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Subdomain Competition, Cooperativity, and Topological Frustration in the Folding of CheY

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The folding of multidomain proteins often proceeds in a hierarchical fashion with individual domains folding independent of one another. A large single-domain protein, however, can consist of multiple modules whose folding may be autonomous or interdependent in ways that are unclear. We used coarse-grained simulations to explore the folding landscape of the two-subdomain bacterial response regulator CheY. Thermodynamic and kinetic characterization shows the landscape to be highly analogous to the four-state landscape reported for another two-subdomain protein, T4 lysozyme. An on-pathway intermediate structured in the more stable nucleating subdomain was observed, as were transient states frustrated in off-pathway contacts prematurely structured in the weaker subdomain. Local unfolding, or backtracking, was observed in the frustrated state before the native conformation could be reached. Nonproductive frustration was attributable to competition for van der Waals contacts between the two subdomains. In an accompanying article, stopped-flow kinetic measurements support an off-pathway burst-phase intermediate, seemingly consistent with our prediction of early frustration in the folding landscape of CheY. Comparison of the folding mechanisms for CheY, T4 lysozyme, and interleukin-1β leads us to postulate that subdomain competition is a general feature of large single-domain proteins with multiple folding modules.
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Introduction

The mechanism by which a protein adopts its unique native fold remains a fundamental question in biology. Knowledge of the factors controlling how a polypeptide sequence dictates its own structure not only is necessary for efforts in structure prediction and protein design but also will further our understanding of the thermodynamics governing protein stability and conformational dynamics. Failure of a protein to reach the native state can be deleterious. Partially unfolded or misfolded intermediates can lead to protein self-association and the formation of pathogenic oligomers and aggregates.[1]

A protein need not always fold to a well-structured, highly ordered native state, however. It has recently been discovered that large protein segments lacking a well-structured fold can also be functional. Intrinsically unstructured proteins contain highly conserved disordered regions that can be involved in target binding and recognition.[2] In many disordered segments, target binding can then induce a population shift to a well-structured fold. A fundamental step in understanding the conformational landscape of proteins as it relates to folding, aggregation, and functional dynamics is the characterization of folding pathways and the nature of intermediates populated en route from the unfolded ensemble to the native state.

A number of experimental techniques have proven valuable for elucidating folding pathways. Phi-value analysis of mutations can determine which regions of a protein are natively structured in the folding transition state.[3] Nuclear magnetic resonance (NMR) and pulse-labeling hydrogen exchange have been used to monitor the development of site-specific

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Abbreviations used: NSHX, native-state hydrogen exchange; T4L, T4 lysozyme.

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structure in early kinetic intermediates during folding reactions.[4–6] Rare thermodynamic intermediates have been probed using native-state hydrogen exchange (NSHX), which reports on the structure of high-energy, partially unfolded conformations in equilibrium with the native state.[7,8] Recently, these techniques have been increasingly applied to large single-domain proteins with complex folding mechanisms.[9–13]

The folding of multidomain proteins typically proceeds in a hierarchical fashion with individual domains folding independent of one another,[14] which is important for evolutionary domain shuffling.[15] A large single-domain protein, however, can consist of multiple modules whose folding may be autonomous or interdependent in ways we are only beginning to understand.[16] The 164-residue T4 lysozyme (T4L), for instance, consists of an α-helical C-terminal subdomain and a predominantly β-sheet N-terminal subdomain. NSHX has identified an on-pathway "hidden" intermediate structured in the C-terminus but unstructured in the N-terminus, the formation of which is the rate-limiting step in folding as illustrated by the cartoon in Fig. 1a.[10–13,17] Isolated as fragments, the C-terminus folds in the absence of its partner subdomain, whereas the N-terminus does not.[11,18] The C-terminal subdomain thus serves as the folding nucleus in T4L, with the N-terminus rapidly folding once it has formed.

