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Heterogeneous side chain conformation highlights a network of interactions implicated in hysteresis of the knotted protein, Minimal Tied Trefoil (MTT_{Tm})

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Abstract

Hysteresis is a signature for a bistability in the native landscape of a protein with significant transition state barriers for the interconversion of stable species. Large global stability, as in GFP, contributes to the observation of this rare hysteretic phenomenon in folding. The signature for such behavior is non-coincidence in the unfolding and refolding transitions, despite waiting significantly longer than the time necessary for complete denaturation. Our work indicates that hysteresis in the knotted protein, the Minimal Tied Trefoil from *Thermotoga maritima* (MTT_{Tm}), is mediated by a network of side chain interactions within a tightly packed core. These initially identified interactions include proline 62 from a tight β -like turn, phenylalanine 65 at the beginning of the knotting loop, and histidine 114 that initiates the threading element. It is this tightly packed region and the knotting element that we propose is disrupted with prolonged incubation in the denatured state, and is involved in the observed hysteresis. Interestingly, the disruption is not linked to backbone interactions, but rather to the packing of side chains in this critical region.

Keywords

Trefoil knot; hysteresis; SPOUT methyltransferase; 1O6D; Minimal Tied Trefoil *Thermotoga maritima* (MTT_{Tm})

Introduction

Hysteresis is a generally observed phenomenon across many systems in which the output is a function of not only the current input, but also of the function of the history of inputs to the system. The consequence of hysteresis can present itself in a number of ways across a broad range of models that can be presented physically and mathematically. In biology, for example, it is observed in activated T-cells with increased plasticity of their response as the

threshold for their activation is lowered (1). With respect to proteins, hysteresis can be a signature of biphasic behavior or an apparent non-equilibrium behavior in the native state where the energetic barrier is large enough to distinguish two native conformations (2-6). This behavior can be attributed to the varying populations of folded states depending on where you are on the dual basin hysteretic energy landscape of Green Fluorescent Protein (GFP) (7, 8). Hysteresis in the folding of GFP (7-9) is mediated by the coupling of a proline-locking mechanism that alters the packing of the central functional chromophore. The dynamic lag manifests itself as a non-coincidence in the denaturing and refolding “thermodynamic” titrations, after the protein has been denatured for an extended period of time (7). While knots in proteins are an active area of research, the question of hysteresis and knotting is largely unexplored.

Hysteresis in the knotted protein, Minimal Tied Trefoil from *Thermotoga maritima* (MTT_{Tm}, PDB code 1O6D), was predicted and confirmed for this highly stable knotted protein because of the complex topology and high stability of the protein (10, 11). Originally knots were studied in biomolecules, such as nucleic acids and DNA, which are known to be flexible and can diffuse along the polymer chain (12-14). However, due to increased complexity inherent in a polypeptide chain, the same theory of flexibility and diffusion is not directly translatable to proteins (12). Due to the conformational restriction in a knotted protein, the geometric constraints can introduce a variety of other complex structural consequences. Simulations indicate an uncoupling of folding from untying; that is, the protein unties on timescales significantly longer than required for unfolding (10). In the current study, we use a combination of optical and solution Nuclear Magnetic Resonance (NMR) spectroscopies to elucidate differences in the refolded hysteretic protein from the native, under conditions strongly favoring the native state. Furthermore, we highlight molecular interactions we attribute to hysteresis, using ¹H-¹H-¹⁵N-NOESY-HSQC to further visualize the side chain packing in relation to the knot topology. Our results indicate a noticeable switch in the knotting loop where a pronounced difference in the environment for initiating residue F65 is observed. In line with theoretical data (10), we find that the packing of the of the β-like turn with the phenylalanine-histidine pair in the knot element precludes true equilibrium, contributing to hysteresis in MTT_{Tm}. Our results indicate that the interactions that hinder reversible folding are side chain mediated for knot proteins. Consistent with simulations, early formation of this β-like turn requires backtracking, a localized unfolding event needed to allow folding to proceed, as the turn may be in the wrong orientation for proper formation of the knot. Here, we provide the initial experimental evidence supporting theoretical suggestions (11) of non-native interactions of the β-like turn.

Results and Discussion

The structure of MTT_{Tm}

MTT_{Tm} is identified as a member of the SPOUT family of methyltransferases, containing a five stranded β-sheet sandwiched between two α-helices on one side and three α-helices on the other (15-17) (Figure 1). The most distinct feature of the SPOUT family is the topologically unique deep C-terminal knot, classified as a 3₁ knot (trefoil knot). This represents the simplest possible knot found within protein structures, defined by a single

loop threaded once by the backbone, where the chain crosses itself no more than three times (18). The knot loop in MTT_{Tm} is formed by β -strand 3 and α -helix 3 and is subsequently threaded by β -strand 5 (Figure 1). The knotting loop region is anchored by residue F65 through D92 (Figure 1, blue), while the threading β -strand (Figure 1, orange) encompasses residue H114 through L119.

