

New force replica exchange method and protein folding pathways probed by force-clamp technique

Maksim Kouza

Institute of Physics, Polish Academy of Sciences, Aleja Lotnikow 32/46, 02-668 Warsaw, Poland

Chin-Kun Hu

Institute of Physics, Academia Sinica, Nankang, Taipei 11529, Taiwan and Center for Nonlinear and Complex Systems and Department of Physics, Chung Yuan Christian University, Chungli 32023, Taiwan

Mai Suan Li^{a)}

Institute of Physics, Polish Academy of Sciences, Aleja Lotnikow 32/46, 02-668 Warsaw, Poland

(Received 30 July 2007; accepted 14 November 2007; published online 28 January 2008)

We have developed a new extended replica exchange method to study thermodynamics of a system in the presence of external force. Our idea is based on the exchange between different force replicas to accelerate the equilibrium process. This new approach was applied to obtain the force-temperature phase diagram and other thermodynamical quantities of the three-domain ubiquitin. Using the C_α -Go model and the Langevin dynamics, we have shown that the refolding pathways of single ubiquitin depend on which terminus is fixed. If the N end is fixed then the folding pathways are different compared to the case when both termini are free, but fixing the C terminal does not change them. Surprisingly, we have found that the anchoring terminal does not affect the pathways of individual secondary structures of three-domain ubiquitin, indicating the important role of the multidomain construction. Therefore, force-clamp experiments, in which one end of a protein is kept fixed, can probe the refolding pathways of a single free-end ubiquitin if one uses either the polyubiquitin or a single domain with the C terminus anchored. However, it is shown that anchoring one end does not affect refolding pathways of the titin domain I27, and the force-clamp spectroscopy is always capable to predict folding sequencing of this protein. We have obtained the reasonable estimate for unfolding barrier of ubiquitin, using the microscopic theory for the dependence of unfolding time on the external force. The linkage between residue Lys48 and the C terminal of ubiquitin is found to have the dramatic effect on the location of the transition state along the end-to-end distance reaction coordinate, but the multidomain construction leaves the transition state almost unchanged. We have found that the maximum force in the force-extension profile from constant velocity force pulling simulations depends on temperature nonlinearly. However, for some narrow temperature interval this dependence becomes linear, as have been observed in recent experiments. © 2008 American Institute of Physics. [DOI: [10.1063/1.2822272](https://doi.org/10.1063/1.2822272)]

I. INTRODUCTION

Protein ubiquitin (Ub) continues to attract the attention of researchers because there exist many processes in living systems where it plays the vital role. Usually, Ub presents in the form of a polyubiquitin chain that is conjugated to other proteins. Different Ub linkages lead to different biological functions. In the case of Lys48-C and N-C linkages polyubiquitin chain serves as a signal for degradation proteins,^{1,2} whereas in the Lys63-C case it plays completely different functions, including DNA repair, polysome stability, and endocytosis.³⁻⁵

The folding properties of Ub have been studied in detail by experiments^{6,7} as well as by simulations.^{8,9} The unfolding of Ub under thermal fluctuations was investigated experimentally by Cordier and Grzesiek¹⁰ and by Chung *et al.*,¹¹ and studied theoretically.¹²⁻¹⁴ There is strong evidence that

thermal unfolding pathways are reverse of the folding ones. The mechanical unfolding of Ub has been studied by several groups.¹⁵⁻¹⁸ It was found that the unfolding may proceed through rare intermediates, but the overall behavior remains two state, although a three-state scenario was also reported.¹⁹ The mechanical unfolding pathways are very different from the thermal pathways, and depend on what terminal is kept fixed.¹⁴

Recently Fernandez and Li²⁰ have applied the force-clamp technique to probe refolding of Ub under quench force f_q , which is smaller than the equilibrium critical force separating the folded and unfolded states. This kind of experiment has a number of advantages over the standard ones. In the force-clamp technique, one can control starting conformations which are well prepared by applying the large initial force of several hundreds of piconewtons. Moreover, since the quench force slows down the folding process, it is easier to monitor refolding pathways. However, this begs the important question as to whether the experiments with one end of the protein anchored probes the same folding pathways as

^{a)} Author to whom correspondence should be addressed. Electronic mail: masli@ifpan.edu.pl.

a free-end protein. Recently, using a simple Go-type model, it has been shown that fixing the N terminal of Ub changes its folding pathways.²¹ If it is so, the force-clamp technique in which the N terminal is anchored is not useful for prediction of folding pathways of the free-end Ub.

When one studies thermodynamics of a large system like multidomain ubiquitin the problem of slow dynamics occurs due to the rough free energy landscape. This problem might be remedied using the standard replica exchange method in the temperature space in the absence of external force²²⁻²⁴ as well as in the presence of it.²⁵ However, if one wants to construct the force-temperature phase diagram, then this approach becomes inconvenient because one has to collect data at different values of forces. Moreover, the external force increases unfolding barriers and a system may get trapped in some local minima. In order to have better sampling for a system subject to external force, we propose a new replica exchange method in which the exchange is carried out not in the temperature space but in the force space, i.e., the exchange between different force values. This procedure would help the system to escape from local minima efficiently.

In this paper we address three topics. First, we develop a new version of the replica exchange method to study thermodynamics of a large system under the force. The basic idea is that for a given temperature we perform simulation at different values of force and the exchange between them is carried out according to the Metropolis rule. This new approach has been employed to obtain the force-temperature phase diagram of the three-domain ubiquitin. Within our choice of force replicas it speeds up computation about four times compared to the conventional simulation. Second, question we ask is under what conditions the force-clamp technique still gives the same folding pathways as for the free-end Ub. Third, we determine the temperature dependence of the unfolding force, the unfolding barriers, and the location of the transition state of the single Ub and of the three-domain Ub (the three-domain Ub will also be referred to as trimer).

Because the study of refolding of 76-residue Ub by all-atom simulations is beyond present computational facilities, the Go modeling is an appropriate choice. The Go-type model²⁶ was proved^{14,27} to give not only qualitative but also quasiquantitative agreement with existing refolding and unfolding experiments. Therefore we will use it to study the folding and unfolding of a single and a multidomain Ub. In agreement with an earlier study,²¹ we show that fixing N terminal of the single Ub changes its folding pathways. Our new finding is that anchoring C terminal leaves them unchanged. More importantly, we have found that for the three-domain Ub with either end fixed, each domain follows the same folding pathways as for the free-end single domain. Therefore, to probe the folding pathways of Ub by the force-clamp technique one can either use the single domain with C terminal fixed or several domains with either end fixed. In order to check if the effect of fixing one terminus is valid for other proteins, we have studied the titin domain I27. It turns out that the fixation of one end of a polypeptide chain does not change the refolding pathways of I27. Therefore the force clamp can always predict the refolding pathways of the

single as well as multidomain I27. Our study suggests that the effect of the end fixation is not universal for all proteins, and the force-clamp spectroscopy should be applied with caution.

The third part of this paper was inspired by the recent pulling experiments of Yang *et al.*²⁸ Performing the experiments in the temperature interval between 278 and 318 K, they found that the unfolding force (maximum force in the force-extension profile) f_u of Ub depends on temperature linearly. In addition, the corresponding slopes of the linear behavior have been found to be independent of pulling velocities. An interesting question that arises is if the linear dependence of f_u on T is valid for this particular region, or it holds for the whole temperature interval. Using the same Go model,²⁶ we can reproduce the experimental results of Yang *et al.*²⁸ on the quasiquantitative level. More importantly, we have shown that for the entire temperature interval the dependence is not linear, because a protein is not an entropic spring in the temperature regime studied.

As a part of the third topic, we have studied the effect of multidomain construction and linkage on the location of the transition state along the end-to-end distance reaction coordinate, x_u . It is found that the multidomain construction has a minor effect on x_u but, in agreement with the experiments,¹⁵ the Lys48-C linkage has the strong effect on it. Using the microscopic theory for unfolding dynamics,²⁹ we have determined the unfolding barrier for Ub.

To summarize, in this article we have obtained the following novel results. We have proposed a new replica exchange method, in which the exchange is carried out between different external forces at a given temperature. It is shown that the force-clamp technique probes the same folding pathways as the free-end Ub, if one keeps the C terminal fixed or uses the multidomain Ub with whatever terminal anchored. Contrary to the α/β -protein ubiquitin, anchoring one end has a minor effect on refolding pathways of the titin domain I27. We attribute this to its β -sandwich structure, which is mechanically more stable. If Ub is pulled at Lys48 and C then, in the Bell approximation, we obtained the parameter $x_u \approx 0.61$ nm. This estimate agrees well with the experimental result of Fernandez group.¹⁵ We propose to fit simulation data with different theoretical schemes to determine the distance between the transition state and the native state x_u for biomolecules. Our estimate for unfolding barrier of Ub is in reasonable agreement with the experiments. It has been demonstrated that the temperature dependence of the unfolding force is linear for some narrow interval, but that this dependence should be nonlinear in general.

II. MODEL

Three-domain model of ubiquitin. Since the native conformation of polyubiquitin is not available yet, we have to construct it for Go modeling. The native conformation of single Ub, which has five β strands (S1–S5) and one helix (A), was taken from the PDB [PI: 1ubq, Fig. 1(a)]. We assume that residues i and j are in native contact if r_{0ij} is less than a cutoff distance d_c taken to be $d_c = 6.5$ Å, where r_{0ij} is the distance between the residues in the native conformation.

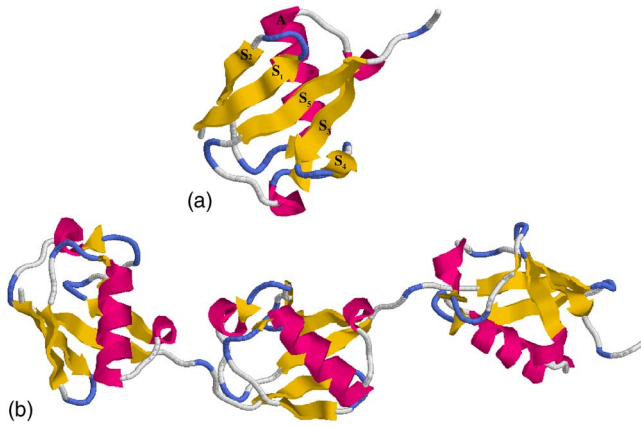


FIG. 1. (Color online) (a) Native state conformation of Ub taken from the PDB (PDB ID: 1ubq). There are five β strands: S1 (2–6), S2 (12–16), S3 (41–45), S4 (48–49), and S5 (65–71), and helix A (23–34). (b) The native conformation of the three-domain Ub was designed as described in Sec. II. There are 18 inter- and 297 intradomain native contacts.

