Observation of Membrane Proteins Through An Electron Beam

Technological and instrumental advancements in electron microscopy have facilitated comprehension of structures of biological components. For example, electron crystallography of membrane proteins is now an established technique to analyze their structures in lipid bilayers which are close to the natural biological environment. By utilizing JEOL cryo-electron microscopes with originally developed helium cooled specimen stages, structures of membrane proteins were analyzed at a resolution better than 0.3 nm. Here some examples, such as water channels and gap junction channels, are discussed by focusing on their biological mechanisms through structural analyses of membrane proteins. Based on observation of structures of membrane proteins through an electron beam, we are trying to establish a new research field named structural physiology.

Introduction

Electron microscopy made it possible to observe structures of biological components struggling against radiation damage by an electron beam. The induced problem caused by damage was avoided by staining and shadowing methods with many technological variations and these techniques have given us important structural knowledge about biological cells and tissues. Higher resolution analysis is however required to understand molecular mechanisms of biological functions, especially membrane proteins which were known to be key molecules for sensing and regulating signal transduction, and for keeping energy metabolism and functioning other activities of cells. While observation of these molecular structures at high resolution is essential, radiation damage to bio-molecules strongly restricts the resolution and forces us to average image signal to improve signal to noise ratio (S/N). As a powerful averaging technique using a computer, single particle analysis of cryo-electron microscopy is very widely used for structural studies of biological macromolecular complexes. The method gives us three-dimensional (3D-) structures without crystals, which are generally difficult to be grown, and is actively used for studying many important biological targets. Although the single particle method is growing into the most important technique in electron microscopy by achieving surely important structural analyses in a wide range of biological fields, there are some examples of inconsistent structures which were analyzed from a same sample by the same method. The analyzed resolution is also rather limited because of several difficulties of the method so far, while the single particle method has potential ability to reach a real atomic resolution.

On the other hand, electron crystallography is particularly a good candidate for structure analyses of membrane proteins in detail because of the following several reasons, while a rather smaller number of groups are so far interested in this method. 1) Structure can be analyzed in a membrane that gives environment for membrane proteins similar to native conditions. 2) Structure can be analyzed even by poor crystals while the resolution is strongly related to the quality of crystals. 3) Both sides of the crystal are kept open and have less influence from artificial crystal packing. This feature enables to use the freeze-trapping technique which was developed and actually used for studying the gating mechanism of Nicotinic Acetylcholine receptor (AChR) by N.Unwin [1]. 4) Phase for structure analysis is calculated directly from images and gives a better map than that of X-ray crystallography. Thus images and diffraction patterns of well-ordered, planar and tubular protein-lipid crystals are yielding atomic models, which show us how the proteins are designed in biological cells and function in native environment.

Electron crystallography for the first time gave us a real image of a membrane protein, bacteriorhodopsin whose structure was analyzed by R. Henderson and N. Unwin in 1975 [2]. Whereas this method is extremely powerful especially for structural study of membrane proteins as explained in the previous sentence and R. Henderson et al. analyzed for the first time an atomic structure of bacteriorhodopsin based on electron crystallography [3], this method has been spreading rather slowly in structural biology field presumably because of technological difficulties as well as difficulty of crystallization of proteins. I however believe electron crystallography could be the core method for establishing structural physiology because it enables us to analyze functional structures of membrane proteins in membrane layers at the high resolution that enables us to discuss physiological functions.

The radiation and dehydration sensitivities of biological molecules give us difficult challenge for high resolution structural study and these difficulties force us to develop an effective and stable cryo-electron microscope with a helium cooled specimen stage and a cryo-transfer system for ice embedded samples. Recent methodological as well as instrumental advances, and the inclusion of tomographic and cryo-sectioning techniques, are enabling detailed information to be obtained from increasingly more disordered and complex membrane assemblies, extending the potential of this approach.

