Supplementary Figures

Supplementary Fig. S1a
Supplementary Fig. S1. FRET efficiency histograms and corresponding 2D histograms including relative fluorescence lifetimes, $\tau_{DA}/\tau_D$, versus the FRET efficiency, $E$, for R15 (a) and R17 (b). $\tau_{DA}$ and $\tau_D$ are the donor lifetimes in the presence and absence of the acceptor, respectively. The straight red lines are the dependences $E(\tau_{DA}/\tau_D)$ expected for a single fixed distance, while the curved red lines result from the distance distribution of a Gaussian chain. The peaks at high, intermediate, and zero FRET efficiencies correspond to the native, unfolded, and “donor only” subpopulations, respectively. Dashed lines indicate the centers of the peaks corresponding to the unfolded state. In the 2D histograms, peak heights are color-coded: blue indicates the lowest value, corresponding to the background emission level, and red is the highest value. Data were recorded at the GdmCl concentration indicated at the top of each panel.
Supplementary Fig. S2. FRET efficiency histograms from measurements in the microfluidic mixer show the progress of the folding reaction along the observation channel of the device (times elapsed after mixing and distances from the beginning of the observation channel are indicated in the panels). The GdmCl concentration in the observation channel is 0.03 M. Data shown in Fig. 2 are taken under conditions corresponding to the top panel.
Supplementary Fig. S3. The root mean squared end-to-end distance, \( \langle r^2 \rangle \), of the chain segments of unfolded R15 and R17 as a function of solvent viscosity (adjusted by the addition of glucose and glycerol as indicated) for different GdmCl concentrations, color coded as in Fig. 3. The axis scale chosen for \( \langle r^2 \rangle \) is similar to that used for plotting the dependence of this parameter on GdmCl concentration (Fig. 1c), to show the relative magnitude of the observed changes. The values of \( \langle r^2 \rangle \) remain essentially unchanged upon addition of the viscogens, indicating that the viscogens do not alter the equilibrium distribution of the unfolded polypeptide chain.
**Supplementary Fig. S4.** Solvent viscosity dependence of folding and unfolding times of R16 (c) and R17 (d) corresponding to crossing of the late transition state at $\Delta G = 1.5$ kcal/mol ($\tau_u, \tau_f$) and at $\Delta G = 0$ ($\tau_{mp}$) with linear fits. A quantitative analysis of these dependencies is reported in Fig. 4b as inverse relative unfolding rates versus relative viscosities. Color code is the same as in Fig. 5 (see Methods and ref. 8 for details of this analysis). The shaded areas along the fits indicate confidence intervals (confidence level 90%) for the unfolding, refolding, and midpoint data sets of each domain (see Methods for details and Supporting Table S1 for the resulting values).
Supplementary Fig. S5 Schematic representation of the dependence of folding ($\tau_f$) and unfolding ($\tau_u$) times on solvent viscosity, $\eta$, as described by equation 2, assuming that for both processes the contribution of internal viscosity at the transition state ($\sigma_f$ and $\sigma_u$, respectively) is the same (cf. Fig. 5). E.g., $\tau_f$ can be related to $\sigma$ via $\eta_0/\sigma = \tau_f(\eta_0)/\tau_f - 1$, where $\tau_f(\eta_0)$ is the overall relaxation time of the process at a reference solvent viscosity $\eta_0$, typically ~1 mPa s.
**Summary of the kinetic parameters describing the folding and dynamics of R15, R16, and R17 spectrin domains.** The values for all parameters are given in the absence of GdmCl. The values of $\sigma$ in the unfolded state were calculated assuming the relationship $\sigma = \tau_i/\tau_s$, in analogy with the case illustrated in Methods (Calculation of $\sigma$ and the upper limits of $\tau_f$, for R16 and R17 in the late transition state) and Supplementary Fig. S5. Correspondingly, we obtain very large values of $\sigma$ in the unfolded state of the spectrin domains in the absence of denaturant, ranging from ~10 mPa s to ~20 mPa s, which indicates that unfolded state reconfiguration is completely dominated by internal friction. However, since the absolute timescale of unfolded state dynamics is still only in the sub-microsecond range, it does not limit the millisecond folding kinetics for the spectrin domains, even in cases where $\sigma_{TS}$ is very small. Note, however, that according to the Kuhn theorem, the relative contribution of internal friction to chain dynamics (as expressed by $\sigma$) will strongly depend on the segment length probed and is expected vanish in the limit of very long chains. Therefore, an absolute comparison of $\sigma$ between unfolded and more structured conformations has to be treated with caution. Values of $\sigma$ and $\tau_i$ in 0 M GdmCl were calculated as explained in Methods.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_r$ (ns)</th>
<th>$\tau_i$ (ns)</th>
<th>$\tau_i/\tau_r$ (%)</th>
<th>$\sigma_{\text{unfolded}}$ (mPa s)</th>
<th>$\tau_f$ early TS (ms)</th>
<th>$\tau_f$ late TS (ms)</th>
<th>$\sigma_f$ early TS (mPa s)</th>
<th>$\sigma_f$ late TS (mPa s)</th>
<th>$\tau_f/\tau_s$ early TS (%)</th>
<th>$\tau_f/\tau_s$ late TS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R15</td>
<td>210 ± 24</td>
<td>200 ± 25</td>
<td>95 ± 16</td>
<td>18 ± 8</td>
<td>0.13 ± 0.01</td>
<td>(16 ± 9)·10^{-3}</td>
<td>1.1 ± 0.08</td>
<td>0.14 ± 0.08</td>
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<tr>
<td>R16</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>9.2 ± 0.4</td>
<td>7.3 ± 0.5</td>
<td>4.8 ± 1.0</td>
<td>3.8 ± 1.0</td>
<td>0.09 ± 0.01</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>R17</td>
<td>123 ± 6</td>
<td>111 ± 7</td>
<td>90 ± 7</td>
<td>9 ± 3</td>
<td>113 ± 12</td>
<td>96 ± 11</td>
<td>6.6 ± 2.0</td>
<td>5.6 ± 2.0</td>
<td>0.2 ± 0.3</td>
<td>&lt; 0.03</td>
</tr>
</tbody>
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$\tau_r$: total reconfiguration time of the unfolded chain (see Eq. 1, main text)
$\tau_i$: internal friction time of the unfolded chain (see Eq. 1, main text)
$\tau_s$: reconfiguration time of the unfolded chain in the absence of internal friction (see Eq. 1, main text)
$\sigma$: internal viscosity (see Eq. 2, main text)
$\tau_f$: folding time (see Eq. 2, main text)
$\tau_{fi}$: solvent-independent contribution to the folding time due to internal friction (see Eq. 2, main text)
$\tau_{fs}$: solvent-dependent contribution to the folding time (see Eq. 2, main text)
Supplementary Methods

