Consensus for the Fip35 folding mechanism?

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Recent advances in computational power and simulation programs finally delivered the first examples of reversible folding for small proteins with an all-atom description. But having at hand the atomistic details of the process did not lead to a straightforward interpretation of the mechanism. For the case of the Fip35 WW-domain where multiple long trajectories of 100 µs are available from D. E. Shaw Research, different interpretations emerged. Some of those are in clear contradiction with each other while others are in qualitative agreement. Here, we present a network-based analysis of the same data by looking at the local fluctuations of conventional order parameters for folding. We found that folding occurs through two major pathways, one almost four times more populated than the other. Each pathway involves the formation of an intermediate with one of the two hairpins in a native configuration. The quantitative agreement of our results with a state-of-the-art reaction coordinate optimization procedure as well as qualitative agreement with other Markov-state-models and different simulation schemes provides strong evidence for a multiple folding pathways scenario with the presence of intermediates.

INTRODUCTION

Computer models of protein folding have been around now for almost two decades [1, 2]. Such models evolved dramatically, going from simple lattice models [3] and implicit solvent implementations [4] to fully atomistic calculations [5]. In the quest for microscopic models of protein folding, several groups focused on small proteins like WW domains which can be very informative yet much easier to treat. Recently, an important breakthrough in this direction was delivered by D. E. Shaw Research. By using in-house technology with optimized software they delivered first examples of reversible folding for some mini-proteins [6]. For the case of the Fip35 WW-domain they made available two trajectories each of which of 100 µs in length. These calculations raised a number of controversial interpretations on the actual folding mechanism.

For this system, no agreement was found on whether the folding process proceeds via on-pathway intermediates or downhill folding. The original work applied an optimization procedure to obtain a reaction coordinate for the folding process [7], concluding that no relevant barriers are present in the folding process [6]. This interpretation was challenged by others. In particular, Krivov demonstrated the presence of intermediates by calculating a novel optimized reaction coordinate [8]. This view was also supported, at least at a qualitative level, by Pande and Baker groups which independently analyzed the same data with Markov-state-models [9, 10].

All approaches have found that the folding process is more likely to proceed via the formation of the first hairpin (β1) followed by the second one (β2) but the conceptual disagreement on the presence of intermediates or a downhill scenario is pretty strong. Unfortunately, these types of analysis [6, 8, 10] are not very intuitive, making an objective evaluation of the results hard. On one side, optimization procedures like the ones applied by Shaw and Krivov, tend to hinder the physical meaning of the obtained reaction coordinates while clustering of high-dimensional spaces strongly suffer from thermal fluctuations [11] and limited sampling [10].

In an effort to bridge the gap between the use of more intuitive coordinates and the application of Markov-state-models, we recently extended an approach derived from single-molecule spectroscopy to study molecular simulations [12]. Aiming at identifying the most robust features of Fip35 folding, we present here an extension of this framework for the analysis of D. E. Shaw data. The entire analysis is based on conventional order parameters time series, like root mean square deviations (RMSD). This overcomes the problem of working in complex multidimensional spaces as in the case of coordinate optimizations [6, 8], k-means clustering [9] or contact maps comparisons [10]. Still, the approach provides a good assessment of the kinetics thanks to the application of complex networks analysis, allowing the development of simple Markov-state-models reproducing the time scales of the original MD trajectory [11, 12]. Our results reinforce the interpretation that Fip35 folding proceeds via intermediates.

METHODS

Markov state models from conventional order parameter analysis

In this section we are going to explain the tools that were used to analyze the Fip35 data set. The main idea behind local fluctuations analysis is to build a Markov-state-model using as input the time series of a general order parameter. This is done by looking at the fluctuations of the coordinate within a predetermined time window. The approach was initially developed for single molecule experiments [13, 14], but in a recent paper we applied and
extended this technique to study conventional order parameter time series from molecular simulations [12]. The main motivation was to develop a tool to analyze those time series in a more rigorous way, going beyond straightforward histogram analysis. In fact, the great advantage of this strategy is to characterize order parameter time series on the base of the kinetics, something that was definitively impossible by using free-energy projections and histogram analysis. The approach takes advantage of the work done in complex network analysis [16, 17] and Markov-state-models [18] in molecular systems but it overcomes some of the problems, e.g. avoiding to work in high dimensional spaces. The downside here is that the framework is based on simple coordinates, therefore if they miss some relevant aspects of the system there is no way to recover that type of information.

The steps to be covered are very similar to any other Markov-state-model: (i) microstate building, (ii) transition network building, (iii) kinetic lumping. Although already discussed elsewhere in detail [12, 16, 17], below we provide the essentials to better follow the paper. A code to reproduce the presented analysis is freely distributed at the website raolab.com.

