Review

Potential of cryo-EM for high-resolution structural analysis of gap junction channels

Atsunori Oshima[1, 2, ¶]

[1]Cellular and Structural Physiology Institute (CeSPI), Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

[2]Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

¶To whom correspondence should be addressed: Atsunori Oshima

Cellular and Structural Physiology Institute (CeSPI)

Department of Basic Medicinal Sciences, Graduate School of Pharmaceutical Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Tel: +81-52-747-6837

Fax: +81-52-747-6795

E-mail: atsu@cespi.nagoya-u.ac.jp

ABSTRACT

Gap junction family proteins form conduits connecting the cytoplasm of adjacent cells, thereby enabling electrical and chemical coupling to maintain physiological homeostasis. Gap junction proteins comprise two gene families, connexins in chordates and innexins in pre-chordates. Their channel structures have been analyzed by electron or X-ray crystallography, but only a few atomic structures have been reported. Recent advances in single-particle cryo-electron microscopy (cryo-EM) will help to elucidate these structures further. Here the structural biology of gap junction channels utilizing crystallography and single-particle cryo-EM is overviewed to shed light on the functional mechanisms of cell-cell communication that are essential for multicellular organisms.

Introduction:

Intercellular communication is an indispensable event for multicellular organisms, from invertebrates to chordates. The main machinery mediating this function is the gap junction. Gap junctions, an assembly of communicating channels that penetrate two adjoining cell membranes – referred to as gap junction channels, facilitate the transfer of small molecules such as nucleotides, second messengers, ions, and peptides between cells [1]. This function is associated with many biological processes, such as cardiac development, immune system function, fertility [2], and electrically mediated neuronal synchronization [3]. Malfunction of gap junction communication leads to many human diseases, such as hearing loss, skin disorders, neuropathies, cataracts, and cardiovascular disease [4]. Early evidence of gap junctions in association with electrical synapses was provided by the giant fiber motor neuron of cray fish [5], marking the dawn of research on intercellular channels.

The machinery of the intercellular channels is formed by subunit families with four transmembrane domains, connexin in chordates and innexin in pre-chordates, which have little similarity in terms of their amino acid sequences. It remains unclear whether these two protein families are in a relationship of evolutionary convergence or divergence [6-8]. Pannexin and leucine-rich repeat-containing 8 (LRRC8) protein families also have weak homology with innexin [9,10]; these are not thought to form gap junction channels, but rather to function as single membrane channels [10,11]. Until a few years ago, the only high-resolution structure of gap junction channels was for a channel composed of connexin 26 (Cx26) [12●●,13●●]. It has been by no means easy to obtain the crystals of gap junction proteins diffracting to high-resolution possibly due to flexibility in the cytoplasmic domains. Given the 3D crystals of Cx26 composed of fully docked junction channels [12●●,13●●], it is unavoidable to have crystal-packing contacts mediated by the cytoplasmic domains that have not yet been resolved for connexin channels. Recent advances in cryo-EM have promoted structural studies without crystallization, which led to atomic resolution of the structures of *Caenorhabditis elegans* innexin-6 (*Ce*-INX6) and LRRC8 [14●●,15-17]. All these proteins have four transmembrane helices, TM1 ~ TM4, and two extracellular loops, E1 and E2, and the cytoplasmic domains contain the N-terminus, the C-terminus, and

the cytoplasmic loop. This review highlights structural studies focusing on connexins and innexins as the main components forming gap junction channels, and specifically discusses the N-terminal conformation, which has been implicated as essential to channel function.

