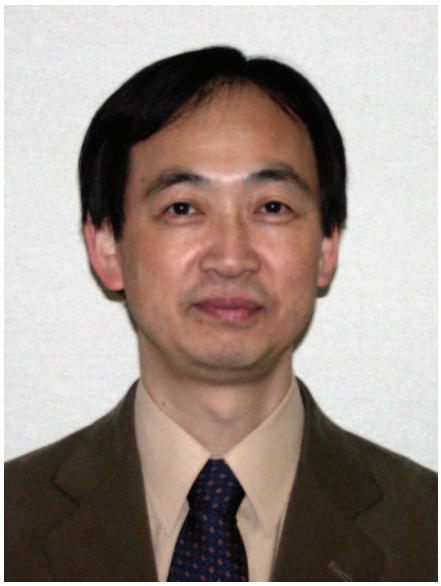
Profile of Chikashi Toyoshima

When water is suddenly cooled to temperatures below −140°C, by careful methods such as plunging it into a bath of liquid nitrogen, it forms an amorphous structure that is more like a glass than a crystal. Called vitreous ice, this substance cannot be found in nature but can be used to preserve samples for use with an electron microscope. When biophysicist Chikashi Toyoshima first heard of this technique in graduate school at the University of Tokyo, he knew immediately it was a powerful tool to help reveal the structure of biological samples that would otherwise be crushed in the vacuum of electron microscopes. He traveled across the Pacific to learn the technique in California and eventually used the technique to determine the structure of membrane proteins in tubular crystals, providing images of these biological structures for the first time.

Throughout his career, Toyoshima has combined knowledge of mathematics, physics, and biology to reveal atomic structures of biological samples. Now a professor at the Institute of Molecular and Cellular Biosciences at the University of Tokyo and elected to the National Academy of Sciences as a Foreign Associate in 2005, Toyoshima continues to investigate the structures of proteins through a variety of methods. In his Inaugural Article, published in a recent issue of PNAS (1), Toyoshima and his colleagues presented a crystal structure of Ca^{2+}-ATPase and discussed the role that proton countertransport plays in maintaining the enzyme's structural integrity.



Chikashi Toyoshima

The Ease of Physics

Toyoshima grew up in post-World War II Japan in the small town of Honjo, in the prefecture of Akita. Elementary-school education in Honjo was "extraordinary," Toyoshima says, describing the system as "enthusiastic in developing children's ability in science, writing, sports, and arts. In a way, it was very experimental." Toyoshima and his brother, who was 3 years ahead of him in school, worked on science experiments with their mother, a high school home economics teacher. They did quite well in science research contests throughout Akita, Toyoshima says.

Toyoshima also showed an early flair for crafty innovation, and he spent his free time constructing plastic and wooden models of planes and ships. Later, toy electronics became his pastime. "These hobbies helped me a lot in doing science," he says. The skills he practiced as a youth have paid off as a researcher in a highly technical field. "Small tools can make difficult tasks much easier. You can think of new ways to tackle problems. If you want to do something that other people did not do, you will perhaps need new tools," he explains.

Toyoshima's first career decision was whether to become a doctor. His brother pursued medicine in college, so Toyoshima decided he was then free to choose another branch of science. "I thought it not necessary to have two medical doctors in the family," Toyoshima says, "and at the time, to be a physicist was a rather fashionable thing." Physics' rigor and difficulty only added to its cachet, he adds. In high school, Toyoshima performed well in his studies of modern Japanese, and his teacher suggested he study literature and writing in college. Toyoshima thought, however, that literature would be much more difficult than physics, at least when it came to the job hunt.

Toyoshima decided to apply to the University of Tokyo, arguably the top university in the country. In his application, he was required to declare his interest in one of the three main science colleges: technology, biology, or medicine. He chose technology, which was the largest division and included the physics department, the department on which he set his sights. He was admitted in 1973 on his first trial. Looking ahead, he soon sought out biophysics texts and diligently studied them to determine whether he had the ability to survive in that rigorous environment. "I decided it was not *too* difficult," he says.

