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Crystal Structures of Monomeric Actin Bound to Cytochalasin D

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Abstract

The fungal toxin cytochalasin D (CD) interferes with the normal dynamics of the actin cytoskeleton by binding to the barbed end of actin filaments. Despite its widespread use as a tool for studying actin-mediated processes, the exact location and nature of its binding to actin has not been previously determined. Here we describe two crystal structures of an expressed monomeric actin in complex with CD, one obtained by soaking preformed actin crystals with CD, and the other by co-crystallization. The binding site for CD, in the hydrophobic cleft between actin subdomains 1 and 3, is the same in the two structures. Polar and hydrophobic contacts play an equally important role in CD binding, and six hydrogen bonds stabilize the actin-CD complex. Many unrelated actin-binding proteins and marine toxins target this cleft, and the hydrophobic pocket at the front end of the cleft (viewing actin with subdomain 2 in the upper right corner). CD differs in that it binds to the back half of the cleft. The ability of CD to induce actin dimer formation and actin-catalyzed ATP hydrolysis may be related to its unique binding site, and the necessity to fit its bulky macrocycle into this cleft. Contacts with residues lining this cleft appear to be crucial to capping and/or severing. The co-crystallized actin-CD structure also revealed changes in actin conformation. A rotation of $\sim 6^\circ$ of the smaller actin domain (subdomains 1 and 2) with respect to the larger domain (subdomains 3 and 4) results in small changes in crystal packing that allow the D-loop to adopt an extended loop structure, instead of being disordered as it is in most crystal structures of actin. We speculate that these changes represent a potential conformation that the actin monomer can adopt on the pathway to polymerization or in the filament.

Keywords

actin; cytochalasin; domain motions; fungal toxin; crystal structure; filament capping

Introduction

The cytochalasins are a family of fungal metabolites widely used to study the role of actin in cellular processes, and to probe basic aspects of actin polymerization *in vitro*. Cytochalasins bind to the barbed or fast-growing end of filamentous actin (F-actin) with high affinity ($K_d \approx 2$ nM) (reviewed in Cooper¹). Cytochalasin binding inhibits, but does not completely stop, association and dissociation of actin monomers at the barbed end.^{2, 3} Monomeric actin (G-actin) also binds cytochalasin D (CD), but with lower affinity than F-actin ($K_d \approx 2$ –20 μ M, depending on the divalent cations present).⁴ Cytochalasin D induces actin dimer formation in

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the presence of magnesium, and eliminates the lag phase in polymerization, presumably due to accelerated nucleation by the dimers.^{4,5} ATP hydrolysis is also stimulated by CD,⁶ a feature that may contribute to the effect of CD on actin in the cell.³ Despite the wide use of this compound in both cellular and *in vitro* experiments, the structure of cytochalasin D bound to actin has not yet been reported, perhaps due to its tendency to mediate oligomer formation. The marine macrolide toxins, in contrast, depolymerize F-actin to a stable monomeric complex suitable for crystallization.⁷

Our strategy was to bind CD to an expressed cytoplasmic actin that was rendered non-polymerizable by virtue of two point mutations in subdomain 4 (A204E/P243K, referred to as AP-actin).⁸ This AP-actin retains all other biochemical⁸ and structural properties⁹ characteristic of tissue-purified G-actin. Here we report two crystal structures of AP-actin·CaATP complexed to CD, one obtained by soaking the compound into pre-formed protein crystals, and the other obtained by *de novo* co-crystallization. The CD binding site is the same in the two structures. However, the co-crystallized structure also exhibits large changes in actin conformation, including a rotation between the large (subdomains 3 and 4) and small (subdomains 1 and 2) domains of actin, and an ordering of the D-loop. These results are discussed with regard to how CD binding compares to that of other toxins, as well as the implications of the conformational changes observed in the actin structure.