**Microstate building**

Microstates were defined for every trajectory snapshot by looking at the local fluctuations of the order parameter coordinate within a time window \( t_w \) centered in the snapshot itself [12, 14]. Two time points belonged to the same microstate if they had comparable distributions of the order parameter within \( t_w \) according to a Kolmogorov-Smirnov test [19]. That is, if the condition \( D \leq \zeta \sqrt{2/t_w} \) was fulfilled, where \( D \) is the maximum difference of the two cumulative distributions and \( \zeta \) corresponds to a certain confidence level. Being \( t_w \) and \( \zeta \) related, we fixed the latter value to 0.5 and let \( t_w \) vary as done in Ref. [12]. Comparisons were made along the trajectory using the leader algorithm in a way that every time point was associated to a microstate [14, 20]. As shown by others [14] and us [12], the methodology is robust for a reasonable wide range of time windows. For example, for values of the time window up to 13 ns the mean first passage time to the folded state steadily increases till a plateau. Then, between 13 and 24 ns the mean first passage time fluctuates around 4.6 \( \mu s \). Finally for larger windows, this value tends to slightly increase together with larger fluctuations but still in agreement with previous calculations [8]. In the following, we fix the time window to 18 ns. In the general case, short time windows are unable to capture significantly well the coordinate distribution leading to faster kinetics [12] while long ones result in too many fast fluctuations to neighboring states inside a single distribution. Given the \( \mu s \) time scales of the folding process, this would happen only in cases when windows of hundreds of ns are selected.

**The configuration-space-network**

The resulting time series of microstates was mapped onto a configuration-space-network [12, 16, 17, 21, 22]. Microstates represent network nodes and a link between them exists if they were successively visited along the molecular trajectory. Detailed balance was imposed for each link by making an average of the number of transitions in both directions. This was only partially necessary because the original trajectories mostly satisfied detailed balance already.

**Kinetic lumping by network clusterization**

Protein conformational states were defined by applying a kinetic lumping scheme. This was done by running a clusterization procedure on the configuration-space-network, the Markov-Clustering-Algorithm (MCL) [23]. This approach assures that the obtained network clusters represent meaningful free-energy basins with preserved system kinetics [12, 17]. Being interested on the characterization of the folding mechanism, a granularity parameter of 1.2 was used to focus on the highest barriers only [12, 17]. The obtained states were used to build a Markov-state-model, schematically representing all relevant slow transitions of the system.

**First passage time distributions**

The kinetic similarity between the original trajectory and the Markov-state-model was investigated by comparing the distribution of the first-passage-times (fpt) [11, 12] to the folded state. This is the distribution of times to reach the native state from any other snapshot of the trajectory [11]. For the Markov-state-model the fpt was calculated on trajectories generated by running a random walk. Arrival times depend on the definition of the target only and not on the detailed decomposition of the trajectory. For this study we used the definition of the native state as obtained by MCL.

**Molecular simulation data**

The simulation data was directly obtained from D. E. Shaw Research and published in Ref. [6]. It is an all-atom molecular dynamics simulation in explicit water (TIP3P water model) at the protein’s in silico melting temperature (395K). It was calculated using the Anton supercomputer with the modified Amber ff99SB-ILDN force field [24] carried out in the NVT ensemble using the...
Nose-Hoover thermostat with a relaxation time of 1.0 ps \[6, 24\]. The simulation data consisted of two trajectories, each of length 100 \(\mu\)s.

RESULTS

RMSD analysis of the folding mechanism: identification of putative intermediate states

Conventional order parameters for protein folding include RMSD with respect to the native state \[25\], number of native contacts \[4\] or radius of gyration \[26\]. RMSD is certainly one of the most obvious choices when it comes to monitor folding to a known structure because it requires minimal a priori knowledge (i.e. the native structure). The projected free-energy landscape onto the RMSD from the native state \((R_{\text{all}})\) shows two clear minima, corresponding to the folded and unfolded states (Fig. 1a). To improve on this simple description we projected the landscape onto two coordinates instead of one. Given the triple stranded topology of Fip35, we expect that the RMSD coordinates from the first (residues 7 – 23, \(R_{\beta_1}\)) and second (residues 18 – 29, \(R_{\beta_2}\)) hairpin to provide additional information on the process. Fig. 1b shows a 2D projection onto these two new coordinates. The folded and unfolded states are clearly visible at regions around \((1, 1)\) and \((7, 5)\) respectively. In agreement with the 1D projection, this plot provides some further information on the presence of other states like the darker regions at around \((2, 1)\) and \((1, 3.5)\). Those regions might represent intermediate steps to the folding process, a property that cannot be validated by Fig. 1b due to the lack of information on the kinetics \[16, 27\].