Electron crystallographic structures of connexins

The structures of gap junctions are good targets for electron microscopy because they exhibit the characteristic features of self-assembly. Early studies examined the three-dimensional (3D) structures of native gap junction channels from rodents using electron crystallography, and the proposed movement for channel function was a twist and tilt of each subunit [18,19] (Fig. 1A). While this was supported by an atomic force microscopy (AFM) study [20], a functional study with electrophysiology provided the alternative interpretation that each subunit can move individually, resulting in voltage-dependent closure [21]. Split gap junctions comprising undocked hemichannels isolated from rodents were reconstructed in 3D, showing six protrusions in the extracellular domain [22]. Recombinant Cx43 channels were also studied by electron crystallography at 7 Å resolution, revealing the separate transmembrane helices [23]. The functional state of this structure was unclear, however, as the pore exhibited a widely open pathway when oleamide, a drug known to be a gap junction blocker [24], was added to the crystallization buffer. The evidence of a density in the pore was observed in the 3D reconstruction of the Cx26M34A mutant, which exhibits functional, but decreased, permeability and conductance compared with the wild-type channel [25●-28]. It has been suggested that the N-terminus of Cx26 physically blocks the pore [25●,29], but this model remains unproven because this type of closure has yet to be shown in any wild-type connexin (Fig. 1B).

X-ray crystal structures of Cx26

The atomic structure was first determined for wild-type Cx26 using X-ray crystallography [12●●] (Fig.1C). This structure provided answers to many questions. The transmembrane helix assignment was a controversial point proposed by multiple models [30,31], but was explicitly solved. The

two extracellular loops have three intramolecular disulfide bonds, forming an anti-parallel beta sheet in E2 and a short alpha helix in E1. The extracellular docking surface between apposed hemichannels was flatter than expected for engagement by the anti-parallel β-strands of the E1 and E2 loops [32]. The N-terminal arrangement in the pore cavity assumes the conformation of a funnel formed by the N-terminal helix. The helical arrangement of the connexin N-terminus is consistent with the proposed model based on NMR structures of the N-terminal peptides [33-35]. The conformation of the X-ray structure was interpreted to be an open form as there is no obstacle in the pore pathway. This model does not contain the Met1 residue, however, and therefore an alternative interpretation has been presented based on a molecular dynamics (MD) simulation study that the average equilibrated structure generated by MD simulations is more likely to be an open structure of Cx26, and the N-terminus of Cx26 is less stable than observed in the X-ray structure [36].

X-ray crystal structures of Cx26 in the presence and absence of Ca^{2+} in the crystallization buffer were recently reported [13●●]. Comparison of the two conditions suggests that Ca^{2+} ions are coordinated by Glu42, Gly45, and Glu47, which are closely positioned residues in the pore pathway close to the extracellular side. The Ca^{2+}-bound and Ca^{2+}-free structures of Cx26 are mostly identical, and a conformational change, e.g., a Ca^{2+}-induced subunit rotation [19], was not observed (Fig. 1A). The two structures exhibited different electrostatic surface potentials in the pore, suggesting that the electrostatic effects function as a switch to regulate the charge selectivity of permeants depending on Ca^{2+} binding (Fig. 1D). The N-terminus and most of the cytoplasmic domains were not resolved in these structures. It will be of interest in the future to see the N-terminal distribution in the presence and absence of Ca^{2+} in complete models of connexins. The closure induced by Ca^{2+} may be similar to the loop-gating evoked in a voltage-dependent manner [37]. While the N-terminal portion, specifically the charge distribution, is considered to function as a sensor for voltage-dependent channel closure [38●], it remains to be elucidated if channel closure by electrostatic distribution also accounts for the voltage-dependent channel closure.

Cryo-EM structures of *Ce*-INX6 by electron crystallography and single-particle analysis

Although 3D reconstruction of an invertebrate gap junction channel from native tissue of crayfish was reported in 1991, the negative-staining EM imaging was limited to low-resolution structural analysis [39]. The oligomeric number of innexin channels was believed to be the same as that in connexin, a dodecameric channel [39,40]. The structural studies of innexin channels aiming for high-resolution were initiated a few years ago. A 3D reconstruction of a recombinant innexin channel at 10 Å resolution was obtained from 2D crystals of *Ce*-INX6 using electron crystallography [41] (Fig. 2A). It was demonstrated that *Ce*-INX6 with an N-terminal deletion construct (*Ce*-INX6ΔN) is missing amino acid residues 2 through 19, contains 16 subunits, and therefore has a larger pore diameter than Cx26. This structure revealed four additional densities on the channel pore (Fig. 2A), reminiscent of the plug density in the Cx26M34A channel [25]. While this might be indicative of a loss of functional permeability due to the N-terminal deletion, it was impossible to provide a plausible interpretation for those densities because of the low-resolution (10Å) and the lack of an available atomic model of innexin [41].

