Cryo-electron microscopy for structure analyses of membrane proteins in the lipid bilayer.

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Asymmetric distributions of materials, such as ions, solutes, chemicals, and information across the bio-membrane, which are generated by membrane proteins, are crucial hallmarks of all living organisms. Since the appropriate asymmetric distribution depends on the precise function of membrane proteins in the lipid bilayer, structural and functional analyses of the membrane-embedded forms of these proteins are crucial toward elucidating their mechanisms.

In this review, we provide remarkable examples of structural analyses of membrane proteins in lipid bilayers based on electron crystallography of two-dimensional crystals. We also discuss the recent development of

single-particle cryo-electron microscopy and propose a conceptual strategy for structural analysis of membrane proteins in the lipid bilayer.

Introduction

The inside and outside environments of living cells and cell organelles are strictly discriminated by the membrane, which is highly waterproof and electrochemically sealed, providing a specialized permeability barrier. Diffusion of water-soluble materials, including water, ions, nutrients, and chemicals across the membrane, and also signal transduction through the membrane are therefore substantially restricted and/or regulated. Active or passive translocation of small molecules is mediated by membrane proteins integrated within the lipid bilayer [1]. The amphiphilic properties of membrane proteins allow for their stable positioning in the hydrophobic lipid bilayer while at the same time facing the solvent on either side of the cell membrane. These complex structural features of membrane proteins make their analysis particularly challenging for structural biologists because the protein molecules must be solubilized with detergent before they can be isolated and crystallized. Nevertheless, our knowledge of how these proteins work has greatly advanced during the last decade due to the marked increase in the number of X-ray crystal structures of membrane proteins [2], as well as recent innovative changes in structure analyses by single-particle cryo-electron microscopy (EM) [3]. Except for type I three-dimensional (3D) crystals in which protein molecules are packed within a detergent-lipid layer [4], however, our understanding of the precise molecular mechanisms of membrane proteins in a physiological setting remains incomplete.

Since the determination of the first 3D structure of a membrane protein,

bacteriorhodopsin, in 1975 [5], electron crystallography of two-dimensional (2D) crystals has been successfully applied for studying the 3D structure of membrane proteins [6]. Unlike 3D crystals that can be analyzed by X-rays, electron crystallography relies on 2D crystals in which membrane proteins are reconstituted in a lipid bilayer to form single-, double-, or a triple- (found only in one case [7]) layered 2D ordered arrays for structure analysis in a membrane that provides an environment close to the native condition. This review highlights recent advances in electron crystallographic studies of membrane proteins, and describes the important contribution of the lipid bilayer as more than a simple fluidic “stage” for the membrane protein. We also provide examples of structural analysis using relatively “bad” 2D crystals, for which no diffraction patterns were available and the structure had to be analyzed based only on their images and more generally by combining with electron tomography. Although the final resolution was limited, taken together with functional studies, these structures provide important information for understanding how membrane proteins function in their physiological environment.

Two-dimensional crystals in the lipid bilayer

An advantage of electron crystallography over other methods is its potential to analyze the membrane protein structure in the lipid bilayer. Specific lipid requirements for the structural integrity and proper function of membrane proteins are frequently observed. X-ray structures of membrane proteins containing bound lipids (or detergents as their mimetics) have provided some insight into the specific interactions of particular lipids with membrane proteins [8]. Nonspecific interactions between lipids and membrane proteins are more difficult to study, and are thus poorly characterized. In this respect,

some high-resolution structural analyses by electron crystallography visualized the annular lipids attached to the transmembrane portion of the membrane proteins as well as lipid molecules forming the bilayer, providing general information regarding the interactions of lipids with the membrane proteins [9-11] and the formation of the lipid bilayer [12]. The lipid bilayer may also exert lateral pressure that affects the structural integrity and stability of the membrane proteins [8]. Moreover, in addition to the individual effects of a particular phospholipid, the lipid bilayer as a hydrophobic stage for the membrane protein has environmental effects on membrane protein function, as exemplified by the water channel structures described below.

