Mechanical unfolding and refolding pathways of ubiquitin

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Mechanical unfolding and refolding of ubiquitin are studied by Monte Carlo simulations of a Gō model with binary variables. The exponential dependence of the time constants on the force is verified, and folding/unfolding lengths are computed, with good agreement with experimental results. Furthermore, the model exhibits intermediate kinetic states, as observed in experiments. Unfolding and refolding pathways and intermediate states, obtained by tracing single secondary structure elements, are consistent with simulations of previous all-atom models and with the experimentally observed step sizes.

Understanding folding and unfolding pathways remains one of the major challenges in protein science. Thermal and chemical denaturation have been studied for decades, and in recent years new experimental techniques, where single molecules are manipulated by atomic force microscopy and optical tweezers, collectively referred to as force spectroscopy [1, 2, 3, 4, 5, 6], have given a new perspective on this problem. In typical experiments a protein is pulled by its ends, and the unfolding and refolding processes are monitored by measuring its end-to-end length as a function of time. In force clamp experiments [5, 6] the force is kept constant by a feedback system. To unfold a molecule, the force is suddenly increased from a small to a large value, and the reverse is done to let it refold. Typically, unfolding and refolding turn out to be two-state processes with a characteristic time which depends exponentially on the force according to an Arrhenius–like law, with a distance from the extended to the folded state. The folding time turns out to follow an Arrhenius–like law, with a distance from the native to the transition state equal to $x_u = 0.24$ nm and $x_f = 0.96$ nm. They did not find an unfolding intermediate, and attributed this to the lack of non–native interactions. They distinguished three cases, where the force is applied to (a) both termini, (b) N–terminus only, and (c) C–terminus only. In cases (a) and (c) they obtained that the secondary structure elements (SSEs) break in the order $\beta_1 \rightarrow \beta_2 \rightarrow \beta_3 \rightarrow \beta_4 \rightarrow \alpha_1$, while in (b) they found $\beta_3 \rightarrow \beta_5 \rightarrow \beta_4 \rightarrow \beta_1 \rightarrow \beta_2 \rightarrow \alpha$.

Li et al. [10] verified, using molecular dynamics (MD) simulations of a $C_\alpha$ Gō model, that unfolding and refolding times depend exponentially on the force, with $x_u = 0.24$ nm and $x_f = 0.96$ nm. They did not find an unfolding intermediate, and attributed this to the lack of non–native interactions. They distinguished three cases, where the force is applied to (a) both termini, (b) N–terminus only, and (c) C–terminus only. In cases (a) and (c) they obtained that the secondary structure elements (SSEs) break in the order $\beta_1 \rightarrow \beta_2 \rightarrow \beta_3 \rightarrow \beta_4 \rightarrow \alpha_1$, while in (b) they found $\beta_3 \rightarrow \beta_5 \rightarrow \beta_4 \rightarrow \beta_1 \rightarrow \beta_2 \rightarrow \alpha$.

Kleiner and Shakhnovich [11] found, using MC simulations of an all–atom Gō model, that unfolding starts with the separation of $\beta_1, \beta_2$ and $\beta_3$ from the rest of the structure and from each other, followed by the separation of $\beta_3$ and $\beta_4$ from $\alpha_1$ and from each other, and finally by the unfolding of the helices. Their typical trajectory shows a plateau in the end–to–end length, which they associate to an unstable intermediate where $\beta_1$ and $\beta_2$ are unfolded and $\beta_3$ is about to unfold.

Szymczak and Cieplak [12, 13] observed, in MD simulations of a $C_\sigma$ Gō model, that, during folding, the hairpin $\beta_1 \beta_2$ forms at the beginning if both extremities are left free, while it forms at the end if the N-terminus is held fixed.

Motivated by the discrepancies between these results we have studied the mechanical unfolding and refolding of ubiquitin by means of MC simulations of a simplified Gō model with binary variables [14]. We have recently developed this model as a generalization of a model originally proposed by Wako and Saitō [15] in a purely thermodynamic version and subsequently reconsidered by Muñoz, Eaton and coworkers [16, 17], who used a kinetic version of the model to analyze experimental results. In the last few years it has been shown

The refolding also exhibits a rich behaviour [6], with an initial rapid elastic recoil, followed by an intermediate state characterized by large length fluctuations, and a final transition to the folded state. The folding time turns out to follow an Arrhenius–like law, with a distance from the extended to the transition state estimated as $x_f = 0.8$ nm [6].

