

Role of topology in protein thermal stability

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For several decades, experimental and computational studies have been used to investigate the potential functional role of knots in protein structures. A property that has attracted considerable attention is thermal stability, i.e., the extent to which a protein retains its native conformation and biological activity at high temperatures, without undergoing denaturation or aggregation. Thermal stability is quantified by the melting temperature T_m , an equilibrium property that corresponds to the peak of heat capacity in differential scanning calorimetry (DSC) experiments. Experimental and computational studies report conflicting effects of knotting on protein thermal stability [M. A. Soler and P. F. N. Faísca, *PLoS One* **8**, e74755 (2013); Y.-C. Chuang, I.-C. Hu, P.-C. Lyu, and S.-T. D. Hsu, *J. Mol. Biol.* **431**, 857 (2019)]. Here, we use extensive Monte Carlo simulations of a simple C-alpha model of protein YibK, with energetics modeled by the Go potential, to show that T_m does not depend on the topological state of the protein. Our simulations further support the view that the discrepancy between the experimental and computational results stems from a pronounced separation of timescales for unknotting and unfolding that is inherent to deeply knotted proteins like YibK. In particular, the timescale separation implies that the complete unfolding-untying transition may not be accessible within the duration of a DSC experiment, whose apparent T_m measurements likely reflect a nonequilibrium distribution lacking unfolded states that are also unknotted.

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I. INTRODUCTION

A knotted protein is a protein whose polypeptide chain is self-tied, forming a physical (i.e., open) knot in its native structure. Although the first knotted protein was reported in 1977 [1], it was not until 2000 that Taylor's computational methodology [2] (which enables a systematic determination of the topological state of open polymer chains) brought these complex proteins into the spotlight. Knotted proteins make up 1% of all protein entries (225k) in the Protein Data Bank (PDB) [3] and are statistically rare compared to compact random loops of the same size [4]. All three domains of life contain knotted proteins [5], including Archaea, a group of single-celled organisms believed to have inhabited the planet for around 3.8 billion years. A recent survey that analyzed 700k knotted proteins predicted by the computer artificial intelligence system AlphaFold (AF) [6], and 42 proteomes (16 bacteria, 9 fungi, 4 plants, and 13 animals), reported that

the percentage of knotted proteins in any given proteome is around 0.4% [7].

The minimum number of crossings in all possible planar projections of a given chain is an incomplete topological invariant used to classify both topological knots (which are closed curves in space) and protein knots. The 3_1 (or trefoil) knots, a class that features three crossings, is by far the most prevalent protein knot, followed by the 4_1 and 5_2 knots, with four and five crossings, respectively [3,7]. The most complex protein knot has seven crossings (it is a 7_1 knot) and was predicted by AF [6]. It is an 89-residue-long bacterial protein (termed Q9PR55) whose atomic structure was recently experimentally resolved, validating AF's predictions [8].

In the context of structural biology, the design principle “form follows function” means that a protein's structure is adapted to successfully perform its specific task. It highlights the fact that protein structure is not arbitrary but is shaped by the specificities of its role, which ultimately contributes to the organism's survival. The fact that knotted proteins are statistically rare does not imply a lack of function for the knot; rather, it indicates that folding into a knotted native structure is costly (e.g., it leads to slow folding and topological frustration [9]) and therefore unlikely to be tolerated unless compensated by specific advantages. If knotting were purely neutral or deleterious, one would expect it to be entirely suppressed by evolution. The fact that knotted proteins have been conserved throughout evolution [10] (and, in many cases, have likely

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evolved from knotted precursor structures in organisms dating back billions of years [11]) suggests that they may confer some advantage to their carriers. Thus, it is not surprising that a large body of theoretical and experimental work has been devoted to determining the potential functional roles of knots in proteins (reviewed in Refs. [11,12]), alongside establishing how they fold into their intricate native structures (reviewed in Refs. [11,13–15]). While significant advancements have been made regarding folding and knotting mechanisms, a consensus has not been reached concerning the function of protein knots [12]. Knots can potentially influence proteins at the level of physical properties, structure, and their biological function. A recent study found that the 5_2 knot in protein UCHL-1 is critical for the preservation of secondary structure [16]. Previous studies have reported that a knotted structure helps shape and form the binding site of enzymes [17–19]. Additionally, one study has shown that the knotted region is directly involved in enzymatic activity [20]. Indeed, for the protein TrmD (an example of an important class of enzymes, the SPOUT and SAM synthases), it was shown that the knot regulates catalysis, being critical to protein function [20]. Examples of physical properties are thermal stability, which measures a protein's resistance to melting and is quantified by the melting temperature; kinetic stability, which measures the protein's resistance to unfolding at a certain temperature and is quantified by the unfolding rate constant; and mechanical stability, a measure of the protein's ability to withstand physical forces and deformations without unfolding or losing its native structure. Simulation studies using models with different levels of resolution suggested that knots in proteins enhance kinetic stability [21,22], while an enhancement of mechanical stability has been reported for several model systems based on *in vitro* experiments [8,15,23] and computational studies [22,24].