Aquaporins

As a water channel, aquaporin-4 (AQP4) mediates rapid and highly selective water permeation. Its structure has been determined at 2.8 Å resolution by electron crystallography [13], and at 1.8 Å resolution by X-ray crystallography [14], analyzed in a lipid-associated form of 2D crystals and in a detergent-solubilized micelle form, respectively (Fig. 1a,b). Although these protein structures themselves are, regardless of the analysis procedure, essentially identical based on the low value of the root mean square deviation of 0.607 Å between the main chain structures, the water structures in the channel pore exhibit clear differences. In the X-ray structure, water molecules in the pore appear as a continuous stream of electron density (Fig. 1b). Counterintuitively, the electron crystallography structure (Fig. 1a) has superior density in this region, despite the apparently lower resolution data, with discrete peaks unambiguously revealing seven water molecules arranged in single file through the pore [13,15,16]. One water density at the narrowest constriction named ar/R had a weak density, presumably because the constriction in the static structure has a smaller

diameter than that of water molecules, and the eighth water molecule was assigned to the narrowest part of the channel pore in the 2D crystal structure (Fig. 1a). In addition, the B-factor of the observed seven waters was lower than $40 \text{ \AA}^2$, and some of them showed significantly lower B-factor (W1: $4.6 \text{ \AA}^2$, W2: $35.3 \text{ \AA}^2$, W4: $34.8 \text{ \AA}^2$, W5: $13.2 \text{ \AA}^2$, W6: $2.9 \text{ \AA}^2$, W7: $5.9 \text{ \AA}^2$, W8: $11.6 \text{ \AA}^2$) in comparison to an average value of $49.6 \text{ \AA}^2$ for protein part [13], suggesting that the water molecules were strictly set in energetically favorable positions in the channel pore.

A demanding task for the water channel is the rapid permeation of water molecules with strict exclusion of any ions, including protons. Exclusion of the proton permeation is particularly puzzling as protons can easily translocate according to the Grotthaus mechanism by changing hydrogen-bonding partners of water molecules through a so-called water wire. Therefore, proton exclusion requires separation of the hydrogen bond that causes a large potential barrier. A hydrogen-bond isolation mechanism was proposed based on the electron crystallographic analysis of AQP1 (Fig. 1c) [17], and was structurally proven by the above-described AQP4 structure (Fig. 1d) [13]. The positions of water molecules visualized in the AQP4 pore indicate interatomic distances between the waters closest to the Asn-Pro-Ala (NPA) center, W4-W5 and W5-W6 of 3.9 and 3.8 $\text{\AA}$, respectively. These distance are too long to permit hydrogen bond formation, thus precluding proton transfer (Fig. 1d).

We propose that the presence of the membrane layer might be one reason for the clearly separated densities of water molecules in the AQP4 structure analyzed by electron crystallography (Fig. 1c). There are two short helices in the AQP structure, termed HB and HE, that penetrate the middle part of the membrane. Highly-conserved NPA motifs are located at the N-terminal sides of these helices (*i.e.*, faced together at

the middle part of the membrane layer) and oxygen of the water in this exact position is coordinated with amide groups of asparagine residues of the NPA motifs. Because the lipid bilayer has a characteristic distribution of the dielectric constant, which produces a large dipole moment of the half membrane-penetrating short helix in the membrane environment as discussed by Sengupta et al. [18], the water molecule is forced to orient with its oxygen atom facing the NPA side of the channel. In cooperation with the electrostatic field formed by the two short helices having a large dipole moment, arrangement of the carbonyl group in the channel pore serves as a binding site in the otherwise perfectly hydrophobic narrow channel, thus enabling the AQP to permeate 3 billion water molecules per second. Similar short helical arrangements are found in ion channels [19,20], thus the enhanced helical dipole moment in the lipid bilayer is an important structural determinant for at least some channels.

Structures analyzed from non-ideal crystals

Like the 2D crystals of AQPs, electron diffractions obtained directly from sufficiently large and coherent 2D crystals allow for atomic model building. Many samples are restricted to only small and moderately disordered 2D crystals, which give no electron diffraction patterns. Nevertheless, electron crystallography enables us to analyze 3D structures from only electron micrographs [21]. Although the analyzable resolution is restricted by the quality of the 2D crystals, important structures have also been determined from such “bad” 2D crystals at a resolution where the α -helical arrangement has become visible. For example, we could analyze the structures of the connexin-26 gap junction channel [7], voltage-gated Na^+ -channel [22], and gastric H^+, K^+ -ATPase [23-27].