Then the single Ub has 99 native contacts. We constructed the three-domain Ub [Fig. 1(b)] by translating one unit by the distance $a=3.82 \text{ \AA}$ along the end-to-end vector, and slightly rotating it to have nine interdomain contacts (about 10% of the intra-domain contacts).

The Go-type modeling. The energy of the Go-type model for the single as well as multidomain Ub is as follows:²⁶

$$E = \sum_{\text{bonds}} K_r (r_i - r_{0i})^2 + \sum_{\text{angles}} K_\theta (\theta_i - \theta_{0i})^2 + \sum_{\text{dihedral}} \{K_\phi^{(1)} [1 - \cos(\phi_i - \phi_{0i})] + K_\phi^{(3)} [1 - \cos 3(\phi_i - \phi_{0i})]\} + \sum_{i>j-3}^{\text{NC}} \epsilon_H \left[5 \left(\frac{r_{0ij}}{r_{ij}} \right)^{12} - 6 \left(\frac{r_{0ij}}{r_{ij}} \right)^{10} \right] + \sum_{i>j-3}^{\text{NNC}} \epsilon_H \left(\frac{C}{r_{ij}} \right)^{12}. \quad (1)$$

Here $\Delta\phi_i = \phi_i - \phi_{0i}$, $r_{i,i+1}$ is the distance between beads i and $i+1$, θ_i is the bond angle between bonds $(i-1)$ and i , ϕ_i is the dihedral angle around the i th bond, and r_{ij} is the distance between the i th and j th residues. Subscripts “0,” “NC,” and “NNC” refer to the native conformation, native contacts, and nonnative contacts, respectively.

The first harmonic term in Eq. (1) accounts for chain connectivity and the second term represents the bond angle potential. The potential for the dihedral angle degrees of freedom is given by the third term in Eq. (1). The interaction energy between residues that are separated by at least three beads is given by 10–12 Lennard-Jones potential. A soft sphere repulsive potential [the fifth term in Eq. (1)] disfavors the formation of non-native contacts. We choose $K_r = 100\epsilon_H/\text{\AA}^2$, $K_\theta = 20\epsilon_H/\text{rad}^2$, $K_\phi^{(1)} = \epsilon_H$, and $K_\phi^{(3)} = 0.5\epsilon_H$, where ϵ_H is the characteristic hydrogen bond energy and $C = 4 \text{ \AA}$.

Since for the trimer $T_F = 0.64\epsilon_H/k_B$ (see below), which is very close to $T_F = 0.67\epsilon_H/k_B$ obtained for the single Ub by the same model,¹⁴ we will use the same energy unit $\epsilon_H = 4.1 \text{ kJ/mol}$ as in our previous work. This unit was obtained by equating the simulated value of T_F to the experimental

$T_F = 332.5 \text{ K}$.³⁰ The force unit is then $[f] = \epsilon_H/\text{\AA} = 68.0 \text{ pN}$. Most of our simulations were performed at temperature $T = 285 \text{ K} = 0.53\epsilon_H/k_B$.

The dynamics of the system is obtained by integrating the following Langevin equation:^{31,32}

$$m \frac{d^2 \mathbf{r}}{dt^2} = -\zeta \frac{d\mathbf{r}}{dt} + \mathbf{F}_c + \mathbf{\Gamma}, \quad (2)$$

where m is the mass of a bead and ζ is the friction coefficient $\mathbf{F}_c = -dE/d\mathbf{r}$. The random force $\mathbf{\Gamma}$ is a white noise, i.e., $\langle \mathbf{\Gamma}(t)\mathbf{\Gamma}(t') \rangle = 2\zeta k_B T \delta(t-t')$. It should be noted that the folding thermodynamics does not depend on the environment viscosity (or on ζ) but the folding kinetics depends on it. Most of our simulations (if not stated otherwise) were performed at the friction $\zeta = 2(m/\tau_L)$, where the folding is fast. Here $\tau_L = (ma^2/\epsilon_H)^{1/2} \approx 3 \text{ ps}$. The equations of motion were integrated using the velocity form of the Verlet algorithm³³ with the time step $\Delta t = 0.005\tau_L$. In order to check the robustness of our predictions for refolding pathways, limited computations were carried out for the friction $\zeta = 50(m/\tau_L)$ which is believed to correspond to the viscosity of water³⁴. In this overdamped limit we use the Euler method for integration and the time step $\Delta t = 0.1\tau_L$.

In the constant force simulations, we add an energy $-\mathbf{f} \cdot \mathbf{R}$ to the total energy of the system [Eq. (1)], where R is the end-to-end distance and f is the force applied to both the termini or to one of them. We define the unfolding time τ_U as the average of first passage times to reach a rod conformation. Different trajectories start from the same native conformation but with different random number seeds. In order to get the reasonable estimate for τ_U , for each value of f we have generated 30–50 trajectories.

In order to probe folding pathways, for i th trajectory we introduce the progressive variable $\delta_i = t/\tau_U^i$, where τ_U^i is the unfolding time.¹⁴ Then one can average the fraction of native contacts over many trajectories in a unique time window $0 \leq \delta_i \leq 1$ and monitor the folding sequencing with the help of the progressive variable δ .

In the constant velocity force simulation, we fix the N terminal and pull the C terminal by force $f = K_r(\nu t - r)$, where r is the displacement of the pulled atom from its original position³⁵ and the spring constant K_r is set to be the same as in Eq. (1). The pulling direction was chosen along the vector from fixed atom to pulled atom. The pulling speeds are set equal to $\nu = 3.6 \times 10^7$ and $4.55 \times 10^8 \text{ nm/s}$ which are about five to six orders of magnitude faster than those used in experiments.²⁸

III. NEW FORCE REPLICA EXCHANGE METHOD AND ITS APPLICATION

A. Force replica exchange method

The equilibration of long peptides at low temperatures is a computationally expensive job. In order to speed up computation of thermodynamic quantities we extend the standard replica exchange method (with replicas at different temperatures) developed for spin²² and peptide systems²³ to the case when the replica exchange is performed between states with different values of the external force $\{f_i\}$. Suppose for a

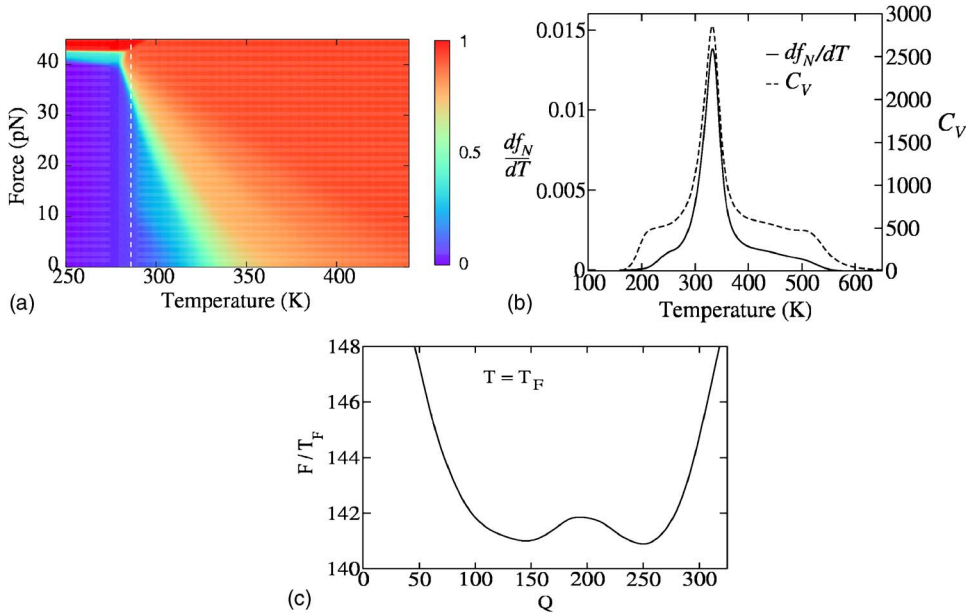


FIG. 2. (Color online) (a) The T - f phase diagram obtained by the extended replica exchange and histogram method. The force is applied to termini N and C. The color code for $1-f_N$ is given on the right. The folded state occurs at low forces and low temperatures. The vertical dashed line denotes $T=0.85T_F \approx 285$ K, at which most of simulations have been performed. (b) Temperature dependence of the specific heat C_V (right axis) and df_N/dT (left axis) at $f=0$. Their peaks coincide at $T=T_F$. (c) The dependence of the free energy of the trimer on the total number of native contacts Q at $T=T_F$.

given temperature we have M replicas $\{x_i, f_i\}$, where $\{x_i\}$ denotes coordinates and velocities of residues. Then the statistical sum of the extended ensemble is

$$Z = \int \cdots \int dx_1, \dots, dx_M \exp\left(-\sum_{i=1}^M \beta H(x_i)\right) = \prod_{i=1}^M Z(f_i). \quad (3)$$

The total distribution function has the following form:

$$P(\{x, f\}) = \prod_{i=1}^M P_{\text{eq}}(x_i, f_i), \quad (4)$$

$$P_{\text{eq}}(x, f) = Z^{-1}(f) \exp(-\beta H(x, f)).$$

For a Markov process the detailed balance condition reads as

$$P(\dots, x_m f_m, \dots, x_n f_n, \dots) W(x_m f_m | x_n f_n) = P(\dots, x_n f_n, \dots, x_m f_m, \dots) W(x_n f_n | x_m f_m), \quad (5)$$

where $W(x_m f_m | x_n f_n)$ is the rate of transition $\{x_m, f_m\} \rightarrow \{x_n, f_n\}$. Using

$$H(x, f) = H_0(x) - \mathbf{f}\mathbf{R}, \quad (6)$$

and Eq. (5) we obtain

$$\frac{W(x_m f_m | x_n f_n)}{W(x_n f_n | x_m f_m)} = \frac{P(\dots, x_m f_m, \dots, x_n f_n, \dots)}{P(\dots, x_n f_n, \dots, x_m f_m, \dots)} = \frac{\exp[-\beta(H_0(x_n) - \mathbf{f}_m \mathbf{R}_n) - \beta(H_0(x_m) - \mathbf{f}_n \mathbf{R}_m)]}{\exp[-\beta(H_0(x_m) - \mathbf{f}_m \mathbf{R}_m) - \beta(H_0(x_n) - \mathbf{f}_n \mathbf{R}_n)]} = \exp(-\Delta), \quad (7)$$

with

$$\Delta = \beta(\mathbf{f}_m - \mathbf{f}_n)(\mathbf{R}_m - \mathbf{R}_n). \quad (8)$$