These experimental results have prompted a series of computational studies [8, 9, 10, 11, 12, 13] aimed at reproducing the general behaviour and elucidating the details of the unfolding and refolding pathways and the nature of the intermediate state. Irb¨ack and coworkers [8, 9] suggest, on the basis of Monte Carlo (MC) simulations of an all–atom model, that the most probable unfolding pathway corresponds to $\beta_1 \beta_2 \rightarrow \beta_1 \beta_2 \rightarrow \beta_2 \beta_5 \rightarrow \beta_3 \beta_4 \rightarrow \alpha_1$, i.e., the contacts between $\beta$–strands 1 and 5 are the first to yield, followed by those between strands 1 and 2, and so on until finally the $\alpha$–helix yields. Furthermore, in the typical unfolding intermediate found in that paper, $\alpha_1, \beta_5, \beta_3$ and $\beta_4$ are still folded, in marked contrast with [5].

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that the model equilibrium thermodynamics can be solved exactly [18], and that it successfully describes the kinetics of protein folding [19, 20, 21, 22, 23]. Our generalized model for protein mechanical unfolding [14], has been shown to exhibit the typical response of proteins to external loading, allowing one to estimate the unfolding length of a titin domain, in excellent agreement with the experimental value, and with a very limited computational effort. In the model a protein made up of \(N+1\) aminoacids is described as a chain of \(N\) peptide bonds: a binary variable \(m_i\) is associated to each bond and can live in two states (native and unfolded, \(m_i = 1, 0\)). Given the \(m_i\) values one can identify native–like stretches (which can be as short as a single aminoacid and as long as the whole chain) delimited by unfolded bonds. A stretch goes from bond \(i\) to \(j\) if and only if \((1-m_i) \prod_{i<k<j} m_k (1-m_j) = 1\), and to each stretch we associate (i) a native length \(l_{ij}\) taken from the pdb [24], and (ii) an orientational variable \(\sigma_{ij} = \pm 1\), where \(+1(-1)\) means parallel (antiparallel) to the applied force [25]. Detailed definitions have been given in [14], where the parameter choice is also described. The energy scale for the ubiquitin model turns out to be \(\epsilon/k_B = 156.6 K\), and the force unit is fixed so as to match the experimental characteristic unfolding force \(f_u \approx 35\) pN [6, 25]. The unfolding and refolding kinetics are studied by MC simulations with single–variable–flip Metropolis dynamics: the model time scale \(\tau_0\) corresponds to a single MC step, the temperature is taken to be \(T = 300\) K. In order to monitor the unfolding of SSEs, we define the order parameter for each SSE as the fraction of its peptide bonds in the native (folded) state: \(m_{g_i} = \frac{1}{\sum_{s=1}^{5} m_{s}}\sum_{s=1}^{5} m_{s}\), with \(s = 1 \ldots 5\), and where \(\beta\)-strand \(s\) includes aminoacids from \(i_s\) to \(f_s\), and similarly we define \(m_{s,j}\), \(r = 1, 2\) for the helices.

In the present work we consider the protein unfolding induced by a force clamp [5]. Typically, in such experiment, the average unfolding time is given by the Arrhenius’ law \(\langle \tau_u \rangle = \tau_0 \exp [-f x_u/(k_B T)]\), where \(f\) is the external force and \(x_u\) the unfolding length. We start from the completely folded state, with \(f = 0\), and then we apply a constant force \(f\) and sample the unfolding time \(\tau_u\) over 1000 trajectories. In fig. 1 the mean unfolding time is plotted as a function of the external force \(f\). The force dependence is clearly exponential at small forces and saturates at larger forces, as noticed in [6]. From a fit of the data in fig. 1 to the Arrhenius’ law we find \(x_u = 1.8 \pm 0.1\) Å, in excellent agreement with the experimental value found in [5]. The fit is performed in the same range of forces considered in [5], \(50 < f < 250\) pN. In ref. [2], the ubiquitin unfolding process has been shown to depend on the pulling vector, relative to the structure. Following that idea, we applied the force to the portion of molecule spanning from the 48th aminoacid (Lys48) to the C terminus (aminoacids 48–76). We found bistability, signaling the unfolding transition, at \(f \sim 100\) pN, in reasonable agreement with the average unfolding force of 85 pN measured in [2]. We then measured \(\langle \tau_u \rangle\) as a function of \(f\) (fig. 1). From the fit we get \(x_u' = 4.1 \pm 0.5\) Å, which is larger than \(x_u\) of the whole molecule, signalling a softer structure, in agreement with ref. [5]. This value of \(x_u'\) is slightly larger than that