In response to food intake, the pH of our stomach reaches ~1. This highly acidic environment is indispensable for digestion, but conversely, too much acidification of the stomach induces gastric ulcers or gastroesophageal-reflux diseases. The gastric proton pump, H^+,K^+ -ATPase, is a membrane protein responsible for gastric acid secretion, and therefore a prominent drug target for acid-related diseases [28]. Gastric H^+,K^+ -ATPase has the remarkable task of pumping protons against a million-fold gradient, ranging from approximately pH 7 in the parietal cells to 1 in the stomach. Maintaining a potent concentration gradient of six orders of magnitude is hardly met by any other biologic pump in nature. How does the proton pump manage to resist a million-fold H^+ gradient without ever going in reverse?

By determining the 6.5 Å resolution electron crystallographic structure of H^+,K^+ -ATPase in an *E2P* conformation, we observed a novel inter-subunit interaction between the N-terminal tail of the β -subunit and the P-domain [23] (Fig. 2a). This inter-subunit interaction stabilizes the *E2P* conformation, and prevents reverse reaction of the transport cycle, which was confirmed by kinetic analysis of N-terminus deletion mutants [23,29]. Thus, the N-terminal tail of the β -subunit functions as a “ratchet”, to ensure that the transport cycle of the H^+,K^+ -ATPase only proceeds in the forward direction. The proton pump, therefore, resists the massive proton pressure across the parietal cell membrane.

Another key requirement for generation of the steep proton gradient is the transport stoichiometry. Because of the limited free energy available for ATP hydrolysis, the stoichiometry of the transport cation must vary from $2H^+/2K^+$ to $1H^+/1K^+$ per hydrolysis of each ATP molecule as the luminal pH decreases [30]. A strong density peak located in the transmembrane cation-binding site in the Rb^+ -bound

EM structure is not present in Rb^+ -free structure, or weaker in K^+ -bound structure, and thus most likely to represent a single bound Rb^+ ion (Fig. 2b) [26]. Measurement of radioactive $^{86}\text{Rb}^+$ binding suggests that the binding stoichiometry varies depending on the pH, and approximately half the amount of Rb^+ is bound under acidic crystallized conditions compared with that at neutral pH. These data represent structural evidence of the $1\text{H}^+/1\text{K}^+/1\text{ATP}$ transport mode of H^+, K^+ -ATPase that is prerequisite for generation of the 10^6 -fold proton gradient across the membrane.

Although ~ 7 Å resolution EM maps of H^+, K^+ -ATPase cannot resolve individual amino acid residues, the arrangement of cytoplasmic domains and transmembrane helices are well defined (Fig. 2c) [25,27]. Therefore, according to its molecular envelope and contour levels of the EM density maps, a superimposable homology model was generated based on the atomic model of closely-related Na^+, K^+ -ATPase [31]. A bound inhibitor (Fig. 2c) [25], or even much smaller rubidium ion at the cation binding site (Fig. 2b) [26], was determined by careful analysis of EM maps, and supported by functional studies. We thus believe that, by corroborating with functional analysis, it is possible to discuss the molecular mechanisms from so-called medium-resolution structures.

Molecular tomography

Due to the instability of some membrane proteins and/or their complexes, their 2D crystals are sometimes insufficiently ordered for electron crystallographic processing, despite numerous attempts to improve crystallinity [32,33]. The recently reported structural analysis of bovine F_1F_0 ATP synthase provides an attractive alternative for structural analysis of such large membrane complexes by a combination of electron

tomography, subtomogram averaging, and electron crystallography [34].

Cryo-electron tomography is a powerful method for bridging ultrastructural compartmentalization and the structural analysis of individual protein molecules or a complex of proteins, by visualizing 3D images (tomograms) of cells and cellular organelles [35]. As conceptually similar to single-particle analysis, *in situ* structures of a protein or its complex can be obtained in subtomogram averaging, by averaging the 3D volumes extracted from tomograms [36].