This gives us the following Metropolis rule for accepting or rejecting the exchange between replicas f_n and f_m :

$$W(x f_m | x' f_n) = \begin{cases} 1, & \Delta < 0 \\ \exp(-\Delta), & \Delta > 0. \end{cases} \quad (9)$$

B. Application of the force replica exchange method to construction of the temperature-force phase diagram of three-domain ubiquitin

Since the three-domain Ub is rather long peptide (228 residues), we apply the replica exchange (RE) method to obtain its temperature-force phase diagram. We have performed two sets of the RE simulations. In the first set we fixed $f=0$ and the RE is carried out in the standard temperature replica space,²³ where 12 values of T were chosen in the interval $[0.46, 0.82]$ in such a way that the RE acceptance ratio was 15%–33%. This procedure speeds up the equilibration of our system nearly tenfold compared to the standard computation without the use of RE.

In the second set, the RE simulation was performed in the force replica space at $T=0.53$ using the Metropolis rule given by Eq. (9). We have also used 12 replicas with different values of f in the interval $0 \leq f \leq 0.6$ to get the acceptance ratio about 12%. Even for this modest acceptance rate our new RE scheme accelerates the equilibration of the three-domain ubiquitin about fourfold. One can expect better performance by increasing the number of replicas. However, within our computational facilities we were restricted to parallel runs on 12 processors for 12 replicas. The system was equilibrated during first $10^5 \tau_L$, after which histograms for the energy, the native contacts, and end-to-end distances were collected for $4 \times 10^5 \tau_L$. For each replica, we have generated 25 independent trajectories for thermal averaging. Using the data from two sets of the RE simulations and the extended

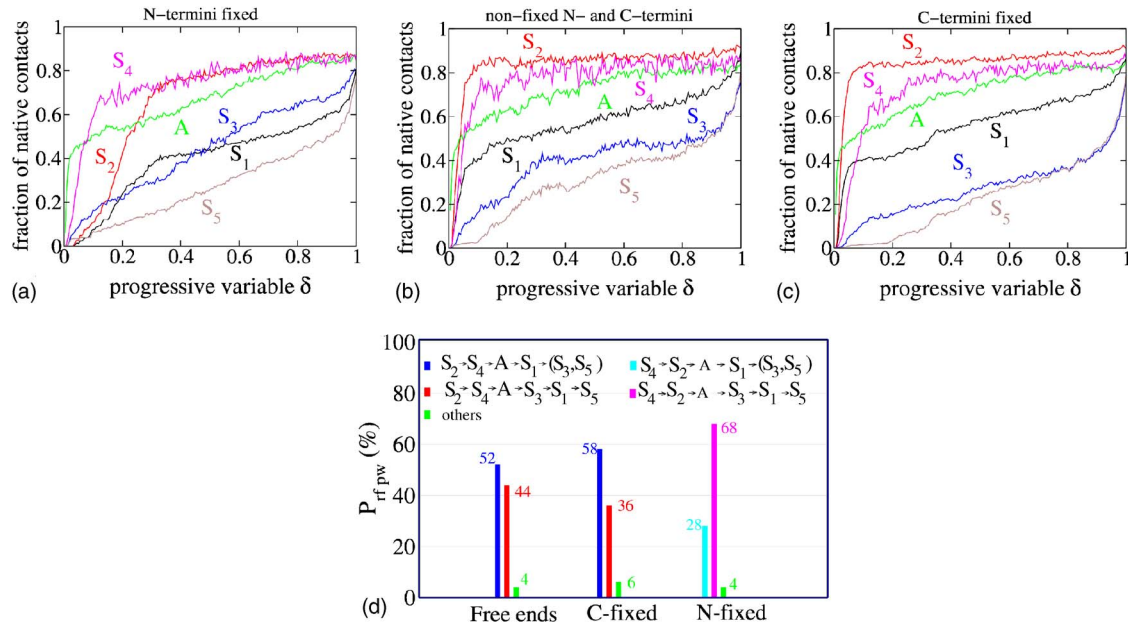


FIG. 3. (Color online) The dependence of native contacts of β strands and helix A on the progressive variable δ when (a) the N terminal is fixed, (b) both ends are free, and (c) C terminal is fixed. The results are averaged over 200 trajectories. (d) The probability of refolding pathways in three cases. Each value is written on top of the histograms.

reweighting technique³⁶ in the temperature and force space³⁷ we obtained the T - f phase diagram and the thermodynamic quantities of the trimer.

The T - f phase diagram [Fig. 2(a)] was obtained by monitoring the probability of being in the native state f_N as a function of T and f , where f_N is defined as an averaged fraction of native contacts (see Ref. 14 for more details). The folding-unfolding transition (the yellow region) is sharp in the low temperature region, but it becomes less cooperative (the fuzzy transition region is wider) as T increases. The folding temperature in the absence of force [peak of C_v or df_N/dT in Fig. 2(b)] is equal to $T_F = 0.64\epsilon_H/k_B$ which is a bit lower than $T_F = 0.67\epsilon_H/k_B$ of the single Ub.¹⁴ This reflects the fact that the folding of the trimer is less cooperative compared to the monomer due to a small number of native contacts between domains. One can ascertain this by calculating the cooperativity index Ω_c (Ref. 38 and 39) for the denaturation transition. From simulation data for df_N/dT presented in Fig. 2(b), we obtain $\Omega_c \approx 40$ which is indeed lower than $\Omega_c \approx 57$ for the single Ub (Ref. 14) obtained by the same Go model. According to our previous estimate,¹⁴ the experimental value $\Omega_c \approx 384$ is considerably higher than the Go value. The underestimation of Ω_c is not only a shortcoming of the off-lattice Go model⁴⁰ but also a common problem of much more sophisticated force fields in all-atom models.²⁴ Rigid lattice models with side chains give better results for the cooperativity^{40,41} but they are less realistic than the off-lattice ones. Although the present Go model does not provide the realistic estimate for cooperativity, it still mimics the experimental fact that folding of a multidomain protein remains cooperative observed for not only Ub but also other proteins.

Figure 2(c) shows the free energy as a function of native contacts at $T = T_F$. The folding/unfolding barrier is rather low (≈ 1 kcal/mol) and is comparable with the case of single

ubiquitin.¹⁴ The low barrier is probably an artifact of the simple Go modeling. The double minimum structure suggests that the trimer is a two-state folder.

IV. CAN THE FORCE-CLAMP TECHNIQUE PROBE REFOLDING PATHWAYS OF PROTEINS?

A. Refolding pathways of single ubiquitin

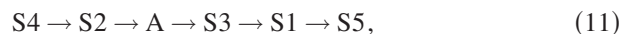
In order to study refolding under small quenched force we follow the same protocol as in the experiments.²⁰ First, a large force (≈ 130 pN) is applied to both termini to prepare the initial stretched conformations. This force is then released, but a weak quench force f_q is applied to study the refolding process. The refolding of a single Ub was studied^{14,21} in the presence or absence of the quench force. Fixing the N terminal was found to change the refolding pathways of the free-end Ub,²¹ but the effect of anchoring the C terminal has not been studied yet. Here we study this problem in detail, monitoring the time dependence of native contacts of secondary structures (Fig. 3). Since the quench force increases the folding time but leaves the folding pathways unchanged, we present only the results for $f_q = 0$ (Fig. 3). Interestingly, the fixed C-terminal and free-end cases have the identical folding sequencing

$$S_2 \rightarrow S_4 \rightarrow A \rightarrow S_1 \rightarrow (S_3, S_5). \quad (10)$$

This is the reverse of the unfolding pathway under thermal fluctuations.¹⁴ As discussed in detail by Li *et al.*,¹⁴ Eq. (10) partially agrees with the folding⁶ and unfolding¹⁰ experiments, and simulations.^{8,9,42} Our new finding here is that keeping the C terminal fixed does not change the folding pathways. One should keep in mind that the dominant pathway given by Eq. (10) is valid in the statistical sense. It occurs in about 52% and 58% of events for the free-end and C-anchored cases [Fig. 3(d)], respectively. The probability of

observing an alternate pathway ($S_2 \rightarrow S_4 \rightarrow A \rightarrow S_3 \rightarrow S_1 \rightarrow S_5$) is $\approx 44\%$ and is 36% for these two cases [Fig. 3(d)]. The difference between these two pathways is only in sequencing of S_1 and S_3 . Other pathways, denoted in green, are also possible but they are rather minor.

In the case when the N terminal is fixed (Fig. 3) we have the following sequencing:



which is, in agreement with Ref. 21, different from the free-end case. We present folding pathways as the sequencing of secondary structures, making comparison with experiments easier than an approach based on the time evolution of individual contacts.²¹ The main pathway [Eq. (11)] occurs in $\approx 68\%$ of events [Fig. 3(d)], while the competing sequencing $S_4 \rightarrow S_2 \rightarrow A \rightarrow S_1 \rightarrow (S_1, S_5)$ (28%) and other minor pathways are also possible. From Eq. (10) and (11) it follows that the force-clamp technique can probe the folding pathways of Ub if one anchors the C terminal but not the N one.

In order to check the robustness of our prediction for refolding pathways [Eqs. (10) and (11)], obtained for the friction $\zeta = 2(m/\tau_L)$, we have performed simulations for the water friction $\zeta = 50(m/\tau_L)$ (see Sec. II). Our results (not shown) demonstrate that although the folding time is about 20 times longer compared to the $\zeta = 2(m/\tau_L)$ case, the pathways remain the same. Thus, within the framework of Go modeling, the effect of the N-terminus fixation on refolding pathways of Ub is not an artifact of fast dynamics, occurring for both large and small frictions. It would be very interesting to verify our prediction using more sophisticated models. This question is left for future studies.

