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Kinetic folding studies of the P22 tailspike beta-helix domain reveal multiple unfolded states

M.L. Spatara, C.J. Roberts, A.S. Robinson *

Department of Chemical Engineering, University of Delaware, Newark, Delaware 19716, United States

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ABSTRACT

The beta-helix is an important protein fold in many pathogens, and is a challenging system for folding pathway prediction because it primarily is stabilized by non-local interactions along the primary sequence. A useful experimental model of this fold is a monomeric truncation of P22 tailspike protein, the beta-helix domain (bhx). This report describes a systematic *in vitro* study of the chemical denaturation and refolding of bhx. Results from equilibrium chemical denaturation experiments were consistent with a two-state folding mechanism, but showed only partial reversibility. Stopped-flow fluorescence studies showed a single unfolding step, but two refolding steps. The slow refolding step could be partly attributed to proline isomerization, based on an increased rate during refolding in the presence of PPIase and an increased relative amplitude of this step with increasing delay time in double-jump refolding experiments observed over delays of 5–100 s. However, double-jump refolding experiments with delay times longer than 100 s along with size exclusion chromatography and dynamic light scattering of refolding samples showed that the overall refolding yield decreased as bhx was unfolded for longer periods of time. Furthermore, the losses resulted from aggregate formation during refolding. This suggests that a change occurs over time in the unfolded or denatured state ensemble that increases the propensity for aggregation upon the shift to more native-favoring conditions. Alternatively aggregate nuclei may be able to form even under high denaturant conditions, and these subsequently grow and consume monomer when placed under native-favoring conditions.

1. Introduction

In protein folding, the formation of beta-sheet structure often involves interactions between residues that are far from each other in the primary sequence. For this reason, beta-sheet formation is often more difficult to predict than alpha-helix formation. However, the formation of beta-sheet structures during folding has gained recent attention, partially due to the prevalence of that structure in amyloid and other protein aggregates present in disease states [1]. One of the proposed model structures for amyloid is the parallel beta-helix [2,3], a coiled structure in which each rung of the helix contributes a beta-strand to each of three parallel beta-sheets that run along the helix surface. The beta-helix structure is being increasingly discovered as a native protein fold, particularly in proteins associated with infectious disease such as viral adhesin and tail proteins [4]. Based on sequence information, more than 97% of the passenger domains from autotransporters – secreted virulence proteins of gram-negative pathogenic bacteria – are predicted to adopt a beta-helix structure even though they show little sequence homology [5]. A detailed understanding of beta-helix formation is obviously important given this fold's utility as a model for protein aggregates in human disease, association with pathogenic systems, and ability to form robustly from diverse and non-repetitive protein sequences.

The current work focuses on the tailspike protein from the P22 bacteriophage. Fig. 1 shows the crystal structure of the tailspike trimer [6–8]. Tailspike has been used as a model system to study the folding of large, multimeric proteins, and its folding pathway has been extensively characterized both *in vitro* and *in vivo* [9–17]. Although tailspike is thermally stable and resistant to aggregation at temperatures up to 80 °C in its mature, trimeric form [18], it is highly susceptible to aggregation during folding, particularly *in vitro*, at elevated temperature, or in amino acid variants [12]. The aggregation is believed to occur through folding intermediates, which are destabilized under these conditions [12]. During refolding, tailspike adopts the majority of its secondary structure quickly, and in a monomeric state. It is believed that the successful formation of the beta-helix structure in the monomeric state is the critical step in determining the partitioning between a productive folding pathway or misfolding and aggregation [19]. Unfortunately, the complexity and irreversibility of the tailspike folding and assembly pathway, including competing aggregation events, make it difficult to study the beta-helix formation as an isolated event. For this reason, we are studying the *in vitro* folding of a truncated form of tailspike, comprising the beta-helix domain of the protein (bhx). The approximate location of the truncation points (residues 109 and 544) in the full-length structure

* Corresponding author. Tel.: +1 302 831 0557; fax: +1 301 831 6262.
E-mail address: asr@udel.edu (A.S. Robinson).

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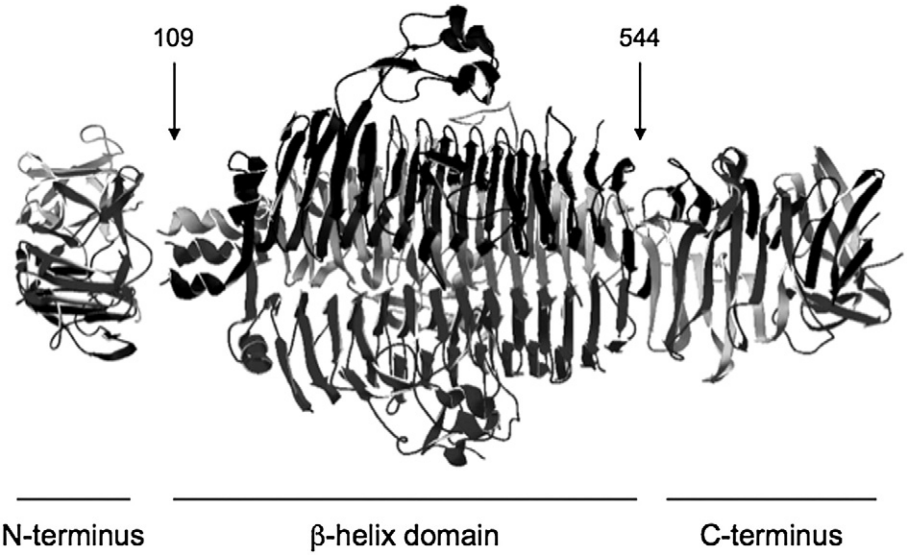

