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Intermediates: ubiquitous species on folding energy landscapes?

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Although intermediates have long been recognised as fascinating species that form during the folding of large proteins, the role that intermediates play in the folding of small, single-domain proteins has been widely debated. Recent discoveries using new, sensitive methods of detection and studies combining simulation and experiment have now converged on a common vision for folding, involving intermediates as ubiquitous stepping stones en route to the native state. The results suggest that the folding energy landscapes of even the smallest proteins possess significant ruggedness in which intermediates stabilized by both native and non-native interactions are common features.

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Introduction

One of the major advances in studies of protein folding over the past 15 years was the discovery that, in the absence of complicating factors such as proline isomerization, most small proteins (<100 amino acids) fold to their native structure in a cooperative two-state transition [1]. In such (un)folding pathways, only the native and denatured states are significantly populated. The simplicity of the folding mechanism of this class of protein has had enormous impact on the development of our understanding of folding, focusing attention on the structures of transition state ensembles [2], driving attempts to capture the essential features of folding using computer-based models [3], and underpinning attempts to delineate mathematical relationships between protein sequence, structure and folding rate constants [4]. However, two-state folding not only precludes a detailed view of different stages in folding pathways, but also masks the underlying complexity of a process that involves finding a single structure, stabilized by a myriad of specific interatomic interactions, from the astronomical number of possible alternatives available. For these reasons, the characterization of intermediates (metastable states that are transiently populated on the folding free energy landscape) has proved attractive to experimentalists and theoreticians alike [5].

It is generally accepted that proteins greater than 100 amino acids in size fold via one or more intermediate states that act as stepping stones to the native state. However, the role or even the presence of metastable states during the folding of smaller proteins has proved highly controversial [6], even though two-state behaviour does not preclude the existence of high-energy states along the folding pathway [7]. What role, then, do intermediates play in folding? Are they off-pathway species that might be the progenitors of human disease, or misfolded states that slow the rate of folding [8]? Alternatively, could intermediates play a role in correct folding, even for proteins that apparently fold efficiently without them?

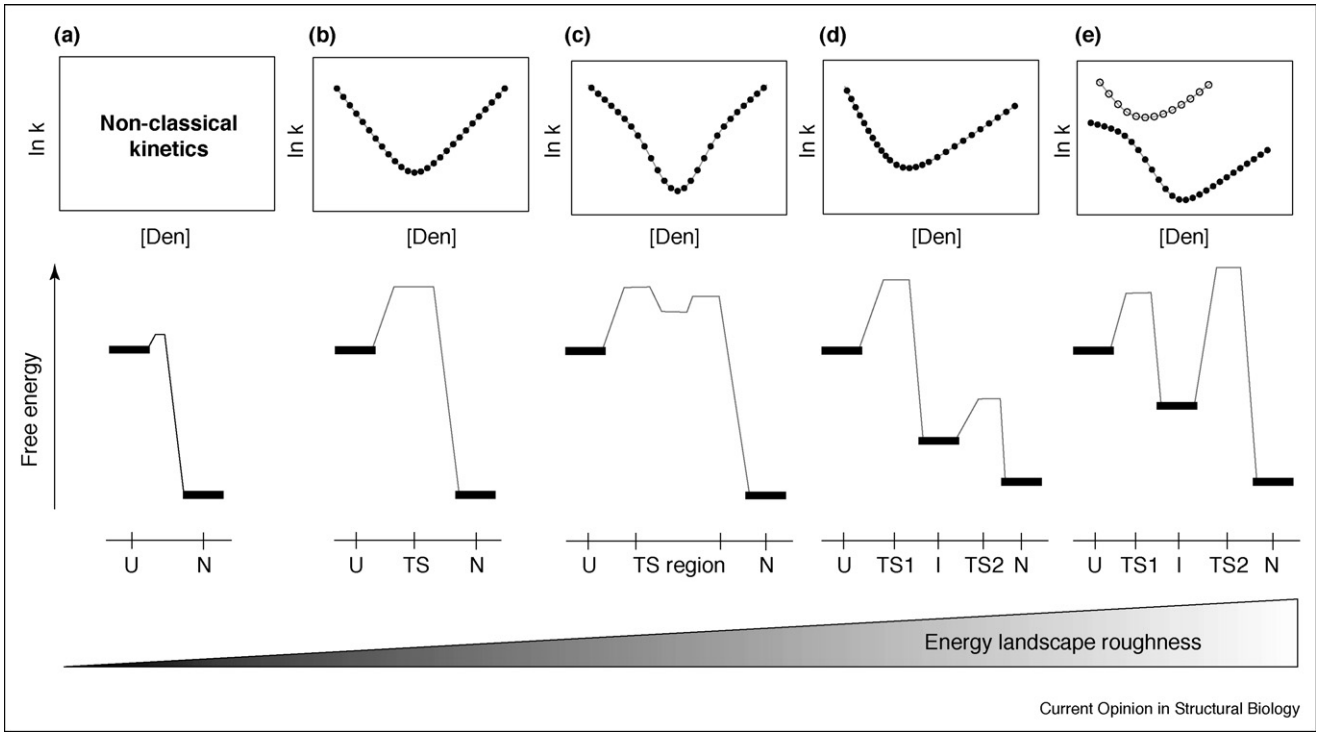
Here, we summarize recent advances in experimental data that address these questions and the insights gained. We also discuss the emerging view that potentially all proteins fold via intermediate states.

