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# Interdomain Linker Determines Primarily the Structural Stability of Dystrophin and Utrophin Tandem Calponin-Homology Domains Rather than Their Actin-Binding Affinity

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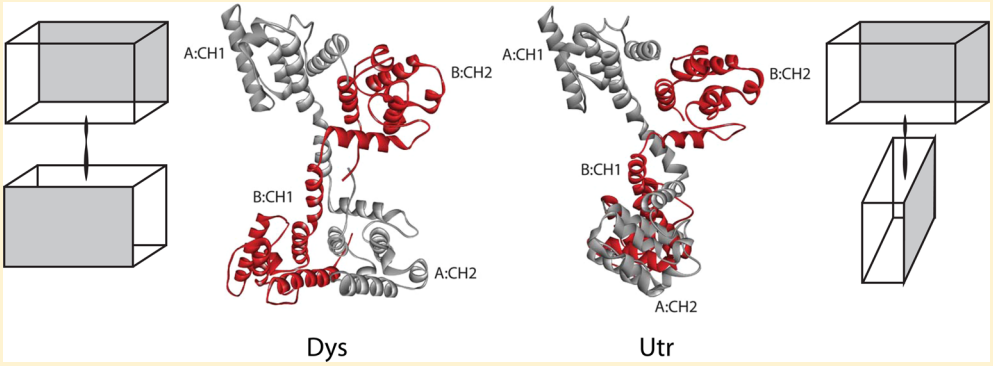
Supporting Information




**ABSTRACT:** Tandem calponin-homology (CH) domains are the most common actin-binding domains in proteins. However, structural principles underlying their function are poorly understood. These tandem domains exist in multiple conformations with varying degrees of inter-CH-domain interactions. Dystrophin and utrophin tandem CH domains share high sequence similarity (~82%), yet differ in their structural stability and actin-binding affinity. We examined whether the conformational differences between the two tandem CH domains can explain differences in their stability and actin binding. Dystrophin tandem CH domain is more stable by ~4 kcal/mol than that of utrophin. Individual CH domains of dystrophin and utrophin have identical structures but differ in their relative orientation around the interdomain linker. We swapped the linkers between dystrophin and utrophin tandem CH domains. Dystrophin tandem CH domain with utrophin linker (DUL) has similar stability as that of utrophin tandem CH domain. Utrophin tandem CH domain with dystrophin linker (UDL) has similar stability as that of dystrophin tandem CH domain. Dystrophin tandem CH domain binds to F-actin ~30 times weaker than that of utrophin. After linker swapping, DUL has twice the binding affinity as that of dystrophin tandem CH domain. Similarly, UDL has half the binding affinity as that of utrophin tandem CH domain. However, changes in binding free energies due to linker swapping are much lower by an order of magnitude compared to the corresponding changes in unfolding free energies. These results indicate that the linker region determines primarily the structural stability of tandem CH domains rather than their actin-binding affinity.

Among various structural domains used by muscle and signaling proteins that bind to F-actin, tandem calponin-homology (CH) domains form the most common actin-binding domains.[1−3] These tandem domains are composed of two structurally similar CH domains connected by a linker helix (Figure 1A). Each CH domain is made up of ~125 residues with six to seven $\alpha$-helices connected by flexible loops. Structurally, tandem CH domains can be in an extended, open form with minimal interactions between the two CH domains, for example, dystrophin[4] and utrophin,[5] or in a closed form with significant interactions between the two CH domains, for example, fimbrin,[6] plectin,[7] and $\alpha$-actinin.[8] Individual CH domains from all tandem CH domains have similar calponin-like structure, and the difference is in the relative orientation of two CH domains around the connecting central linker helix (Figure 1), thus modulating inter-CH-domain interactions. In this manuscript, we probed how this conformational variability imposed by the linker determines the structure and function of tandem CH domains.

We specifically studied the tandem CH domains of dystrophin and utrophin, because they are highly similar in amino acid sequence (~82%)[9] and in the structures of individual CH domains (RMSD < 1 Å) (Figure 1C). Hence, the structural and functional differences between these two tandem CH domains can be predominantly attributed to the differences in their inter-CH-domain interactions. In addition, these proteins are involved in the trigger and treatment of a highly morbid muscle disease Duchenne/Becker muscular

