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Utrophin ABD binds to F-actin in an open conformation

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ABSTRACT

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. 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.

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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 spectroplakin family and other F-actin bundling or cross-linking proteins such as filamin and fimbrin. High-resolution atomic structures have been determined for members of each of these groups [2–4]. Furthermore, in an attempt to understand the structural and functional determinants for actin binding, several of these proteins have also been analysed in complex with F-actin by electron microscopy (EM); however, analysis of the utrophin ABD by this route has resulted in a number of conflicting models, reviewed in [5]. Part of the controversy may have arisen because in the original crystal structure of the utrophin ABD [6], despite being a monomer in solution the ABD crystallised as a dimer. Moreover, because of the orientation of the individual chains within the dimer it was suggested that a three-dimensional domain swap may have occurred (Fig. 1). The crystal structures of utrophin and dystrophin ABD reveal dimers in an extended conformation with CH domain 1 (CH1) of one crystallographic dimer interacting with CH domain 2 (CH2) of the other crystallographic dimer (Fig. 1A and B, red and blue structures) [6,7]. The α-actinin ABD on the other hand, which shares considerable sequence and structural homology [4,8], crystallised as a monomer with CH1 and CH2 from the same molecule in close apposition and in an orientation similar to CH1 and CH2 from opposite dimers of the utrophin or dystrophin structure (Fig. 1A and B, green structure).

Nonetheless the idea that the utrophin ABD could exist in an open extended and closed compact form was attractive, and to an extent supported by some of the available data [9]. One of the main arguments against the open conformation stemmed from available crystal structures. With the exception of utrophin and its close homologue dystrophin, all other tandem CH domains that had been crystallised, did so in a closed conformation. Because of the apparent domain swap in the utrophin and dystrophin crystals, possibly induced by the crystallisation at low pH, it has been argued that the extended conformation is an artefact of crystallisation and does not reflect a true state in solution [5], whereas more recent studies do suggest an open conformation [10]. We have therefore examined the binding properties of utrophin with F-actin using native utrophin that is allowed to adopt any conformation (open or closed) and in a closed conformation by introducing

Abbreviations: ABD, actin binding domain; CH, calponin homology; CD, circular dichroism; DSC, differential scanning calorimetry; EM, electron microscopy; F-actin, filamentous actin; NTCB, 2-nitro-5-thiocyanobenzoic acid; SDS-PAGE, sodium dodecyl sulphate poly-acrylamide electrophoresis; T_m, melting temperature; UTR261, utrophin residues 1-261

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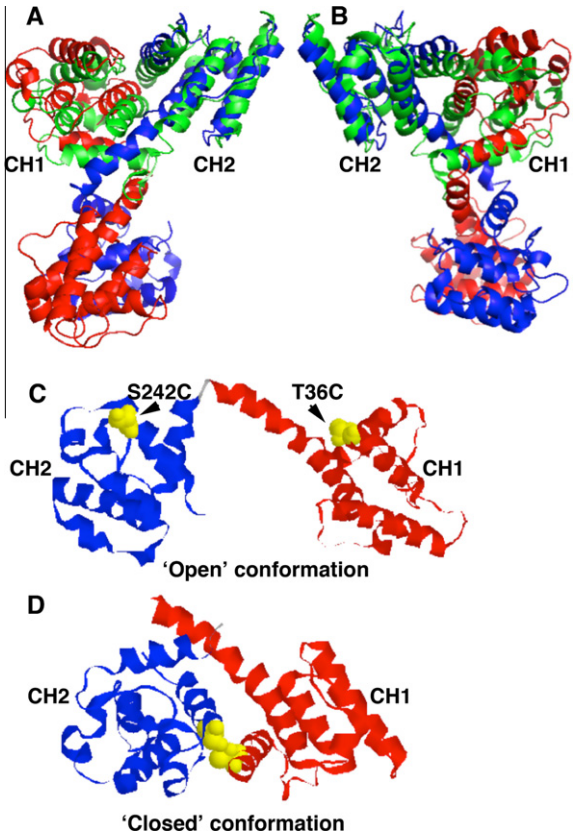

Fig. 1. The utrophin ABD structure. (A and B) Ribbon diagrams of two different views of the UTR261 crystal structure 1QAG comprising a dimer with two molecules shown in red and blue. These are overlayed with the structure of α-actinin 1WKU to demonstrate the apparent two-dimensional domain swap. Annotation to CH1 and CH2 refer to the corresponding CH domains of the UTR261 structure and the α-actinin fit. (C and D) Images of utrophin monomers in the open conformation and closed conformation derived from the structure in (A) and (B). The position of threonine 36 and serine 241 are shown in yellow spacefill.

cysteine residues into each CH domain to form an inter-CH domain disulphide that locks the two CH domains together. The utrophin ABD is easily expressed in bacteria, highly soluble (up to mM concentrations) and is stable. Furthermore, unlike dystrophin and α-actinin the primary sequence contains no cysteine residues making it an ideal model to introduce cysteine residues to address functional changes.

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 UTR261^{T36C}, and then the S242C mutation was introduced into UTR261^{T36C} to generate UTR261$^{T36C/S242C}$. UTR261^{T36C} and UTR261$^{T36C/S242C}$ 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-thiocyanobenzoic acid (NTCB) was carried out as described previously [11]. Oxidation or reduction of UTR261$^{T36C/S241C}$ was achieved overnight in 20 mM Tris (pH 8.0) in the presence of 4 mM *o*-phenanthroline and 1 mM $CuSO_4$ 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 UTR261$^{T36C/S241C}$ and oxidised UTR261$^{T36C/S241C}$ 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]. UTR261^{T36C} and UTR261$^{T36C/S241C}$ 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 UTR261^{T36C} as template, the second site was mutated to give UTR261$^{T36C/S242C}$. 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 UTR261$^{T36C/S242C}$ protein was susceptible to cleavage by NTCB. Furthermore, chemical oxidation of UTR261$^{T36C/S242C}$ 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 (19.2 ± 2.2 μM; [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 UTR261$^{T36C/S242C}$ (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

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