Native topology determines force-induced unfolding pathways in globular proteins

D. K. Klimov* and D. Thirumalai*

Department of Chemistry and Biochemistry and Institute for Physical Science and Technology, University of Maryland, College Park, MD 20742

Communicated by Bruce J. Berne, Columbia University, New York, NY, April 21, 2000 (received for review February 21, 2000)

Single-molecule manipulation techniques reveal that stretching unravels individually folded domains in the muscle protein titin and the extracellular matrix protein tenasin. These elastic proteins contain tandem repeats of folded domains with β-sandwich architecture. Herein, we propose by stretching two model sequences (S1 and S2) with four-stranded β-barrel topology that unfolding forces and pathways in folded domains can be predicted by using only the structure of the native state. Thermal refolding of S1 and S2 in the absence of force proceeds in an all-or-none fashion. In contrast, phase diagrams in the force-temperature (f,T) plane and steered Langevin dynamics studies of these sequences, which differ in the native registry of the strands, show that S1 unfolds in an all-or-none fashion, whereas unfolding of S2 occurs via an obligatory intermediate. Force-induced unfolding is determined by the native topology. After proving that the simulation results for S1 and S2 can be calculated by using native topology alone, we predict the order of unfolding events in Ig domain (Ig27) and two fibronectin III type domains (FnII and FnIII). The calculated unfolding pathways for these proteins, the location of the transition states, and the pulling speed dependence of the unfolding forces reflect the differences in the way the strands are arranged in the native states. We also predict the mechanisms of force-induced unfolding of the coiled-coil spectrin (a three-helix bundle protein) for all 20 structures deposited in the Protein Data Bank. Our approach suggests a natural way to measure the phase diagram in the (f,C) plane, where C is the concentration of denaturants.

Although these computational studies have been successful in showing how the folding scaffolds of a protein respond to force, a simple framework for predicting the force-induced unfolding pathways has not yet emerged. In this paper, we take a step in this direction. We first studied in detail the equilibrium phase diagrams in the force-temperature plane (f,T) of two model off-lattice sequences with β-sandwich topology (just as in Ig27, FnII and FnIII). Then, we investigated the forced unfolding kinetics by using steered Langevin dynamics (SLD). From these results, we propose that force-induced unfolding characteristics (pathways and pulling speed dependence) can be predicted by using only the structure of the native state. This idea is applied to force-induced unfolding of Ig27, FnII, and FnIII. The results are in near quantitative agreement with experiments and/or SMD simulations. We also predict unfolding mechanisms for spectrin, for which only limited experimental data are available.

Materials and Methods

Off-Lattice Models for β-Barrel Proteins. We use coarse-grained continuum representations for proteins in which only the positions of Cα-carbons are retained (12). Sequences S1 and S2 are modeled as chains of 46 connected beads of three types, hydrophobic B, hydrophilic L, and neutral N. The potential energy includes the contributions from bond-length and bond-angle potentials, the dihedral angle potential $V_{DIA}(\phi)$, and nonbonded potentials (12). $V_{DIA}(\phi)$ is given by $A(1 + \cos \phi) + B(1 + \cos 3\phi)$. Along the β-strands trans-states are preferred, and accordingly, we chose $A = B = 1.2\epsilon_h$, where $\epsilon_h = 1.25$ kcal/mol is the strength of hydrophobic interactions. In the turn regions (i.e., in the vicinity of a cluster of N residues), $A = 0$ and $B = 0.2\epsilon_h$. The interaction between a pair of hydrophobic residues is

$$V_{\mu}(r) = 4\lambda\epsilon_h \left( \frac{a}{r} \right)^{12} - \left( \frac{a}{r} \right)^{6},$$

where $\lambda$ is a random factor unique for each pair of B residues (12) and $a = 3.8$ Å is the residue size. When the sequence is subject to external force $f$, the total energy is written as $E_{\text{tot}} = E_p - (f\cdot r)$, where $r$ is the end-to-end vector.

Abbreviations: AFM, atomic force microscopy; SMD, steered molecular dynamics; SLD, steered Langevin dynamics; TS, transition state.

