Folding Proteins Caught in the Act

Biochemists have developed the fastest techniques yet to spy on proteins in the first stages of curling into their final three-dimensional structures—acts that happen in less than a microsecond

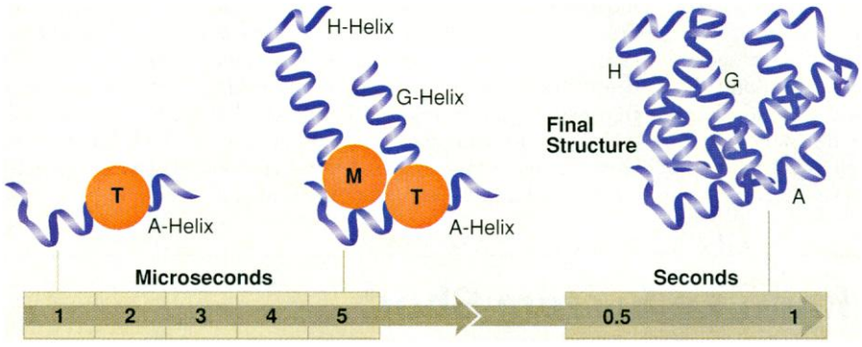
The folding of a protein into its characteristic three-dimensional (3D) shape is a biological equivalent of the big bang: The end result is evident everywhere, in every living cell, but the beginnings are shrouded in mystery. Biochemists have long been able to witness the final flurry of twisting, bending, and bunching that takes place as proteins fold into the complex structures they prefer. But like cosmologists searching for clues to the birth of the universe, chemists have been unable to trace the first, most basic steps in protein folding, many of which occur in less than a millisecond. By the time researchers using traditional chemical methods can catch a glimpse of proteins changing their shape, the main events are over, and the protein is already close to its final configuration.

In recent months, however, researchers have developed a handful of new techniques for coaxing proteins to fold on a hair trigger, then snapping freeze-frame images of the shape-shifting process. In an advance published last month in the *Proceedings of the National Academy of Sciences (PNAS)*, for example, researchers at the University of Illinois describe the fastest such technique to date. Using lasers and fluorescent tags, they have spied on some of the earliest events in protein folding, such as the curling of a protein's ribbonlike collection of amino acids into a tightly wound helix—an act that takes place in less than 1 millionth of a second, or 1 microsecond.

"They're looking at time scales that people have never been able to address before," marvels Jay Winkler, a chemist at the California Institute of Technology (Caltech) in Pasadena. This and other techniques are finally giving researchers "a look in the window of the early protein-folding universe," says protein-folding theorist Peter Wolynes of the University of Illinois, Urbana-Champaign. In time, researchers hope to use these sightings to come up with a set of rules for why proteins fold the way they do—rules that may one day open the way to tailoring the shapes and functions of designer proteins. And Wolynes and others believe that such rapid snapshots of the folding process may help theorists settle one of the fundamental debates of the field: whether the intermediate structures that proteins adopt on the way to their final shape are necessary steps or merely mistakes that must be corrected.

Getting the kinks in

Folding is crucial to the myriad roles proteins play in the cell, because part of each protein's unique biochemical character is embodied in its complex, 3D pattern of kinks and loops, called its native state. Because this structure keeps water-loving amino acids near the surface and water-fleeing aminos buried in the protein's core, it represents a lower energy state in the watery environment of the cell than does a disorganized coil of amino acids, the protein's denatured state. Heat, cold, or acidic conditions denature proteins, but in more congenial conditions, they twist into their preferred structures in a flash.



SOURCE: BALLEW *ET AL.*

Protein's progress. A disorganized ribbon of amino acids first curls into a single helix *(left)*, then acquires some 3D structure *(center)*, before folding into its final shape *(right)*; this process is seen by tracking fluorescent tryptophan (T), which is influenced by methionine (M).

The problem in seeing the earliest events in this process was not a lack of high-speed chemical tracking techniques; for years, chemists have used ultrafast lasers to watch reactions that happen in femtoseconds, millionths of a billionth of a second, which is several orders of magnitude faster than the time it takes for a protein to fold. Rather, the difficulty has been in coaxing a collection of identical protein molecules to start folding at the same time, as it's difficult to pin down the exact timing of particular twists and turns if the molecules aren't moving in synchrony.