View of a Protein

For the next couple of years, Toyoshima studied standard physics but also took classes in the biochemistry and botany departments. "In a way, I was not a good physics student," he says of his cross-disciplinary approach. In the middle of their third year, students were permitted to visit laboratories and conduct summer research projects. Toyoshima visited Setsuro Ebashi's laboratory, where he met Taki Wakabayashi, a research associate who had just returned from doing electron microscopy (EM) work at the Medical Research Council Laboratory of Molecular Biology (MRC LMB) in Cambridge, England. Wakabayashi was interested in how muscle thin filaments were regulated by intracellular levels of calcium, which controls the contraction cycle of muscles. He used three-dimensional image reconstruction from EM images to help elucidate the structure and regulation of filaments.

Wakabayashi's description of the EM work appealed to Toyoshima. "I thought it was a very strong technique. It's revealing because you can see what the protein really is," says Toyoshima. He chose to carry out a small project in Ebashi's laboratory, and after finishing his undergraduate degree in 1978, Toyoshima stayed with Ebashi's group. He continued to work on microscopy of muscle thin filaments and myosin heads with Wakabayashi for his master's and doctoral research in biophysics.

EM at the time posed special difficulties for examining biological specimens. Because electrons are reflected by air molecules, EM samples require placement in a vacuum. Standard specimen preparation at the time involved preserving the protein in a salt of a heavy metal, such as uranyl acetate. But this process damaged biological structures. "The protein is pressed down, flattened very severely," Toyoshima explains.

Some researchers began turning to new technology as a way of alleviating these problems. One such technology, cryomicroscopy, involves placing the specimen in water and cooling it very rapidly to temperatures such as that of liquid nitrogen. This process forms vitreous ice, Toyoshima says, and preserves

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This is a Profile of a recently elected member of the National Academy of Sciences to accompany the member's Inaugural Article on page 14489 in issue 41 of volume 102.

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the specimen in a snapshot of its solution state, even in the vacuum. Also, the low temperatures help protect fragile biological specimens against radiation damage from the electron microscope.

Toyoshima chose to study this cryomicroscopy technique for his postdoctoral research. In 1986, he moved to northern California to work with biophysicist Nigel Unwin at Stanford University (Stanford, CA). Unwin studied nicotinic acetylcholine receptors, which are arranged in a helical array. At the time, no methods existed for analyzing tubular crystals with EM. Toyoshima worked to develop mathematical methods for disentangling the superimposed information from a projection image, or electron micrograph, of the tubular structure (2). A year after Toyoshima moved to California, Unwin returned to his previous home in the MRC LMB in England, and 6 months later, in 1988, Toyoshima followed him.

Three Paths to the Ion Pump

At the MRC LMB, Toyoshima also worked with biophysicist David Stokes, who was studying Ca^{2+}-ATPase. Toyoshima had honed his method of analyzing tubular structures on the acetylcholine receptors and now wanted to explore a different protein (3, 4). Stokes had tubular crystals of calcium ATPase, but he also had tiny three-dimensional crystals of the protein. No one had actually been able to analyze these small crystals with x-ray crystallography because the methods required a much larger sample. Even EM techniques were not capable of fully analyzing these crystals because the samples needed to be formed from

