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On the Use of Elevated Temperature in Simulations To Study Protein Unfolding Mechanisms

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Abstract: In protein unfolding simulations, elevated temperature, significantly exceeding the melting temperature T_m, provides an important means to accelerate unfolding to a computationally accessible time range. This procedure is based on the assumption that protein thermal unfolding has Arrhenius behavior and therefore that increasing temperature does not alter the protein unfolding pathways. However, in nature, proteins can show non-Arrhenius behavior and, in practice, overly fast unfolding in high-temperature simulations can result in difficulties in identifying unfolding intermediates and distinguishing their relative stabilities. In this paper, we describe simulations of two WW domains, small protein domains that have a three-stranded β-sheet structure. Simulations were carried out at several temperatures ranging from 300 K to 500 K, starting from folded structures. The results demonstrate the temperature dependence of the unfolding pathways, showing that to obtain unfolding pathways corresponding to those observed in experiments, the elevation of the simulation temperature has to be controlled. Based on trajectory analysis, we proposed a qualitative criterion for judging when an elevated temperature is acceptable or not, namely, that the temperature must be such that the native folded state is sampled substantially before protein unfolding begins. While depending on force field parameters and protein fold complexity, this criterion can be quantified to obtain the upper bound of an "acceptable elevated temperature", which was observed to be dependent on the thermostabilities of the two WW domain proteins.

Introduction

In principle, molecular dynamics simulations to study protein unfolding and folding mechanisms should be conducted at the protein melting temperature (T_m) where the protein reaches folding/unfolding equilibrium. In practice, due to the long time scale (>10 μs for the fastest folding protein) of equilibrium unfolding and the limitations of computer power, highly elevated temperature (much above T_m) has been used in most unfolding simulations[1−8] to enable unfolding in an accessible computational time (<100 ns). The basis for the use of elevated temperature is the assumption that protein thermal unfolding shows Arrhenius behavior. That is, as stated in Beck and Daggett's review,[9] "increasing temperature does not alter the pathway of unfolding, only the rate". However, proteins can show non-Arrhenius behavior.[10−14] It has been observed that increasing temperature can slow down folding[11,12] and change intermediate states.[13,14] Two explanations for non-Arrhenius behavior are kinetic traps and the temperature dependence of hydrophobic interactions. For simple two-state folding, the temperature dependence of the hydrophobic effect is the main reason.[10] On the other hand, at very high temperature, protein structures can unfold very rapidly, and a large number of intramolecular interactions can be disrupted simultaneously. As a result, it is not possible to detect any order in the unfolding events or an unfolding pathway or funnel. In particular, when long-lived intermediates are expected, high

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temperature can shorten their lifetime and they can thereby go undetected.

A range of simulation temperatures can be used as in the replica exchange method (REMD) to fully sample the free energy landscape.[15−17] Folding/unfolding pathways can be assessed at an appropriate sampling temperature. Furthermore, if sampling is complete, then T_m can be found by considering heat capacity, and protein kinetics and unfolding pathways can be computed from the shapes and heights of free energy barriers between minima. The problem is that such complete sampling is hard to achieve for real systems. Another problem is that the T_m computed from REMD has often been found to be higher than experimental values due to force fields being parametrized at room temperature.[17] This can lead to overestimation of protein stability.

Consequently, the following questions arise: To what extent can temperature be elevated to accelerate unfolding without changing unfolding pathways or to reproduce the unfolding pathways observed at T_m? In other words, what is the 'acceptable elevated temperature' for simulating the unfolding of a given protein? And what criteria can be used to estimate whether a trajectory at an elevated temperature will give insights into the early stages of the unfolding process of the protein itself? When unfolding simulations are used to study the relative stabilities of two proteins, is the acceptable elevated temperature for each protein a good measure for relative protein stability? To address these questions, and being motivated by our study of the relative stabilities of WW domains and mutants by molecular dynamics simulations,[18] we studied the unfolding of two WW domains: one from formin binding protein 28 (FBP28) and one from Yes kinase-associated protein 65 (YAP65). Both of these WW domains consist of ca. 40 residues and have a three-stranded β-sheet. FBP28 has been studied extensively by both experiment and computation. It is the only WW domain that has been observed to exhibit biphasic folding/unfolding kinetics with a stable intermediate state.[14,17,19,20] Nguyen et al. reported that FBP28 exhibits folding/unfolding kinetics that are tuneable by temperature, being a two-state folder with T_m of 337 K and exhibiting a stable intermediate below T_m at 312.5 K.[14] The origin of the biphasic kinetics has been attributed to a unique hydrophobic packing that is absent in other WW domains.[19,20] However, in contradicting studies, other authors have found that FBP28 exhibits two-state behavior at a range of temperatures below T_m.[8,21] A stable intermediate state that consists of only the first and the second native β-strands has been detected in both experimental and computational works.[14,17,19,20] Despite the controversy, consensus has been reached on the relative stabilities of the two β-hairpins: the first is more persistent in unfolding processes.[8,22]

