

Ultrarapid mixing experiments shed new light on the characteristics of the initial conformational ensemble during the folding of ribonuclease A

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The earliest folding events in single-tryptophan mutants of RNase A were investigated by fluorescence measurements by using a combination of stopped-flow and continuous-flow mixing experiments covering the time range from 70 μ s to 10 s. An ultrarapid double-jump mixing protocol was used to study refolding from an unfolded ensemble containing only native proline isomers. The continuous-flow measurements revealed a series of kinetic events on the submillisecond time scale that account for the burst-phase signal observed in previous stopped-flow experiments. An initial increase in fluorescence within the 70- μ s dead time of the continuous-flow experiment is consistent with a relatively nonspecific collapse of the polypeptide chain whereas a subsequent decrease in fluorescence with a time constant of \approx 80 μ s is indicative of a more specific structural event. These rapid conformational changes are not observed if RNase A is allowed to equilibrate under denaturing conditions, resulting in formation of nonnative proline isomers. Thus, contrary to previous expectations, the isomerization state of proline peptide bonds can have a major impact on the structural events during early stages of folding.

burst phase | continuous-flow | proline isomers | stopped-flow

The protein folding process is frequently described in terms of funnel-shaped energy landscapes in which the depth of the funnel represents the interaction free energy of the chain and the width represents the conformational chain entropy (1–4). The funnel is commonly depicted without an energy barrier, sharing the view with other protein folding scenarios that proteins fold without encountering enthalpic barriers in the early stages of the process (5–7). In this picture, the molecules are disordered and the individual trajectories are very different from each other, which plausibly led to the claim that very localized alterations, such as nonnative proline isomers, have no impact on the early folding events (6). Based on the same expectations, nonfoldable (reduced or truncated) protein analogs were explored to characterize the unfolded state of foldable proteins under folding conditions (5, 6, 8, 9). It is difficult to address this problem experimentally because, even if specific interactions that direct the folding process along specific pathways are at work early in the folding process, the conformations may have only marginal stability and are characterized by an overall topology in certain segments rather than by well defined backbone structure. Consequently, they can be detected only with specific probes that report locally on the implicated segments. Furthermore, when an early intermediate forms, one often can detect only a so-called “burst phase,” i.e., a change of signal during the dead time of the kinetic measurement above and beyond that expected by simply changing the solution environment of the reporter group. However, the interpretation of such a burst phase is ambiguous, and it was suggested that these burst phase signals in general, and in bovine pancreatic RNase A in particular, represent a nonspecific collapse of the unfolded polypeptide chain in response to the changing solvent conditions (5–7).

To investigate the nature of the conformational ensembles in the unfolded protein and the early folding events in RNase A, we combined ultrafast continuous-flow (CF) and conventional stopped-flow (SF) mixing techniques to monitor folding of a single-tryptophan variant of RNase A, Y92W, which contains a sensitive fluorescence probe in a region of the protein implicated in early folding events (10). This mutant has been reported to exhibit a major burst phase in double-jump stopped-flow fluorescence experiments (11). The specific questions addressed here are as follows. (i) Is there any kinetic phase, detectable by CF experiments, that occurs in the dead time of the SF instrument? (ii) Can the SF burst phase be explained by a simple readjustment of the unfolded polypeptide chain to the new solution conditions, i.e., a nonspecific conformational change (5–7)? (iii) Is the unfolded ensemble of a foldable protein structurally equivalent to that of an unstable (nonfoldable) analog under native conditions? (iv) Do nonnative proline isomers have a measurable impact on the distribution of the conformations in the unfolded protein and on the dynamics of early folding events?

To understand the rationale of these experiments, it is necessary to review some of the structural and folding characteristics of RNase A. RNase A has four disulfide bonds (Fig. 1) and four prolines, Pro-93 and -114 being in the cis conformation (12). The population of unfolded states with all X-Pro peptide groups in their native isomeric state (the “very fast-folding” form, U_{vf}) is a small fraction (\approx 2%) of the species in the equilibrium unfolded protein (U_{eq}) in which each of the four X-Pro peptide groups has been equilibrated between the cis and trans isomeric states (13). To study U_{vf} exclusively, a double-jump procedure was developed for SF experiments in which the protein was unfolded for a short period (1–2 s), which is sufficiently long for complete unfolding of the protein, but short enough to prevent the prolines from isomerizing, before refolding is initiated by a second jump (13–15). This procedure, which populates U_{vf} to the extent of \approx 99%, was also adopted for CF measurements to follow the folding of U_{vf} on a microsecond time scale.

