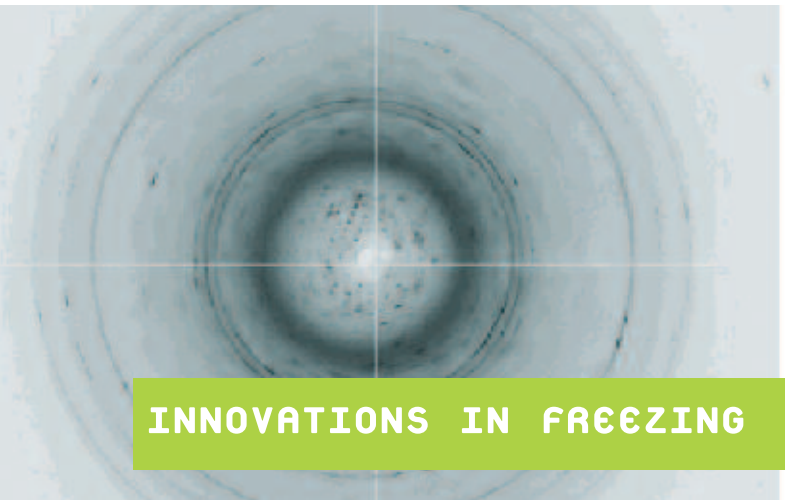

INNOVATIONS IN FREEZING

Provided

Robert Thorne

One would think that simply spending time in Ithaca during the winter months would make a person an expert on freezing. After all, freezing is something that occupies our minds for six months of the year as we worry about soaring fuel costs, frozen pipes, icy roads, ski conditions, and simply our own body temperatures in homes with lowered thermostats. It is also something that occupies the minds of the scientists in the research groups of Robert E. Thorne and Sol M. Gruner, Physics/Laboratory of Atomic and Solid State Physics (LASSP), who are trying to understand the physics of freezing and how to do it better. It turns out that freezing is critical to more than just utility bills and winter sports; it is also of great importance to proteomics.

IT TURNS OUT THAT FREEZING IS CRITICAL TO MORE THAN JUST UTILITY BILLS AND WINTER SPORTS; IT IS ALSO OF GREAT IMPORTANCE TO PROTEOMICS.

Proteomics is the study of proteins. While genomics has been getting most of the headlines lately, proteomics is a rapidly growing discipline of at least equal promise. Proteins are not only the basic building blocks of living organisms, but also the chemicals that do most of the work. Much more complicated than genomics—there are only about 22,000 genes in the human genome, versus nearly 400,000 proteins in the proteome—proteomics' aim is to understand how the proteome differs from cell to cell, and how it changes in response to biochemical interactions within the genome and the varying cellular environments found in different parts of an organism's body at different stages of its life cycle and under different environmental conditions. There are two key areas of proteomics: functional proteomics, the study of what proteins do; and structural proteomics, the study of how the structure of a protein enables it to perform a function (also referred to more broadly as structural biology).

The structure of DNA, base pairs of four nucleotides forming the rungs of a double-helical ladder, is familiar to most. The structure of proteins is not only unfamiliar, but also vastly more complicated and varied. Proteins are chain molecules consisting of a unique sequence of thousands of amino acids, of which there are 20 varieties. The specific sequence of amino acids comprising each protein is encoded in DNA, and cellular machinery uses this information to manufacture proteins.

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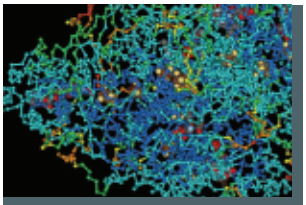
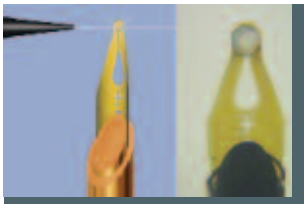


Protein structure

Under proper conditions, a protein will, through a process known as protein folding, assume its proper functional shape or conformation. It is only by coiling and folding into the correct three-dimensional shape that proteins are able to perform their biological function. Although only one conformation is active, a specific sequence of proteins can nevertheless assume more than one stable conformation, depending upon the conditions under which the protein is formed. Since protein folding is incompletely understood, it is still impossible to predict a protein's functional conformation solely from the DNA that encodes it. Scientists therefore resort to other techniques, one of the more successful of which is x-ray crystallography. Protein crystals are bombarded by x-rays, such as those generated at CHESS, which are scattered by the crystal. The pattern of those scattered x-rays provides information that helps scientists unravel protein structure.