Detecting intermediates

Effect on kinetic chevron plots

A powerful method of detecting intermediates formed during folding or unfolding is to analyse the denaturant dependence of the folding and unfolding kinetics using chevron analysis. In the absence of a significant transition state barrier (<2 k_BT), folding is predicted to occur in a downhill manner [9], ruling out analysis of folding using chevron analysis (Figure 1a). By contrast, the presence of a single transition state barrier on an otherwise smooth energy landscape results in classical V-shaped chevron plots in which the logarithm of both the folding and unfolding rate constants depend linearly on the denaturant concentration (Figure 1b). Such a scenario is indicative of two-state folding, for which chymotrypsin inhibitor *2* (CI2) remains the paradigm example [7]. Deviations from linearity in either branch of the chevron plot (Figure 1c,e) are consistent with the transient population of metastable states on the folding energy landscape. Where no direct experimental evidence can be found for the accumulation of an intermediate during folding (such as the absence of a burst phase change in the signal amplitude), non-linearity in the chevron plot can be interpreted as reflecting either a gradual change in the position of the rate-limiting transition state on the reaction coordinate (Hammond effects), as classically observed for *Thermus thermophilus* ribosomal protein S6

Figure 1



Example chevron plots (top) and free energy diagrams (bottom) for different folding scenarios. Each chevron plot shows the denaturant ([Den]) dependence of the observed rate constant (ln k). **(a)** Barrier-less folding. **(b)** Classical two-state folding, in which intermediates are not populated. **(c)** Folding via a high-energy intermediate. The switch between early and late transition states as a function of denaturant concentration results in non-linearity of the folding and unfolding branches of the chevron plot. **(d)** Population of a late folding intermediate, subsequent to the rate-limiting transition state. Such proteins might display two-state kinetics and the population of intermediates is typically inferred from native state hydrogen exchange experiments. **(e)** Folding via the population of a stable intermediate in the first millisecond of folding. This is revealed by the so-called 'rollover' in the folding branch of the chevron plot determined using stopped-flow methods (closed circles). In the case where the intermediate accumulates rapidly during folding, a second chevron plot reflecting the formation and unfolding of the intermediate can be obtained using ultra-rapid mixing experiments (open circles). The positions of the denatured state (U), intermediate state (I), early and late transition states (TS1 and TS2, respectively) and native state (N) are shown on an arbitrary scale.

[10], or a denaturant-dependent switch between distinct transition states on a sequential pathway [11]. The latter mechanism necessarily invokes the transient formation of a high-energy intermediate that is on-pathway for folding, as shown in Figure 1c. Kiefhaber and co-workers have fitted the folding and unfolding kinetics of 23 small proteins to the latter model, suggesting that folding via high-energy intermediates is a common occurrence in folding energy landscapes [11,12].

Intermediates can also be formed after the rate-limiting transition state has been traversed. Such species might not be detected using chevron analysis (Figure 1d), unless the intermediate is unusually stable. Such late intermediates can be revealed, however, by measuring conformational fluctuations from the native state using, for example, native state hydrogen exchange or NMR relaxation methods (see [13,14] and reviews published elsewhere in this issue). These techniques have been used to identify hidden intermediate states that occur after the rate-limiting transition state in cytochrome *c* [13], barnase [15], a redesigned apo-cytochrome b_{562} [16,17], the third PDZ domain from PSD-95 [18], acyl-coenzyme A binding protein (ACBP) [19•] and T4 lysozyme [20]. In addition, recent simulations indicate that the Fyn Src homology 3 (SH3) domain might also unfold via such an intermediate [21]. Native state hydrogen exchange has also been used to identify misfolded off-pathway intermediates in the folding energy landscape of 179-residue apoflavodoxin [22]. From a physiological viewpoint, such species might be important in the control of signalling pathways [23], or might provide a link between the protein folding and aggregation energy landscapes [8,24].

When stable intermediates are formed before the rate-limiting transition state for folding, a distinct rollover in the folding branch of the chevron plot results (Figure 1e). In this scenario, the intermediate accumulates in the first millisecond of folding and the kinetic rollover is accompanied by a change in signal amplitude in the burst phase