BIOPHYSICS

Elusive vibrations

At last, a method to measure long-range vibrations in proteins may provide clues to the functional relevance of these motions.

Structural changes to proteins are absolutely critical for biological function, but how these structural changes come about is still somewhat of a mystery. One hypothesis suggests that proteins flex and stretch as a result of long-range vibrational motions mediated by interconnected webs of amino acid residues. The well-known phenomenon of allostery, where binding of a molecule or a mutation at a site distant to the active site has an effect on enzyme function, supports this hypothesis.

Researchers, however, have not had at their disposal a suitable approach to physically measure these long-range vibrations. "There's been a lot of skepticism about whether these motions actually exist," says Andrea Markelz of the University at Buffalo, State University of New York. Although a handful of studies have reported X-ray scattering or Raman spectroscopy measurements attributed to long-range vibrational motions in proteins, Markelz notes, these measurements can be quite complicated to perform, and the preliminary results were never further explored with follow-up experiments.

Markelz has long suspected that the right technique would allow these elusive long-range protein vibrations to be detected. And now her team—including postdoc Gheorghe Acbas, graduate student Katherine Niessen and crystallography collaborator Edward Snell—has a method to do so.

The team used a technique based on terahertz near-field microscopy, which allows vibrational measurements to be made under fully hydrated, room-temperature conditions. Terahertz-frequency light (found on the electromagnetic spectrum between infrared and microwave frequencies) induces different vibrational modes in a protein. "Just about everything in the cell absorbs this kind of light very well, so it's been hard to actually see these internal modes of the protein," says Markelz. The key to singling

NANOBIOTECHNOLOGY

NANOPORES SENSE PROTEIN MODIFICATIONS

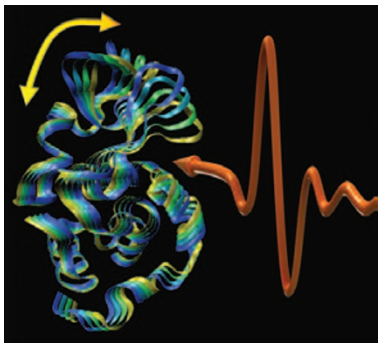
Protein nanopores can detect post-translational modifications in proteins.

With so much attention on using nanopores to sequence long stretches of DNA, it is easy to forget that there are more uses for a pore than DNA sequencing. Hagan Bayley, a co-founder of Oxford Nanopore Technologies, appreciates this well. With the company now beta-testing a commercial sequencing solution based on nanopores, his laboratory at the University of Oxford has been devoting energy to look beyond DNA. Bayley asked, "If we could sequence G, A, T and C, could we sequence proteins?"

By measuring the conductance that occurs across a nanopore when a voltage is applied, one can identify molecules on the basis of how they block this ionic current. Bayley's group uses a pore formed by the α-hemolysin protein in cell membranes. Early work showed that the approach is extremely sensitive to small molecular differences, but protein studies had largely been restricted to short unfolded fragments, says Bayley.

As a step toward studying full-length proteins, Bayley's group showed last year that the protein thioredoxin could be unfolded while being threaded through the pore. The researchers tacked on a short piece of negatively charged DNA—a string of 30 cytidines—to one end of the protein and then pulled it into the pore under an electric field, forcing it to unfold. They found that ionic current changes in discrete stages reflecting the entrance of the DNA leader, the unfolding of one end of the protein under the leader's pull, and finally the spontaneous unraveling and diffusion of the remainder of the protein.

The team went on to ask whether they could use this method to detect protein differences such as post-translational modifications (Rosen *et al.*, 2014). They chose phosphorylation for its importance in regulating protein function and signaling in the cell, and engineered potential modification sites near one end of the thioredoxin protein. Each amino acid mutation changed the average drop in current and was



Long-range vibrations in lysozyme are observed upon excitation with terahertz light. Image courtesy of A. Markelz and K. Niessen.

out long-range vibrational modes over a sea of background noise, the team discovered, was to align the protein molecules via crystallization to create a strong signal. "You have to come up with some way to subtract out that background," explains Markelz. "If every single protein is aligned, then we have a special direction where we're going to see a difference in the light versus any other direction."

In this first proof-of-principle report, Markelz's team studied a protein called chicken egg white lysozyme. They identified long-range motions thought to be important for its antibacterial activity of cleaving cell-wall carbohydrates. The researchers' next task is to perform further experiments, such as using mutations or inhibitors to isolate particular vibrational modes, to get at the mechanism and biological function of these motions. "That's been our hope and dream all along," says Markelz.

Markelz also hopes that traditional structural biology labs will be able to perform these sorts of dynamic measurements in the near future, once the technology becomes a bit more affordable. "The terahertz near-field microscope is definitely not something you buy off the shelf yet," she notes. "It is a tabletop technique, but it does require an ultrafast laser." However, these have become turnkey lasers and are rapidly dropping in price. It will certainly be interesting to watch whether the approach can be coupled with traditional structural biology tools to uncover new insights into protein function.

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RESEARCH PAPERS

Acbas, G. *et al.* Optical measurements of long-range protein vibrations. *Nat. Commun.* **5**, 3076 (2014).

associated with a certain level of noise; plotting these two features of the current could distinguish between phosphorylated and unphosphorylated proteins after both were run through the same nanopore. The same was true for molecules phosphorylated at one, both or neither of two neighboring positions. Despite overlaps between these isoforms on plots of current feature distribution, the identities of the vast majority of molecules could be clearly distinguished. Unlike mass spectrometry approaches, which analyze ensembles of protein fragments, the nanopores allow single molecules to be analyzed.

The team has studied only one protein so far but believes that the method will work on others. Bayley also points to a promising approach published last year (Nivala *et al.*, 2013), which uses a molecular motor to maintain force while the entire protein is drawn through a pore, whereas Bayley's DNA leader method only applies force to unfold one end of the protein (modifications further from this end cannot be distinguished during subsequent passive diffusion across the pore).

The next step will be to study proteins in the cell, which adds complications. "When we deal with real cells, which we're in the process of doing, we're going to have to extract the proteins of interest and concentrate them," before adding DNA leaders and sending them through pores, says Bayley.

As for using nanopores to sequence proteins, he is not very optimistic. "I'm not going to rule it out," says Bayley. "But there are 20 amino acids. It's a much more difficult problem than sequencing DNA." Sequence context also multiplies the complexity of the problem. But the unknown trajectory of nanopore research is what he likes about his protein work: "It has one foot in reality and another stepping into the future," he says.

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RESEARCH PAPERS

Nivala, J. *et al.* Unfoldase-mediated protein translocation through an α-hemolysin nanopore. *Nat. Biotechnol.* **31**, 247–250 (2013).

Rosen, C.B. *et al.* Single-molecule site-specific detection of protein phosphorylation with a nanopore. *Nat. Biotechnol.* **32**, 179–181 (2014).