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A Single Mutation at Residue 25 Populates the Folding Intermediate of *E. coli* RNase H and Reveals a Highly Dynamic Partially Folded Ensemble

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Understanding the nature of partially folded intermediates transiently populated during protein folding is important for understanding both protein folding and misfolding. These ephemeral species, however, often elude direct experimental characterization. The well-characterized protein ribonuclease H (RNase H) from *Escherichia coli* populates an on-pathway intermediate identified in both bulk studies and single-molecule mechanical unfolding experiments. Here, we set out to trap the transient intermediate of RNase H at equilibrium by selectively destabilizing the region of the protein known to be unfolded in this species. Surprisingly, a single change at Ile25 (I25A) resulted in the equilibrium population of the intermediate under near-native conditions. The intermediate was undetectable in a series of heteronuclear single quantum coherences, revealing the dynamic nature of this partially unfolded form on the timescale of NMR detection. This result is in contrast to studies in which the structures of trapped intermediates are solved by NMR, indicating that they are well packed and native-like. The dynamic nature of the RNase H intermediate may be important for its role as an on-pathway, productive species that promotes efficient folding.

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Introduction

Partially folded intermediates are known to play an important role in the mechanism of protein folding. For some proteins, intermediates appear to play a productive role, aiding in the formation of the native fold, while for others, they constitute an off-pathway species. In addition to their importance in folding mechanisms, partially folded forms may also play crucial biological roles. There are many examples of proteins with unstructured regions whose disordered–ordered folding transitions are important for binding and other regulatory events.[1,2] For example, the active form of the steroidogenic acute regulatory protein appears to be a molten globule,[3] a state usually associated with early folding intermediates. Such intermediates have also been implicated in the formation of aggregation-prone species.[4] In spite of their clear importance in both folding and function, these partially folded intermediates are usually not amenable to detailed structural and biophysical studies due to their low populations at equilibrium and transient nature.

Here, we set out to trap the intermediate of ribonuclease H (RNase H) from *Escherichia coli*, a small (17.5 kDa), monomeric enzyme known to fold through such a partially folded kinetic intermediate[5–7] (throughout this article, wild-type RNase H refers to the *E. coli* variant with all three cysteines replaced by alanine). Recent single-molecule experiments conclusively demonstrated that this species is on-pathway and obligatory.[8] Characterization of the intermediate by ϕ-value analysis and quenched flow hydrogen exchange suggest that the intermediate is composed of native-like topology in the core and a relatively unstructured periphery (Fig. 1a).[5,6] This partially folded ensemble appears to play a robust and important role in the folding trajectory of

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Abbreviations used: RNase H, ribonuclease H; ANS, 1-anilino-8-naphthalene sulfonic acid; HSQC, heteronuclear single quantum coherence.