The thermal unfolding of YAP65 has been studied experimentally and has been reported to be a two-state process at and below $T_m = 323$ K[12] with transition states alterable by temperature.[12,23] Compared with FBP28, YAP65 has been less studied by simulation and its atomic level unfolding details are less clear. One of the few simulations predicted that the unfolding of YAP65 begins with the loss of the third strand.[24]

Our simulations demonstrate that the trajectories of the WW domains generated at different temperatures show different unfolding pathways. Analysis of the trajectories suggests criteria for determining suitable elevated temperatures for unfolding simulations. The qualitative criterion is that the simulation temperature be such that the majority of independent simulations starting in the native folded state sample the native state substantially before the unfolding process begins. This can only happen if the temperature is low enough for the native state to be a local energy minimum. We call this temperature the "acceptable elevated temperature". It is dependent on the stability of the protein. We further propose a procedure for quantitatively applying this criterion based on computation of the native contact percentage and the radius of gyration of the protein in simulations at room temperature and elevated temperature.

Methods

Molecular Dynamics Simulation. Two WW domains, FBP28 and YAP65, were simulated by using the AMBER8 program[25] with the TIP3P explicit water model and the AMBER ff03 force field.[26] The first structure from the NMR ensemble (PDB entry 1e01) of FBP28 was used; this has 37 residues. The coordinates of the NMR structure of YAP65 were provided by Dr. Maria Macias; this has 39 residues. The simulation protocol was as described in ref 18.

The simulations were carried out at several temperatures ranging from 300 K to 500 K starting from the native folded states. At each temperature, three to five different simulations were conducted by varying the speed of heating of the system at the beginning of the simulations. The length of the simulations was chosen according to the simulation temperature. It was 20 ns for simulations at 300 K. Simulations at higher temperatures were run for at least 12 ns. They were stopped either after unfolding was completed or after 72 ns if no unfolding occurred. The coordinates were saved every 10 ps.

Trajectory Analysis. The unfolding process was monitored by computing the variation in the percentage of native contacts (Q_N) present during the simulations. A contact was defined to be present when the van der Waals spheres of any backbone atoms from two different residues were within 1 Å of each other. The van der Waals parameters were taken from the AMBER force field.[26] This definition yielded 30 native contacts in FBP28 and 46 native contacts in YAP65. The greater number of native contacts in YAP65 is due to contacts in and between the N- and C-termini of the domain.

Each simulation trajectory was also analyzed by plotting the two-dimensional population histograms defined by percentage of native contacts (Q_N) (X-axis) and the radius of gyration (Rg) (Y-axis). The Z-values were plotted as the negative logarithm of the population in the bins defined by the Q_N and Rg coordinates. A more negative Z-value corresponds to a state with higher population and thereby with lower free energy. The bin size of the percentage of native contacts was 2% (less than 1 contact for both FBP28 and YAP65). The bin size of the radius of gyration was 0.075 Å. Because such small bin sizes were used, different members of the population in the same x,y coordinate bin had almost identical conformations.