Development of stable and effective cryo-electron microscope

For high-resolution data collection, 2D-crystals are embedded in a thin layer of amorphous ice and/or a sugar solution for which trehalose is recommended [4]. The specimen is then mounted on the cryo-stage of an electron microscope using a cryo-transfer device. In
this case, the thickness of the ice layer has to be optimized for high resolution data collection, because the flatness of 2D-crystals is crucially important especially for data collection in the direction perpendicular to the tilting axis at a highly tilted condition as shown in Fig. 1. To make the crystal flat, a thinner ice layer is better as shown in Fig. 2 but to the contrary, such a thin layer causes dehydration problem of sample. Namely, the best specimen preparation condition forms a very narrow hitting target and thus many trials are generally required for high resolution data collection, especially a resolution better than 0.3 nm. It is impossible to accurately regulate the best thin layer formation without dehydration because sample condition and other conditions, such as density of crystals, temperature and humidity, easily change the thickness of the water layer on the specimen grid. Therefore a cryo-electron microscope (cryo-EM) with a quick specimen exchange system and a stable stage, by which we can try many specimen preparation conditions, is inevitable. By the way, a multiple specimen loading system is not useful for this purpose because the optimization of water layer requires feedback procedure. We therefore developed a cryo-electron microscope with a stable helium stage as well as an effective cryo-transfer system by which we can take a 0.2 nm resolution image of a new sample just after finishing observation of the previous specimen in shorter than 10 minutes and therefore my own record of specimen exchange frequency in a day was 34 times.

The resolution of an image of a biological macromolecule is usually limited to a value much worse than 0.3 nm. This limitation is not due to the resolution of the instrument but to damage to the specimen by the electron beam. The irradiation damage to biological crystals was found to be reduced to about 1/10 to 1/20 with respect to the value at room temperature when the specimen was cooled to below 20 K and 8 K, respectively. We have therefore developed a super fluid helium stage that can achieve a resolution of 0.26 nm in 1991 [5]. Thermal shield by liquid Nitrogen and Helium tank is gold plated to minimize radiational heat. The pot cooling specimen is cooled down to 1.5 K by superfluid helium and we call this a 1.5 K pot. Although our target resolution of electron micrographs is some thing like 0.3 nm, an instrument yielding better resolution would be highly beneficial, because biological molecules consist mainly of light atoms that exhibit small atomic scattering factors in a high-resolution range. A resolution better than 0.25 nm might be required to identify water molecules. Then we improved the instrumental resolution to 0.2 nm and overcome the operational difficulties [6]. We could develop the third generation cryo-EM which is a mature instrument for electron crystallography (Fig. 3). We modified an effective cryo-transfer system for ice embedded specimens to an automatic one which is a user friendly system and is tested in our third generation cryo electron microscope. After the third generation cryo-EM, the fourth generation cryo-EM equipped with an automatic cryo-transfer system, a field emission gun and an omega filter was developed and the fifth generation cryo-EM was also developed mainly for the single particle method (Fig. 3). From the second generation cryo-EM, we made quite a bit effort to improve resolution as well as overcome operational difficulties of the cryo-EM with a top-entry type helium stage. Recently we developed a new cryo-microscope, the sixth generation cryo-EM with an outer control tilting device for electron tomography and some other analyses. We also improved the sixth generation cryo-EM to accommodate a eucentric top entry helium stage and this seventh generation cryo-EM is used for taking tomographic images (Fig. 3).

