Testing simplified protein models of the hPin1 WW domain

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The WW domain of the human Pin1 protein for its simple topology and the large amount of experimental data is an ideal candidate to assess theoretical approaches to protein folding. The purpose of the present work is to compare the reliability of the chemically-based Sorenson/Head-Gordon (SHG) model and a standard native centric model in reproducing through molecular dynamics simulations some of the well known features of the folding transition of this small domain. Our results show that the Gō model correctly reproduces the cooperative, two-state, folding mechanism of the WW-domain, while the SHG model predicts a transition occurring in two stages: a collapse followed by a structural rearrangement. The lack of a cooperative folding in the SHG simulations appears to be related to the non-funnel shape of the energy landscape featuring a partitioning of the native valley in sub-basins corresponding to different chain chiralities. However the SHG approach remains more reliable in estimating the Φ-values with respect to Gō-like description. This may suggest that the WW-domain folding process is stirred by energetic and topological factors as well, and it highlights the better suitability of chemically-based models in simulating mutations.

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I. INTRODUCTION

The WW domains are a family of fast-folding, compact, modular domains featuring a triple-stranded, antiparallel beta-sheet owing their name to the presence of two highly conserved Triptophanes (W). Recent studies suggested that these domains may fold at a rate close to the speed limit for β-sheet formation offering the opportunity to investigate the pathways of β-sheet kinetics.

In particular, the human Pin1 protein WW domain, due to the availability of several structural, thermodynamical and kinetic experimental data, represents an excellent target to test computational techniques and theoretical approaches.

The structure of this domain, resolved through NMR and X-ray diffraction (Fig. 1), is characterized by hydrophobic clusters providing the largest contribution to the thermodynamic stability.

Cluster 1 (CL1) involves residues Leu7, Trp11, Tyr24 and Pro37, the second cluster (CL2) comprises Tyr23, Phe25 and Arg14. The stability of the molecule also derives from a network of hydrogen-bonds whose central element is the highly conserved Asn26 located on strand β2 and acting both as donor and acceptor in bonds with Pro9, Trp11, Ile28 and Thr29, thus linking strands β1 and β3. The presence of two loops, Loop I (L1) plays a key role in substrate recognition as it binds to the phosphate of the pS-P motif of the Proline-rich ligands, Loop II (L2) instead, gives an important contribution to thermal stability. Thermal denaturation experiments and simplified statistical physics approaches have shown that the Pin1 WW domain folds following cooperative two-state mechanism at the temperature $T_M = 332$ K. The mutagenesis analysis performed by the same authors identified the mutations on Ser16, Ser18 and Ser19 in Loop I as maximally destabilizing for the transition state, so that the formation of L1 appears to be the rate-limiting step in the folding/unfolding process. Loop II (L2) is involved in the formation of the transition state only at high temperatures. Due to the ability of inducing conformational changes in Proline-rich, phosphorilated substrates, Pin1 is a potential regulator of the cell-cycle, and maybe involved in pathologies like Liddle’s syndrome, muscular dystrophy and Alzheimer’s disease.

The aim of this work is the comparison of two off-lattice protein descriptions: the Gō-model which customary allows studying the influence of the native state structure on the folding process, and a model proposed by Sorenson and Head-Gordon (hereafter referred to as SHG), mainly based on the primary and secondary structural information. The conceptual justification of topology-based or native centric models relies on the observation that the topology of native states can play a crucial role in selecting some features of the folding mechanism. The main experimental finding supporting the above statement can be summarized as follows (Baker): (a) the similarity shared by transition-state conformations and folding mechanisms of proteins having structurally related native states despite their low sequence homology, and (b) the correlation that certain simple topological properties, such as contact order, may have with protein folding rates. The Gō-force field is independent of the native state only at high temperatures.
even if recent works\cite{21,22,23,24,25,26} provide growing evidence that it can be confidently used for the characterization of transition states of real small, fast-folding (sub-millisecond)\cite{22,28} proteins that are characterized by a low level of energetic frustration. However, topological models might not correctly reproduce the folding process when chemical interactions play a relevant role. The SHG model, which is instead based on the chemical and physical properties of amino-acids such as hydrophobicity, is in principle better applicable to proteins with a higher level of energetic frustration. Moreover, requiring the knowledge of primary and secondary structures only, the model has a greater predictive power, and in this sense, could be considered closer to an ab initio representation. The above arguments motivate a detailed comparison between the two protein models to assess their applicability and potentialities in the study of biomolecules.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Backbone representation of the NMR structure of Pin1 WW domain (pdb-id = 1NMV, Left) and simulated structure $\Gamma_0$ (Right). Residues in the three $\beta$-strands are coloured in blue, those belonging to loops L1 and L2 in yellow. Side-chains of residues participating in CL1 (Leu7, Trp11, Tyr24, Pro37) are shown in green stick representation, those involved in CL2 (Arg14, Tyr23, Phe25) in magenta. Figures were drawn with RASMOL.}
\end{figure}

\section{II. Theory and Methods}

When native state topology plays the relevant role in driving the folding process, many molecular details of protein structures can be mapped onto simplified coarse-grained models encoding the overall topology through the knowledge of the native contacts. These models, neglecting side chains and peptide groups, reduce a protein chain to its backbone, where aminoacids are assimilated to beads centred on their $\alpha$ carbon atoms. The G"{o} energy function, mimicking a perfect funnel landscape, assigns to the native state the lowest energy by simply promoting the formation of native interactions. Here we employ the force field proposed by Clementi et al.\cite{22} with distance cutoff $R_c = 6.5$ Å\cite{29} to to identify native contacts in the structure 1NMV.pdb. A native contact means that the distance, $R_{ij}$, of C$_\alpha$ atoms relative to residues $i$ and $j$ ($|i-j| \geq 3$) is less than $R_c$ in the native state. This pair undergoes an attractive LJ-interaction

$$V_{nat}(r_{ij}) = \epsilon \left[ 5 \left( \frac{R_{ij}}{r_{ij}} \right)^{12} - 6 \left( \frac{R_{ij}}{r_{ij}} \right)^{10} \right] \quad (1)$$

with equilibrium distance $R_{ij}$. When two residues are not in a native contact ($R_{ij} > R_c$) they interact through a repulsive potential

$$V_{nnat}(r_{ij}) = \frac{2\epsilon}{3} \left( \frac{\sigma}{r_{ij}} \right)^{12}$$

with $\sigma = 4.5$ Å. These non-native interactions, besides ensuring the self-avoidance of the chain, generally enhance the cooperativity of the overall folding process\cite{30}. A further bias towards the native secondary structure is introduced through a bending and a dihedral potential. The former is modeled as an harmonic function and allows only small oscillations around the native angles $(\theta^0_i)$ formed by three consecutive residues