**Received:** July 2, 2015
**Revised:** August 7, 2015
**Published:** August 19, 2015





DOI: 10.1021/acs.biochem.5b00741
*Biochemistry* 2015, 54, 5480−5488

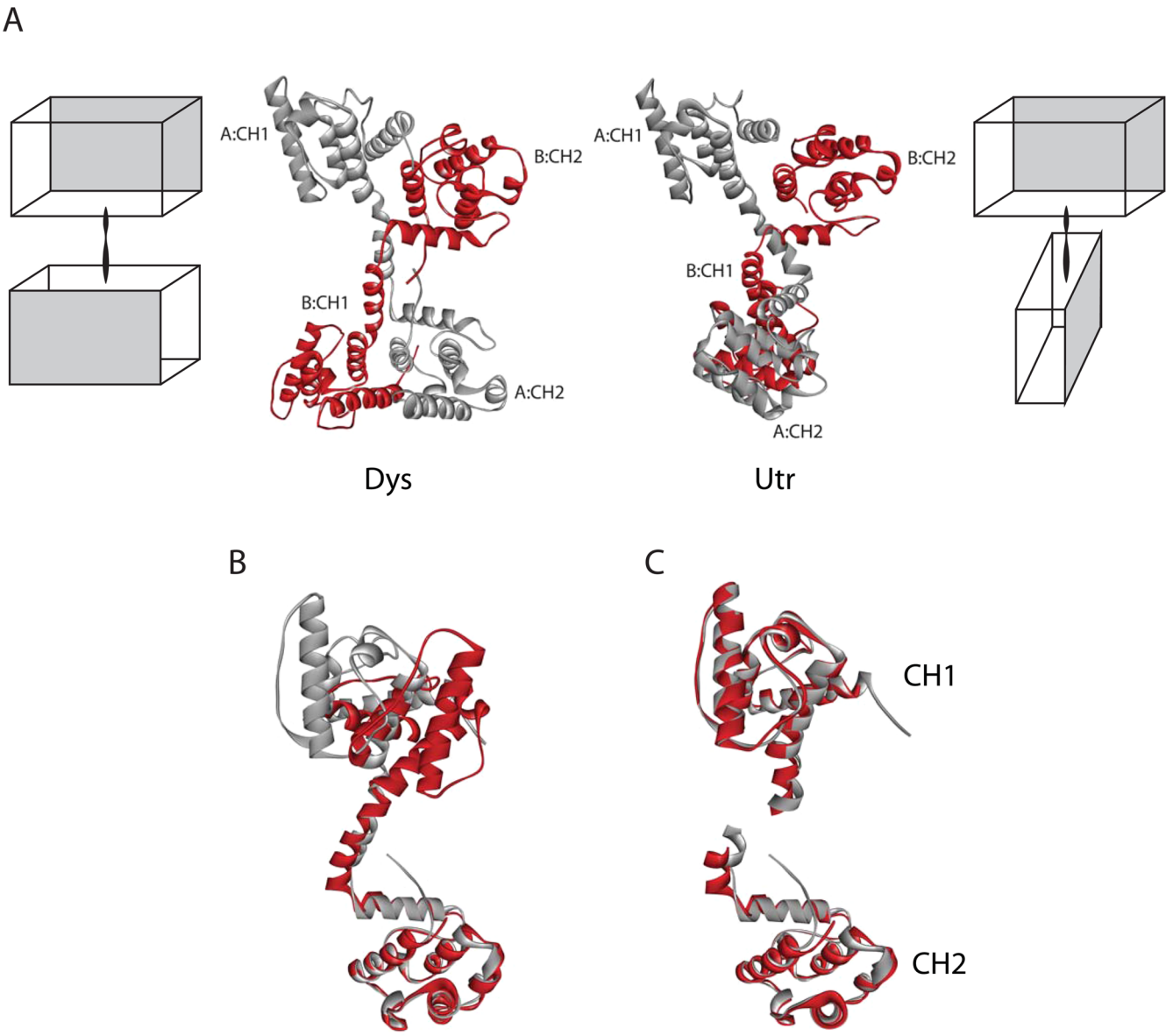



**Figure 1.** Structural analysis of dystrophin (Dys) and utrophin (Utr) tandem CH domains. (A) X-ray crystal structures of the tandem CH domains of Dys (1DXX.pdb) and Utr (1QAG.pdb). Although both tandem CH domains are monomers in solution, they crystallize as antiparallel domain-swapped dimers. Monomers in each dimer are colored gray and red. The relative orientation of the two CH1−CH2 interfaces between monomers is parallel in Dys tandem CH domain, whereas they are perpendicular in Utr tandem CH domain. (B) Structural alignment of the monomers of Dys (gray) and Utr (red) tandem CH domains from X-ray structures shown in panel (A). The two tandem CH domains differ in the relative orientation of CH domains around the central linker helix. (C) Structural alignment of the N-terminal CH1 and the C-terminal CH2 domains from the two tandem CH domains. Gray and red represent the corresponding domains from Dys and Utr tandem CH domains. The CH1 domains in Dys and Utr have identical structures (RMSD: 0.85 Å); similarly, the CH2 domains have identical structures (RMSD: 0.97 Å). Structure alignments were performed using the MultiProt program[46] (http://bioinfo3d.cs.tau.ac.il/MultiProt/). Molecular structures were drawn using the Accelrys Discovery Studio Visualizer program (http://accelrys.com/products/discovery-studio/visualization-download.php).

dystrophy.[10−13] Studying the structure−function relationship of dystrophin and utrophin will help in understanding how these two important muscle proteins work. Such knowledge will further help in understanding the effects of disease-causing genetic mutations on protein structure and function, since most disease-causing missense mutations occur in the dystrophin tandem CH domain.[14−16] Studying the structure−function of tandem CH domains will also help in improving the efficacy of mini- and microdystrophins and utrophins that are currently being explored to replace the loss of functional dystrophin in human patients.[12,13] These miniaturized versions of dystrophin and utrophin were designed to improve the success of gene therapy, however, these constructs have been found to have decreased stability and functionality compared to the full-length proteins.[17−19] All these constructs contain tandem CH domains in common. Therefore, determining the structural principles that govern the stability and function of tandem CH domains may lead to optimizing dystrophin and utrophin-based constructs for gene and protein therapies.

Earlier structural studies showed that both dystrophin and utrophin tandem CH domains crystallize as antiparallel domain swapped dimers, where each monomer is in an extended, open conformation with no significant interactions between the two CH domains[4,5] (Figure 1A). When we compared these



DOI: 10.1021/acs.biochem.5b00741
*Biochemistry* 2015, 54, 5480−5488

structures, the main difference is in the relative orientation of two CH domains around the connecting central linker helix (Figure 1A and B). The corresponding CH domains from dystrophin and utrophin have identical structures (Figure 1C). Similar conformational differences have also been proposed in recent solution structural studies on the monomeric proteins.[20,21] Although both tandem CH domains crystallize as domain-swapped dimers, we and others have shown that both proteins are monomers in solution.[2,4,5,9,15,20−26] To determine the solution conformation of dystrophin tandem CH domain, we recently used pyrene excimer fluorescence technique,[20,27] and the results indicated that it predominantly exists in a closed conformation with significant interactions between the two CH domains, contrary to the open conformation observed in the X-ray structure (Figure 1). Parallel EPR studies on utrophin tandem CH domain indicated that it predominantly exists in an open conformation,[21] analogous to that observed in the crystal structure (Figure 1). In this manuscript, we probed how these conformational differences between dystrophin and utrophin tandem CH domains determine their functional differences.