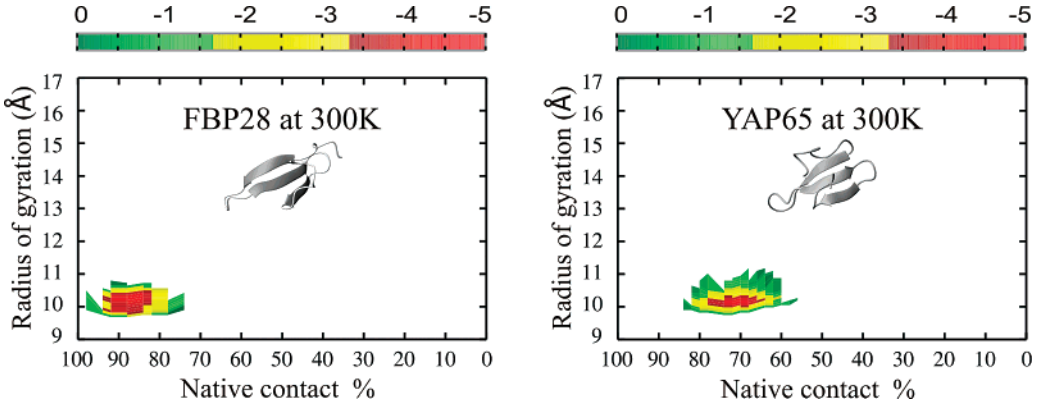

Figure 1. FBP28 and YAP65 simulated at 300 K for 20 ns. Shown are the two-dimensional population histograms defined by the percentage of native contacts (Q_N) and radius of gyration (Rg). The simulations sampled very narrow regions around the proteins' native starting structures, which were defined as the native state regions of $Q_N = 82-100\%$, Rg = 9.4−10.4 Å for FBP28 and $Q_N = 64-100\%$, Rg = 9.5−10.5 Å for YAP65.

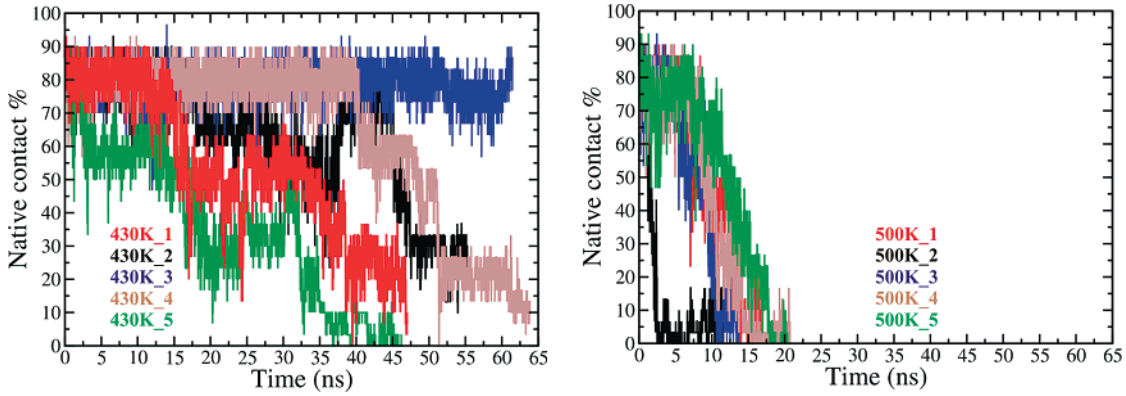

Figure 2. Time development of percentages of native contacts in the simulations of FBP28 at 430 K and 500 K. Five simulations were run at each temperature. At 430 K, four (430K_1 430K_2, 430K_4, and 430K_5) of the five simulations left less than 30% native contacts at the ends (47 ns, 55 ns, 64 ns, and 46 ns), while the third simulation (430K_3) still maintained more than 80% native contact at the end (61 ns). At 500 K, less than 10% native contacts were left at the end of all the five simulations (of 17 ns, 12 ns, 13 ns, 21 ns, and 21 ns duration).

Most of the simulation trajectories were also projected onto the two-dimensional population histograms defined by percentages of native contacts between the $\beta1$ and $\beta2$ strands (Q_{12}) and the $\beta2$ and $\beta3$ strands (Q_{23}).

Results and Discussion

Simulations at 300 K Define Native State Regions. Both the WW protein domains maintained their folded states during the 20 ns long simulations at 300 K. Figure 1 shows the two-dimensional population histograms defined by percentage of native contacts (Q_N) and radius of gyration (Rg). Both protein domains sampled very narrow regions around their native starting states, peaking at $Q_N = 88\%$, Rg = 10.2 Å for FBP28 and at $Q_N = 70\%$, Rg = 10.1 Å for YAP65. The fraction of native contacts maintained in YAP65 is lower due to the lower stability of native contacts in and between the N- and C-termini that are absent in the FBP28 structure. We then used the Q_N with the highest population (88% for FBP28 and 70% for YAP65) and the Rg of the starting structure (9.9 Å for FBP28 and 10.0 Å for YAP65) to define a native state region where Q_N should not be smaller than the peak Q_N minus 6% and Rg should stay within 0.5 Å of the starting Rg. The 6% window was chosen based on the population distribution of FBP28, beyond which the population showed a large drop from 268 structures with 82% native contacts to 0 structures with 80% native contacts and 79 structures with 78% native contacts. As a result, the native state region is at $Q_N = 82-100\%$, Rg = 9.4−10.4 Å for FBP28 and at $Q_N = 64-100\%$, Rg = 9.5−10.5 Å for YAP65. The native state region accounts for 85% of structures in the trajectory for FBP28 and 92% for YAP65.