Results

Cytochalasin D stimulates ATP hydrolysis by AP-actin

Binding of CD to monomeric AP-actin was demonstrated by its ability to enhance the rate of hydrolysis of bound ATP, similar to that observed with tissue-purified actin.⁶ Addition of CD to AP-actin greatly stimulated hydrolysis of bound CaATP or MgATP, with more rapid hydrolysis observed with MgATP (Figure 1A, B). Cytochalasin B, a related fungal metabolite which differs from CD in having a larger 14-membered macrocyclic ring and different substituents on this ring, did not enhance the rate of CaATP hydrolysis (Figure 1A, filled triangles), presumably due to its higher K_{app} for stimulation compared to that of CD.⁶ The stimulation of MgATP hydrolysis increased with CD concentration (Figure 1C). After 10 min, approximately half of the nucleotide was hydrolyzed in the presence of 5 μ M CD. In contrast, hydrolysis of half of the bound MgATP by AP-actin in the absence of CD required approximately 24 hrs (Figure 1B, open circles).⁹

Cytochalasin D binding site on actin

A crystal of AP-actin·CaATP was soaked into a solution containing CD. It diffracted to 2.5Å resolution, and was isomorphous with a reference crystal that contained the same amount of ethanol as was needed to solubilize CD (Table 1). The asymmetric unit contained one molecule each of AP-actin, ATP, and CD, two calcium ions, and 249 water molecules (Figure 2A). The CD structure was very well-defined in an isomorphous difference map, despite the relatively modest resolution of the overall structure (Figure 2C).¹⁰ Cytochalasin D binds in the cleft between subdomains 1 and 3, at the barbed or growing end of actin. CD did not induce ATP hydrolysis when diffused into pre-grown crystals, even after several days of soaking. Electron density in residual Fo-Fc and 2Fo-Fc maps, prior to building in a model for the ATP, showed the presence of the gamma-phosphate of ATP in the CD-bound actin structure. The isomorphous difference Fourier map of the CD-bound data versus the reference data also shows no detectable hydrolysis in the CD-containing crystal.

When ligands are soaked into preformed protein crystals, there exists the possibility that crystal packing forces may prevent the protein from either adopting its optimum ligand-binding conformation or from expressing a ligand-induced conformational change. To address this

possibility, we co-crystallized AP-actin and CD by growing crystals of AP-actin in the presence of CD (see Methods). A combination of micro- and macro-seeding ultimately resulted in a crystal that diffracted to 1.8Å resolution. Data were complete to a resolution of 2.06Å. The refined model consists of one molecule each of AP-actin, ATP and CD, two calcium ions, and 271 water molecules (Figure 2B). As with the soaked crystal, CD binds in the cleft between subdomains 1 and 3. Its position and stereochemistry were well-defined in residual Fo-Fc (Figure 2D) and 2Fo-Fc maps before any model for CD had been included in the model of the complex. The ATP nucleotide in the co-crystallized complex showed no evidence of hydrolysis, in spite of taking 2 days for the crystals to fully form, as determined from Fo-Fc and 2Fo-Fc maps prior to the inclusion of any model for the nucleotide.

The structures of CD in these complexes are similar to the one previously determined by small molecule crystallography.¹¹ The RMSD for the superposition of the CD molecule from the co-crystallized structure and the small molecule crystal structure is 0.29Å, while the comparable RMSD using the ligand-soaked crystal structure is 0.24Å. The RMSD for the superposition of the two CD structures determined here is 0.16Å. Of the two C=C double bonds at C13 and C19 in the macrocycle, both adopt the trans conformation, while the six-membered ring that forms part of the isoindolone core adopts a boat conformation (Figure 3B). The phenyl ring adopts a gauche conformation around the C3-C10 bond.

The CD-binding site is the same in both structures. CD binds along the length of the largely hydrophobic cleft between subdomains 1 and 3, with the concave face of the bicyclic isoindolone ring facing the actin cleft (Figures 2 and 4). The cleft is ≈20Å long and ≈10Å at its widest, with CD oriented such that its macrocycle is placed close to the back face of actin.