Some years ago, we used Monte Carlo simulations of a simple lattice model to find that a knotted topology does not affect protein's thermal stability [25]. However, this contradicts a recent experimental study that reported a significant enhancement in the thermal stability of protein YibK, which features a deep trefoil knot [26]. In lattice models, the discretization of conformational space restricts backbone geometry, hindering the study of specific proteins like YibK, which is only possible in the context of a continuum off-lattice model. Additionally, the reduced conformational freedom inherent to lattice models means that they cannot accurately capture entropic effects. Thus, the purpose of the present work is to revisit the question of whether or not thermal stability is affected by the presence of an open knot in the native structure of protein YibK. Our results strongly support the view that knots play no role in enhancing protein thermal stability or any other thermodynamic equilibrium property.

II. MODEL AND METHODS

A. The C_α Go model

Proteins are represented by a simple C_α model (Fig. 1). Accordingly, residues are reduced to hard spherical beads of uniform size, centered on the C_α atoms. Consecutive C_α atoms are connected by rigid sticks representing pseudobonds on

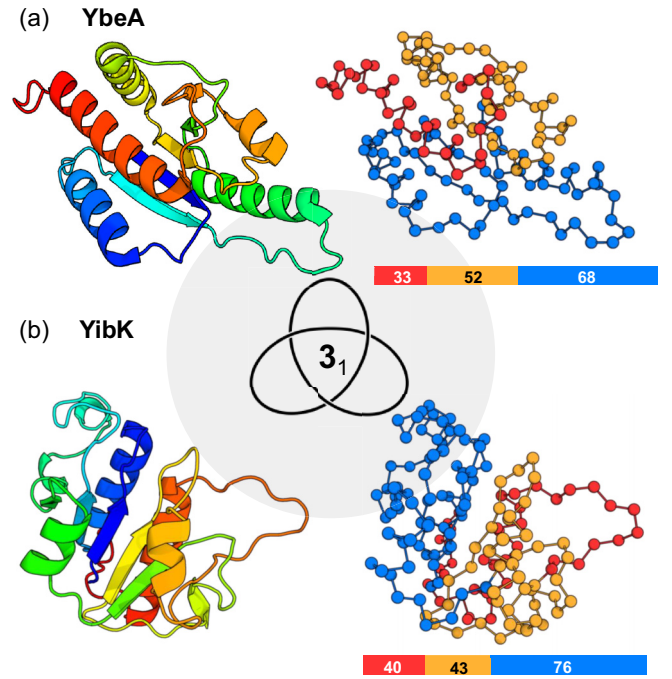


FIG. 1. Model systems used in the present study. Cartoon representation (left) and bead and stick representation (right) of the native structure of proteins YbeA (PDB ID: 1ns5, chain A) [panel (a)] and YibK (PDB ID: 1mxi, chain A) [panel (b)] with the knotted core colored in orange and the knot tails (number of residues that must be removed from the chain termini to untie the knot) colored in red (C-terminus) and blue (N-terminus). Each bead represents a C_α atom and rigid sticks represent pseudobonds connecting pairs of C_α atoms. The size (measured in number of beads) of the knot tails and knotted core is indicated. In both proteins, the knotted topology results from threading the C-terminus through the knotted core.

the amide planes. We adopt a radius of 1.7 Å for the beads, which is the van der Waals radius of C_α atoms [27]. For the length of each stick, we adopt the distance between the C_α atoms of the respective bonded residues in the protein's native conformation, these being approximately 2.9 Å for *cis* bonds and 3.8 Å for *trans* bonds. Two nonbonded residues are said to be in contact in the native conformation if the smallest distance between any two heavy atoms, one belonging to each residue, is ≤ 4.5 Å, this cutoff being chosen because it is slightly larger than twice the average van der Waals radius of heavy atoms in proteins.

