Utrophin ABD binds to F-actin in an open conformation

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1. Introduction

Calponin homology (CH) domains are found primarily, but not exclusively, in proteins that interact with the F-actin cytoskeleton. In most cases a functional actin binding domain (ABD) comprises two structurally equivalent but functionally distinct CH domains [1]. Tandem-CH domain ABDs are found in a large number of F-actin binding proteins with roles as structural linkers including the spectrin family of proteins, the spectraplakin family and other F-actin bundling or cross-linking proteins such as filamin and fimbrin. High-resolution atomic structures have been determined in solution and when bound to F-actin. The data suggest the amino-terminal utrophin actin binding domain is in an open conformation when bound to F-actin. Locking the actin binding domain in a closed conformation using engineered cysteine residues in each calponin homology domain reduced the affinity for F-actin without affecting the stoichiometry furthermore differential scanning calorimetry experiments revealed a reduction in melting temperature on binding to actin. The data suggest the amino-terminal utrophin actin binding domain is in an open conformation in solution and when bound to F-actin.

Structural analyses of actin binding regions comprising tandem calponin homology domains alone and when bound to F-actin have revealed a number of different conformations with calponin homology domains in 'open' and 'closed' positions. In an attempt to resolve these issues we have examined the properties of the utrophin actin binding domain in open and closed conformations in order to verify the conformation when bound to F-actin. The actin binding domain in a closed conformation using engineered cysteine residues in each calponin homology domain reduced the affinity for F-actin without affecting the stoichiometry Furthermore differential scanning calorimetry experiments revealed a reduction in melting temperature on binding to actin. The data suggest the amino-terminal utrophin actin binding domain is in an open conformation in solution and when bound to F-actin.
2. Methods

2.1. Purification and characterisation of UTR261 cysteine mutants

The double cysteine mutant construct was generated by QuikChange mutagenesis of UTR261, first the T36C mutation was generated to give UTR261T36C, and then the S242C mutation was introduced into UTR261T36C to generate UTR261T36C/S242C. UTR261T36C and UTR261T36C/S242C are expressed in soluble form in Escherichia coli BL21(DE3) and purified under the same conditions as wildtype UTR261 [8]. Cleavage of UTR261 by 2-nitro-5-thiobenzonic acid (NTCB) was carried out as described previously [11]. Oxidation or reduction of UTR261T36C/S242C was achieved overnight in 20 mM Tris (pH 8.0) in the presence of 4 mM o-phenanthroline and 1 mM CuSO4 or 1 mM Tris(2-carboxyethyl)phosphine hydrochloride, respectively, proteins were then dialysed back into 20 mM Tris for functional studies.

2.2. High speed co-sedimentation actin binding assays

Rabbit skeletal muscle actin was purified as described previously [8]. High speed co-sedimentation of 5 µM F-actin in the presence of increasing concentrations of UTR261, reduced UTR261T36C/S242C and oxidised UTR261T36C/S242C were carried out as previously described [8].

2.3. Fluorescence spectroscopy and differential scanning calorimetry

Tryptophan fluorescence spectroscopy was measured using a Shimadzu RF-5301PC spectrofluorophotometer. Protein samples were excited at 296 nm and fluorescence emission data were recorded between 300 and 450 nm. Differential scanning calorimetry (DSC) experiments were carried out in a N-DSC II differential scanning calorimeter from Calorimetry Sciences Corp. (Provo, UT), at scanning rate of 1 K/min under 3.0 atm of pressure. DSC samples contained 10 µM UTR261 (wildtype or mutants) using buffer conditions identical to those described previously [3]. UTR261T36C and UTR261T36C/S242C samples under reducing conditions were kept with 1.0 mM DTT at all times and diluted 10-fold with DTT-free buffer immediately before loading into calorimeter. Where stated 10 µM F-actin or 20 µM F-actin + 20 µM phalloidin were also added.