$$V_\phi(\theta_i) = \frac{1}{2} k_\phi (\theta_i - \theta^0_i)^2$$

with harmonic constant $k_\phi = 20\epsilon$. The most important determinant of the secondary structure is the dihedral potential arising from the torsional energy. Each dihedral angle, identified by four consecutive beads, contributes to the potential with the terms

$$V_\phi(\phi_i) = k^{(1)}_\phi \left[ 1 - \cos(\phi_i - \phi^0_i) \right] + k^{(3)}_\phi \left[ 1 - \cos 3(\phi_i - \phi^0_i) \right]$$

where $\phi^0_i$ is the value of angle $i$ in the native structure, $k^{(1)}_\phi = \epsilon$ and $k^{(3)}_\phi = 5\epsilon$. Finally, consecutive residues interact with each other through the potential harmonic in their distance $r_{i,i+1}$

$$V_b(r_{i,i+1}) = \frac{k_b}{2} (r_{i,i+1} - b)^2 \quad (2)$$

which maintains the chain connectivity, with $b_i$ being the native bond-length and $k_b = 1000/r_b^2$. Therefore, the global G"{o}-potential reads

$$V_{Tot} = \sum_{i=1}^{N-1} V_h(r_{i,i+1}) + \sum_{i=1}^{N-2} V_\phi(\theta_i) + \sum_{i=1}^{N-3} V_\phi(\phi_i) + \sum_{i,j>i+2} \left\{ V_{nat}(r_{ij}) \Delta_{ij} + (1 - \Delta_{ij}) V_{nnat}(r_{ij}) \right\}$$

where $\Delta_{ij} = 1(0)$ if the contact is native (non-native). G"{o} models of the type just outlined may produce a gradual folding behaviour being incapable to reproduce the typical kinetic cooperativity of two-state folders. Experimental studies suggest\cite{31,32} that the origin of cooperativity lies in specific interactions appearing only after
the assembly of native-like structures. These particular interactions can be modeled by imparting an extra energetic global stabilization to the native state through a different analytical form of the energy function when the chain visits the native basin. In the present work we need to implement the rescaling method to make the folding transition highly cooperative in agreement with the experiments on the WW domain. The interaction forces on the residues are thus computed according to the following rule

$$F_{\text{conf}} = \begin{cases} -\nabla V_{T\alpha} & \text{for } Q < Q_{\text{th}} \\ -\nabla V_h - \rho \nabla (V_{T\alpha} - V_h) & \text{for } Q \geq Q_{\text{th}} \end{cases}$$

(3)

where $Q$ is the fraction of formed native contacts and $\rho = 2$ is the scaling factor. The force rescaling determines a higher free energy barrier between the folded and unfolded state in correspondence of the folding temperature resulting in a higher cooperativity. Therefore, the residence times in the folded and unfolded state are expected to be significantly longer.

The SHG model is an off-lattice minimal model that generalizes a previous model introduced by Thirumalai and co-workers. This approach represents α-carbons with beads of three possible flavors hydrophobic (B), hydrophilic (L) and neutral (N), according to table [I].

### Table I: Dictionary for the translation of three-letter code of the 20 natural amino-acid into the three-flavor code [I].

| A.A. F | A.A. F | A.A. F | A.A. F |
|--------|--------|--------|--------|
| Ala B | Met B | Gly N | Asn L |
| Cys B | Val B | Ser N | His L |
| Leu B | Thr B | Gln L | Lys L |
| Ile B | Tyr B | Glu L | Arg L |
| Phe B | Pro B | Asp L | 

The driving force responsible for the collapse onto a compact structure is the attraction between B-beads, whereas the repulsion between L and N beads determines the rearrangements of the compact structure into the native topology. The long-range interactions between residues which may be far apart in sequence space is modelled through the potential:

$$V_{LR} = \sum_{i,j,i \neq j \geq 3} \epsilon_h S_1 \left( \frac{\sigma}{r_{ij}} \right)^{12} - 2 S_2 \left( \frac{\sigma}{r_{ij}} \right)^6$$

(4)

where $\epsilon_h$ (1.65 Kcal mol$^{-1}$ see below) sets the energy scale and $\sigma = 4.0$ Å. The attractive forces between hydrophobic residues is attained by setting $S_1 = S_2 = 1$ for BB pairs, while the interactions involving the LL and LB pairs are characterized by $S_1 = 1/3$ and $S_2 = -1$. This interaction is repulsive and the $r^{-6}$ term, which accounts for the hydration shell around the hydrophilic residues, makes the potential longer ranged than the usual $r^{-12}$. The forces involving neutral residues are also repulsive and amount to an excluded volume potential by setting $S_1 = 0$ and $S_2 = 0$. The secondary structure arises as a result of bending and dihedral interactions, which surrogates side-chain packing and hydrogen-bonding. The analytic expression of the dihedral potential is

$$V_{dh} = \sum_{i=1}^{N-3} \left[ A_i (1 + \cos \phi_i) + B_i (1 - \cos \phi_i) + C_i (1 + \cos 3\phi_i) + D_i (1 - \cos (\phi_i + \pi/4)) \right]$$

(5)

where $\phi_i$ indicates the angle between the two adjacent planes identified by the positions of four consecutive beads. The information on secondary structures is systematically stored in the coefficients $A, B, C$ and $D$ that determine a bias on the angles reflecting the propensity of residues to form a specific secondary motif. Indeed, each dihedral, in the chain, is defined to be either helical (H: $A_i = 0$, $B_i = C_i = D_i = 1.2\epsilon_h$), extended (E: $A_i = 0.9\epsilon_h$, $C_i = 1.2\epsilon_h$, $B_i = D_i = 0$), or turn (T: $A_i = B_i = D_i = 0$, $C_i = 0.2\epsilon_h$). Therefore, the primary structure must be complemented with the auxiliary sequence, of “E,H,T” symbols assigning the appropriate set of coefficients. The decoupling between primary and dihedral sequence, not present in similar models, increases the possibilities in the modulations of relative strengths between local and non-local interactions which results in a finer structural tuning. The Head-Gordon force field is completed by a bond angle interaction modelled as a harmonic potential

$$V_\theta = \sum_{i=1}^{N-2} \frac{k_\theta}{2} (\theta_i - \theta_0)^2$$

(6)

