How to picture a protein

Tracking site-specific cell functions is a lot easier with a transmission electron micrograph of the site.

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Because so many of today's drugs work by fitting into pockets, pores, and channels in protein molecules, wouldn't it be great to have an atom-by-atom map? Unfortunately, protein molecules are large and complex, so data collection is a lengthy process. To complicate matters further, proteins are susceptible to radiation damage from the X-rays and electrons used to generate the data. By the time the data collection is finished, the irradiated sample may have morphed into something completely different. X-ray crystallography is the method of choice for many types of structural studies, but producing a high-resolution data set often requires milligram quantities of sample. If the entire sample weighs only a few micrograms, you're out of luck. Enter transmission electron microscopy (TEM).

Transmission electron microscopy

As the name suggests, TEM uses electron beams to produce images in an analogous fashion to photon beams in a light microscope. In contrast to scanning electron microscopy, in which electron beams are reflected from the sample into a detector or camera, TEM beams pass through the sample to produce images or diffraction patterns.

In contrast to X-rays, electron beams focus in on a very small spot, so tiny samples are actually preferred. Transmission electron microscopes collect both images and diffraction patterns in the same region of the sample, an advantage over typical X-ray crystallographic studies, which are limited to diffraction patterns. Images taken using low beam dosages can be enhanced using software, so collecting lots of data causes less radiation damage to the sample. Cooling the sample to cryogenic temperatures not only reduces radiation damage further, but improves the signal-to-noise ratio by reducing the atomic vibrations in the sample. (Reference 1 provides a good general overview of TEM applications in molecular biology.)

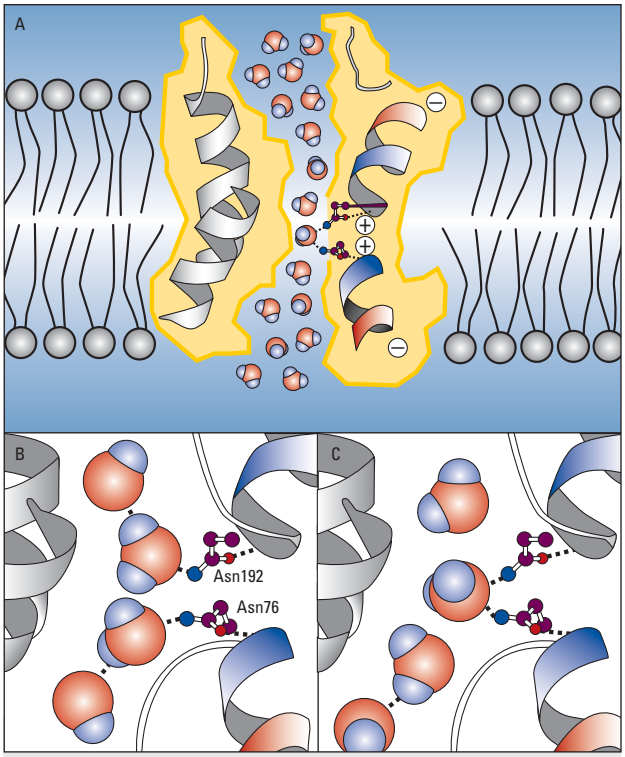

Figure 1. Red blood cell membrane pores lined with AQP1 block the passage of protons but allow H_2O to pass through (A). One or two asparagine residues (B and C, respectively) near the center of the pore form hydrogen bonds with water molecules, breaking the chain along which protons are transported. (Reprinted with permission from reference 2. Copyright 2000, Macmillan Publishers Ltd.)

Sample prep is the big drawback in analyzing proteins using TEM. Most biological materials contain a lot of water. Electron microscopy is done in a vacuum (electron beams are easily scattered by any kind of gas molecules in their path), so the water must be removed from the sample or fixed in place so it will not evaporate. This must be done without altering the sample from its natural state any more than necessary. For example, samples must be frozen quickly so that ice crystals do not destroy them. Flat sheets one molecule thick are preferred, because the TEM image is a superposition of all the structures in the beam path. If the beam passes through only one structure at a time, this simplifies data analysis later. Data are taken from different crystal planes by tilting the sample through a series of angles, collecting images and diffraction patterns at each angle. Fortunately, many electron microscopes are equipped with tilting stages for just this purpose.

