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The C-Terminal Domain of the Utrophin Tandem Calponin-Homology Domain Appears To Be Thermodynamically and Kinetically More Stable Than the Full-Length Protein

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Supporting Information

ABSTRACT: Domains are in general less stable than the corresponding full-length proteins. Human utrophin tandem calponin-homology (CH) domain seems to be an exception. Reversible, equilibrium denaturant melts indicate that the isolated C-terminal domain (CH2) is thermodynamically more stable than the tandem CH domain. Thermal melts show that CH2 unfolds at a temperature higher than that at which the full-length protein unfolds. Stopped-flow kinetics indicates that CH2 unfolds slower than the full-length protein, indicating its higher kinetic stability. Thus, the utrophin tandem CH domain may be one of the few proteins in which an isolated domain is more stable than the corresponding full-length protein.

The classical definition of a domain is that it can fold independently.[1] Experiments with numerous proteins have shown that isolated domains are in general less stable than the corresponding full-length proteins.[2] It is rare to observe an isolated domain more stable than the full-length protein, although it is not theoretically impossible.[3−6] In this manuscript, we show that the utrophin tandem calponin-homology (CH) domain (Figure 1A) may be one such protein, where its isolated C-terminal CH domain (CH2) is both thermodynamically and kinetically more stable than the full-length protein.

Using the methods described in the Supporting Information, we obtained high yields of the pure full-length tandem CH domain and its CH2 (Figure 1B). Isolated CH2 is a well-folded and well-structured protein, similar to the tandem CH domain. Its circular dichroism (CD) spectrum showed two negative bands at 208 and 222 nm (Figure 1C) characteristic of an α-helical protein and is consistent with the known crystal structures (Figure 1A). In addition, the native fluorescence of CH2 (N_CH2) was blue-shifted with respect to that of its unfolded state (U_CH2) (Figure 1D), similar to the full-length protein (N_tandem CH vs U_tandem CH). The blue shift in the tryptophan emission maximum indicates the burial of tryptophan residues from the solvent. Thus, CH2 has a well-folded structure in solution, similar to that of the full-length protein. The increase in fluorescence upon unfolding indicates that the tryptophan fluorescence is quenched in the native state by the neighboring amino acids, as observed in other proteins.[7,8]

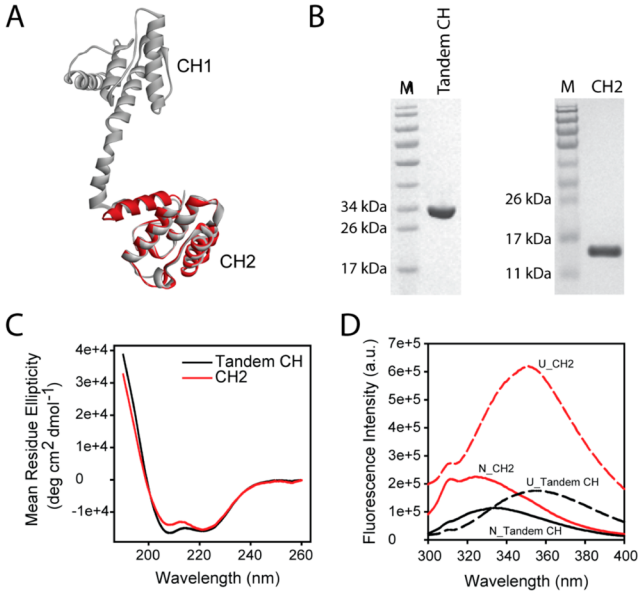

Figure 1. (A) X-ray crystal structures of the utrophin tandem CH domain [Protein Data Bank (PDB) entry 1QAG] and its isolated CH2 (PDB entry 1BHD), colored gray and red, respectively. Isolated CH2 has the same structure as the full-length protein (root-mean-square deviation of 0.83 Å). (B) Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of the purified full-length tandem CH domain and its CH2. Lanes labeled M contained the molecular mass markers. (C) Circular dichroism (CD) spectra of the full-length tandem CH domain (black) and CH2 (red). (D) Fluorescence spectra of the native (N) and unfolded (U) states of the full-length tandem CH domain (black) and CH2 (red). All the experiments were performed at 1 μM protein concentration in phosphate-buffered saline (pH 7).

