Absolute comparison of simulated and experimental protein-folding dynamics

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**Protein folding is difficult to simulate with classical molecular
dynamics. Secondary structure motifs such as α-helices and β-
hairpins can form in 0.1–10 μs (ref. 1), whereas small proteins
have been shown to fold completely in tens of microseconds[2]. The
longest folding simulation to date is a single 1-μs simulation of
the villin headpiece[3]; however, such single runs may miss many
features of the folding process as it is a heterogeneous reaction
involving an ensemble of transition states[4,5]. Here, we have used a
distributed computing implementation to produce tens of thou-
sands of 5–20-ns trajectories (700 μs) to simulate mutants of the
designed mini-protein BBA5. The fast relaxation dynamics these
predict were compared with the results of laser temperature-
jump experiments. Our computational predictions are in excellent
agreement with the experimentally determined mean folding
times and equilibrium constants. The rapid folding of BBA5 is
due to the swift formation of secondary structure. The conver-
gence of experimentally and computationally accessible timescales
will allow the comparison of absolute quantities characterizing
in vitro and *in silico* (computed) protein folding[6].**

To establish a statistically favoured mechanism, map the free
energy surface, and to compare absolute rate and equilibrium
constants with experimental data requires multiple simulations of
any folding reaction. Such multiple sampling has only been
achieved for peptide systems[7–10]. As an alternative, simulations in
the past have been compared to relative quantities such as Phi-
values[11], or high temperature unfolding rates[2]. The prevailing view
is that current molecular dynamics cannot find the native state at
the free energy minimum owing to limitations of timescale and
force field accuracy. For example, performing a molecular dynamics
simulation for 10 μs with a 2-fs time step on a simple system in
implicit solvent, such as our model protein BBA5, would require
decades for a typical modern CPU. Our approach is to compare
directly experimental rate and equilibrium constants with distrib-
uted computing simulations of the same quantities from thousands
of short (5–20 ns) folding trajectories totalling hundreds of micro-
seconds. Absolute quantities such as rate constants provide strong
constraints on barriers and stringent folding model tests, but are
more difficult to calculate precisely than relative quantities. For
instance, agreement between simulation and experiment within a
factor of two would be noteworthy for even small-molecule rate
prediction. To allow experiment and theory to meet on the micro-
second timescale, we have chosen to study mutants of the fast-
folding, designed mini-protein BBA5. The choice of a 23-residue
protein with strong secondary structure propensities and a small
hydrophobic core reduces the impact of force field inaccuracies,

and allows kinetics to be resolved both experimentally and
computationally.

Although folding during a 20-ns simulation is improbable,
collecting a very large number of trajectories has allowed us to
observe such improbable events (Fig. 1). Given a small, two-state
protein that obeys exponential kinetics with a mean folding time of
10 μs, roughly 10 out of 10,000 individual molecules should have
folded conformations after 10 ns (ref. 10). Individual trajectories
may not be arbitrarily short. Each simulation must first relax from
the initial condition to the unfolded state under native conditions.
For BBA5 this relaxation is extremely rapid—the initial extended
ensemble collapses to a compact unfolded ensemble within 10 ns.
The unfolded ensemble under native conditions has a significant
degree of α-helical structure (in both simulation and experiment).
Our simulations also satisfy the constraint that each simulation
should not be substantially shorter than the minimal passage time
through the transition state (about 10 ns, from peptide diffusion
data[12,13]). The Folding@Home distributed computing project[14], a
cluster of over 30,000 volunteer computers distributed around
the world, has been used for several months to accumulate
approximately a million CPU days of simulation time (http://
folding.stanford.edu/). We observed folding in over 100 indepen-
dent trajectories. Calculations and laser temperature-jump exper-
iments indicate that our BBA5 mutants are moderately cooperative
folders, have a very low melting temperature, and fold on a time-
scale of several microseconds. Furthermore, the simulations charac-
terize the timescale for the formation of secondary structure, and
demonstrate folding by means of a heterogeneous transition state
(Supplementary Information).