Water gate secrets revealed

Several structure analysis techniques are available, depending on the degree of detail needed and how amenable the sample is to being crystallized in a sheet. Electron crystallography is the most ambitious, producing 2-D structures at atomic resolution, which can then be assembled into the full 3-D crystal structures. This is not only the most thorough method in terms of data analysis, but it is also demanding in terms of the sample required. Crystals that are at least 1–2 μm across work best, and specimens must be very thin and flat. A full data set can require several hundreds to several thousands of images and diffraction patterns, which might be produced at the rate of about 30 per day, on a good day.

One group of researchers used electron crystallography to find out how a molecular gatekeeper works (*2*). Basic biology fact: Cells are mostly water (with other stuff floating around in it) surrounded by lipid membranes that keep what's inside the cell separate from what's outside. However, the membranes of some

cells, including red blood cells, are very permeable to water—otherwise they wouldn't function. One member of the aquaporin protein family, AQP1, forms tetramers that line the water pore walls in red blood cell membranes. For years, biologists had wondered how AQP1 distinguishes between water, which passes through, and hydrated protons, which do not.

To perform the crystallographic study, the researchers reconstituted purified AQP1 protein with pure phospholipids in the absence of divalent cations. Then they deposited the resulting 2-D crystals on carbon-coated molybdenum electron microscope grids. Electron micrographs were recorded and digitized using a scanner. Electron diffraction patterns were collected using a slow-scan charge-coupled device camera. The AQP1 crystals were relatively small and contained a significant number of lattice defects, two factors that limited the resolution of the final structure to 3.8 Å. The best 135 diffraction patterns and 103 images were selected from a substantially larger data set.

Solving the AQP1 structure revealed a constriction at the center of the pore formed by the AQP1 tetramer. Water molecules enter the pore in a single-file hydrogen-bonded chain, like a class of first graders holding hands on a field trip (Figure 1). If the water chain is uninterrupted, protons pass from water molecule to water molecule along the chain. However, in AQP1, asparagine (Asn) residues attached to the protein's helices protrude into the pore and snag a passing water molecule by forming a hydrogen bond between the water's oxygen atom and either one or two amido groups from the Asn residues. In the process, the water molecule is reoriented so that it cannot form hydrogen bonds with the other water molecules passing through. Thus, when a proton traveling along the water chain encounters this break, it cannot travel any farther. Individual neutral water molecules, however, can pass through unimpeded.

Another research group has since refined the AQP1 structure to a resolution of 2.2 Å using multiple-wavelength anomalous X-ray diffraction (a technique that requires a synchrotron X-ray source) and crystallographic model building (*3*). Their model reveals a smaller pore constriction, relocates it about 8 Å from the location determined using TEM, and elucidates the structural basis for the protein's selectivity toward water.

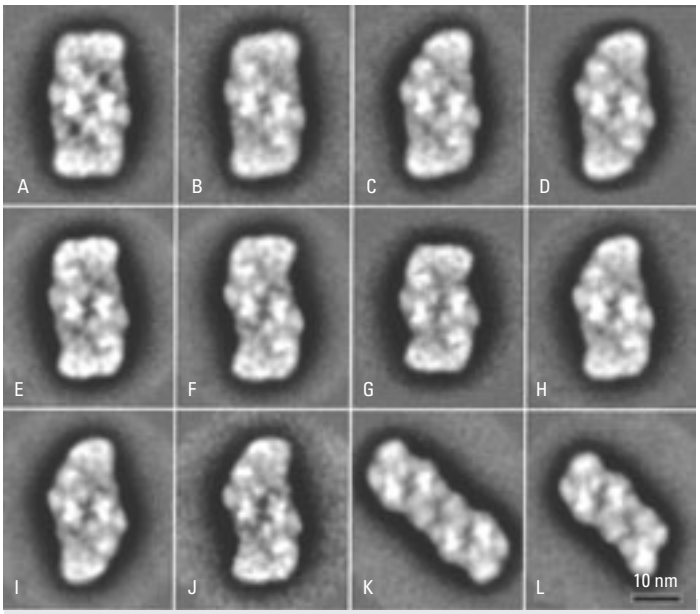

Figure 2. Spinach knows the secret of extracting oxygen from water. A series of averaged single-particle images of the C_2S_2-type PSII–LHCII protein–pigment light-harvesting supercomplex extracted from spinach thylakoid membranes highlights the structural changes that occur when various extrinsic proteins are removed. These changes affect the plant cell's ability to use sunlight to extract oxygen from water. (Reprinted with permission from reference 4.)