*To whom reprint requests should be addressed: E-mail: thirum@glue.umd.edu or klimov@glue.umd.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Simulations. The dynamics of stretching are obtained by integrating the Langevin equation in the overdamped limit (approximately at water viscosity). Two types of SLD simulations were carried out. (i) A constant force was applied to the termini. (ii) To mimic the AFM experiments, the carboxyl-terminal was pulled along the z axis with the force

\[ f = -k(z - v), \]  

where \( v \) is the pulling speed and \( k \) is the spring constant, which is equal to the constant used in the bond-length potential (12). The pulling speed was varied from \( 6 \times 10^4 \) \( \mu \text{m/s} \) to \( 6 \times 10^5 \) \( \mu \text{m/s} \). These speeds are about four to five orders of magnitude faster than experimental values (1, 13) but are about 10^5 times slower than the values used in SMD simulations (10, 11). Thermodynamic functions are computed by using a version of the multiple histogram method in which \( T \) and \( f \) are varied (8).

Predicting Unfolding Pathways by Using PDB Structures. Using the results of the SLD study of S1 and S2, we developed a method for predicting the unfolding events on application of force to the termini of a protein. There are several steps in the application of our method. (i) From the PDB native structure of a given protein, a coarse-grained model is created. In this model, backbone is represented by Cα-carbons, and side chains are given by the positions of heavy atoms. (ii) The secondary structure blocks (i.e., strands and helices) are assigned in the coarse-grained model by using the secondary structure information in the headers of PDB entries. We assume that residues i and j form a tertiary contact, if the distance between any pair of heavy atoms in their side chains is less than 5.2 Å. By using this definition, the tertiary contact map is obtained, and the stabilization energy for each secondary structure block is computed as the energy of tertiary interactions of the respective block with other secondary structure blocks in the native structure. We use several contact potentials available in the literature (14–16) to recalculate the stabilization energies of all the remaining blocks. The stabilization energies of terminal blocks are once again compared to determine the next secondary structure block to unfold. If both terminal blocks have approximately equal stabilization energies, their simultaneous unfolding takes place. This procedure is repeated until the entire native structure is unfolded. Note that this method is valid when secondary structure blocks in the native state do not form subdomains, and forced unfolding may proceed only via unraveling of terminal blocks. With this method, the breakup of hydrogen bonds (11, 17) cannot be predicted. The method allows us to compute the order of unfolding events and energy profiles, the pathways and the structure of the transition state (TS), and the equilibrium forces \( f_{eq} \) for the force-induced unfolding.

Pulling Speed Dependence of Unfolding Forces. The pulling speed dependence of forces is obtained with \( f_p = f_{eq} \ln(v/v_{min}) \), where \( v_{min} = k_u \Delta \) (\( k_u \) is the unfolding rate at \( f = 0 \), and \( \Delta \) is the maximum extension). For Ig27, 10FnIII, and 10FnIII, \( k_u \) was taken from chemical denaturant experiments (6, 18, 19), and \( \Delta \) was calculated from AFM experiments. For spectrin, we use \( \Delta E/RT = M \) (where \( M \) is the number of residues) and the folding time \( \tau_f \approx \tau_M M^{-1/2} (\tau_M \approx 10^{-10} \text{s}; \text{ref. 20}) \) to obtain \( k_u \approx 2.2 \times 10^{-3} \text{s}^{-1} \). For unfolding of strand A in titin and helix A in spectrin, we use \( \Delta E/\Delta \approx 3.9 \text{ kcal/mol and 3.8 nm for titin and 6.8 kcal/mol and 11 nm for spectrin, respectively.} \)

Results

Phase Diagrams for S1 and S2. We first studied the unfolding of off-lattice models of proteins. The phase diagrams for S1 and S2, both with β-sandwich native topology (Fig. 1 a and c), are dramatically different (Fig. 1 b and d). There is a small green region in Fig. 1b (S1) that represents states with intermediate values of the fraction of native contacts, \(<Q>\). This small region suggests that force-induced unfolding would be an all-or-none process. In contrast, the phase diagram for S2 (Fig. 1d) shows a broad region of intermediate states along the edge of stability of the native state (red region). This broad region indicates that unfolding must occur via an intermediate. The conclusion that S1 must unfold in an all-or-none manner and that S2 must unfold through an intermediate, which naturally emerges from the equilibrium phase diagram, is verified explicitly by SLD simulations (see below).