FBP WW Domain Unfolds by Different Pathways at 430 K and 500 K. At 430 K, complete unfolding of FBP28 occurred in four out of five simulations. Figure 2 shows the time development of the percentages of native contacts in the five simulations. In the first simulation (referred as 430K_1), before the percentage of native contacts (Q_N) fell to 20%, there was a stage from 17 to 30 ns with a stable Q_N of around 50−60%, indicating a stable intermediate on the unfolding path. The two-dimensional population histogram in Figure 3 shows a second intermediate apart from the native state. This implies two energy minima separated by a clear energy barrier. This result is in agreement with the reported three-state behavior of FBP28[14,17,19,20] at temperatures below T_m.[14] The intermediate retains the $\beta1$ and $\beta2$ strands but has no $\beta3$ strand. This is shown in Figure 4 in a two-dimensional

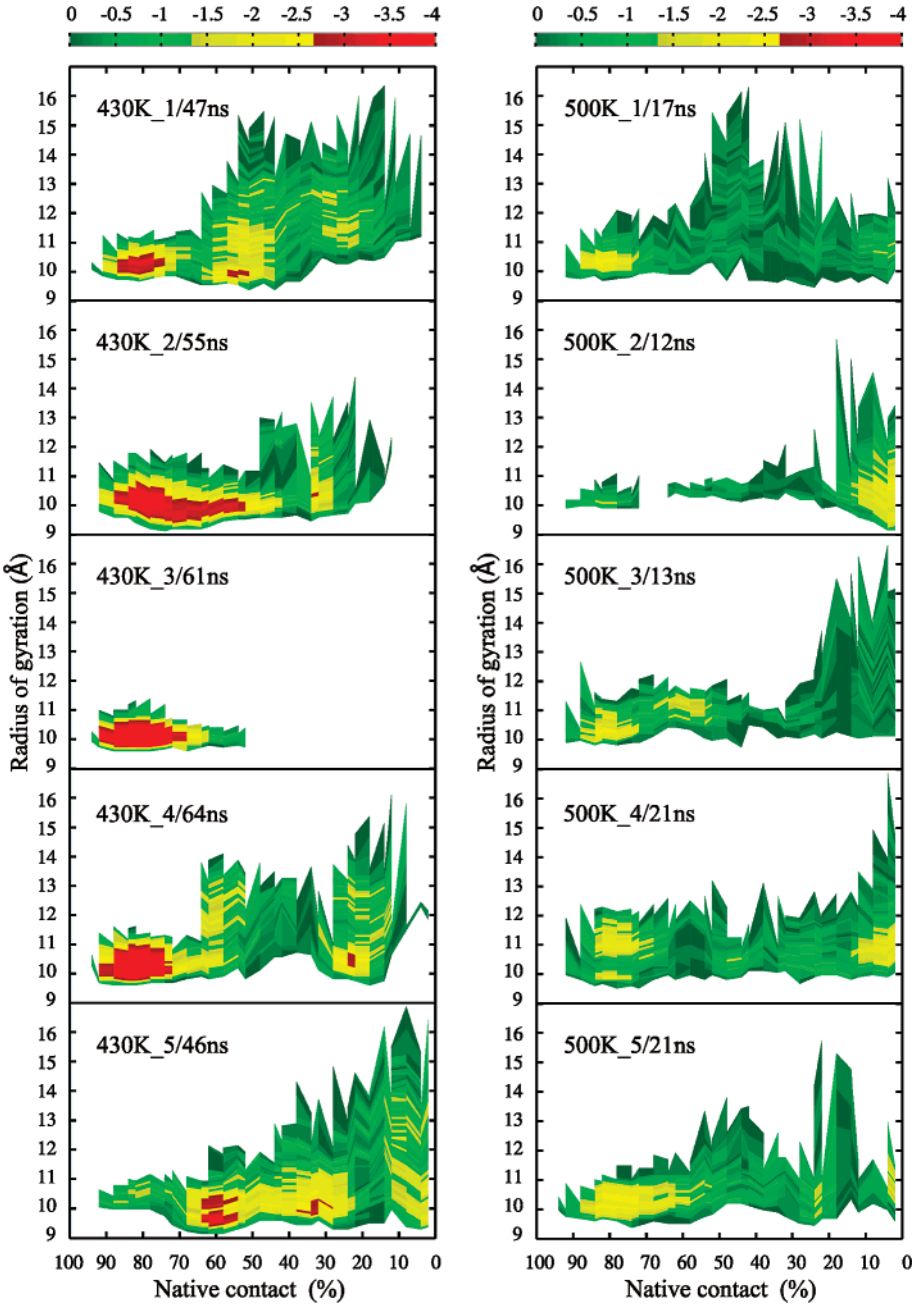

Figure 3. Two-dimensional population histograms of FBP28 simulated at 430 K and 500 K. The *X*-axis and the *Y*-axis are defined by the percentage of native contacts (Q_N) and radius of gyration (Rg). Five simulations were run at each temperature. In 430K_1, one minimum is in the native state region and the second minimum at $Q_N = 50-60\%$ and Rg = ca. 10 Å, indicating a stable intermediate state before complete unfolding. In 430K_2, there is a single and broad minimum that spans from the native state region to the region of $Q_N = 50\%$ before complete unfolding. In 430K_3, the protein did not unfold. In 430K_4, the