Response to Comment on "Force-Clamp Spectroscopy Monitors the Folding Trajectory of a Single Protein"

Science moves forward when new techniques uncover unanticipated results, and the field of protein folding is no exception. Indeed, our force-clamp spectroscopy measurements of the folding of ubiquitin chains (*1*) revealed trajectories that departed from the expected two-state folding reactions observed with chemical denaturation techniques (*2*). However, the mechanical and chemical studies of protein folding involve very different endpoints and therefore are not directly comparable. An important difference is that these two experimental approaches result in very different changes in the length of the folding protein. A mechanically stretched and unfolded polyprotein begins its folding trajectory from a well-defined point at which the polypeptide can be extended to the point of losing its secondary structure. For example, at a stretching force of 110 pN, ubiquitin is extended by ~86% of its contour length (*1*, *3*). By contrast, a chemical folding trajectory begins from an unfolded state that is far more compact and less well defined (*4*, *5*). Although the trajectory of a protein that folds after chemical denaturation involves changes in the end-to-end distance of at most a few nanometers (*2*, *6*), force-clamp spectroscopy monitors folding trajectories that can be up to several hundred nanometers in length. Even the more steplike final folding contraction [see figure 5 in (*1*)] of a single ubiquitin involves a reduction in length of more than 15 nm and appears rate-limited.

The asymmetry observed between the stepwise unfolding and the folding trajectories reveals a more complex energy landscape than that monitored by chemical denaturation experiments. This is not surprising, given that extension of the unfolded protein to near its contour length drives the protein much further away from the native state and thereby explores new regions of the folding landscape. From this perspective, the classical view of barrier crossing in protein folding may only apply to small extensions away from the native state (*7*).

This debate also raises the more general question of how relevant the available experimental methods are to in vivo protein folding. In view of the force of gravity and the need of living organisms to perform mechanical work, mechanical stretching is very likely to have played a role in the evolution of proteins. By contrast, the large changes in temperature or chemical denaturants commonly employed in protein-folding studies (*2*) are not found in living cells. Furthermore, chemical or thermal denaturation experiments typically define folding through changes in fluorescence of a tryptophan residue or fluorescence resonance energy transfer (FRET) pairs. Although such measurements provide accurate kinetic information, they do not reveal to what degree the folding proteins have recovered their native form. By contrast, the recovery of mechanical stability monitored by force-clamp spectroscopy (*1*) provides an excellent indication of whether the native state has been reached, given that natively folded proteins exhibit mechanical resistance before unfolding.

Although the mechanical folding trajectories observed by force-clamp spectroscopy still defy explanation, we do not agree with the proposal advanced by Sosnick (*8*) that the folding trajectories of a ubiquitin chain represent the incongruous collapse of aggregating protein modules, driven mostly by their forced intimacy. Simple collapse due to aggregation would not lead to the correct folding of the individual ubiquitins in the chain, which is our main observation. Furthermore, the folding of contiguous protein modules is likely to be a common theme in the function of modular proteins such as titin (*9*), tenascin (*10*), spectrin (*11*), ubiquitin (*3*), and many others. Evolutionary pressure on these proteins must have resulted in mechanisms that effectively avoid the entanglement of folding neighbors (*12*). From this perspective, the mechanical folding trajectories captured by force-clamp spectroscopy reflect much more closely the folding of such modular proteins in vivo, compared with those obtained by means of thermal or chemical manipulations of isolated monomers.

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1 July 2004; accepted 22 September 2004