| \(\alpha_1\) | \(\alpha_2\) | \(\beta_3\) | \(\beta_4\) | \(\beta_5\) | \(\beta_6\) | \(\beta_7\) |
|---|---|---|---|---|---|---|
| \(1\times\) | 0.36 | 0.00 | 0.02 | 0.05 | 0.07 | 0.05 |
| \(2\times\) | 0.64 | 0.02 | 0.04 | 0.1 | 0.15 | 0.11 |
| \(3\times\) | 1.00 | 0.98 | 0.57 | 0.86 | 0.945 | 0.94 |
| \(4\times\) | 0.98 | 0.96 | 0.43 | 0.83 | 0.91 | 0.90 |
| \(5\times\) | 0.95 | 0.90 | 0.14 | 0.17 | 0.64 | 0.55 |
| \(6\times\) | 0.93 | 0.85 | 0.055 | 0.09 | 0.36 | 0.365 |
| \(7\times\) | 0.95 | 0.89 | 0.06 | 0.10 | 0.45 | 0.645 | 0.36 |

### Table I: Probability that the row–index SSE unfolds before the column–index one, with \(f = 100\) pN and \(m_u = 1/3\).

![FIG. 1: Average unfolding time \(\tau_u\) as a function of the force \(f\), applied to the whole molecule (□) and to the portion of molecule spanning from the 48th to the 76th aminoacid (○). Lines are exponential fits. Inset: Refolding time as a function of the quenched force \(f_1\), and with \(f_0 = 100\) pN. The line is an exponential fit to the data.](image)
FIG. 2: (color online). Order parameters \( m_{\beta_{12}} \) (black), \( m_{\beta_{43}} \) (red) and \( m_{\beta_3} \) (green) as functions of time, with \( f = 100 \) pN, for a typical trajectory. Inset: end–to–end length \( L \) (in Å) of the model molecule as a function of time.

This intermediate state \( \beta_1 \) and \( \beta_2 \) are unfolded, \( \beta_3 \) and \( \beta_4 \) are folded and \( \beta_5 \) fluctuates (as it already did in the folded state). The presence of the intermediate state is also signalled by a plateau in the end–to–end length, see inset of fig. 2. This state appears in all but a few trajectories. Since its lifetime is widely varying we argue that when we do not see it in a trajectory, this is just due to the time resolution limit. The unfolding pathway is then unique, exhibiting an intermediate state with a fluctuating lifetime. We have checked that the distribution of the time difference \( t_{\beta_3} - t_{\beta_1} \), which measures the time elapsed between the unfolding of the hairpins \( \beta_1 - \beta_2 \) and \( \beta_3 - \beta_4 \), exhibits a single peak (data not shown), while in the case of two different pathways one would expect a two–peaked distribution.

Our pathway is consistent with the all–atom models \cite{8,11} and the C\(_\theta\) Gō model by Li et al. \cite{10} (except for the case in which the force is applied to the N–terminus only). In the experimental reference \cite{5} the intermediate was attributed to the partial unfolding of the strands \( \beta_1 \) and \( \beta_2 \) and of the \( \alpha \)–helix, although this conclusion was based only on the length of the single strands, their unfolding trajectories being not experimentally accessible. On the contrary, in ref. \cite{8} the intermediate state were identified to be composed by \( \beta_3 \), \( \beta_4 \) and \( \beta_5 \) and the \( \alpha \)–helix, as found in the present work, though \( \beta_5 \) is fluctuating here. Also in ref. \cite{11} it was found that in the intermediate state \( \beta_1 \) and \( \beta_2 \) are unfolded, while \( \beta_3 \) separates from \( \beta_1 \) along the plateau which characterizes the intermediate. The apparent discrepancy between theoretical and experimental scenarios for the intermediate state can be reconciled if one considers that the only direct information which comes from the experiments is that in the first unfolding step a portion of the size of 28 aminoacids unfolds \cite{5}. The hairpin \( \beta_1 - \beta_2 \), plus the loop which connects it to the \( \alpha \)–helix, measures 22 aminoacids, and the remaining 6 aminoacids can be attributed to the fluctuations of the strand \( \beta_5 \), that we observe in the intermediate state.