with a constant $k_\theta = 20\epsilon_h/(rad)^2$ so that large deviations from the equilibrium value $\theta_0 = 1.8326$ rad are unlikely, and bond angles result basically fixed. Also in this model, stiff springs with equilibrium distance $r_0 = 3.8$ Å, maintain the chain connectivity mimicking the presence of covalent peptide bonds between successive aminocids. This stiff interaction allows to keep the bond length approximately fixed, while being less computationally demanding than the RATTLE algorithm used in previous works to enforce fixed bond lengths. The SHG model retains only the minimal number of elements needed to capture the essential features of protein molecules, however, some strong determinants such as hydrogen bonding and side chains are missing. These limitations should be compensated through a design strategy for optimising the sequence. Here, we used the sequence LBBNN-BLBLB-NLNNN-LBBBB- LLNLL-BNBBB-LBNN proposed in Ref. [I] for the hPin1 WW domain and designed via a threading approach based on energy gap maximization (42). The secondary structure propensity, selecting the native-like dihedral angles, is encoded in the auxiliary sequence TTTT-TEEEE- TTTT-TEEEE-TTTT-EETTT-EET, built directly through the information contained in the PDB file 1NMB.pdb.
to control the temperature, we performed constant temperature MD simulations within the isokinetic scheme using dimensionless quantities. The temperature was measured in units of $\epsilon_h/R = 1070.96$ K, time in units of $t = \sigma (\epsilon_h/M)^{1/2} = 4.44$ ps, ($\sigma = 4.0$ Å is the equilibrium length of Lennard-Jones interactions, $M = 110$ is the average aminoacid mass), energy in units $\epsilon_h$, specific heat in units $R = 1.9872 \times 10^{-3}$ Kcal mol$^{-1}$K$^{-1}$ and the radius of gyration in units $\sigma$. The energy scale $\epsilon_h$ was set to 1.65 Kcal mol$^{-1}$ to reach a denaturant temperature compatible with experimental data $T = 332$ K. For the Gō-model the same units apply, except for the energy scale set to $\epsilon = 0.66$ Kcal mol$^{-1}$. During the simulations, we monitored the difference from the native state or reference state through the overlap $Q$, representing the fraction of formed native contacts

$$Q = \frac{\sum_{i,j \geq i+3} \Theta(R_c - r_{ij})}{\sum_{i,j \geq i+3} \Theta(R_c - R_{ij})}$$

where, the sum runs over all pairs of native contacts, $r_{ij}$ and $R_{ij}$ are the distances of residues $i$ and $j$ in the current and in the reference conformation respectively and $\Theta(u)$ indicates the unitary step function. A value $Q \cong 1$, indicates that the conformation is native-like, while values close to zero refer to denatured states. We also considered, as further reaction coordinates of the folding/unfolding process, the gyration radius and the root mean square distance (RMSD) between the current and reference conformations after an optimal superposition performed according to Karplus’s algorithm. The thermodynamics of the folding/unfolding transition was obtained via the weighted histogram method. This technique offers the possibility to gain a better sampling of the conformation space than ordinary methods. The procedure consists in storing bidimensional histograms of the number of contacts $N(E, Q)$ as a function of the energy $E$ and coordinate $Q$ at each temperature run. Such histograms are then optimally combined to reconstruct the best estimate of the density of states $\Omega(E, Q)$, which, in turn, will be used to compute the thermodynamics of the system. The knowledge of $\Omega(E, Q)$ can be also employed to derive the probability that, at temperature $T$, the protein states are characterized by energy $E$ and reaction coordinate $Q$.

$$P_T(E, Q) = \Omega(E, Q) \exp\{-\beta(E - F)\}$$

where $\beta = 1/RT$ and $F$ is the total free energy of the system coming from the normalization of $P_T(Q, E)$. The sum of $P_T(E, Q)$ over all possible energies $E$ provides the probability for the system to have a specific value $Q$ at temperature $T$, which in turn, by reversing the Boltzmann’s weight, gives the potential of mean force along the reaction coordinate $Q$

$$W_T(Q) = -RT \ln[P_T(Q)] .$$

We computed the specific heat profile as a function of the temperature: its peaks and shoulders locate those temperatures at which the main structural chain rearrangements occur. A detailed characterization of the folding/unfolding process can be obtained by measuring the probability of native contact formation as the temperature is varied

$$P_{ij}(T) = \langle \Theta(R_c - r_{ij}) \rangle$$

where the average $\langle \cdots \rangle$ is taken over time assuming the dynamics to be ergodic. $P_{ij}(T)$ typically features a sigmoidal shape, keeping values close to 1 at low temperatures and decreasing to zero at high temperatures. The knowledge of probabilities $P_{ij}(T)$ allows for a classification and ranking of native contacts according to their “thermodynamic relevance” thus suggesting possible reaction pathway, key residues and folding nucleus.

### A. $\Phi$ values

The comparison of Gō and the SHG models on the WW domain provide the opportunity to study the relevance of topological versus energetic frustration in the folding mechanism. This can be accomplished by $\Phi$ values computation and by the further comparison with experimental data. $\Phi$-values measure the perturbation effects of a site-directed mutation which, by altering the free energy difference among native, transition and unfolded states may affect the thermodynamics and the kinetics of the reaction. A prevalence of topological or energetic frustration may be argued from a better fit with the experiments of the Gō-derived or SHG-derived $\Phi$-values respectively. The $\Phi$-values can be computed through a kinetic approach from the folding and unfolding rates of the mutant and wild-type protein:

$$\Phi = \frac{RT \log(k_W^T/k_f)}{RT \log([k_W^T/k_f] \cdot [k_W^m/k_W^U])} \ (7)$$

where $R$ is the ideal gas constant, $T$ is the absolute temperature and $k_f$ and $k_u$ are the folding and unfolding rates respectively. The denominator of the above expression is just the total stability change $\Delta\Delta G^0$. The use of equation $7$ is computationally demanding as it requires a simulation for each mutation. This motivates the use of a thermodynamic strategy for the $\Phi$-value evaluation:

$$\Phi = \frac{\Delta\Delta G^T}{\Delta\Delta G^0} = \frac{\Delta\Delta G^{TS} - \Delta\Delta G^U}{\Delta\Delta G^F - \Delta\Delta G^U} \ (8)$$

where $\Delta\Delta G^T$ is the change in stability of the free-energy barrier between the native and denatured state. Equation $8$ is equivalent to equation $7$ when Kramer-like theory applies.

If the effect of the mutations is sufficiently small, then, following Ref. the $\Phi$-values can be derived by a free
energy perturbation (FEP) approach:

\[ \Phi = \frac{\log(\exp(-\Delta E/RT))_{TS} - \log(\exp(-\Delta E/RT))_{U}}{\log(\exp(-\Delta E/RT))_{F} - \log(\exp(-\Delta E/RT))_{U}} \]  

(9)

where the Boltzmann factors depend on the energy difference between the mutant and the wild type (WT) and the averages are computed over WT-conformations of the folded (F), transition state (TS) and unfolded (U) ensembles.

