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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]

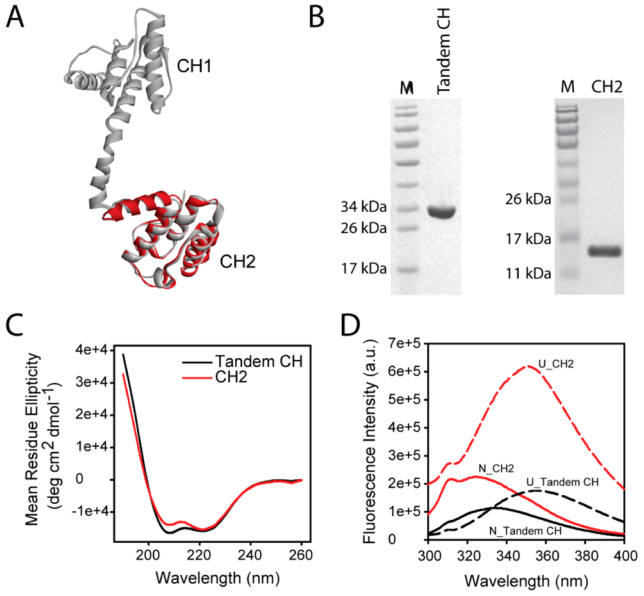

Figure 1. (A) X-ray crystal structures of the utrophin tandem CH domain [Protein Data Bank (PDB) entry 1QAG] and its isolated CH2 (PDB entry 1BHD), colored gray and red, respectively. Isolated CH2 has the same structure as the full-length protein (root-mean-square deviation of 0.83 Å). (B) Sodium dodecyl 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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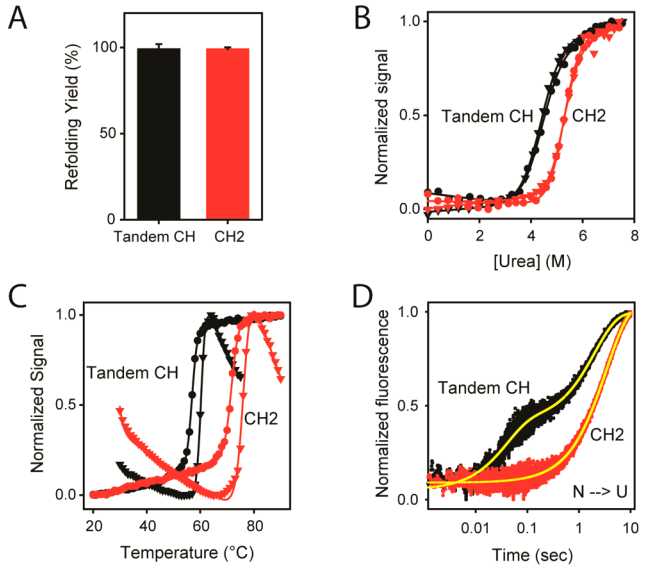

Figure 2. (A) Refolding yields of the two proteins, indicating that their folding is completely reversible. (B) Changes in the CD signal at 222 nm (circles) and protein fluorescence (triangles) as a function of urea concentration. (C) Changes in the CD signal at 222 nm (circles) and protein fluorescence (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.

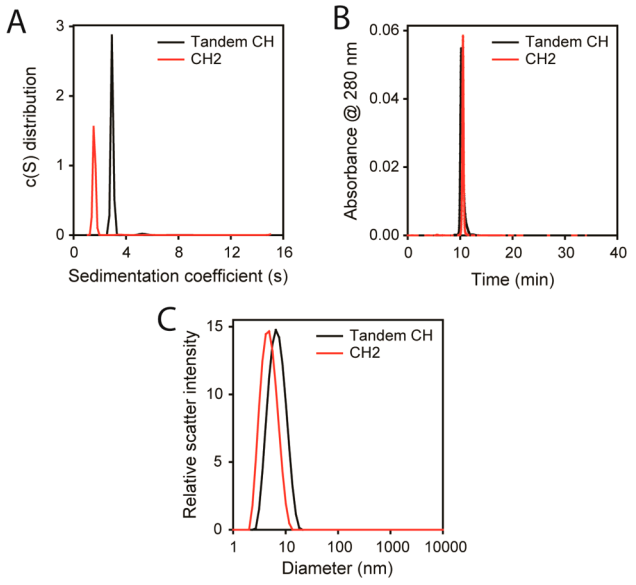

Figure 3. (A) Sedimentation coefficient distributions obtained from analytical ultracentrifugation (AUC). (B) Size-exclusion chromatograms (SEC). (C) Scattering intensity profiles obtained from dynamic light scattering (DLS). In all panels, black and red colors indicate the data for the tandem CH domain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