B. Refolding pathways of three-domain ubiquitin

The time dependence of the total number of native contacts, Q , R , and the gyration radius R_g , is presented in Fig. 4 for the trimer. The folding times τ_f were ≈ 553 and 936 ns for the free-end and N-fixed cases, respectively. The fact that anchoring one end slows down refolding by a factor of nearly 2 implies that diffusion-collision processes⁴³ play an important role in the Ub folding. Namely, as follows from the diffusion-collision model, the time required for formation contacts is inversely proportional to the diffusion coefficient D of a pair of spherical units. If one of them is idle, D is halved and the time needed to form contacts increases accordingly. The similar effect for unfolding was observed in our recent work.¹⁴

From the biexponential fitting, we obtain two time scales for collapsing (τ_1) and compaction (τ_2), where $\tau_1 < \tau_2$. For R , e.g., $\tau_1^R \approx 2.4$ ns and $\tau_2^R \approx 52.3$ ns if two ends are free, and $\tau_1^R \approx 8.8$ ns and $\tau_2^R \approx 148$ ns for the fixed-N case. Similar results hold for the time evolution of R_g . Thus, the collapse is much faster than the barrier limited folding process. Monitoring the time evolution of the end-to-end extension and of the number of native contacts, one can show (results not shown) that the refolding of the trimer is staircaselike, as observed in the simulations^{14,44} and the experiments.²⁰

Figure 5 shows the dependence of the number of native contacts of the secondary structures of each domain on δ for

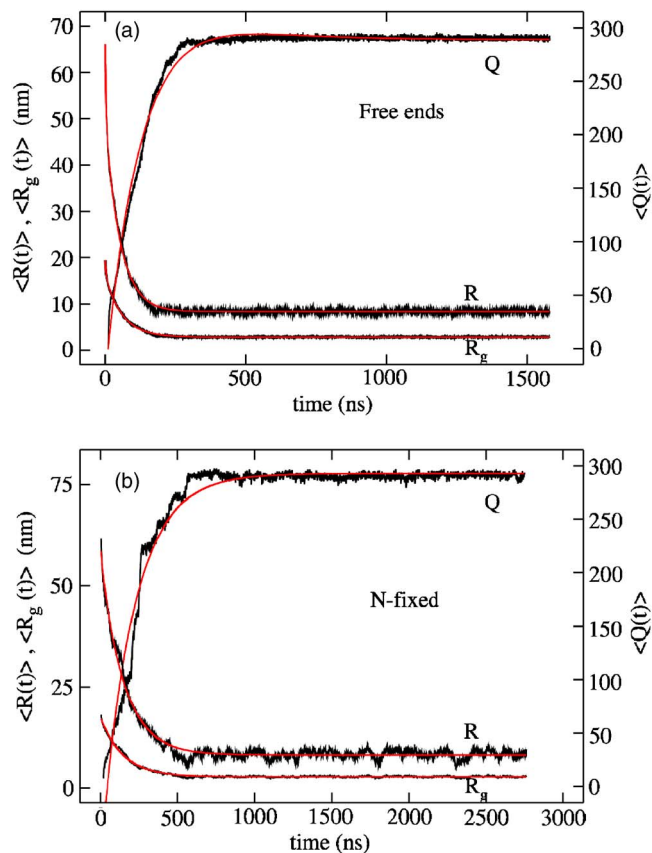


FIG. 4. (Color online) (a) The time dependence of Q , R , and R_g at $T = 285$ K for the free-end case. (b) The same as in (a) but for the N-fixed case. The smooth line is a biexponential fit $A(t) = A_0 + a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$. Results for the C-fixed case are similar to the N-fixed case and are not shown.

three situations: both termini are free and one or the other of them is fixed. In each of these cases the folding pathways of three domains are identical. Interestingly, they are the same, as given by Eq. (10), regardless of we keep one end fixed or not. As evident from Fig. 6, although the dominant pathway is the same for three cases its probabilities are different. It is equal the 68%, 44%, and 43% for the C-fixed, free-end, and N-fixed cases, respectively. For the last two cases, the competing pathway $S_2 \rightarrow S_4 \rightarrow A \rightarrow S_3 \rightarrow S_1 \rightarrow S_5$ has a reasonably high probability of $\approx 40\%$.

The irrelevance of one-end fixation for refolding pathways of a multidomain Ub may be understood as follows. Recall that applying the low quenched force to both termini does not change folding pathways of single Ub.¹⁴ So in the three-domain case, with the N end of the first domain fixed, both termini of the first and second domains are effectively subjected to external force, and their pathways should remain the same as in the free-end case. The N terminal of the third domain is tethered to the second domain but this would have much weaker effect compared to the case when it is anchored to a surface. Thus this unit has almost free ends and its pathways remain unchanged. Overall, the “boundary” effect gets weaker as the number of domains becomes bigger. In order to check this argument, we have performed simulations for the two-domain Ub. It turns out that the sequencing is roughly the same as in Fig. 5, but the common tendency is

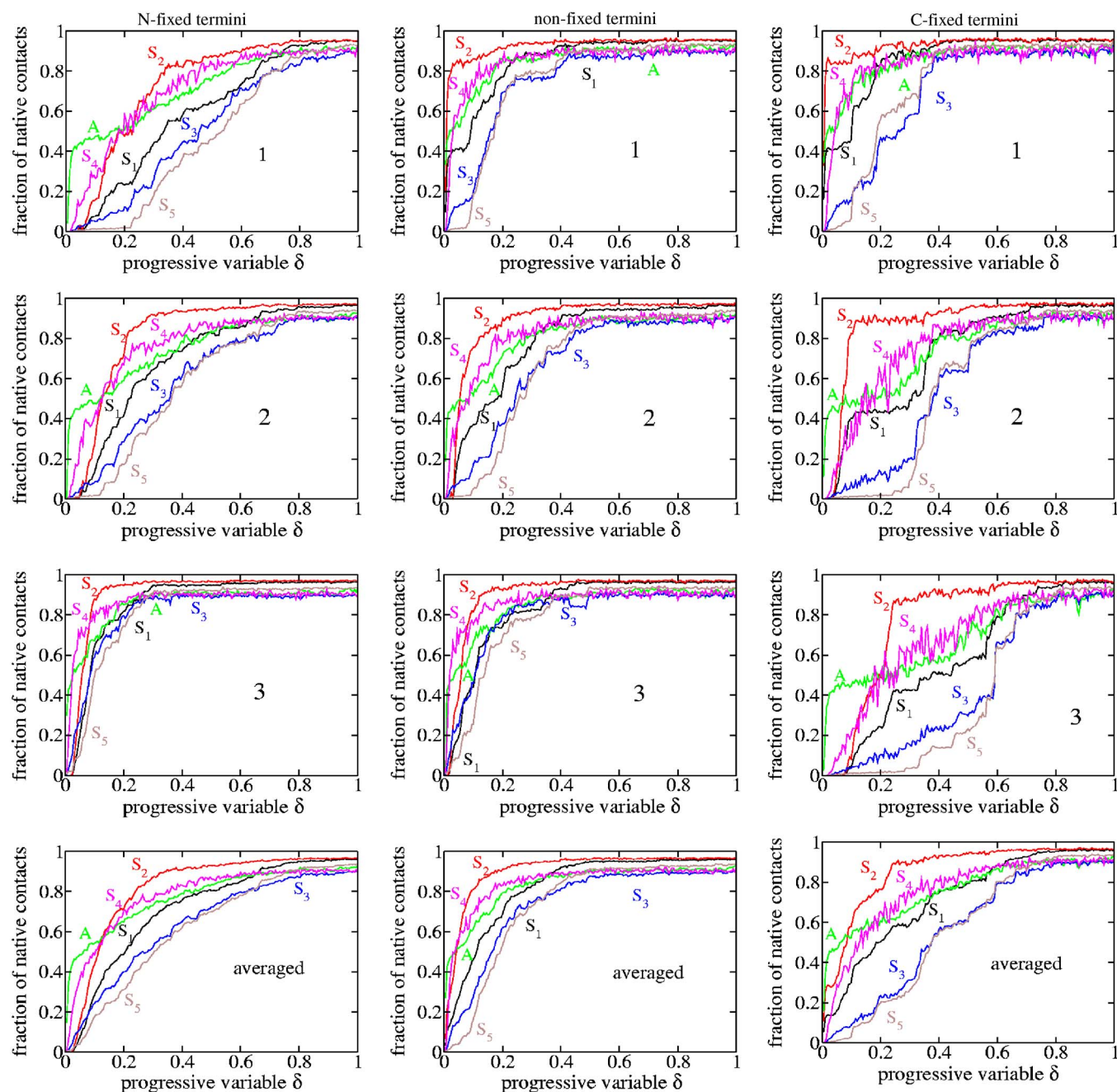


FIG. 5. (Color online) The same as in Fig. 3 but for the trimer. Numbers 1, 2, and 3 refer to the first, second, and third domains. The last row represents the results averaged over three domains. The fractions of native contacts of each secondary structure are averaged over 100 trajectories.

less pronounced (results not shown) compared to the trimer case. Thus we predict that the force-clamp technique can probe folding pathways of free Ub if one uses either the single domain with the C terminus anchored or the multidomain construction.

Although fixing one end of the trimer does not influence folding pathways of individual secondary structures, it affects the folding sequencing of individual domains (Fig. 7). We have the following sequencing: (1,3)→2, 3→2→1, and 1→2→3 for the free end, N terminal fixed, and C terminal fixed, respectively. These scenarios are supported by typical snapshots shown in Fig. 7. It should be noted that the domain at the free end folds first in all of three cases in statistical sense (also true for the two-domain case). As fol-

lows from the bottom of Fig. 7, if two ends are free then each of them folds first in about 40 out of 100 observations. The middle unit may fold first, but with much lower probability of about 15%. This value remains almost unchanged when one of the ends is anchored, and the probability that the nonfixed unit folds increases to $\geq 80\%$.