In the present paper, the \( \Phi \)-values are computed according to equation 9 using a method developed in Ref. [22] that can be summarized in the following steps:

1. Determination of the folding temperature \( T_f \) from the specific heat plot.

2. Analysis of the free energy profile at temperature \( T_f \) plotted as a function of a suitable reaction coordinate. The free energy profile of a two-state folder typically shows a double-well shape which allows to choose three windows of the reaction coordinate identifying the folded, transition state, and unfolded ensembles respectively.

3. Dynamic simulation at \( T = T_f \) and storage of conformations belonging to the F, TS and U ensembles.

4. Choice of mutations and computation of FEP \( \Phi \)-values.

Structural information about the native-likeness of the transition state was also gained from the so-called structural \( \Phi \)-values [49]:

\[ \Phi_{\text{struc}}(i) = \frac{1}{N_j \in C(i)} \sum_{j \in C(i)} P_{FS}(i, j) / \sum_{j \in C(i)} P_F(i, j) \]  

(10)

where \( P_F(i, j) \) and \( P_{FS}(i, j) \) are the frequencies of the native contact \( i - j \) in the folded and transition ensembles respectively, and the sums run over the set \( C(i) \) of native contacts in which residue \( i \) is involved.

III. RESULTS

We report on the thermodynamic properties and contact formation patterns observed in unfolding/refolding equilibrium MD simulations of the WW domain. We first analyze the simulations based on Gō-model and then we discuss the corresponding scenario in the SHG-model approach. Since the implementation of the SHG model requires a well designed sequence we employed the 6-40 truncated sequence already optimized in Ref. [11]. For the sake of a consistent comparison with Gō-simulations, the corresponding fragment was extracted from the NMR structure stored in the PDB file 1NMV [4].

Gō-model

A folding simulation was performed through a gradual cooling of a random coil structure from a temperature \( T = 1.5 \) down to 0.5 in forty steps. The specific heat profile, Fig. 2, is characterized by a single narrow peak at temperature \( T_f = 1.0 \) suggesting a possible two state process.

![Figure 2: Heat capacity as a function of temperature in Gō-model simulations: folding (solid), unfolding (dotted). Inset: thermal behaviour of energy; dotted lines represent quadratic fits of the baselines.](image)

The same conclusion can be drawn from the ratio of van’t Hoff over the calorimetric enthalpy changes amounting to 0.74 without and 0.99 with standard baseline subtraction [50].

The folding/unfolding processes are reversible in temperature as shown by the agreement between specific heat plots. The other observables used to characterize the folding transition such as, RMSD, overlap and gyration radius exhibit an abrupt change in correspondence to the folding temperature \( T_f \) (Fig. 3). Free energy profile (Fig 4) as a function of the overlap, around the folding temperature, clearly features two distinct wells identifying the folded and unfolded ensembles separated by a barrier corresponding to the transition state conformations.

The shape of the free-energy plot suggests a choice of overlap windows for the sampling of conformations in the three ensembles F, U and TS (see caption of Fig. 4) for the computation of \( \Phi \)-values (Methods). In figure 6 we compare our single site simulated \( \Phi \)-values (Eq. 9) with the experimental data by Gruebele [7]. In the Gō-like approach, a mutation can be modeled as the removal of a single native contact [22] or in alternative, as an average over all possible removals of contacts involving the same residue. We followed the second strategy considering only contacts \( |i - j| \geq 3 \). In this scheme we cannot evaluate \( \Phi \)-value of Ser18 because it lacks such contacts.
FIG. 3: Structural parameters monitored during the Gö-model folding simulations. Triangles are the reduced gyration radius; filled circles indicate reduced RMSD; open circles refer to the fraction of native contacts $Q$ magnified by a factor 4. Each point in the plots corresponds to an average of $6 \times 10^6$ conformations sampled every $10^{-3}$ time-steps. RMSD and $Q$ are computed using the PDB structure as a reference.

FIG. 4: Free energy profiles at different temperatures as a function of the overlap $Q$ (Lower panel). Vertical lines indicate the the boundaries of the sampling windows for $F$ ($0.85 < Q < 1.00$), $U$ ($0.20 < Q < 0.35$) and $TS$ ($0.60 < Q < 0.65$) see text. Upper panel: time evolution of $Q$ at the folding temperature $T = 1$ oscillating between the minima of the free energy wells.

The theoretical $\Phi$-values in figure vary in the range $[0.0, 0.5]$, whereas the experimental ones are distributed in a much wider interval. This feature is an expected result of the very limited energetic frustration of the Gö-force fields.

The discrepancy is reflected by the modest value of the linear correlation coefficient $r = 0.54$, (see regression line...
in the figure 5, inset). Of course we cannot exclude that a possible improvement of Φ-value accuracy might be achieved either by employing other mutation implementations or by using alternative contact maps accounting for the high flexibility of the native structure of peptides and small proteins [5]. Despite this not high correlation, the theoretical Φ-values provide a qualitative indication about the molecule regions that are still native-like in the transition state. The plot in fact indicates that the sites most sensitive to mutations are those in the region of loops L1 and L2 in agreement with experimental results (see Figure 4).

The picture provided by the structural Φ-values (Fig. 5t) is consistent with that derived from the perturbation method, in fact also in this case the highest Φ-values correspond to residues located in L1 (Ser 19), L2 (Thr 29) or in the neighborhood of the first hydrophobic cluster CL1 (Pro 8). The low Φ-values pertain mainly to residues in strands β1 and β2 suggesting that these two regions are unlikely to be in contact in the transition state.

A. SHG-model

Ten independent folding simulations starting from random-coil conformations, were performed through a gradual cooling schedule from temperature \( T = 1.0 \) to \( T = 0.01 \) in 40 steps. The final structures were further relaxed by a steepest descent cycle until the maximal total force per monomer reached a value smaller than \( 10^{-8} \) Kcal mol\(^{-1}\)Å\(^{-1}\). We obtained different folds and chose the conformation with lowest energy \( (E = -19.0035\epsilon) \) and lowest RMSD (4.74 Å) from the PDB structure as the reference structure \( \Gamma_0 \) (Fig. 1). However, the simulations revealed also the existence of another degenerate minimum with the same energy and specular to \( \Gamma_0 \) resulting in much higher RMSD.