Another interesting characteristic of RNase A is its biphasic fluorescence-detected unfolding kinetics (16, 17). At low pH, the fast phase corresponds to the unfolding of the three-dimensional structure and can also be detected by absorbance and CD; the slow phase is silent in absorbance (17, 18) and CD (16) and insensitive to GuHCl concentrations (16, 18), and corresponds to the isomerization of proline residues next to the fluorescence reporters (Tyr, or Trp in mutants) (11, 16, 17, 19). Because the sole tryptophan in the mutant proteins studied here is next to Pro-93, it reports on the isomeric state of Pro-93 in the unfolded

Abbreviations: CF, continuous-flow; SF, stopped-flow.

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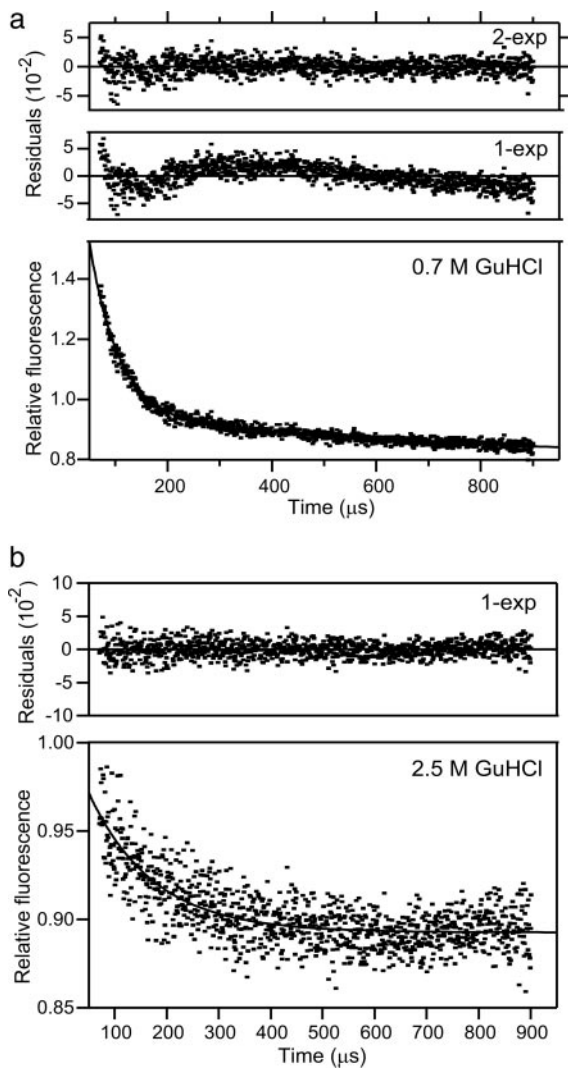


Fig. 4. CF fluorescence traces measured in double-jump refolding experiments on Y92W RNase A at a final concentration of 0.7 M GuHCl (a) and 2.5 M GuHCl (b). Residuals obtained by double-exponential and single exponential-functions are shown above each trace.

26 and 84 are not in contact with Trp-92. Thus, preventing formation of the 26–84 disulfide bond in Y92Wdes is unlikely to have any direct effect on the fluorescence of Trp-92, and the difference in fluorescence properties of the states formed within the dead time of the CF experiment must reflect a more global effect of the modification. The initial fluorescence increase in Y92W thus represents a conformational change of a subdomain, or even the whole protein, which occurs only if the 26–84 disulfide bond is intact. However, more direct structural information, based on FRET or small-angle x-ray scattering (SAXS) data, will be needed to determine whether the conformational change involves compaction of the whole protein or a subdomain. Also, it is uncertain whether this initial conformational change occurs within tens or hundreds of nanoseconds, as expected for random polymer collapse, or on the microsecond time scale, reflecting a more specific folding event. Nevertheless, our evidence for long-range (tertiary) structural perturbations due to elimination of the 26–84 disulfide bond clearly indicates the limitations of using fully reduced proteins as nonfoldable analogs as a reference for investigating the properties of early folding intermediates (6, 8).