As shown by experiments²⁰ and simulations,^{14,27} one can use the dependence of refolding times τ_F on the quenched force to find the distance between the denatured state and transition state x_f along the end-to-end distance reaction coordinate. Namely, in the Bell approximation⁴⁵ $\tau_F = \tau_F^0 \exp(x_f f / k_B T)$, where τ_F^0 is the folding time in the absence of the external force. Then from Fig. 8 we obtain $x_f = 0.74 \pm 0.07$ nm for the three-domain Ub. Within the error

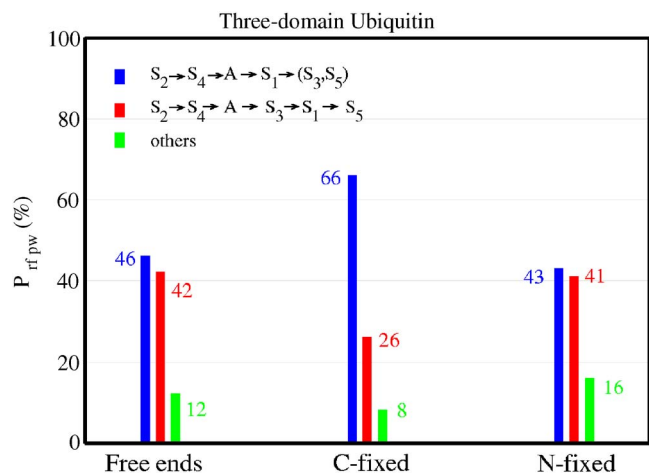


FIG. 6. (Color online) The probability of different refolding pathways for the trimer. Each value is shown on top of the histograms.

bars this value coincides with $x_f = 0.96 \pm 0.15$ nm obtained by the same Go model for the single Ub,¹⁴ and with the experimental result $x_f \approx 0.80$ nm.²⁰ Our results suggest that the multidomain structure leaves x_f almost unchanged.

C. Is the effect of fixing one terminus on refolding pathways universal?

We now ask if the effect of fixing one end on refolding pathway, observed for Ub, is also valid for other proteins? To

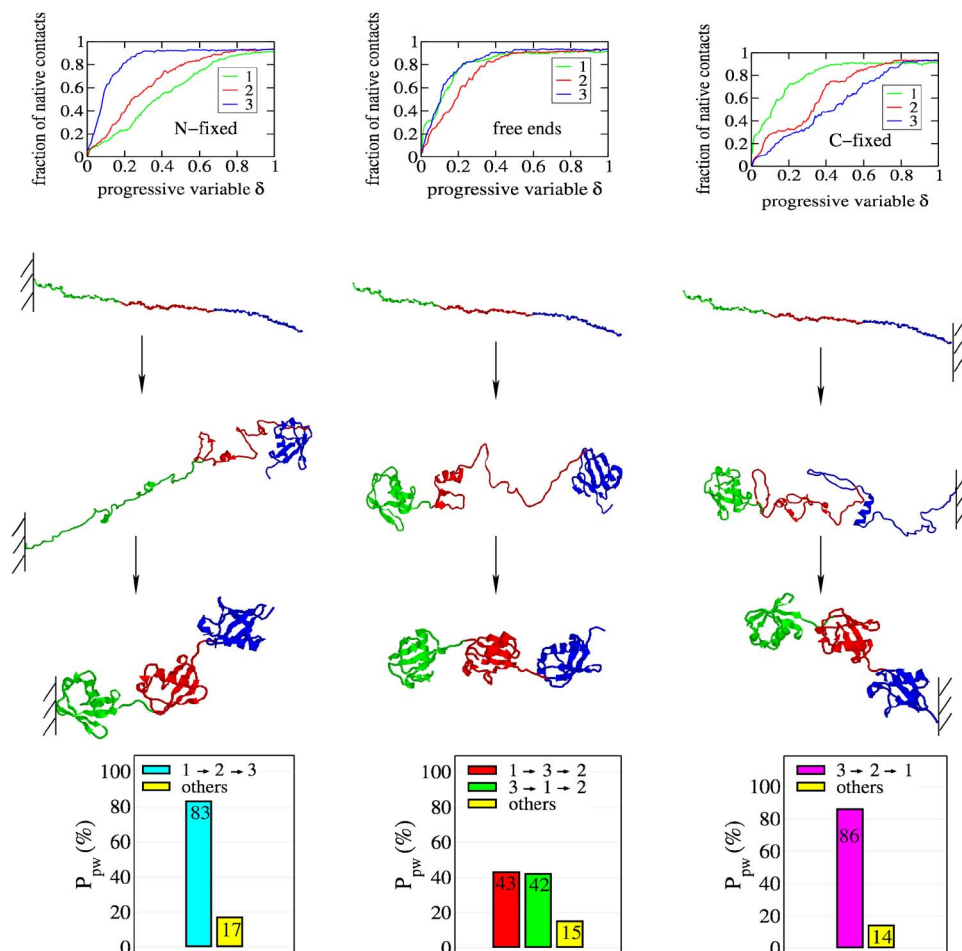


FIG. 7. (Color online) The dependence of the total number of native contacts on δ for the first, second, and third domains. Typical snapshots of the initial, middle, and final conformations for three cases when both two ends are free or one of them is fixed. The effect of anchoring one terminus on the folding sequencing of domains is clearly evident. In the bottom we show the probability of refolding pathways for three cases. Its value is written on top of the histograms.

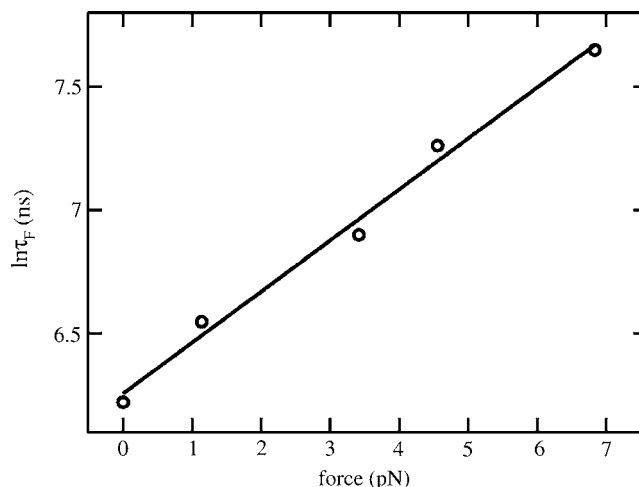


FIG. 8. The dependence of folding times of the trimer on f_q at $T=285$ K. τ_f was computed as the median first passage times of 30–50 trajectories for each value of f_q . From the linear fit $y=6.257+0.207x$, we obtained the average distance between the unfolded and transition states $x_f = 0.74 \pm 0.07$ nm.

answer this question, we study the single domain I27 from the muscle protein titin. We choose this protein as a good candidate from the conceptual point of view because its β -sandwich structure [see Fig. 9(a)] is very different from α/β structure of Ub. Moreover, because I27 is subject to mechanical stress under physiological conditions,⁴⁶ it is instructive to study refolding from extended conformations

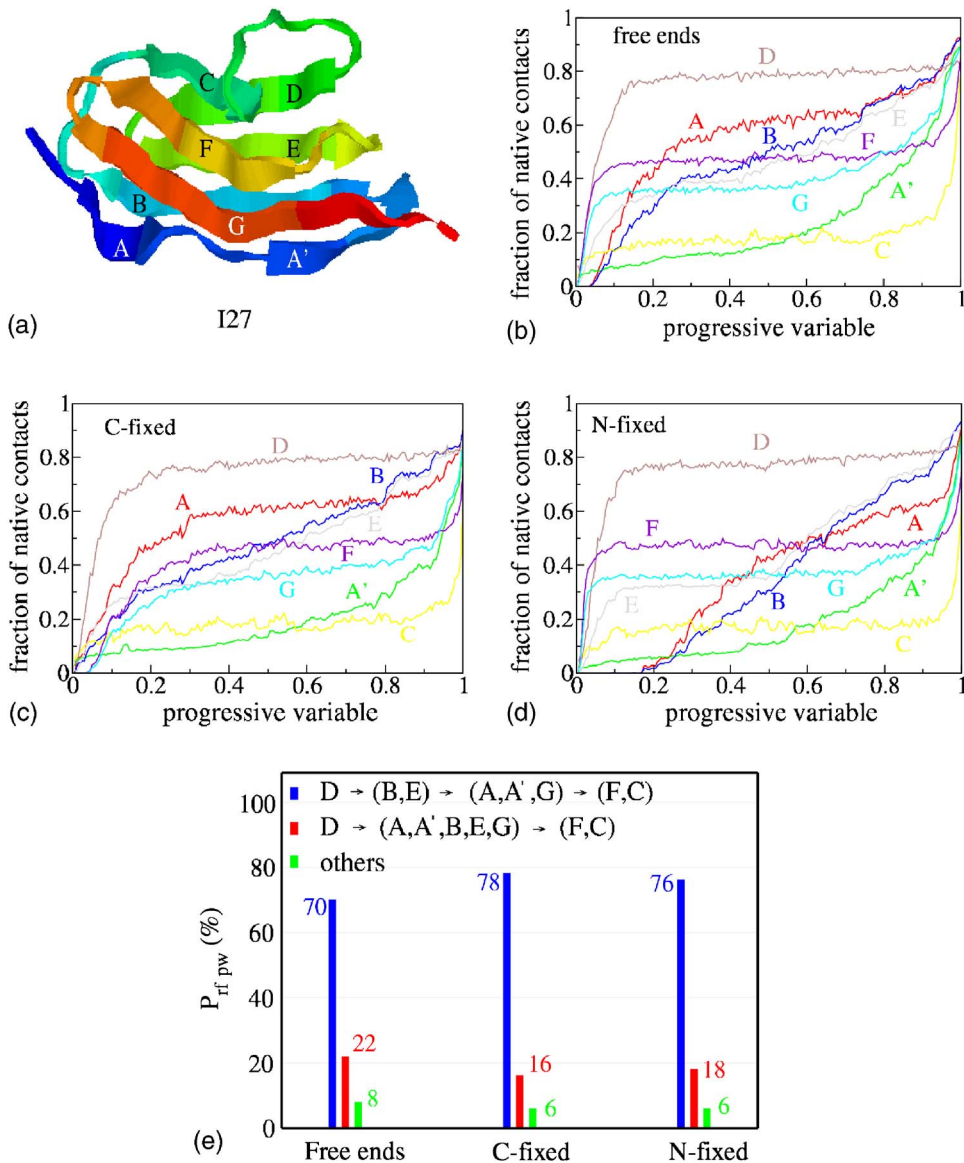


FIG. 9. (Color online) (a) Native state conformation of Ig27 domain of titin (PDB ID: 1tit). There are eight β strands: A (4–7), A' (11–15), B (18–25), C (32–36), D (47–52), E (55–61), F (69–75), and G (78–88). The dependence of native contacts of different β strands on the progressive variable δ for the case when (b) two ends are free, (c) the N terminus is fixed, and (d) the C terminal is anchored. (e) The probability of observing refolding pathways for three cases. Each value is written on top of the histograms.

generated by force. There have been extensive unfolding (see recent review⁴⁷ for references) and refolding²⁷ studies on this system, but the effect of one-end fixation on folding sequencing of individual secondary structures has not been considered either theoretically or experimentally.