Structural details of MTT_{Tm} indicate that there are unique features in the loop connecting β -strand 3 and α -helix 2. This tight loop resembles a β -turn, as it is a short loop in a restricted conformation. Prototypically, β -turns consist of four critically packed residues (labeled i , $i+1$, $i+2$, and $i+3$) and are defined by either having an inter-backbone hydrogen bond between the CO of residue i and the NH of residue $i+3$, or a distance of less than 7 Å between the C α atoms of residue i and $i+3$ (19). In the case of MTT_{Tm} , the packing of the β -like turn is mediated by a hydrogen bond between the CO of L61 and the OH of S64, contributing to the restricted conformation of the turn (Figure 5). The *trans*-proline and glycine (P62 and G63) in between residues L61 and S64 orient them into a position that allows them to hydrogen bond forming a β -like turn (19). This creates a critical network of contacts that stabilizes the native state.

Characterization of hysteresis in MTT_{Tm}

MTT_{Tm} has been observed to undergo the unique phenomenon of hysteresis, which is dependent on the time scales allowed for denaturing. The hysteretic behavior of MTT_{Tm} is most clearly evident by evaluating thermodynamic titrations in denaturant (10). To further investigate the molecular details of hysteresis, we expanded upon previous work by evaluating the influence of incubation time in denaturant with spectroscopic methods, including residue specific details by NMR. The subsequent thermodynamic effects of the varied incubation times were evaluated under NMR conditions (Figure 2). MTT_{Tm} was incubated for 24 hours before refolding, a significantly longer time than ten times the $t_{1/2}$ -value obtained from kinetics (Supplemental Figure S1). From these denaturing conditions, we obtain two superimposable titration curves for unfolding and refolding (Figure 2, Top). This suggests that the knot is still intact in MTT_{Tm} , even after incubating for 24 hours under denaturing conditions. This is supported by previously published *in vitro* and *in silico* studies indicating that the knot persists in the denatured state much longer than initially thought (20, 21).

Hysteresis was initially identified in MTT_{Tm} after a six-month incubation in strong denaturant (10), we evaluate the time frame in which hysteresis is observed under our new NMR conditions (see methods below). After denaturing MTT_{Tm} for four weeks, hysteresis was clearly evident (Figure 2, Bottom). In addition to shifting the midpoint to lower denaturant, a new biphasic behavior emerges, indicating that there is a third state in the hysteretic protein. Due to the emergence of a third state and a shifting midpoint we wanted to evaluate whether the native protein and the hysteretic protein have the same native structure.

Previous NMR studies investigating the effects of hysteresis in the highly stable GFP revealed chemical shift heterogeneity in the native-like trapped hysteric protein (7). Using a similar approach, we acquired both ^1H - ^{15}N HSQCs of native and hysteretic protein to obtain

residue specific information. To probe the dynamic solution state, MTT_{Tm} was assigned using a suite of NMR experiments. The chemical shifts are well dispersed in both native and hysteretic protein (Figure 3). The overlaid spectra are indistinguishable under these conditions, indicating that hysteresis is not attributable to perturbations in the backbone interactions. This is indicative that the hysteretic protein retains its native backbone structure despite hysteresis. Given the constrained geometry of the knotted MTT_{Tm} , it is reasonable that backbone limitations will influence both the position and relative dynamics of side chains, particularly those that have steric restrictions. As assessment of backbone interactions indicate that they are not principally involved in hysteresis, examination of side chain interactions by NMR proved more revealing. Figure 4 presents 1H - 1H - ^{15}N -NOESY-HSQC 2D strip plots of key residues, with the most pronounced heterogeneity observed in F65. The heterogeneity in F65 is clearly observed as two distinct peaks, indicating that F65 populates two slowly exchanging conformations. F65 is in close proximity to H114, allowing the aromatic rings to form stabilizing π -stacking interactions (22-24). This π -stacking interaction can account for the presence of two conformations, one of which is stabilized, identified as the more dominant peak in the 1H - 1H - ^{15}N -NOESY-HSQC (Figure 4). H114 is in a critical position as it is the initiating residue of the threading β -strand 5 (Figure 5). Interestingly, the knotted region is adjacent to the constrained β -like turn facilitating crosstalk through the network of contacts and the knot through the π -stacking of F65 and H114.