Two mutants of BBA5 (Fig. 1a), a 23-residue β-hairpin/turn/
α-helix motif inspired by the zinc finger fold, were constructed on
the basis of the sequence developed by the Imperiali group, Ac-
YRVpSYDFSRSDELAKLLRQHAG-NH$_2$ (ref. 15). BBA5 is a 'mini-
protein' containing all three elementary units of secondary struc-
ture, and folds without obligate metal-ion binding[16]. A D-proline
residue favours the formation of a type II′ β-turn, stabilizing a
hairpin centred about residues 4–5. The amino-terminal β-hairpin
and carboxy-terminal α-helix interact to form a small hydrophobic
core. Native state simulations at 278 K reveal flexibility in the
relative orientation of the hairpin and the helix. Our single mutant
replaces a phenylalanine at position 8 with a tryptophan, which acts
as a fluorescent probe and as a larger aromatic residue for inter-
action with side chains in the helix. The double mutant additionally
replaces valine 3 with a tyrosine. Both mutants show the same basic
fold in simulations, and have proton NMR spectra with dispersion
similar to BBA5. BBA5 mutants were chemically synthesized,
purified and buffered in 10 mM phosphate at pH 7 for all experi-
ments. To study the folded mutants, homology models were
constructed from the structure of BBA5 (see Methods).

Circular dichroism and fluorescence spectra of BBA5 mutants
were measured to quantify the folding equilibrium (Fig. 2). The
folding transition was weakly cooperative, as expected for a mini-
protein (midpoint below 0 °C). To assess secondary structure
changes during folding, far-ultraviolet circular dichroism spectra
were collected from −15 °C to 90 °C. Both mutants fold with
increased helical content at low temperature (local minimum at
222 nm). Their circular dichroism spectra closely resemble BBA5
(ref. 15). The N-terminal 1–10 hairpin fragment by itself has a
random coil spectrum without cooperative transition (Fig. 2a). The
C-terminal 11–23 helix fragment has higher secondary structure
propensity. To assess solvent exposure of the core, tryptophan
fluorescence as a function of wavelength was measured at tempera-
tures from −2.5 °C to 60 °C. On unfolding at higher temperature,
the fluorescence intensity of the double mutant displayed a redshift
of 11 nm, indicating increased tryptophan solvent exposure. The
fluorescence and circular dichroism spectra have identical melting
transitions, as well as isosbestic/isodichroic points where all spectra

intersect. This indicates that the BBA5 mutants fold thermodynamically by means of a two-state mechanism. The calculated equilibrium constant K_{eq} for the double mutant is shown as a function of temperature in Fig. 2c (see also Methods).

To investigate experimental folding kinetics, laser temperature jumps of $11 \pm 1\,°C$ were performed on both BBA5 mutants with final temperatures between 293 and 303 K. Relaxation to the new equilibrium concentration was monitored at 100-ns intervals by following the temporal shift of the fluorescence spectrum with a sub-microsecond, real-time multichannel fluorescence wavelength detector[17]. The double mutant response could be resolved above the 400-ns instrument response function (Fig. 2d; see also Methods).

The same $1.5 \pm 0.7\,\mu s$ relaxation rate was detected whether analysing intensity changes or wavelength shifts of the time-resolved fluorescence spectrum. Standard definitions for the folding rate and the equilibrium constant of a two-state folding protein resulted in a folding rate of $7.5 \pm 3.5\,\mu s$ at 298 K (see Methods). For the single mutant we can establish a limit $\tau < 10\,\mu s$. The kinetic data are entirely compatible with single exponential folding kinetics, although we cannot exclude processes of small amplitude, or speed greater than the 400-ns resolution of our apparatus. Such processes could become relevant for fast folding over a low barrier (for example, a subpopulation of protein molecules might fold directly without need for further thermal excitation to the barrier).

