BIOPHYSICS

Unfolding to force

Applying tiny forces to single molecules at high speed reveals the mechanics behind molecule unfolding.

The cell is no longer viewed only as a biochemical entity. In an increasing manner, the focus of research is on trying to understand the physical processes that go on inside and around the cell as well. Cells use molecules to sense and exert forces that are important in biological function. Adhesion molecules on the cell surface sense tension in the surrounding tissue, motor proteins apply force to move cargo in the cell, and proteins in the muscle mechanically move the tissue and allow motion. Both large and small mechanical effects are ultimately mediated by tiny forces that take place within and between individual molecules.

Over the past decades, methods based on optical tweezers and atomic force microscopy (AFM) have allowed researchers to apply small forces to single molecules and measure the molecule's response, relating protein mechanics, structure and folding. Combinations of AFM experiments with molecular dynamic simulations have led to rich atomic-level descriptions of processes such as receptor-ligand binding or the detailed unfolding of muscle proteins and have pushed forward the emerging field of single-molecule mechanics.

But up until now, the two methodologies, experimental force spectroscopy and *in silico* simulations, have not been easy to compare side by side because the experimental methods could not attain the pulling speeds of simulations.

High-speed AFM methods have, however, existed for quite some time, explains Simon Scheuring, who works at the INSERM (the French National Institute of Health and Medical Research) at Aix-Marseille University. These methods are based on using small cantilevers with high resonance frequency as well as fast scanners, feedback controllers and electronics. In the past, high-speed AFM has been used for imaging experiments, not for measuring forces, says Scheuring. In a paper recently published by his group, Scheuring and colleagues adapted the methodology to perform high-speed force spectroscopy measurements.

Scheuring and collaborators used a short cantilever and a small piezoelectric actuator like those used in previous high-speed AFM setups, but they added a few optimizations of

STRUCTURAL BIOLOGY

ELECTRON CRYSTALLOGRAPHY GOES 3D WITH MicroED

Electron diffraction using three-dimensional (3D) crystals may expand the reach of this technique.

Though X-ray crystallography has long been a workhorse technique to solve protein structures at atomic resolution, many proteins just do not behave when researchers attempt to grow large crystals. The emerging technique of serial femtosecond crystallography, in which a stream of tiny microcrystals is analyzed by femtosecond pulses from an extraordinarily powerful X-ray free-electron laser beam, is addressing this in part. But given that there are only a few facilities with such laser beams in the world, with extremely limited hours for biological research, alternative methods are needed to tackle these crystallization-recalcitrant proteins.

Tamir Gonen of Janelia Farm in Ashburn, Virginia, USA thinks micro electron diffraction (MicroED) may just fit the bill. His group recently reported the method, which may expand the reach of electron diffraction studies to include 3D protein crystals.

Traditional electron crystallography uses an electron microscope to capture diffraction patterns from very thin, two-dimensional (2D) crystals. Data can be obtained from such small crystals because electrons interact with atoms more strongly than do X-rays. But because of damage by the electron beam, only one snapshot can be taken of each 2D crystal. Diffraction images must be captured from hundreds of 2D crystals, and data must be merged to solve a protein structure at high resolution. However, the traditional method of performing electron crystallography does not work for 3D crystals. "There was no way of indexing the resulting data and actually getting to a structure," says Gonen. "That's where people got stuck."

Gonen's team figured out how to solve protein structures by electron diffraction from 3D crystals. The crystals they use are six orders of magnitude smaller than what is

High-speed force spectroscopy pulls molecules at high speeds to study their unfolding. Image courtesy of F. Rico.

their own. For instance, they tilted the surface to which the molecule is attached to on one end, reducing hydrodynamic drag and laser interferences in the force measurements. They also improved the electronics and data-collection processes. This allowed them to exert pulling forces at a speed of 4 millimeters per second, more than two orders of magnitude faster than in conventional AFM.

The technique offers two main advantages, says Scheuring. "One can do very fast velocity force spectroscopy experiments, or one can pull relatively slowly and use the high sampling resolution to see intermediate folding steps." Using this approach, the team studied how the muscle protein, Titin, unfolds in response to forces exerted at high and low speeds. Because of the high sampling resolution, they could decipher the detailed molecular dynamics that take place as Titin's domains unfold and refold. They also measured the dynamic force spectrum for this protein and found that it was not linear, something that had been previously predicted by simulations but that had not been experimentally confirmed.

"Our hope is that this type of work will stimulate new theory," says Scheuring, "and that we will be able to go back to back between the experiments and simulations." Scheuring is planning to apply the method to study ligand-receptor interactions now and envisions a future, not too far away, in which these types of measurements will be performed directly on molecules in cells and tissues.

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Rico, F. *et al.* High-speed force spectroscopy unfolds Titin at the velocity of molecular dynamics simulations. *Science* **342**, 741–743 (2013).

needed for X-ray crystallography. They apply a very low electron dose, 200 times lower than is typical in electron crystallography. This allows them to take multiple diffraction snapshots of each 3D crystal by rotating the crystal in one-degree increments. "When you have so much data out of a single crystal, you can then go back and index and get the structure," explains Gonen. The approach also benefits from the use of a very high-sensitivity camera that can detect single electrons, Gonen notes. This MicroED method allowed the team to solve the structure of the enzyme lysozyme at 2.9 Å resolution using electron diffraction data from just three crystals.

Though this work provides a strong proof-of-principle demonstration, more polishing is needed before the technique becomes as routine as X-ray crystallography. High on the list of needs for optimizing the pipeline is a simpler way of processing data. Gonen is collaborating with others to adapt user-friendly X-ray diffraction data-processing programs for MicroED. He also notes that better methods for freezing crystals for MicroED are needed, as are methods for phasing (such as using heavy-atom doping).

But because 3D microcrystals are much easier to produce than the large crystals generally needed for X-ray diffraction, MicroED may reveal itself to be quite suitable for obtaining structures of membrane proteins and other proteins that are notoriously difficult to crystallize. Gonen encourages researchers with interesting targets to send 3D microcrystals to his lab for analysis. Any electron microscope can be operated in diffraction mode, and such microscopes are widely available, so the method should become usable in many labs. "I think that in the next five to ten years, this could become a very powerful method for determining protein structure from very small crystals," says Gonen.

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Shi, D. *et al.* Three-dimensional electron crystallography of protein microcrystals. *eLife* **2**, e01345 (2013).