Tech News

The Ever Folding Protein Landscape

Check the cover of any biochemistry textbook or peer-reviewed journal, odds are you'll see protein structures, cartoon representations that reduce the molecules' tens of thousands of individual atoms into simplified sculptures resplendent in coils and ribbons of red, blue, green and yellow.

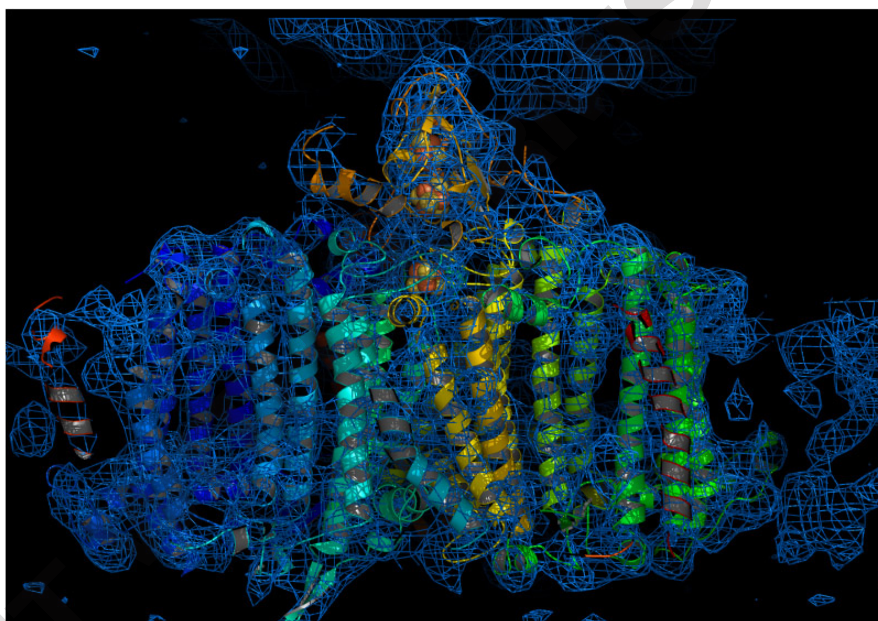
Generally solved using X-ray crystallography, nuclear magnetic resonance spectroscopy, or cryoelectron microscopy, such simplified structures help researchers conceptualize macromolecular protein complexes, visualize the impact of mutations, and even design targeted drug therapies. There's just one problem with these structures: Proteins are not as rigid as their structural depictions imply. Instead, they breathe and flex, folding and unfolding in response to changing cellular conditions, post-translational modifications, and simple thermodynamics.

"People have this idea that when a protein folds in the cell, it folds once and that's the end of it. But that's not true," explains Martin Gruebele, who studies protein folding at the University of Illinois at Urbana-Champaign. "Proteins fold and unfold and fold and unfold in the cell.... They continuously go back and forth."

While the pretty pictures published on book covers and journals are indeed accurate, they only tell part of the story. These images don't represent every possible form of the molecule, or perhaps even the most biologically interesting ones. Rather these are the most stable or crystallizable states, what North Carolina State University physicist Keith Weninger calls "landmarks in a conformational landscape."

And that's just in vitro; what a protein looks like in vivo may differ even more. "Living cells are amazing things," Weninger says. "They maintain non-equilibrium conditions; the system keeps gradients that shouldn't exist, and very non-equilibrium flows, and those are hard to reproduce outside of a cell. Those conditions can affect biology, which is why people want to develop high-resolution methods to look at protein structure in cells."

Until recently researchers had no way to capture such data. But today, using a crop of new fluorescence and NMR techniques, researchers are finally catching glimpses of protein structures and even folding events in cells rather than in a test tube.



The Photosystem membrane protein complex, deduced using femtosecond X-ray protein nanocrystallography. Image courtesy of Petra Fromme and Raimund Fromme, ASU.