Carbon Sandwich technique

When we attempted structure analysis of bacteriorhodopsin, we encountered a serious difficulty of image shift which could be induced by specimen charging, especially at tilted specimen conditions, resulting in a low probability to have good images. We took more than 10,000 bad images to get about 200 good images as shown in Fig. 4. This is the most serious problem for image data collection from the two-dimensional crystals of biological macromolecules. The image shift extinguishes optical diffraction spots perpendicular to the tilt axis even in a medium or low resolution area (Fig. 4). Almost all of the images from a tilted specimen prepared on a one-layer carbon support tend to be deteriorated by the image shift caused by beam-induced specimen charging. The success ratio for obtaining high resolution images from tilted specimens is therefore only 2% or so. To overcome this serious problem, the carbon sandwich preparation method, in which crystals are put between two sheets of carbon film, was investigated. When we used carbon sandwiched specimens, the ratio of images which showed clear asymmetric features in diffraction patterns due to the image shift was significantly decreased as compared to that obtained when the specimens were supported by a single carbon film. Thus, the carbon sandwich preparation method was confirmed to overcome the most difficult problem and contribute to more prompt structural analysis by electron crystallography [7].

Another benefit of the carbon sandwich technique was observed as shown by structure analysis of water channel aquaporin-(AQP)0. By the carbon sandwich technique, dehydration problem was minimized and the structure of AQP0 was analyzed at 0.19 nm [8]. At such a high resolution, lipid molecules in the AQP0 crystal were clearly discriminated as shown in Fig. 5.
Fig. 3 History of development of JEOL cryo-electron microscopes with helium stage. From the first generation cryo-EM to the seventh generation one.

Development of cryo-EM: 1st - 7th generations

1st G 1986
Kyoto U

2nd G 1988
PERI

3rd G 1994
Kyoto U

4th G 2001
Harima, Tokyo

5th G 2004
Tokyo

6th G 2006
Kyoto U

7th G with U-SET system

Helium stage

Fig. 4 At untilted condition, good quality images are relatively easy to be taken but at highly tilted condition, images tend to shift as shown in the upper optical diffraction patterns. We threw away more than 10,000 blurred images caused by the problem of charging for taking 200 good images. The lower photograph shows piling up EM films, all of which are bad images.

Fourier transforms of Bacteriorhodopsin images

Untilted

60°
Water channel aquaporin-1

Water is the most abundant molecule in any cells on the earth. The cell membranes therefore require an effective water channel function. In 1992, a 28kD membrane protein, which was eventually named AQP1, was identified in red blood cells and the water channel function was clearly shown in *Xenopus oocytes* [9] because only a limited amount of water can penetrate a simple lipid bilayer. The cell membrane exquisitely regulates entry and exit of ions because ion concentration and its dynamic change are strongly related with cell signalling functions. The water channels, therefore, need to keep ionic conditions in a cell, while these channels permeate a large amount of water. The pH regulation in the cell was well known to be also crucially important for cell functions. The AQP1 molecule attained effective water selective transport keeping the strict selectivity and it gave us puzzling questions. An atomic model analyzed at 0.38 nm resolution by electron crystallography gave an answer to such puzzling questions and revealed a molecular contrivance of water selective channel [10]. The atomic model is interestingly a first structure of human source membrane proteins. For accomplishing the effective water channel functions, the structure showed peculiar structural determinants including an unusual fold (AQP fold) as shown in Fig. 6.

The handedness of the structure of aquaporin-1 was carefully examined and the right handed helical bundle structure of AQP1 was confirmed by using bacteriorhodopsin structure which was analyzed at an atomic resolution [11] before publication of a paper [12], because relatively low resolution, such as 0.6 nm, did not allow anyone to construct an atomic model but just assign a helix on a rod shape density and required confirmation of the handedness. The handedness was directly confirmed at a resolution of 0.38 nm or better at which an atomic model was constructed as shown in Fig. 6 in 2000 [10].