The folding of CH2 and that of the full-length protein are completely reversible, as shown by their 100% refolding yield (Figure 2A). Complete folding reversibility implies that we can use denaturant melts to measure their equilibrium thermodynamic stability.

When equilibrium protein unfolding was assessed using urea, CH2 unfolded at urea concentrations higher than those at which the full-length protein unfolded (Figure 2B). The urea

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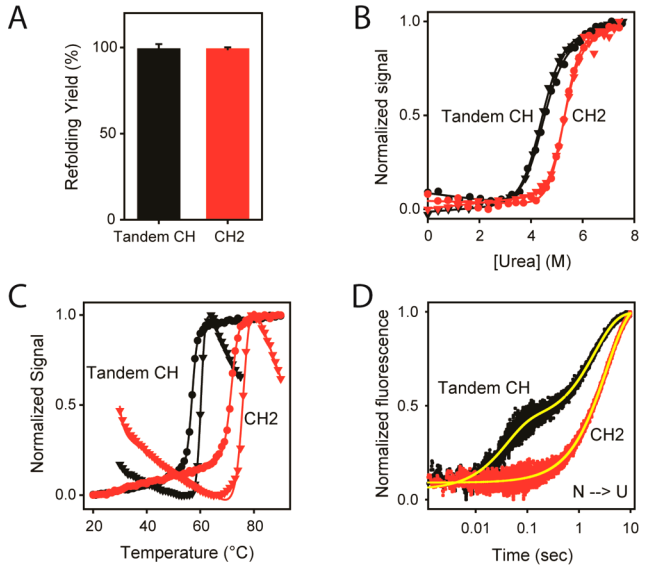

Figure 2. (A) Refolding yields of the two proteins, indicating that their folding is completely reversible. (B) Changes in the CD signal at 222 nm (circles) and protein fluorescence (triangles) as a function of urea concentration. (C) Changes in the CD signal at 222 nm (circles) and protein fluorescence (triangles) as a function of solution temperature. (D) Unfolding kinetics measured by total protein fluorescence. In all panels, black and red colors indicate the data for the tandem CH domain and isolated CH2, respectively.

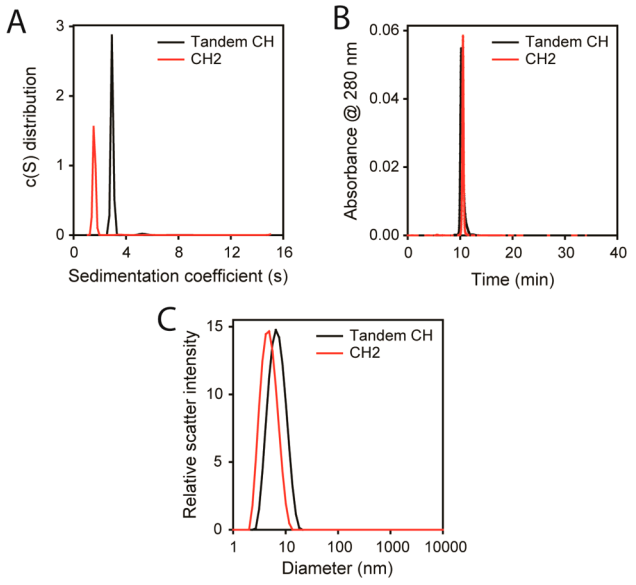

Figure 3. (A) Sedimentation coefficient distributions obtained from analytical ultracentrifugation (AUC). (B) Size-exclusion chromatograms (SEC). (C) Scattering intensity profiles obtained from dynamic light scattering (DLS). In all panels, black and red colors indicate the data for the tandem CH domain and isolated CH2, respectively.

melts recorded with CD at 222 nm and protein fluorescence as the signals exactly overlapped for CH2 and for the tandem CH domain. Globally fitting these melts to a two-state unfolding model[9] resulted in unfolding free energies (ΔG_{unf}) of 10.48 ± 0.59 kcal/mol for CH2 and 6.49 ± 0.27 kcal/mol for the full-length protein. These values indicate that CH2 is thermodynamically more stable than the full-length protein by 3.99 ± 0.65 kcal/mol.

With temperature, CH2 melted with a T_m of 71.5 ± 0.0 °C, whereas the full-length protein melted with a T_m of 56.9 ± 0.0 °C when CD was used as the signal (Figure 2C). When the same thermal melt was monitored using fluorescence as the signal, CH2 and the full-length protein melted with T_m values of 76.1 ± 0.1 and 60.3 ± 0.0 °C, respectively (Figure 2C). These temperature melts are irreversible, and hence, the T_m values should be considered as only a qualitative measure of the higher stability of CH2 compared to that of the full-length protein.