Despite the large value of RMSD, \( \Gamma_0 \) correctly displays the topology of a triple-stranded, antiparallel β-sheet, that however lacks the typical twist of the PDB structure making loop L2 almost perpendicular to loop L1 (see Figure 1). As a result, the folded structure is much more compact than the real protein and has a much larger number of native contacts (71 versus 41). The fact that 22 out of the 41 PDB contacts are also present in the folded structure is an indication of the satisfactory structural performance of the SHG simulation.

Structure \( \Gamma_0 \) was then denaturated through ten independent runs with the same but inverse temperature schedule, involving a thermalization stage of \( 6 \times 10^4 \) time steps (\( \Delta t = 0.005 \)) at each temperature followed by a run over the same length, where control parameters were measured to assess the unfolding progress. The course of both folding and unfolding simulations was monitored through the analysis of the energy, the specific heat, RMSD from \( \Gamma_0 \), the overlap and radius of gyration \( R_g \).

Both the folding and unfolding specific heat plots (Fig. 6) are characterized by the presence of a main peak and a shoulder. The peaks of the folding and unfolding thermograms \( P_f \) and \( P_u \) are located at \( T_f = 0.36 \) and \( T_u = 0.33 \) respectively whereas the shoulders \( S_f \) and \( S_u \) correspond to \( T_{sf} = 0.24 \) and \( T_{su} = 0.28 \). The folding process appears not to be fully reversible probably due to the fact that the sequence, although designed, is not yet a good folder.

The existence of the shoulder in the folding \( C_v \) plot is a signature of a non cooperative folding mechanism in which, an initial collapse is followed by a structural chain rearrangement characterized by a significant increase in the number of native contacts unaffacting the overall compactness of the molecule (see Fig. 7).

This is confirmed by the thermal fluctuation of the structural overlap

\[
\text{var}_Q(T) = \langle Q^2 \rangle - \langle Q \rangle^2
\]

featuring the highest peak, not in correspondence of the main peak \( P_f \) but at the temperature \( T_{sf} \) of the shoulder (inset of Fig. 7).

The marked difference between the folding and unfolding specific heat suggests the opportunity to consider the free energy profiles \( W_T(Q) \) to better determine the folding temperature. The profiles (lower panel of Fig. 8) indicate that the transition is characterized by the presence of two wells separated by a barrier and the temperature where these two wells are evenly populated is \( T = 0.237 \). This confirms that, the peak of \( C_v \) is mainly related to the Θ-collapse, whereas the shoulder corresponds to the folding transition. In fact, kinetic simulations at temperature \( T = 0.237 \) show that the time evolution of \( Q(t) \) exhibit jumps between the two free energy wells (upper panel of Fig 8). The double-well shape of the
FIG. 7: Noncooperativity of the SHG folding simulation yielding our best final structure $\Gamma_0$. After a first collapse the radius of gyration remains constant whereas the structural difference from the reference structure $1-Q$ keeps on decreasing at temperatures corresponding to the shoulder of the $CV$ plot signalling a massive structural rearrangement. Insert: temperature dependence of fluctuations of the structural overlap. The main peak, located at the same temperature as the heat capacity shoulder, corresponds to the folding temperature.

FIG. 8: Free energy profile as a function of the overlap $Q$ at the folding temperature $T = 0.237$ (lower panel). The vertical lines mark the boundaries of the Folded ($0.78 < Q < 0.90$), Transition State ($0.53 < Q < 0.61$) and Unfolded ($0.30 < Q < 0.47$) ensembles. The upper panel shows the typical temporal evolution of the overlap in a sub-interval of a simulation at folding temperature. The overlap was sampled every $5 \times 10^3$ time-steps.

The free-energy profile again allows to sample conformations in the folded (F), transition state (TS) and unfolded (U) ensemble used to implement the perturbation technique for $\Phi$-value computation (Methods). The plot in figure 9 shows the $\Phi$-values restricted to the set of residues mutated by Gruebele [5].

FIG. 9: Experimental (solid circles) and computed (open circles) $\Phi$-values. $\Phi$-values were computed from the conformations sampled in a SHG simulation at folding temperature using the perturbation method. The two profiles show a qualitative agreement although the correlation coefficient of the regression line (see inset) is $r = 0.65$.

For each site we tested the effect of all the possible single mutations allowed by the model, namely two hydrophobicity shifts and two shifts in the secondary structural bias for each of the two dihedral angles flanking the residue under examination. For each site we chose the least perturbative mutations. Theoretical $\Phi$-values feature two major peaks in correspondence with loop L1 and loop L2 which is a qualitative resemblance with experiments. A more quantitative comparison is provided by the correlation coefficient between theoretical and experimental data amounting to $r = 0.65$.

The set of native conformations collected during the kinetic simulation provides a structural characterization of the ensemble F whose most interesting feature is the clustering of native-basin conformations in two main subsets characterized by non overlapping distributions of RMSD from the reference structure $\Gamma_0$. This is a further indication of the high level of frustration of the free-energy landscape associated to the sequence and it is in agreement with the findings by Miller and Wales [52] about the glass-like structure of the energy landscape in a closely related model [35]. This partitioning of the native basin is evident in figure 10, where we plot the free energy versus the RMSD which is a structural indicator more sensitive than the overlap. A finer analysis reveals that the structures in the two sub-basins of the native valley correspond to different chiralities but similar energies. The absence of such a partitioning in the same plot for the Gō force field, indicates that this feature is mainly peculiar of the model rather than this specific protein.
To clarify how the landscape properties affect the reversibility of the folding process we studied the folding/unfolding transition from the contact formation probabilities $P_{ij}$. In particular, as the plots $P_{ij}(T)$ are typically sigmoid, a contact can be regarded as broken/formed in correspondence of the temperature, where the absolute value of the slope of the probability curve is maximal. This allows to identify the contacts whose formation/breakdown occurs at a given temperature.

To analyze the folding/unfolding process, we considered three temperature windows corresponding to different regions of the specific heat plots. The first window ($T < 0.15$) refers to the pre-transition baseline of the $C_v$ plot, the second window ($0.15 \leq T \leq 0.30$) insists on the shoulder of the $C_v$ plot, the third window ($T > 0.30$) refers to the native conformation (pdb-id = 1NMV). The native valley shows a single basin as opposed to the partitioning in two sub-valleys typical of the Gō model.}

FIG. 10: Upper panel: low-temperature free energy profiles of the SHG model as a function of the RMSD from the reference conformation $\Gamma_0$. The native valley appears to be partitioned in two main sub-basins separated by a barrier. The sub-basin corresponding to the RMSD range [0.25–1.00] is populated by conformations with the same chirality as the PDB structure, whereas the sub-valley in the range [2.80–6.50], corresponds to the opposite chirality. Lower panel: low-temperature free energy profiles of the Gō model as a function of the RMSD from the native conformation (pdb-id = 1NMV). The native valley shows a single basin as opposed to the partitioning in two sub-valleys typical of the Gō model.