Single-Molecule Fret

To watch proteins go through their thermodynamic acrobatic routines in vivo, both Gruebele and Weninger have turned to fluorescence resonance energy transfer (FRET, also called Förster resonance energy transfer). FRET uses the efficiency of non-radiative energy transfer between two fluorophores as a measure of the distance between them.

Imagine you position fluorophores at either end of a peptide that is capable of folding into a hairpin. One fluorophore absorbs blue light and emits green while the other absorbs green light and emits red. In an unfolded, extended conformation, irradiation of your peptide with blue light will produce a green fluorescent signal, as expected. But, if the molecule folds properly, and the two fluorophores are brought together in close proximity, then excitation with blue laser light will yield red fluorescence, as the green fluorophore passes its energy to the second dye.

The efficiency with which FRET occurs is a sensitive measure of the distance between the two points, leading some to call the technique a "molecular ruler." As with physical rulers, the resulting measurements can be used to pin down molecular structures. For instance, in one recent study Weninger and his team used 34 separate FRET distance measurements to "constrain" a structure of the synaptotagmin 1-SNARE complex, a multicomponent assembly that could not be solved by other methods. "To our knowledge, this is the first experimentally derived model of a synaptotagmin–SNARE complex, which has resisted crystallization and NMR analysis," the authors wrote. (1)

But that work was done in vitro; studying structure in vivo, Weninger says, is more challenging. "It's noisier, less controlled, so it's harder to interpret the data, make measurements, get good signal-to-noise ratios, and so on." Nevertheless, Weninger has begun migrating his studies into cells, using FRET to monitor the folding of a protein called SNAP-25.

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SNAP-25 is a SNARE protein, a family of polypeptides implicated in membrane fusion. As a monomer, SNAP-25 remains relatively unfolded. But when the protein enters a membrane fusion complex with other SNARE proteins, SNAP-25, well, snaps into a tightly folded helical bundle conformation. It's a molecular event akin to an unfolded tangle of yarn suddenly ordered itself into a skein.

To monitor the transition, Weninger and student John Sakon first analyzed the crystal structure of the SNARE complex in order to create two dual-labeled forms of SNAP-25, one with two fluors (Cy3, the donor, and Cy5, the acceptor) separated by 1.5 nm (a "high-FRET" variant) in the folded conformation, and one where the distance was 8 nm ("low-FRET").

"What we often do is we take the crystal structure ... and then we design places to put FRET labels based on that structure," explains Weninger. "Then we watch a real protein move and we can interpret the FRET signal as changes away from that conformation."

To watch that movement, Weninger and Sakon injected relatively small numbers of the proteins into mammalian cells and tracked individual molecules using TIRF microscopy in a process known as single-molecule FRET (smFRET). This single-molecule (as opposed to "ensemble") approach allows studies on the behavior of individual molecules, each of which may have distinct properties, rather than the averaged behavior of all labeled molecules. "The benefit of single molecules is that it gives you sensitivity to [molecular] subpopulations," notes Weninger. Another benefit of smFRET: the ability to witness "transient dynamic processes" — that is, unsynchronized molecular outliers.

The flipside, Weninger says, is that smFRET requires a brighter excitation source and more sensitive detectors. Those effects combine to raise experimental background, making signal — already just a fraction of that in an ensemble experiment — harder to detect. At the same time, those signals are fleeting, as the bright excitation induces more rapid photobleaching. "The question is whether you can measure those smaller signals in the context of the experiment," he says.

In this case, Weninger and Sakon were able to measure both the speed of folding — they detected fluorescence emission from the FRET acceptor less than a second after injection — and the trajectories (that is, motion) of individual molecules. Their results showed that SNAREs from distinct cellular trafficking pathways readily substitute for each other in vivo.

University of North Carolina researcher Gary Pielak uses in-cell NMR to study protein dynamics in vivo. Image courtesy of Lars Sahl of UNC Chemistry..

"The single-molecule approach provides insights into these transient pathway-crossing events," Weninger says.