Comparison of native and hysteretic protein 1H - 1H - ^{15}N -NOESY-HSQC spectra indicate no significant deviations between the two spectra, with the exception of F65 (Figure 4). A unique difference in the hysteretic protein is observed as a loss in heterogeneity in the side chain conformation compared to native. This change in conformation upon refolding is similar to other observations of “aromatic ring switches” where a subtle change in ring packing, swap in hydrogen bonding (25) or stacking interactions lead to heterogeneity. More importantly, while overall backbone interactions appear intact, subtle disruption of side chain interactions are evident, as noted by the differences presented here (Figure 4). Consistent with the coupling of long range geometric constraints observed in GFP (7), the observed conformational differences in the F65 side chain are attributable to the interplay between the specific network of contacts bridging the complex topology of the knot to the tight β -like turn.

Conclusions

While theoretical and experimental work has shown that over time knots will eventually untie, this event takes significantly longer than initially anticipated (10, 20, 21). As untying the knot of MTT_{Tm} is distinctly decoupled from the unfolding mechanism (10), the results presented here suggest a specific network of side chain contacts as the cause of hysteresis. We hypothesize that hysteresis in MTT_{Tm} is attributable to the specific packing of side chain contacts bridging the complex topology of the knot to the β -like turn, leading into the knot, mirroring some of the complex behavior of the proline-linked chromophore hula-twist packing challenges leading to hysteresis in GFP (7). The π -stacking interaction between H114 (Figure 5, orange) and F65 (Figure 5, blue) allows for direct cross talk through the adjacent β -like turn (Figure 5, green), which could “lock” P62 in a *trans*-conformation in the

native state. The “locking” represents the introduction of a geometrical constraint in the denatured state by the knot, which inhibits the isomerization of P62 from a *trans*- to *cis*-conformation. Hence, there is no hysteresis after denaturing and refolding in a short period of time because the proline is held in the *trans*-conformation by the knot. However, if MTT_{Tm} denatures for an extended time the packing of β-like turn together with the knot can destabilize, so that P62 can eventually isomerize to a thermodynamic distribution of interconverting the *cis-trans* conformations in the denatured state. This then allows for refolding into a “native-like” protein, with the proline in a *cis*-conformation rather than *trans*. Thus, the time dependent isomerization state of the proline may contribute to the observed hysteretic shift in the thermodynamic titration. Interestingly previous simulation studies indicate that early formation of the P62 β-like turn “precludes the correct route towards folding” (11). It has yet to be seen if experimental manipulation of the β-like turn would allow for the recovery of the native packing and relieve hysteresis, which is an area of active study. Importantly, simple *cis-trans* proline isomerization will not lead to hysteresis on its own. When proline isomerization is packed in close proximity to complex topology in proteins, hysteretic bistability can occur. In the case of MTT_{Tm}, the β-like turn is packed against the knotting loop, which stabilizes this structure in the denatured state and precludes true equilibrium and causes the observed hysteretic behavior.

Methods

Expression and purification of MTT_{Tm}

MTT_{Tm} was grown in BL21-DE3 *E. coli* cells at 37° C, expressed, harvested, and lysed by sonication. The supernatant was purified by Ni-Affinity chromatography and gel filtration by an S-200 sepharose column. Protein purity was assessed by SDS-PAGE. The most well dispersed ¹H-¹⁵N HSQC was obtained using 75 mM sodium acetate at pH 5.6, and 1% (v/v) glycerol plus reducing agent. This buffer condition is different than used previously (10), and was used throughout this work.

Thermodynamic titrations experiments

Circular dichroism (CD) measurements were accomplished using an Aviv Circular Dichroism spectrometer Model 215 instrument to monitor the secondary structure of MTT_{Tm} from a wavelength scan from 200-260 nm with a spectral bandwidth of 1 nm at 25° C. Thermodynamic titrations were performed at a final protein concentration of approximately 5 μM. All samples were incubated for 24 hours. For refolding titrations, MTT_{Tm} was denatured for 24 hours and 4 weeks, respectively in varying concentrations of guanidine hydrochloride (Gnd-HCl).

The native and refolded thermodynamic data was fit to a two-state equation according to:

$$S = \frac{S_N + S_D \times K_{D-N}}{1 + K_{D-N}} \quad (1)$$

$$K_{D-N} = \exp \left(\frac{-\Delta G_{D-N}^{H_2O} + (m_{D-N} \times [D])}{RT} \right) \quad (2)$$

where [D] is Gnd-HCl concentration, m_{D-N} is the linear dependence of G_{D-N} on denaturant concentrations and G_{D-N} is of folding in H_2O . And S_N and S_D are the signal of the native and denatured states, respectively. The fitted denatured protein gives a midpoint (MP) at 3.9 ± 0.1 in Gnd-HCl, an m_{D-N} equal to 5.6 ± 0.2 and a global stability of G_{D-N} of 21.2 ± 0.1 kcal/mol. The refolded protein gives a MP at 3.8 ± 0.1 in Gnd-HCl, an m_{D-N} equal to 5.7 ± 0.2 and a global stability of 21.8 ± 0.1 kcal/mol.