The critical function of AQP1 is exceptionally high water permeability, 2 billion water molecules per monomer per second. Almost all residues within a central 2 nm zone in the pore are highly hydrophobic, while one might expect that AQP1 has hydrophilic pore because of the water channel. A narrow part of the pore with about 0.3 nm in diameter is located at the middle of the membrane where short helices HB and HE interact with each other especially with proline 77 and 193 of the conserved NPA sequences. Despite the enormous capacity for water conductance, the AQP1 pore also exhibits marked selectivity. Water molecules were found to be strongly oriented in the channel interior, through alignment of their dipoles with the electric field exerted by the protein, causing water molecules to rotate by 180 degrees upon passage [10]. Two major interaction sites for water molecules were identified inside the channel:

![Fig. 6](image)
the NPA and ar/R regions. The two highest enthalpic barriers for water molecules are located directly adjacent to the NPA region. This, together with the water rotation that is centered also here, renders the NPA region a major selectivity filter as shown in Fig. 7. This mechanism was named as hydrogen bond isolation mechanism [10]. Contiguous hydrogen-bonded water chains are known to be efficient proton conductors. Aquaporin must prevent proton conduction along its pore, to maintain the proton gradient across the cell membrane that serves as a major energy storage mechanism. Water regulation is crucially important for all cells and therefore for all life forms on the earth. Unexpected structural features, such as right handed helical bundle and hydrophobic channel wall, were revealed to facilitate the water transport through the channel. All of these findings were impossible to predict without structure analysis at high resolution.

**Structure analysis of AQP4**

AQP4 is the predominant water channel in the mammalian brain and we determined the AQP4 structure by electron crystallography of double-layered, two-dimensional (2D-) crystals. Analysis of the AQP4 structure was complicated by variations in the double-layered 2D crystals in terms of lateral alignment and distance between the two layers. Despite the high resolution of 0.32 nm, electron diffraction data proved not sufficiently sensitive to distinguish between different crystal variants. Phase data extracted from images even at medium resolution, on the other hand, were sensitive enough to discriminate between crystals with different arrangements of the two layers. The use of a helium-cooled electron microscope [6] and the carbon sandwich specimen preparation technique, which significantly increases the yield of good images [7], allowed us to also take an image of each crystal that produced a high-resolution diffraction pattern. Classification based on the image data, which provided phase information to better than 0.6 nm, identified one predominant crystal type, which accounted for about 70% of the analyzed crystals that yielded a high-quality electron diffraction pattern. In this crystal type, the two layers have a spacing of 4.5 nm (molecule center to molecule center). The final intensity data set at 0.32 nm resolution was used to determine the AQP4 structure by molecular replacement using the AQP1 structure.

Glial cells contain characteristic orthogonal arrays in the plasma membrane, which are especially prominent in glial end feet surrounding vascular capillaries in the brain. Immunogold labeling experiments showed that these arrays consist of AQP4 [13]. While AQP4 and AQP1 both function as very fast water-selective pores, AQP4 has distinctive biological characteristics as it forms orthogonal arrays in intact membranes. Furthermore, AQP4 exists in glial cells as a full-length protein starting with Met1 (AQP4M1) and an alternative shorter splicing isoform that starts with Met23 (AQP4M23). The structure allows us to discuss how the expression ratio between the long and short AQP4 splicing variant can determine the size of in vivo orthogonal arrays as shown in Fig. 8a [14].