The threshold force required to unfold the native state (determined from the edges of the red region) for S1 is larger than that required for S2 at all temperatures. At \( T = 300 \text{ K} \), the equilibrium unfolding force for S1 is about 28 pN, whereas for S2, it is only 14 pN. We deduce that the native state of S1 is more stable than that of S2. Equilibrium folding and unfolding simulations in the absence of force show that the free energy of stability of S1 is larger than that of S2 by a factor of 2.5 (4.3 kcal/mol vs. 1.7 kcal/mol). Thus, the phase diagrams can be used to obtain the free energies of stability in the absence of force.

Stretching Under Constant Force. To probe the structural transitions on stretching, we performed SLD simulations by applying a constant force \( f_p \) along the z axis. For S1, the stretched state is reached in a single kinetic step, in which all strands unfold nearly simultaneously (Fig. 2a). There is, however, considerable heterogeneity in the time scales in which individual molecules (green curves in Fig. 2a) unfold. The strands unfold by an unzipping process in a cooperative manner without populating intermediates. A qualitatively different unfolding mechanism is found for S2 (Fig. 2b). The normalized extension, \(<r_3(t)> > 1/L \) (\( L \) is the contour length), shows a biexponential behavior (blue line in Fig. 2b), indicating that S2 unravels by a two-step process. The unfolding kinetics is further revealed by examining the extension profiles for 20 individual trajectories (green curves in Fig. 2b). On a short time scale, the polypeptide chain unfolds from the native state N to a stable on-pathway intermediate I (the green region in Fig. 1d represents the area of stability of I). The transition \( N \rightarrow I \) is reversible, i.e., on turning off the force, the chain spontaneously reaches the folded state without any evidence of hysteresis. Individual trajectories reach I (Fig. 2b inset) by unzipping strand D. Fig. 2b shows that I is obligatory, i.e., all of the molecules reach S (stretched state) through I. The I → S transition also shows a broad distribution of unfolding times. Remarkably, individual transitions I → S occur over a narrower time interval compared with the events leading to I (compare with Fig. 2b inset). The unfolding of I, in which three strands are pulled apart, occurs by a shearing-type motion, which is dynamically more cooperative (occurs extremely sharply) compared with the unzipping motion.

Pulling Speed Dependence. On application of force, the barrier between N and S is lowered such that the unfolding rates increase (21). Thus, the threshold force and the unfolding rate must depend on the pulling speed \( v \). We performed SLD simulations by fixing one of the termini and pulling the other at a constant \( v \) (Eq. 1). Force-extension profiles for S1 show a single peak at \( f \approx 100 \text{ pN} \) at an extension of about 0.17L (Fig. 3a). We conclude that the TS for force-induced unfolding occurs close to the folded native state, which is reminiscent of the unfolding seen in Ig27 (1, 6). For S2, there are two peaks in the force-extension profile...
(Fig. 3b). The one at \( f \approx 58 \) pN with an extension of about 0.1L represents the breakup of strand D (Fig. 1c), thus creating the stable intermediate I. The dominant peak (\( f \approx 130 \) pN and \( \Delta z \approx 0.44L \)) represents the unraveling of I. From the dependence of the fraction of native contacts \(<Q(\Delta z)>\), the values of \(<Q>\) are color coded according to the scales shown on the right. The green area in b, which corresponds to intermediate \(<Q>\), is extremely narrow. There is a large green region in the diagram for S2 (d). This large area suggests that S2 unfolds through partially unfolded structure I, in which the strand D is stretched, whereas strands A–C maintain their native conformation.

**Native Topology Determines Force-Induced Unfolding Events.** The dramatic differences in the force-induced unfolding between S1 and S2, which are masked in thermal refolding (see legend to Fig. 1), can be predicted in terms of the topology of the native state.