To model protein energetics, we consider the native-centric Go potential [28]. Accordingly, the total energy E of a conformation defined by bead coordinates $\{\vec{r}_i\}$ is given by

$$E(\{\vec{r}_i\}) = \varepsilon \sum_{i,j \geq i+2}^N \left[\left(\frac{|\vec{r}_i - \vec{r}_j| - |\vec{r}_i^{\text{nat}} - \vec{r}_j^{\text{nat}}|}{w} \right)^2 + 1 \right]^{-1} \times \left(\chi_{ij} \chi_{ij}^{\text{nat}} + \chi_{ji} \chi_{ji}^{\text{nat}} + \frac{1}{2} \right) \Delta_{ij}^{\text{nat}}, \quad (1)$$

where N is the chain length measured in the number of beads, $\vec{r}_i^{\text{nat}}$ is the position vector of bead i in the native structure, Δ_{ij}^{nat} is 1 if the $i - j$ contact is present in the native conformation and is 0 otherwise, ε is a uniform intramolecular energy

parameter (taken as -1 in this study, in which energies and temperatures are shown in reduced units), w is the half-width of the inverse quadratic potential well, and the chirality of contact $i - j$ in the conformation under consideration is

$$\chi_{ij} = \Theta((\vec{r}_i - \vec{r}_j) \cdot [(\vec{r}_{j+1} - \vec{r}_j) \times (\vec{r}_{j-1} - \vec{r}_j)]) - \frac{1}{2}. \quad (2)$$

The chirality of the $i - j$ contact in the native conformation is

$$\chi_{ij}^{\text{nat}} = \Theta((\vec{r}_i^{\text{nat}} - \vec{r}_j^{\text{nat}}) \cdot [(\vec{r}_{j+1}^{\text{nat}} - \vec{r}_j^{\text{nat}}) \times (\vec{r}_{j-1}^{\text{nat}} - \vec{r}_j^{\text{nat}})]) - \frac{1}{2}. \quad (3)$$

In Eqs. (2) and (3), Θ is Heaviside's unit step function, which takes the value 1 if its argument is greater than zero and the value 0 otherwise. The chirality factor in Eq. (1) favors the native conformation *vis-à-vis* its mirror conformation. A native contact is considered formed if the distance between the centers of the respective beads differs from the distance between their C_α atoms in the native conformation by less than the half-width of the potential wells, w .

B. Replica-exchange Monte Carlo simulations

The conformational space of the C-alpha model is explored with Metropolis Monte Carlo (MC) [29] by means of a move set that comprises crankshaft and pivot moves. All simulations start from an unfolded (and unknotted) conformation obtained from a high-temperature simulation, and folding progress is monitored using the fraction of native contacts, Q . To sample equilibrium distributions, we use Monte Carlo replica exchange (MC-RE) [30].

To explore the conformational space, two sampling schemes can be deployed: a canonical one in which the move set preserves the linear topology of the chain, and another one that breaks the linear topology of the chain by allowing it to cross itself [31], while preserving the sequential bonding of the protein chain and excluded volume among its units. We have previously shown, using simple C_α protein models (of unknotted proteins Fn-III and β_2 m, shallowly knotted protein MJ0366, and deeply knotted protein Rds3p), that there is no difference in equilibrium properties calculated with a move set that preserves linear topology and one that does not [31]. However, the move set that breaks the linear topology of the chain is extraordinarily advantageous in the case of deeply knotted proteins like the ones studied here, since it provides correct equilibrium results at a much lower computational cost [31]. Thus, unless otherwise stated, we will deploy the sampling method that does not preserve the linear topology of the chain.

The weighted histogram analysis method (WHAM) [32] is used to analyze data from the MC-RE simulations and to produce maximum-likelihood estimates of the density of states, from which expected values for thermodynamic properties are calculated as functions of temperature. An example is the heat capacity, C_V , defined in reduced units as $C_V = (\langle E^2 \rangle - \langle E \rangle^2)/T^2$. The melting temperature, T_m , is determined as the temperature at which the C_V peaks. We measure the cooperativity degree of the transition by the ratio of the full width at half maximum (FWHM) of the C_V peak to the melting temperature, and the half-width of the potential well, w , is adjusted to obtain a simulated FWHM/ T_m ratio between 4%

and 5% [33,34]. This criterion has been successfully used in previous simulations employing a similar potential and sampling [35,36]. WHAM is also used to project the density of states along Q to obtain free energy profiles at a specific temperature.