The relevance of these regions for the folding process emerged by the inspection of some of the folding/unfolding events. One example is shown in Fig. 2. In the two panels, the RMSD with respect to the native state \((R_{\text{all}})\), first hairpin \((R_{\beta_1}, \text{top panel})\) and second hairpin \((R_{\beta_2}, \text{bottom panel})\) are shown as black and gray lines, respectively. This picture shows that at around 30.3 \(\mu\)s an unfolding event is present. Here \(R_{\text{all}}\) rapidly increases (black line) as well as \(R_{\beta_2}\) (gray, bottom panel). This is not the case for \(R_{\beta_1}\) (gray, top panel) which stays low for around 200 ns while the other hairpin is unfolded (the region is highlighted with a gray band). A similar behavior was observed for the folding event at 31.1 \(\mu\)s, with the second hairpin being native for a time
span of roughly 150 ns before the complete folding event (right gray band). In the folding/unfolding process the two hairpins evolve in an uncorrelated manner. Consequently, the RMSD coordinates $R_{\beta 1}$ and $R_{\beta 2}$ provide independent information on the folding mechanism, suggesting the presence of on-pathway intermediates. It is important to note, while the total RMSD is able to report on the presence of these states (the RMSD in the gray band regions of Fig. 2 is lower than the completely unfolded state), this coordinate does not have the sensitivity to discriminate between partially folded states with the first hairpin formed from the ones with the second hairpin formed. For this reason, we do not think that $R_{\text{tot}}$ represents a good coordinate for a local fluctuations analysis.

Local fluctuations analysis: kinetics assessment of the intermediate states

Given these observations, we chose $R_{\beta 1}$ and $R_{\beta 2}$ coordinates as probes for a more insightful kinetic analysis of the folding mechanism. This was done by performing a joint local fluctuations analysis of these coordinates (see the Methods section for details). Being this approach developed for a single coordinate, here we extended the framework to account for multiple order parameters, such as $R_{\beta 1}$ and $R_{\beta 2}$. To do so, the local fluctuations of each coordinate were first analyzed separately using the standard approach. Then, the obtained states for each coordinate were merged into a set of “combined” states. That is, given at a certain time the states corresponding to $R_{\beta 1}$ and $R_{\beta 2}$, being respectively $A$ and $B$, the new combined state is $(A, B)$. This strategy includes the contribution of two coordinates in a simultaneous way.

Application of this technique resulted in the identification of six states with a population larger than (or equal to) 1.0%. The cumulative population of these states is of about 99.3%, indicating that they well characterize the sampled conformational space. In this representation the native (N) and unfolded states (U) have a population of 59.0% and 32.0%, respectively. The remaining four states have a much smaller population of few percents. The six states were used to build a reduced Markov-state-model whose transition network is shown in Fig. 3. Interestingly, all states stay on-pathway from U to N. Specifically, two major folding intermediates were found just preceding the fully folded state: I1 and I2. These intermediates correspond to two independent folding routes with different relative populations. We calculated this explicitly by looking along the original trajectory which states were preceding the folding state. Of the total 10 folding events 7 and 2 events followed the I1 and I2 routes, respectively. A third pathway was followed only once.

To check that the reduced Markov model of Fig. 3 was able to correctly reproduce the original dynamics of the MD trajectory, a first passage time analysis to the native state was computed. In Fig. 4 the distributions of the first passage times corresponding to the original trajectory and the six-states Markov model are shown as black and gray lines, respectively. Interestingly, the two distributions present a very similar decay in the long times.
regime, corresponding to a folding time of around 4.3 μs. The ability of the Markov model to reasonably reproduce the folding time of the original trajectory is remarkable. Specifically, it indicates that the kinetic lumping via network clusterization correctly partitioned the whole free-energy landscape. When this would not be the case [11], a much faster kinetics usually appears [12,27].

Structural analysis of Fip35 folding intermediates

In Fig. 5 a free-energy projection of the native, unfolded, I1 and I2 states onto the \(R_{\beta_1}\) and \(R_{\beta_2}\) coordinates is shown. The four states occupy well defined regions of the map. However, the distributions of intermediate states are rather broad producing large overlaps with both the native and unfolded states (compare also with Fig. 1 and check references [16,27]). Besides this, the two intermediate states have a good degree of nativeness: for the case of I1 (I2) the value of \(R_{\beta_1} (R_{\beta_2})\) was most of the time below 2 Å.