Single-particle cryo-EM is a powerful tool for studying high-resolution structures without crystallization, which allowed us to obtain an atomic model of wild-type *Ce*-INX6 (Fig. 2B) [14●●]. GraDeR [42], a method for excluding free detergent micelles by density gradient ultra-centrifugation, was critical for improving the quality of the cryo-EM images, resulting in the high-resolution structural analysis. The structure not only confirmed the hexadecameric subunits of *Ce*-INX6, but also revealed most of the cytoplasmic domains, which were ambiguous in earlier structures of connexins. A number of helix-turn-helix motifs are formed by the cytoplasmic loop and C-terminus, referred to as the cytoplasmic dome, in a single *Ce*-INX6 subunit, making up a dome configuration in an octameric hemichannel. In Cx43, nuclear magnetic resonance spectroscopy (NMR) has provided insights into the solution structures of the cytoplasmic loop and carboxyl-terminal domain peptides that contain a couple of alpha helices in a random coil [43, 44], which may show a similarity to *Ce*-INX6. The N-terminal portion carries a short helix and has a funnel conformation (Fig. 2C), consistent with the Cx26 X-ray structure by Maeda et al. [12●●]. Although the six residues at the N-terminal end are not completely resolved, the 3D density map

looks like an open channel because the most constricted region in the pore has a diameter of over 10 Å. Interestingly, a part of the C-terminal loop in the cytoplasmic dome covers the N-terminal funnel, suggesting that it contributes to regulating channel activity (Fig. 2D). *Ce*-INX6 and Cx26 are correlated to each other in the arrangement of the extracellular docking interface with an anti-parallel beta sheet in E2 and a short alpha helix in E1. Whereas only E1 of *Ce*-INX6 is involved in the interactions between two opposed hemichannels, both E1 and E2 of Cx26 contribute to these interactions with more hydrogen bonds (Fig. 2E). The tightness of docking interaction may therefore not be identical to each other.

High-resolution structures clearly show that the resolved part of Cx26 is relevant to *Ce*-INX6 regarding not only the monomeric but also oligomeric structures, despite having different subunit numbers. In connexin, cytoplasmic pH and aminosulfonates are intrinsic modulators of channel function [2,37]. A direct interaction between the C-terminus and the second half of the cytoplasmic loop is implicated for Cx43 [43]. Whether the arrangement of the cytoplasmic domains is analogous between the innexin and connexin gap junction families should be carefully considered, however, because the lengths and sequences of the cytoplasmic loop and C-terminal domains are not consistent. The recent studies of LRRC8 may help to clarify this issue [15]. LRRC8 proteins function as single membrane anion channels and have low sequence similarity with innexins and pannexins [10]. The resolved monomeric and oligomeric arrangements of LRRC8 highly correlate with the hemichannel parts of Cx26 and *Ce*-INX6, and its cytoplasmic domains comprising helix-turn-helix are common with *Ce*-INX6 [15]. In this regard, the working model of functional regulation by the cytoplasmic dome of the *Ce*-INX6 structure (Fig. 3B) may be partially shared with LRRC8 family proteins.

The atomic model of *Ce*-INX6 provides an additional interpretation to the *Ce*-INX6ΔN mutant structure at low-resolution [41]. Given that the N-terminal residues from 2 to 19 are deleted in the *Ce*-INX6ΔN mutant, the N-terminal funnel found in the pore of the wild-type *Ce*-INX6 should be lost in the *Ce*-INX6ΔN structure. The density found in the *Ce*-INX6ΔN pore cavity is therefore not derived from the polypeptide. In a cryo-EM study, an unassigned density was observed in the middle of a large circular