Membrane-reconstituted bovine F_1F_0 ATP synthase forms small vesicular 2D crystals in which the enzyme complex is complete and active. The crystals are too imperfectly packed for application of ordinary image-based electron crystallographic analysis. Jiko *et al.* obtained cryo-electron tomograms of these 2D crystals and determined the 24 Å resolution structure of the matrix-exposed part of the complex by subtomogram averaging (Fig. 3a). The tomographic volume was also utilized for crystallographic analysis by extracting the slice volume of a coherently ordered crystalline plane from an electron tomogram, which enhances the signal-to-noise ratio of the diffraction data in comparison to the analysis of whole projection images (Fig. 3bc). Because noise originating from additional crystal layers or surrounding buffer was removed *in silico* by selecting z-slices. These combinations of structural analysis methods of membrane-reconstituted forms of the full F_1F_0 ATP synthase complex reveal that each complex is inclined 16° relative to the crystal *ab*-plane, consistent with the membrane bending effect of the F_1F_0 ATP synthase dimer in the mitochondria cristae [37,38], and further demonstrate that the monomeric complex is sufficient to deform lipid bilayers [34], neither of which could have been concluded by observing the protein in the absence of the membrane.

Image-based structural analysis in the membrane

Recent technical improvements in single-particle cryo-EM that have greatly advanced crystal-free structural analysis are mainly attributable to methodological development [39] and direct electron detection devices [40]. The advantages are that crystals are not required (this often a bottleneck for membrane proteins) and the potential to acquire distinguishable multiple conformations in the absence of crystal lattice contacts [41].

Membrane proteins are usually analyzed in a solubilized form with either detergent, amphiphilic polymer [42], or in a nanodisk-reconstituted form when the target molecule is sufficiently large [43]. A method for removing the free detergent micelles, termed GraDeR [44], was also developed, because detergent micelles usually give high-background images. Together with X-ray crystallography, single-particle cryo-EM will greatly contribute to our knowledge of protein structures. It is increasingly recognized that structural analysis of membrane proteins embedded in a bilayer yields novel insight into function, ensuring that electron crystallography of 2D crystals remains an important method of analysis. The process of 2D crystallization is time consuming and difficult, however, and we are attempting to develop a novel method termed Image-Based Structural Analysis (IBSA), by which we can analyze membrane protein structures in a membrane embedded form [16]. While IBSA is conceptually similar to single-particle cryo-EM, membrane-reconstituted proteins are observed instead of a purified, solubilized form of the membrane protein samples. First, a low-dose image of a membrane in which the target molecules are reconstituted is obtained under a tilting condition, followed by the acquisition of a highly defocused image of the same specimen area in a non-tilted condition to obtain a higher contrast.

Reconstituted membrane protein particles in high-contrast non-tilted images are then used for classifying those in low-dose tilted images, to analyze the 3D structure of the membrane-embedded form of the membrane proteins. As discussed above, analysis of the membrane proteins in their physiological setting may shed light on their precise molecular mechanism.

Conclusion

Membrane proteins can fully express their physiological functions in the lipid bilayer, unlike in detergent micelles or solution. Methods for high-resolution structural analysis by X-ray and single-particle cryo-EM of detergent-solubilized targets are now established. However, electron crystallography remains an important alternative method for analyzing the membrane protein structure in the physiological membrane environment. In addition to its role as a simple fluidic stage for membrane proteins, the lipid bilayer has considerable effects on membrane proteins, and its inclusion is therefore indispensable for gaining a precise understanding of the mechanisms of membrane proteins in the cell membrane.

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References

- of special interest
- of outstanding interest

- [1] Widdas, WF: **Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer.** *J Physiol* 1952, **118**: 23-39.
- [2] Shi Y: **A glimpse of structural biology through X-ray crystallography.** *Cell* 2014, **159**: 995-1014.
- [3] Cheng Y: **Single particle cryo-EM at crystallographic resolution.** *Cell* 2015, **161**: 450-457.
- [4] Toyoshima C, Nakasako M, Nomura H, Ogawa H: **Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 Å resolution.** *Nature* 2000, **405**: 647-655.
- [5] Henderson R, Unwin PN: **Three-dimensional model of purple membrane obtained by electron microscopy.** *Nature* 1975, **257**: 28-32.
- [6] Fujiyoshi Y, Unwin N: **Electron crystallography of proteins in membranes.** *Curr Opin Struct Biol* 2008, **18**: 587-592.
- [7] Oshima A, Tani K, Hiroaki Y, Fujiyoshi Y, Sosinsky GE: **Three-dimensional structure of a human connexin26 gap junction channel reveals a plug in the vestibule.** *Proc Natl Acad Sci U S A* 2007, **104**:10034-10039.