Traditionally, researchers have tried to trigger this simultaneous folding with a sophisticated version of bench-top chemistry known as stopped-flow mixing. In one common version, researchers use hydrochloric acid to unwind a purified sample of a protein, then add a basic solution to counteract the denaturant and trigger the proteins to fold. Unfortunately, "it takes a few milliseconds for the two solutions to mix," explains Martin Gruebele, a chemist at the University of Illinois, Urbana-Champaign. So some protein molecules start to fold while others are still waiting, "and you lose the time resolution on your experiment," says Gruebele. For sharper resolution, researchers needed to create a trigger that sounds the starting gun for all proteins to fold at the same nanosecond.

In their new *PNAS* experiment, Gruebele and his Illinois colleagues R. M. Ballew and J. Sabelko accomplished just that with the help of low temperatures and a laser. They studied folding in an oxygen-carrying protein called apomyoglobin, for which the 3D structure is known in detail. First, they denatured the protein by "supercooling" the sample in a few drops of liquid water, keeping the water ultrapure and free of air bubbles and other crystal nucleation sites so that it could be chilled to −10 degrees C without freezing. To prompt the proteins to refold, the team next quickly heated the water, using a laser that fires a single 8-nanosecond pulse of infrared light. The water absorbed the light pulse almost immediately, transforming the light energy to heat. Within just a few nanoseconds, the solution heated up by an average of 20 degrees, enough to trigger the protein to begin refolding.

To watch its progress, Gruebele and colleagues used a second laser to fire a series of femtosecond pulses of ultraviolet (UV) light at the protein solution. Some of the UV photons are absorbed by the amino acid tryptophan, which converts part of the energy into heat and re-emits the rest as a photon of longer wavelength UV light. Conventional detectors pick up this fluorescence, which provides a clue to the tryptophan's location because its brightness is influenced by its neighbors. If the amino acid methionine is nearby, tryptophan's fluorescence is dimmed,

as the methionine essentially steals tryptophan's light energy and converts it instead to invisible vibrational and electronic energy.

Gruebele and his colleagues used this dimming effect to track protein folding by using genetically engineered *Escherichia coli* bacteria to express the tryptophan and methionine amino acids at different places in the protein sequence. Then they ran their experiment many times on different versions of the protein, gauging how the fluorescence changed when folding began.

Seeing is believing

The results offer tantalizing clues to the sequence of events in protein folding. Apomyoglobin's final shape includes a trio of helixes on different parts of the molecule (see diagram on p. 29), with the so-called H and G helixes spiraling parallel to one another and roughly perpendicular to the A helix. So the researchers engineered their protein to express tryptophan toward one end of the A helix and the methionine on a nearby site on the H helix. They found that the tryptophan stopped fluorescing after a 5-microsecond delay, suggesting that it is at this point that the A and H helixes converge. And using a similar scheme, the researchers concluded that the A helix winds itself into a coil in less than a single microsecond.

Although this sequence of events applies to just one protein, the result is a crucial first step toward understanding the rules of protein folding, says Wolynes. Taken together, the two results suggest that "local" interactions between neighboring amino acids compel the protein to very rapidly adopt some secondary structure—the coil in the A helix—before more "global" interactions push it to acquire the 3D structure that juxtaposes the A and H helixes, says Wolynes. That counters the views of some theorists, who suggested that global structure forms either before or at the same time as local structure, notes Wolynes. "It says that local signals are more important than we thought," he says.

William Eaton, who heads the laboratory of chemical physics at the National Institute of Diabetes and Digestive and Kidney Diseases in Bethesda, Maryland, calls the new experiment "just absolutely A^{++} work." He notes, however, that this particular technique won't work for all proteins, because only a relatively small number denature in supercooled water. But researchers have been making recent progress on other fast triggers for protein folding. Eaton and his colleagues, for example, presented preliminary work at a conference earlier this year on a new fluid-mixing technique with a time resolution down to 80 microseconds. Also earlier this year, Winkler, Harry Gray, and their colleagues at Caltech reported that pumping certain proteins with extra electrons triggers folding with a time resolution of about 40 microseconds (*Science*, 15 March, p. 1558).