The binding pocket is bounded by three helices (residues 137–145, 338–348 and 350–355) that define its front half, while an underlying beta-strand (residues 131–136) defines its base (Figure 4). The back half of this cleft is defined by a loop and a short helix of subdomain 3 (residues 165–176) that connect two β-strands in this subdomain. This half of the cleft is bordered on the other side by the helical C-terminal region of the protein and a loop (residues 107–113) from subdomain 1. Of all the interacting residues, Ile136, Tyr169, Ala170, Pro172, Met355 and Phe375 make the most extensive contacts with CD (Figure 3A). This is based on the absolute surface area of the residue buried upon ligand binding, and is consistent with the number of van der Waals contacts made by each residue. There are six hydrogen bonds between the protein and ligand (Figure 3 and Table 2). Five of these are mediated by the isoindolone core and substituents on its rings, while one is water-mediated. With the exception of the Tyr143 side-chain, all of the hydrogen bonds involve actin main chain atoms. The C7 hydroxyl group and the amide group of the 5-membered lactam ring make extensive contacts with actin. The large number of interactions that involve the isoindolone core and substituents on its bicyclic ring suggest a key role for this moiety in stabilizing the actin-CD interaction. The macrocyclic ring predominantly makes van der Waals contacts. Only one hydrogen bond between the hydroxyl group at C18 and the backbone carbonyl of Ala170 involves the macrocyclic ring.

Based on the solvent accessible surface area buried by CD binding, both hydrogen bonds and hydrophobic interactions appear equally important in driving complex formation. Complex formation leads to the burial of 637Å² of the accessible hydrophobic surface on both ligand and protein in the soaked structure and 644Å² in the co-crystallized structure. Thus, 79–80% of the accessible surface area of the ligand is buried upon complex formation. We conservatively estimate that binding is accompanied by a solvation free energy change of approximately 10–20 kcal/mol (see Methods) which is roughly of the same magnitude as the enthalpic contribution made by the six hydrogen bonds between the ligand and protein observed in our structures.

Inter-domain and local shifts in actin upon CD binding

The co-crystallized actin-CD structure reveals several unusual features of actin. There is a shift of the smaller actin domain (comprised of subdomains 1 and 2) with respect to the larger domain (subdomains 3 and 4) (Figure 5A–C). The RMSD for the superposition of all C α atoms of actin from the co-crystallized complex on an AP-actin-ATP structure (PDB Id: 2HF4)⁹ is 1.07Å. However, the RMSDs for the superposition of their large and small domains, independent of each other, are 0.41Å and 0.43Å respectively, which are on the order of the 0.35Å estimated coordinated error (Table 1).

The significance of these changes, evaluated with the program DYNDOM¹², suggests that the smaller domain as a whole moves as a consequence of CD binding (Table 3). The magnitudes of the rotation angles suggest that the rotation of the small domain, while modest in the soaked CD-bound structure, is clearly manifested in the co-crystallized CD-bound actin. Actin co-crystallized with CD was also compared to the AP-actin-ATP structure (PDB Id: 2HF4)⁹ using error-scaled difference distance matrices (ESCET)¹³ (Figure 5D). The upper right half of the plot shows the absolute distance differences between equivalent C α atoms of the two structures, while the lower left half shows error-scaled distance differences between them. Blue represents a contraction in one protein relative to the other, while red represents an expansion, and color gradients indicate the magnitudes of the changes. The lower left half of Figure 5D shows only those changes that are greater than twice the estimated radial positional error associated with each element of the difference distance matrix. The plot shows that the smaller domain of actin and the larger domain move essentially as rigid units relative to each other. The results obtained from our analysis using both DYNDOM and ESCET are largely consistent.

Binding of CD thus generates a twist that results in a ‘backward’ motion of subdomain 1, and a simultaneous ‘forward’ motion of subdomain 2. A consequence of this twist is a shift in the geometry of the active site residues that define the ATP-binding cleft. A superposition of the ATP molecule from the co-crystal structure on that of the native structure exactly superposes their large domains, suggesting that the ATP molecule is bound to and oriented primarily by the large domain. Changes in the ATP-binding cleft include a movement of the sensor loop (residues 70–78), and negligible shifts of the P1 and P2 loops (residues 11–22 and 150–159, respectively) and Lys213 and Tyr206 side-chains. We also carried out a superposition of the soaked and co-crystallized complexes using only their corresponding CD molecules. Local changes in the vicinity of the CD-binding cleft included the position of the loop spanning residues 107–114. The remaining residues of sub-domain 1 of the two actin molecules neatly superposed. However, the larger domains of actin from the two structures were significantly shifted, reaffirming the domain-level nature of the change induced by CD binding that is observed in the co-crystallized structure.