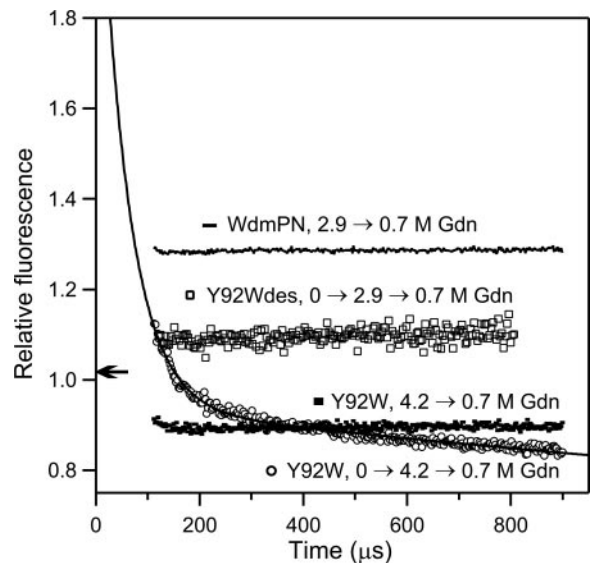


Fig. 5. Submillisecond folding kinetics of the Y92W and Y92Wdes proteins, and the respective trace of the WdmPN peptide. Fluorescence change during refolding of GuHCl-denatured proteins and WdmPN at 0.7 M GuHCl in CF measurements. The fluorescence is normalized to a relative value of 1.0 for the corresponding unfolded conditions (2.9 M GuHCl for Y92Wdes RNase A in double-jump experiments and for WdmPN and 4.2 M GuHCl for Y92W in both single and double-jump experiments). Note that the unfolding conditions are different in the double-jump experiments with Y92W RNase A (Fig. 2) and Y92Wdes (here). Therefore, the fluorescence values of the WdmPN peptide that are scaled relative to their respective unfolding conditions are different in Figs. 2 and 5. The arrow indicates the fluorescence of the WPN-peptide, which is a control for the initial value expected for refolding from the equilibrated unfolded state, U. Traces are: thin solid line, WdmPN-peptide; open squares, U_{vf} of Y92Wdes RNase A; filled squares, U of Y92W RNase A. The dead time in these experiments is 120 μ s. For easier comparison of the kinetics of the proteins under different conditions, the 0.7 M GuHCl trace for U_{vf} of Y92W RNase A (open circles), truncated at 120 μ s, are also shown from Fig. 2. The kinetic traces are reproducible to within a 1–2% of the reference signal.

The Folding of a Slow-Folding Species. We now consider the question whether the initial collapse and the subsequent folding events detected for Y92W (but not for the nonfolding analog, Y92Wdes) are related to the stability and foldability of the protein species, and whether they reflect early folding events that are obligatory for rapid conformational folding. Nonnative proline isomers are known to inhibit rapid folding of RNase A (24, 25). If refolding of Y92W is studied by single-jump SF measurements starting from the equilibrium-unfolded form, U_{eq} , the amplitudes of the fast phases are too small to be detected by fluorescence (11). To address these questions, we performed a single-jump CF experiment (Fig. 5, filled squares) under conditions identical to the second jump of the double-jump experiment (4.2 M GuHCl, pH 2, to 0.7 M GuHCl, pH 5). There is a small decrease in the fluorescence signal (to ≈ 0.9) in the dead time of the CF measurement (compared with the expected initial value of ≈ 1.0 estimated by using the WPN tripeptide, as indicated by the arrow in Fig. 5), consistent with a minor conformational adjustment within the unfolded ensemble. However, neither the initial collapse (found to be absent in previous stopped-flow dynamic light-scattering measurements) (26), nor any other early folding events were detectable during refolding from U_{eq} (Fig. 5). Thus, the rapid ($<70 \mu$ s) collapse observed for the U_{vf} species of Y92W is absent both in the case of Y92Wdes (a nonfolding species) and U_{eq} of Y92W (a foldable species), indicating that equilibrium foldability and rapid collapse of the polypeptide chain are not correlated. By contrast, conditions that lead to a fluorescence enhancement during the

CF dead time (refolding from U_{vf} at low GuHCl concentrations) also give rise to a fluorescence decay on the 100- μ s time scale, suggesting that these conformational events may be linked. However, it is not clear whether this connection is obligatory or not. Our findings clearly show that, contrary to prior expectations (6), nonnative proline isomers have a large impact both on the distribution of the conformations in the unfolded ensemble and on the dynamics of folding at early stages of folding.