As predicted by Evans and Ritchie (21), the average threshold unfolding force depends logarithmically on the pulling speed (Fig. 3c). The unfolding forces, namely, \( f_{p,S1} \) (for S1) and \( f_{p,S2} \) (for unfolding I in S2), may be written as \( f_{p,\alpha} = f_{eq,\alpha} \ln(v/v_{min}) \) (\( \alpha = S1 \) or S2). Here, \( f_{eq,\alpha} \) is the equilibrium unfolding force (obtained from the phase diagram) and the minimum pulling speed \( v_{min} = k_u \Delta z \) (\( k_u \) is the unfolding rate at \( f = 0 \), and \( \Delta z \) is the extension in going from N to S). We also show the scaled results for titin (1) and the recombinant tenascin segment TnFnIII (5). The parameterization proposed here (which is natural when the overall spring constant of the protein is comparable to the stiffness of cantilever) produces a scaling plot in which the results of simulations for model sequences and proteins lie essentially on one line. Thus, scaling the experimental data by \( v_{min} \) gives a direct measurement of \( f_{eq} \). From the dependence of \( f_{eq} \) on denaturant concentration, which is easier to change than \( T \) in experiments, the phase diagram (Fig. 1) for the various proteins can be measured.
ics of on-pathway intermediate that exists in all unfolding trajectories. The dynam-
ics of S1 and S2.}

If there are two strands of nearly equal energies, we predict that they may nearly simultaneously unfold or that there may be parallel unfolding pathways. With this method, we successfully reproduced the simulated force-induced unfolding characteristics of S1 and S2.

**Predicting Force-Induced Unfolding Characteristics for β-Sandwich Proteins.** We applied this method to predict the unfolding pathways of three β-sandwich proteins, Ig27 (PDB code 1tit), 9FnIII (1fnf), and 10FnIII (1fnf), as well as the α-helical repeats of spectrin (1aj3). For all these modules, unfolding occurs by a shearing-type motion just as in I → S for S2. For Ig27 of the I band of titin, we find that the strand A unfolds first followed by A’ and G (Fig. 4a). Strands C, D, and E retain their structural integrity until late in the unfolding process. These observations are in accord with SMD simulations (10). An estimate of the energy barrier, with Q as a reaction coordinate (ref. 23; Fig. 4e), shows that force-induced unfolding TS is highly structured and occurs close to the native state (1, 2, 6, 17).

From the pulling speed dependence of the forces required to unfold individual domains of titin (1) and the unfolding rate at \( f = 0 \) (ref. 6; see legend to Fig. 3c), we predict that the equilibrium unfolding force is about 18 pN. A recent AFM experiment has shown that there is a “hump” in the force-extension curves (at \( f = 100 \) pN) on stretching tandem of repeats of individual Ig domains of human cardiac titin (17). The hump corresponds to an initial event in which strand A starts to shear away from the rest of the module (10, 17). Using various statistical potentials (see Materials and Methods), we find that the equilibrium unfolding force for A to unravel is \( f_{eq} \approx 7 \) pN. With this value, we predict that the initial intermediate in Ig27 module should occur at \( f_0 \approx f_{eq} \ln(v/v_{min}) \approx 80 \) pN at \( v = 0.5 \) \( \mu \text{m/s} \).

Our method predicts that, in 10FnIII, strand G unravels first. This unraveling is followed by A, B, and F. On unfolding of B, the major barrier is overcome (Fig. 4e). The structure containing strands E, C, and D remains intact until late in the unfolding process. The calculations also predict a minor barrier between S
and the conformation containing strands C and D. The order of unfolding sketched here is in excellent agreement with SMD simulations (11). Interestingly, the unfolding TS for 10FnIII is structurally more disordered than that for Ig27. More importantly, we predict that the unfolding TS is about halfway between N and S states (Fig. 4c). Refolding experiments with $f = 0$ on the isolated FnIII lead to the same conclusion (18, 19), implying that $N_t$ is less stable than 10FnIII (19). Although the location and the structure of the unfolding TS for 9FnIII is rather similar to those for 10FnIII, the energy barrier is smaller; therefore, the unfolding rate for 9FnIII is greater than for 10FnIII.