AQP4 is also expressed in glial lamellae of the hypothalamus, where it may play a role in osmo-, thermo- and glucose-sensing. In glial lamellae the plasma membrane forms large junctions between individual layers, which have been shown to contain AQP4. Interestingly, another water channel, AQP0, forms the "thin junctions" between fiber cells. Structure analysis of AQP4 by double layered 2D-crystals revealed that the molecule contains a short α helix in an extracellular loop, which mediates weak but specific interactions between AQP4 molecules in the adjoining membrane [14]. This finding suggests a previously unexpected role for AQP4 in cell adhesion as shown in Fig. 8b. This notion was corroborated by expression of AQP4 in L-cells, which resulted in clustering of the cells. Our AQP4 structure thus enables us to propose models for the size regulation of orthogonal arrays and channel-mediated cell adhesion as observed in glial lamellae of the hypothalamus. AQP4 membrane junctions may reduce the water permeability of glial cell plasma membranes, because the tight tongue-into-groove packing of the two crystalline layers results in a partial blockage of the extracellular pore entrances (Fig. 8b). While the packing of the AQP4 tetramers in the junctions must create resistance for water flowing across the two membranes, rapid water flow through the channels may also reduce the adhesion between adjoining membranes. This may establish the basis for a role of AQP4 in osmo-sensing. For example, a high AQP4M1/AQP4M23 expression ratio would produce small AQP4 arrays providing weak adhesion between membranes, which could easily be separated and thus react to small water flows resulting from small osmotic differences. A low AQP4M1/AQP4M23 expression ratio, on the other hand, would result in extensive AQP4 arrays providing relatively strong adhesion between membranes that would withstand large water flows associated with large osmotic differences. Although further experiments are needed to elucidate the interplay of the two functions in aquaporins and potentially other membrane channels with adhesive properties, we propose to name "adhennels" for adhesive water and ion channels [14].

**Higher resolution analysis of AQP4**

Based on the electron crystallographic structure of AQP1, the hydrogen bond isolation mechanism was proposed to explain why AQPs are impermeable to protons despite their very fast water conduction. The mechanism by which AQPs exclude protons remained, however, is controversial. Therefore we present the structure of AQP4 by electron crystallography of double-layered two-dimensional crystals improving resolution from the previous 0.32 nm to 0.28 nm with an accompanying improvement in data quality resulting in the
The ability to identify seven individual water molecules in the channel as shown in Fig. 9a [15]. In addition, the Fo-Fc map showed an additional spherical density at the ar/R constriction site. Since the side chains of AQP4 around the ar/R region were represented by clear density (Fig. 9a) and the atoms of the protein molecule had low temperature factors in this region, we assigned an eighth water molecule to the spherical density at the ar/R site. The narrow diameter at this constriction would make it an unfavorable position for a water molecule, potentially explaining the weak density for the water at this position. The two neighboring water molecules on either side of the ar/R constriction, which form hydrogen bonds with the unstable water molecule in the constriction, showed higher temperature factors (35 Å 2) compared to those of all the other water molecules in the channel (2.9 Å 2 to 13.2 Å 2 ) (Fig. 9b). The temperature factor of each water molecule in the channel is indicated at the right side of Fig. 9b.

In X-ray crystallography, a resolution of 0.28 nm would be considered too low to see water and lipid molecules, but our density map, which explains the Hydrogen bond isolation mechanism, showed higher temperature factors (35 Å 2) compared to those of the surrounding main chain atoms, but the difference is usually less than 20 Å 2 . Another possible reason why we could observe water molecules in the AQP4 channel could be attributed to the data collection at liquid helium temperature. We collected all data for this structure analysis at stage temperature of 4.2K and such a low temperature could give clearly the lowest energy positions for water molecules in the channel.

The eight water molecules in the AQP4 channel are in a single-file arrangement (Fig. 9a, b). From the measured distances between successive water molecules in the channel (Fig. 9a), all water molecules appear to form hydrogen bonds with their neighbors (red dotted lines in Fig. 9b), except for the water at the NPA site and the one below it. These two waters thus seem to be separated from the other water molecules in the channel, lending support to the hydrogen bond isolation mechanism. The inside surface of water channels is largely hydrophobic except for a narrow, hydrophilic belt formed by the oxygen atoms of the main chain carbonyl groups of the amide groups of the NPA motifs is 1.7 Å 2 , while those of the surrounding main chain atoms are in the range of 30 Å 2 to 40 Å 2 . The large difference in the B factors between the water and protein atoms might be due to the characteristic features of electron crystallography and/or the enhancement of the effect of the helical dipole moments due to the lipid environment. In X-ray structures of AQP s in detergent micelles, the water molecules sometimes also displayed lower temperature factors compared to those of the surrounding main chain atoms, but the difference is usually less than 20 Å 2 .