Ensemble Fret

While Weninger and Sakon are focused on understanding the folding properties of single protein molecules, Gruebele's group uses FRET to study all the labeled proteins in a cell en masse, an ensemble measurement that works because he synchronizes their folding behavior with heat.

Gruebele's heat source is an infrared laser tuned to excite the molecular bonds in water. By applying the laser to rapidly heat and cool cells expressing temperature-sensitive proteins tagged on either end with thermostable fluorescent proteins (in this case, a GFP donor and mCherry recipient), he can correlate changes in FRET efficiency between the two fluors with the degree to which the protein between them is folded in vivo.

"The [temperature] jump gets the protein to fold or unfold, while most other proteins are unaffected," says Gruebele. He likens the process to giving cells "a slight fever."

Gruebele and his team applied their approach, called "fast relaxation imaging" (FreI), to a "low-melting-temperature triple mutant" form of the metabolic enzyme, phosphoglycerate kinase (PGK) in human cells. Cells expressing labeled PGK variants were imaged on glass slides using a customized inverted microscope with lasers for heating as well as exciting both donor and acceptor fluors. Folding was assessed colorimetrically, by assessing the relative intensity of red and green fluorescence in transfected cells. Increasing the temperature in this proof-of-principle experiment from 27oC to 31oC had no effect on FRET efficiency (that is, the cellular color remained static), as the protein remains stable in this temperature range. But bumping the thermostat from 39oC to 43oC resulted in protein unfolding within seconds — an effect the team could resolve pixel by pixel.

With proof-of-principle in hand, the team is now looking to assess how protein stability and dynamics vary throughout the cell. According to Gruebele, preliminary results indicate that proteins fold faster and are more stable in the nucleus than in the cytoplasm — the protein's "melting temperature" in vitro is about 38oC, compared to 43oC in the nucleus and 41oC in the endoplasmic reticulum. "That seems like a relatively small temperature difference," Gruebele notes. "But a human being at 38oC is in good shape; at 43oC you are on your death bed."

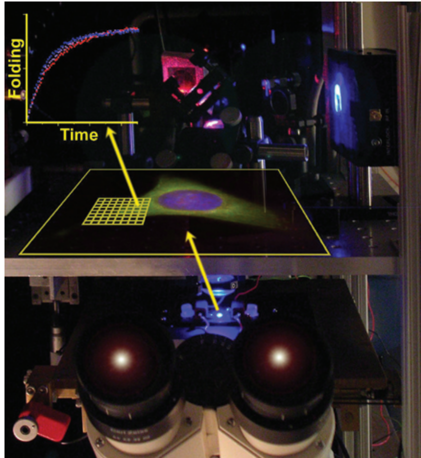
In-Cell NMR

Gary Pielak, a professor of chemistry, biochemistry, and biophysics at the University of North Carolina, Chapel Hill, uses a very different physical process to study protein dynamics in vivo. In fact, Pielak's technique might seem more at home in a chemistry building than a biology lab: nuclear magnetic resonance.

For years, Pielak has been interested in the impact macromolecular crowding has on protein stability. As an assistant professor studying proteins in dilute buffers in a test tube, a colleague pointed out that his model system probably did not accurately reflect life inside a cell. "This bugged me," he recalls. He's spent the past 15 years trying to resolve the problem.

Inside a cell macromolecular concentrations are on the order of 300 gm/L or higher — three times more concentrated than an egg white and certainly more concentrated than the buffers Pielak previously used. Researchers have assumed that such a high volume occupancy, in and of itself, can constrain reactions, motion, and folding in vivo, simply by raising local concentrations, restricting motion, and limiting available space. "That is going to favor folded, collapsed states over unfolded ones, because unfolded structures take more volume," he says.

That was the theory anyway; but Pielak wanted to know if it was true. To find out, he and his team created a mutant, isotopically labeled protein that was mostly



Martin Gruebele's team uses this micrscope setup to study protein folding in vivo using ensemble FRET. The inset shows time-resolved folding data for one representative pixel. Photo courtesy of Apratim Dhar.

unfolded in vitro, except in the presence of salt. They then asked whether macromolecular crowding in bacterial cells would push this protein's equilibrium toward a more folded state.