C. Molecular dynamics simulations

The molecular dynamics (MD) simulations were performed using GROMACS 2024.3 [37], with the AMBER 14SB force fields [38], and the TIP3P water model [39,40]. The system consisted of a protein, 8808 water molecules, and 4 Cl^- ions to equilibrate the overall system charges. We performed a two-step minimization procedure using the steepest descent method and an initialization protocol with two additional steps, in which temperature (NVT) and pressure (NPT) baths are turned on and equilibrated for 100 ps segments, with an integrator step of 1 fs. The nonbonded interactions were computed using a single cutoff of 1.0 nm, with the neighbor list updated every 20 steps. van der Waals interactions were truncated beyond this cutoff, while long-range electrostatics were calculated using the particle-mesh Ewald method [41], with a Verlet cutoff of 1.0 nm and a Fourier grid spacing of 0.125 nm. Bond lengths involving hydrogen atoms in solute and water molecules were constrained using the LINCS [42] and SETTLE [43] algorithms, respectively. In the NVT ensemble, the v-rescale thermostat [44] was employed to maintain a temperature of 310 K (with a coupling constant of 0.1 ps). In the NPT ensemble, the v-rescale thermostat [44] (coupling constant of 1.0 ps) was used in combination with the Parrinello-Rahman barostat [45] (coupling constant of 2.0 ps and isothermal compressibility of $4.5 \times 10^{-5} \text{ bar}^{-1}$). For the MD relaxation step, we run for 100 ns with the integration step adjusted to 2 fs.

D. Topological state

The topological state (knotted or unknotted) of a sampled conformation is determined using the Koniaris-Muthukumar-Taylor algorithm [2].

III. RESULTS AND DISCUSSION

A. Model systems

Proteins YbeA (from *Escherichia coli*, PDB ID: 1ns5) and YibK (from *Haemophilus influenzae*, PDB ID: 1mxi and PDB ID: 1j85) are homodimeric enzymes that belong to the $\alpha\beta$ -knot superfamily of MTases. In this work, we consider the monomer identified as chain A in the corresponding PDB files. The native structure of both proteins features a trefoil knot. In a knotted protein, the region of the polypeptide chain encompassing the knot is termed the knotted core. Protein knots are classified as deep or shallow, depending on the number of residues that must be removed from one of the termini to unknot the chain. If the latter is larger (or smaller) than 20, the knot is deep (or shallow) [14,46,47]. YbeA has a chain length of 155 amino acids, and its knotted core extends from residue 69 to residue 121. Consequently, it is necessary to remove 68 residues from the N-terminus (or 34 residues from the C-terminus) to untie the knot that is classified as

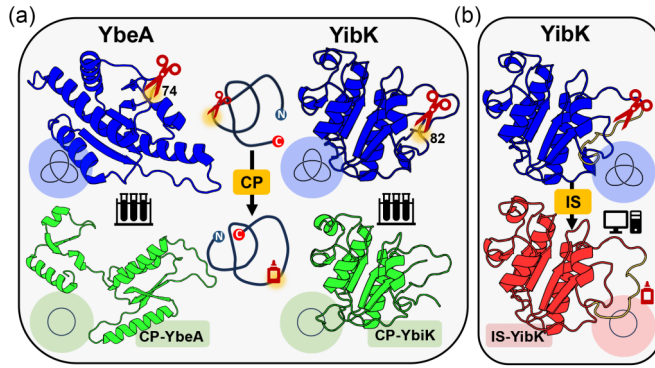


FIG. 2. Control systems used in the present study. Unknotted control systems CP-YbeA (PDB ID: 1ns5) and CP-YibK (PDB ID: 6ahw) obtained experimentally by Hsu and co-workers through circular permutation of YbeA (PDB ID: 1ns5) and YibK (PDB ID: 1mxi), respectively [panel (a)], and *in silico* unknotted control system IS-YibK, obtained through computer modeling from YibK (PDB ID: 1mxi) [panel (b)]. The knotted structures are all shown in blue, the unknotted structures obtained experimentally by CP are shown in green, and the unknotted structure obtained *in silico* is shown in red.

deep. The native structure of YibK also embeds a deep trefoil knot. Indeed, YibK has a chain length of 160 residues and a knotted core that extends from residue 77 to residue 120, requiring removal of 76 residues from the N-terminus (or 40 residues from the C-terminus) to untie the knot. Despite their structural similarity, these bacterial proteins share only 19% of sequence identity.