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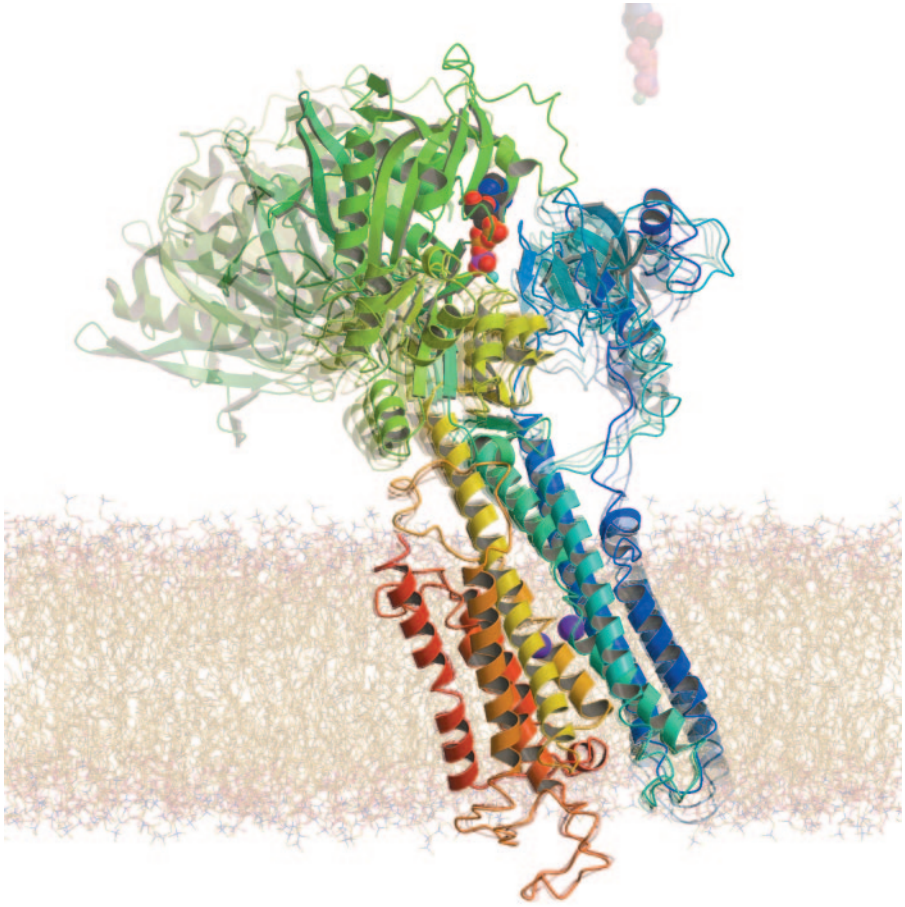
one or two layers of two-dimensional crystals. "At that time, crystallization for x-ray crystallography and EM were two different things," explains Toyoshima. Crystallization for EM involved reconstituting the protein in lipid bilayers. Researchers believed that this method would not produce samples useful for x-ray crystallography because the stacks of two-dimensional crystals could not grow large enough, and the lipids were too heterogeneous to be useful.

A major step forward was achieved by a graduate student in the laboratory at



Ca^{2+}-ATPase in lipid bilayer.

that time, Haruo Ogawa, who found a paper on the use of organic anions (5). Toyoshima was intrigued. "I wondered if it might be possible to make crystals large enough for x-rays. In fact, we became to be able to make large crystals, but they were extremely thin and there was no way to analyze them," he says. Another insight was necessary.

Toyoshima realized that he faced three possible approaches to the problem, and he decided to try all of them. He worked simultaneously to develop new electron crystallography technology for analyzing very thin stacks of two-dimensional crystals, to grow the crystal thick enough for analysis with standard x-ray crystallography, and to decrease the number of stackings to allow analysis with standard EM. "I thought I should try all three ways to tackle the problem because I didn't know which one would succeed," he says.

"There were three unusual things in the crystallization," Toyoshima explains, citing that "usually, the first barrier in analyzing membrane proteins is obtaining enough pure proteins to crystallize them." Yet Ca^{2+}-ATPase can be found in high concentrations in the sarcoplasmic reticulum membrane, so it was relatively simple to purify with standard methods. "One special thing was the use of sodium butyrate, which was obviously inspired by Ken Taylor's work. The second thing was the inclusion of lipids. It was necessary because the ATPase died in purification without lipids. I think I had a good sense in handling proteins because I was trained in Ebashi's lab for muscle proteins. But I had to learn how to handle lipids and to make it as reproducible as possible," he explains. To avoid oxidation of lipids, the researchers used dialysis buttons of their own design, which was the third unusual feature. "As a whole, we may be the first to use the crystallization method for EM to make x-ray crystals," says Toyoshima.