First, a researcher needs protein crystals. Protein crystals are grown by taking a droplet that contains a high concentration of a protein dissolved in water and allowing the protein to precipitate out as a crystal. The next step is to collect the crystal. This task has become more challenging since the size of crystals

THERE ARE TWO KEY AREAS OF PROTEOMICS: FUNCTIONAL PROTEOMICS, THE STUDY OF WHAT PROTEINS DO; AND STRUCTURAL PROTEOMICS, THE STUDY OF HOW THE STRUCTURE OF A PROTEIN ENABLES IT TO PERFORM A FUNCTION (ALSO REFERRED TO MORE BROADLY AS STRUCTURAL BIOLOGY).


Crystal held by a MicroMount™

that can be studied by modern x-ray sources has shrunk from a fraction of a millimeter to as small as five micrometers. It is at this point in the process that Thorne made an innovation: a simple microfabricated plastic device for scooping protein crystals off the surface on which they were grown and then holding them during freezing and x-ray crystallography (see illustration above). He first disclosed the innovation to the Cornell Research Foundation, the predecessor of the Cornell Center for Technology, Enterprise, and Commercialization (CCTEC), several years ago. Thorne's idea was to use an extremely thin piece of plastic that is almost transparent to x-rays and to give it sufficient rigidity to effectively hold the crystal by curving it. Thorne also incorporated wicking apertures to help draw liquid away from the crystal and markings for automatically locating and identifying the sample under analysis. Thorne started a company, MiTeGen, to sell the resulting crystal mounts under the trade name MicroMounts™.

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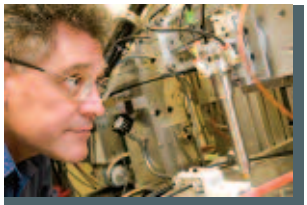
With help from the community as he put together packaging, logos, prototypes, website, and company, Thorne's transformation from faculty scientist to faculty scientist–entrepreneur was rapid. More inventions followed, including x-ray–transparent multiwell plates for improved growth of protein crystals, thin polymer capillaries in which crystals can be both grown and analyzed, a set of microtools ranging from saws to baskets to rulers to chisels for use with tiny samples and crystals, and an improvement to equipment used for freezing small samples.

Once a protein crystal has been picked up using a MicroMount™ or similar device, the crystal is analyzed using x-rays. Unfortunately, a typical protein crystal at room temperature survives only a fraction of the x-ray dose required for a complete high resolution data set before it becomes irrevocably radiation-damaged. To inhibit the radiation damage, protein crystals are typically flash cooled at atmospheric pressure by plunging them into liquid nitrogen (77° K or -196° C). Cryocooling also reduces the thermal motion within the crystal, enabling the collection of higher quality data.

...THORNE MADE AN INNOVATION: A SIMPLE MICROFABRICATED PLASTIC DEVICE FOR SCOOPING PROTEIN CRYSTALS OFF THE SURFACE ON WHICH THEY WERE GROWN AND THEN HOLDING THEM DURING FREEZING AND X-RAY CRYSTALLOGRAPHY.