To explore the role of the knotted topology in determining equilibrium properties, it is necessary to consider control systems [9]. A suitable control system is a similar, yet unknotted protein that must be used for comparison. Since knotted/unknotted pairs do not exist in nature, they must be designed or engineered. In the present study, we consider two control systems obtained experimentally and one control system created through computational modeling. The *in vitro* control systems were engineered by Hsu and co-workers through a process known as circular permutation [26,48]. A circular permutant (CP) is an engineered protein that results from linking the C- and N-terminus of the polypeptide chain after disrupting the protein's backbone at some selected site [49]. In the case of protein YbeA, an unknotted control system was obtained by creating a CP after breaking the chain at the knotted core in position 74, while for YibK the chain was broken at position 82, also in the knotted core, to create the CP [Fig. 2(a)]. In the present study, the resulting unknotted structures are designated by CP-YbeA (PDB ID: 5zyo) and CP-YibK (PDB ID: 6ahw). The *in silico* control structure was obtained for YibK (PDB ID: 1mxi) by manually passing the loop containing residues 114–125 across the knotted core (amino acids 81–85) [Fig. 2(b)]. The manipulated structure was then subjected to an MD simulation, in which segments 1–80, 86–113, and 126–156 were positionally restrained 1000 kJ/mol nm², while the two loops (81–85 and 114–125) were allowed to relax. The final relaxed and refined structure is named IS-YibK.

For all structures, we computed the gyration radius (R_g), which is a measure of structural compactness, the root-mean-

TABLE I. Properties of the model proteins considered in this study, where K stands for knotted and U stands for unknotted. The RMSD of the unknotted controls is measured against the corresponding knotted structure.

Protein	Topological state	RMSD (Å)	R_g (Å)	No. of native contacts
YbeA	K	–	15.5	542
CP-YbeA	U	16.6	21.8	410
YibK	K	–	15.9	552
CP-YibK	U	0.35	15.5	561
IS-YibK	U	0.48	16.6	500

square-deviation (RMSD) to the wild-type native structure, which is a measure of structural similarity, and the number of native contacts, which is a measure of the system's energy in the context of the adopted model. These properties are reported in Table I. The native structure of CP-YbeA exhibits significant structural rearrangements, which are visible in the cartoon representation [Fig. 2(a)], and results in a substantially high RMSD ($\gg 3$ Å). There is also a significant loss of compactness, resulting in a high gyration radius and a substantial loss ($\sim 25\%$) of native contacts. On the other hand, both the unknotted CP-YibK and IS-YibK share a high degree of structural similarity with YibK (RMSD $\ll 2$ Å). However, while IS-YibK has fewer native contacts than YibK ($\sim 11\%$), the circular permutant exhibits a nearly identical degree of compactness and essentially the same number of native contacts (the difference between the two model systems being only 2%), which makes it the best control system considered here.

For each knotted protein and corresponding control system, we measured several equilibrium properties as a function of temperature.

B. Protein YbeA and its control system

Results reported in Figs. 3(a)–3(d) show that YbeA folds cooperatively in a two-state manner, while the control CP-YbeA folds noncooperatively. This lack of cooperativity is particularly evident from the rather gentle slope of the internal energy curve [Fig. 3(a)], and from the broadness of the heat capacity curve [Fig. 3(d)]. In addition, the melting temperature of CP-YbeA is significantly ($\sim 15\%$) lower than that of YbeA. We have also measured the dependence of the free energy on the fraction of native contacts (Q) at the melting temperature for the knotted-unknotted pair. We find that the behavior of the folding transition changes from two-state (for YbeA) to downhill (or barrierless) (for CP-YbeA) [Fig. 4(a)]. These findings, in particular the decreased thermal stability of CP-YbeA, cannot be attributed solely to its unknotted topological state. Indeed, untying the knot in YbeA introduced drastic structural changes and a significant loss of native interactions, which collectively account for the observed behavior.

C. Protein YibK and its control systems

To isolate the role of the topological state in thermal equilibrium properties, particularly thermal stability, we now

