Energy landscapes of fast-folding proteins pushing the limits of atomic force microscope (AFM) pulling

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Protein folding is a complex diffusive process on a high-dimensional energy surface (1, 2). Gaining detailed insight into the folding energy landscape is an experimental challenge. First, most measurements can observe only one coordinate and provide one-dimensional (1D) projections (Fig. 1*A*). Second, even though a single-molecule experiment can yield folding/unfolding rate constants of a protein, those rates are determined by two distinct properties: barrier height and intramolecular diffusion constant. Recent advances in single-molecule fluorescence resonance energy transfer (FRET) have allowed us to measure the time the protein spends when it transits from the folded to the unfolded conformation (3). Since by definition, the transition state is the most rarely populated state in a protein's time trajectory, transition path times must be extremely short and are in fact in the range of microseconds (3–6). Since transition path times mostly depend on the intraprotein diffusion constant, they can be used to separate contributions of diffusion constant and barrier height to the measured rate constants. In an elegant study combining transition path measurements with molecular dynamics (MD) simulations, Chung et al. (7) showed that changing pH from 7.5 to 3.2 increases folding rates of the computationally designed protein α_3D 15-fold by merely affecting protein diffusion while leaving the transition barrier height unchanged.

In their paper "Modulation of a protein-folding landscape revealed by AFM-based force spectroscopy notwithstanding instrumental limitations," Edwards et al. (8) use single-molecule force spectroscopy to gain more insight into the nature of the pH-dependent changes of this protein's energy landscape. Single-molecule force spectroscopy provides an elegant way to control and study the folding pathways and kinetics of proteins. For example, using optical tweezers in combination with deconvolution techniques, folding energy profiles could be extracted directly (9–11). Atomic force microscopy (AFM)-based force spectroscopy has provided important insight into folding/unfolding of mechanically stable proteins (12–15), but owing to the slow diffusion of the large cantilevers (thus limiting force resolution), application to fast near-equilibrium folding proteins in a force range below 15 pN has been challenging. Edwards et al. (8) combine many instrumental improvements they have developed over the years (16–19) to study near-equilibrium folding of α_3D. In brief, they 1) removed gold coating from the AFM cantilevers to reduce drift (16); 2) used shorter (L = 40 μM, compared with a standard long cantilever with L = 100 μM) cantilever and used focused ion beam milling to modify the shape of cantilevers to reduce friction and thus, dramatically increased force and temporal resolution (17); and 3) used a mechanically stable and defined attachment chemistry and polyethylene glycol (PEG)-coated cantilever (18). These improvements open the path for AFM-based methods to study energy landscapes for fast-folding proteins and molecular bonds in general.

Tethering an AFM cantilever to the ends of a protein and applying a mechanical load may seem to be a very direct access to control the conformation of the protein. However, it is important to note that the readout in such an experiment is just the position of the AFM cantilever tip (x_{meas}), while the coordinate

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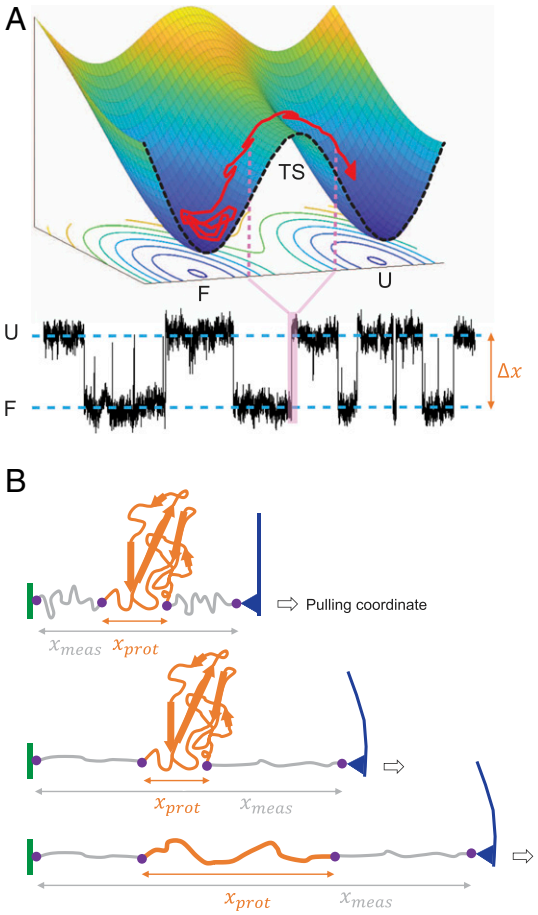