Dystrophin and utrophin tandem CH domains differ in their structural stability and actin-binding function. Dystrophin tandem CH domain is more stable by ∼4 kcal/mol than the utrophin tandem CH domain,[9] but binds to F-actin weaker than the utrophin tandem CH domain.[22,24] Available solution structural information can explain these differences in stability and function. Since dystrophin tandem CH domain predominantly exists in a closed conformation,[20] favorable inter-CH-domain interactions that exist might compete with the protein binding to actin. In contrast, since utrophin tandem CH domain predominantly exists in an open conformation,[21] it might be easier for it to bind to actin. Higher stability of dystrophin tandem CH domain might be due to increased inter-CH-domain interactions when compared to the utrophin tandem CH domain. To test these hypotheses, in particular, to examine the role of conformational differences around the central linker helix (Figure 1), we swapped the interdomain linkers between dystrophin and utrophin tandem CH domains. Our expectation was that the linker swapping will decrease stability with increased actin binding for dystrophin tandem CH domain with utrophin linker (DUL), and will increase stability with decreased actin binding for utrophin tandem CH domain with dystrophin linker (UDL). Our experimental results indicate that the conformational differences around the interdomain linker accounts for the differences in structural stability of dystrophin and utrophin tandem CH domains; however, has relatively less effect on their actin-binding affinity.

## MATERIALS AND METHODS

**Cloning, Expression, and Purification of Tandem CH Domains and Their Linker Swapped Mutants.** Plasmid vectors for dystrophin tandem CH domain (residues 1−246) and utrophin tandem CH domain (residues 1−261) were cloned using the DNA coding for both tandem CH domains into pET28a plasmid using NdeI and *Hin*dIII restriction endonuclease sites. Ligation mix was transformed into DH5$\alpha$ by heat shock. Plasmids were amplified using Qiagen miniprep kit and constructs were confirmed by DNA sequencing. Constructs for linker swapped mutants were generated using quick mutagenesis protocol (Qiagen). Constructs were confirmed by DNA sequencing, and were transformed into BL21 (DE3). Proteins were expressed and purified using protocols described before.[9,15,20,22−24]

**Circular Dichroism (CD).** Dystrophin and utrophin tandem CH domains and their linker-swapped mutants (1 $\mu$M protein concentration) in phosphate buffered saline (PBS) (100 mM $NaH_2PO_4$, 150 mM NaCl, pH 7) were used for measuring CD (Chirascan Plus, Applied Photophysics, UK). Mean residue ellipticity (MRE) of the proteins were calculated from the measured CD values in millidegrees.[28]

**Fluorescence.** Dystrophin and utrophin tandem CH domains and their linker-swapped mutants (1 $\mu$M) in PBS buffer were used for measuring intrinsic protein fluorescence of aromatic amino acids (PTI QuantaMaster fluorometer). The samples were excited at 280 nm.

**Denaturant Melts.** For urea denaturation melts, 1 $\mu$M protein in PBS buffer was used. Changes in far UV CD signal @ 222 nm and protein fluorescence (excitation 280 nm, emission 360 nm) were monitored as a function of increasing concentration of urea. Denaturant melts were fit to a two-state equilibrium unfolding model using Santoro−Bolen linear extrapolation equations[29,30] to determine the Gibbs free energy of unfolding, $\Delta G_{unf}$, and $m$-value, the linear slope of the variation of $\Delta G_{unf}$ with denaturant concentration.

**Refolding Yields.** Refolding yields for all four proteins were determined by diluting the denatured proteins in 8 M urea (10 $\mu$M protein concentration) 10 times into PBS buffer. Samples were then centrifuged at ∼30 000g to remove any aggregates, and supernatants were subjected to protein concentration quantification using peptide absorbance at 280 nm. Molecular extinction coefficients for all variants were calculated from their amino acid sequence using PROTPARAM software in ExPASY (http://www.expasy.org/).

**Actin Binding Affinity of Tandem CH Domains.** Skeletal muscle G-actin (Cytoskeleton, Denver, CO) was polymerized (7 $\mu$M) and incubated with varying concentrations of the binding partner proteins (tandem CH domains or their linker-swapped mutants) for 5 min at room temperature. The above mix (final volume 100 $\mu$L) was centrifuged at 100 000$g$ for 30 min (sw55Ti rotor, Beckman Optima LE80K) and pellets were solubilized in 30 $\mu$L SDS-PAGE loading buffer. Half of this was boiled, subjected to SDS-PAGE, and stained with coomassie blue. The intensity of the individual bands was determined using Quantity One software on Biorad Gel Doc XR, and were corrected by multiplying with the correction factors obtained from BSA standard curve to account for the differential staining of dye to proteins.[22,31] Ratio of the intensities was used to determine the fraction bound of F-actin using the formula

$$\begin{aligned}\text{fraction actin bound} &= (\text{corrected band intensity of bound protein} \\ &\quad \times \text{molecular weight of actin})/(\text{corrected band} \\ &\quad \text{intensity of actin} \times \text{molecular weight of bound protein})\end{aligned} \quad (1)$$

Free protein concentration was calculated using the formula

$$\begin{aligned}\text{free protein} &= \text{total protein added} - (\text{fraction actin bound} \\ &\quad \times \text{concentration of total actin added})\end{aligned} \quad (2)$$

The binding data were fit to the equation

$$\text{fraction actin bound} = B_{max}x/(K_d + x) \quad (3)$$



DOI: 10.1021/acs.biochem.5b00741
*Biochemistry* 2015, 54, 5480−5488

where $x$ is the free protein concentration, $B_{max}$ is the maximal number of binding sites, and $K_d$ is the dissociation constant.[22]

## RESULTS

**Dystrophin and Utrophin Tandem CH Domains Differ in Their Structural Stability and Actin-Binding Function.** Although dystrophin and utrophin tandem CH domains are highly similar in primary amino acid sequence (~82%)[9] and in the structures of individual CH domains (Figure 1), our previously published results indicated that they differ in their structural stability and actin-binding function.[9,22,24] Urea denaturant melts indicated that dystrophin tandem CH domain melted at higher denaturant concentrations and is more stable than the utrophin tandem CH domain by ~4 kcal/mol (Figure 2A).[9] Despite being stable, dystrophin tandem CH domain binds to F-actin ~30 times weaker than the utrophin tandem CH domain (Figure 2B), as evident from the actin binding curves determined using high-speed cosedimentation assays.[22,24] We examined whether these stability and functional differences can be explained in terms of the conformational differences around the central linker helix by swapping the linkers between dystrophin and utrophin tandem CH domains.