The contact map shows that the first contacts formed during folding are located in the intermediate part of sheet $\beta_1$-$\beta_2$ and in the region of sheet $\beta_2$-$\beta_3$ most distant from loop 2. The formation of these contacts is responsible for the collapse of the molecule into a compact but not completely folded conformation. The map also shows that the shoulder of the $C_v$ plot is characterized by the zipping of sheets $\beta_1$-$\beta_2$ and $\beta_2$-$\beta_3$ towards loop 1 and loop 2 respectively. During the process there also occurs the locking of $\beta_1$-$\beta_3$, $\beta_2$-tail and head-$\beta_2$ contacts (hereafter, the term 'head' and 'tail' indicate the amino-terminal region $\text{Lys}6$–$\text{Gly}10$ and the carboxy-terminal residues $\text{Asn}36$–$\text{Asn}40$ respectively). These contacts are not present in the PDB structure and they arise as a consequence of the higher compactness of $\Gamma_0$, so that their formation probabilities are always below 50%. The folding is completed by the appearance of a few contacts between residues very far from each other along the protein chain.

During the unfolding reaction no "native" (with respect to $\Gamma_0$) contact breaks down in the low temperature window because the heating schedule enables the protein to escape easily from kinetic traps making the process much less gradual than folding. This reflects on the smaller number of contacts broken in the shoulder region as compared to the number of contacts formed in the same temperature range during folding. In particular, the cleavage occurs of $\beta_1$-$\beta_3$, $\beta_2$-tail and head-$\beta_2$ contacts, whereas the dissolution of the contacts of loop 1 and loop 2 is delayed to the region of the peak of the $C_v$ plot where most $\beta_1$-$\beta_2$ and $\beta_2$-$\beta_3$ contacts also disappear.

The comparison of the two contact maps thus reveals that the sequences of the molecular events in the fold-
ing and unfolding processes are basically reverse to each other even if the unfolding is a more abrupt phenomenon occurring in a narrower temperature window.

IV. DISCUSSION

Our results indicate how the different approaches of structure-based and sequence-based description exemplified by G° and SHG model are appropriate to simulate complementary features of the folding process. The G° model, in fact, being based on the influence of the native state topology on the folding process is independent from the amino-acid sequence and it completely disregards the chemical properties of the molecule. The SHG model, on the other hand, is a minimal model, where the chemical features of amino-acids are partially included determining the folding driving force.

Our simulations showed that the G° model with angular bias and rescaling can correctly reproduce the reversible, cooperative, two-state mechanism of unfolding of hPin1 WW domain. The reversibility, indeed appears from the almost perfect superposition of the CV plots of folding and unfolding. Several elements, on the other hand, suggest a cooperative, two-state mechanism: the CV plots show a single sharp peak, the ratio of the van’t Hoff to calorimetric enthalpy is close to 1 $\kappa_C \approx 1$, and the indicators used to monitor the similarity with the native state exhibit a sharp sigmoidal thermal behaviour and the barrier between the two free-energy wells at the folding temperature is very high $\Delta G^{\text{bar}}$. The results from the simulation using the SHG model were rather ambiguous. The simulated thermograms featured not only a peak, but also a shoulder at lower temperature. This is the signature of a non cooperative folding involving a collapse into a compact, only partially structured globule, followed by a rearrangement into a native conformation. This scenario is confirmed by the thermal behaviour of the structural parameters (overlap, gyration radius and RMSD from native structure) used to monitor the folding reaction. The results are consistent with the findings by Nymeyer et al. and by Guo and Brooks III, in their simulations on the model by Honeycutt and Thirumalai. The SHG formulation, although being an improvement of the latter model, still retains some of its drawbacks. Indeed, the conformations of the native ensemble (F), sampled at the folding temperature, can be clustered in two groups with non-overlapping RMSD distributions and opposite chiralities. The existence of two distinct clusters of native-like conformations can be easily explained by examining the low-temperature free-energy profiles as a function of the RMSD (from reference structure $\Gamma_0$): the native basin appears to be partitioned in sub-basins separated by barriers. The partitioning of the native basin is likely a feature that the SHG model inherited from Thirumalai model. Miller and Wales, in fact, analyzed the disconnectivity graph of the potential energy surface of Thirumalai’s force field drawing the conclusion that the energy hypersurface is not a single funnel, but it contains low-energy minima separated by high barriers.

Presumably, the reason for the degeneration of the native state of the SHG model relies in the symmetry of the dihedral potential $V_\Phi$, Eq. [4] In particular, the sequence designed to represent the hPin1 WW domain, contains only "Extended" or "Turn" symbols, so that $V_\Phi$ is a polynomial in $\cos(\phi)$ and becomes symmetric for the inversion $\phi \rightarrow -\phi$. The symmetry of the $V_\Phi$ term, however, is not the only reason for the poor performance of the SHG model. In fact, we find that the energy histograms of the Folded- and Unfolded- state ensembles are significantly overlapping thus suggesting the existence of many low-energy, non-native conformations.

We suspect that this is an effect of the only approximated maximization of the energy gap between the native conformation and the decoy set used in the sequence optimization procedure. This would call for further refinements of the threading procedure.

Despite the several drawbacks, the SHG model enabled the computation of perturbation $\Phi$-values in qualitative agreement with experimental data. The linear correlation coefficient between theoretical and experimental $\Phi$-values ($r = 0.65$) is actually better than the one yielded by the G° simulation ($r = 0.54$). The explanation of these result must be sought in the partial incorpotation of the chemistry in the SHG description. Indeed, real mutations are chemical transformations of the molecule and they are better simulated by a chemically-based model such as the SHG rather than by a topological model. In the G° model, in fact, mutations are generally simulated by the removal of native contacts, however, they may affect all the interactions a residue is involved in. The SHG model, conversely, offers the possibility to treat mutations in a more realistic way because it implements shifts in the hydrophobic character of residues or changes in the secondary structural bias of dihedral angles. Moreover, the better agreement of experimental data with the SHG-computed $\Phi$-values may show that the folding mechanism of hPin1 WW domain is controlled not only by topological but also by energetic factors.

The significant differences, beyond statistical errors, between the $\Phi$-values profiles yielded by the two models, in our opinion, reflect the different strategies upon which the two models are built.

