Supplementary information

Protein sample and experimental measurements
The Pin WW domain gene was incorporated into a GST fusion protein expression system (PGEX-2T vector). Fip35 was made by site-directed mutagenesis. The sequence of the protein is: GSKLPPGWEKRMSRDGRVYYFNHITNASQFERPSG. The protein was expressed and purified as described in [1].

Thermal titrations were carried out on a JASCO J-715 spectropolarimeter equipped with a Peltier temperature controller and an external photomultiplier tube. Lyophilized protein samples were dissolved in 10 mM phosphate buffer at pH 7 with a protein concentration near 10 μM. Thermal titration curves were obtained by monitoring the circular dichroism (CD) signal at 227 nm, and the tryptophan fluorescence intensity excited at 295 nm. The temperature range from 279 K to 379 K was examined. The titration signals were fitted with the two-state model as described in [2] to extract the equilibrium constant $K_{eq}(T)$.

Relaxation kinetics were measured with a home-built laser T-jump instrument. Protein solutions with a final concentration of about 100 μM were prepared in 20% D_2O/80% H_2O buffer at pH 7 (pH without isotope effect correction). The details of the laser T-jump setup and data analysis are described in [2].

Folding kinetics results
The thermal denaturation data from CD and fluorescence can be fitted by a two-state model. The fitted melting temperature of 351 K is the same for the two probes, indicating that the protein behaves like a two-state folder near the thermal denaturation midpoint. Fip35 is one of the fastest folders in the pin WW domain mutant family. When its temperature is lowered below the thermal denaturation midpoint, it becomes an incipient downhill folder. The kinetic signature of this transition is a new fast phase (the so-called molecular phase with rate coefficient k_m) that appears in addition to the slower activated kinetics (with rate coefficient k_a) when the temperature is lowered. This happens because the very low barrier at optimum temperature allows fast diffusion of proteins over the barrier to be observed, in addition to the slower activated kinetics. At 352 K (near the thermal denaturation midpoint), a single relaxation rate k_{obs} is observed $(8.1 \pm 0.2 \ \mu s)^{-1}$. Based on a two-state analysis ($K_{eq} = k_f/k_u$, and $k_{obs} = k_u + k_f$), the folding rate determined is $k_f = k_{obs} K_{eq} /(1+K_{eq}) \approx (17.7 \ \mu s)^{-1}$. As the temperature decreases, the increased native bias reduces the folding barrier and, thus, the folding rate accelerates. In addition, the molecular phase appears. Thus at 337 K, a double exponential decay fits the relaxation kinetics better than a single exponential decay. The molecular rate k_m and the activated rate k_a are $(1.5\pm0.3 \ \mu s)^{-1}$ and $(11.4\pm1.4 \ \mu s)^{-1}$ respectively. The folding rate is estimated to be $k_f \approx k_a K_{eq} /(1+K_{eq}) = (13.3 \ \mu s)^{-1}$. (This latter value is an approximation because the two-state analysis is no longer accurate for incipient downhill folding.) Measurement at lower temperature is not possible because of the low signal to noise ratio resulting from a lower population change upon T-jump.

Molecular dynamics simulations

All equilibration steps and production runs were performed in the NVT ensemble, using a periodic box size obtained from a 100 ps NPT equilibration of the 2F21 structure. Both the folded and unfolded states were minimized for 3000 steps and equilibrated for 100 ps in the NVT ensemble with all backbone atoms restrained prior to production runs. A temperature of 337 K was maintained using Langevin dynamics, with a damping constant of 5 ps^{-1} (for the folded state) or 0.1 ps^{-1} (for the unfolded state); the latter was chosen to minimally perturb the folding kinetics. A time step of 2 fs was used, with the lengths of all bonds involving hydrogens constrained. Short-range nonbonded interactions were cut off at 8.0 Å, with shifting beginning at 7.0 Å. Bonded and short-range nonbonded interactions were evaluated every time step, and full electrostatics evaluated using the particle mesh Ewald method every third time step.

Simulations were performed on the abe cluster at the National Center for Supercomputing Applications, using a modified version of NAMD tuned for performance on abe and similar clusters (multicore nodes of x86 compatible processors with an InfiniBand interconnect). Specific optimizations included modification of the RATTLE [3] and SETTLE [4] implementations to use streaming SIMD extensions (SSE) intrinsics allowing performance of multiple floating point operations per processor cycle, optimization of the load balancer for the abe network topology, the use of pencil decomposition for PME, and application of processor affinities for efficient scheduling of multiple threads on each node. These changes allowed efficient scaling to 329 cores (47 nodes, 7 processors per node) while simulating the 30,000 atom system, yielding a performance of 100 ns/day. All optimizations are available in the NAMD CVS repository. The new version of NAMD will be described in a forthcoming publication.

MD trajectory analysis

For Fig. 1, native contacts for each residue were defined as other residues separated by more than one residue in the primary sequence which are within 4.0 Å in the crystal structure. Secondary structure was calculated using STRIDE [5]. Clustering analysis was performed using the g_cluster utility of GROMACS 3.3 [6], with the grooms clustering method [7] and a cutoff Cα-RMSD of 6.4 Å, which corresponded to the midpoint between two maxima in the pairwise frame-frame RMSD distribution. The presence of fluctuations in the secondary structure content, even after the cluster distribution has stabilized, appears to be due to transient local rearrangements in the secondary structure content of a few helical turns, rather than significant structural rearrangements. This is a consequence of the large RMSD cutoff used to obtain a manageable number of clusters.

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