Three-dimensional reconstruction of Cx26 using 2D crystal images reveals new

architecture, a plug in the vestibule, of gap junction channel.

[8] Lee G: **How lipids affect the activities of integral membrane proteins.** *Biochim Biophys Acta* 2004, **1666**: 62-87.

[9] Mitsuoka K, Hirai T, Murata K, Miyazawa A, Kidera A, Kimura Y, Fujiyoshi Y: **The structure of bacteriorhodopsin at 3.0 Å resolution based on electron crystallography: Implication of the charge distribution.** *J Mol Biol* 1999, **286**: 861-882.

[10] Hite RK, Li Z, Walz T: **Principles of membrane protein interactions with annular lipids deduced from aquaporin-0 2D crystals.** *EMBO J* 2010, **29**: 1652-1658.

[11] Hite RK, Gonen T, Harrison SC, Walz T: **Interaction of lipids with aquaporin-0 and other membrane proteins.** *Pflugers Arch* 2008, **456**: 651-661.

[12] Gonen T, Cheng Y, Sliz P, Hiroaki Y, Fujiyoshi Y, Harrison SC, Walz T: **Lipid-protein interactions in double-layered two-dimensional AQP0 crystals.** *Nature* 2005, **438**: 633-638.

●●[13] Tani K, Mitsuma T, Hiroaki Y, Kamegawa A, Nishikawa, Tanimura Y, Fujiyoshi Y: **Mechanism of aquaporin-4's fast and highly selective water conduction and proton exclusion.** *J Mol Biol* 2009, **389**: 694-706.

Structure of AQP4 at 2.8 Å resolution by electron crystallography of double-layered 2D crystals reveals eight water molecules in the channel, the arrangement of which provides structural evidence for the hydrogen-bond isolation mechanism.

[14] Ho JD, Yeh R, Sandstrom A, Chorny I, Harries WEC, Robbins RA, Miercke LJW, Stroud RM: **Crystal structure of human aquaporin 4 at 1.8Å and its mechanism of conductance.** *Proc Natl Acad Sci U S A* 2009, **106**: 7437-7442.

- [15] Tani K, Fujiyoshi Y: **Water channel structures analysed by electron crystallography.** *Biochim Biophys Acta* 2014, **1840**: 1605-1613.
- [16] Fujiyoshi Y: **Development of the field of structural physiology.** *Proc Jpn Acad Ser B Phys Biol Sci* 2015, **91**: 447-468.
- [17] Murata K, Mitsuoka K, Hirai T, Walz T, Agre P, Heymann JB, Engel A, Fujiyoshi Y: **Structural determinants of water permeation through aquaporin-1.** *Nature* 2000, **407**: 599-605.
- [18] Sengupta D, Behera RN, Smith JC, Ullmann GM: **The α helix dipole: screened out?** *Structure* 2005, **13**: 849-855.
- [19] Doyle DA, Morais Cabral J, Pfuetzner RA, Kuo A, Gulbis JM, Cohen SL, Chait BT, MacKinnon R: **The structure of the potassium channel: molecular basis of K^+ conduction and selectivity.** *Science* 1998, **280**: 69-77.
- [20] Payandeh J, Gamal El-Din TM, Scheuer T, Zheng N, Catterall WA: **Crystal structure of a voltage-gated sodium channel in two potentially inactivated states.** *Nature* 2012, **486**: 135-139.
- [21] Glaser RM, Downing K, Derosier D, Baumeister W, Frank J (Eds): *Electron crystallography of biological macromolecules*. Oxford Univ Press; 2007
- [22] Tsai CJ, Tani K, Irie K, Hiroaki Y, Shimomura T, McMillan DG, Cook GM, Schertler GFX, Fujiyoshi F, Li XD: **Two alternative conformations of a voltage-gated sodium channel.** *J Mol Biol* 2013, **425**: 4074-4088.
- [23] Abe K., Tani K, Nishizawa T, Fujiyoshi Y: **Inter-subunit interaction of gastric H^+,K^+ -ATPase prevents reverse reaction of the transport cycle.** *EMBO J* 2009, **28**: 1637-1643.