Discussion

A primary objective of this study was to gain a better understanding of the structural basis of the kinetically unresolved spectral changes (burst phase) observed in SF measurements of many proteins, including RNase A. These signals were suggested to reflect a solvent-dependent chain contraction or collapse of the ensemble of denatured states when the unfolded polypeptide is transferred from a high concentration of denaturant (good solvent) into a lower concentration of denaturant (poor solvent) (5–7). Alternatively, SF burst phases and nonlinear relationships between the logarithm of the folding rate and the denaturant concentration (rollover) were explained in terms of a partially structured intermediate, formed in a rapid barrier-crossing event and stabilized by decreasing denaturant concentration (27–29).

Ultrafast mixing experiments have made it possible to kinetically resolve the fast processes responsible for the burst phase of several proteins (30–35), but ambiguities remain in the interpretation of the results. A mechanism with a discrete free energy barrier preceding the rate-limiting barrier for folding is the simplest mechanism consistent with an exponential decay on the submillisecond time scale preceding the rate-limiting phase (30). On the other hand, if chain collapse is governed by diffusion on a complex free energy surface, its kinetics is more likely to be nonexponential (36–38). However, it has been argued that mono-exponentiality is not diagnostic for barrier crossing, because other mechanisms can also approximate exponential behavior (39, 40). Furthermore, a simple chain contraction may also give rise to (small) free energy barriers due to, e.g., main-chain bond rotation and solvent viscosity (41). Qui *et al.* recently reported that both full-length cytochrome *c* and two large fragments (nonfoldable analogs) showed monoexponential kinetics with an apparent activation energy (9). Although these results are consistent with a nonspecific chain-contraction, another possibility is that the cytochrome *c* fragments exhibit a barrier-limited initial folding event similar to the whole protein but cannot continue folding to the native state (9). Here, we obtained further insight into these issues by detecting the two processes separately in CF measurements: an initial fluorescence increase in $<70 \mu$ s that may correspond to an initial contraction of the polypeptide chain and a subsequent multiexponential fluorescence decrease that corresponds to succeeding folding events and accounts for the SF burst phase (the missing amplitude in the SF experiment is associated with a decrease of fluorescence).

The earliest conformational event during folding of RNase A, which gives rise to a large enhancement in Trp-92 fluorescence above the level expected for the unfolded protein under refolding conditions, occurred within the 70- μ s dead time of our CF experiment (Fig. 2), indicating that the time constant of this process is of the order of 10 μ s or less. Because we can estimate only an upper limit for the time constant of this process, we cannot rule out the possibility that it reflects a nonspecific collapse of the polypeptide chain induced by the change in solvent conditions; on the basis of polymer theory and/or computer simulations, this hydrophobic chain collapse has been estimated to occur in the 10- to 100-ns range (41–44), as confirmed by recent T-jump measurements on a denatured protein (36). However, our observation that the amplitude of the burst-phase event is highly sensitive to denaturant concentration (an increase from 0.7 to 1.2 M GuHCl results in a

70% decrease in amplitude; see Figs. 2 and 3) is indicative of a more specific structural event. Although we have no information on the overall dimensions of the state formed within the CF dead time, the large increase in fluorescence indicates that Trp-92 is largely removed from the solvent, which requires formation of a sizeable cluster of hydrophobic side chains. Because this cluster formation involves at least partial compaction of the chain, we refer to this process as “initial chain collapse,” but this term is not meant to be synonymous with “random polymer collapse.” Although the time constant of the initial phase in Y92W RNase A is shorter than that of the earliest detected folding events in other proteins studied by CF mixing (30–33, 45), it may be comparable to the folding times for some of the small proteins with ultrafast two-state folding reactions (46). Thus, the time scale of the earliest fluorescence changes detected here does not violate the “speed limit” (46) for protein folding.

With a time constant in the range of 60 to 140 μ s at low denaturant concentrations, the second process detected in the refolding of the U_{vf} ensemble of Y92W (Fig. 2) is comparable to the earliest phase detected for several other proteins studied by CF mixing, including cytochrome *c* ($\tau = 50$ – 90μ s; refs. 30 and 41), SNase (75 μ s; ref. 33) and acyl-CoA binding protein (80 μ s; ref. 32), and somewhat faster than the initial phases of the bacterial immunity protein, Im7 (330 μ s; ref. 34), and protein G (600 μ s; ref. 31). The fact that these times are long compared to the initial phase of RNase A, and clearly much longer than the 10- to 100-ns time scale of a nonspecific chain collapse (36) suggests that they reflect more specific folding events.