CH2 is kinetically more stable than the tandem CH domain. CH2 unfolds slower than the full-length protein (Figure 2D). CH2 unfolds with a single rate constant of 0.29 ± 0.00 s^{-1}, whereas the full-length protein unfolds with two rate constants of 25.82 ± 0.22 and 0.49 ± 0.00 s^{-1} (relative amplitudes of 38 and 62%, respectively). Comparison of the rate constant with the maximal amplitude (0.29 vs 0.49 s^{-1}) or the amplitude-weighted average rate constant (0.29 vs 0.78 s^{-1}) indicates that CH2 unfolds slower than the tandem CH domain.

The obvious question that arises is whether isolated CH2 is a monomer in solution. Any stable oligomer formation increases the stability of CH2, which could explain why CH2 appears to be more stable than the full-length protein. To exclude such a possibility, we used analytical ultracentrifugation (AUC), size-exclusion chromatography (SEC), and dynamic light scattering (DLS). Figure 3A shows the sedimentation coefficient (s) distributions obtained from the sedimentation velocity experiment in AUC. Both CH2 and the full-length protein showed single distributions, indicating the homogeneity of the samples. The molecular masses estimated from s values[10] were 13.1 and 30.0 kDa for CH2 and the full-length protein, respectively, which are close to the expected values (13.4 and 31.8 kDa, respectively). Figure 3B shows the elution profiles from SEC. Both CH2 and the full-length protein eluted as a single species, indicating the homogeneity of the samples.[11] Figure 3C shows the scattering profiles obtained from DLS measurements. As seen with AUC and SEC, we observed single peaks indicating the presence of single species in solution.[12] For CH2, the molecular mass calculated from the hydrodynamic diameter was 13.5 kDa, which is close to the expected value (13.4 kDa). All these results indicate that CH2 is a monomer in solution. We also confirmed that CH2 retains its monomeric nature during the denaturant melt (Figure S1 of the Supporting Information).

Similar to our results presented here, two recent experimental studies suggest the presence of more stable domains. In the first study, deleting internal repeats increases the stability of a leucine-rich repeat protein[5] by 1.4-fold. This has been attributed to the breakage of the destabilizing interdomain interactions between individual repeats. In the second study, the sum of the free energies of two isolated C2 domains of synaptotagmin I is higher than that of the full-length protein.[6] Similar to our findings for utrophin CH2, one of the isolated C2 domains of synaptotagmin I is more stable by 1.4-fold than the corresponding tandem C2 domain under certain experimental conditions (Table 3 of ref 6). Such negative coupling between the two domains has been shown to be important for protein function.[3,6] Our study along with these two experimental studies indicates that it is possible for isolated domains to be more stable than the corresponding full-length proteins.

Are there any caveats in the conclusions drawn here that CH2 is more stable than the full-length protein? For both CH2 and the full-length protein, the denaturant melts measured

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using CD and fluorescence exactly overlap (Figure 2B), implying that they may be two-state folders. However, the *m*-value obtained from fitting the data in Figure 2B was -1.49 ± 0.06 kcal mol^{-1} (M [urea])$^{-1}$ for the tandem CH domain, which did not match the *m*-value of -1.84 kcal mol^{-1} (M [urea])$^{-1}$ estimated from the accessible surface area (ASA) of the protein's crystal structure[13] (Figure 1A). Further, its unfolding kinetics (Figure 2D) could not be fit to a single-exponential function but to a double-exponential function, indicating the presence of an intermediate between the native and unfolded states. When such intermediates exist, fitting the denaturant melt to a two-state equation often results in a decreased *m*-value and hence a decreased ΔG_{unf}.[14] How the true ΔG of the tandem CH domain factors into the difference in free energy between CH2 and the full-length protein needs to be probed, which require high-resolution NMR experiments to characterize the stability of the intermediate state.[15] Preliminary evidence suggests that this intermediate has CH1 unfolded and CH2 folded, because the slowest unfolding rate constant of the tandem CH domain (0.49 ± 0.00 s^{-1}) is similar to that of CH2 (0.29 ± 0.00 s^{-1}) (Figure 2D).