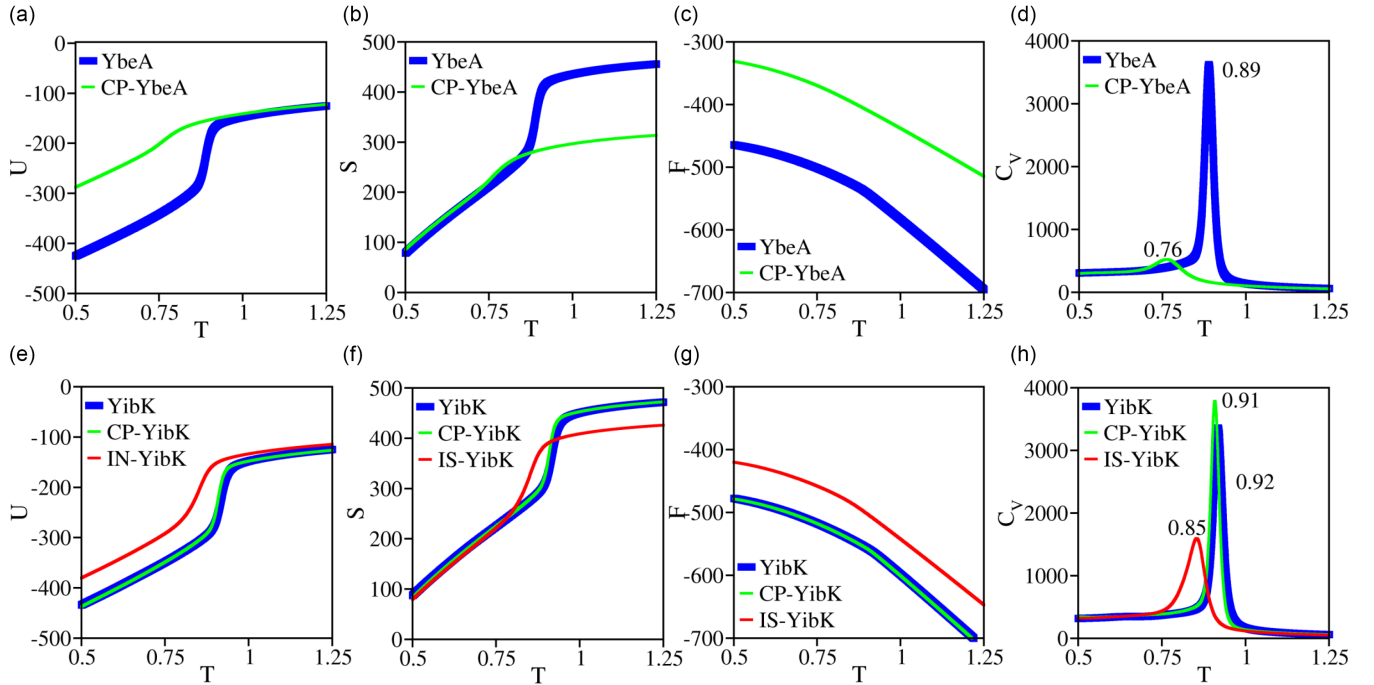


FIG. 3. Equilibrium properties as a function of temperature obtained from Monte Carlo simulations. Dependence of the internal energy (U), entropy (S), free energy (F), and heat capacity (C_V) on temperature (T) for the knotted (K) and unknotted (U) model systems of YbeA [panels (a)–(d)] and YibK [panels (e)–(h)]. The melting temperature, T_m , is indicated on the corresponding heat capacity curves.

examine YibK and its control systems. In this case, the two-state nature of the folding transition is conserved across the three systems [Figs. 3(e)–3(h)]. A minor, yet non-negligible difference is observed between YibK and its *in silico* control system, IS-YibK. In particular, for the latter, the melting temperature is 7% lower than that of YibK [Fig. 3(h)]. Since the number of native contacts is not conserved in the unknotted protein, we cannot ascribe its lower thermal stability to its unknotted topological state. In fact, when one compares YibK and CP-YibK, which are nearly structurally identical and share almost the same number of native contacts, their thermodynamic behavior is practically identical, with the thermal stability differing by only 1%. In line with these findings, the similarity between the free energy profiles [Fig. 4(b)] is

also significantly higher for YibK and its unknotted circular permutant.

D. Comparison with experimental data

The results reported in the previous section show that as the similarity between the structures of a knotted protein and its unknotted control increases, differences in thermodynamic equilibrium behavior diminish. Indeed, simulations indicate that an unknotted control preserving the same native geometry (and, consequently, distances between atoms) would exhibit similar thermodynamic behavior. From a physics perspective, this is expected: In our model, the Hamiltonian depends only on the native contact map (atomic positions and corresponding interactions) and is independent of the topological state. All native interactions are identical between the knotted and unknotted structures, and non-native interactions are neutral by construction. Therefore, if the native contact maps of knotted and unknotted structures are identical (or nearly identical as in the case of YibK and its control system CP-YibK), an identical (or nearly identical) density of states is expected, and, consequently, an identical (or nearly identical) thermodynamic behavior as well.