Our study has further implications with respect to the characteristics of the unfolded ensemble of the protein at a very early stage of the folding process. The facts (i) that experiments with the unfolded analog, Y92Wdes, showed the microsecond fluorescence signals in Y92W to be a nonlocal phenomenon, and (ii) that localized changes could diminish these effects, suggest that the same nonlocal interactions are preserved in most of the molecules and play a role in the majority of the folding trajectories. Therefore, these experiments suggest that the initial unfolded state is a relatively heterogeneous ensemble containing sufficient specific interactions to guide the polypeptide along specific folding pathways. In particular, our experiments showed that nonnative proline isomers of RNase A influence both early and late stages of the process. Our data also show that the presence of the initial chain collapse ($<70 \mu$ s) is not an obligatory step for rapid conformational folding (faster than 100 ms), because the initial collapse is eliminated by an increase in GuHCl concentration from 0.7 to 1.2 M, which results in only a modest (≈ 2 -fold) decrease in the folding rate.

Finally, a plausible explanation for the absence of the initial collapse upon the elimination of a single disulfide bond can be proposed. The absence of the 26–84 disulfide bond increases the entropy of the polypeptide chain, and thus prevents the hydrophobic residues from associating. The nonnative proline isomers that also diminished the initial collapse may exert their effect in a similar manner. NMR investigations showed that a peptide fragment of RNase A forms a turn with a *cis*-Pro-93 but not with a *trans*-Pro-93 (20, 47). Thus, the entropy of the polypeptide chain is higher in U_{eq} than in U_{vf} .

In summary, the SF burst phase in fluorescence that is detected in the folding of a tryptophan-containing variant of RNase A was resolved as a multiexponential decay preceded by an initial fluorescence increase with a time constant faster than 70 μ s. Experiments with an unfolded analog (Y92Wdes) revealed that this initial fluorescence increase represents a relatively global conformational change. Thus, our result clarified a widely debated, controversial issue by separating the SF burst phase signal from the initial contraction of the polypeptide chain during folding of RNase A. Surprisingly, nonnative proline

isomers influence not only late stages of folding, but also the initial stages of the process, suggesting that the initial unfolded state (a relatively heterogeneous ensemble of conformations) contains sufficient residual interactions to guide the polypeptide along specific folding pathways, which supports the notion that even the initial contraction of the polypeptide chain is a non-random process.