Fig. 1. P22 tailspike protein structure. Ribbon diagram of full-length, trimeric tailspike protein. Arrows indicate the approximate locations of residues 109 and 544, the truncation points for bhx.

is shown in Fig. 1. The bhx truncation has a molecular weight of 47.2 kDa, and exists primarily as a monomer at low concentrations [20]. Preliminary studies have demonstrated that bhx is spectroscopically similar to full length tailspike, retains partial enzymatic activity, has folding reversibility [20], and readily forms fibrous aggregates [21]. These observations indicate that the bhx truncation is structurally similar to the helix domain in the full-length protein and can act as a good model for a structured, monomeric folding intermediate.

2. Experimental

2.1. Materials

All chemicals used were obtained from major commercial suppliers. The cyclophilin enzyme was purchased from Sigma. Ultrapure urea was obtained from MP Biomedicals and used in all spectroscopic experiments.

2.2. Protein expression

Chemically competent *E. coli* BL21(DE3) cells (Novagen) were transformed with a pET11a plasmid containing the DNA encoding residues 109–544 of P22 tailspike (1tyr: pdb.org) under control of the T7 promoter [22]. Cells were selected on LB-Ampicillin (100 μg/mL) plates. For protein expression, an individual colony was selected and grown to an OD_{600} of approximately 0.6 at 20 °C before expression was induced by addition of 1 mM IPTG. Following induction for 18 h at 20 °C, cells were harvested by centrifugation at 4000 ×*g* for 20 min, resuspended in lysis buffer (50 mM Tris, pH 7.6, 25 mM NaCl, 2 mM EDTA, 20 mM $MgSO_4$, 20 μg/ml DNase, 100 μg/ml lysozyme, 0.1% Triton-X 100) and subjected to two freeze/thaw cycles (−80 °C/20 °C). Cellular debris was removed by centrifugation at 13,000 ×*g* for 30 min and the resulting supernatant was used for subsequent purification.

2.3. Protein purification

Purification of beta helix protein (bhx) was performed as previously described for the full length tailspike protein [23]. Purified bhx was applied to a Superdex 75 size exclusion column (GE Healthcare) and eluted isocratically in 100 mM phosphate buffer, pH 7, to remove any larger multimers formed during the purification prior to folding experiments. Protein concentration was determined by absorbance at 280 nm (1 OD_{280} = 1.33 mg/mL) [22]. Protein purity was >99% as determined by SDS–PAGE with silver staining.

2.4. Equilibrium folding/unfolding

Bhx was diluted to a concentration of 0.35 mg/mL in 100 mM phosphate buffer, pH 7, containing either 0 or 6 M urea and incubated in a 10 °C water bath for 1 h. The bhx samples were then diluted ten-fold into final urea concentrations between 0 and 6 M urea with a final protein concentration of 0.035 mg/mL and incubated for 1 h before measuring the fluorescence spectra. Samples incubated for longer times (up to 20 h) did not show a change in behavior compared to the 1 h samples. Spectra were measured at approximately 16 °C in a PC1 spectrofluorometer (ISS, Illinois) using excitation and emission slit widths of 0.5 and 1.0 mm, respectively, and an excitation wavelength of 280 nm. The emission was measured from 300 to 400 nm, and the spectra were quantified using the spectral center of mass (COM) [24], defined as

$$\mathrm{COM} = \left(\frac{\sum I_\nu \cdot \nu}{\sum I_\nu}\right)^{-1} \tag{1}$$

where ν is the wavenumber, and I_ν is the intensity at a given wavenumber, ν.

2.5. Stopped-flow folding/unfolding

Kinetic folding and unfolding experiments were performed in a model SX.18MV-R stopped-flow fluorescence instrument (Applied Photophysics, Leatherhead, U.K.). Unfolding and refolding were initiated by rapid dilution of native or denatured bhx into varying final urea concentrations. All experiments were performed at 15 °C in 100 mM phosphate buffer, pH 7. Intrinsic fluorescence was measured with an excitation wavelength of 280 nm, and the integrated emission signal was measured above a 305 nm cutoff filter. Kinetic traces were fit to the equation:

$$I(t) = I_\infty + \sum_i A_i \cdot e^{-k_i \cdot t} \tag{2}$$

where I is the fluorescence intensity, A_i is the amplitude of the ith phase, k_i is the rate constant of the ith phase, t is time in seconds, and I_∞ is the signal at infinite time. Quality of fit was evaluated by magnitude and randomness of residuals.