Our choice of a tractable protein, a united atom model, an implicit solvent treatment, and the use of distributed computing techniques allowed aggregate simulation of over $700\,\mu s$. Folded state simulations were started from homology models (see Methods). We acquired 2,500 single-mutant folded simulations (10 ns) at both 278 K and 298 K. We also acquired 2,500 double-mutant folded simulations (10 ns) at 278 K, 378 K and 478 K. Twenty-five alternative double-mutant models generated 8,750 trajectories (5 ns) at 278 K. Folding trajectories were started from extended initial conformations. A total of 15,000 single-mutant folding simulations (20 ns) were acquired at both 278 K and 298 K; 9,000 and 8,500 double-mutant folding simulations were acquired, respectively, at 278 K (20 ns) and 338 K (10 ns). Considering all 32,500 folding trajectories, we observed the expected β-hairpin in over 1,100 independent trajectories and the α-helix in over 21,000 independent trajectories. We selected successful folding trajectories that
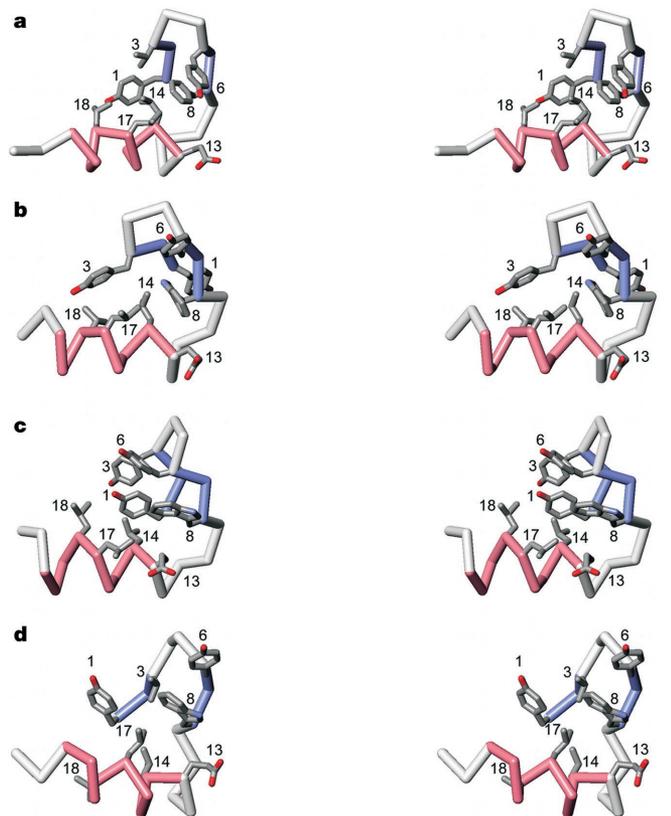
Figure 1 Folding results. Stereo diagrams showing the Cα backbone (blue, 1–3 and 6–8; red, 11–21) and selected side chains (Tyr 1, Val/Tyr 3, Tyr 6, Phe/Trp 8, Glu 13, Leu 14, Leu 17 and Leu 18) for folding trajectories terminating with low r.m.s.d.$_{Cα}$ to BBA5. **a**, NMR structure of BBA5. **b**, Double mutant at 2.2 Å. **c**, Double mutant at 2.4 Å. **d**, Single mutant at 2.5 Å. See Supplementary Information for examples of folding trajectories.
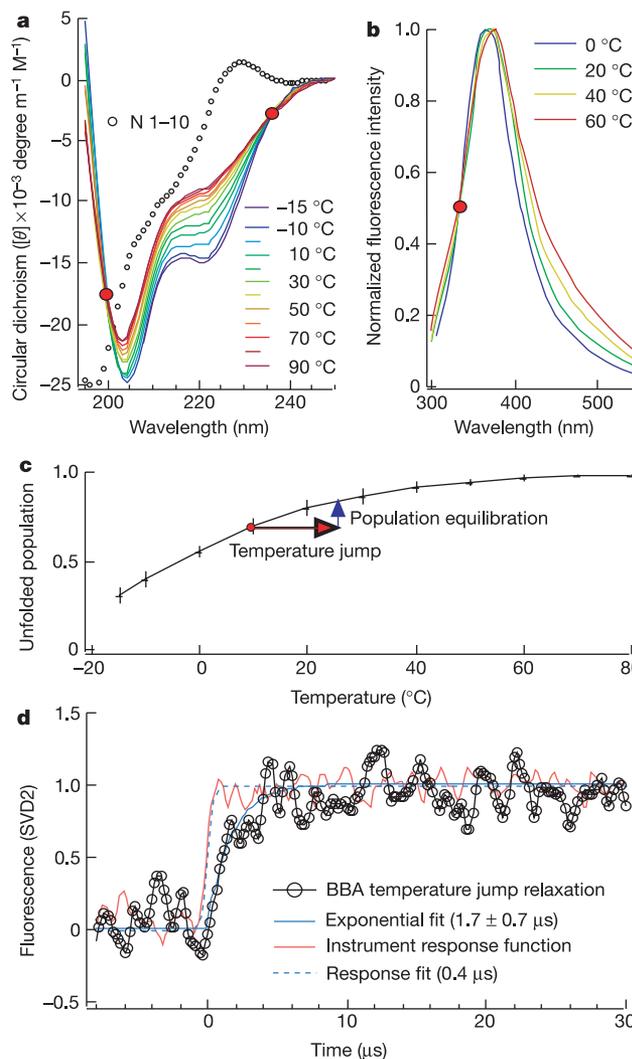
Figure 2 BBA5 double mutant thermodynamics and kinetics. **a**, Circular dichroism spectra from −15 °C to 90 °C. Isodichroic points (red circles) and the largest singular values (S2/S1 = 0.10, S3/S1 = 0.02, S4/S1 = 0.02, S5/S1 = 0.01; see Methods) indicate thermodynamic two-state behaviour. The N-terminal fragment is a random coil. Circular dichroism spectra of the single mutant (not shown) are very similar. **b**, Intensity normalized fluorescence spectra. The emission peak shifts from 366 nm to 377 nm and fluorescence intensity decreases by 2.5 as temperature increases from 0 °C to 60 °C. The 330-nm isosbestic point is further evidence of a two-state mechanism. **c**, Unfolded protein population with error bars. **d**, Temperature-jump relaxation kinetics and tryptophan instrument response at an average temperature of 298 K. The kinetics are normalized second singular value components of the time-evolving fluorescence spectrum ($\Delta\nu \approx 2$ nm). The single exponential fit with a relaxation time constant of 1.7 µs yields a deconvoluted relaxation time constant of 1.5 µs.

