PROTEIN STRUCTURE

NMR Maps Giant Molecules As They Fold and Flutter

OXFORD, UNITED KINGDOM—Thirty years ago, the late Cyrus Levinthal, a protein chemist at the Massachusetts Institute of Technology, posed one of the most daunting riddles in structural biology: How do polypeptides—linear chains of up to 300 or more amino acids—arrive at the intricately folded, three-dimensional conformations characteristic of biologically active proteins? Levinthal ruled out the possibility that proteins randomly fold until they stumble upon their native state. A typical protein, he calculated, would take many orders of magnitude longer than the age of the universe to reach that state if it had to randomly search out all possible conformations. Most proteins, however, fold up on time scales ranging from milliseconds to seconds.

At the time he posed what has become known as the Levinthal paradox, protein chemists could do little more than scratch their heads over the problem. But over the decades since then, powerful analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy have begun to unravel the paradox by giving structural biologists a glimpse into the dynamics of proteins. At a meeting* here late this summer on the biological uses of NMR, researchers presented some of the fruits of new NMR techniques that are providing much higher levels of resolution (see next story). Using these new methods, researchers are opening a window on the intricate steps in the folding process and learning that proteins may be much more flexible and dynamic than previously thought—a finding that could have profound implications for how these macromolecules carry out their functions.

NMR exploits the fact that many atomic nuclei behave like magnets. When a magnetic nucleus encounters a strong external magnetic field, its orientation is restricted by quantum mechanics to a small number of directions, each with a different energy level. These energy levels depend on the type of nucleus and its environment—what other atoms are nearby in the molecule. If the sample is then exposed to radio waves of varying frequencies, some of the radio photons will have just the right amount of energy to cause a nucleus to jump from one energy level to the next and so will be absorbed. As the nucleus drops back to a lower energy level, it emits a photon. Researchers can record these photons as an NMR spectrum, which carries a wealth of detail about the structure of the molecule.

In recent years, NMR spectroscopists have found ways to extract even more detail. These new techniques rely on heavy-isotope tags and rapid pulses of radio waves to generate highly resolved, multidimensional spectra. "There have been huge advances recently," said Christopher Dobson of Oxford University's Center for Molecular Sciences at the meeting.

Magnetic machine. An NMR spectrometer at Oxford University's Centre for Molecular Sciences.

Protein paradox. The key strength of NMR for studying protein dynamics is its ability to map proteins in solution—their native environment. "The behavior of proteins in solution is largely inaccessible by other structural techniques, such as crystallography," says Mark Williams of Britain's National Institute for Medical Research in London.

At the meeting, Jane Dyson at The Scripps Research Institute in La Jolla, California, presented NMR evidence supporting a possible solution to Levinthal's paradox. Levinthal's own proposal was that polypeptides follow a specific sequence of folding steps, determined by their amino acid sequence. But in recent years, this view has fallen from favor, in part because theoretical models showed that the fastest route would be for the protein to collapse rapidly into a conformation that roughly approximates the native state, then edge more slowly toward its precise final conformation. "Yesterday, it seemed that it was impossible for proteins to fold to their native states," says Martin Karplus of Louis Pasteur University in Strasbourg, France, a leading theoretician in this field. "Today, we think protein folding should be easy."

Dyson's studies provide what may be glimpses of these partly folded intermediate states. She and her co-workers studied the behavior of apomyoglobin—a modified version of the protein myoglobin, which carries oxygen to muscle tissues—in solutions of various acidities, or pH. At a pH close to neutral, apomyoglobin is a highly compact globule consisting of eight tightly wound helices. But at very low pH (high acidity), the protein unfolds into a relatively unstructured "random coil." By gradually increasing the pH, Dyson hoped to simulate the stages of folding in slow motion.

NMR spectra of the solutions showed that as the pH was increased, the polypeptide began to develop structures found in the native state. For example, at pH 2.3, about 12% of the molecule was in the form of helices, while at pH 4.1 about 35% was tightly wound. Moreover, the first helix formed even at very low pH, and the pH 4.1 form, which corresponded to an intermediate state called a "molten globule," consistently contained three of the final eight helices.

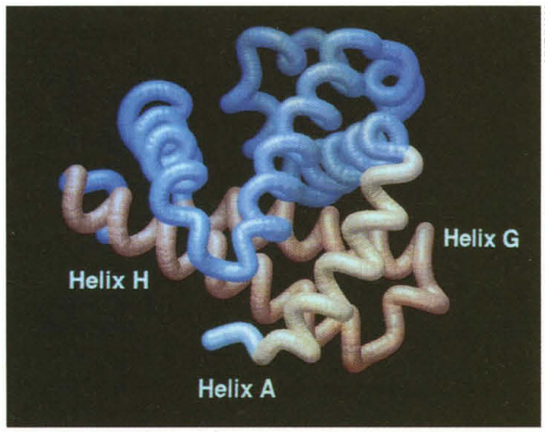
Dyson and others caution that these pH-dependent forms of apomyoglobin might not exactly represent the protein's folding mechanism, because they are stable equilibrium states, while a protein reaches its native state through a series of extremely rapid folds. Nevertheless, Dyson says, the results are "snapshots of how the protein might become more compact as it folds."

Dyson says her results are consistent with the idea that protein folding follows neither a random process nor a strictly defined pathway: "Apomyoglobin doesn't fold by sampling every single conformation, but by starting off with certain regions that are more inclined to make helices. As things move along, these helices interact to make a more compact form." Thus, the solution to the Levinthal paradox, Dobson says, may be that each polypeptide in a solution is free to

* NMR in Molecular Biology, Oxford, United Kingdom, 23–28 August.

find its own way through the various stages in the folding process: "Each molecule does something differently."