1. Leopold, P. E., Montal, M. & Onuchic, J. N. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 8721–8725.
2. Wolynes, P. G., Onuchic, J. N. & Thirumalai, D. (1995) *Science* **267**, 1619–1620.
3. Dill, K. A. & Chan, H. S. (1997) *Nat. Struct. Biol.* **4**, 10–19.
4. Schonbrun, J. & Dill, K. A. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 12678–12682.
5. Sosnick, T. R., Shtilerman, M. D., Mayne, L. & Englander, W. S. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 8545–8550.
6. Qi, P. X., Sosnick, T. R. & Englander, W. S. (1998) *Nat. Struct. Biol.* **5**, 882–884.
7. Krantz, B. A., Mayne, L., Rumbley, J., Englander, W. S. & Sosnick, T. R. (2002) *J. Mol. Biol.* **324**, 359–371.
8. Klein-Seetharaman, J., Oikawa, M., Grimshaw, S. B., Wirmer, J., Duchardt, E., Ueda, T., Imoto, T., Smith, L. J., Dobson, C. M. & Schwalbe, H. (2002) *Science* **295**, 1719–1722.
9. Qui, L., Zachariah, C. & Hagen, S. J. (2003) *Phys. Rev. Lett.* **90**, 168103.
10. Montelione, G. T. & Scheraga, H. A. (1989) *Acc. Chem. Res.* **22**, 70–76.
11. Sendak, R. A., Rothwarf, D. M., Wedemeyer, W. J., Houry, W. A. & Scheraga, H. A. (1996) *Biochemistry* **35**, 12978–12992.
12. Wlodawer, A., Svensson, L. A., Sjölin, L. & Gilliland, G. L. (1988) *Biochemistry* **27**, 2705–2717.
13. Houry, W. A., Rothwarf, D. M. & Scheraga, H. A. (1995) *Nat. Struct. Biol.* **2**, 495–503.
14. Schmid, F. X., Graf, R., Wrba, A. & Beintema, J. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 872–876.
15. Houry, W. A., Rothwarf, D. M. & Scheraga, H. A. (1996) *Biochemistry* **35**, 10125–10133.
16. Schmid, F. X. (1982) *FEBS Lett.* **139**, 190–192.
17. Juminaga, D., Wedemeyer, W. J. & Scheraga, H. A. (1998) *Biochemistry* **37**, 11614–11620.
18. Iwaoka, M., Wedemeyer, W. J. & Scheraga, H. A. (1999) *Biochemistry* **38**, 2805–2815.
19. Rehage, A. & Schmid, F. X. (1982) *Biochemistry* **21**, 1499–1505.
20. An, S. S. A., Lester, C. C., Peng, J.-L., Li, Y.-J., Rothwarf, D. M., Welker, E., Thannhauser, T. W., Zhang, L., Tam, J. P. & Scheraga, H. A. (1999) *J. Am. Chem. Soc.* **121**, 11558–11566.
21. Cerovsky, V., Welker, E. & Scheraga, H. A. (2003) *J. Pept. Res.* **61**, 140–151.
22. Shastry, M. C. R., Luck, S. D. & Roder, H. (1998) *Biophys. J.* **74**, 2714–2721.
23. Peterman, B. F. (1979) *Anal. Biochem.* **93**, 424–444.
24. Neira, J. L. & Rico, M. (1997) *Fold. Des.* **2**, R1–R11.
25. Brandts, J. F., Halvorsen, H. R. & Brennan, M. (1975) *Biochemistry* **14**, 4953–4963.
26. Noppert, A., Gast, K., Zirwer, D. & Damaschun, G. (1998) *Fold. Des.* **3**, 213–221.
27. Roder, H. & Colon, W. (1997) *Curr. Opin. Struct. Biol.* **7**, 15–28.
28. Shastry, M. C. R., Sauder, J. M. & Roder, H. (1998) *Acc. Chem. Res.* **31**, 717–725.
29. Myers, J. K. & Oas, T. G. (2002) *Annu. Rev. Biochem.* **71**, 783–815.
30. Shastry, M. C. & Roder, H. (1998) *Nat. Struct. Biol.* **5**, 385–392.
31. Park, S.-H., Shastry, M. C. R. & Roder, H. (1999) *Nat. Struct. Biol.* **6**, 943–947.
32. Teilum, K., Maki, K., Kragelund, B. B., Poulsen, F. M. & Roder, H. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 9807–9812.
33. Maki, K., Cheng, H., Dolgikh, D. A., Shastry, M. C. R. & Roder, H. (2004) *J. Mol. Biol.* **338**, 383–400.
34. Capaldi, A. P., Shastry, M. C. R., Kleanthous, C., Roder, H. & Radford, S. E. (2001) *Nat. Struct. Biol.* **8**, 68–72.
35. Uzawa, T., Akiyama, S., Kimura, T., Takahashi, S., Ishimori, K., Morishima, I. & Fujisawa, T. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 1171–1176.
36. Sadqi, M., Lapidus, L. J. & Munoz, V. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 12117–12122.
37. Sabelko, J., Ervin, J. & Gruebele, M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 6031–6036.
38. Leeson, D. T., Gai, F., Rodriguez, H. M., Gregoret, L. M. & Dyer, R. B. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 2527–2532.
39. Parker, M. J. & Marqusee, S. J. (1999) *J. Mol. Biol.* **293**, 1195–1210.
40. Hagen, S. J. (2003) *Proteins* **50**, 1–4.
41. Eaton, W. A., Munoz, V., Hagen, S. J., Jas, G. S., Lapidus, L. J., Henry, E. R. & Hofrichter, J. (2000) *Annu. Rev. Biophys. Biomol. Struct.* **29**, 327–359.
42. Camacho, C. J. & Thirumalai, D. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6369–6372.
43. Gutin, A. M., Abkevich, V. I. & Shakhnovich, E. I. (1995) *Biochemistry* **34**, 3066–3076.
44. Onuchic, J. N., Luthey-Schulten, Z. & Wolynes, P. G. (1997) *Annu. Rev. Phys. Chem.* **48**, 545–600.
45. Hagen, S. J. & Eaton, W. A. (2000) *J. Mol. Biol.* **297**, 781–789.
46. Kubelka, J., Hofrichter, J. & Eaton, W. A. (2004) *Curr. Opin. Struct. Biol.* **14**, 76–88.
47. Montelione, G. T., Arnold, E., Meinwald, Y. C., Stimson, E. R., Denton, J. B., Huang, S.-G., Clardy, J. & Scheraga, H. A. (1984) *J. Am. Chem. Soc.* **106**, 7946–7958.

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