3. Results and discussion

Based on the previous studies of de Pereda and colleagues on the plectin ABD [3], and using a notional closed conformation of the utrophin ABD derived from the crystallographic dimer (Fig. 1C and D), we identified threonine 36 in CH1 and serine 242 in CH2 that would be close together in a predicted closed conformation. UTR261 T32 was mutated to cysteine, and then using this UTR261T36C as template, the second site was mutated to give UTR261T36C/S242C. DNA sequencing of the mutated construct confirmed the presence of both cysteine substitutions, which was further demonstrated by chemical cleavage at the cysteines with NTCB. As can be seen from Fig. 2B, compared to UTR261 which contains no cysteines, the UTR261T36C/S242C protein was susceptible to cleavage by NTCB. Furthermore, chemical oxidation of UTR261T36C/S242C revealed a mobility shift on non-reducing SDS-PAGE consistent with the formation of the intra-chain disulphide, with no evidence of inter-chain disulphide formation leading to dimerisation (Fig. 2C). The latter was also confirmed by analytical gel filtration, with the oxidised protein eluting as a monodisperse peak with a calculated mass of 28kDa (data not shown).

Analysis of the F-actin binding properties of wild type and cysteine mutants of UTR261, in either reduced or oxidised form as shown in Fig. 3. UTR261 bound to F-actin with similar stoichiometry ($B_{max}$, 1:1) and dissociation constant as reported previously [12,13]; however, introduction of the two cysteine residues did have an effect on the dissociation constant but without affecting the stoichiometry. Threonine 36 is within the conserved KTFT motif, also termed ‘ABS1’ in earlier mapping studies of actin binding regions within the amino-terminal actin binding domains of dystrophin and utrophin [8]. Whether this region is in direct contact with F-actin or is simply required for structural integrity of CH1 remains equivocal. CD spectra of UTR261 and cysteine mutants showed no significant changes in overall secondary structure (data not shown); however, there was a reduction in tryptophan fluorescence on introduction of T36C and S242C but there was little difference between reduced and oxidised UTR261T36C/S242C (Fig. 4). The reduction in affinity for F-actin could be due to an effect of T36C on ABS1 or this structurally conserved region, and the drop in tryptophan fluorescence
may result from cysteine quenching [14] of the nearby W40, and W128 which is close in the structure. Furthermore, as determined by DSC, the $T_m$ for all reduced proteins in solution is within 3°C (Table 1), suggesting that there are not large scale structural changes. The double cysteine mutants were also slightly red shifted compared to UTR261. The oxidised form of the double cysteine mutant, however bound to F-actin with an even lower affinity (74.8 ± 19 M reduced, 123 ± 14 M oxidised) suggesting that either the open form of the ABD bound to actin better, or that a greater degree of flexibility was required for the interaction with F-actin which was inhibited by locking the two CH domains closed.

In order to test further the conformation of UTR261 when bound to F-actin we carried out differential scanning calorimetry on UTR261 and cysteine mutants, either alone or in the presence of F-actin (Table 1, Fig. 5). UTR261 denatured in DSC experiments as a single peak with $T_m = 53.3$ °C (Table 1, Fig. 5A). In studies conducted under otherwise identical conditions the $T_m$ of UTR261 was much lower than the $T_m$ of the plectin ABD either in solution or when in complexed with actin: 63.9 °C vs 59.1 °C [3]. The plectin ABD $T_m$ were interpreted by Garcia-Alvarez et al., to suggest that uncomplexed plectin ABD was in a closed state and plectin ABD in complex with F-actin was in an open state [3]. The $T_m$ of UTR261 in complex with F-actin increased (rather than decreased as in the case of plectin) but only slightly to 55.5 °C. These observations, along with those of the recent studies performed using spin labelling [10] suggest that UTR261 adopts an open conformation in solution. To verify this we have used UTR261T36C/S242C with two cysteines introduced at T36 and S242 positions, which based on the prediction from Fig. 2, the formation of disulphide bond should lock UTR261 in the closed state. In DSC experiments oxidised UTR261T36C/S242C denatured at much higher temperature than UTR261 (Table 1, Fig. 5D). The $T_m = 68.1$ °C was as high as that of the analogous plectin ABD T74C/S277C mutant in the oxidised, i.e. closed state [3]. This similarity suggests that we have also succeeded in locking UTR261T36C/S242C in the closed conformation.