The B-factor of the water molecule associated with the amide groups of the NPA motifs is 1.7 Å 2 , while those of the surrounding main chain atoms are in the range of 30 Å 2 to 40 Å 2 . The large difference in the B factors between the water and protein atoms might be due to the characteristic features of electron crystallography and/or the enhancement of the effect of the helical dipole moments due to the lipid environment. In X-ray structures of AQP s in detergent micelles, the water molecules sometimes also displayed lower temperature factors compared to those of the surrounding main chain atoms, but the difference is usually less than 20 Å 2 . Another possible reason why we could observe water molecules in the AQP4 channel could be attributed to the data collection at liquid helium temperature. We collected all data for this structure analysis at stage temperature of 4.2K and such a low temperature could give clearly the lowest energy positions for water molecules in the channel.

The eight water molecules in the AQP4 channel are in a single-file arrangement (Fig. 9a, b). From the measured distances between successive water molecules in the channel (Fig. 9a), all water molecules appear to form hydrogen bonds with their neighbors (red dotted lines in Fig. 9b), except for the water at the NPA site and the one below it. These two waters thus seem to be separated from the other water molecules in the channel, lending support to the hydrogen bond isolation mechanism. The inside surface of water channels is largely hydrophobic except for a narrow, hydrophilic belt formed by the oxygen atoms of the main chain carbonyl groups of
Gly209, Ala210, Ser211, His95, Gly94 and Gly93 and the nitrogen atoms of the side chain amide groups of Asn213 and Asn97 of the NPA motifs. The line of mechanically stable carbonyl groups provides "a guide rail" of hydrogen bonding partners for the hydrogen atoms of the permeating water molecules as illustrated by the red ovals in the schematic drawing shown in Fig. 9b. Each water molecule in the single file can thus form two or three hydrogen bonds. Since water in bulk solution usually forms three or four hydrogen bonds with neighboring water molecules, water molecules entering the channel only have to sacrifice a single hydrogen bond, an energy cost of about 3 kcal (per mole). The arrangement of carbonyl and amide groups in the AQP4 channel thus dramatically lowers the energy barrier for water molecules entering the narrow AQP channel and allows for the very fast water permeation through the otherwise hydrophobic channel.

The NPA motifs and the arrangement of the carbonyl groups in the hydrophobic channel together with the arrangement of the two short pore helices HB and HE are crucially important to break the hydrogen bond network, which prevents proton conduction while maintaining fast water permeation. The ar/R constriction site is important for blocking H$_3$O$^+$ but not for the separation of hydrogen bonds. Our higher-resolution structure of AQP4 supports the hydrogen bond isolation mechanism, which has previously been proposed based on the lower-resolution structure of AQP1 [10] to explain the puzzling mechanism by which water channels can conduct water at very high speed while completely blocking proton permeation.

**Gap junction channel**

Gap junctions contain intercellular communication channels that allow a wide variety of solutes with different sizes to be transferred between the cytoplasm of adjacent cells. These solutes include ions, metabolites, nucleotides, peptides, and secondary messengers. Gap junction channels have critical roles in many biologically important processes including cardiac...
development, fertility, the immune system, and electrical signaling in the nervous system. The diversely expressed Connexin26 (Cx26) is the second smallest member of the conserved mammalian gap junction protein family.