configuration in a transmembrane domain of a rotor ring of an ATP synthase [45], which was estimated to be sub-stoichiometric amounts of phospholipids [46]. One possibility is that the density in the *Ce*-INX6ΔN pore corresponds to lipid molecules as the channels were embedded in a lipid bilayer in 2D crystals. When constructing an oligomeric form of the *Ce*-INX6ΔN channel, it might be possible for lipids to fortuitously remain inside the pore unless excluded by the N-terminal funnel. Interestingly, a wide space is observed between the adjacent subunits in the transmembrane region in the high-resolution cryo-EM structure of *Ce*-INX6 where unassigned densities like carried-over lipids or detergent micelles were inserted close to the N-terminal funnel [14●●](Fig. 2D). High-resolution structures of the *Ce*-INX6 gap junction channels reconstituted in the lipid bilayer are awaited for further investigation of the contribution of lipids to channel function.

The N-terminus is essential for channel function in both connexin and innexin channels

The N-terminus of connexin is thought to be essential for channel function in association with the voltage-dependent gating mechanism (summarized in [47]). Specifically, the voltage sensor is located in the N-terminus of connexin [48]. This suggests that the N-terminal portion should reside in the pore vestibule because this is the only position at which the difference in voltage across the adjacent cells can be sensed [38●] (Fig. 3A). The structural organization of the N-terminal funnel observed in the Cx26 X-ray structure is consistent with these interpretations. Despite the lack of sequence similarity, the *Ce*-INX6 structure also has an N-terminal funnel, suggesting that this conformation could be generally shared among gap junction family proteins. The partially resolved N-terminus of LRRC8 faces the pore [15], which, if it is not helical, is similar to Cx26 and *Ce*-INX6 [12●●,14]. All of the N-terminal deletion constructs of Cx37 are no longer able to form conducting channels [49], consistent with findings from functional innexin studies [41,50]. These findings suggest that an intact N-terminus is necessary for normally opening gap junction channels. Recent X-ray structures of Cx26 by Bennet et al. do not visualize the N-terminal portion [13●●]; nor does the electron crystallographic structure of Cx43 [23]. It

is possible that the N-terminus of connexin is flexible and can assume variable and unexpected conformations.

The movement of the N-terminus during a functional cycle remains to be further elucidated. Functional studies with site-directed mutagenesis demonstrated the possibility that voltage-dependent closure is initiated by movement of the voltage sensor, namely the N-terminus of connexin, toward the cytoplasmic side [48,51] (Fig. 3A). This movement is inconsistent with the idea that the N-termini aggregate to act as a physical blockage [25,12], and cannot be accounted for by electrostatic distribution in the structures without the N-terminal portion [13●●]. The individual model suggests that the behavior of the N-terminus does not have to be concerted, but can be independent [21]. The discrepancy of these interpretations is basically attributed to the lack of a high-resolution structure in a closed state representing a physiological condition. The pore size, estimated from the structures above to be over 10 Å in diameter, should be completely shut when the channel is closed. One key point that has been so far neglected in considering the functional interpretation of gap junction channels is the possible presence of lipid molecules. It should be noted that all high-resolution structures of gap junction channels by X-ray and cryo-EM are in a solubilized form surrounded by detergent micelles that would have removed most of the lipids carried over from cells. The cryo-EM structure of *Ce*-INX6 indicates that lipid molecules could possibly be distributed between adjacent subunits in the transmembrane domains *in vivo*, which is close to the N-terminal funnel [14●●] (Fig. 2D). Nanodisc reconstitution [52] may be a possible method for obtaining a high-resolution structure of a gap junction channel in the lipid bilayer that will provide insight into understanding the N-terminal arrangements of connexins and innexins under more physiological conditions.

Gap junction channels exhibit different types of gating properties. One alternative mechanism that should be mentioned is loop gating in which the extracellular loop of connexin assumes a configuration to form a narrow pathway for closure of unopposed hemichannels [53] and docked junction channels [54]. The properties were also evaluated by MD simulations with the X-ray structure of Cx26 [55]. To date, however, there is no evidence from high-resolution structures of gap junction channels to

reconcile these models. In the structure of LRRC8, the extracellular domain may act as a selectivity filter as the pore diameter is constricted by basic residue side-chains such as arginine, histidine, and lysine in E1 [15]. As multiple basic residues are not found in the corresponding region of Cx26, a high-resolution structure of an undocked gap junction hemichannel would be required to elucidate the mechanism of loop gating.