A final issue that deserves some discussion is the quality of the structural prediction using the SHG model. The SHG is a minimal model based on chemical properties of the system and a good outcome of the simulation is not a priori guaranteed. The simulations show that, apart from chirality problems, the best final structure $\Gamma_0$ (Fig. 1) presents the correct topology of a three-stranded antiparallel $\beta$-sheet of the hPin1 WW domain, even if the structure appears to be more compact. This however, does not prevent the correct formation of both hydrophobic clusters. Moreover, $\Gamma_0$ shares 22 of the 41 native contacts of the PDB structure.
V. CONCLUSIONS

We performed folding and unfolding simulations of the WW domain of hPin1 protein that represents an excellent candidate to test folding algorithms and models due to the availability of a large amount of structural, thermodynamical and kinetic experimental data. The purpose of the work was to compare the performance of the Gō and SHG models that represent two different strategies to the folding problem. Our simulations indicated that for the specific WW domain considered in this work, the Gō model with angular bias and rescaling, correctly reproduces the cooperative, two-state, reversible folding mechanism, whereas the SHG model does not. The reasons for the limitations of the SHG model must be sought in the insufficient optimization the sequence and in the non-funnel shape of the landscape. As a consequence, the present version of the SHG model does not allow reliable predictions of the folding mechanism. The satisfactory performance of the SHG model in the computation of Φ-values, however, clearly shows the importance of incorporating the chemical properties of the sequence in a protein model. Our work, highlighting the limits of the SHG model, is thus intended to be a starting point for a further refinement of the model, in the firm belief that coarse-grained, minimal models represent viable alternatives to computationally demanding all-atom simulations in investigations of large-sized, slow-folding proteins.

[1] Ferguson, N., C. M. Johnson, M. Macias, H. Oschkinat, and A. R. Fersht. (2001). Ultrafast folding of ww domains without structured aromatic clusters in the denatured state. Proc. Natl. Acad. Sci. USA 98:13002–13007.
[2] Karianicolas, J., and C. L. Brooks-III. (2003). Structural basis for biphasic kinetics in the folding of the ww domain from a formin-binding protein: Lessons for protein design? Proc. Natl. Acad. Sci. USA 100:3954–3959.
[3] Verdecia, M. A., M. E. Bowmann, K. P. Lu, T. Hunter, and J. P. Noel. (2000). Structural basis for phosphoserine-proline recognition by ww domains. Nature Struct. Biol. 7:639–643.
[4] Bayer, E., S. Goetttsch, J. W. Mueller, B. Griewel, E. Guiberman, L. M. Mayr, and P. Bayer. (2003). Structural analysis of the mitotic regulator hip1 in solution: Insights into domain architecture and substrate binding. J. Biol. Chem 278:26183–93.
[5] Jäger, M., H. Nguyen, J. C. Crane, J. W. Kelly, and M. Gruebele. (2001). The folding mechanism of a beta-sheet: the ww domain. J. Mol. Biol. 311:373–393.
[6] Bruscolini, P., and F. Cecconi. (2005). Analysis of pin1 ww domain through a simple statistical mechanics model. Biophys. Chem. 115:153–158.
[7] Garnier, L., J. W. Wills, M. F. Verderame, and M. Sudol. (1996). Ww domains and retrovirus budding. Nature 381:744–745.
[8] Sudol, M. (1996). Structure and function of the ww domain. Prog. Biophys. Mol. Biol. 65:113–132.
[9] Gō, N., and H. A. Scheraga. (1976). On the use of classical statistical mechanics in the treatment of polymer chain conformations. Macromolecules 9:535–542.
[10] Sorensen, J. M., and T. Head-Gordon. (2000). Matching simulation and experiment: a new simplified model for simulating protein folding. J. Comp. Bio. 7:469–481.
[11] Brown, S., N. J. Fawzi, and T. Head-Gordon. (2003). Coarse-grained sequences for protein folding and design. Proc. Natl. Acad. Sci. USA 100:10712–10717.
[12] Micheletti, C., J. R. Bansar, A. Maritan, and F. Seno. (1999). Protein structures and optimal folding from a geometrical variational principle. Phys. Rev. Lett. 82:3372–3375.
[13] Munõz, V., E. R. Henry, J. Hofrichter, and W. A. Eaton. (1998). A statistical mechanical model for beta-hairpin kinetics. Proc. Natl. Acad. Sci. USA 95:5872–5879.
[14] Alm, E., and D. Baker. (1999). Prediction of protein-folding mechanisms from free-energy landscapes derived from native structures. Proc. Natl. Acad. Sci. USA 96:11305–11310.
[15] Fersht, A. R. (2000). Transition-state structure as a unifying basis in protein-folding mechanisms: contact order, chain topology, stability, and the extended nucleus mechanism. Proc. Natl. Acad. Sci. USA 97:1525–1529.
[16] Baker, D. (2000). A surprising simplicity to protein folding. Nature 405:39–42.
[17] Martinez, J. C., M. T. Pisabarro, and L. Serrano. (1998). Obligatory steps in protein folding and the conformational diversity of the transition state. Nature Struct. Biol. 5:721–729.
[18] Martinez, J. C., and L. Serrano. 1999. The folding transition state between sh3 domains is conformationally restricted and evolutionarily conserved. Nature Struct. Biol. 6:1010–1016.
[19] Riddle, D. S., V. P. Grantcharova, J. V. Santiago, E. Alm, I. Ruczinski, and D. Baker. 1999. Experiment and theory highlight role of native state topology in sh3 folding. Nature Struct. Biol. 6:1016–1024.
[20] Chiti, F., N. Taddei, P. M. White, M. Bucchini, F. Magherini, M. Stefani, and C. M. Dobson. 1999. Mutational analysis of acylphosphatase suggests the importance of topology and contact order in protein folding. Nature Struct. Biol. 6:1005–1009.
[21] Plaxco, K. W., K. T. Simons, and D. Baker. 1998. Contact order, transition state placement and the refolding rates of single domain proteins. J. Mol. Biol. 277:985–994.
[22] Clementi, C., H. Nymeyer, and J. N. Onuchic. 2000. Topological and energetic factors: what determines the structural details of the transition state ensemble and "en-route" intermediates for protein folding? J. Mol. Biol. 298:937–953.
[23] Galitskaya, O. V., and A. V. Finkelstein. 1999. A theoretical search for folding/unfolding nuclei in three-dimensional protein structures. Proc. Natl. Acad. Sci. USA 96:11299–11304.
[24] Zhou, Y., and M. Karplus. 1999. Folding of a model three-helix bundle protein: a thermodynamic and kinetic analysis. J. Mol. Biol. 293:917–951.
[25] Ding, F., N. V. Dokholyan, S. V. Buldyrev, H. E. Stanley,
and E. I. Shakhnovich. 2002. Direct molecular dynamics observation of protein folding transition state ensemble. *Biophys. J.* 83:3525–3532.