Transfer efficiencies and fluorescence lifetimes.
Transfer efficiencies were obtained from $E = n_A / (n_A + n_D)$, where $n_D$ and $n_A$ are the numbers of donor and acceptor photons in the burst, respectively, corrected for background, channel crosstalk, acceptor direct excitation, differences in quantum yields of the dyes, and detection efficiencies$^{61}$. Average fluorescence lifetimes were estimated as the mean detection time of the photons in a burst after donor excitation. For a fixed inter-dye distance $r$, the mean donor lifetime in the presence of acceptor is given by $\tau_{DA} = \tau_{DA}(r) = \tau_D(1 - E(r))$, where $\tau_D$ is the lifetime in the absence of acceptor, and the transfer efficiency at a certain inter-dye distance $r$ is $E(r) = 1/(1 + R_0^2 / r^2)$, with the Förster radius $R_0$ calculated for the respective values of the refractive index of the solution$^{62}$ (straight line in Fig. S2 bottom panels/2D histograms). For a Gaussian chain, the mean FRET efficiency $\langle E \rangle$ is calculated as $\langle E \rangle = \int E(r)P(r)dr$ $^{35,36,63,64}$, where $P(r)$ is the probability density function of $r$, defined as

$$P(r) = 4\pi r^2 \left( \frac{3}{2\pi \langle r^2 \rangle} \right)^{3/2} \cdot e^{-3r^2/2\langle r^2 \rangle}$$

(S1).

For such a distribution of $r$, the lifetime in the presence of the acceptor is

$$\tau_{DA} = \int_0^\infty tI(t)dt / \int_0^\infty I(t)dt \quad \text{with} \quad I(t) = I_0 \int_0^\infty P(r)e^{-t/\tau_{DA}(r)}dr,$$

where $I$ is the time-dependent fluorescence emission. The resulting value of $\tau_{DA}$ is represented by the curved lines (Fig. S2). Analogous calculations with the $P(r)$ for a wormlike chain give essentially indistinguishable results.
Supplementary References