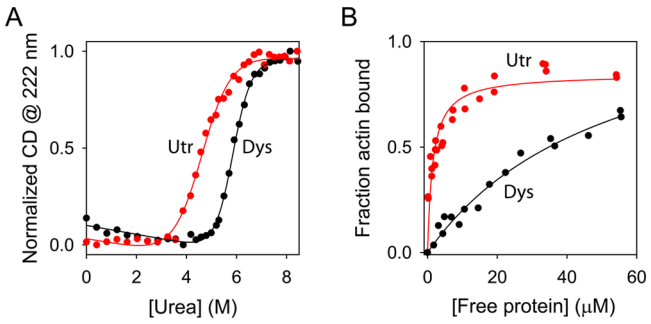



**Figure 2.** Thermodynamic stability and actin binding of Dys and Utr tandem CH domains. (A) Denaturant melts. Utr tandem CH domain unfolds at lower denaturant concentrations, indicating its lower stability, compared to Dys tandem CH domain. (B) Actin binding curves obtained from cosedimentation assays. Utr tandem CH domain strongly binds to actin compared to Dys tandem CH domain.

**Linker Swapping Did Not Perturb the Secondary and Tertiary Structures of Dystrophin and Utrophin Tandem CH Domains.** Linker of dystrophin tandem CH domain differs by five amino acids from that of the utrophin tandem CH domain (Figure 3). Our previous work has shown that single amino acid mutations in tandem CH domains can drastically change the protein structure.[9,15] Therefore, before proceeding to examine the effect of linker on the stability and function of tandem CH domains, we need to show that the linker swapping, which resulted in five amino acid mutations (Figure 3), did not change the protein structures. Linker-swapped mutants, dystrophin tandem CH domain with utrophin linker (DUL) and utrophin tandem CH domain with dystrophin linker (UDL), were expressed as soluble proteins and were purified to homogeneity. Theoretical molecular weights were consistent with the protein bands observed on SDS-PAGE (Figure S1). Circular dichroism (CD) was used to monitor the linker effect on the secondary structure of proteins (Figure 4A and B). CD spectra were identical between the parent proteins (black curves) and the corresponding linker-swapped mutants (red curves). Negative peaks at 208 and 222 nm are characteristic of $\alpha$-helical proteins.[28] The observed CD spectra for dystrophin and utrophin tandem CH domains are consistent with their $\alpha$-helical X-ray crystal structures (Figure 1). More importantly, DUL has identical CD spectrum as that of the dystrophin tandem CH domain (Figure 4A). Similarly, UDL has identical CD spectrum as that of the utrophin tandem CH domain (Figure 4B). No change in CD spectra indicates that the linker swapping did not perturb the secondary structures of proteins.


**Figure 3.** Amino acid sequences of the linker regions connecting the two CH domains in dystrophin (black) and utrophin (red) tandem CH domains. These linkers were swapped to generate linker mutants, dystrophin tandem CH domain with utrophin linker (DUL) and utrophin tandem CH domain with dystrophin linker (UDL).

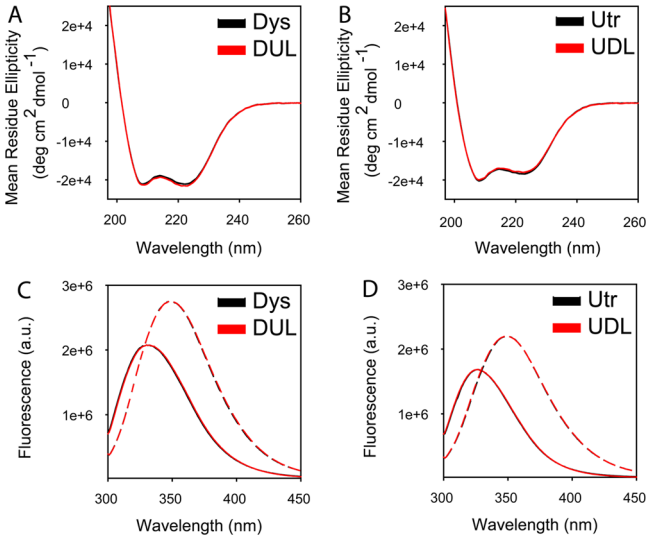



**Figure 4.** Linker swapping did not alter the structure of tandem CH domains. (A) Circular dichroism (CD) spectra of the Dys tandem CH domain (black) and DUL (red). (B) CD spectra of the Utr tandem CH domain (black) and UDL (red). (C) Fluorescence spectra of the native (N) and unfolded (U) states of the Dys tandem CH domain (black) and DUL (red). (D) Fluorescence spectra of the native (N) and unfolded (U) states of the Utr tandem CH domain (black) and UDL (red). The CD and fluorescence spectra were identical between the linker-swapped mutants and the corresponding parent proteins.

Effect of linker swapping on protein tertiary structure was monitored by comparing fluorescence spectra of the parent proteins and the corresponding linker-swapped mutants (Figure 4C and D). Protein fluorescence originates from the side chains of aromatic residues, in particular, tryptophans and tyrosines, and hence can be considered as a probe for protein tertiary structure. Dystrophin tandem CH domain contains eight tryptophans and five tyrosines distributed across the protein structure, whereas utrophin tandem CH domain has six



DOI: 10.1021/acs.biochem.5b00741
Biochemistry 2015, 54, 5480−5488

tryptophans and four tyrosines. None of the five mutations in the linker is an aromatic amino acid (Figure 3). For all the four proteins, fluorescence spectrum of the native state is blue-shifted with respect to the corresponding unfolded state. Such a blue shift is characteristic of well-folded and well-structured proteins.[32] Fluorescence spectra of the unfolded states were higher in intensity compared to the respective native states, indicating fluorescence quenching by neighboring amino acids in the native states.[33,34] More importantly, measured spectra of DUL and UDL are identical to that of dystrophin and utrophin tandem CH domains, respectively. These spectral comparisons indicate that the linker swapping did not significantly perturb the tertiary structure of proteins, similar to no effect of linker swapping on their secondary structures.

**Linker Region Accounts for the Differences in Thermodynamic Stabilities of Dystrophin and Utrophin Tandem CH Domains.** Equilibrium denaturant melts were used for determining thermodynamic stability of the four proteins, and urea was used as the denaturant to destabilize protein structure. Since unfolding/folding reversibility is a prerequisite for using denaturant melts to determine the equilibrium thermodynamic stabilities, we subjected the unfolded proteins in high denaturant to refolding by diluting the denaturant 10 times into native buffer. All the four proteins refolded by ∼100% (Figure S2), indicating that their unfolding is reversible.