To verify that the structural effect associated with increased $T_m$ of oxidised UTR261T36C/S242C is due to S-S cross-linking and not cysteine mutation per se, we ran DSC on reduced UTR261T36C/S242C with two cysteines introduced at T36 and S242 positions, which based on the prediction from Fig. 2, the formation of disulphide bond should lock UTR261 in the closed state. In DSC experiments oxidised UTR261T36C/S242C denatured at much higher temperature than UTR261 (Table 1, Fig. 5D). The $T_m = 68.1$ °C was as high as that of the analogous plectin ABD T74C/S277C mutant in the oxidised, i.e. closed state [3]. This similarity suggests that we have also succeeded in locking UTR261T36C/S242C in the closed conformation.
of UTR261T36C in the presence of F-actin (Fig. 5D) was very similar as in solution ie presumed to be open. Interestingly, unmodified UTR261 and thus, it is likely that it binds F-actin in the same state indicating that there are no major changes in the conformation of F-actin, the relatively small amplitude of the effect (2.2 °C) as a result of its strong stabilizing 0.2 °C. WT = wildtype sequence, UTR261 1C = UTR261T36C and UTR261 2C = UTR261T36C/S242C.

equilibrium towards the closed state. Overall, DSC analysis of UTR261, UTR261T36C and UTR261T36C/S242C preparations demonstrated that similar to plectin ABD, utrophin ABD can adopt two conformations, closed and open. However, in contrast to plectin ABD which exists in a predominantly closed state in solution, unmodified utrophin ABD in solution is likely to be in a predominantly open state.

In the presence of F-actin the $T_m$ of UTR261 increased to 55.5 °C (Table 1, Fig 5A). While this increase reflects UTR261 binding to F-actin, the relatively small amplitude of the effect (2.2 °C) indicates that there are no major changes in the conformation of UTR261 and thus, it is likely that it binds F-actin in the same state as in solution ie presumed to be open. Interestingly, unmodified plectin ABD also bound F-actin in the open state [3]. The behaviour of UTR261T36C in the presence of F-actin (Fig. 5D) was very similar to that of UTR261. In the DSC profile of F-actin complexed with oxidised UTR261T36C/S242C (Fig. 5D), the main peak with $T_m = 69.7 °C$ (associated with melting of F-actin) has a shoulder at ~67 °C. This shoulder likely represents melting of UTR261T36C/S242C. To resolve the peaks of F-actin and oxidised UTR261T36C/S242C we have repeated this experiment in the presence of phalloidin. As reported before [15] and seen in Fig. 5, phalloidin increases the melting temperature of F-actin by ~10 °C as a result of its strong stabilizing effect on the inter-subunit contacts in the actin filaments. In the sample with phalloidin we have also doubled the amount of F-actin to check if the actin effect on UTR261 conformation is saturated. It can be seen clearly that melting profiles of oxidised UTR261T36C/S242C in the presence and absence of phalloidin-F-actin are very similar (Table 1, Fig 5D). Thus, oxidised UTR261T36C/S242C binds phalloidin-F-actin in the closed state. To verify that phalloidin does not alter the interaction of UTR261 with F-actin we also performed DSC on WT UTR261 in the presence of phalloidin-F-actin. Results showed that the effects of F-actin and phalloidin-F-actin on the conformation of WT UTR261 are similar (Table 1, Fig 5A).

The melting profile of the reduced UTR261T36C/S242C in the complex with F-actin (Fig. 5C) was very similar to that of oxidised UTR261T36C/S242C (Fig. 5D). Again, to resolve reduced UTR261T36C/S242C and F-actin peaks we repeated the experiment with phalloidin. As one can see, in both scans reduced UTR261T36C/S242C melts as a single peak with $T_m$ ~68 °C (Table 1, Fig. 5C). Thus, the vast majority of the reduced UTR261T36C/S242C molecules adopt the closed conformation on F-actin, while in the absence of F-actin more molecules (~60%) are in the open state (Fig. 5D). These results indicate that for UTR261T36C/S242C mutant F-actin favours the closed state. Whilst the vast majority of WT UTR261 binds F-actin in the open state, we cannot exclude that a small fraction may be in the closed state on F-actin.

In other tandem-CH domain ABD structures such as those for α-actinin or filamin, mutations in the inter-CH domain interface affect actin binding [16,17]. In all cases the mutations in the CH1:CH2 interface region do not alter the gross structural conformation, in that both α-actinin 4 and filamin B crystal structures adopt a compact structure whether or not the mutations are present. However the presence of the mutations does increase the affinity of both the α-actinin and filamin ABDs for F-actin [16,17]. The α-actinin mutants appeared to retain their compact shape as determined by analytical ultracentrifugation [16], whereas the filamin B mutants are also associated with a reduction in the melting temperatures for this ABD. This would argue at the very least in favour of inter-CH domain rearrangement on binding to F-actin, or even the possibility of the proteins adopting an open conformation as shown previously [18]. By contrast the cysteine mutants in utrophin increased the melting temperature and reduced actin binding suggesting that a loss of CH1–CH2 flexibility reduced their affinity for F-actin. The highest resolution cryo-EM