only minimum is in the native state region. In 430K_5, the native state region was sampled very briefly. At 500 K, the protein unfolded in all three simulations without significantly sampling its native state region or intermediate region to unfolding.

population histogram defined by percentages of native contacts between $\beta1$ and $\beta2$ (Q_{12}) and $\beta2$ and $\beta3$ (Q_{23}). In addition, the small radius of gyration of the intermediate state (Rg around 10 Å) implies that although native contacts between $\beta2$ and $\beta3$ were lost, non-native contacts were formed. In fact, the $\beta3$ strand makes a U-turn in these structures. These non-native contacts were also observed in the experiments[14] by Nguyen and co-workers and the simulations by Mu and co-workers.[17] In addition, Mu and co-workers' computations showed that the intermediate structure has a much lower free energy of dimerization than the native structure and is likely to be the initial structure to form amyloid fibrils.[17,21]

Among the other three unfolding trajectories at 430 K (referred to as 430K_2, 430K_4, and 430K_5), 430K_4 exhibits two-state kinetics with the native states (Q_N=70−90%) and the unfolded states (Q_N<30%) being the only well populated states in the free energy landscape defined by Q_N and Rg as shown in Figure 3. However, in Figure 4 showing the two-dimensional population histogram defined by Q_{12} and Q_{23}, the states maintaining the $\beta1$-$\beta2$ contacts but with the $\beta2$-$\beta3$ contacts lost formed a second intermediate