We used CD at 222 nm for measuring secondary structure unfolding with urea. For measuring unfolding of the tertiary structure, we used intrinsic protein fluorescence. Denaturant melts for a given protein measured using both CD and fluorescence (Figure 5) were globally fitted to a Santoro−Bolen

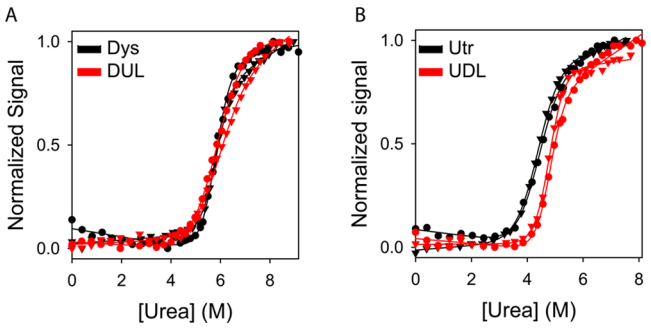



**Figure 5.** Structural stability of tandem CH domains and their linker-swapped mutants probed using denaturant melts. (A) Normalized changes in the CD signal at 222 nm (circles) and in the intrinsic protein fluorescence (triangles) of Dys tandem CH domain (black) and its linker mutant DUL (red) as a function of increasing urea concentration. (B) Normalized changes in the CD signal at 222 nm (circles) and in the intrinsic protein fluorescence (triangles) of Utr tandem CH domain (black) and its linker mutant UDL (red) as a function of increasing urea concentration. Both CD and fluorescence denaturant melts were globally fitted to obtain Gibbs free energy $\Delta G_{unf}$ and *m*-value, slope of the linear variation of $\Delta G_{unf}$ with urea concentration. The fit parameters are shown in Table 1.

two-state unfolding model[29,30] to obtain the Gibbs free energy of unfolding, $\Delta G_{unf}$, and the slope of linear variation of $\Delta G_{unf}$ with denaturant concentration, known as the *m*-value. The goodness of the fits indicates the validity of our global analysis. The fit values are summarized in Table 1. These values represent the apparent or ensemble averaged thermodynamic parameters of the system, since we are using a two-state unfolding model to fit the denaturant melts of a two-domain protein.[35]

The $\Delta G_{unf}$ values for the four proteins show interesting results (Table 1). Upon linker swapping, dystrophin tandem CH domain stability decreased from 10.83 ± 0.32 kcal/mol to 6.50 ± 0.19 kcal/mol, whereas utrophin tandem CH domain stability increased from 6.49 ± 0.27 kcal/mol to 10.40 ± 0.64 kcal/mol, indicating that the interdomain linker plays a significant role in determining the thermodynamic stability of tandem CH domains. More importantly, within the experimental error, dystrophin tandem CH domain with utrophin linker (DUL) has similar stability as that of the utrophin tandem CH domain, and utrophin tandem CH domain with dystrophin linker (UDL) has similar stability as that of the dystrophin tandem CH domain. These comparisons indicate that the stability difference between dystrophin and utrophin tandem CH domains predominantly originates from the linker region connecting the two CH domains.

In addition to $\Delta G_{unf}$ values, *m*-values can be used to determine the role of linker in the conformation of tandem CH domains. Dystrophin tandem CH domain has a higher *m*-value of −1.87 ± 0.13 (kcal/mol)/M [urea] compared to the utrophin tandem CH domain (*m* = −1.49 ± 0.16 (kcal/mol)/M [urea]) (Table 1). Upon linker swapping, the dystrophin tandem CH domain *m*-value decreased from −1.87 ± 0.13 to −1.12 ± 0.14 (kcal/mol)/M [urea], whereas utrophin tandem CH domain *m*-value increased from −1.49 ± 0.16 to −2.22 ± 0.14 (kcal/mol)/M [urea]. These changes in *m*-values can be interpreted as follows. Denaturant *m*-values are in general a measure of the difference in accessible surface area (ASA) between the native and unfolded states of a protein.[36] Earlier studies indicated that dystrophin tandem CH domain predominantly exists in a closed conformation,[20] whereas utrophin tandem CH domain predominantly exists in an open conformation.[21] Since the closed conformation is expected to bury more surface area compared to an open conformation because of the increased inter-CH-domain interactions, dystrophin tandem CH domain is expected to show a higher *m*-value compared to the utrophin tandem CH domain. Observed experimental *m*-values are consistent with this view (Table 1). Similar analogy can be used to interpret the change in *m*-values upon linker swapping. Dystrophin tandem CH domain with utrophin linker (DUL) has a decreased *m*-value compared to the parent dystrophin tandem CH domain (Table 1), implying that the DUL is more in an open conformation compared to dystrophin tandem CH domain. In the case of utrophin, UDL has increased *m*-value compared to the utrophin tandem CH domain, implying that UDL is more in a closed conformation compared to the utrophin tandem CH domain. These *m*-value comparisons between the parent proteins and their linker-swapped mutants suggest that the dystrophin linker makes the tandem CH domains to exist more in a closed conformation whereas utrophin linker makes the tandem CH domains to exist more in an open conformation. This interpretation implies that the linker modulates inter-CH-domain interactions in tandem CH domains by switching the conformational preferences between closed and open conformations. This role of linker also explains actin-binding results discussed in the next section.