A possible explanation of why isolated CH2 appears to be more stable than the tandem CH domain could be the effects of neighboring polypeptide chains on domain stability. The domains may differ in their stabilities when they are linked compared to when they are isolated.[16] A simple tethering can decrease the domain stability.[4] Assuming that CH2 in the tandem CH domain has the same stability as that in isolation, the denaturant melt of the tandem CH domain could not be fit to a three-state folding model (Figure S2 of the Supporting Information). The fit indicates that CH2 is less stable when connected to CH1. In addition, CH2 unfolds 1.7 times faster when in the tandem CH domain than when it is isolated (Figure 2D). This stability difference seems to originate from the increase in the compactness of isolated CH2. The *m*-value for isolated CH2 obtained from fitting the data in Figure 2B to a two-state folding model was -1.99 ± 0.12 kcal mol^{-1} (M [urea])$^{-1}$, which is much higher than the *m*-value of -1.06 kcal mol^{-1} (M [urea])$^{-1}$ estimated from the ASA of its crystal structure (Figure 1A). This adds isolated CH2 to the group of proteins observed before[13] whose experimental *m*-values deviate significantly from the *m*-values estimated from the ASA. The structural origins of these differences in the *m*-values and free energies of CH2 when it is isolated and when it is connected to CH1 need to be further probed.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2, materials, and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Supporting Information

Materials and Methods

Protein expression and purification

The expression plasmid for the full-length tandem CH domain of utrophin (residues 1-261) was cloned from the corresponding cDNA into pET28a (Invitrogen) using the restriction sites NdeI and HindIII. The expression plasmid for the isolated CH2 (residues 147-261) was generated by sub-cloning the corresponding cDNA into pET-SUMO using the restriction sites BamH1 and Xho1. Plasmids were amplified using Qiagen miniprep kit and constructs were confirmed by DNA sequencing. Proteins were expressed in competent BL21(DE3) *Escherichia coli* cells, and purified using the protocols we described earlier [(1-3)]. The SUMO tag at the N-terminus of CH2 was cleaved using Ulp1 protease.

Circular dichroism (CD)

Proteins (1 μM) in PBS buffer (0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7) were used for measuring CD using a Chirascan Plus spectrometer (Applied Photophysics, UK). Mean residue ellipticity (MRE) was calculated using the formula,

MRE = CD in millidegrees / (pathlength in millimeters × the molar concentration of protein × the number of residues)

(Eq. 1)

Protein fluorescence

Fluorescence spectra of native (1 μM in PBS buffer) and unfolded states (1 μM in PBS buffer containing 8 M urea) of full-length tandem CH domain and CH2 were recorded by exciting the samples at 280 nm (PTI QuantaMaster Fluorometer).

Refolding yields

Refolding of the full-length tandem CH domain and CH2 was initiated from their denatured states (10 μM protein) in PBS buffer containing 8 M urea, and the denaturant was diluted 10-fold in PBS buffer. Samples were centrifuged at 30,000 g to precipitate any protein aggregates. Supernatants were subjected to protein quantification using absorbance at 280 nm. Molar extinction coefficients were calculated from their amino acid sequences using PROTPARAM software (http://expasy.ch). Protein concentration in the supernatant was used to determine the refolding yield.

Denaturant melts

Full-length tandem CH domain and CH2 (1 μM) in PBS buffer were used to monitor changes in the CD signal at 222 nm (ChirascanPlus spectrometer; Applied Photophysics, UK) and protein fluorescence with excitation at 280 nm (PTI QuantaMaster Fluorometer) as a function of urea (Nacalai Tesque, 35940-81) concentration. For this experiment, buffer samples with different urea concentrations were initially prepared and the protein was added from a stock solution. The samples were allowed to equilibrate for an hour. The data were normalized from 0 to 1 and fit to a two-state model [4, 5] or a three-state model [6] using SigmaPlot software (Systat Software Inc) to obtain the Gibbs free-energy of unfolding, ΔG_{unf} values.

Thermal melts

For full-length tandem CH domain and CH2 (1 μM in PBS buffer), changes in the far-UV CD signal at 222 nm (ChirascanPlus spectrometer, Applied Photophysics, UK) and protein fluorescence with excitation at 280 nm (PTI QuantaMaster Fluorometer) were monitored as a function of increasing temperature at a rate of 1°C/min. The data were fit to a two-state unfolding model using SigmaPlot software to determine the midpoint temperature (T_m) values.