To figure that out, the team expressed isotopically (15^{N} and 19^{F}) labeled protein at high levels in bacteria — so high, in fact, that he calls his technique "in cell NMR" rather than in vivo, as the expression far exceeds normal levels. The cell slurry was then placed in an NMR tube — Pielak compares the mixture to "a melted vanilla milkshake"— and bombarded with radio frequency radiation to assess the chemical environment of the protein backbone, such as how exposed it is to buffer.

To their surprise, the team found that the protein was no more folded in vivo than in vitro, even under "hyperosmotic" conditions that forced the cells to take up more salt. "This idea that crowding will always stabilize proteins, is just not true," Pielak concludes. Instead, he suggests a competition could be occurring between the influence of crowding, which would tend to favor the folded protein, and non-specific interactions between the unfolded protein and other macromolecules in the cell, which favor an unfolded form.

"Although there is an excluded volume effect [caused by crowding], the non-specific interactions favor the denatured state, and they win," he says.

Several other recently developed methods also have the potential to shed new light on the ways in which proteins fold in cells. In February 2011, for instance, Henry Chapman of the Center for Free-Electron Laser Science at the University of Hamburg, Germany, described a technique called "femtosecond X-ray protein nanocrystallography," a high-speed approach that bombards a moving stream of nanoscale protein crystals smaller than one-micron in size with intense bursts of X-ray energy (each pulse is one billion times brighter than synchrotron radiation). Chapman and his colleagues used three million diffraction patterns generated in this manner to produce an 8-Angstrom structure of photosystem-I.

Ultimately, the goal is to tune the laser to image individual proteins, says Chapman, though he admits "it's by no means a sure thing." For one, focusing the laser to so small a spot requires new engineering. And it isn't clear how matter will behave under such intense radiation. If it works, though, it could make in vitro folding studies possible, he says, assuming it were possible to synchronize their motion in some way.

Another potentially useful technology: photoactivatable probes. Jennifer Lippincott-Schwartz, a Distinguished NIH Investigator at the National Institute of Child Health & Human Development, has developed photoactivatable fluorescent proteins — molecules that are "dark" unless they first are activated by a pulse of light — and applied them to a superresolution microscopy technique called PALM.

Photoactivatable FRET probes, she says, could be used as FRET acceptors in, for instance, pulse-chase experiments that track specific molecular populations over time. Or they could be combined with PALM to localize particular complexes with nanometer-precision.

Of course, it's not the technique that matters so much as the insight it provides. And according to Weninger, the problem right now is that most of the insights structural biology has so far provided have been based on unmodified bacterially expressed proteins studied in isolation. "We want to look at how the structure of proteins is related to function in a fully functioning living system," he says. "[But] those kinds of complicated interactions are difficult to reproduce in vitro."

References

1. **U.B. Choi et al.** 2010. Single-molecule FRET-derived model of the synaptotagmin 1-SNARE fusion complex. Nat Struct Mol Biol, 17:318-24.

Written by Jeffrey M. Perkel.

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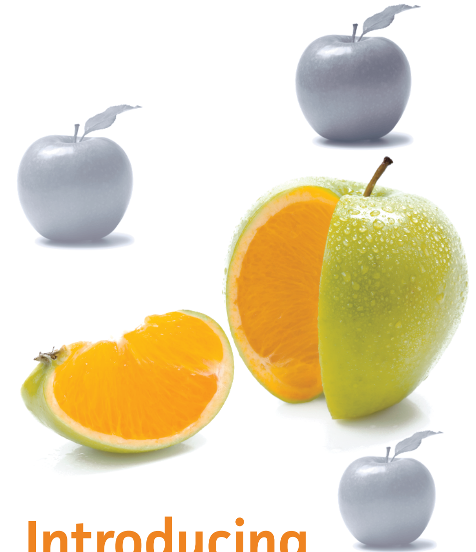

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