As follows from Fig. 9(b), if two ends are free then strands A, B, and E fold at nearly the same rate. The pathways of the N-fixed and C-fixed cases are identical, and they are almost the same as in the free-end case except that strand A seems to fold after B and E. Thus, keeping the N terminus fixed has much weaker effect on the folding sequencing as compared to the single Ub. Overall the effect of anchoring one terminus has a little effect on the refolding pathways of I27, and we have the following common sequencing:

$$D \rightarrow (B,E) \rightarrow (A,G,A') \rightarrow F \rightarrow C \quad (12)$$

for all three cases. The probability of observing these main pathways varies between 70% and 78% [Fig. 9(e)]. The second pathway, $D \rightarrow (A,A',B,E,G) \rightarrow (F,C)$, has considerably lower probability. Other minor routes to the folded state are also possible.

Because the multidomain construction weakens this effect, we expect that the force-clamp spectroscopy can probe refolding pathways for a single and poly-I27. More importantly, our study reveals that the influence of fixation on refolding pathways may depend on the native topology of proteins.

V. SOME PROBLEMS OF UNFOLDING OF UBIQUITIN

A. Estimation of x_u and the unfolding barrier ΔG^\ddagger

In experiments one usually uses the Bell formula⁴⁵

$$\tau_U = \tau_U^0 \exp(-x_u f / k_B T) \quad (13)$$

to extract x_u for two-state proteins from the force dependence of unfolding times. Equation (13) is valid if the location of the transition state does not move under external force. Recently, assuming that x_u depends on external force and using the Kramers theory, Dudko *et al.* have tried to go beyond the Bell approximation and proposed the following force dependence for the unfolding time:²⁹

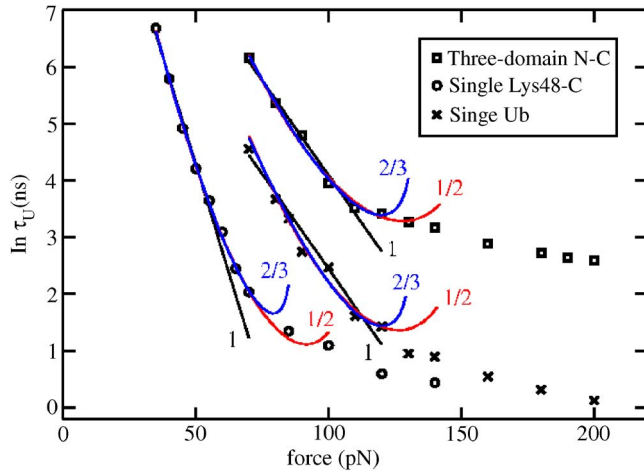


FIG. 10. (Color online) The semilog plot for the force dependence of unfolding times at $T=285$ K. Crosses and squares refer to the single Ub and the trimer with the force applied to N and C terminals, respectively. Circles refer to the single Ub with the force applied to Lys48 and C terminal. Depending on f , 30–50 folding events were used for averaging. In the Bell approximation, if the N and C terminals of the trimer are pulled then we have the linear fit $y=10.448-0.066x$ (straight line) and the distance between the native and transition states $x_u \approx 0.24$ nm. The same value of x_u was obtained for the single Ub (Ref. 14). In the case when we pull at Lys48 and C terminal of single Ub the linear fit (straight line) at low forces is $y=11.963-0.168x$ and $x_u=0.61$ nm. The correlation level of fitting is about 0.99. The curves marked by 1/2 and 2/3, correspond to the fits with $\nu=1/2$ and 2/3, respectively [Eq. (14)].

$$\tau_U = \tau_U^0 \left(1 - \frac{\nu x_u f}{\Delta G^\ddagger}\right)^{1-1/\nu} \exp \left\{ -\frac{\Delta G^\ddagger}{k_B T} \left[1 - (1 - \nu x_u f / \Delta G^\ddagger)^{1/\nu}\right] \right\}. \quad (14)$$

Here ΔG^\ddagger is the unfolding barrier and $\nu=1/2$ and $2/3$ for the cusp⁴⁸ and linear-cubic free energy surface,⁴⁹ respectively. Note that $\nu=1$ corresponds to the phenomenological Bell theory [Eq. (13)]. One important consequence, following from Eq. (14), is that one can apply this technique to estimate not only x_u but also G^\ddagger for $\nu=1/2$ and $2/3$. This will be done in this section for the single Ub and the trimer.

1. Single Ub

Using the Bell approximation, we have already estimated $x_u \approx 2.4$ Å,¹⁴ which is consistent with the experimental data $x_u=1.4-2.5$ Å.^{15,16,50} With the help of an all-atom simulation Li and Makarov *et al.*⁵¹ have shown that x_u does depend on f . At low forces, where the Bell approximation is valid,¹⁴ they obtained $x_u=10$ Å, which is noticeably higher than our and the experimental value. Presumably, this is due

to the fact that these authors computed x_u from equilibrium data, but their sampling was not good enough for such a long protein as Ub.

We now use Eq. (14) with $\nu=2/3$ and $\nu=1/2$ to compute x_u and ΔG^\ddagger . The regions, where the $\nu=2/3$ and $\nu=1/2$ fits work well, are wider than that for the Bell scenario (Fig. 10). However, these fits cannot cover the entire force interval. The values of τ_U^0 , x_u , and ΔG^\ddagger obtained from the fitting procedure are listed in Table I. In accord with Ref. 29 all of these quantities increase with decreasing ν . In our opinion, the microscopic theory ($\nu=2/3$ and $\nu=1/2$) gives too high a value for x_u compared to its typical experimental value.^{15,16,50} However, the latter was calculated from fitting experimental data to the Bell formula, and it is not clear how much the microscopic theory would change the result.

In order to estimate the unfolding barrier of Ub from the available experimental data and compare it with our theoretical estimate, we use the following formula:

$$\Delta G^\ddagger = -k_B T \ln(\tau_A / \tau_U^0), \quad (15)$$

where τ_U^0 denotes the unfolding time in the absence of force and τ_A is a typical unfolding prefactor. Since τ_A for unfolding is not known, we use the typical value for folding $\tau_A=1$ μs.^{52,53} Using $\tau_U^0=10^4/4$ s (Ref. 54) and Eq. (15) we obtain $\Delta G^\ddagger=21.6k_B T$ which is in reasonable agreement with our result $\Delta G^\ddagger \approx 17.4k_B T$, followed from the microscopic fit with $\nu=1/2$. Using the GB/SA continuum solvation model⁵⁵ and the CHARMM27 force field⁵⁶ Li and Makarov^{51,57} obtained a much higher value $\Delta G^\ddagger=29$ kcal/mol $\approx 48.6k_B T$. Again, the large departure from the experimental result may be related to poor sampling or to the force field they used.

2. The effect of linkage on x_u for single Ub

One of the most interesting experimental results of Carrion-Vazquez *et al.*¹⁵ is that pulling Ub at different positions changes x_u drastically. Namely, if the force is applied at the C terminal and Lys48, then in the Bell approximation $x_u \approx 6.3$ Å, which is about two and half times larger than the case when the termini N and C are pulled. Using the all-atom model Li and Makarov⁵¹ have shown that x_u is much larger than 10 Å. Thus, a theoretical reliable estimate for x_u of Lys48-C Ub is not available. Our aim is to compute x_u employing the present Go-type model²⁶ as it is successful in predicting x_u for the N-C Ub. Figure 10 shows the force dependence of unfolding time of the fragment Lys48-C when the force is applied to Lys48 and C terminus. The unfolding time is defined as the averaged time to stretch this fragment. From the linear fit ($\nu=1$ in Fig. 10) at low forces we obtain

TABLE I. Dependence of x_u on fitting procedures for the three-domain Ub and Lys48-C. $\nu=1$ corresponds to the phenomenological Bell approximation [Eq. (13)]. $\nu=1/2$ and $2/3$ refer to the microscopic theory [Eq. (14)]. For comparison we also show the data for the single Ub which are taken from our previous work (Ref. 14). For the single and three-domain Ub the force is applied to both termini.

	Single			Lys48-C			Three domain		
ν	1/2	2/3	1	1/2	2/3	1	1/2	2/3	1
τ_U^0 (μs)	13 200	1289	9.1	4627	2304	157	1814	756	47
x_u (Å)	7.92	5.86	2.4	12.35	10.59	6.1	6.21	5.09	2.4
ΔG^\ddagger ($k_B T$)	17.39	14.22	...	15.90	13.94	...	13.49	11.64	...