We now turn to the analysis of refolding, by considering a protein which is initially completely unfolded, at equilibrium with a large force \( f_0 \). Then, at \( t = 0 \), the stretching force is quenched to a low value \( f_1 \), and the folding trajectory of the protein is monitored. In fig. 3 a typical trajectory is shown: the order parameter \( m \) (fraction of native peptide bonds \cite{14} ) and end–to–end length \( L \) are plotted as functions of time. It is worth noting that, after the quench at \( t = 0 \), we observe a slow elastic recoil, where the length jumps to a value \( L = 100 \) Å, while the order parameter \( m \) increases more gradually, indicating that the molecule is not yet structured. After this recoil, the length and the order parameter exhibit large fluctuations. This stage is followed by another abrupt contraction in the length, where the protein reaches its equilibrium length for the small force applied. This is accompanied by a marked increase in the order parameter, which shows that the molecule is now fully folded. This behaviour is the same that was found by Fernandez and Li \cite{6} in their experimental observation of refolding of ubiquitin under force quenching. It is worth stressing that in a subset of trajectories, they found that the last transition can be split in two stages, however the order of refolding of the SSEs during these two stages could not be sampled. We find that the refolding pathway is similar to the reverse unfolding one, see table II. The helices and the strands \( \beta_3, \beta_4 \) are the first SSEs to refold, \( \beta_1 \) and \( \beta_2 \) fold at the final stage of the process, while \( \beta_5 \) folds randomly between \( \beta_3 - \beta_4 \) and \( \beta_1 - \beta_2 \): thus in addition to the fast elastic recoil the model exhibits also a refolding intermediate (see the insets in Fig. 3). It is tempting to associate this intermediate to the two stages observed in ref. \cite{6} in the last transition. Finally, we observe that the average refolding time depends also exponentially on the force \( \ln \left< \tau_f \right> \sim x_f \), as shown in the inset of Fig. 1 and the corresponding folding length is \( x_f = 6.7 \pm 0.8 \) Å, in reasonable agreement with the experimental value \( x_f = 8 \) Å \cite{6} and with the value obtained by Li et al. \cite{10}.

In conclusion, we have shown that a simple Gō model with binary variables can account for the main features observed in the mechanical unfolding and refolding of ubiquitin. This model is, to the best of our knowledge, the simplest one with sufficient details to allow the study of specific molecules. We believe that this model may be a useful tool to investigate the interplay between the protein native structures and their unfolding and refolding pathways. Finally, we want to stress that the ubiquitin refolding cannot be investigated by all-atom

| \( \alpha_1 \) | \( \alpha_2 \) | \( \beta_2 \) | \( \beta_3 \) | \( \beta_4 \) |
|---|---|---|---|---|
| \( \times \) | 0.875 | 1.00 | 0.99 | 0.98 | 0.98 |
| \( \alpha_2 \) | 0.025 | \( \times \) | 1.00 | 1.00 | 1.00 | 0.975 |
| \( \beta_2 \) | 0.00 | 0.00 | \( \times \) | 0.54 | 0.37 | 0.14 | 0.14 |
| \( \beta_1 \) | 0.01 | 0.00 | 0.56 | \( \times \) | 0.365 | 0.13 | 0.13 |
| \( \beta_3 \) | 0.01 | 0.00 | 0.63 | 0.635 | \( \times \) | 0.09 | 0.085 |
| \( \beta_4 \) | 0.02 | 0.025 | 0.86 | 0.87 | 0.91 | \( \times \) | 0.97 |
| \( \beta_5 \) | 0.02 | 0.025 | 0.86 | 0.87 | 0.915 | 0.03 | \( \times \) |
FIG. 3: Refolding under constant force: molecular order parameter $m$ and length $L$ as functions of the time, $f_0 = 232$ pN, $f_1 = 23.2$ pN. Simulations because of the huge computation time required.

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[24] In our model the native length $l_i$ is independent of the force. This simplification leads to overall lengths slightly smaller than the experimental ones, as the extensibility of the native stretches is not taken into account.
[25] Our assumption on orientations corresponds to a reduction in configuration space and hence in the entropy of unfolded bonds: as a consequence we have to rescale the “natural” force unit $\epsilon/\sigma (1 \text{nm})$ by a factor 5.4, in such a way that the force $f_u$ where the molecule elongation is half of its maximum value, corresponds to the experimental unfolding force $f_u = 35$ pN [6].