**Force-Induced Unfolding of Spectrin.** Finally, we predict the unfolding pathways for the triple-helix spectrin for which AFM measurements of only the unfolding forces are available (13). Here, helix A unfolds first and is followed by a simultaneous unraveling of B and C helices (Fig. 4d). There is only one energy barrier for the unfolding process. The calculation of the energy profile for unfolding (Fig. 4c) indicates that unfolding TS of spectrin occurs close to the native state. Remarkably, on unraveling of helix A, the stability of the remaining structure is greatly compromised, which suggests that there should be no intermediate for spectrin unfolding. These conclusions were unchanged when we applied our method to all 20 PDB structures for spectrin and used different statistical potentials (see Materials and Methods). Using the estimates $f_{eq} = 4.4$ pN and $v_{max} = 8.1 \times 10^{-2}$ μm/s (see Materials and Methods), we predict that the force required to unfold spectrin domain at $v = 0.8$ μm/s is 40 pN (experimental value is ~30 pN; ref. 13). Rief et al. (13) have reported the unfolding forces for spectrin at two pulling speeds (0.5 μm/s and 8 μm/s). From these measurements and using our parameterization of the dependence of $f$ on $v$ (Fig. 3c), we can calculate the unfolding rate when $f = 0$. We find that $k_d(f = 0) \approx 10^{-4} \text{ s}^{-1}$, which is only a factor of 20 smaller than the value obtained by using scaling arguments. Because $f$ scales only logarithmically with $v$, this difference results in a relatively small error in predictions for $f$.

**Conclusions**

In this article, we have provided a general framework for predicting the force-induced unfolding characteristics (pathways, pulling speed dependence of the unfolding forces, and location of TSs). Our approach is based on the hypothesis that unfolding by stretching can be understood by using only the topology of the native state. This hypothesis was firmly established by studying in detail the unfolding of two off-lattice model sequences with β-sandwich architecture similar to that of Ig27 and fibronectin domains. The remarkable differences between the stretching behaviors of two model sequences, which are not evident in thermal refolding calculations, are predicted quantitatively with our method. The success of our method in nearly quantitatively predicting the pulling speed dependence and location of TSs in Ig27, 10FnIII, 9FnIII, and spectrin confirms the validity of our theoretical model. Thus, given the native state of a protein, one can predict the force profiles for tandem repeats of identical protein modules.

Our method for predicting the characteristics of force-induced unfolding also suggests a way to measure the phase diagram of the sort shown in Fig. 1. In AFM and optical tweezer experiments, it is easier to alter the concentration of denaturants, $C$, than temperature. We propose that, by measuring the unfolding forces as a function of $C$, the equilibrium phase diagram in the plane of ($f$,$C$) can be obtained. Such experiments (especially when combined with fluorescence resonance energy transfer measurements) can provide a detailed map of the energy landscape of proteins.

Although the model devised herein is in nearly quantitative agreement with the experiments conducted thus far, we should...
emphasize its certain limitations. In particular, the location of the unfolding TS and associated barriers can be affected by the entropy of the unfolded regions of the chain. However, under tension, the fluctuations in these regions are expected to be small compared with the case of thermally induced (or denaturant-induced) unfolding.

One of the major issues is the relationship between the sequence of events in thermally induced and force-induced unfolding of proteins. The unfolding pathways may be similar in the limit of very slow pulling speeds. However, it is less clear whether unfolding pathways remain unaltered at pulling speeds normally used in experiments. Recent study of forced unfolding of β-hairpin indeed suggests that unfolding events depend on the pulling speed (25). This difference is probably exaggerated in SMD simulations because of very large pulling speeds. It would be desirable to carry out experimental and computational studies to compare thermal and forced unfolding pathways. Our preliminary results on S1 and S2 sequences suggest that there are differences in the thermal and forced unfolding.

We have focused only on the stretching of proteins in this article. The theory and the computational protocol is general and can be used to describe the stretching of other biomolecules and complexes. In particular, applications to RNA stretching can be made as long as the crystal structure is known. Such applications would be particularly useful given that much less is known about the folding landscape of RNA (26).

Note Added in Proof. We have applied our computational protocol to obtain the unfolding force for T4 lysozyme. Using the statistical potentials derived in ref. 16, we found that \( f \approx 73 \) pN at the pulling speed \( V = 1 \mu \text{m/s} \). The experimental value is 64 ± 16 pN (7).

We are grateful to Prof. G. H. Lorimer and Dr. M. Rief for penetrating discussions. This work was supported in part by National Science Foundation Grant CHE99-75150.