possessed both native secondary structure and low alpha carbon position root mean square deviation (r.m.s.d.$_{C\alpha}$) with BBA5 (see Methods). Of 9,000 double-mutant folding trajectories at 278 K, 16 were folded after 20 ns (Fig. 3). Trajectories that fold quickly are not unphysical[10]; the fact that the average unfolded molecule explores conformational space for microseconds before finding the native conformation does not preclude individual molecules from folding quickly. Quite the opposite, the basic principles of two-state protein kinetics indicate that we must expect to observe a small fraction of molecules to fold on short timescales[18].

Our simulations were consistent with the experimentally determined folding rate and stability. Our two-state assumption was justified by the thermodynamic data. The interpolated simulated folding time at 298 K was about 6 μs for the double mutant, and we computed unfolding rates slightly higher than the folding rates. This is to be compared with the experimental folding time of 7.5 ± 3.5 μs and K_{eq} of 0.25 ± 0.1 (that is, the unfolding rate k_u, is about four times faster than the folding rate, k_f). The primary uncertainty in the computed rate was not the fit, but sensitivity to the folding criteria; varying the r.m.s.d.$_{C\alpha}$ cutoff by ±0.5 Å resulted in expected folding times at 298 K of 3–13 μs. Simulations of the single mutant at 298 K were more difficult to resolve and generated an expected folding time of 16 μs (7–43 μs), which was roughly consistent with experimental observation ($\tau < 10$ μs). A total of 10,000 trajectories at a timescale of tens of nanoseconds seem sufficient to provide a reasonable statistical estimate of the folding rate for this rapidly folding, two-state mini-protein.

It is important to address directly limitations in terms of methods and modelling. First, to discriminate folded structures, we rely on qualitatively reasonable but arbitrary criteria. Also, our simulations do not represent structure prediction, as our folded criterion relies on the Cα positions of BBA5. Besides methodological limitations, it is imperative to address whether established methods, such as the optimized potentials for liquid simulations (OPLS) parameter set, the generalized Born surface area solvent model, and stochastic dynamics within Tinker are able to sample native-like states without further optimization. The observed native state flexibility may be an indication that our force field was insufficiently accurate to describe completely the native structure free energy minimum. Finally, given a 400-ns instrument response function and a 1.5-μs relaxation rate, the uncertainty in the experimental folding rate is still large.