Stopped-flow folding/unfolding kinetics

Unfolding kinetics of the full-length tandem CH domain and CH2 were monitored using an Applied Photophysics stopped-flow assembly attached to a ChirascanPlus spectrometer. Native proteins (10 μM) were diluted 10-fold into PBS buffer containing urea (9 M final urea concentration) to initiate the protein unfolding. Total protein fluorescence with excitation at 280 nm was used as the signal. An average of 20 traces was fit to exponential functions using SigmaPlot to determine the rate constants. The equation used for fitting the kinetic data to a multi-exponential function was

$$y = y_0 + \sum_{i=1}^{n} a_n \left(1 - \exp\left(-k_n t\right)\right) \qquad \text{(Eq. 2)}$$

where k_n and a_n represent the rate constants and the corresponding signal amplitudes. In the above equation, n = 1 for single-exponential and n = 2 for two-exponential functions. The amplitude-weighted average rate constant was determined using the equation

$$\langle k \rangle = \left(\left(\sum_{i=1}^{n} |a_n| / k_n \right) \Big/ \sum_{i=1}^{n} |a_n| \right)^{-1} \qquad \text{(Eq. 3)}$$

Analytical ultracentrifugation (AUC)

Full-length tandem CH domain and CH2 (20 μM each) were subjected to sedimentation velocity AUC in PBS buffer at 50,000 rpm using a Beckman XL-A analytical ultracentrifuge. Absorbance at 280 nm was used to record the meniscus shift data. Raw data was analyzed using SEDFIT software. The density and viscosity of the buffer were calculated using SEDNTERP software. Data were fitted to a continuous sedimentation distribution model.

Size exclusion chromatography (SEC)

The analysis was performed using 50 μM of either the full-length tandem CH domain or the CH2. Volume injection for each sample was 20 μl. Agilent 1100 HPLC equipped with Tosoh TSKgel G3000SW xl column was used. Column was equilibrated with two column volumes of mobile phase (0.1 M sodium phosphate, 0.1 M sodium sulfate, 0.3 M sodium chloride, pH 6.8 at 1 ml/min flow rate). Protein peaks were monitored using absorbance at 280 nm.

Dynamic light scattering (DLS)

Oligomerization of the full-length tandem CH domain and CH2 was checked using DLS. Both proteins (50 μM in PBS buffer) were subjected to zeta-sizer (Nano ZS, Malvern). An average of 11 runs was used for the calculation of mean diameter.