A comparison of the structure of the soaked AP-actin-CD complex with its native isomorph revealed only local changes. These include a minor widening of the CD-binding cleft and local shifts in the positions of Tyr133, Tyr169 and Met355 side-chains (Figures 3A and 4). Local shifts along the protein backbones of Tyr166, Glu167 and Phe375 are also observed.

D-loop ordering

The torsional motion described above results in changes in crystal packing contacts, which allows the D-loop (residues 40–49) to adopt an ordered, extended conformation (Figure 5), the second striking feature of the structure. The D-loop has been proposed to play a critical role in actin polymerization¹⁴, but is disordered in most structures of actin. This is the sixth instance where it has been found to be ordered; the other instances being the complex structures of actin-DNase I¹⁵, β -actin-profilin¹⁶, TMR bound actin-ADP¹⁷, WASP-actin-DNase I¹⁸ and yeast

actin-gelsolin segment I¹⁹. The open and extended D-loop conformation is stabilized by extensive contacts with 3 neighboring actin molecules, including an actin monomer that is translated by ~54Å along the crystallographic b axis (cyan in Figure 6). The relationship between neighboring molecules along this axis is very similar to that seen among longitudinally related monomers in an actin filament. The D-loop bridges the cleft between subdomains 1 and 3 of the 'longitudinally' adjacent monomer in the crystal, but does not penetrate this cleft as is often seen in filament models of actin²⁰.

Residues from the D-loop do not directly interact with CD from the symmetry-related molecule (Figures 6B and C). Contacts stabilizing the D-loop include a stacking interaction between the His40 side-chain and the Tyr169 side-chain of the longitudinally placed symmetry-related monomer, which in turn partially stacks on the phenyl ring of CD (Figure 6C). This conformation of the D-loop is stabilized by 5 hydrogen bonds and several van der Waals interactions with the three adjacent actin monomers.

Discussion

Here we define the binding site for CD on actin, as well as provide evidence for novel conformational changes in actin itself. The binding site for CD on actin was identified by soaking the compound into pre-existing crystals of AP-actin, as well as by *de novo* co-crystallization of AP-actin with CD. The CD binding site was largely identical in the two structures, but differs slightly in its location from other toxins that target the same hydrophobic cleft between subdomains 1 and 3 (described in detail in a subsequent section). This cleft is also targeted by a number of actin-binding proteins (reviewed in Dominguez¹⁴).

Unexpected conformational changes were observed in the actin from the co-crystallized actin-CD complex. There was an ~6° rotation of subdomains 1 and 2, with respect to the larger domain composed of subdomains 3 and 4. This can be described as a 'backward' motion of subdomain 1 and a simultaneous 'forward' motion of subdomain 2 (see Figure 5). A subtle change in crystal packing caused by this rotation was found to be compatible with an ordered, extended D-loop conformation, a region which is disordered in most structures of actin. The D-loop adopts conformations that approximate a β -hairpin in the structures of actin bound to DNase I¹⁵ and profilin¹⁶, and forms a right-handed α -helical conformation in TMR-bound ADP-actin¹⁷. The D-loop adopts a more irregular and non-periodic, extended conformation in our co-crystallized CD-bound form, attesting to the conformational plasticity of this region.

The different conformations of actin that have been observed in crystals, and the packing contacts between adjacent monomers in crystals, have often been related to potential conformations that actin can adopt in the filament. Do the changes seen here reveal a conformation that might be relevant for the F-actin structure? Recent modeling of the F-actin structure by Maeda and co-workers, based on new higher resolution X-ray fiber diffraction data, suggests that actin polymerization is accompanied by a flattening of the actin conformation, caused by a propeller-like relative rotation of the two domains on either side of the nucleotide binding cleft.^{21; 22} Their filament model also suggests that the D-loop should not be compact but extended.²¹ Although the details of the changes involved have not yet been published, the simultaneous forward motion of subdomain 2 and backward motion of subdomain 1, relative to the larger domain, that we observed in the actin-CD co-crystal, along with the extended D-loop conformation, would appear to generate a conformation that more closely resembles actin in this new filament model.