There is a growing body of knowledge about rapid protein dynamics. The fastest helices in apomyoglobin form in approximately 50 ns (ref. 19), and a collapsed hydrophobic core in the same molecule forms in about 10 μs (ref. 20). It has been suggested that helices and turns commonly form on the timescale of about 100 ns and 1 μs, respectively[1]. For the BBA5 single mutant, we can make the rough estimate that α-helix formation above the level in the

unfolded state population has a relaxation time of about 500–800 ns (from 278 to 298 K). We further estimate that the β-hairpin has a relaxation time of about 1.7–1.5 μs (from 278 to 298 K). The β-hairpin forms more quickly in the double mutant with a relaxation time of only about 0.5 μs at 278 K, which demonstrates the sensitivity of simulated folding kinetics to a single mutation and explains why the estimate of the double-mutant folding rate exceeds the single mutant estimate. These folding rates approach the theoretical maximal folding rate estimated from protein chain diffusion[12]. The ability to simulate an approximately 10-μs process by sampling many shorter trajectories puts any number of rapidly folding proteins within our reach. Simulated rates could complement and explain quantities correlated with rate, such as contact order[21].

The two principal challenges of simulated protein folding—to build accurate models and to adequately sample conformational space—are not easily decomposed. Here, we have addressed an improved means of sampling; using many thousands of independent trajectories, we have explored the conformational space of two BBA5 mutants. We have observed their folding many times, a process that takes microseconds. We have studied the behaviour of ensembles of thousands of trajectories. Helical structure in the unfolded state, fragment secondary structure propensity, rate of helix formation, rate of hairpin formation, and the rate for folding were all consistent with the available experimental evidence. The ability to investigate directly folding dynamics and heterogeneity with molecular dynamics will bring molecular dynamics to the same prominence in protein folding that it has already achieved in structural modelling. □

Methods

Protein preparation

BBA5 mutants and fragments were synthesized with C-terminal amidation and N-terminal acetylation by Bioworld or the UIUC Biotech Center to 70% purity. High-performance liquid chromatography (HPLC) purification using a Vydac C18 reversed-phase analytical column yielded over 90% purity, verified by mass spectrometry. The samples were lyophilized after purification. To calculate concentrations, extinction coefficients at 280 nm were estimated for the mutants and fragments using the extinction coefficients of tyrosine and tryptophan residues. C-terminal fragment concentrations were determined by amino-acid composition analysis, using the Moore–Stein method[22].

Model preparation

The BBA5 double mutant model 1 was generated by homology to BBA1 (Protein Data Bank code 1HCW)[16] with 74% sequence identity. All applicable torsion values from the most representative member of the BBA1 NMR ensemble were taken for an initial conformation. Mutant residues used the Tinker[23] default torsions of 180° except for Trp 8 where a side-chain torsion angle χ1 of 0° relieved a steric clash. No memory Broyden–Fletcher–Goldfarb–Shanno (BFGS) quasi-Newton energy optimization[24] to r.m.s. gradient convergence, followed by 100 ps of molecular dynamics, provided a numerically stable initial conformation. The other double mutant models (2–26) were generated by homology to BBA5 (91% identical to the double mutant)[15]. We took each of the 25 NMR structures, removed Val 3 and Phe 8, placed Tyr 3 and Trp 8 simultaneously into their most favoured rotamers (holding the rest of the protein fixed) using a self-consistent mean field method[25], and minimized each structure including a generalized Born surface area energy term[26] at each step. To obtain an initial unfolded structure, a fully unfolded structure was generated using Tinker (backbone torsions $\varphi,\psi = -135,135$). A total of 100 steps of minimization relieved a clash between the side chain and carbonyl of D-Pro 4. This extended structure was equilibrated for 100 ps using molecular dynamics, providing a numerically stable denatured structure. The single mutant and fragment simulations were started from similarly prepared extended conformations.