Note that the two constructs in which CH1 is from utrophin, UDL and utrophin tandem CH domain, show higher *m*-values compared to the corresponding proteins in which CH1 is from dystrophin, dystrophin tandem CH domain and DUL,


DOI: 10.1021/acs.biochem.5b00741
*Biochemistry* 2015, 54, 5480−5488

**Table 1. Equilibrium Stability and Actin Binding Parameters Obtained from Denaturant Melts (Figure 5) and Actin Cosedimentation Assays (Figure 6)[a]**

| | Dys | DUL | Utr | UDL |
|---|---|---|---|---|
| $\Delta G_{unf}$ (kcal/mol) | 10.83 ± 0.32 | 6.50 ± 0.19 | 6.49 ± 0.27 | 10.40 ± 0.64 |
| *m*-value ((kcal/mol)/M [urea]) | −1.87 ± 0.13 | −1.12 ± 0.14 | −1.49 ± 0.16 | −2.22 ± 0.14 |
| $K_d$ ($\mu$M) | 47.05 ± 13.92 | 26.29 ± 3.10 | 1.53 ± 0.88 | 2.55 ± 0.42 |
| $B_{max}$ | 1.17 ± 0.16 | 0.62 ± 0.03 | 0.85 ± 0.03 | 0.57 ± 0.02 |

[a]The $\Delta G_{unf}$ and *m*-values were obtained by globally fitting three CD and three fluorescence melts, whereas $K_d$ and $B_{max}$ values were determined by globally fitting three, independent data sets.

respectively. This is because *m*-value is a measure of the ASA between native and unfolded states, which in turn is related to the number of amino acids in a protein.[36] Utrophin CH1 is longer in primary structure by 15 amino acids than that of dystrophin CH1 whereas both CH2 domains of dystrophin and utrophin are similar in length. Hence, utrophin CH1 is expected to have a higher *m*-value compared to the dystrophin CH1 domain. This explains why the *m*-values of proteins with utrophin CH1 are higher compared to the corresponding proteins with dystrophin CH1.

**Role of Linker in the Actin Binding Function of Dystrophin and Utrophin Tandem CH Domains.** High-speed cosedimentation assays were used to determine actin binding.[26,37] In this method, a fixed concentration of actin was incubated with varying concentrations of actin-binding protein. Actin along with its bound protein was pelleted using high-speed centrifugation, and the pellets were loaded on SDS-PAGE to determine the relative concentration of bound protein with respect to actin. Figure 6A and B shows SDS-PAGE for the two parent proteins, dystrophin and utrophin tandem CH domains, and Figure 6C and D shows the results for the respective linker-swapped mutants, DUL and UDL. These raw data indicate that the relative trend in protein concentration that is bound to actin did not change significantly upon linker swapping. DUL essentially has a similar pattern of binding as that of dystrophin tandem CH domain (Figure 6A and C), whereas the binding of UDL is similar to that of utrophin tandem CH domain (Figure 6B and D). Quantitative results obtained by digitizing the protein bands on SDS-PAGE are shown in Figure 6E and F. These curves were fit to the binding equation (eq 3 in Materials and Methods) to determine the dissociation constants ($K_d$). The fit values are shown in Table 1.

Dystrophin tandem CH domain with utrophin linker (DUL) binds to actin twice strongly compared to the parent dystrophin tandem CH domain (Table 1). The $K_d$ values were 47.05 ± 13.92 $\mu$M for dystrophin tandem CH domain and 26.29 ± 3.10 $\mu$M for DUL. This increase can be understood in terms of the conformational differences between the two proteins inferred from denaturant melts (Table 1). The denaturant *m*-values and $\Delta G_{unf}$ values indicate that dystrophin tandem CH domain predominantly exists in a closed conformation, whereas DUL predominantly exists in an open conformation (see the discussion on *m*-values in the previous section). These experimental values indicate that the tandem CH domain with open conformation binds to F-actin more efficiently compared to that with closed conformation. This interpretation is consistent with recent studies which suggested that the opening of tandem CH domains controls their actin binding.[38] Similar differences in actin binding affinity can be observed between utrophin tandem CH domain and UDL. Utrophin tandem CH domain binds to F-actin with a $K_d$ of 1.53 ± 0.88 $\mu$M, whereas UDL binds with a $K_d$ of 2.55 ± 0.42 $\mu$M. Again,

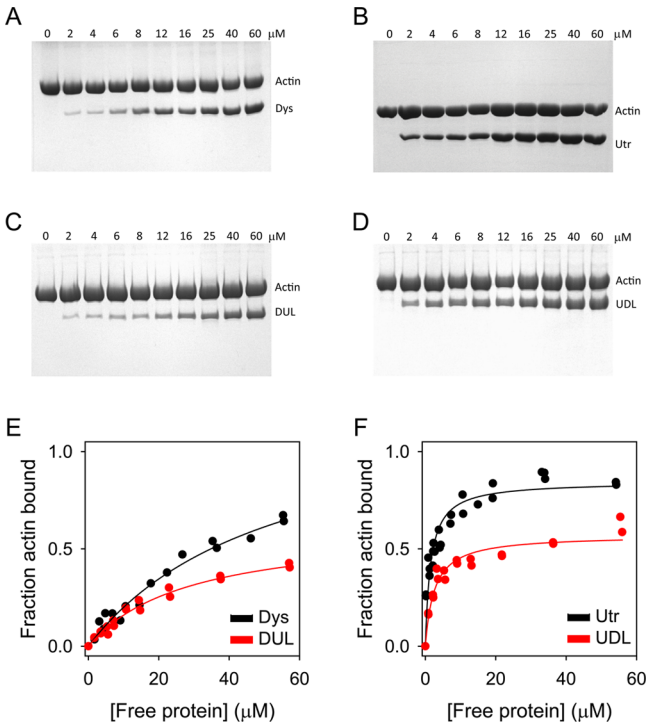



**Figure 6.** Actin binding of dystrophin and utrophin tandem CH domains and their linker-swapped mutants, DUL and UDL. (A–D) Actin binding cosedimentation assays of the four proteins. Figures show SDS–PAGE of the pellets from high-speed centrifugation performed at a fixed concentration of F-actin (7 $\mu$M) and with varying concentrations of the tandem CH domains. (E and F) Actin binding curves of the four proteins obtained from band intensities on SDS-PAGE shown in panels (A)–(D), after correcting for differential staining of the dye to proteins. These binding curves were fit to determine the $K_d$ and $B_{max}$ values, and the fit parameters are shown in Table 1.

these binding affinities are consistent with the denaturant *m*-values and $\Delta G_{unf}$ which indicate that utrophin tandem CH domain predominantly exists in an open conformation whereas UDL predominantly exists in a closed conformation.

Note that the two proteins where CH1 is from utrophin, utrophin tandem CH domain, and UDL show higher $K_d$ values compared to the proteins where CH1 is from dystrophin, dystrophin tandem CH domain and DUL. This is consistent with our recent experimental results[22] which indicate that the CH1 domain predominantly determines the actin-binding function of tandem CH domains.