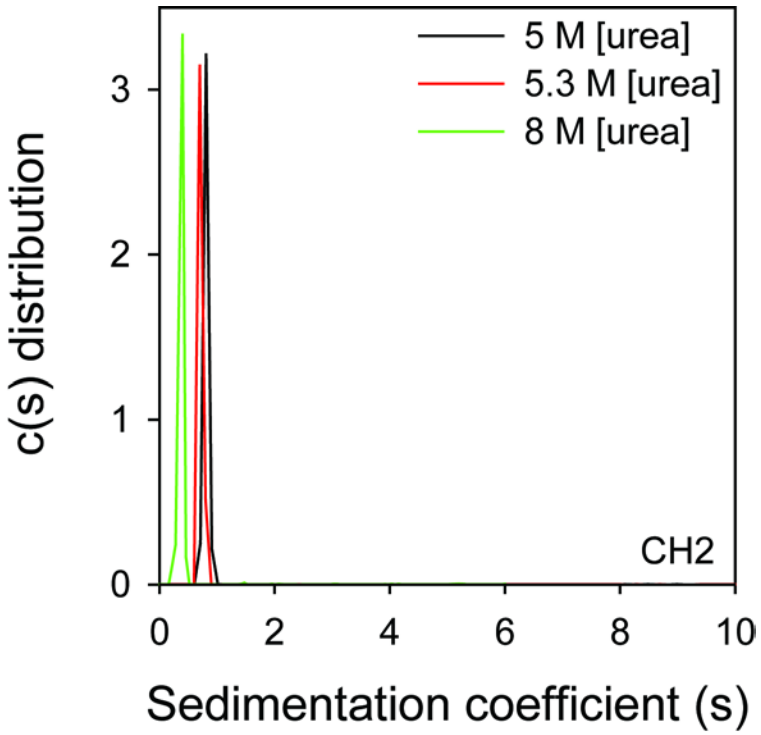

Figure S1. Sedimentation coefficient distributions of CH2 at varying concentrations of urea obtained from analytical ultracentrifugation (AUC). The three concentrations of urea, 5 M, 5.3 M, and 8 M correspond to the conditions where CH2 is 33%, 50%, and 100% unfolded respectively in the denaturant melt (Fig. 2B). CH2 is a monomer at all urea concentrations. The molecular weights estimated from these s values were 13.4 ± 0.8 KDa, which closely match with the expected value (13.4 KDa). The s value shifts to lower values with the increase in urea concentration due to increased viscosity and density of the solution.

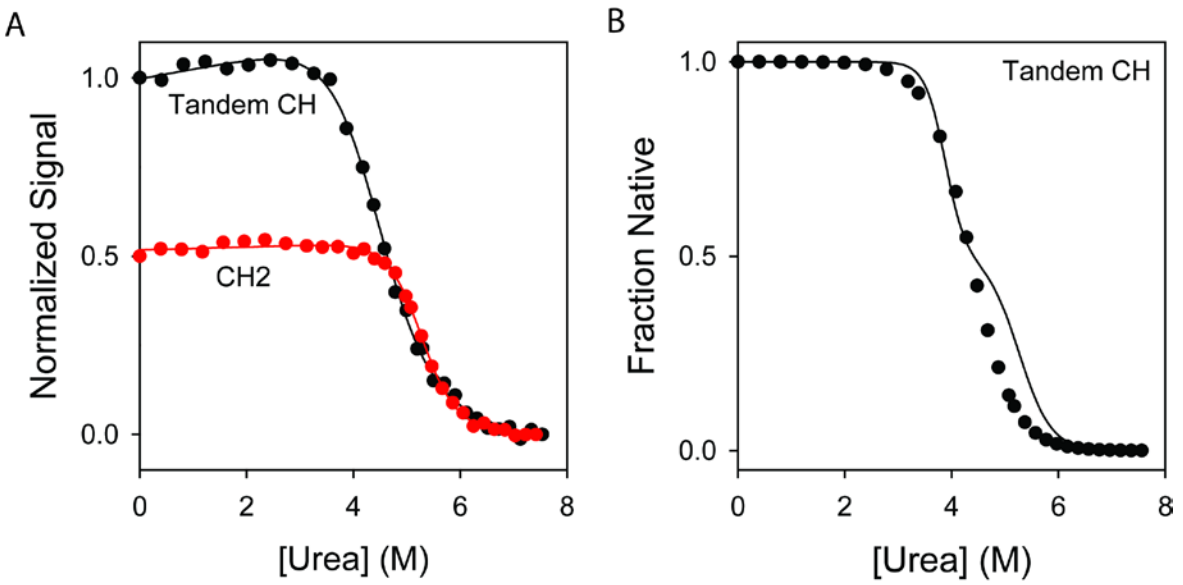

Figure S2. (A) Denaturant melts of CH2 and the tandem CH domain as measured by CD signal at 222 nm (Fig. 2B), and normalized with respect to their relative signals. Consistent with the crystal structures (Fig. 1A), the native CD of CH2 is half that of the tandem CH. The latter part of the denaturant melt of tandem CH domain does not exactly overlap with that of the isolated CH2. Isolated CH2 melts at a slightly higher denaturant concentration compared to the tandem CH domain. Solid lines indicate the fits to a 2-state folding model. The obtained fit parameters were ΔG_{unf} = 6.49 ± 0.27 kcal/mol, m = -1.49 ± 0.06 kcal/mol/M [urea], C_m = 4.36 ± 0.25 M [urea] for tandem CH domain, and were ΔG_{unf} = 10.48 ± 0.59 kcal/mol, m = -1.99 ± 0.12 kcal/mol/M [urea], C_m = 5.27 ± 0.43 M [urea] for CH2 domain. (B) Denaturant melt of the tandem CH domain (circles), and its fit to a three-state folding model assuming that the CH2 in the tandem CH domain has the same stability as that of isolated CH2 (black solid line). The data was generated from the 2-state fit shown in panel A after subtracting the native and unfolded baselines. The latter part of the denaturant melt could not be satisfactorily fitted to the 3-state folding model, indicating that CH2 in the tandem CH domain has a decreased stability compared to when it is isolated.

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