PERSPECTIVES: STRUCTURAL BIOLOGY

Unraveling a Membrane Protein

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Before the advent of the atomic force microscope (AFM), students of protein folding who wanted to unfold a protein had either to heat it or expose it to chemical denaturants. Now, by manipulating a protein with optical tweezers or the tip of an AFM, the experimenter can unfold the protein and measure the force needed to do so (*1*).

Studying how proteins are folded and inserted into membranes is an especially challenging problem for scientists (*2*). Much of our understanding of membrane proteins comes from work on bacteriorhodopsin, the light-driven proton pump of *Halobacterium salinarum*. Bacteriorhodopsin, the classic model membrane protein, is composed of seven closely packed transmembrane α helices (A through G) and is assembled (in groups of three) into a two-dimensional hexagonal lattice called the purple membrane. Now, on page 143 of this issue, Oesterhelt and his colleagues (*3*) affix the tip of an AFM to the carboxyl terminus of bacteriorhodopsin, which is exposed on the cytoplasmic surface of the membrane (see the figure). As the tip is withdrawn, the protein is mechanically stretched, causing it to unfold in a stepwise manner.

Previous studies on soluble proteins have used a "smash-and-grab" method where the AFM tip is pressed into the sample and the protein is picked up randomly by adsorption. Owing to various technical difficulties, it has not been possible to obtain an image of the sample after application of mechanical force. In their approach, Oesterhelt and co-workers take advantage of the topology of the purple membrane. Because the purple membrane is asymmetric, AFM imaging can distinguish the cytoplasmic surface from the extracellular surface (*4*). The ordered nature of the purple membrane's two-dimensional hexagonal lattice permitted recording of the image of the membrane's cytoplasmic surface after a single molecule of bacteriorhodopsin had been pulled out of the membrane. In each case a "hole" in the remainder of the array was found, corresponding to the "missing" bacteriorhodopsin.

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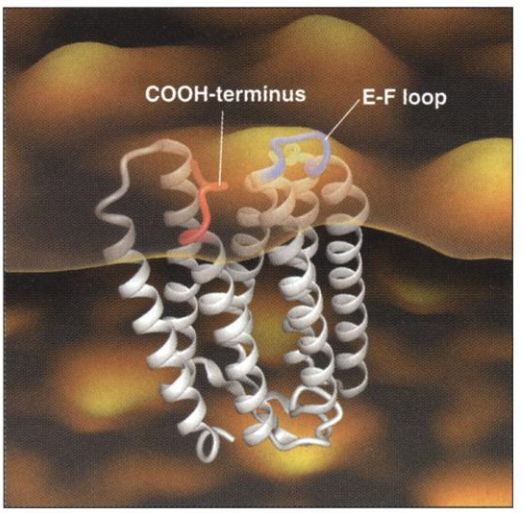
Much is already known about the structure of bacteriorhodopsin (*5*). Folding studies with synthetic peptides consisting of one or more of the seven transmembrane α helices (*6*) suggest that each helix represents a semi-autonomous folding unit. These studies also demonstrate that the folding and insertion of bacteriorhodopsin (and possibly other α-helical membrane proteins) into membranes can happen spontaneously.

One of the several important findings of the Oesterhelt group concerns the specificity of the interaction of the AFM tip with the protein. The asymmetric nature of the purple membrane ensures that, as the AFM tip approaches the membrane's cytoplasmic surface, more than 80% of the bacteriorhodopsin is inaccessible—only the loops connecting helices A and B, C and D, E and F, and the carboxyl terminus extending from helix G are exposed (see the figure). However, the investigators introduced a further element of specificity that has been largely absent from previous AFM studies. They genetically engineered a single cysteine amino acid residue into the carboxyl terminus that extends beyond the G helix on the cytoplasmic surface (see the figure). This permits a strong and specific attachment between the protein and the gold AFM tip. Consistent with this position for tip attachment, the force-extension spectra show that the protein was extended by more than 70 nm indicating complete unfolding of the seven helices. Proteolytic cleavage within the E-F loop yields spectra that extend no further than 45 nm, consistent with an interaction between the AFM tip and the carboxyl terminus of the truncated bacteriorhodopsin.