Fig. 1. (*A*) Schematics of a free energy landscape of protein folding. A 1D projection is highlighted by the black dashed line. A diffusive transition from the folded (F) to the unfolded (U) state passing the transition state (TS) is drawn in red. *Lower* shows a schematic single-molecule equilibrium time trace, where the protein molecule is transitioning between the folded (F) and the unfolded (U) states. The time span marked in pink highlights the transition path, which is the very short time span during which the protein actually transitions from the folded to the unfolded state. The cyan dashed lines mark the average positions of the folded and unfolded states on the time trace, and the orange vertical arrow represents the difference in length between the two states. (*B*) The protein construct is tethered between a fixed surface (on the left; green) and a movable AFM cantilever (deep blue) by means of biochemical modifications (purple circles). The position of the cantilever constitutes the coordinate x_{meas}, while the length of the protein along the pulling coordinate is denoted by x_{prot}. *Top*, *Middle*, and *Bottom* show different states of the protein (folded, unfolded) at different pulling forces and the respective values for x_{meas} and x_{prot}.

we are mostly interested in is the end-to-end distance of the protein (x_{prot}). This is illustrated in Fig. 1*B*. Note that the two coordinates x_{meas} and x_{prot} are interconnected but still distinct from each other. For example, applying load to the folded protein (Fig. 1 *B*, *Top* and *Middle*) will mostly lead to stretching of the gray elastic linkers, thus increasing x_{meas}, while the more rigid folded protein core will only slightly stretch, thus leaving x_{prot} unchanged. In fact, the elastic energy from linker stretching adds to the protein energy landscape and can even create its own apparent barrier (20–22). Hence, it is important to deconvolve the "true" protein energy landscape from the apparent landscape obtained from the fluctuations of the cantilever. Edwards et al. (8) showed using dynamic energy landscape reconstruction that, at both high and low pH, the energy barrier stays the same, and consistent with the earlier single-molecule FRET study, the observed changes in kinetics are not related to changes in barrier height. They find that the reconstructed energy landscape is identical to a simple energy landscape obtained from the stretching energies of the elastic linkers, showing that the landscape measured does not contain information about the protein-folding energy landscape beyond the equilibrium free energy of folding.

However, being able to resolve the folding transitions directly, the authors found that the folding/unfolding kinetics become faster when the pH drops from 6.2 to 4.2. To gain insight into the nature of these altered kinetics, they then measured force-dependent folding rate constants and found that they are independent of pH, but unfolding rate constants are much more sensitive to force at low vs. high pH. In other words, the position of the transition state moves away from the folded state at low pH, making this protein more compliant. Interestingly, molecular dynamics (MD) simulations had shown a similar effect (7) that now finds experimental confirmation.

While the increase in rate constants upon pH drop is qualitatively consistent with the earlier transition path measurements by Chung et al. (7), the drop in the AFM experiments is rather 5- than 15-fold. Many interesting questions remain for future experiments. What is the relative contribution of the effect of the pH-dependent compliance change vs. the change in energy landscape roughness? Will the AFM measurements be able to increase in resolution so that the protein energy landscape can be directly reconstructed? The technical progress that this paper constitutes promises more breakthroughs in this exciting field of modern physical chemistry.

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