking similarity, indicating they may share a similar structural motif. While such overall similarity can be observed at the current resolution (12–15 Å), it is probable that the α -helices currently observed in these domains will require adjustment with improving resolution, as happened, for example, with early studies of the nicotinic acetylcholine receptor [45,46]. Ultimately, channel gating requires a structural rearrangement of the ion conduction pathway, but the precise molecular nature of these conformational changes and how ligand-binding communicated to the channel pore will remain largely speculative without a sufficiently high-resolution reconstruction [47]. Furthermore, at the current marginal resolution limit, when no secondary structure elements can be rigorously identified in the majority of the density map, no mechanism exists to unambiguously validate fits of crystal structures of small domains into the large cryo-EM map. These limitations can lead to ambiguities in fitting, such as in recent docking studies of RyR1 [12,48].

It has been demonstrated that single-particle cryo-EM is a powerful method for producing reliable structures of macromolecular assemblies at resolutions as high as 3–5 Å [15], however integral membrane proteins such as ligand-gated Ca²⁺ release channels have struggled just to reach intermediate resolutions (10–15 Å). Certainly the imaging of these specimens themselves pose a challenge, as the presence of detergent or other solubilizing agents has the side-effect of reducing image contrast, and other experimental issues such as preferred orientation and particle distribution problems abound. However, emerging evidence indicates that the primary reason for this limited resolution is the structural flexibility of the specimen. A feature shared by both of these channels, is a large number of molecular modulators, each of which is clearly going to modify the structure of the channel. Recent studies with, for example, the ribosome, have demonstrated that with a suitably large population of particle images, it is possible to characterize the entire dynamic pathway of a

complex molecular assembly [49]. Such methods can readily be applied to ion channels as well, and may prove to be the path forward to moderately better resolutions.

While it has been demonstrated that solubilized ion channels can be reconstituted into lipid vesicles and remain functional, it is still quite possible that the solubilized form of the channels is undergoing more conformational variability. The lipid bilayer may act as a stabilizing agent. Study of reconstituted channels thus offers another potential way forward for this field [50].

With recent advances, such as direct electron detectors and phase plates, more rigorous data analysis strategies, and new specimen preparation methods, cryo-EM technology continues to improve rapidly, offering hope that high-resolution structures of these important ion channels may finally be achieved in the near future.

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Highlights

- The complete 3D structures of IP₃R/RyR channels have been solved by single-particle cryo-EM at 10–17 Å resolution
- Structure validation is critical for low-contrast images of detergent-containing cryospecimens.
- Tilt-pair analysis is a powerful tool for validating the low-resolution veracity of structures.
- Subclassification of particles can characterize molecular motion, but very large data sets may be required.
- The “gold-standard” FSC resolution test is not susceptible to exaggeration due to bias or conformational variability

Figure 1. Surface representation of 3D maps of Ca^{2+} release channels determined by single-particle cryo-EM

(a, c) RyR1 (EMD-1275) [2]; (b, d) IP₃R1 (EMD-5278) [4]. (a, b) Structures of the tetrameric channels are viewed from the cytoplasmic side with one putative subunit is depicted in blue; (c, d) the two opposing subunits are viewed along membrane plane to allow visualization of internal features in the channel structure. The TM regions of RyR1 and IP₃R1 channels are quite similar, however the CY portions, aside from the difference in their overall dimensions, have quite different architectural arrangements including the most notable features such as a central plug in the IP₃R1 structure, and distinctive clamp-shaped regions in the RyR1 structure.

Figure 2. Validation of cryo-EM structures with different software packages

(a) 3D reconstructions of RyR1 (EMD-1275 [2] and EMD-5014 [3]) were filtered to the same ~ 12 Å resolution and rendered at a contour levels corresponding to a molecular mass of ~ 2.4 MDa. (b) 3D reconstructions of IP₃R1 were calculated in each software package using the same cryo-EM images [5], the final maps were filtered to match (~ 20 Å) and rendered at equivalent thresholds.

Figure 3. Tilt-pair analysis of images from vitrified IP₃R1

The tilt-pair parameter plots for 42 tilt pairs of particle images, recorded with a tilt angle of 10° in recently published study [5]. Surface representations of 3D maps used to determine orientations of particles from cryo-EM images: (a) EMDB-5278 [4]; (b) from [51]; (c) from [52]; (d) EMDB-1061 [53]. The plot in (a) clearly shows that the particles are clustered at the expected location based on the experimental tilt geometry, thus confirming veracity of cryo-EM map and its self-consistency [4]. The same tilt-pair validation failed with three cryo-EM structures (b–d) published a decade earlier thus demonstrating that these maps are in no way consistent with the current particle images.