[26] Ozkan, S. B., K. A. Dill, and I. Bahar. 2003. Computing the transition state populations in simple protein model. *Biopolymers* 68:35–46.

[27] Kubicke, J., J. Hofrichter, and W. A. Eaton. (2004). The protein folding ’speed limit’. *Curr. Opin. Struct. Biol.* 14:76–88.

[28] Gruebele, M. (1999). The fast protein folding problem. *Annu. Rev. Phys. Chem.* 50:485–516.

[29] Miyazawa, S., and R. L. Jernigan. 1985. Estimation of effective interresidue contact energies from protein crystal structures: quasi-chemical approximation. *Macromolecules* 18:534–552.

[30] Clementi, C., and S. S. Plotkin. 2004. The effects of nonnative interactions on protein folding rates: theory and simulations. *Protein Sci.* 13:1750–1766.

[31] Northey, J. G. B., A. A. D. Nardo, and A. R. Davidson. (2002). Hydrophobic core packing in the sh3 domain folding transition state. *Nature Struct. Biol.* 9:126–130.

[32] Di Nardo, A. A., D. M. Korzhnev, P. J. Stogios, A. Zarrine-Afsar, L. E. Kay, and A. R. Davidson. (2004). Dramatic acceleration of protein folding by stabilization of a nonnative backbone conformation. *Proc. Natl. Acad. Sci. USA* 101:7954–7959.

[33] Kaya, H., Z. Liu, and H. S. Chan. 2005. Chevron behavior and isostable enthalpic barriers in protein folding: successes and limitation of simple go-like modeling. *Biophys. J.* 89:520–535.

[34] Honeycutt, J. D., and D. Thirumalai. 1992. The nature of folded states of globular proteins. *Biopolymers* 32:695–709.

[35] Veitshans, T., D. Klimov, and D. Thirumalai. 1996. Protein folding kinetics: timescales, pathways and energy landscapes in terms of sequence-dependent properties. *Fold. Des.* 2:1–22.

[36] Sorenson, J. M., and T. Head-Gordon. 1999. Redesigning the hydrophobic core of a model β-sheet protein: destabilizing traps through a threading approach. *Proteins: Struct. Funct. Gen.* 37:582–591.

[37] Shakhnovich, E. I. 1998. Protein design: a perspective from simple tractable models. *Fold. Des.* 3:R45–R58.

[38] Evans, D. J., W. G. Hoover, B. H. Faucil, B. Moran, and A. J. C. Ladd. 1983. Non-equilibrium molecular dynamics via gauss’s principle of least constraint. *Phys. Rev. A.* 28:1016–1021.

[39] Kabsch, W. 1976. Solution for best rotation to relate two sets of vectors. *Acta Crystallogr.* 32:922–923.

[40] Ferrenberg, A. M., and R. H. Swendsen. 1989. Optimized monte carlo data analysis. *Phys. Rev. Lett.* 63:1195–1198.

[41] Guo, Z., and C. L. Brooks-III. 1997. Thermodynamics of protein folding: a statistical mechanical study of a small all-β protein. *Biopolymers* 42:745–757.

[42] Cecconi, F., C. Micheletti, P. Carloni, and A. Maritan. 2001. The structural basis of antiviral drug resistance and role of folding pathways in hiv-1 protease. *Proteins Struct. Funct. Genet.* 43:365–372.

[43] Micheletti, C., F. Cecconi, A. Flammini, and A. Maritan. 2002. Crucial stages of protein folding through a solvable model: Predicting target sites for enzyme-inhibiting drugs. *Protein Sci.* 11:1878–1887.

[44] Scala, A., N. V. Dokholyan, S. V. Buldyrev, and H. E. Stanley. 2001. Thermodynamically important contacts in folding of model proteins. *Phys. Rev. E* 63:032901.

[45] Vendruscolo, M., E. Paci, C. M. Dobson, and M. Karplus. (2001). Three key residues form a critical contact network in a protein folding transition state. *Nature* 409:641–645.

[46] Abkevich, V. I., A. M. Gutin, and E. I. Shakhnovich. (1994). Specific nucleus as the transition state for protein folding: evidence from the lattice model. *Biochemistry* 33:10026–10036.

[47] Onuchic, J. N., H. Nymeyer, A. E. Garcia, J. Chahine, and N. D. Socci. (2000). The energy landscape theory of protein folding: Insights into folding mechanisms and scenarios. *Advances in Protein Chemistry* 53:87–152.

[48] Fersht, A. R. (1998). Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding. 3rd edition. W. H. Freeman, USA.

[49] Settanni, G., F. Rao, and A. Caflisch. (2005). ø-value analysis by molecular dynamics simulations of reversible folding. *Proc. Natl. Acad. Sci. USA* 102:628–633.

[50] Nagarathan, A. N., R. Perez-Jimenez, J. M. Sanchez-Ruiz, and V. Munoz. (2005). Robustness of downhill folding: Guidelines for the analysis of equilibrium folding experiments on small proteins. *Biochemistry* 44:7435–7440.

[51] Cavalli, A., M. Vendruscolo, and E. Paci. (2005). Comparison of sequence-based and structure-based energy functions for the reversible folding of a peptide. *Biophys. J.* 88:3158.

[52] Miller, M. A., and D. J. Wales. (1999). Energy landscape of a model protein. *J. Chem. Phys.* 111:6610–6616.

[53] Kaya, H., and H. S. Chan. (2000). Polymer principles of protein calorimetric two-state cooperativity. *Proteins: Struct. Funct. Gen.* 40:637–661.

[54] Knott, M., H. Kaya, and H. S. Chan. (2004). Energetic of protein thermodynamic cooperativity: contributions of local and nonlocal interactions. *Polymer* 45:623–632.

[55] Chan, H. S. (2000). Modeling protein density of states: Additive hydrophobic effects are insufficient for calorimetric two-state cooperativity. *Proteins: Struct. Funct. Gen.* 40:543–571.

[56] Nymeyer, H., A. E. García, and J. N. Onuchic. (1998). Folding funnels and frustration in off-lattice minimalist protein landscapes. *Proc. Natl. Acad. Sci. USA* 95:5921–5928.

[57] Kwiecinska, J. I., and M. Cieplak. (2005). Chirality and proteins folding. *J. Phys.: Condens. Matter* 17:S1565–S1580.