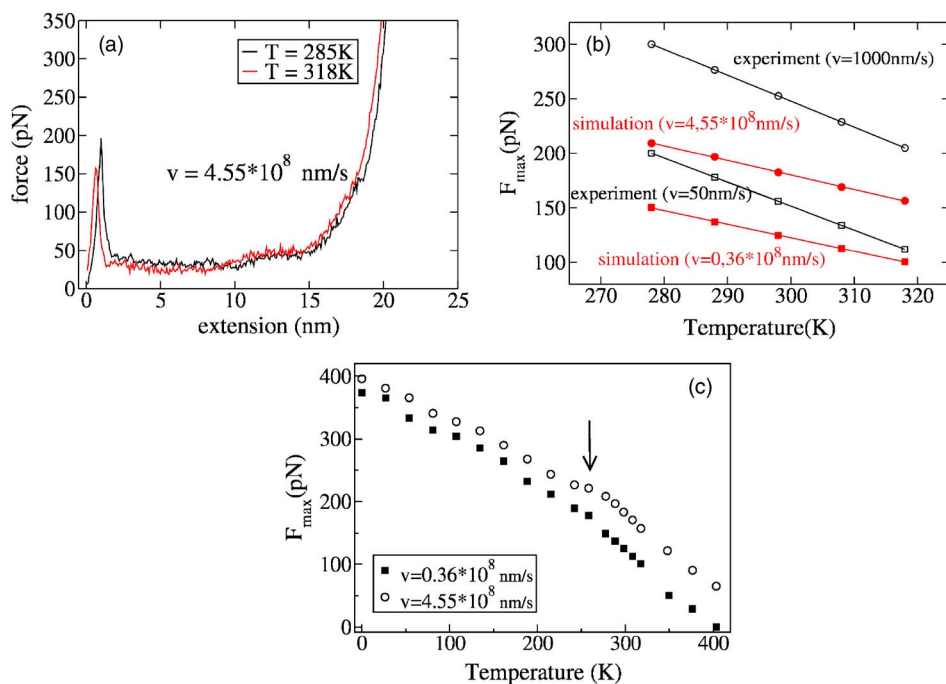


FIG. 11. (Color online) (a) The force-extension profile obtained at $T = 285$ K and 318 K at the pulling speed $v = 4.55 \times 10^8$ nm/s. f_{\max} is located at the extension ≈ 1 and 0.6 nm for $T = 285$ and 318 K, respectively. The results are averaged over 50 independent trajectories. (b) The dependence of F_{\max} on temperature for two values of v . The experimental data are taken from Ref. 28 for comparison. The linear fits for the simulations are $y = 494.95 - 1.241x$ and $y = 580.69 - 1.335x$. For the experimental sets we have $y = 811.6 - 2.2x$ and $y = 960.25 - 2.375x$. (c) The dependence temperature of F_{\max} for the whole temperature region and two values of v . The arrow marks the crossover between two nearly linear regimes.

$x_u \approx 0.61$ nm which is in good agreement with the experiment.¹⁵ The Go model is suitable for estimating x_u for not only Ub but also for other proteins⁵⁸ because the unfolding is mainly governed by the native topology. The fact that x_u for the linkage Lys48-C is larger than that of the N-C Ub may be understood using our recent observation⁵⁸ that it anticorrelates with the contact order (CO).⁵⁹ Defining contact formation between any two amino acids ($|i-j| \geq 1$) as occurring when the distance between the centers of mass of side chains $d_{ij} \leq 6.0$ Å (see also Ref. 60), we obtain COs equal to 0.075 and 0.15 for the Lys48-C and N-C Ub, respectively. Thus, x_u of the Lys48-C linkage is larger than that of the N-C case because its CO is smaller. This result suggests that the anticorrelation between x_u and CO may hold not only when proteins are pulled at termini,⁵⁸ but also when the force is applied to different positions. Note that the linker (not linkage) effect on x_u has been studied for protein L.⁶¹ It seems that this effect is less pronounced compared to the effect caused by changing pulling direction studied here. We have carried out the microscopic fit for $\nu = 1/2$ and $2/3$ (Fig. 10). As in the N-C Ub case, x_u is larger than its Bell value. However, the linkage at Lys48 has a little effect on the activation energy ΔG^\ddagger (Table I).

3. Determination of x_u for the three-domain ubiquitin

Since the trimer is a two-state folder [Fig. 2(c)], one can determine its averaged distance between the native state and transition state x_u along the end-to-end distance reaction coordinate using kinetic theory.^{29,45} We now ask if the multidomain structure of Ub changes x_u . As in the single Ub case,¹⁴ there exists a critical force $f_c \approx 120$ pN separating the low force and high force regimes (Fig. 10). In the high force region, where the unfolding barrier disappears, the unfolding time depends on f linearly (fitting curve not shown) as predicted theoretically by Evans and Ritchie.⁶² In the Bell approximation, from the linear fit (Fig. 10) we obtain x_u

≈ 0.24 nm which is exactly the same as for the single Ub.¹⁴ The multidomain construction of Ub does not affect x_u , but this may be not the case for other proteins.⁶³ The values of τ_U^0 , x_u , and ΔG^\ddagger , extracted from the nonlinear fit (Fig. 10), are presented in Table I. For both $\nu = 1/2$ and $\nu = 2/3$, ΔG^\ddagger is a bit lower than that for the single Ub. In the Bell approximation, the value of x_u is the same for the single and three-domain Ub but it is no longer valid for the $\nu = 2/3$ and $\nu = 1/2$ cases. It would be interesting to perform experiments to check this result and to see the effect of multiple domain structure on the free energy landscape.

B. Dependence of unfolding force of single Ub on T

Recently, using the improved temperature control technique to perform the pulling experiments for the single Ub, Yang *et al.*²⁸ have found that the unfolding force depends on T linearly for $278 \text{ K} \leq T \leq 318 \text{ K}$, and the slope of linear behavior does not depend on pulling speeds. Our goal is to see if the present Go model can reproduce this result at least qualitatively, and more importantly, to check whether the linear dependence holds for the whole temperature interval where $f_{\max} > 0$.

The pulling simulations have been carried out at two speeds following the protocol described in Sec. II. Figure 11(a) shows the force-extension profile of the single Ub for $T = 288$ and 318 K at the pulling speed $v = 4.55 \times 10^8$ nm/s. The peak is lowered as T increases because thermal fluctuations promote the unfolding of the system. In addition the peak moves toward a lower extension. This fact is also understandable, because at higher T a protein can unfold at lower extensions due to thermal fluctuations. For $T = 318$ K, e.g., the maximum force is located at the extension of ≈ 0.6 nm, which corresponds to the plateau observed in the time dependence of the end-to-end distance under constant force.^{14,18} One can show that, in agreement with Chyan *et al.*,⁵⁰ at this maximum the extension between strands S_1

and S_5 is ≈ 0.25 nm. Beyond the maximum, all of the native contacts between strands S_1 and S_5 are broken. At this stage, the chain ends are almost stretched out, but the rest of the polypeptide chain remains natively like.

The temperature dependence of the unfolding force, f_{\max} , is shown in Fig. 11(b) for $278 \text{ K} \leq T \leq 318 \text{ K}$, and for two pulling speeds. The experimental results of Yang *et al.* are also presented for comparison. Clearly, in agreement with experiments²⁸ linear behavior is observed and the corresponding slopes do not depend on v . Using the fit $f_{\max} = f_{\max}^0 - \gamma T$ we obtain the ratio between the simulation and experimental slopes $\gamma_{\text{sim}}/\gamma_{\text{exp}} \approx 0.56$. Thus, the Go model gives a weaker temperature dependence compared to the experiments. Given the simplicity of this model, the agreement between theory and experiment should be considered reasonable, but it would be interesting to check if a fuller accounting of non-native contacts and environment can improve our results.

As evident from Fig. 11(c), the dependence of f_{\max} on T ceases to be linear for the whole temperature interval. The nonlinear temperature dependence of f_{\max} may be understood qualitatively using the simple theory of Evans and Ritchie.⁶¹ Assuming the Bell [Eq. (13)] for the unfolding time and a linearly ramped force $f = K_r vt$ (See Sec. II) the unfolding force is given by $f_{\max} = (k_B T/x_u) \ln(K_r v x_u \tau_U^0/k_B T)$. A more complicated microscopic expression for f_{\max} is provided in Ref. 29. Since τ_U^0 is temperature dependent (x_u also displays a weak temperature dependence⁶⁴), the resulting dependence should be nonlinear. This result can also be understood by noting that the temperatures considered here are low enough so that we are not in the entropic limit, where the linear dependence would be valid for the wormlike model.⁶⁵ The arrow in Fig. 11(c) separates two regimes of the T dependence of f_{\max} . The crossover takes place roughly in the temperature interval where the temperature dependence of the equilibrium critical force changes the slope (Fig. 2). At low temperatures, thermal fluctuations are weak and the temperature dependence of f_{\max} is weaker compared to the high temperature regime. Thus the linear dependence observed in the experiments of Yang *et al.*²⁸ is valid, but only in the narrow T interval.

VI. CONCLUSIONS

We have adapted the standard temperature RE method to the case when the force replicas are considered at a fixed temperature. One can extend the RE method to cover both temperature and force replicas, as has been done for all-atom simulations⁶⁶ where pressure is used instead of force. One caveat of the force RE method is that the acceptance depends on the end-to-end distance [Eqs. (8) and (9)], and becomes inefficient for long proteins. We can overcome this by increasing the number of replicas, but this will increase CPU time substantially. Thus, the question of improving the force RE approach for long biomolecules remains open.

It has been clearly shown that the secondary structures have the same folding pathways for all domains, and this is probably the reason why poly-Ub folds cooperatively. The folding sequencing of individual domains depends on

whether one end is kept fixed or not. We predict that the force-clamp technique gives the same folding pathways for individual secondary structures as the free-end case, if one anchors either the C terminal of the single Ub or performs the experiments on the polydomain Ub. It would be very interesting to verify our prediction experimentally. Another exciting challenge is to see if the force-clamp technique can probe the folding sequencing of other biomolecules using a multidomain construction.

We have demonstrated that the anchoring of one terminal has a minor effect on refolding pathways of I27. This is probably due to the rigidity of its β -sandwich structure. It would be interesting to check if this conclusion holds for other β proteins. The question of to what extent the force-clamp technique is useful for predicting pathways for α proteins is left for future studies.

We have shown that, in agreement with the experiment of Carrion-Vazquez *et al.*,¹⁵ the Lys48-C linkage changes x_u drastically. From the point of view of biological function, the linkage Lys63-C is very important, but the study of its mechanical properties is not as interesting as the Lys48-C because this fragment is almost stretched out in the native state. Similar to titin and RNA, x_u and ΔG^\ddagger are sensitive to the parameter v , and one has to be very careful in the interpretation of experimental data. When comparing theoretical results with experiments, the same fitting procedure should be used. The weak interdomain interaction has a negligible effect on x_u in the Bell approximation, but changes become substantial in the nonlinear approximation ($\nu=1/2$ and $2/3$). The validity of this observation for other biomolecules requires further investigation. Using the microscopic fitting with $\nu=1/2$ we have obtained the reasonable value for ΔG^\ddagger for the single Ub. This result suggests that the Go modeling is useful for estimating unfolding barriers of proteins.

