Supporting Information

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SI Text

Preparation of NMR Samples. Mutagenesis, protein expression, and purification were performed as previously described (1), except that the rich media were replaced by isotope-enriched minimal media with supplements. For $^1$H-, $^{13}$C-, and $^{15}$N-labeled protein samples, the M9 minimal media contain 0.8 g/L $^{15}$N-enriched NH$_4$Cl and 6 g/L unlabeled glucose in 99% D$_2$O, supplemented with 1% (vol/vol) $^1$H-, $^{13}$C-, and $^{15}$N-labeled BioExpress (Cambridge Isotope Laboratories). For $^1$H-, $^{13}$C-, and $^{15}$N-labeled protein samples, the M9 media contain 0.8 g/L $^{15}$N-enriched NH$_4$Cl and 4 g/L $^{13}$C-enriched glucose in 99% D$_2$O, supplemented with 1% (vol/vol) $^1$H-, $^{13}$C-, and $^{15}$N-labeled BioExpress.

Backbone Assignment. A standard set of triple-resonance experiments were performed at 299 K, and a subset of these experiments was performed at 306 K besides 299 K to minimize line broadening caused by chemical exchange. The monomer and dimer states were distinguished by distinct concentration dependence of relative peak intensities. The resonances corresponding to K14 in monomers were significantly broadened by chemical exchange and invisible in triple-resonance experiments, and instead were assigned by observing the exchange cross-peaks between monomers and dimers in a $^{15}$N ZZ-exchange experiment performed at 287 K and 900 MHz. K14E mutation did not significantly perturb the chemical shifts and the assignments of wild-type EC1–EC2 domains (ECAD12) were directly used for the K14E mutant.

$^{15}$N ZZ-exchange Experiments. The pulse sequence was adapted from the heteronuclear single-quantum coherence (HSQC)-based $^{15}$N ZZ-exchange experiment (2) by replacing the reverse insensitive nuclei enhanced by polarization transfer (INEPT) period at the end with a transverse relaxation-optimized spectroscopy (TROSY) detection period. The pulse sequence diagram is depicted in Fig. S7. A set of 2D spectra was recorded at six mixing times $T_{mix} = 20, 100, 200, 300, 400$, and $500$ ms. A composite ratio of exchange and autocorrelation peak intensities, which has quadratic time dependence, was used to extract kinetic on- and off-rate constants as described previously (3). The main benefit of using the composite ratio is to reduce the effects of differences in $R_1$ relaxation rates of monomeric and dimeric species, as well as differences in the transfer efficiencies during the preparation period and line widths during the detection period. The expression is given below:

$$ \Xi(T_{mix}) = \frac{I_{MD}(T_{mix})I_{DM}(T_{mix})}{I_{MM}(T_{mix})I_{DD}(T_{mix}) - I_{MD}(T_{mix})I_{DM}(T_{mix})} \simeq 4k_{off}k_{on}|M|T_{mix}^2. $$

where $k_{off}$ is the auto- or cross-peak intensity with $i$ and $j$ standing for initial and final states, respectively; monomers and dimers are denoted by $M$ and $D$, respectively; and $[M]$ represents the equilibrium concentration of monomers. The data were globally fit to Eq. S1 using the nonlinear least-squares fitting procedure implemented in Mathematica 8.0 (Wolfram Research). $[M]$ was calculated from the relative equilibrium populations of monomers and dimers determined from two $^1$H-$^{15}$N HSQC spectra as described in Materials and Methods of the main text and the total protein concentration determined from absorbance at 280 nm. Uncertainties in the fitting parameter were estimated from the SD of the fitted parameters for I38 and D90.

TROSY-Selected $^1$H Carr–Purcell–Meiboom–Gill Experiments. The pulse sequence is depicted in Fig. S8. It was constructed from a 2D TROSY sequence by inserting a Carr–Purcell–Meiboom–Gill (CPMG) period before detection and was based on several previously published $^1$H CPMG sequences (4, 5). The TROSY effects are preserved throughout the entire sequence, including both frequency encoding periods and the CPMG period. $R_2$ relaxation rate constants were calculated from peak intensities, $I_d$, determined from a series of 2D spectra with the spacing between 180° pulses ranging from 5 to 0.25 ms, using the formula

$$ R_{2eff}^d = -\frac{1}{T_{rel}} \ln \left( \frac{I_d}{I_0} \right), $$

where $I_d$ is the peak intensity from a reference 2D spectrum recorded without the CPMG relaxation period and $T_{rel}$ is the relaxation delay. Data were recorded with 20- and 40-ms constant relaxation periods and the recycle delay was 2.5 s. For a given residue in a given oligomeric state, either data acquired with 20- or 40-ms relaxation period was chosen based on the desired percentage decay in peak intensities, usually ~50%. Measurement uncertainties were estimated from replicate spectra for all samples except for the wild-type sample at 97 μM. Due to the low sensitivity at 97 μM protein concentration, the uncertainties were estimated from the baseline noise.