Conclusions

Cryo-EM is an important technique for elucidating the structural and functional basis of gap junction channels. The atomic structure of *Ce*-INX6 suggests that gap junction channels may be amenable to single-particle cryo-EM due to their high symmetry and the large size of the molecular complex. The flexible cytoplasmic domains, including the N-terminus, are thought to be important modulators of channel function, but are not well resolved for connexin channels. A possible strategy to determine the flexibility is to use a monoclonal antibody or nanobody against the cytoplasmic domains, which is often used for crystallographic and cryo-EM studies of G-protein-coupled receptors [56,57]. This strategy may also be useful for labeling a particular subunit in a heteromeric subunit complex, as utilized for the acetylcholine and gamma-amino butyric acid receptors [58,59], as long as the subunit stoichiometry is constant. Using nanodiscs may be an effective method, as reconstituting the TRPV1 channel into lipid nanodiscs allowed for its detailed structural analysis in a more stable environment [60]. For fundamental comprehension of the structure and function of gap junction channels in the native environment, protein-lipid interactions should also be considered. Structures of undocked hemichannels of gap junction channels are required to investigate the docking mechanism of unopposed hemichannels in addition to understanding the loop gating mechanism. Single-particle cryo-EM will be a key and essential method for future research of gap junction channels.

Conflict of interest

The author declares no conflicts of interest associated with this manuscript.

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Annotations

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● This study suggests that the N-terminus of connexin is located in the pore to sense the voltage field together with functioning as a voltage-sensor. Movement of the N-terminus toward the cytoplasmic side is also suggested.

Figure legends

Figure 1

Structural studies of connexins implicating the gating mechanism of gap junction channels.

(A) Subunit twist and rotating model. The conformational change occurs in response to the presence and absence of Ca^{2+} [19].

(B) Electron crystallographic 3D reconstruction of Cx26M34A corresponding to a hemichannel portion showing a density in the pore [28]. An atomic model of the Cx26 (pdb code: 2zw3) is superimposed.

(C) Atomic model of Cx26 determined by X-ray crystallography (pdb code: 2zw3) [12]. (left) Ribbon model of Cx26 where the connexin subunits are color coded. (right) Top view of a Cx26 hemichannel part represented by a ribbon model is superimposed on a surface representation. The N-terminal helices are emphasized by a cartoon representation.

(D) Electrostatic surface potential of Cx26 in the presence (left) and absence (right) of Ca^{2+} [13]. Positive surface is shown in blue and negative surface is shown in red.

Figure 2

Structures of *Ce*-INX6 channels determined by cryo-EM.

(A) 3D reconstruction of the *Ce*-INX6ΔN channel at 10 Å resolution by electron crystallography [41]. The four bulb densities are colored in yellow in the pore and in green on the cytoplasmic side. The gap between the transmembrane domains of the adjacent subunits is indicated by a red arrow.

(B) Single-particle cryo-EM structures of *Ce*-INX6 [14]. (left) Density map for the hemichannel part where the ribbon model is superimposed. (right) Density map for the whole gap junction channel part with a ribbon model. Red arrows indicate the space between the adjacent subunits as in (A).

(C) Top view of an atomic model of the *Ce*-INX6 hemichannel [14]. The N-terminal funnel is colored in magenta surrounding the pore (red).

(D) Distribution of the cytoplasmic loop (orange) and the N-terminal funnel [14]. The unassigned density (green) between the adjacent subunits could represent the assembly of carried over lipids or detergent micelles.

(E) Extracellular junction interfaces of *Ce*-INX6 (left) [14] and Cx26 (right) [12]. Cartoon or ribbon representation shows one hemichannel, and the molecular surface of an opposed hemichannel is colored in grey. Side chains of amino acid residues which contribute to hydrogen bond are represented as stick style, and hydrogen bonds are shown in red dotted lines. For *Ce*-INX6, the E1 outer lobe and inner lobe are marked with E1 in and E1 out, respectively.