The similarity of the contacts between longitudinally placed actin monomers in current published models of the actin filament²⁰, and symmetry related contacts between actin monomers in the crystal, is striking (Figure 7). This similarity has been also observed in other

crystal forms^{23; 24}, highlighting the likelihood that these contacts will be recapitulated in the F-actin filament. What has not yet been seen is the full rotation of the monomers that will likely need to occur to generate F-actin. To observe this, native contacts between monomers may need to be stabilized by cross-linking prior to crystallization.

Effect of CD on ATP hydrolysis and polymerization

Experiments described here and by others⁶ show that CD enhances ATP hydrolysis, but this effect is not observed in either the CD-soaked or the co-crystallized complex. The enhancement is, however, much less pronounced with CaATP than with MgATP (Figure 1). Moreover, the CD-soaked crystal may not allow the domain motions necessary for hydrolysis. The occurrence of ATP in the co-crystallized complex may thus be explained by the low rates of its hydrolysis in the presence of Ca²⁺ ions and may also suggest that rate limited conformational changes or actin dimerization are essential for this process to occur. Despite the absence of ATP hydrolysis, the co-crystallized complex offers tantalizing insights into the nature of this process. As mentioned previously, small shifts at the active site are observed as part of the larger process of movement of the actin domains. Previous high resolution structures of non-vertebrate actins have allowed the tentative identification of the nucleophilic water molecule.¹⁹ This putative nucleophilic water is bound to and positioned by the Gln137 and His161 side-chains. In our structure of the co-crystallized complex, this water is attached to Gln137 and appears to move together with its side-chain as part of the larger inter-domain movements consequent upon CD binding. This water molecule is placed at a distance of 4.95Å from the γ-P atom in the AP-actin·CaATP structure⁹ (PDB Id: 2HF4), 4.47Å in the structure of the soaked complex, and 4.27Å away from the γ-P atom in the co-crystallized complex. This movement of the nucleophilic water may explain the enhanced rate of ATP hydrolysis observed upon CD binding. Vorobiev *et al.*¹⁹ also noted that this nucleophilic water was placed at variable distances from the gamma phosphoryl group depending on the identity of the bound cation, and used this to explain the differential rates of ATP hydrolysis in the presence of different cations. There are also very small shifts in the positions of residues of the P1 and P2 loops. The sensor loop (69–78) moves significantly in tandem with the smaller domain as part of the larger inter-domain rotation, but the significance of this shift remains unclear.

Our structures are consistent with biochemical data which show that cytochalasins target the barbed end of the actin filament with a stoichiometry of one cytochalasin molecule per filament, and inhibit association and dissociation of monomers from this end (reviewed in Cooper¹). Current models of the actin filament, based on the actin monomer structure fit to X-ray fiber diffraction data, propose that the hydrophobic cleft between subdomains 1 and 3 is involved in inter-subunit contacts along the filament.²⁵ In more recent versions of this model,²⁰ the D-loop (residues 39–49) of one monomer projects into the cleft of an adjacent monomer along the filament long axis (Figure 8).¹⁴

Actin-binding proteins that cap or disrupt filaments can do so either by displacing an existing monomer-monomer interaction or by blocking access to an incoming monomer. A superposition of any of our structures onto a monomer within the filament suggests that CD binding to the barbed end would sterically hinder access to the cleft, thereby capping the filament (Figure 8). Its small size, the existence of a single CD binding site on actin, and the almost buried nature of the CD-actin subunit contacts in the filament are consistent with a capping mechanism of depolymerization.¹

Comparison of CD binding with that of other toxins

The crystal structures of monomeric actin bound to a number of natural small molecule inhibitors of actin dynamics have recently been solved (reviewed in Allingham *et al.*²⁶). Almost all classes of these compounds target a hydrophobic patch on the front face of actin between

subdomains 1 and 3, and the front part of the cleft between these subdomains. Interest in these molecules as potential candidates for cancer treatment has increased,^{27; 28} and, therefore, understanding the structural basis of the recognition of actin and toxin has become critical. A potential practical use of our AP-actin crystals is to use them as a means to readily determine the structure of novel actin-binding compounds that are synthesized, by soaking these compounds into preformed crystals of AP-actin. The compounds would thus not need to depolymerize actin to a monomer in order for their structure to be determined.