The relaxation dispersion curves were fit to a two-site exchange model described by the Carver–Richards equation (6),

$$ R_x^d = R_{20} + R_x(t_{cp}), $$

in which

$$ R_x(t_{cp}) = \frac{1}{2} \left( k_{ex} - \frac{1}{t_{cp}} \right) \cosh^{-1} \left[ D_+ \cosh(\eta_+ - D_- \cosh(\eta_-)) \right], $$

where

$$ D_+ = \frac{1}{2} \left[ 1 + \sqrt{1 + \frac{\psi + 2\Delta \sigma^2_{\eta}}{(\psi^2 + \zeta^2)^{1/2}}} \right], $$

$$ \eta_+ = \frac{t_{cp}}{\sqrt{2}} \left[ \left( \psi^2 + \zeta^2 + 1/2 \right)^{1/2} \right]. $$

$\psi = k_{ex} - \Delta \sigma_{\eta}$, $\zeta = -2\Delta \sigma_{\eta}k_{ex}(1 - 2p_m)$, $k_{ex} = k_1 + k_{-1}$ is the sum of forward and reverse rate constants, $\Delta \sigma_{\eta}$ is the $^1$H chemical shift difference between major and minor conformations, $R_{20}$ is the intrinsic transverse relaxation rate in the absence of chemical exchange, $p_m$ is the population of the minor conformational state, and $t_{cp}$ is the spacing between 180° pulses in the CPMG period.

The fitting was performed by $\chi^2$ minimization using Mathematica 8.0 (Wolfram). The exchange-free transverse relaxation rates, $R_{20}$, at 600 and 800 MHz were not constrained with respect to each other. The uncertainties in the fit parameters were estimated from Monte Carlo simulations (7) with 500 iterations.

$^{15}$N $R_2$ Hahn Echo Experiments. $^{15}$N $R_2$ relaxation rates shown in Fig. S5A were measured using the Hahn echo sequence, which preserves transverse relaxation associated with chemical exchange, as previously described (8). The data were recorded at 600-MHz $^1$H frequency and 299 K on a wild-type sample containing 374 μM protein. Data were recorded at relaxation delays of 0, 9.84, 19.68, 29.04, 38.72, and 48.40 ms and fit to an exponential function to yield $R_2$ relaxation rate constants.
Fluorescence-Based and NMR-Based Experiments for Measuring the Association and Dissociation Kinetics of the K14E Mutant. Previous results from surface plasmon resonance and analytical ultracentrifugation experiments have shown that the binding kinetics of the K14E mutant are on the order of ∼1 h. To measure the exact on- and off-rates, we concentrated the protein to 200–300 μM and allowed the samples to reach equilibrium by incubating at 299 K overnight.

The kinetic experiment monitored by intrinsic Trp fluorescence was initiated by diluting the sample to a concentration much lower than the dissociation constant. The dilution was performed by manually adding buffer and the dead time is approximately a few seconds. The increase in quantum yield upon dissociation presumably comes from the separation of two Trp2s located at the dimer interface. The dissociation reaction follows the first-order rate equation because the reverse reaction is essentially precluded by the low protein concentration. The effects of photobleaching were quantified using a control experiment on a wild-type sample, in which the decay in intensity is solely due to photobleaching. The data were fit to a linear function because the initial regime of an exponential decay can be approximated by a linear function.

The NMR experiment was performed on a sample with an initial concentration of 234 μM. The experiment was initiated by 1:5 dilution of the sample and the signal intensities were monitored by a series of 1H-15N TROSY experiments with acquisition time ∼5 min for each experiment. Both the decrease in dimer intensities and increase in monomer intensities are plotted and fitted to integrated rate equations. This procedure is necessary because the dissociation reaction cannot be treated as first-order reaction due to the fact that the final protein concentration is comparable to the dissociation constant. The relatively high final concentration is due to the lower sensitivity of NMR experiments compared with fluorescence-based experiments.