However, the experimental measurements carried out for YibK and CP-YibK do not support the findings from simulations, indicating that the knotted topology enhances the protein's thermal stability. Indeed, a decrease in the melting temperature of 22%, measured with differential scanning calorimetry (DSC), or 27%, measured with far-ultraviolet circular dichroism signals, was reported [26] for the unknotted protein.

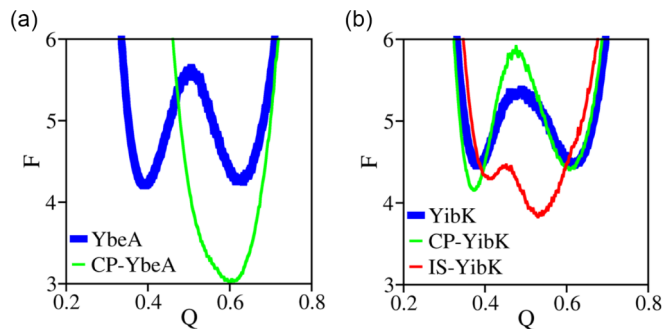


FIG. 4. Free energy profiles obtained from Monte Carlo simulations. Dependence of the free energy (F) on the fraction of native contacts Q at the melting temperature (T_m) for the YbeA [panel (a)] and YibK [panel (b)] model systems.

Part of the decrease in thermal stability observed experimentally may be due to a 13% reduction in the dimer interface per chain in the dimeric version of CP-YibK. A fraction of the decreased stability could then be attributed to the weakened dimer interface, featuring fewer intermolecular hydrogen bonds and salt bridges [26]. Because experimental measurements were performed on the dimer while simulations focused on the monomer, a direct comparison between experimental and computational data is not straightforward. Furthermore, the thermal unfolding experiments were not demonstrated to be reversible, unlike the simulations. If unfolding is irreversible, the reported experimental melting temperatures reflect apparent rather than true thermal stability. If the control system CP-YibK used in experiments is more prone to aggregation than the knotted YibK, this could also easily suppress the experimental melting temperature. Although simulations provide a controlled framework to isolate the role of protein topology in thermal stability, they rely on approximations at the level of both protein representation and the Hamiltonian. Ultimately, this means that the simulation results should be interpreted with care rather than providing definitive thermodynamic measurements. Despite the limitations inherent in both experimental and computational approaches, their results provide different views of the role of topology in determining YibK's thermal stability. In the following, we discuss a possible, fundamental reason for this discrepancy.

A few years ago, Capraro and Jennings [50] reported the existence of two distinct timescales for unfolding and untying, upon chemical denaturation, in the deeply knotted protein MTTTm (PDB ID: 106d), which belongs to the same family as YibK and has a comparable chain length. They found that the transition to the untied states required at least 6 months, whereas unfolding occurred on a timescale of approximately 2 weeks. A previous study by Jackson and co-workers reported that the knotted topology is retained in the unfolded state of YibK, following chemical denaturation [51]. These experimental observations are consistent with insights from computational simulations based on lattice models that also reported the occurrence of knotted conformations in thermally denatured states of deeply knotted lattice proteins [25].

As discussed in the Model and Methods section, achieving equilibrium distributions for complex polymeric systems, such as deeply knotted proteins, is particularly challenging when using off-lattice Monte Carlo (MC) simulations with a move set that preserves the chain's linear topology, starting from unfolded, unknotted conformations. For instance, in simulations of the deeply knotted protein Rds3p, which is 51 amino acids shorter than YibK, the linear topology-preserving move set required an order of magnitude more MC steps to reach equilibrium compared to simulations that allow chain crossings [31]. In contrast, for unknotted proteins, such as Fn-III and β 2-microglobulin, the computational cost is independent of the move set employed [31]. The fact that, for unknotted proteins, the equilibration time is independent of the move set, whereas deeply knotted proteins exhibit a strong dependence, indicates that knotting introduces a significant degree of topological frustration, which delays the equilibration time, when chain crossings are forbidden. We repeated the analysis of the equilibration time for YibK and