Unfolding possibilities. A bacteriorhodopsin monomer embedded in the purple membrane, visualized by atomic force microscopy (AFM). Shown are the E-F loop (blue) and 5 amino acids of the 23–amino acid carboxyl terminus (red), which is exposed on the cytoplasmic surface of the membrane (*13*). In experiments where the protein was extracted from the membrane with an Si3N4 AFM tip, the tip preferentially attached to the E-F loop or to the carboxyl terminus. In the Oesterhelt study, a cysteine residue was engineered into the carboxyl terminus, enabling specific attachment of the gold-coated AFM tip to the cysteine.

Previous demonstrations of forced unfolding by AFM have been with proteins that have repeating globular domains that occur naturally (such as titin or spectrin) or that have been genetically engineered that way (for example, polylysozyme) (*1*, *7*). These globular domains undergo a one-step stretch-induced unfolding without detectable intermediates [however, see (*8*)], as would be expected if one or more of the secondary structural elements unfolded independently of the other elements in the domain. In the case of bacteriorhodopsin, Oesterhelt *et al.* offer compelling evidence for a more complicated unfolding pathway. Upon retraction of the AFM tip from the surface of bacteriorhodopsin, they observed four ruptures that vary in distance and force between protein molecules. They attribute these ruptures to the sequential unfolding of pairs of helices: first the F and G helices, then the D and E helices, followed by B and C, and ending with the unfolding of the A helix and complete extraction of the protein from the membrane. This sequential unfolding of the secondary structural elements of bacteriorhodopsin demonstrates that unfolding of this protein is not a two-state process but rather involves several steps. This conclusion, based on unfolding, nicely complements previous conclusions based on the reverse process, that is, folding (*6*).

It is already clear that force-induced unfolding can yield useful information about how proteins fold, unfold, and even misfold (*9*). The Oesterhelt study opens up an exciting new avenue to understanding how membrane proteins such as bacteriorhodopsin fold and become inserted into the membrane. All sorts of questions remain to be answered. Is the forced unfold-

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ing reversible? If bacteriorhodopsin is not completely extracted from the membrane and the AFM tip is brought back to the membrane surface, will the unfolded helices resume their folded states within the membrane, and, if so, under what conditions? Can one conduct repeated cycles of partial unfolding and refolding?

Theoretical considerations (*10*) indicate that force-induced unfolding of "two-state folders" (proteins that fold in one step) should occur cooperatively, whereas the unfolding of "non–two-state folders" (proteins that fold in two or more steps) should proceed by the formation of intermediates. Other considerations (*11*) predict that the force needed to rupture weak interactions will depend on how quickly the force is applied. This dependence on speed has been observed for the unfolding of spectrin (*7*). It remains to be seen if it also applies to the unfolding of the individual elements of bacteriorhodopsin.

Perhaps there are also lessons here for future AFM studies of force-induced unfolding and folding of soluble proteins. First, investigating the purple membrane in which the bacteriorhodopsin molecules of the two-dimensional lattice all point in the same direction offers clear advantages because only a limited portion of the protein is accessible to the AFM tip. For some soluble proteins (such as the bacterial heat shock protein GroEL) this is also the case (*12*). Second, a biophysically rigorous interpretation of the information that is available in the force curves requires the specific attachment of the AFM tip to a unique site on the protein. As Oesterhelt *et al.* demonstrate, this too can now be accomplished with genetic engineering.

All-in-all, the results reported by Oesterhelt and co-workers offer an exciting new approach to tackling a long-standing and difficult problem—how proteins fold and become inserted into membranes.