Another interesting result emerges from actin-binding studies. The $B_{max}$ values of linker-swapped mutants are lower than those of parent tandem CH domains (Table 1). $B_{max}$ represents the maximum number of molecules that can bind to one molecule of actin. These values are close to one in the case



DOI: 10.1021/acs.biochem.5b00741
*Biochemistry* 2015, 54, 5480–5488

of dystrophin and utrophin tandem CH domains, consistent with cryo-EM studies on their actin-bound conformations.[39,40] However, upon linker swapping, both DUL and UDL show substoichiometric $B_{max}$ values close to 0.6, implying that only 6 molecules of DUL or UDL can bind to 10 molecules of actin. This might be due to new steric constraints imposed by CH2 due to linker swapping for the subsequent binding of another tandem CH domain next to it. Earlier cryo-EM studies indicate that the main difference between tandem CH domains which bind to actin in closed conformation vs those which bind in an open conformation is in the relative orientation of CH2 with respect to CH1.[2] This proposed role of steric constraints on $B_{max}$ value needs to be further probed.

**Linker Swapping Affects Predominantly the Thermodynamic Stability of Dystrophin and Utrophin Tandem CH Domains Rather than Their Actin-Binding Affinity.** We compared the relative effect of linker on binding and unfolding free energies. The $K_d$ values in Table 1 represent the equilibrium dissociation constants, and have been used to calculate the binding free energies, $\Delta G_{bind}$. Between dystrophin tandem CH domain and DUL, the change in binding free energy, $\Delta\Delta G_{bind} = -RT\ln(K_a^{Dys}/K_a^{DUL}) = -RT\ln(K_d^{DUL}/K_d^{Dys})$ was 0.35 ± 0.14 kcal/mol. In this equation, $K_a$ is the association constant, which is inverse of the dissociation constant, $K_d$. This $\Delta\Delta G_{bind}$ is much lower by an order of magnitude than the change in unfolding free energy upon linker-swapping, $\Delta\Delta G_{unf}$ = 4.33 ± 0.37 kcal/mol (Table 1) (propagation of errors were calculated using formulas described before[41]). Similarly, the change in binding free energy between UDL and utrophin tandem CH domain was $\Delta\Delta G_{bind}$ = 0.30 ± 0.28 kcal/mol (calculated from $K_d$ values in Table 1). This is again much lower by an order of magnitude than the corresponding change in unfolding free energy, $\Delta\Delta G_{unf}$ = 3.91 ± 0.69 kcal/mol (Table 1). These comparisons between binding and unfolding free energies clearly indicate that the main effect of interdomain linker is on the thermodynamic stabilities of tandem CH domains, rather than on their actin-binding affinities.

## DISCUSSION

Despite the widespread occurrence of tandem CH domains in numerous muscle and signaling proteins, structural determinants of their actin binding are poorly understood. The major difference between tandem CH domains is in their conformational variability around the central linker region connecting the two CH domains (Figure 1).[2,3] How this variability controls the properties of tandem CH domains is much debated over the past 25 years.[42] Here, we examined how the interdomain linker influences the structural and functional properties of tandem CH domains by swapping the linkers between dystrophin and utrophin tandem CH domains. Earlier results from us and others indicated that dystrophin tandem CH domain predominantly exists in a closed conformation,[20,27] whereas utrophin tandem CH domain predominantly exists in an open conformation.[21] Upon linker swapping, dystrophin tandem CH domain with utrophin linker (DUL) has significantly decreased stability compared to dystrophin tandem CH domain (Table 1), and possibly changes its conformational preference from a closed conformation to an open conformation (interpreted from *m*-values). In contrast, utrophin tandem CH domain with dystrophin linker (UDL) has significantly increased stability compared to utrophin tandem CH domain, and possibly changes its conformational preference from an open conformation to a closed conformation. Difference in the linker regions accounts for the stability difference between dystrophin and utrophin tandem CH domain (Figure 2A; Table 1). However, the linker swapping did not significantly affect the actin-binding function when compared to its effect on stability (Table 1). The $K_d$ values do change by approximately twice, but the change in binding energy ($\Delta\Delta G_{bind} \sim 0.3$ kcal/mol) upon linker swapping is much lower by an order of magnitude (more than ten times) compared to the change in thermodynamic stability differences ($\Delta\Delta G_{unf} \sim 4$ kcal/mol). These $\Delta\Delta G$ values indicate that the major effect of linker is on the thermodynamic stability rather than on the actin-binding affinity of tandem CH domains.

The above $\Delta\Delta G$ values can be understood as follows. The nonfunctional unfolded state population is given by [unfolded] = [native]$\exp(-\Delta G_{unf}/RT)$, where $R$ is the gas constant and T is absolute temperature in Kelvin. The unfolding stability difference of 4 kcal/mol changes the unfolded state population by 822 times upon linker mutation. Similarly, the actin-bound native population is given by $[native]_{bound} = [native]_{unbound} \exp(-\Delta G_{bind}/RT)$. A change of $\Delta G_{bind}$ by 0.3 kcal/mol merely changes the actin-bound population by 1.7 times. These numbers again indicate that the major effect of linker is on modulating the folded native state population, rather than affecting the amount of actin-bound native molecules.

The amino acid differences between the two linker regions (Figure 3) can explain how linker swapping affects the conformational preferences of dystrophin and utrophin tandem CH domains. In the case of dystrophin linker, the amino acid residues are more hydrophobic than that of the utrophin linker. In addition, utrophin linker has three aspartic acids that are distributed uniformly across the sequence with a DVM repeating motif (Figure 3). These negatively charged amino acids that are three residues apart will result in charge−charge repulsion, which will favor the open conformation for those proteins with utrophin linker. Consistently, the experimental denaturant *m*-values indicate that the two proteins with utrophin linker, utrophin tandem CH domain and DUL, prefer to exist in an open conformation, whereas the two proteins with dystrophin linker, dystrophin tandem CH domain and UDL, prefer to exist in a closed conformation.