The first 3D structure of gastric H^+,K^+ -ATPase at 6.5 Å resolution reveals a novel

inter-subunit interaction that prevents a physiologically unfavorable reverse reaction.

[24] Abe K, Tani K, Fujiyoshi, Y: **Structural and functional characterization of H^+,K^+ -ATPase with bound fluorinated phosphate analogs.** *J Struct Biol* 2010, **170**: 60-68.

[25] Abe K, Tani K, Fujiyoshi, Y: **Conformational rearrangement of gastric H^+,K^+ -ATPase with an acid suppressant.** *Nat Commun* 2011, **2**: 155.

●●[26] Abe K, Tani K, Friedrich T, Fujiyoshi, Y: **Cryo-EM structure of gastric H^+,K^+ -ATPase with a single occupied cation-binding site.** *Proc Natl Acad Sci U S A* 2012, **109**: 18401–18406.

A single bound Rb^+ is visualized in the 2D crystal structure of H^+,K^+ -ATPase, supporting a transport stoichiometry variation hypothesis.

[27] Abe K, Tani K, Fujiyoshi, Y: **Systematic comparison of molecular conformations of H^+,K^+ -ATPase reveals an important contribution of the A-M2 linker for the luminal gating.** *J Biol Chem* 2014, **289**: 30590-30601.

[28] Shin JM, Munson K, Vagin O, Sachs G: **The gastric HKATPase: structure, function, and inhibition.** *Pflugers Arch* 2009, **457**: 609–622.

[29] Dürr KL, Abe K, Tavrız NN, Friedrich T: **E2P state stabilization by the N-terminal tail of the H,K -ATPase β -subunit is critical for efficient proton pumping under *in vivo* conditions.** *J Biol Chem* 2009, **284**: 20147–20154.

[30] Rabon EC, McFall TL, Sachs G: **The gastric $[H,K]ATPase:H^+/ATP$ stoichiometry.** *J Biol Chem* 1982, **257**: 6296–6299.

[31] Shinoda T, Ogawa H, Cornelius F, Toyoshima C: **Crystal structure of the sodium potassium pump at 2.4 Å resolution.** *Nature* 2009, **459**: 446–450.

[32] Tani K, Arthur CP, Tamakoshi M, Yokoyama K, Mitsuoka K, Fujiyoshi Y, Gerle C:

Visualization of two distinct states of disassembly in the bacterial V-ATPase from *Thermus thermophilus*. *Microscopy (Tokyo)* 2013, **62**: 467-474.

[33] Shimada S, Shinzawa-Itoh K, Amano S, Akira Y, Miyazawa A, Tsukihara T, Tani K, Gerle C, Yoshikawa S: **Three-dimensional structure of bovine heart NADH: ubiquinone oxidoreductase (complex I) by electron microscopy of a single negatively stained two-dimensional crystal.** *Microscopy (Tokyo)* 2014, **63**: 167-174.

[34] Jiko C, Davies KM, Shinzawa-Itoh K, Tani K, Maeda S, Mills DJ, Tsukihara T, Fujiyoshi Y, Kühlbrandt W, Gerle C: **Bovine F1Fo ATP synthase monomers bend the lipid bilayer in 2D membrane crystals.** *eLife* 2015, **4**: e06119

[35] Lučić V, Rigort A, Baumeister W: **Cryo-electron tomography: the challenge of doing structural biology in situ.** *J Cell Biol* 2013, **202**: 407-419.

[36] Oda T, Yanagisawa HA, Kamiya R, Kikkawa M: **A molecular ruler determines the repeat length in eukaryotic cilia and flagella.** *Science* 2014, **346**: 857-860.

[37] Davies KM, Anselmi C, Wittig I, Faraldo-Gomez JD, Kuhlbrandt W: **Structure of the yeast F1Fo-ATP synthase dimer and its role in shaping the mitochondrial cristae.** *Proc Natl Acad Sci U S A* 2012, **109**: 13602-13607.

•[38] Allegretti M, Klusch N, Mills DJ, Vonck J, Kühlbrandt W, Davies, KM: **Horizontal membrane-intrinsic α -helices in the stator a-subunit of an F-type ATP synthase.** *Nature* 2015, **521**: 237-240.

Single particle cryo-EM of an F-type ATP synthase dimer at 6.2 Å resolution reveals the architecture of the whole complex, including four long, horizontal membrane-intrinsic α -helical structures in the a-subunit.