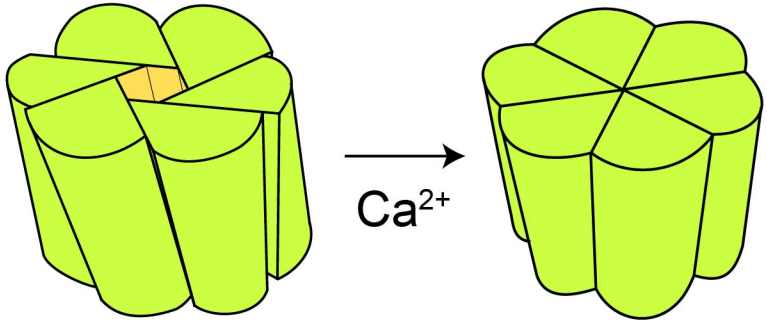
Figure 3

Schematic representation of Cx26 (A) and *Ce*-INX6 (B) hemichannels based on structural and functional studies.

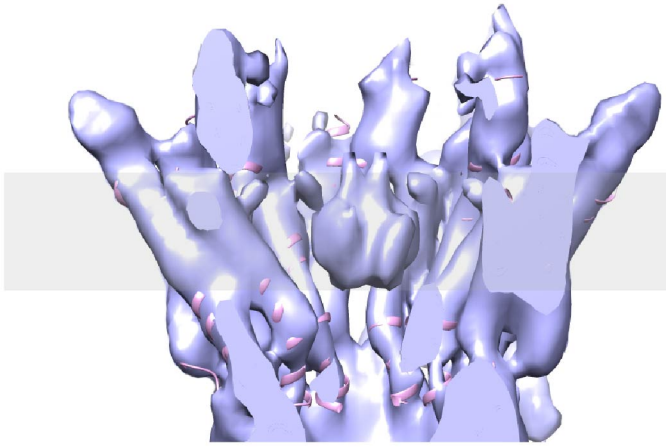
(A) For connexin channels, it has been proposed that the channel is closed depending on the transjunctional voltage when the N-terminus of connexin moves toward the cytoplasmic side (red arrow, [51]). The N-terminal funnel and density in the pore are derived from X-ray and electron crystallography [12, 25]

(B) The cryo-EM structure of *Ce*-INX6 [14] demonstrates that movement of the N-terminal funnel could be regulated by the cytoplasmic dome (C-dome). The interacting site between the N-terminal loop and C-dome is indicated by orange circles.

(A)



(B)