Finally, we have reproduced an experiment²⁸ of the linear temperature dependence of unfolding force of Ub on the quasiquantitative level. Moreover, we have shown that for the whole temperature region the dependence of f_{\max} on T is nonlinear, and the observed linear dependence is valid only for a narrow temperature interval. This behavior should be common for all proteins because it reflects the fact that the entropic limit is not applicable to all temperatures.

ACKNOWLEDGMENTS

M.S.L. thanks D.K. Klimov, G. Morrison, M. Schlierf, and D. Thirumalai for very useful discussions. This work was supported by Polish KBN Grant No. 1P03B01827, National Science Council (Taipei) Grant No. NSC 96-2911-M 001-003-MY3, Academia Sinica (Taipei) Grant No. AS-95-TP-A07, and National Center for Theoretical Sciences in Taiwan.

¹J. S. Thrower, L. Hoffman, M. Rechsteiner, and C. M. Pickart, *EMBO J.* **19**, 94 (2000).

²T. Kirisako, K. Kamei, S. Murata, M. Kato, H. Fukumoto, M. Kanie, S. Sano, F. Tokunaga, K. Tanaka, and K. Iwai, *EMBO J.* **25**, 4877 (2006).

³R. M. Hofmann and C. M. Pickart, *Cell* **96**, 645 (1999).

⁴J. Spence, R. R. Gali, G. Dittmar, and D. Finely, *Cell* **102**, 67 (2000).

⁵J. M. Galan and R. Haguenaer-Tsapis, *EMBO J.* **16**, 5847 (1997).

⁶H. M. Went and S. E. Jackson, *Protein Engineering Design and Selection*

- 18**, 229 (2005).
- ⁷ T. R. Sosnick, B. A. Kratz, R. S. Dothager, and M. Baxa, *Chem. Rev.* (Washington, D.C.) **106**, 1862 (2006).
- ⁸ A. Fernandez, *J. Phys. Chem.* **114**, 2489 (2001).
- ⁹ J. M. Sorenson and T. Head-Gordon, *Proteins: Struct., Funct., Genet.* **46**, 368 (2002).
- ¹⁰ F. Cordier and S. Grzesiek, *J. Mol. Biol.* **315**, 739 (2002).
- ¹¹ H. S. Chung, M. Khalil, A. W. Smith, Z. Ganim, and A. Tomakoff, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 612 (2005).
- ¹² D. O. V. Alonso and V. Daggett, *Protein Sci.* **7**, 860 (1998).
- ¹³ A. Irback and S. Mitternacht, *Proteins: Struct., Funct., Bioinf.* **65**, 759 (2006).
- ¹⁴ M. S. Li, M. Kouza, and C. K. Hu, *Biophys. J.* **92**, 547 (2007).
- ¹⁵ M. Carrion-Vazquez, H. Li, H. Lu, P. E. Marszalek, A. F. Oberhauser, and J. M. Fernandez, *Nat. Struct. Biol.* **10**, 738 (2003).
- ¹⁶ M. Schlierf, H. Li, and J. M. Fernandez, *Proc. Natl. Acad. Sci. U.S.A.* **101**, 7299 (2004).
- ¹⁷ D. K. West, D. J. Brockwell, P. D. Olmsted, S. E. Radford, and E. Paci, *Biophys. J.* **90**, 287 (2006).
- ¹⁸ A. Irback, S. Mitternacht, and S. Mohanty, *Proc. Natl. Acad. Sci. U.S.A.* **102**, 13427 (2005).
- ¹⁹ J. Brujic, R. I. Hermans, K. A. Walther, and J. M. Fernandez, *Nat. Phys.* **2**, 282 (2006).
- ²⁰ J. M. Fernandez and H. Li, *Science* **303**, 1674 (2004).
- ²¹ M. Cieplak and P. Szymczak, *J. Chem. Phys.* **124**, 194901 (2006).
- ²² K. Hukushima and K. Nemoto, *J. Phys. Soc. Jpn.* **65**, 1604 (1996).
- ²³ Y. Sugita and Y. Okamoto, *Chem. Phys. Lett.* **314**, 141 (1999).
- ²⁴ P. H. Nguyen, G. Stock, E. Mittag, C. K. Hu, and M. S. Li, *Proteins: Struct., Funct., Bioinf.* **61**, 795 (2005).
- ²⁵ P. C. Li, L. Huang, and D. E. Makarov, *J. Phys. Chem. B* **110**, 14469 (2006).
- ²⁶ C. Clementi, H. Nymeyer, and J. N. Onuchic, *J. Mol. Biol.* **298**, 937 (2000).
- ²⁷ M. S. Li, C. K. Hu, D. K. Klimov, and D. Thirumalai, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 93 (2006).
- ²⁸ Y. Yang, F. C. Lin, and G. Yang, *Rev. Sci. Instrum.* **77**, 063701 (2006).
- ²⁹ O. K. Dudko, G. Hummer, and A. Szabo, *Phys. Rev. Lett.* **96**, 108101 (2006).
- ³⁰ S. T. Thomas, V. V. Loladze, and G. I. Makhatadze, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10670 (2001).
- ³¹ M. P. Allen and D. J. Tildesley, *Computer Simulations of Liquids* (Oxford Science, Oxford, UK, 1987).
- ³² M. Kouza, C. F. Chang, S. Hayryan, T. H. Yu, M. S. Li, T. H. Huang, and C. K. Hu, *Biophys. J.* **89**, 3353 (2005).
- ³³ W. C. Swope, H. C. Andersen, P. H. Berens, and K. R. Wilson, *J. Chem. Phys.* **76**, 637 (1982).
- ³⁴ T. Veitshans, D. K. Klimov, and D. Thirumalai, *Folding Des.* **2**, 1 (1997).
- ³⁵ H. Lu, B. Isralewitz, A. Krammer, V. Vogel, and K. Schulten, *Biophys. J.* **75**, 662 (1998).
- ³⁶ A. M. Ferrenberg and R. H. Swendsen, *Phys. Rev. Lett.* **63**, 1195 (1989).
- ³⁷ D. K. Klimov and D. Thirumalai, *J. Phys. Chem. B* **105**, 6648 (2001).
- ³⁸ D. K. Klimov and D. Thirumalai, *Folding Des.* **3**, 127 (1998).
- ³⁹ M. S. Li, D. K. Klimov, and D. Thirumalai, *Phys. Rev. Lett.* **93**, 268107 (2004).
- ⁴⁰ M. Kouza, M. S. Li, C. K. Hu, E. P. O'Brien, Jr., and D. Thirumalai, *J. Phys. Chem. A* **110**, 671 (2006).
- ⁴¹ M. S. Li, D. K. Klimov, and D. Thirumalai, *Physica A* **350**, 38 (2005).
- ⁴² A. Fernandez, *Proteins* **47**, 447 (2002).
- ⁴³ M. Karplus and D. L. Weaver, *Nature (London)* **260**, 404 (1976).
- ⁴⁴ R. R. Best and G. Hummer, *Science* **308**, 498 (2005).
- ⁴⁵ G. I. Bell, *Science* **100**, 618 (1978).
- ⁴⁶ H. P. Erickson, *Science* **276**, 1090 (1997).
- ⁴⁷ M. Sotomayor and K. Schulten, *Science* **316**, 1144 (2007).
- ⁴⁸ G. Hummer and A. Szabo, *Biophys. J.* **85**, 5 (2003).
- ⁴⁹ O. K. Dudko, A. E. Filippov, J. Klafter, and M. Urbakh, *Proc. Natl. Acad. Sci. U.S.A.* **100**, 11378 (2003).
- ⁵⁰ C. Chyan, F. Lin, H. Peng, J. Yuan, C. Chang, S. Lin, and G. Yang, *Biophys. J.* **87**, 3995 (2004).
- ⁵¹ P. C. Li and D. E. Makarov, *J. Chem. Phys.* **121**, 4826 (2004).
- ⁵² M. S. Li, D. K. Klimov, and D. Thirumalai, *Polymer* **45**, 573 (2004).
- ⁵³ B. Schuler, E. A. Lipman, and W. A. Eaton, *Nature (London)* **419**, 743 (2002).
- ⁵⁴ S. Khorasanizadeh, I. D. Peters, T. R. Butt, and H. Roder, *Biochemistry* **32**, 7054 (1993).
- ⁵⁵ D. Qui, P. S. Shenkin, F. P. Hollinger, and W. C. Still, *J. Phys. Chem. A* **101**, 3005 (1997).
- ⁵⁶ A. D. MacKerell, D. Bashford, M. Bellott, R. L. Dunbrack, J. D. Evanseck, M. J. Field, S. Fischer, J. Gao, H. Guo, S. Ha, D. Joseph-McCarthy, L. Kuchnir, K. Kuczera, F. T. K. Lau, C. Mattos, S. Michnick, T. Ngo, D. T. Nguyen, B. Prodhom, W. E. Reiher, B. Roux, M. Schlenkrich, J. C. Smith, R. Stote, J. Straub, M. Watanabe, J. Wiorcikiewicz-Kuczera, D. Yin, and M. Karplus, *J. Phys. Chem. B* **102**, 3586 (1998).
- ⁵⁷ P. C. Li and D. E. Makarov, *J. Phys. Chem. B* **108**, 745 (2004).
- ⁵⁸ M. S. Li, *Biophys. J.* **93**, 2644 (2007).
- ⁵⁹ K. W. Plaxco, K. T. Simons, and D. Baker, *J. Mol. Biol.* **277**, 985 (1998). http://depts.washington.edu/bakerpg/contact_order/
- ⁶⁰ D. K. West, E. Paci, and P. D. Olmsted, *Phys. Rev. E* **74**, 061912 (2006).
- ⁶¹ E. Evans and K. Ritchie, *Biophys. J.* **72**, 1541 (1997).
- ⁶² M. S. Li, "Free energy landscapes of multi-state proteins," preprint.
- ⁶³ A. Imparato, A. Pelizzola, and M. Zamparo, *Phys. Rev. Lett.* **98**, 148102 (2007).
- ⁶⁴ J. F. Marko and E. D. Siggia, *Macromolecules* **28**, 8759 (1995).
- ⁶⁵ D. Paschek and A. E. Garcia, *Phys. Rev. Lett.* **93**, 238105 (2004).