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PERSPECTIVES: ASTRONOMY

New Stars on the Block

Ray Jayawardhana

Much glory in astronomy comes from probing the farthest reaches of the universe for the most exotic beasts. But poking around closer to home for more prosaic objects has its own rewards—such as finding small groups of relatively isolated young stars destined for the limelight. Over the past few years, one such group, known as the TW Hydrae Association, has attracted quite a following, as evidenced by the high attendance of a special session during the recent American Astronomical Society meeting.

The reason for this widespread interest is that the TW Hydrae group, and others like it in the solar neighborhood, may tell us a lot about the birth of stars and planetary systems. The TW Hydrae Association, in particular, appears to be at the age at which planet formation is believed to occur. It may even be possible to take a picture of a newborn planet around one of these stars.

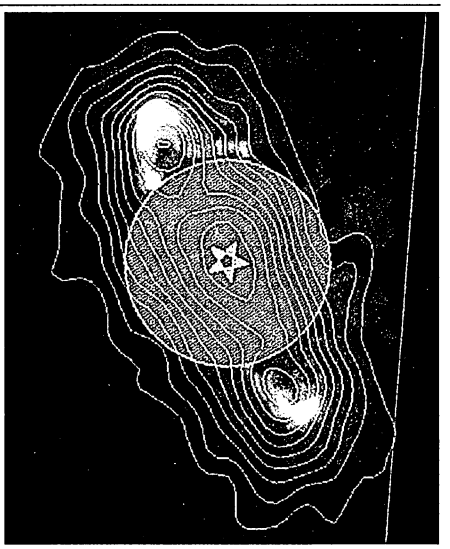
The star TW Hydrae first caught astronomers' attention back in 1978 (*1*). George Herbig noted that it had the earmarks of a young low-mass star, or so-called T Tauri star, including variability in brightness, a strong Hα emission line in its spectrum, and a high abundance of lithium (an element that is easily fused in nuclear reactions and thus does not survive in older stars). Most T Tauri stars are found in clouds of gas and dust, the presumed sites of their birth, such as the Orion nebula. Curiously, TW Hydrae is not.

Subsequent work added further evidence for TW Hydrae's youth and indications for the presence of a circumstellar disk and revealed four other stars in the same region of the sky with similar characteristics (*2*, *3*). Three years ago, Kastner *et al.* (*4*) suggested on the basis of strong x-ray emission from all five systems that the group forms a physical association at a distance of roughly 150 light-years. Since then, at least seven more stars have been identified as candidate members of the TW Hydrae Association, on the basis of the same signatures of youth and the same motion across the sky as the original five members (*5*).

The group consists mostly of low-mass stars, typically a few tenths of the mass of the sun, and includes several binary systems as well as one remarkable quadruple system, HD 98800, in which two pairs of stars appear to orbit a common center of gravity. There is only one higher mass star, HR 4796A, which is twice as massive as the sun and about 20 times as luminous. The TW Hydrae stars are estimated to be roughly 10 million years (My) old (*4*, *6*), older than most T Tauri stars in star-forming regions, which are usually only about 1 My old.

The origin of the TW Hydrae Association remains a bit of a mystery. There is no obvious parent cloud (*1*, *2*), and the stars are dispersed across some 20° on the sky and 60 light-years in radial distance, making it difficult to determine their birthplace (*7*). Were they born in a low-mass cloud that has since dispersed? Or could these stars be escapees from known star-forming regions (*8*)? The slow velocities of TW Hydrae stars through space favor in situ formation, suggesting that clouds may disperse more quickly than previously thought (*9*).

Being the nearest group of young stars (and three times closer than the nearest previously known star-forming region), the TW Hydrae Association offers a unique opportunity to study the evolution of cir-



Young stars reveal their secrets. This dusky disk around the star HR 4796A, a member of the TW Hydrae Association imaged in the infrared, may be the debris of planet formation.

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