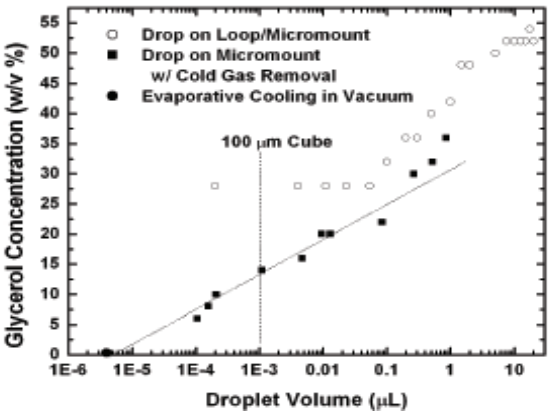

Frank DiMeo

Sol Gruner

Freezing protein crystals successfully and without damage, however, is a tricky business. Proteins crystals form in an aqueous solution and can contain 50 percent or more water by weight. As anyone knows who is lucky enough to be by a northern lake on a frigid night early in the winter, as the ice noisily heaves and cracks, freezing water expands with great force—more than enough to damage the crystals one is trying to protect. The goal of flash freezing is for the water to form amorphous ice rather than crystalline ice, which expands with such force.

Cryoprotectants are typically added to promote this result. Unfortunately, since each protein is unique, a specific cryoprotectant must be formulated for each, a task that proves difficult or impossible in many cases.

The most recent innovation from Thorne's research group is an improved method for flash freezing of small samples. While investigating flash cooling common water-cryoprotectant mixtures by immersion in liquid nitrogen as a function of volume cooled, the researchers noticed that the concentration of cryoprotectant required to prevent ice crystal formation decreased as samples shrunk from 10 to 0.1 microliter, but



then stopped decreasing with further decreases in sample volume. It turns out that there is a layer of cold gas above the liquid nitrogen, which freezes small samples as they pass through it, before they can reach the liquid nitrogen. This is a problem, because cooling by the cold gas is slower than cooling by liquid nitrogen. By removing the cold gas layer (for example, by blowing warm air across the surface of the liquid nitrogen), the freezing rate of small samples increased dramatically below 0.1 microliters, allowing much smaller cryoprotectant concentrations to be used to protect the sample.

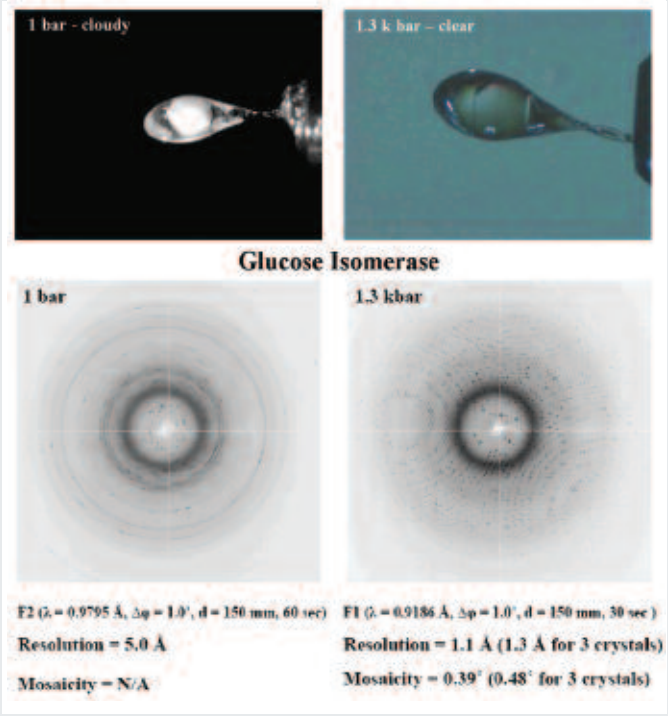
MORE INVENTIONS FOLLOWED, INCLUDING X-RAY–TRANSPARENT MULTIWELL PLATES FOR IMPROVED GROWTH OF PROTEIN CRYSTALS, THIN POLYMER CAPILLARIES IN WHICH CRYSTALS CAN BE BOTH GROWN AND ANALYZED, A SET OF MICROTOOLS RANGING FROM SAWS TO BASKETS TO RULERS TO CHISELS FOR USE WITH TINY SAMPLES AND CRYSTALS, AND AN IMPROVEMENT TO EQUIPMENT USED FOR FREEZING SMALL SAMPLES.