We focus on the structure of Cx26 gap junction channels and used a site specific mutant of human Met34, hCx26M34A, because this mutant expresses in baculovirus infected S19 cells at higher quantities than wild type Cx26 infected cells. The hCx26M34A mutant is a single site mutation at the same position as the hCX26M34T mutant, which can cause prelingual non-sym- 
donamic hereditary deafness [16]. Although the purified hemichannel is hexameric, the 2D arrays obtained by dialysis showed an orthorhombic crystal lattice. A side view of the 3D map reveals that the crystals have a thickness of about 24 nm and contain three lipid bilayers (labeled Mem-1, Mem-2 and Mem-3 in Fig. 11a). Remarkably, the map also shows that the hemichannels re-docked through their extracellular surfaces forming complete gap junction channels (Fig. 11b). This is consistent with published results proposing extensive hydrophobic surfaces in the gap region. In bilayers Mem-1 and Mem-3, the hemichannels show poorer density than in Mem-2 (Fig. 11c, d), presumably because of variability in the molecular packing due to the large cytoplasmic domains of the connexin subunits. The cytoplasmic structures in Mem-1 and Mem-3 may also be deformed by their contact with the carbon film to which the crystals are adsorbed in the sample preparation procedure for cryo-electron microscopy. By contrast, the hemichannels in Mem-2 are protected from any forces such as the surface tension upon specimen drying and mechanical interactions with the carbon film. Therefore, the structural features of the hemichannels in Mem-2 should be the most accurate and, in particular, preserve the structure of the flexible cytoplasmic domains of the connexins. Thus, the description of the gap junction structure is based on the hemichannels in Mem-2 unless noted other- 

wise.

The 3D map shows a novel density in the center of the pore (upper left of Fig. 12) [17]. Since this density was dramatically reduced in the structure of N-terminal deletion mutant, it was confirmed that the plug was formed by N-terminal helices of six subunits in the hemichannel. The plug is located inside of the membrane layer and forms con- 


tacts with the surrounding channel wall as shown in the upper left figure (Fig. 12), which at the con- 

stricted part of the vestibule is formed by the innermost helices 1 (Fig. 12-2). This density strongly suggests that a plug physically blocks the channel within the membrane. Each hemichannel has its own plug, conferring it the ability to gate its pore autonomously. It is possible that the tran- 

s Junctional voltage sensor and the physical gate reside exclusively within a single hemichannel.

Our Cx26 gap junction crystal structure shows that the channel vestibule is blocked by a physical obstruction which we call the "plug". Structure analyzed by X-ray crystallography at higher resolution of the open state [18] provide more structural detail and sup- 

ported our plug gating mechanisms in these widely expressed channels.

Conclusions

The ability of electrons to form images, com- 

bined with advances in cryo-technology, are enabling us to acquire detailed structural and chemical information about membrane proteins in their physiological lipid and ionic settings as shown in Fig. 13 where structures of membrane proteins analyzed by electron crystallography were indicated. This information complements that obtained by x-ray diffraction of proteins in detergent, where biological relevance of the structure is less certain. The development of cryo-electron tomography and real-space averaging methods together with instrumental develop- 
ments such as the seventh generation cryo-EM will be extending the possibilities of obtain- 

ing high resolution information from increasing- 

ly complex protein-lipid arrays, and from mem- 
brane specialisations in situ.

Acknowledgements

These studies were performed in a nice collabo- 

ration with many researchers whose names were recorded as authors in each referenced paper. The author thanks Dr K. Tani for preparation of fig- 
ures. These works were supported by Grants-in-Aid for Specially Promoted Research and NEDO.

References


32 (1975).

658 (1999).

![Fig. 11 Three membrane layered structure of 2D-crystal of gap junction channel, Cx26.](30) JEOL News Vol. 44 No.1 30 (2009)
Fig. 12 Structure of Cx26. Upper left figure indicates sectional image perpendicular to membrane surfaces and numbers (1, 2, and 3) at the upper left show the position of each sectional image parallel to the surfaces.

Fig. 13 Structures of membrane proteins analyzed by cryo-electron microscope with top entry helium stage:

- HK-ATPase: EMBO J, in press
- MGST-1: JMB, 360, 934-945 (2006)
- Cx26: PNAS, 104, 10034-10039 (2007)

Fig. 13 Structures of membrane proteins analyzed by utilizing our cryo-EM systems.