[39] Flank J: **Generalized single-particle cryo-EM – a historical perspective.** *Microscopy (Tokyo)* 2015, *in press*

- [40] Li X, Mooney P, Zheng S, Booth C R, Braunfeld M B, Gubbens S, Agard D A, Cheng Y: **Electron counting and beam-induced motion correction enable near-atomic-resolution single-particle cryo-EM.** *Nat Methods* 2013, **10**: 584–590.
- [41] Fischer N, Konevega AL, Wintermeyer W, Rodnina MV, Stark H: **Ribosome dynamics and tRNA movement by time-resolved electron cryomicroscopy.** *Nature* 2010, **466**: 329-333.
- [42] Liao M, Cao E, Julius D, Cheng Y: **Structure of the TRPV1 ion channel determined by electron cryo-microscopy.** *Nature* 2013, **504**: 107-112.
- [43] Efremov RG, Leitner A, Aebersold R, Raunser S: **Architecture and conformational switch mechanism of the ryanodine receptor.** *Nature* 2015, **517**, 39-43.
- [44] Hauer F, Gerle C, Fischer N, Oshima A, Shinzawa-Itoh K, Shimada S, Yokoyama K, Fujiyoshi Y, Stark H: **GraDeR: Membrane Protein Complex Preparation for Single-Particle Cryo-EM.** *Structure* 2015, **23**: 1769-1775.

A method for removing free detergent micelles using a glycerol density gradient, which can generally be applied to prepare membrane proteins for single-particle cryo-EM.

Figure 1

Water permeation mechanism based on the electron crystallographic structure of AQP4

Comparison of AQP4 structures (surface) determined by electron (a) and X-ray (b) crystallography. Red spheres represent water molecules with densities represented by a mesh pattern (blue: $2F_o - F_c$, green; $F_o - F_c$). To show the channel pathway, two helices (H2 and H5) were removed for clarity. (c) A schematic of the hydrogen bond isolation mechanism. The helical dipole moment is emphasized when the water channel is in the lipid bilayer, which shows a characteristic distribution of dielectric constants (ϵ). The structure of AQP4 with water molecules in the channel pore (d) shows that the hydrogen bonds (dotted lines) formed among the water molecules in the channel are disconnected between W5 (close to NPA motif) and its neighboring waters (W4 and W6), as indicated by the distance between them (indicated in the right).

Figure 2

Electron crystallographic structures of gastric H^+, K^+ -ATPase

(a) EM structure of H^+, K^+ -ATPase in the *E2P* analog state reveals that the inter-subunit interaction (arrowhead) between the P-domain (green) and the β -subunit N-terminus (pink) stabilizes the *E2P* conformation to prevent a reverse reaction of the *E1P* formation, which is accompanied by movement of the P domain (yellow arrow), as indicated in the *E1P* model of SERCA (ribbon). (b) A strong EM density (red: 5σ , green, 4σ , represented by the mesh pattern) is found in the transmembrane region of the Rb^+ -occluded H^+, K^+ -ATPase, which is superimposed on the cation binding site II in the

homology model (ribbon). (c) Overall structure of H⁺,K⁺-ATPase bound with the specific inhibitor SCH28080 (left), and its membrane slice (position is indicated as a red dotted box in the left panel) viewed from the luminal side (right). Colors in the sliced surface indicate contour levels of the EM map (as indicated on the right) and mesh indicates molecular envelope contoured as 1 σ , which is superimposable onto the homology model shown in ribbons.

Figure 3

Electron crystallographic analysis from the z-slice of tomographic volume

(a) Side view of the crystal packing of F₁F₀ ATP synthase. (b) Projection images (left row) calculated from z-slices of the tomographic volume at z-height positions indicated by black bars in (a), their Fourier transform (middle row) and calculated projection maps (right row). (c) Z-slices through the crystal packing in (a) corresponding to the position of the projection maps shown in (b). Protein densities observed in the projection maps perfectly match the features shown in the corresponding z-slices of the crystal-packing model. Dashed orange outlines indicate a pair of F₁F₀ ATP synthases, red boxes indicate the unit cell of the 2D crystal.

Figures

Fig. 1.

Fig. 2.

Fig. 3.