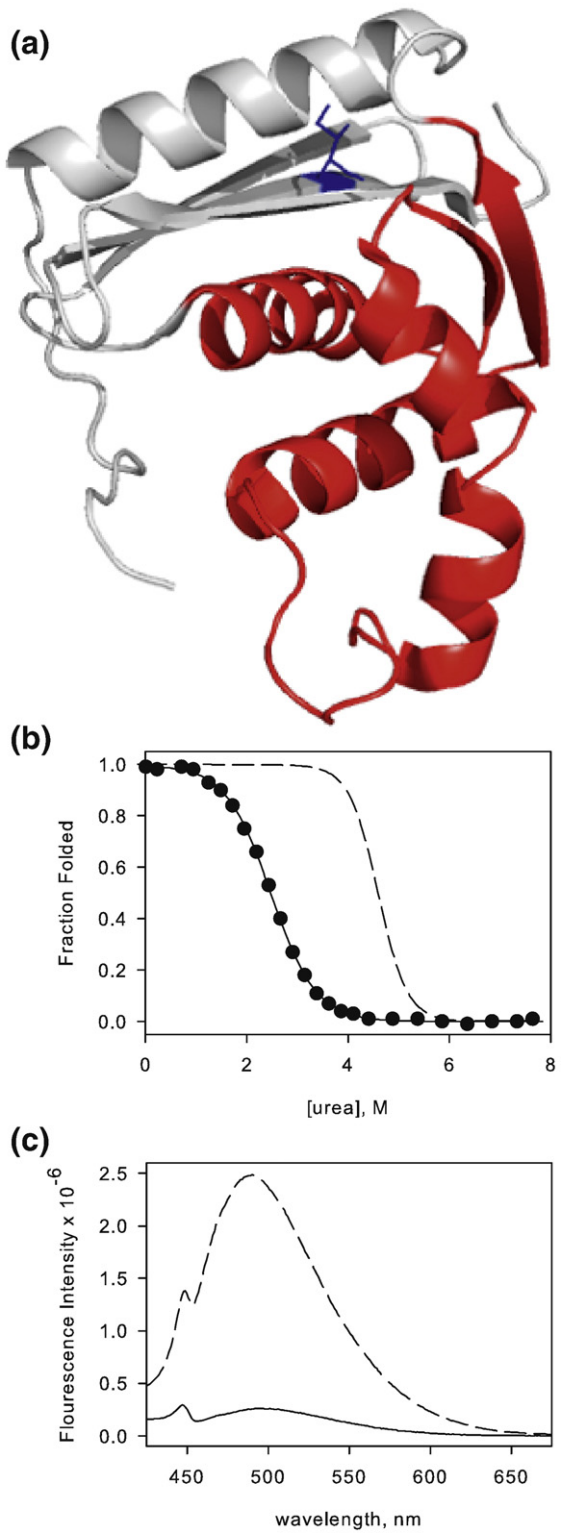

Fig. 1. I25A resembles the RNase H intermediate. (a) Ribbon representation of RNase H (Protein Data Bank code: 1F21) with the core of the protein colored red and the periphery colored gray. Residue I25 is shown as sticks and colored blue. (b) Fraction folded of I25A (filled circles) determined from urea melts overlaid with the wild-type curve (broken line). The continuous line represents the two-state fit for I25A. (c) Fluorescence emission spectra of ANS in the presence of WT (continuous line) and I25A (broken line). The fluorescence emission of ANS in buffer alone has been subtracted from the data.

RNase H, dominating the folding trajectory even in variants that do not transiently populate the state (see accompanying manuscript). Equilibrium native-state hydrogen exchange experiments detect a rare, high-energy partially unfolded form whose structure and energetics mirror this kinetic intermediate, suggesting that the most stable region of the protein is the first to fold.[6,9]

We have used rational design to selectively destabilize the native state of the protein, enriching the population of the high-energy species. The response of RNase H to mutations highlights the differential distribution of stability in the core and periphery that makes this approach possible. Isoleucine 53, located in the core, was mutated to alanine (I53A), destabilizing the protein by ~2 kcal/mol ($\Delta\Delta G_{UN} \sim 2$ kcal mol^{-1}).[10] Native-state hydrogen exchange demonstrated that this destabilizing effect was localized to the core, while residues in the periphery were unaffected, resulting in destabilization of the intermediate. A similar study was carried out in the periphery with the stabilizing mutation D10A,[11] where, although all secondary elements showed an increase in the free energy of unfolding as measured by native-state hydrogen exchange, the difference between unfolding the periphery and the core remained unchanged, suggesting that the two regions are energetically independent. This selective increase in stability causes a decrease in the population of the partially folded form. For both of these variants, the effects in the equilibrium energetics were mirrored in the transient intermediate observed during the folding process. In fact, a severely destabilizing mutation in the core of the protein (I53D) destabilizes the intermediate such that the protein folds in an apparent two-state manner without the obvious accumulation of the intermediate.[7]

In principle, then, we should be able to use mutations that destabilize the periphery of the protein without perturbing the stability of the intermediate to selectively destabilize the native state, enhancing the population of the high-energy intermediate such that we can study it more easily. This strategy has been demonstrated by Bai who, based on native-state hydrogen exchange data, created mutations to selectively destabilize the regions predicted to be unfolded in the intermediate, thereby selectively destabilizing the native state.[12–17] Similarly, Spence *et al.* destabilized regions of the immunity protein Im7 based on results from ϕ-value analysis (monitoring the folding kinetics for engineered site-specific mutations).[18]

In an alternative approach, Bai *et al.* also recently designed a fragment of *Thermus thermophilus* RNase H modeled, in part, on our native-state hydrogen exchange data. By removing two strands believed to be disordered in the intermediate, they obtained a well-folded species amenable to high-resolution NMR spectroscopy. Their results reveal a fragment that folds into a well-packed native-like species, suggesting that the structure of the partially folded intermediate may be a subset of the native structure. These results are at odds with studies that suggest