Theoretical chemists are hopeful that these and related techniques will help resolve the theoretical debate about the meaning of the steps in protein folding. Researchers have known for years that many proteins tend to adopt temporarily stable shapes on their way to their native conformation. Some theorists argue that these "intermediates" are necessary steps along the pathway to proper folding—and so contain clues to the folding process—while others think those steps are merely mistakes, and that proteins can take a variety of twists and turns en route to their final shape.

To distinguish between these theories, it's very important to have experimental verification, theoretical chemists say. By observing whether these intermediates form under a variety of conditions, the new experiments should give researchers clues to just how critical these intermediates are. And that promise itself is likely to trigger a few fast accomplishments of its own.

–Robert F. Service

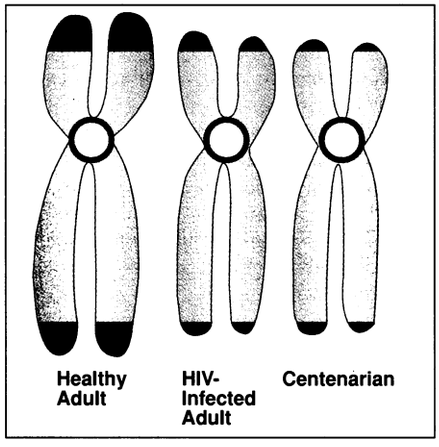
AIDS RESEARCH

Selling the Immune System Short

During the past 18 months, researchers have laid bare two fundamental facts about HIV: It replicates at a blinding rate throughout the course of infection, and viral levels in the body are strong predictors of how quickly a person will progress to full-blown AIDS (*Science*, 28 June, p. 1884). But little noticed amid the excitement surrounding these developments are new findings that may help solve a major outstanding puzzle about the virus: how it causes the immunodeficiency that is the hallmark of the disease.

The key clue comes from a flurry of new studies, the first of which appears in the July issue of *AIDS*, looking at what happens to the telomeres—specialized stretches of genetic material on the chromosome ends—in immune cells from HIV-infected people. In the *AIDS* paper, immunologist Janis Giorgi of the University of California, Los Angeles (UCLA), and her colleagues report that telomeres are significantly shorter in certain immune cells from these people than in controls. Because telomere shortening is considered a sign of cellular senescence, the authors suggest that the battle with the virus is essentially causing premature aging of the immune system, exhausting its ability to fight off pathogens, including HIV itself. "This is a brand-new angle that's never been explored," says co-author Rita Effros of UCLA.

The work, by the UCLA researchers and collaborators at Geron Corp., is attracting widespread attention. "The suggestion that shortened telomeres in [immune cells] of the HIV-infected population might play a role in the functional immunocompetence seen with the infection is intriguing," says Richard Hodes, an immunologist who studies telomeres and also heads the National Institute on Aging.



Premature aging? Telomeres *(red)* in $CD28^-$ $CD8^+$ cells from HIV-infected people resemble those of centenarians.

Giorgi, Effros, and their co-workers got interested in the telomeres of immune cells from HIV-infected people because of an observation Effros and others had previously made while studying the aging immune system. This work had built on the fact that in non–immune cells, telomeres shorten with each cell division, providing a "mitotic clock" that regulates how many divisions can occur. Once the telomeres become too short, cells enter "replicative senescence," at which point they can no longer divide.

Similarly, Effros had found that as people age, for some as yet unknown reason the telomeres of a particular subclass of immune cells—T lymphocytes that bear a CD8 receptor but lack another surface molecule called CD28—shorten. This, she suggested, indicates that the $CD28^-$ $CD8^+$ cells were coming to the end of their replicative life after decades of dividing whenever the immune system revved up to combat invading pathogens. And because CD8 cells play a critical role in clearing viral infections, that could help explain the diminished immune capabilities of the aged.

These immune deficits in some way resemble those of AIDS patients, the researchers realized—for example, both groups may suffer infections such as shingles, caused by