The thermodynamic data for the hysteretic protein was fit to a three-state equation according to:

$$S = \frac{K_{I-N} (Z + K_{D-I})}{1 + K_{I-N} (1 + K_{D-I})} \quad (3)$$

$$Z = \frac{S_I - S_N}{S_D - S_N} \quad (4)$$

where K_{I-N} and K_{D-I} are described as above. Z accounts for the intermediate signal with the native and denatured signal. The state fit of the hysteretic protein give a MP at 3.9 ± 0.1 and 3.6 ± 0.3 , for the transition from $I-D$ and $N-I$ respectively in Gnd-HCl. The values of m_{D-N} were equal to 6.5 ± 0.5 and 3.0 ± 1.0 , for m_{I-D} and m_{N-I} respectively. The overall global stabilities of G were equal to 25.2 ± 0.1 kcal/mol and 10.7 ± 0.1 kcal/mol, for G_{I-D} and G_{N-I} respectively.

NMR Studies

1H - ^{15}N HSQC and 1H - 1H - ^{15}N -NOESY-HSQC were collected on a Varian VNMRs 800 MHz magnet equipped with a triple-resonance cryoprobe at $50^\circ C$. 1H - ^{15}N HSQCs were collected with a total of 256 complex t_1 (^{15}N) and 2048 complex t_2 (1H) points. 1H - 1H - ^{15}N -NOESY-HSQCs were collected with a total of 128 complex t_1 (1H), 256 complex t_2 (^{15}N), and 1024 complex t_3 (1H) points, with a mixing time of 100 ms. Assignments were accomplished using a standard suite of triple resonance experiments consisting of the HNCA, HNCACB, CBCA(CO)NH, HNCO, and HN(CA)CO. The experiments were processed using NMRPipe (26) and visualized and assigned using SPARKY (27).

Molecular Representations

Molecular representations were generated using the crystal structure of MTT_{Tm}, PDB code 1O6D, and PyMOL (28).

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Figure 1.

Crystal structure of the knot protein, MTT_{Tm} (PDB code 1O6D). (A) Viewed from the side, the protein is represented as a cartoon emphasizing the knot with the threading β -strand ($\beta 5$, orange) threading through the knot loop (blue). (B) Same as A, viewed from the top of the protein. (C) Schematic of the protein, viewed in the same orientation as (B) following the same color scheme.

Figure 2.

Thermodynamic titrations of MTT_{Tm} . (Top) Overlay of a denaturing titration curve (black) with a refolding titration curve, after incubation in denaturant for 24 hours (blue). The two titration-curves are superimposable. (Bottom) Overlay of a denaturing titration curve (black) with a refolding titration curve (red), collected after incubation in denaturant for 4 weeks. The titration curves are no longer superimposable.

Figure 3.

Comparison of ^1H - ^{15}N HSQC spectra of native and hysteretic MTT_{Tm} . The spectral overlays of native (black) and hysteretic (red) protein are indistinguishable. The assignments shown in the spectra are highlighted as spheres on the inlayed molecular representation of MTT_{Tm} (L61 and S64 are shown in green, F65 shown in blue, and H114 shown in orange). Additionally, the knot is colored in blue (knotting loop) and orange (the threading β -strand 5), and the β -like turn in green.

Figure 4. ^1H - ^1H - ^{15}N -NOESY-HSQC strip plots highlighting side chain interactions. Shown are 2D strips of residues comparing the native (black) and hysteric (red) proteins. The cyan box in F65 highlights both the heterogeneity of the side chain in MTT_{Tm} (black) and the differences in the side chain signature between native and hysteric protein.

Figure 5.

Schematic of the critical packing interactions around the knot region of MTT_{Tm} . (Top) The knotting loop and threading β -strand 5 are shown in blue and orange, respectively. The two conformations observed in F65 are represented as dashed lines, where the dominant state can interact with H114. The β -like turn is highlighted in dark green. The native *trans*-proline is represented by a dark green proline ring and side chain. The *cis*-conformation of the proline is shown in light green. (Bottom) Crystal structure of the β -like turn, following the same coloring scheme as above, highlighting the hydrogen bond (dashed green line) interaction critical for the loop formation between residue L61 and S64.