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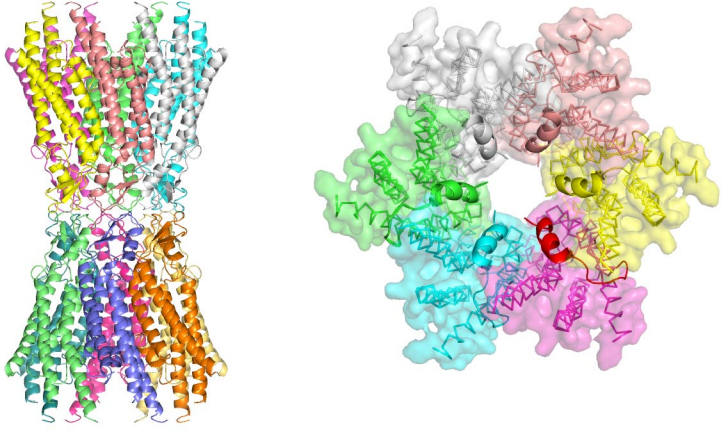
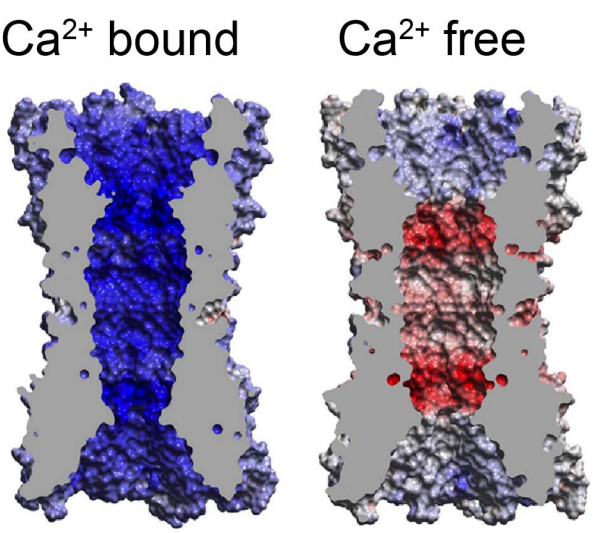
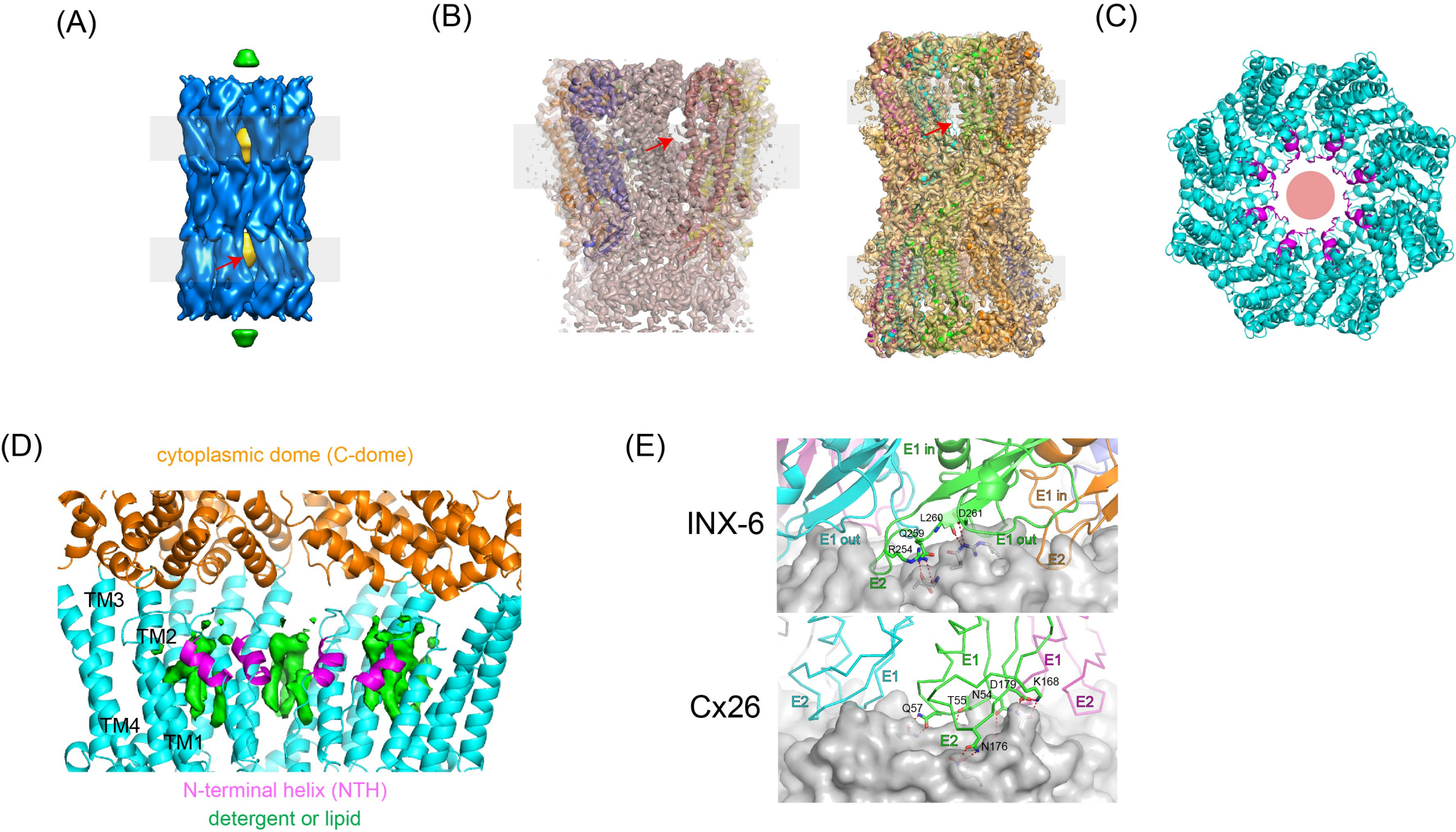
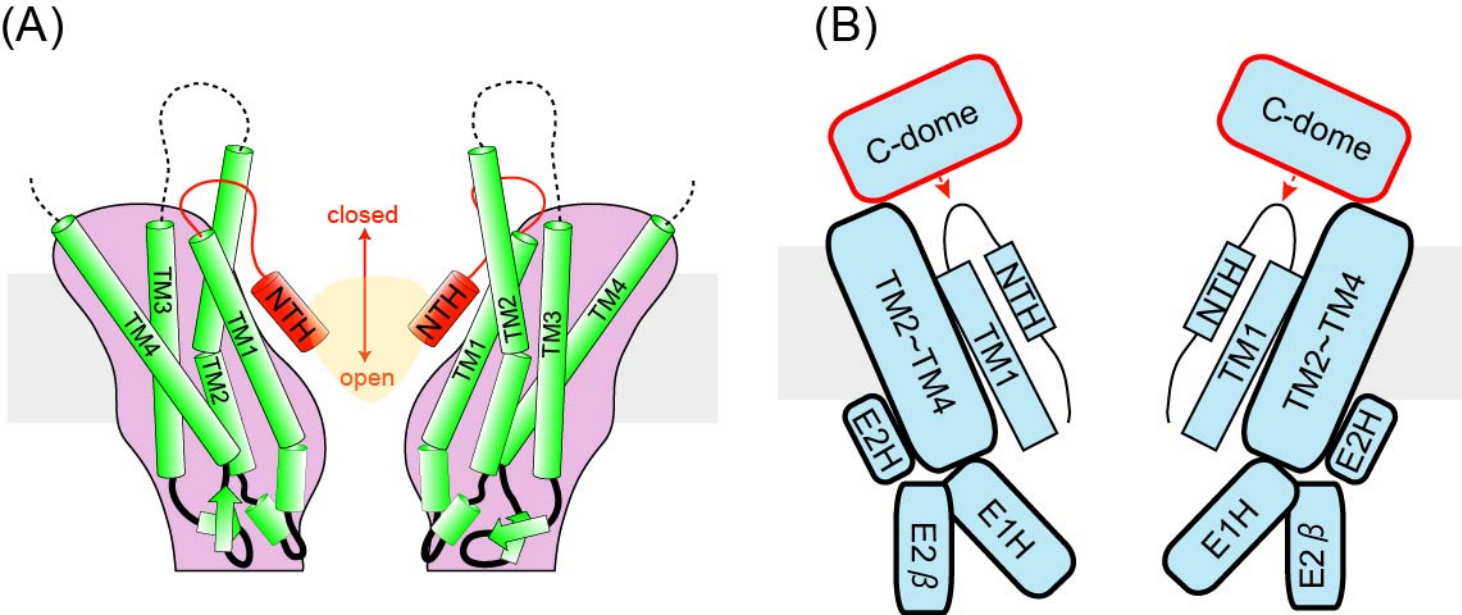


(D)




(A)
(B)
(C)
(D)
cytoplasmic dome (C-dome)
TM3
TM2
TM4
TM1
N-terminal helix (NTH)
detergent or lipid
(E)
INX-6
E1 in
E1 in
L260
D261
Q259
R254
E1 out
E1 out
E2
E2
Cx26
E1
E1
E1
E2
E2
D179
K168
T55
N54
Q57
E2
N176


(A)
closed
open
NTH
NTH
TM3
TM4
TM1
TM2
TM1
TM2
TM3
TM4
(B)
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TM2~TM4
NTH
TM1
E2H
E2β
E1H
C-dome
NTH
TM1
TM2~TM4
E2H
E1H
E2β