New and Notable

Unraveling Bacteriorhodopsin

John van Noort
Physics of Life Processes, LION, Leiden University, 2333 CA Leiden, The Netherlands

How proteins find their highly structured functional state is one of the most intriguing questions in modern biology. It proves to be a hard case to crack, both experimentally and theoretically. In the current view all conformations of a chain of amino acids are represented in a schematic free-energy surface, in which the native state corresponds to the global minimum (Dinner et al., 2000). Although the number of paths that lead to the native state seems almost endless, as originally stated in the Levinthal paradox, more recent theory and experimental evidence points at a limited number of distinct pathways that funnel proteins to their native state. Mapping out this free-energy surface has remained difficult, partially because in most experiments ensemble averaging obscures distinct folding pathways. It is in this niche that recent single-molecule force spectroscopy experiments have the potential to shed new light.

In this issue Janovjak and co-workers report new experiments on unraveling native transmembrane proteins. For their study they use atomic force microscopy (AFM) to pull on a single bacteriorhodopsin molecule. The group has previously shown that individual proteins can be picked up with the AFM tip, pulled out of the membrane, and removed (Oesterhelt et al., 2000). High-resolution imaging confirmed that only a single protein is extracted from the membrane. Furthermore, careful analysis of the recorded force during pulling revealed a discontinuous release, in which each peak in the pulling force can be attributed to single or pairs of the seven transmembrane α-helices that form a single bacteriorhodopsin.

The current report expands on this work by applying a dynamic modulation to the tip while pulling on the molecule. The concept of frequency modulation and lock-in techniques is a universal method to increase the accuracy of measurements by shifting data acquisition to higher frequencies and reducing the bandwidth. As a result the derivative of the original signal is obtained. In some cases this differential signal is directly relevant, like for example in scanning tunneling microscopy, where the derivative of the tunneling current represents the density of states. Janovak et al. now show that a dynamic modulation of the pulling force applied to individual membrane proteins results in two fundamentally new outcomes.

First, by comparing the out-of-phase signal with the in-phase signal, it is shown that viscous contributions can be separated from the elastic response when pulling the peptide out of the membrane. Previous dynamic experiments on polymers like poly(ethylene glycol) and DNA revealed that in these cases elastic interactions dominate the force-extension curves. In this issue, Janovjak et al. report that a significant amount of energy is dissipated during unraveling the membrane protein. The result can be quantified in terms of the relaxation time for this process. Although small values are obtained for the out-of-phase signal, the observation of such energy dissipation allows for quantitative comparison with theoretical models.

Second, compared to quasi-static pulling experiments, new pathways are found in the disruption of the protein. Not only the complete disruption of one or two of the α-helices can be observed but new force peaks are found about halfway of two of the helices. The newly observed energy barriers correlate with predicted kinks in these two α-helices in bacteriorhodopsin. The authors argue that the ability to identify these new unfolding pathways is a result of an effective increase of the pulling rate as the tip moves upward during the oscillation.

Although membrane proteins form a convenient start for AFM-based dynamic force spectroscopy, the method is not restricted to this type of proteins. Other studies in which quasi-static force spectroscopy was applied to globular proteins, like titin, ubiquitine, etc., generally lack details that can be linked directly to the secondary structure of the protein. It will be interesting to see whether dynamic force spectroscopy will be able to reveal more details in unfolding of such globular proteins that do not have stabilizing interactions of neighboring proteins and a lipid bilayer.

The reported approach will further benefit from the development of new, smaller probes for AFM (Viani et al., 1999). The smaller contributions of thermal noise within the detection bandwidth will not only improve the signal/noise ratio of the measurements, it will also enhance both phenomena that distinguish dynamic force spectroscopy on proteins from quasi-static force spectroscopy. The higher resonance frequency allows for a higher modulation frequency, which in turn results in characteristic oscillation times closer to the intrinsic relaxation times of the proteins. Furthermore, higher modulation frequencies will increase the effective pulling rate during the up-swing. This may result in more detail in the unfolding curve, perhaps even revealing new energy barriers to unfolding. Finally, higher pulling rates will also facilitate comparison with detailed molecular dynamics calculations that are, due to the large computational load, limited to times that are much shorter than experimentally accessible (Li and Makarov, 2003).

The experiments take full advantage of the merits of single-molecule techniques that have gained so much popularity in the past decade. The ability to apply a

Submitted November 30, 2004, and accepted for publication December 2, 2004.

Address reprint requests to John van Noort, E-mail: noort@physics.leidenuniv.nl.

0006-3495/05/02/763/02 $2.00

doi: 10.1529/biophysj.104.056242

directed force on biomolecules allows for a detailed interpretation of sequential unfolding of the protein that is hardly possible with other chemical or physical methods of denaturation. Furthermore, it allows full appreciation of the stochastic nature of protein unfolding because no averaging or synchronization is required. Indeed, because each unfolding event is recorded separately, rare unfolding events can be identified and distinguished from more regular unfolding pathways. Whereas many single-molecule studies focus on regular behavior, in this study it is shown that alternative unfolding pathways represent a significant portion of the possible unfolding pathways, resulting in a more complete picture of the unraveling process.

It is not trivial that folding and unfolding of proteins follow the same pathway. Janovjak et al. anticipate refolding experiments of bacteriorhodopsin as well. Such experiments will complement the picture of protein folding. Together, the combined datasets will give an exciting, unprecedented perspective on the forces involved in unraveling and refolding of bacteriorhodopsin, one of the best-studied membrane proteins.

This work was supported by the Netherlands Organization for Scientific Research (NWO).

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