### Table 1

| Proteins | $T_m$ (°C) $m_1$ | $T_m$ (°C) $m_2$ | $T_m$ (°C) $m_3$ |
|----------|-----------------|-----------------|-----------------|
| UTR261 WT | 53.3            | -               | -               |
| UTR261 1C | 52.6            | 68.6            | -               |
| UTR261 2C reduced | 56.3 | 68.1            | -               |
| F-actin | -               | 69.1            | -               |
| UTR261 WT + F-actin | 55.5 | 69.8            | -               |
| UTR261 1C + F-actin | 55.0 | 68.7            | -               |
| UTR261 2C reduced + F-actin | Shoulder at ~67 | 69.7 | -               |
| UTR261 2C oxidised + F-actin | Shoulder at ~67 | 69.6 | -               |
| F-actin-phalloidin | - | 80.0 | -               |
| UTR261 WT + F-actin-phalloidin | 56.2 | 79.8 | -               |
| UTR261 1C + F-actin-phalloidin | 56.5 | 80.6 | -               |
| UTR261 2C reduced + F-actin-phalloidin | - | 80.8 | -               |
| UTR261 2C oxidised + F-actin-phalloidin | - | 79.4 | -               |

The absolute errors in $T_m$ values did not exceed 0.2 °C. WT = wildtype sequence, UTR261 1C = UTR261T36C and UTR261 2C = UTR261T36C/S242C.

Fig. 4. Tryptophan fluorescence of UTR261 and cysteine mutants. Tryptophan fluorescence of 30 μM samples of each of UTR261 (red), UTR261T36C, (green) reduced UTR261T36C/S241C, (dark blue) and oxidised UTR261T36C/S241C (light blue). The introduction of cysteines slightly reduced the fluorescence emission, and furthermore the presence of two cysteines caused a slight red-shift of the spectrum whether the UTR261T36C/S241C was reduced or oxidised.
reconstructions of F-actin and F-actin with a tandem-CH domain ABD – that of fimbrin, however, demonstrate unequivocally that the two CH domains remain in a closed conformation with very little rearrangement required to match the crystal structure[19] and reviewed in [20]. However the situation regarding the utrophin ABD is less clear.

A number of cryo-EM reconstructions of UTR261 with F-actin using different methods of analysis have arrived at different conclusions. The earliest models had been derived from helical reconstructions had proposed that utrophin bound to F-actin in an open conformation, but that there was an induced fit onto actin requiring some rearrangement of the orientation of the CH domains relative to their position in the crystal structure[9]. However using a different method of analysis – iterative helical real space reconstruction, the Egelman group arrived at an alternative model[12]. In this model, although again the utrophin ABD was fitted in an open conformation, it was able to interact with F-actin in two different states depending on whether one or both CH domains were in contact with actin. Furthermore, in the Egelman study, questions were raised over the validity of using helical averaging techniques to derive a reconstruction from heterogeneously decorated actin filaments, and also as to the polarity of the filament used in the reconstructions[12]. However a further reconstruction comprising the utrophin ABD and the first spectrin repeat bound to F-actin, arrived at a third model – that of a closed conformation for the utrophin (and dystrophin) ABD on F-actin[21]. A further reassessment of all the evidence by the Egelman lab provided convincing arguments for utrophin binding to actin in different modes but in an open conformation, see [22] and discussions therein. The actin binding and DSC data presented here indicate that utrophin ABD binds to actin in an open conformation and add further compelling weight to the open conformation hypothesis. More recently, and despite evidence from solution studies and crystal structures of a closed conformation for α-actinin CH domains [4,22], [16] a cryo-EM reconstruction of α-actinin bound to F-actin predicted an open conformation[18]. The use of cysteine mutagenesis has also been employed in a recent electron paramagnetic resonance study by Lin and colleagues[10] to examine the opening and closing of the utrophin CH domains in solution and on binding to actin. Interestingly, in solution they identified a conformation almost identical to that of a single utrophin ABD as seen in the crystal structure (as in Fig. 1C) but in apparent equilibrium with an equally abundant species with a more closed conformation. However on binding to actin, there is only one population evident and this has an even more open conformation [10]. Thus the authors also conclude that utrophin binds to actin in an open conformation, but via an induced fit mechanism, ironically a conclusion also reached from the earliest EM reconstructions a decade earlier[9].

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