Interestingly, pulse-labeling hydrogen exchange identified a second intermediate prior to the rate-limiting step in T4L folding. This kinetic intermediate formed rapidly (within the temporal resolution of the mixing device) and was partially structured in both subdomains.[4,10] Since the N-terminus is unstructured in the on-pathway intermediate, the early intermediate must undergo an N-terminal unfolding event in order to proceed to the native state. Such transient formation of structure that must be undone prior to productive progression of folding to the native state constitutes off-pathway frustration, or "backtracking," in the landscape.

Another multisubdomain protein whose folding has been studied is the 129-residue bacterial chemotactic response regulator CheY. A member of the second most common flavodoxin fold family, CheY consists of five βα-repeats arranged in a central parallel β-sheet surrounded by α-helices (Fig. 1b). Phi-value analysis has identified two folding subdomains in CheY: an N-terminal subdomain that is highly structured in the folding transition state and a C-terminal subdomain that is unstructured in the transition state.[19] It has been observed that van der Waals contacts are weaker in the C-subdomain than in the N-subdomain.[19,20] Helix 4 lacks a strong N-capping residue, and there is a cavity lined by several alanines between this helix and the rest of the protein, which gives rise to flexibility at the $\alpha_4\beta_5$ surface of the protein. Cavity-filling mutants have demonstrated that this flexibility is important for function;[21,22] upon phosphorylation of the loop connecting strand 3 and helix 3, the $\alpha_4\beta_5$ surface undergoes a conformational rearrangement and binds downstream target FliM to modulate flagellar motility.[23–25] The folding mechanism that has emerged for CheY is that the formation of the stable N-terminus is rate limiting and serves to nucleate the weaker C-terminus.

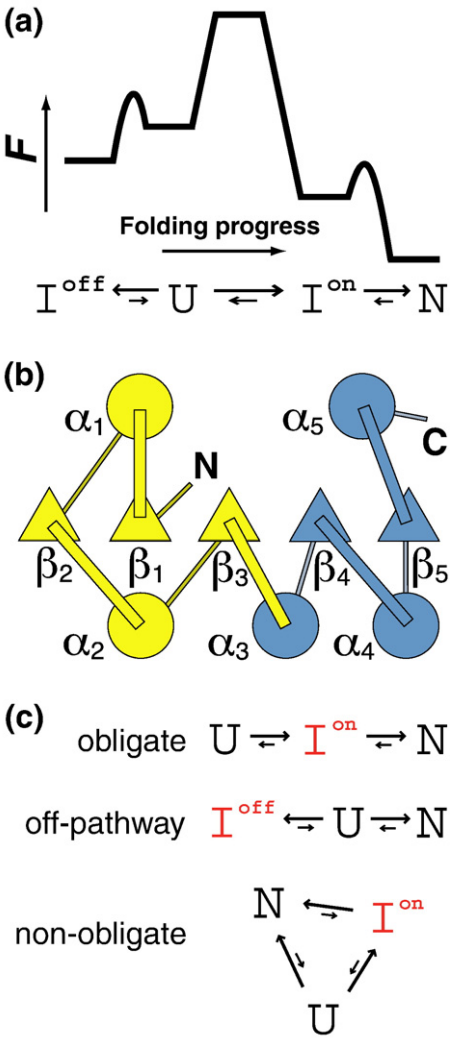

Fig. 1. (a) Generic reaction diagram for four-state folding in proteins with competing subdomains. In T4L, hydrogen exchange studies have identified a thermodynamic on-pathway intermediate with a fully structured C-terminus but an unstructured N-terminus and an early off-pathway kinetic intermediate partially structured in both termini.[10,13] (b) Topology of βα-repeat protein CheY with N-terminal (yellow) and C-terminal (blue) folding subdomains. (c) Three possible classifications for a folding intermediate: obligate on-pathway intermediate, misfolded off-pathway "trap," or on-pathway intermediate in which the rate of direct transfer from *U* to *N* is nonnegligible.

In the present work, we used molecular simulation to explore the landscape of subdomain folding in CheY. Our thermodynamic and kinetic characterization shows the landscape to be highly analogous to the four-state landscape of T4L and establishes a molecular basis for the four-state folding mechanism. Namely, the four-state-like landscape is a consequence of frustration caused by the competition for

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