Sol Gruner, Physics, and graduate student Chae Un Kim bring another innovation to the process: they have eliminated the need for cryoprotectants, increasing cryocooled protein crystal quality. Instead of freezing protein crystals at atmospheric pressure, they cryocool their protein crystals under high pressure. Under these conditions, the water turns into a high density amorphous solid, similar to a glass, which minimizes crystal disruption.

Their process is simple in concept—pressurize a protein crystal in helium at room temperature up to between 100 and 400 MPa (about 14,500 to 58,000 psi), cryocool the crystal to 77° K, then release the pressure while maintaining the low temperature—but complicated in practice, due to the high pressures involved. However, discussions with interested companies have already suggested ways to construct equipment that will make the process practical for structural biologists to use. Protein crystal structure is solved by determining the repetitive three-dimensional electron density distribution of protein molecules in a crystalline arrangement. Each crystal reflection has an intensity (amplitude) and phase, and both are needed to generate a protein structure. The information obtained from x-ray diffraction is not sufficient alone to solve the structure of a protein crystal, since the method provides only intensity data. In a further innovation to their basic method, Gruner and Kim have combined a technique developed by others with their high pressure cryocooling method to obtain both intensity and phase information from a single protein crystal, enabling them to solve the protein's crystal structure. To accomplish this, they added a second pressurization step in krypton or xenon gas, instead of helium. During this additional step, krypton or xenon atoms bind to specific locations in the protein. By taking advantage of the diffraction from these distinct atoms, complete phasing information can be derived. Kim proved the efficacy of this method by solving the structure of porcine pancreas elastase. He used only phase and amplitude data obtained by diffraction of a single crystal cryocooled with Cornell's new process.

Pharmaceutical companies currently use x-ray crystallography to determine exactly how drug lead compounds and their protein targets interact. To date, x-ray crystallography is the most effective technique in the field of structural biology; out of the

Protein crystals are frozen in droplets of water at room pressure (upper left) and 1,300 atomspheres pressure (upper right); the crystalline ice in the left photo is cloudy, while the amorphous ice in the right photo is transparent. The bottom two images are the x-ray diffraction patterns captured from the corresponding protein crystal. The rings on the pattern on the left arise from ice crystals. More importantly, the quality of the diffraction obtained by the pressure-frozen crystal (lower right) is superior to the diffraction from a crystal frozen at room pressure (lower left).



GRUNER AND GRADUATE STUDENT CHAE UN KIM BRING ANOTHER INNOVATION TO THE PROCESS: THEY HAVE ELIMINATED THE NEED FOR CRYOPROTECTANTS, INCREASING CRYOCOOLED PROTEIN CRYSTAL QUALITY.

Frank DiMeo

approximately 35,000 protein structures solved, x-ray crystallography is responsible for about 29,000, while nuclear magnetic resonance has been used to solve most of the rest. The promise of structural biology to improve human health is great, and any method or device that can speed the solving of protein structures will contribute to fulfilling that promise.

Innovations, such as these from the labs of Thorne and Gruner at Cornell, will no doubt play a key role in that success.

Scott S. Macfarlane
Senior Licensing Associate
Physical Sciences

PHARMACEUTICAL COMPANIES CURRENTLY USE X-RAY CRYSTALLOGRAPHY TO DETERMINE EXACTLY HOW DRUG LEAD COMPOUNDS AND THEIR PROTEIN TARGETS INTERACT. ... X-RAY CRYSTALLOGRAPHY IS THE MOST EFFECTIVE TECHNIQUE IN THE FIELD OF STRUCTURAL BIOLOGY.

Thorne started a company, MiTeGen, to sell the resulting crystal mounts under the trade name microMounts™.

For more information:

Cornell Center for Technology, Enterprise, and Commercialization (CCTEC)
20 Thornwood Drive, Suite 105
Ithaca, New York 14850
(607) 257-1081, Fax: (607) 257-1015
Website: www.cctec.cornell.edu