How general is this behavior on the effect of interdomain linker on structural stability and actin binding of tandem CH domains? Although no studies have been performed to date on the specific effect of linker, preliminary results available in the literature suggest a critical role for inter-CH-domain interactions. In the case of utrophin tandem CH domain with two engineered non-native cysteines, one in each domain, the actin binding affinity decreases by 1.6 times when the protein is trapped in a closed conformation by disulfide cross-linking.[43] In terms of stability, utrophin tandem CH domain exists in two conformations before cross-linking, and transitions to a conformation with high thermal stability after cross-linking.[43] Similar observations were made on plectin tandem CH domain.[7] Disulfide cross-linked closed conformation is more stable but weakly binds to actin compared to the open, reduced form. Constraining the closed conformation with disulfide cross-linking has also been shown to impose steric constraints in actin binding. Mutations in α-actinin at the CH1−CH2 interface destabilize the closed conformation but show increased actin-binding.[44] Similarly, mutations at the CH1−CH2 interface in filamin tandem CH domain have decreased stability but increased actin binding.[45] Our results presented


DOI: 10.1021/acs.biochem.5b00741
*Biochemistry* 2015, 54, 5480−5488

here indicate that the linker region connecting the two CH domains plays a major role in modulating inter-CH-domain interactions, and thus affecting the stability and function of tandem CH domains. When the amino acid sequences and three-dimensional atomic structures of multiple tandem CH domains were compared, maximum sequence and structural variability is found in the linker region.[2] It will be interesting to examine how such natural variation in the sequence, structure, and length of the linker region determines the general properties of tandem CH domains, which may further lead to improved understanding of the fundamental structure−function relationship of tandem CH domains.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00741.

Figure S1 shows SDS-PAGE of the purified proteins, and Figure S2 shows refolding yields of the proteins starting from their unfolded states (PDF)

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### Funding

This project was funded by the American Heart Association, Jane and Charlie Butcher grants in Genomics and Biotechnology, and the ALSAM Foundation through the Skaggs Scholars Program.


### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We sincerely thank George Rose for suggesting swapping the linkers, and Steve Winder for many helpful discussions on tandem CH domains. We thank David Bain, Carlos Catalano, and George Rose for their critical reading of the manuscript.

## ■ ABBREVIATIONS

ASA, accessible surface area; CD, circular dichroism; CH, calponin-homology; $\Delta G_{unf}$, Gibbs free energy of unfolding; $\Delta G_{bind}$, Gibbs free energy of binding; DUL, dystrophin tandem CH domain with utrophin linker; Dys, dystrophin; EM, electron microscopy; $K_d$, dissociation constant; $K_a$, association constant; *m*-value, linear slope of $\Delta G_{unf}$ variation with denaturant concentration; $T_m$, melting temperature; UDL, utrophin tandem CH domain with dystrophin linker; Utr, utrophin

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**SUPPORTING INFORMATION**

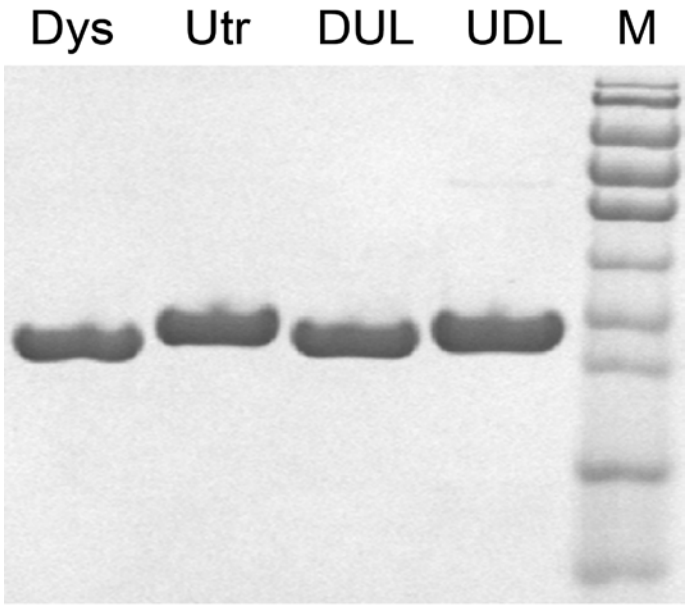


**Figure S1:** SDS-PAGE of purified Dys and Utr tandem CH domains, and their linker-swapped mutants, DUL and UDL. Lane M represents the molecular weight markers (bottom to top: 17, 26, 34, 43, 56, 72, 95, 130, and 170 kDa, respectively).

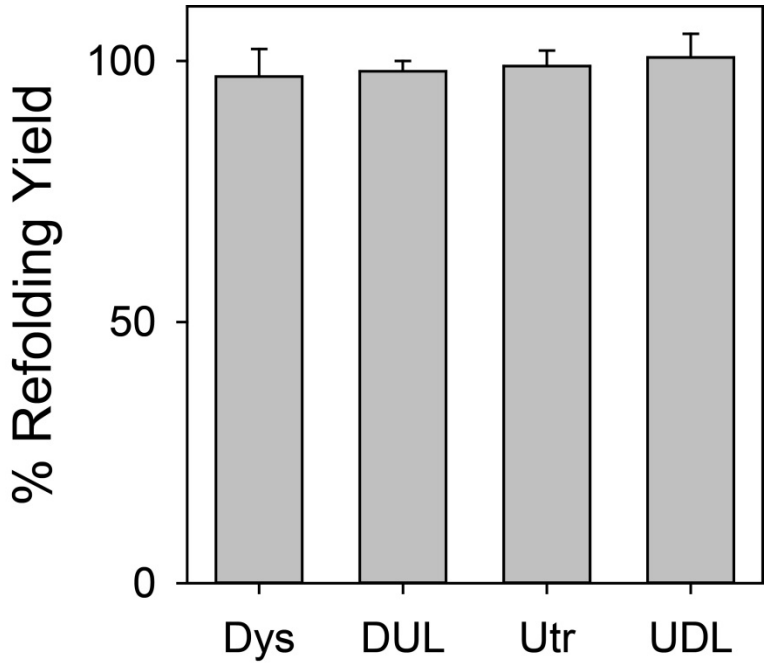


**Figure S2:** Refolding yields of Dys and Utr tandem CH domains and their linker-swapped mutants, DUL and UDL, starting from their unfolded states in 8 M urea. All the four proteins fold reversibly by 100%.