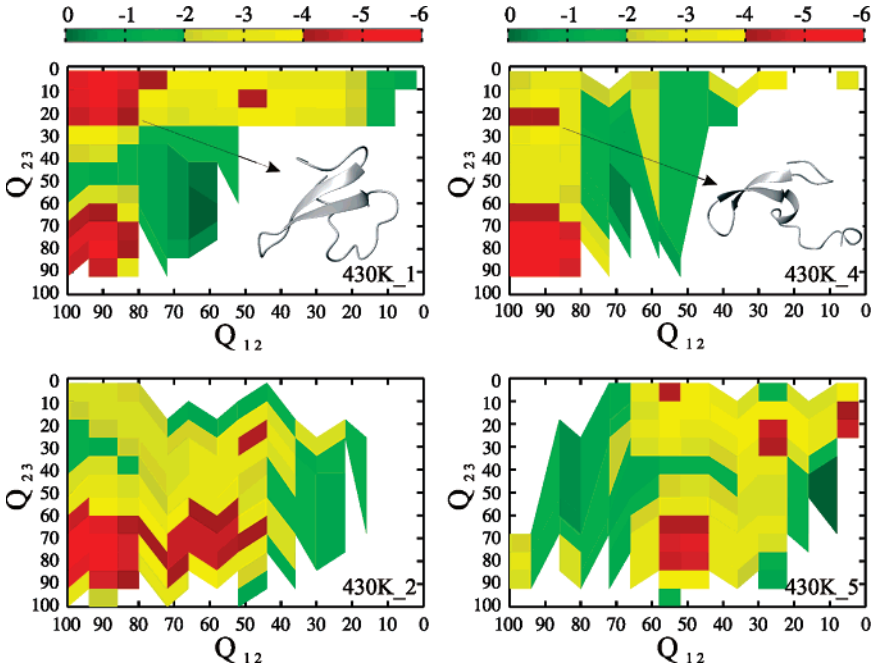

Figure 4. Two-dimensional population histogram defined by native contact percentages between β1 and β2 (Q_{12}) and β2 and β3 (Q_{23}) for FBP28 in the four unfolding trajectories. In 430K_1, the minimum around $Q_{12} = 90\%$, $Q_{23} = 10\%$ indicates a stable state that maintained the native contacts between β1 and β2 but lost the native contacts between β2 and β3. This corresponds to the intermediate state revealed in Figure 3. A similar state was also well populated in 430K_4, corresponding the minimum around $Q_{12} = 90\%$ and $Q_{23} = 20\%$. In 430K_2, the minima span almost the same regions for Q_{12} and Q_{23} at around 60−100%, which corresponds to the single and broad minimum shown in Figure 3. In 430K_5, no minimum is observable in the native state region.

separated from the native state but apparently without a high-energy barrier between these two states. In trajectory 430K_2, the protein sampled a region broad in Q_N (85−50%) and narrow in Rg (around 10 Å) where the native structure is still registered but with shorter β-strands before unfolding (Figures 3 and 4). This indicates a more two-state-like unfolding process. In trajectory 430K_5, the native state was not stable at all, and the well populated states before complete unfolding had ca. 60% of the native contacts.

At 500 K, FBP28 unfolded rapidly in all five simulations (see Figures 2 and 3). The native state region was sampled briefly before complete unfolding took place. No intermediate state was detectable. These trajectories thus suggest two-state unfolding behavior. Note that the trajectories were terminated after unfolding was completed, and therefore the relative sampling of the unfolded states (at $Q_N \sim 0\%$) in the different trajectories and compared to the native state as shown in Figure 3 is not meaningful.

The different results obtained for FBP28 at 430 K and 500 K demonstrate the temperature-dependence of the simulated unfolding of FBP28. At a temperature of 430 K, three-state behavior was observed, whereas it was absent at 500 K. We consider the temperature of 430 K an "acceptable" elevated temperature for studying the unfolding mechanism of FBP28. It should be clear that the five trajectories vary at 430 K, 1 showing no unfolding, 1 showing three-state behavior, 1 showing two-state behavior, and 2 showing intermediate behavior. This is the single molecule vs molecular ensemble problem in molecular dynamics simulations. The ensemble behavior observed in experiments may be reached by running a large number of simulations. Nevertheless, at the temperature of 430 K, even in the trajectories showing two-state behavior, the higher stability of the first β-hairpin was clearly observed. This property of the FBP28 WW domain was hard to detect at the temperature of 500 K.

Criteria for Acceptable Elevated Temperature. Herein, we propose a criterion for judging an elevated temperature based on the analysis of the unfolding trajectories. That is, the majority of the simulations starting from the native state should sample the native state substantially, resulting in a deep and narrow minimum around the native state region in a two-dimensional population histogram defined by native contact percentage and radius of gyration. This can only happen if the temperature is low enough for the native state to be a local energy minimum. Accordingly, as any intermediate on an unfolding pathway is assumed to be less stable than a native state, a lower temperature should be used to detect intermediate states. If the native state is lost too quickly in a trajectory, the simulation temperature is probably too high to be acceptable. As a result, the acceptable elevated temperature of a protein is dependent on its stability.

To quantify this criterion, we computed the number of structures saved during the 10 FBP28 WW domain trajectories simulated that sampled the native state region. The number of structures is 668, 866, 3522, 1931, and 61, respectively, in the five simulations at 430 K and 241, 56, 165, 91, and 255, respectively, in the five simulations at 500 K. We then suggest that, for the simulated systems, an elevated temperature is acceptable only when it leads to sampling of the native state region in at least 500 saved structures. Because the structures were saved every 10 ps,