Spinach structures

Electron crystallography is similar to X-ray crystallography (or NMR, for that matter) in that it produces an atom-by-atom description of structural features, such as molecular bonding sequences, that repeat many times. Other TEM techniques produce structural information on a larger scale—something that X-rays and NMR cannot do. Often, biological molecules or molecular complexes do not have internal symmetry or are not amenable to crystallization. Single-particle averaging, one such TEM method, produces a low-resolution electron density map showing the general structural outline of the molecular complex. This method allows electron micrograph images to be obtained for large numbers of particles. The individual images are compiled into a composite image using computer algorithms to orient, align, and superimpose images from each particle. The resulting composites show features such as RNA helices, peripheral proteins, and bridges between subunits.

Single-particle image analysis recently provided graphic proof of the structural changes that occur when extrinsic proteins are removed from one type of complex found in green plants (*3*). These structural changes have been linked with changes in the ability of plant chloroplasts to extract oxygen from water. The thylakoid membranes of plant chloroplasts contain a protein–pigment complex called photosystem II (PSII), which forms a supercomplex with an "antenna" called light-harvesting complex II (LHCII). The extrinsic proteins in PSII are necessary to stabilize the overall structure and hold it in the proper conformation so that the inorganic cofactors can use sunlight energy to extract the oxygen from water.

PSII membranes were isolated from spinach thylakoid membranes, and the specimens were prepared using various buffers and salt washes and a negative stain. Transmission electron micrographs were obtained with 3000–4000 particle images collected for each treatment type. Glow-discharge grids were used to orient the molecules with their flat sides on the film. The image analysis procedure included repeated cycles of image alignment, multivariate statistical analysis, and particle type classification. Figure 2 shows a series of enhanced composite images of the C_2S_2 type PSII–LHCII supercomplex containing the common features from 100–400 individual particles. Differences in the particle shapes indicate various sample treatments and the effects of removing one CP26 antenna protein area (C, H, J) or two CP26 areas (D, I). Images K and L are C_4- and C_3-type supercomplexes, respectively.

Slices, helices, and icosahedra

Electron tomography works on the same principle as its medical-imaging counterpart, and it is well suited to large structures such as organelles and large macromolecular complexes in which the same structure is not repeated many times. A series of images is obtained as the specimen, usually <300 nm thick, is tilted through a series of angles in 1° increments. This procedure exposes the same region of the sample to a large cumulative dose of electrons over the duration of the data collection, so a low beam current is an unfortunate requirement. As a result, resolution is limited to about 20–70 Å. This method has been used, for example, to examine changes that occur when insect flight muscles are activated (*1*).

Protein structure solution using electron microscopy is not for the faint of heart.

Biological structures that have helical or icosahedral symmetries, however, can be analyzed using single-micrograph techniques (*1*). Sample tilting is not necessary here because all the views are accessible from one micrograph. Thus, you can spend as much time collecting data for one micrograph as you would for a whole series using the tilting methods. For example, filaments and tubular crystals (e.g., acetylcholine receptors and sarcoplasmic Ca–ATPase pumps) contain helical-structure elements that can be resolved to within about 5–11 Å. Many viruses adopt icosahedral symmetry, and their structures can be resolved to within 7–9 Å using the single-micrograph technique.

Protein structure solution using electron microscopy is not for the faint of heart, but as the field of proteomics comes into prominence, more effort is being devoted to developing laboratory methods, instrumentation, and software for data analysis and structure modeling. Incorporating information from other techniques such as NMR and X-ray crystallography expedites the process, and the growing archive of known structures provides a basis on which to begin solving new structures. As with any other emerging method, knowledge builds on itself—the more you know, the more you can find out.

References

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