After much work, Toyoshima obtained large enough crystals in the first state, the $E1{\cdot}2Ca^{2+}$. "But after that, it was relatively easy to crystallize the ATPase in five other states. After establishing the methods, we knew how to crystallize it," he says. Although he eventually managed to make crystals thick enough for x-ray crystallography, his success stemmed from an integration

of all three approaches. "All of them helped. Many divergent efforts converged into a real structure and gave me insight into how the enzyme works. That was a wonderful experience as a scientist," says Toyoshima.

His research, started in 1996 and published over the course of several years from 2000 to 2004, produced the first images researchers had obtained of an ion pump (6–11). The images landed on the cover of *Nature* and even became an image for the journal's calendar. The last structure he published was that of the $E2{\cdot}MgF_4^{2-}$ form, which was solved soon after the first $E1{\cdot}2Ca^{2+}$ form but, in a way, was the farthest form in the reaction cycle. "It took me a really long time to understand why the structure had to be so," he says. He needed the benefit of understanding the structures of the other states first, but when he finally grasped the importance of the conformational changes he saw, "it was a revelation," he says.

Images of Time

Toyoshima moved back to Japan in 1989. First, he joined the Frontier Research Program at RIKEN in Wako, Japan, where he had access to substantial research facilities and equipment. He then moved to the Tokyo Institute of Technology as an associate professor in 1990 and then back to the University of Tokyo in 1994 as a full professor, where he remains.

Nowadays, Toyoshima tends to work primarily on x-ray crystallography and less on microscopy and has also become interested in molecular dynamics simulation (12). "In a way, my laboratory is unique because both experimentalists and theoreticians live in the same laboratory," he says. Describing his laboratory, Toyoshima says, "I started as an electron microscopist. I hired Nakasko to learn x-ray crystallography and Yuji to learn simulation. I want to acquire those techniques as my own tools. For that purpose, I don't think usual collaborations are good enough. I have to learn in person and discuss with the experts every day."

Toyoshima's Inaugural Article (1) uses crystallography techniques to study the pumps in ATPase. The findings help solve the puzzle of countertransport, he says. The Ca^{2+}-ATPase of the sarcoplasmic reticulum is a calcium ion pump in one direction and a proton pump in the opposite direction, but no demonstrated functional role for the countertransport of the protons has been demonstrated. The membrane is "leaky" to protons, Toyoshima explains, asking, "why do protons have to be transferred from this side to the other side? It is a puzzle."

He and his coauthors managed to obtain a 2.4-Å resolution crystal structure of the ATPase in the absence of Ca^{2+}. Their computations showed that stability is provided by the proton binding, and therefore the countertransport is necessary for the structural integrity of the empty Ca^{2+}-binding sites. "You have to have this transfer because otherwise the structure will disintegrate," he says. Furthermore, the results hold not just for Ca^{2+}-ATPase, he says, but also presumably for all P-type ATPases, even those for which the countertransported ions are still unknown.

Having returned to Japan after spending a month this past summer attending meetings and visiting with colleagues in the United States, Germany, and Italy, Toyoshima is happy to be in the laboratory again. "I don't like traveling. I want to do science with my hands," he says. Although Toyoshima has mostly left crystallization experiments to others, he still examines and freezes crystals himself in a cold room and goes to SPring-8 in Hyogo, Japan, to collect diffraction data.

The atomic-level glimpses of the proteins Toyoshima studies bring deeper rewards, he says. "If you look at a structure long enough, you understand why it has to be so. Then suddenly you feel the length of time before you," he says. "The proteins are designed by time. They have evolved. To become this structure we now see, it happened over millions and millions of years. Suddenly you understand. Suddenly you have a different view of time and your life, and also perhaps of the universe."

Regina Nuzzo, *Science Writer*

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