Molecular motion. While a protein in its native conformation usually has a regular and predictable structure, the molecule still has a considerable amount of give. "No protein is totally rigid," says Peter Wright of Scripps. "The emerging view of proteins is that there is significant internal motion." Working with a bacterial enzyme called dihydrofolate reductase (DHFR), Wright has used NMR to investigate how these internal motions might affect the protein's biological function. DHFR catalyzes the conversion of a compound called dihydrofolate to tetrahydrofolate, a coenzyme that enables cells to make numerous organic molecules, including some amino acids. The reaction takes place in a large cleft running along the center of the DHFR enzyme, and also requires a reducing compound called NADPH.



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Fast folders. Helices A, G, and H form first as apomyoglobin folds from a linear chain to an active protein.

Wright and his co-workers exposed the enzyme to nonreactive analogs of dihydrofolate and NADPH to "freeze" the reaction at various steps in the catalytic cycle, and then looked at the enzyme's internal motions at each step. The team found that several regions in the molecule became much less mobile once the inhibitor molecules were bound to the active site. For example, in the unbound state a region called loop 1—which acts as a "cap" over the active site—fluctuates between two slightly different conformations about 30 times per second. But when the inhibitors were bound to the enzyme, these fluctuations stopped. Another highly mobile region near the active site, centered on a glycine molecule in the polypeptide chain, also became much more rigid in the bound state.

"Wright was able to map the changes in protein motion at various steps of the reaction," says Desiree Tsao of the Genetics Institute in Cambridge, Massachusetts. To take things a step further, Wright's collaborator Stephen Benkovic and his colleagues at Pennsylvania State University in University Park made mutant versions of DHFR in which the highly mobile glycine was either replaced with another amino acid or deleted entirely. These changes, Benkovic found, greatly decreased the enzyme's catalytic power—implying that these internal motions play an important role in its biological function.

Exactly what this role might be remains to be determined. But NMR aficionados are confident that their technique will help point the way to solving this mystery, as it has done with the Levinthal paradox. Says Dobson: "NMR is allowing these questions not only to be posed, but to be answered."

–Michael Balter

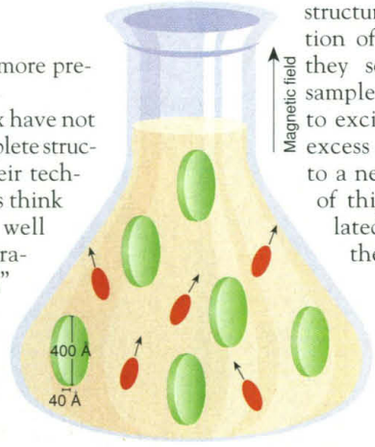
PROTEIN STRUCTURE

Lining Up Proteins for NMR

The tried-and-tested method for mapping the three-dimensional (3D) structure of proteins, x-ray crystallography, has a troublesome shortfall. The technique can pinpoint the location of atoms with extreme accuracy by bouncing off innumerable copies of the protein stacked in a crystal. But many proteins don't readily form such regular assemblies. A rival technique, known as nuclear magnetic resonance (NMR) spectroscopy, can map proteins in their native solution environment and therefore doesn't suffer this problem. But it has never been able to match the peak precision of its rival.

That may soon change. On page 1111, NMR experts Nico Tjandra and Ad Bax of the National Institutes of Health in Bethesda, Maryland, report modifying a long-known NMR technique to dramatically improve NMR's protein-mapping skills. Conventional NMR maps protein structure by determining the identity of atoms in a molecule as well as the distance between given pairs of atoms. With the new technique, which gently aligns the protein molecules in a bath of liquid crystals, researchers can also determine how each bond between neighboring atoms is oriented with respect to the rest of the molecule. By methodically building up a list of such orientations for all neighboring pairs of atoms, researchers should be able to complete a far more precise map of a protein.

So far, Tjandra and Bax have not reported mapping the complete structure of a protein using their technique. But they and others think it is likely to stack up well next to x-ray crystallography. "It's quite impressive," says Stephen Fesik, an NMR spectroscopist at Abbott Laboratories in Chicago, Illinois. "I think this is going to have a big effect on the field." One obvious area of impact, he says, could be in drug design, because designers need detailed structures of proteins so that they can tailor their drugs to interact with them.



Getting oriented. By aligning proteins (red), liquid crystals (green) sharpen NMR's atomic mapping.

All NMR techniques rely on the fact that some atomic nuclei act like tiny bar magnets. When these nuclear magnets are placed in an external magnetic field, they align along the magnetic field lines. If excited by a burst of radio-frequency photons, their magnetic axes precess like wobbling tops around about these lines. As they relax back, they give off a signal at a frequency that betrays their elemental identity. Researchers solve the structure of proteins using a variation of the technique, in which they send radio waves into a sample at frequencies designed to excite particular nuclei. This excess energy can be transferred to a neighboring atom. The rate of this energy exchange is related to the distance between the two atoms.

After painstakingly building up distance information between many hundreds of pairs of nuclei, researchers can calculate a 3D structure of the molecule that fits the data. Extracting that information is not easy, however. In practice, the energy transfer between two atoms is also affected by their constant motion with respect to one another. And there has been no direct way to learn the orientation of the pairs of atoms relative to the rest of the molecule. The resulting structures tend to be fuzzy and imprecise.

Knowing how the bonds are oriented would sharpen the structures considerably. To tease out this extra information, Tjandra and Bax rely on another effect that NMR researchers have known about for decades but have never been able to use to solve