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Unfolding the Secrets of Calmodulin

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Single-molecule experiments are providing an increasingly detailed picture of the function of biomolecules and their assemblies. By watching one molecule at a time, one can observe phenomena that would be lost in macroscopic measurements averaged over large numbers of molecules. Force spectroscopy is particularly well suited to probe changes in biomolecular conformation (1–4). Like other single-molecule methods (5), it monitors the distance between two sites in a macromolecule, but in addition, a variable pulling force is coupled to this distance. Because stability is roughly exponentially sensitive to applied force, conformational equilibria can be probed over a wide free-energy range.

On page xxxx of this issue, Junker, Ziegler, and Rief (1) report the use of atomic force microscopy (AFM) to explore the force-dependent kinetics of calmodulin folding and ligand binding. Calmodulin, a calcium ion (Ca²⁺)–sensing protein ubiquitous in eukaryotes, contains two Ca²⁺-binding domains connected by a flexible linker. Upon Ca²⁺ binding, the domains open and partially expose their hydrophobic interior. This conformational change enables binding to various targets involved in biological processes ranging from apoptosis to muscle contraction (6).

Junker et al. elucidate calmodulin's conformational dynamics at unprecedented resolution. In addition to the now familiar “sawtooth” patterns in the force-extension curves, in which a sharp drop of the force indicates unfolding, the authors directly observe refolding of the protein: They record a remarkable succession of unfolding and folding events near the rupture point, where previously only a single unfolding event would have been recorded. The observation of refolding was made possible by operating closer to equilibrium, taking advantage of improved instrument stability. Together with optical tweezers (2,3) and force-clamp AFM (4), this technique is part of a new generation of force-spectroscopy experiments for observing continuous dynamical trajectories close to equilibrium.

The ability to watch folding or binding dynamics in real time opens the way for direct observations of molecular reaction mechanisms as a sequence of structural events along the microscopic pathways (5). In a classical analysis of bulk experiments that probe changes in population of metastable species, a reaction mechanism would be inferred from the combined effects of perturbations (such as mutations) on the kinetics and thermodynamics (7). For example, macroscopic kinetic measurements have shown that ribonuclease H folds via a stable intermediate (8). However, these experiments did not reveal whether the intermediate was on the pathway between the unfolded and folded states or was an off-pathway trap. A single molecule optical-tweezer experiment demonstrated an on-pathway intermediate by directly observing transitions between the three states near equilibrium (2).

Junker et al. now provide new insights into the mechanisms of coupled folding and binding, a central issue in the function of natively unstructured proteins (9–11). Calmodulin has long served as a textbook example of the preequilibrium scenario in ligand binding (see the figure). In this scenario, unbound proteins are predominantly in the so-called “apo” structure. However,

ligands bind not to these apo structures, but to a preexisting small population of “holo-like” proteins that are structurally similar to the ligand-bound “holo” state. In the case of calmodulin, the folded apo and holo-like states are in rapid exchange in the absence of Ca^{2+} (12,13), and peptide ligands and Ca^{2+} are thought to bind to the already folded holo-like structure. By using force to counteract the stabilizing effects of bound Ca^{2+} , Junker et al. could resolve folding of the calmodulin carboxyl-terminal domain. Their results imply that the preequilibrium scenario does not hold under all conditions.

The authors find that Ca^{2+} stabilizes the protein by increasing the folding rate without affecting the unfolding rate. Thus, under the conditions of high Ca^{2+} concentration in this study, calmodulin folds by a different pathway, in which folding and Ca^{2+} binding occur together, bypassing the folded Ca^{2+} -free apo and holo-like intermediates. This binding-induced folding (11) follows an induced-fit scenario, in which the ligand itself triggers the conformational change (see the figure). At low Ca^{2+} concentration, the preequilibrium mechanism is expected to prevail. These two alternative folding mechanisms have also been seen in molecular simulations (14).

By contrast to Ca^{2+} binding, Junker et al. find that peptide binding to calmodulin has a different effect on the folding energy landscape. The two peptide ligands that they consider bind only to the folded protein and stabilize it by decreasing the unfolding rate, conforming to a preequilibrium mechanism. To demonstrate this mechanism directly, the authors linked one of the peptides, the calmodulin-binding domain of myosin lightchain kinase, to calmodulin in such a way that binding of the peptide induces a change in length of the protein-ligand construct. They observed an obligatory intermediate corresponding to the folded protein in the absence of the ligand, thus confirming the mechanism inferred from their kinetic measurements: Only in the preequilibrium case would it be possible to observe a distinct binding-competent intermediate before ligand binding (see the figure).

Future single-molecule studies may be able to probe the effect of force on the folding mechanism itself. If the pulling direction is not correlated with the motions along the intrinsic unfolding (or unbinding) route, the mechanism should switch from the intrinsic mechanism at low force to a different mechanism at high force (15). This switch may have a role in the mechanical strength of proteins and could make a protein more resistant to unfolding by tensile force. Previous work inferred such a switch by comparing the extrapolation of unfolding kinetics at high force with ensemble measurements at zero force (16). The ability to study folding over a wider range of forces opens the possibility of observing this switch directly.

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Figure 1. Mechanisms of coupled folding and binding

These schematic trajectories portray the extension of a protein (green) with a covalently attached ligand (purple) as a function of time. In a pre-equilibrium scenario, folding precedes binding, resulting in an intermediate; Junker *et al.* observed this scenario for binding of the MLCK peptide to calmodulin. In contrast, in an induced-fit scenario, binding and folding would occur together via an unstructured transition state; Junker *et al.* deduced this scenario for the binding of Ca^{2+} at high concentrations.