From a structural point of view, the two intermediates are characterized by well-defined conformations. Structural superpositions of the native and intermediate states are shown in Fig. 6 (while the unfolded state looks like a disordered bundle of structures). The three states present a reasonable amount of structural homogeneity. For the case of I1, the first hairpin is in its native conformation as well as the turn connecting the two hairpins. On the other hand the second hairpin is unstructured, interacting with the rest of the protein in several non-specific ways. In the I2 intermediate, an inverted situation was found where the second hairpin is native. In this case however, the first hairpin is prone to fold into a specific configuration instead of being unstructured. This conformation resembles the native structure but the formation of the first turn, and consequently the entire hairpin, is shifted by one residue (out-of-register conformations are typical in β-hairpins [16]).

It is striking to see the different amount of disorder in I1 and I2 (Fig. 6) suggesting a qualitative reason for the different statistical relevance of the two folding pathways. In fact, folding through I1 involves the formation of new specific contacts in the \(\beta_2\) region while folding through I2 first requires the disruption of a number of non-native contacts in the \(\beta_1\) region due to the out-of-register conformation. As such, I2 might even be considered per se a misfolded structure.

DISCUSSION

Complex network analysis of Fip35 RMSD local fluctuations provided evidence for three main observations: (i) beyond the native and unfolded states, hidden states were detected; (ii) among those states, two on-pathway intermediates for folding were found; (iii) the different amount of structural disorder in the two intermediates suggest a reason for the prevalence of one pathway with respect to the other.

Previous calculations based on Markov-state-models found multiple pathways and a heterogeneous molecular mechanism. In contrast to us, structural clustering [9] or likelihood methods in conjunction with contact maps [10] were used to build the Markov models. Although it is difficult to compare these approaches in a quantitative way, their predictions are in qualitative agreement with our results. Interestingly, the use of an alternative simulation protocol to probe slow conformational transitions confirmed the presence of two main folding pathways [28].

Given the triple stranded native topology of a WW domain, the presence of these two pathways is not new. The same folding routes were already observed in the past for a 20 residues triple stranded β-sheet peptide in implicit solvent [4,16].

So far, one of the most robust interpretations of Fip35 folding was provided by the analysis of Krivov [8]. Our findings are in excellent agreement with that study. This is quantitatively shown in Fig. 7 where we projected our states to the optimized coordinate developed in that work. This comparison reveals that the two approaches provide very similar results. In Krivov’s profile the native, I1 and unfolded states were identified as peaks of the probability distribution [8]. Strikingly, the distributions arising from our detected states overlap very well with these peaks. For the native state, only a very small fraction of 0.7% was found in the wrong part of the profile.
FIG. 6. Structural superpositions of the native and the two intermediate states as found by the local fluctuation analysis. Each panel contains 25 randomly chosen frames. Structures were superimposed on residues 7–23 and 18–29 for I1 and I2, respectively.

(green peak between the value 18 and 26 of the coordinate) while for I1 there is a perfect agreement. Moreover, the second intermediate I2, which originally could not be directly detected from the profile, was found in the same position as predicted in Ref. [8] (I2 was hidden because parallel pathways cannot be simultaneously displayed in this representation).

Overall, the two approaches provided the same mechanistic understanding. This is encouraging given the strong diversity of the two methods. In fact, Krivov's reaction coordinate was obtained via an optimization procedure starting from an educated guess, i.e. a linear combination of conventional (non-optimized) coordinates. The procedure makes a parameter space search minimizing the flux on top of the barriers of the initial free-energy projection. Better results were obtained when using several (i.e. thousands) of coordinates, e.g. all pairwise inter-atomic distances in a protein [8, 29]. Unfortunately, this makes the optimization procedure highly non-trivial [30]. The strength of this method is to provide kinetically meaningful free-energy profiles with diffusive dynamics. The downsides are the intrinsic limitations of 1D profiles to describe parallel pathways and the non-trivial optimization procedure. The complex network analysis presented here does not run any optimization algorithm but attempts to detect hidden states from the time series of a generic coordinate, e.g. the RMSD. This makes our method much faster. The downsides are again the need of an educated guess for the selection of the coordinate to use as well as potentially larger errors in the resulting kinetic models.

In conclusion, most of the results presented so far on Fip35 folding point to the same direction. We believe that the original interpretation provided by Shaw and co-workers of downhill folding is due to an inappropriate application of the reaction coordinate optimization they used. Being based on commitor probabilities, that approach was designed and tested for two-state processes only [2]. Given the implicit assumption of a two-state scheme, application of this protocol to multi-state systems leads to inaccurate results.

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FIG. 7. Projection of the detected states on the optimal reaction coordinate developed by Krivov [8]. Native, unfolded, I1 and I2 states are shown in green, blue, red and yellow, respectively.

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