Circular dichroism and fluorescence measurements

Fluorescence spectra were excited at 285 nm in a 1-cm quartz cuvette at 100 μM peptide concentration. A thermocouple and circulating water bath measured temperature to ±0.2 °C. Sixty micromolar circular dichroism samples (0.3–2 mm cuvettes) were temperature equilibrated for 1 min for each temperature point. In addition to isosbestic points, singular value decomposition (SVD) was used to verify that the thermodynamic data could be modelled as a function of temperature with only two significant species; the third and higher SVD basis functions were negligible (see legend to Fig. 2): two species account for most of the observed circular dichroism and fluorescence spectral changes with temperature. The second SVD component was fitted to $K_{eq}(T)$, with and without baselines, to estimate the population error from inaccurate baselines (Fig. 2c). $K_{eq} = \exp[-\Delta G_f(T)/RT]$, where $\Delta G_f(T) = mT(T - T_m)$, ΔG_f is folding energy, T is temperature, $R = 8.31$ J K^{-1} mol^{-1} and T_m is the melting temperature of BBAW (-2 ± 3 °C). The resulting unfolded fraction $(1 - K_{eq})/(1 + K_{eq})$ (Fig. 2c) changes from under 25% to over 75% over a 45 °C range.
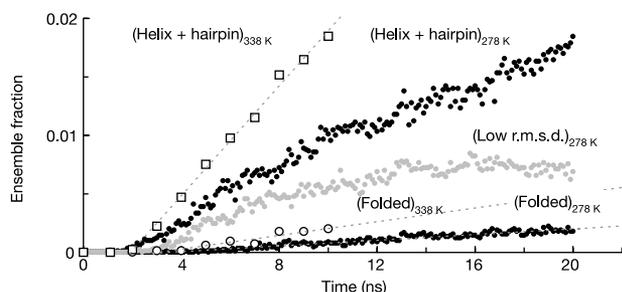
Figure 3 Folded population growth. The growing population of folded double mutant conformations, at 278 K (filled circles, dotted line) and at 338 K (open circles, dotted line), the population with low r.m.s.d.$_{C\alpha}$ (grey circles), and the population with both helix and hairpin at 278 K (filled circles) and 338 K (open squares, dotted line), are shown. The scale of the y axis illustrates the need to simulate thousands of trajectories. Note the increased rate of folding at high temperature.

Temperature jumps

Temperature jumps of 11 °C were induced by a 1.5-µm wavelength infrared pulse lasting 10 ns, generated by a Raman-shifted YAG laser. Free tryptophan in phosphate buffer was used to calibrate the temperature jump before each measurement series. Time-resolved, dispersed fluorescence data was taken by channelling the fluorescence (excited by 288-nm pumping with a frequency-tripled, mode-locked Ti:sapphire laser) by means of a liquid ultraviolet light guide through a monochromator, which imaged 60 nm of the spectrum (340–400 nm) into 15 channels of an array photomultiplier tube[17]. The net unfolding process was monitored by an approximately 11-nm fluorescence redshift. SVD as a function of time also revealed only two significant basis functions in the time-evolving fluorescence spectra. The measured relaxation rate was $k_o = k_f + k_u$, where $K_{eq} = k_f/k_u$. The reported rate of 1.5 µs was obtained by deconvoluting the 400-ns gaussian full-width-at-half-maximum instrument response from a 1.7-µs fit.

Molecular dynamics

Our simulations used software adapted from the Tinker molecular modelling package[23] and the OPLS united atom parameter set[27]. Constant temperature stochastic dynamics modelled the viscous drag of water ($\eta = 91\ \mathrm{ps}^{-1}$). We modelled solvation with the generalized Born/surface area implicit solvent model[26]. The electrostatic calculations used 16 Å cutoffs with 12 Å tapers. The bond lengths were constrained with the RATTLE algorithm allowing time steps of 2 fs[28]. His 21 was given neutral charge. Trajectory conformations were recorded at intervals of 0.1–1 ns.

For analysis, a conformation contained α-helix if it had at least four α-helical residues according to the program Dictionary of Protein Secondary Structure (DSSP)[29] with the default H-bond cutoff parameter of 0.5 kcal mol^{-1}. A conformation contained the native hairpin if it had a hairpin with β-bridges between residues 2–7 and 3–6 according to DSSP. To quantify the presence of expected tertiary structure, we aligned each conformation to the Cα positions of the reported low energy BBA5 NMR structure and calculated r.m.s.d.$_{C\alpha}$ using ProFit. A conformation had low r.m.s.d.$_{C\alpha}$ if it had r.m.s.d.$_{C\alpha}$ with BBA5 < 3.622 Å (one standard deviation above the equilibrated (10 ns) folded double-mutant ensemble mean). A conformation had high r.m.s.d.$_{C\alpha}$ if it had r.m.s.d.$_{C\alpha}$ with BBA5 > 5.0 Å (one standard deviation below the final unfolded ensemble mean). A conformation was folded if it had the helix, the hairpin, and low r.m.s.d.$_{C\alpha}$. A conformation was unfolded if it lacked either the helix or the hairpin, and had high r.m.s.d.$_{C\alpha}$.

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