Fig. S1. $^1$H-$^1$N TROSY of wild-type ECAD12 recorded at 600-MHz $^1$H frequency and 299 K. (A) Full spectrum and (B) an expansion of a crowded region of the spectrum are shown.
Fig. S2. Comparison of the time courses of fluorescence intensity of (A) K14E mutant and (B) wild-type ECAD12 in rapid dilution experiments. To ensure direct comparison of intensities between K14E and wild type, the same scaling factor was applied to the raw data. For the K14E mutant, the blue line represents the initial slope of the fitted curve, and for wild type, the blue line represents a linear fit of the data. (C) Time courses of monomer and dimer concentrations monitored by NMR cross-peak volumes in the rapid dilution experiment. (D) Spectra of ZZ-exchange experiments on the K14E mutant showing residue Q101. “m” and “d” denote monomers and dimers, respectively. No exchange cross-peaks can be observed even at 900-ms mixing time. The results are consistent with no binding detected in the surface plasmon resonance experiments.
Fig. S3. (Continued)
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Fig. S3. $^1$H relaxation dispersion profiles of all assigned residues detected with sufficient sensitivity in wild-type ECAD12. For all residues, the profiles of amide $^1$H are displayed with the exception of Trp 2, for which sidechain $^1$He is displayed. Two datasets with the constant relaxation time set to 20 and 40 ms, respectively, were combined. Both datasets were recorded at 600 MHz and 299 K on a sample containing 374 μM protein.
Fig. S4. $^1$H relaxation dispersion profiles of wild-type ECAD12 at 600- and 800-MHz $^1$H frequencies. The total monomer concentration in the sample was 374 μM. $\tau_{cp}$ is the spacing between 180° pulses.
Fig. S5. (A) $^{15}$N $R_2$ relaxation rates of wild-type ECAD12. The data were recorded at 600 MHz and 299 K. The NMR sample contains signals corresponding to monomers (circle, blue), dimers (square, red), and mixture of monomers and dimers (triangle, black). The relaxation rate of Trp2 side-chain $N_e$ resonance in monomeric state is shown in cyan. The sample concentration is 374 μM. (B) $^{15}$N and $^1$H line widths of cross-peaks corresponding to residues 130–150 in a $^1$H-$^{15}$N TROSY spectrum acquired at 305 K and 600-MHz $^1$H frequency. Residues 130–150 are in cyan in the structure of X-dimer shown in the Inset.
Fig. S6. X-dimer structure of the E89A mutant of ECAD12 (Protein Data Bank ID code 3lni) with residues that display broadened $^1$H or $^{15}$N resonances highlighted by stick representation and mapped to the X-dimer interface, including (A) a full view of the structure and (B) a close-up view of the X-dimer interface.

Fig. S7. Pulse sequence diagram of $^{15}$N ZZ-exchange experiment with TROSY detection. Narrow and wide bars represent 90° and 180° pulses, respectively. All pulse phases are $x$ unless indicated otherwise. The water-selective pulses during the mixing periods are 180° pulses with amplitudes modulated by a cosine function. Other water-selective pulses are 90° pulses with a rectangular shape and duration of 1.5 ms. Quadrature detection in the indirect dimension was achieved by decrementing $\phi_3$ according to States-TPPI (time-proportional phase-incrementation). The phase cycle was $\phi_1 = 4(x, -x) 4(x, 0), \phi_2 = 8(y, -y), \phi_3 = (-x, y, -y, x, -x, 0, x, y), \phi_{rec} = (x, -x, y, -y, x, -x, y, x)$. The phase cycle is for Bruker spectrometers. For Varian spectrometers, $y$ and $-y$ phases should be swapped. Gradients along the z axis have sine amplitude profile, with peak strengths and durations as follows: $g_0 = 10$ G/cm, 1.0 ms; $g_1 = 7.5$ G/cm, 1.0 ms; $g_2 = 1$ G/cm; $g_3 = 15$ G/cm, 1.0 ms; $g_4 = 10$ G/cm, 1.0 ms; $g_5 = 27.5$ G/cm, 0.6 ms.
Fig. S8. Pulse sequence diagram of TROSY-selected $^1$H CPMG experiment. Narrow and wide bars represent $90^\circ$ and $180^\circ$ pulses, respectively. All pulse phases are $x$ unless indicated otherwise. $\Delta = 0.3375$ ms. XY-8 phase alternating scheme ($x, y, x, y, x, x, y$) was used during the INEPT and TROSY periods on both $^1$H and $^{15}$N channels to minimize loss of intensity resulting from chemical exchange. XY-16 phase alternating scheme ($x, y, x, y, x, y, y, x, x, y, y, x$) was used for the $\pi$ pulse train during $T_{relax}$ period. $n$ can be any integer. The phase cycle was $\phi_1 = (y, -y), \phi_2 = -y, \phi_3 = -y, \phi_{rec} = (-x, x)$. Quadrature detection in the indirect dimension was achieved by inverting $\phi_2$ and $\phi_3$ together with gradients $g_3$ and $g_4$ for every $t_1$ increment. The phase cycle is for Bruker spectrometers. For Varian spectrometers, $y$ and $-y$ phases should be swapped. $^1$H carrier was placed at 8.3 ppm for the entire pulse sequence except for the detection period. Gradients along the $z$ axis have sine amplitude profiles, with peak strengths and durations as follows: $g_1 = 12.5$ G/cm, 1.5 ms; $g_2 = 32.5$ G/cm, 3 ms; $g_3 = 30$ G/cm, 0.18 ms; $g_3 = -1.2 \times g_3, 0.9$ ms; $g_4 = 0.8 \times g_3, 0.9$ ms.

| Residue | $k_{en}, s^{-1}$ | $\rho_m$ | $|\Delta \omega_H|$, ppm | $R_{20}, s^{-1}; 600$ MHz | $R_{20}, s^{-1}; 800$ MHz |
|---------|-----------------|----------|-----------------|-----------------|-----------------|
| I7m     | $1,890 \pm 130$ | $0.025 \pm 0.003$ | $0.45 \pm 0.03$ | $14.8 \pm 0.4$ | $14.9 \pm 0.6$ |
| E11     | "               | "        | $0.26 \pm 0.02$ | $29.6 \pm 0.3$ | $29.1 \pm 0.4$ |
| Q101m   | "               | "        | $0.30 \pm 0.02$ | $18.6 \pm 0.3$ | $16.6 \pm 0.5$ |