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Preparation of TNB-Labeled Proteins. The TNB-labeled proteins were obtained by incubating the guanidinium chloride (GdmCl)unfolded protein with a 100-fold molar excess of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) in 6 M GdmCl at pH 8.5. After completion of the reaction, the labeled protein was separated from free dye and GdmCl by the use of a PD-10 column (GE Health Care Life Sciences). All of the proteins were found by mass spectrometry to be >98% labeled, with an expected 197 Da increase in the mass because of the addition of the TNB moiety. Protein concentrations were determined by measurement of the absorbance at 280 nm, using an extinction coefficient of 40,500 M⁻¹⋅cm⁻¹ for the unlabeled proteins. Because the TNB group contributes to the absorbance measured at 280 nm, a correction for its contribution was done for the labeled proteins, as described earlier (1).

The Donors and Acceptors Appear to Rotate Freely Faster than the **Fluorescence Decay Rate of Donor.** The value of R_0 , the Forster's distance, in Eq. 1 can be determined by using the equation:

$$
R_0 = 0.211 \left[Q_D J \kappa^2 n^{-4} \right]^{\frac{1}{6}}.
$$
 [S1]

In Eq. **S1**, Q_D is the quantum yield of donor fluorescence, *J* is the overlap integral, κ^2 is the orientation factor, and *n* is refractive index of the medium (2). Most of these parameters except the

1. Jha SK, Udgaonkar JB (2009) Direct evidence for a dry molten globule intermediate during the unfolding of a small protein. Proc Natl Acad Sci USA 106(30):12289–12294. 2. Lakowicz JR (2006) Energy transfer. Principles of Fluorescence Spectroscopy (Springer, orientation factor can be determined experimentally and have been evaluated for Trp-TNB FRET pair in previous studies (1, 3). The Forster's distance, R_0 of the Trp-TNB FRET pair has been shown to lie between 22 and 23 Å in [GdmCl] ranging between 0 and 6 M for several different proteins, assuming a value of $2/3$ for κ^2 .

The value of κ^2 depends on the relative orientation of the transition dipoles of the donor and the acceptor. At present, there is no direct way to measure κ^2 in nonrigid experimental setup. If the donor and the acceptor are oriented randomly with respect to each other, the value of κ^2 is 2/3 (2). This condition is typically met when at least one of the two chromophores is rotating freely at rates faster than the fluorescence decay.

In the case of RNase H, all of the tryptophan donors and the residues S36 and L136 to which acceptor TNB moiety is attached are on the surface of the protein. Side chains of the surface residues of proteins rotate between different rotamers on the timescale of tens of picoseconds (4). This timescale is faster than the time constant of fluorescence decay of tryptophans (∼4–5 ns). During unfolding, the segmental flexibility of dry molten globule is likely to make the averaging of the orientations of donor and acceptors even faster. Hence, using a value of two-thirds for κ^2 appears reasonable. This is more justified in view of the fact that tryptophan has multiple absorption transition dipoles, and therefore mixed polarizations (5).

New York), 3rd Ed, pp 443–472. 3. Sridevi K, Udgaonkar JB (2003) Surface expansion is independent of and occurs faster

than core solvation during the unfolding of barstar. Biochemistry 42(6):1551–1563.

^{4.} Mccammon JA, Lee CY, Northrup SH (1983) Side-Chain Rotational Isomerization in Proteins - a Mechanism Involving Gating and Transient Packing Defects. J Am Chem Soc 105:2232–2237.

^{5.} Haas E, Katchalski-Katzir E, Steinberg IZ (1978) Effect of the orientation of donor and acceptor on the probability of energy transfer involving electronic transitions of mixed polarization. Biochemistry 17(23):5064–5070.

Fig. S1. TNB labeling does not perturb the secondary structure, stability, and kinetics of unfolding. (A, C, and E) S36C and S36C-TNB; (B, D, and F) L136C and L136C-TNB. A and B show the far-UV CD spectra of the unlabeled native (solid cyan line), TNB-labeled native (solid dark red line), unlabeled unfolded (dashed cyan line), and TNB-labeled unfolded (dashed dark red line) proteins. C and D show the equilibrium unfolding transitions of the unlabeled (cyan circles) and TNB-labeled (dark red circles) proteins as monitored by change in fluorescence. The data for unlabeled proteins is collected at the emission wavelength of 320 nm and for TNB-labeled proteins at 360 nm after excitation at 295 nm. The fraction of unfolded protein is plotted against GdmCl concentration. The black continuous lines through the data represent nonlinear least-squares fits to a two-state native \rightleftharpoons unfolded model. E and F show the dependence of the observed rate constants of unfolding on the concentration of GdmCl as monitored by the change in fluorescence signal at 320 nm for the unlabeled proteins (cyan circles) and at 360 nm for TNB-labeled proteins (dark red circles), after excitation at 295 nm.

Fig. S2. Unfolding rate of (A) I7C, (B) A24C, and (C) I53C as measured by DTNB-labeling (filled triangles), MMTS-labeling (empty squares), and fluorescence (empty circles).