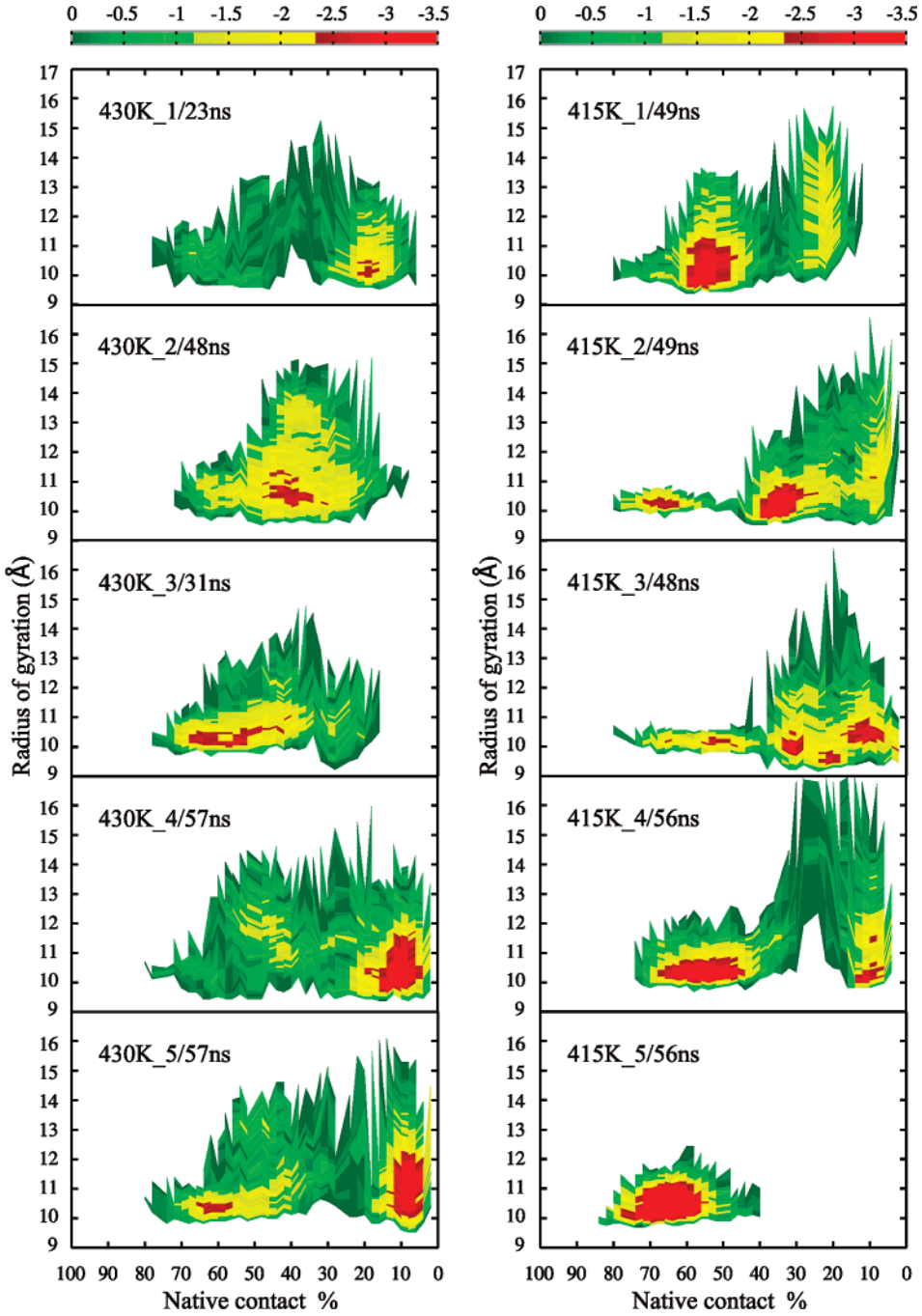

Figure 5. Two-dimensional population histogram of YAP65 simulated at 430 K and 415 K. The *X*-axis and the *Y*-axis are defined by the percentage of native contacts (Q_N) and the radius of gyration (Rg). At 430 K, the protein achieved complete unfolding in all five simulations but the native state region (Q_N=64−100%, Rg=9.5−10.5 Å) was sampled very briefly (far less than 5 ns (see text)). According to the criterion described in the text, 430 K is not an acceptable elevated temperature for unfolding YAP65. At 415 K, sampling of the native state region was substantially increased and reached the time of 5 ns in one (415K_1) of the four unfolding trajectories, indicating that the temperature of 415 K is acceptable.

this implies a total of 5 ns of sampling of the native state. When an unfolding trajectory satisfies this criterion at an elevated temperature of *T*, it means that the trajectory may reveal unfolding mechanisms corresponding to those obtained in experiments, and furthermore, temperature *T* is considered as an "acceptable elevated temperature". But it does not mean that all simulation trajectories obtained at this temperature will satisfy this criterion. Once this happens, as in trajectory 430K_5, it may indicate *T* as being the upper limit of the elevated temperature. In other words, it indicates that a temperature higher than *T* should not be used. In addition, it is worth noting that this empirical quantitative criterion is likely to be highly dependent on the force field used, and it may vary significantly for a different force field.