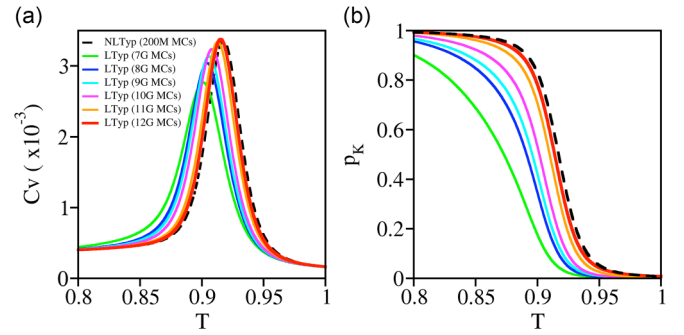


FIG. 5. Dependence of the heat capacity [panel (a)] and of the knotting probability [panel (b)] on the simulation temperature for YibK. The MC simulation that does not preserve the linear topology of the chain (NLTyp) requires 200×10^6 MC steps to equilibrate (the curves do not change for a larger number of MC steps). On the other hand, a simulation that preserves the linear topology of the chain requires 12×10^9 MC steps to equilibrate. Interestingly, a nonequilibrated simulation predicts an apparent T_m that is smaller and a transition that is clearly less sharp (as denoted by the shape of the knotting probability curve) than that predicted by the equilibrated sampling.

found that enforcing linear topology preservation increases the equilibration time by 1.78 orders of magnitude relative to simulations that do not preserve the linear topology of the chain (Fig. 5). Since real protein dynamics also preserves the linear topology of the polypeptide chain, it is plausible that the scan rate (i.e., heating rate) used in the DSC experiments is too fast for the knotted protein to equilibrate at each temperature, and even more so to allow the population of denatured conformations that are also unknotted. Thus, the pronounced separation of timescales for unknotting and unfolding suggests that, for deeply knotted proteins such as YibK, the complete unfolding-untying transition may not be accessible within the time frame of a DSC experiment. In this case, the experimental measurements likely reflect a nonequilibrium distribution lacking unfolded states that are also unknotted; in that case, the measured melting temperature represents an apparent value rather than a true thermodynamic parameter.

IV. CONCLUSIONS

Over the past 20 years, a remarkably large body of theoretical and experimental work has been dedicated to the study of knotted proteins [13–15]. A major line of research has focused on whether knots confer any functional advantage to their carriers [12]. Answering this question will provide insights into the evolutionary significance of knotted proteins and may have an impact on the design of drugs for technological or medical applications.

A major challenge in studying the role of knots in proteins is the construction of a suitable control system, a version of the protein that is nearly identical to the knotted variant but lacks the knot [9]. In the context of lattice models, it is quite straightforward to untie a model protein while introducing minimal changes to the structure [52], and previous simulation efforts in the lattice context have shown that a knotted topology does not enhance the protein's thermal stability [25].

Unfortunately, in more realistic off-lattice models, as well as in real-world proteins, engineering unknotted control systems is not as straightforward.

Here, we conducted extensive Monte Carlo simulations of a simple off-lattice model with energetics governed by a Go potential to show that protein knots can strongly affect the equilibration time and make the equilibrium distribution not accessible in a reasonable timescale. Indeed, by considering the chain's linear topology as either "on" or "off", our simulations corroborated that knots impose this limitation, with the move set that preserves the linear topology requiring almost two orders of magnitude more Monte Carlo steps to equilibrate. Additionally, by comparing the knotted protein YibK with a nearly identical control system experimentally engineered [26], our results show that, if equilibrium is indeed reached, knotting does not enhance the thermal stability of this deeply knotted protein. This finding contradicts experimental data analyzing the same pair of structures, which reported a moderate decrease in thermal stability for the unknotted one [26]. Based on results from the simulations conducted here, and previous experimental results [50,51] and computational data [25], we argue that the experimental enhancement of thermal stability in YibK likely reflects nonequilibrium effects. The persistence of knotted conformations in the denatured ensembles leads to a strong separation of timescales between unfolding (couple of weeks) and unknotting (at least 6 months) [50], and suggests that standard thermal denaturation experiments may not be able to access unfolded and unknotted states, leading to an apparent melting temperature that does not correspond to an equilibrium distribution. Overall, our findings indicate that protein knots do not intrinsically enhance thermal stability (or any other thermodynamic equilibrium property) and suggest that research in this topic should focus on nonequilibrium properties instead.

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P.F.N.F. designed the research, prepared the figures, and wrote the manuscript. J.N.C.E. developed the code and performed all the calculations. All authors analyzed the data. A.N., B.P.T., and M.M. prepared the *in silico* unknotted version of YibK.

The authors declare no competing interests.

DATA AVAILABILITY

The data that support the findings of this article are not publicly available. The data are available from the authors upon reasonable request.

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