We then applied this criterion to simulations of the YAP65 WW domain. The unfolding simulations were first performed at 430 K, and complete unfolding occurred in all five simulations, lasting for 23 ns, 48 ns, 31 ns, 57 ns, and 57 ns, respectively. The two-dimensional population histograms defined by percentage of native contacts (Q_N) and radius of

gyration (Rg) are shown in Figure 5. We can see that the minimum corresponding to the native state of YAP65 is notable in 430K_3 and 430K_5 but absent in the other three simulations (430K_1, 430K_2, and 430K_4). The native state region was sampled 65, 47, 371, 77, and 241 times, respectively. By the criterion proposed above, the temperature of 430 K is not acceptable for studying the unfolding mechanisms of YAP65, and a lower temperature should be used. We then set up simulations at 370, 400, and 415 K. No unfolding was observed in the one simulation at 370 K and the three simulations at 400 K in simulation times of 72 ns. Unfolding was observed at 415 K. Five simulations were carried out at 415 K, lasting for 49 ns, 49 ns, 48 ns, 56 ns, and 56 ns, respectively. Unfolding was completed in four (415K_1, 415K_2, 415K_3, and 415K_4) of the five simulations. In Figure 5, we can see that the shape of the landscape defined by Q_N and Rg appears to differ in simulations at 415 K and at 430 K. Overall, the sampling of the native state region was substantially increased when the temperature decreased from 430 K to 415 K, reaching 162, 453, 233, 506, and 2353 times, respectively. Moreover, trajectory 415K_4 satisfied the criterion of a total of 5 ns sampling of the native state region before complete unfolding. This indicates that the temperature of 415 K is acceptable for simulating the unfolding of YAP65 and may be the upper limit of an elevated temperature as the other three unfolding trajectories (415K_1, 415K_2, and 415K_3) did not satisfy the criterion. The lower acceptable temperature required for YAP65 than FBP28 is consistent with the lower stability of YAP65. In addition, trajectory 415K_4 showed a clear two-state unfolding, and the unfolding began with the loss of the third strand, which is consistent with experiment[12,23] and our previous simulation.[24]

The acceptable temperature for YAP65 is found to be 15 K lower than that of FBP28 in these simulations. This is remarkably consistent with the 14 K lower T_m of YAP65 compared to FBP28, considering that the simulation temperature is elevated about 100 K above the T_m values. As well as being dependent on the force field used, it is worth noting that the acceptable temperature should also depend on the fold complexity of a protein and the level of detail used to define the unfolding pathway of a protein. In this study, the unfolding pathways of the WW domains are defined at the residue−residue contact level because the WW domains have a simple structure consisting of a single secondary structure element, the β strands. For other proteins, such as chymotrypsin inhibitor 2 (CI2)[6,7] and barnase,[2] that have complex structures consisting of both α and β elements, the acceptable temperature may vary with different levels of detail of the unfolding pathways, e.g., whether contacts are followed between secondary structure elements or between residues. More details require a lower simulation temperature.

Our observations for simulating protein unfolding also have parallels to observations of protein folding.[27] Marianayagam and co-workers[27] found that short folding simulations can only give the correct folding mechanism when started from an equilibrated denatured state ensemble, i.e. realistic sampling of the unfolded state is necessary prior to folding, just as sampling of the folded native state is necessary prior to unfolding in this work.

Conclusions

Standard molecular dynamics simulations of two WW domains have been carried out with an explicit solvent model starting from the native folded state at several temperatures ranging from 300 to 500 K. The simulated trajectories have durations of 12−72 ns and exhibit temperature-dependence consistent with experimental data. For the FBP28 WW domain and the given simulation model and force field, 430 K appears to be an acceptable elevated temperature to accelerate unfolding and allow observation of the unfolding pathways observed in experiments around the T_m of 337 K. The native folded state of the FBP28 WW domain was observed to be a significant local minimum only when the temperature did not exceed the acceptable elevated temperature. Based on this observation and the assumption that a lower temperature is needed to observe intermediate states, we propose a qualitative criterion for accepting an elevated temperature as that the native folded state must be sampled substantially before the unfolding starts. This can only happen when the temperature is low enough for the native state to be a local energy minimum. We further quantified this criterion and applied it to the YAP65 WW domain and found a lower acceptable elevated temperature of 415 K, which is in good agreement with its 14 K lower T_m than the FBP28 WW domain.

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