Here we show that CD binds to the back half of the hydrophobic cleft between subdomains 1 and 3, and that its binding to actin is stabilized by both polar and hydrophobic interactions. In contrast, the large macrolide ring of the macrolide toxins rests against a hydrophobic patch on the front face of actin close to the hydrophobic cleft, while the aliphatic tail of the toxin inserts into the front portion of this cleft (Figures 4 and 9). It has been hypothesized that the initial attachment of the toxin to the exposed hydrophobic pocket of an actin monomer in the filament is crucial for the severing action of these toxins which is brought about by the subsequent displacement of the D-loop of the adjacent monomer by the aliphatic tail.²⁹ Consistent with this hypothesis, changes in the macrolide ring, as from swinholide A to misakinolide A, result in a loss of severing activity.³⁰ The non-macrolide marine toxin bistramide A spans almost the entire length of the hydrophobic cleft when bound to actin.³¹ Its binding involves an extensive network of hydrogen-bonding contacts as well as hydrophobic interactions. Recent evidence suggests that bistramide A actively severs filaments.³² These data also support the notion that hydrophobic interactions at the front end of the cleft are crucial for filament severing.

The different toxin binding modes become evident when the actin-bound structures of the inhibitors are superimposed (Figure 10). The entire CD molecule (orange) overlaps with the central amide bonded γ -amino acid and an ether ring of bistramide (cyan). This is consistent with CD occupying the back half of the cleft. There is very little overlap between the binding sites of CD and the macrolide toxin jaspisamide A (green), and accordingly only a small segment of the aliphatic chain and the terminal N-methylvinyl formamide (MVF) moiety of jaspisamide A overlaps with the isoindolone ring of CD and its benzyl substituent. (The molecular structures of bistramide and jaspisamide are shown in Supplementary figures 1 and 2, respectively)

Despite their disparate overall regions of binding, there is considerable similarity in the binding of the terminal N-MVF moiety of jaspisamide A and CD (Figure 10B). The substituted isoindolone core of CD is involved in several hydrogen bonds. These include two bonds involving the C7 hydroxyl group (one of which is water-mediated), and one each involving the carbonyl oxygen and nitrogen of the lactam ring (Figure 3B and Table 2). Although the jaspisamide tail does not extend into the back half of the cleft, three of these four interactions are observed in the actin-jaspisamide A structure, two of which are water-mediated. All four of these hydrogen bonds are conserved in the actin-bistramide A structure, which involve the two amide bonds of bistramide A. Hydrogen bond interactions involving the backbone amide groups of Ile136 and Ala170, and the hydroxyl group of the Tyr143 side chain are conserved in most barbed end targeting macrolide toxins. Toxins such as reidispongiolide C and ulapualide A, which lack the N-MVF moiety or possess additional interfering groups, display significantly reduced capping and severing activity,²⁹ demonstrating the significance of the above interactions in the binding of barbed end targeting toxins, including CD, to actin. Both bistramide A and CD differ from other barbed end targeting toxins in that their binding is characterized by a significant number of polar contacts, which may be related to their ability to target the back half of the cleft.

Comparison of CD binding with actin binding proteins

The structures of numerous actin-binding proteins that target the barbed end are available in complex with the actin monomer. These include gelsolin,³³ cofilin,³⁴ vitamin D-binding protein,^{35; 36} profilin,¹⁶ the formin homology 2 domain of formin,³⁷ and WASP-homology domain 2.¹⁸ All of these proteins, with the exception of profilin, bind to actin by inserting an α -helix into the front section of the hydrophobic cleft (reviewed in Dominguez¹⁴). Although the binding footprints of these proteins on actin are much larger than that of the toxins, there is some overlap between the binding sites of these proteins and that of CD, which can be narrowed down to a loop (167–173) in the back half of the actin cleft, residues on the hinge helix, and residues from the helical segments between 345 and 356 that line the cleft. It is interesting to note that of all the proteins listed above, profilin alone has limited contacts with the front portion of the cleft. Indeed, nearly all of its interactions are limited to the back half of the cleft. Profilin is known to preferentially bind to ADP-actin monomers and cap nucleation of new filaments. Therefore, contacts with this region of the cleft may be sufficient to function as an effective cap. Significantly, profilin does not contact the hinge helix, although it has been proposed that it triggers conformational changes which lead to the opening of the major cleft and exchange of ADP for ATP in ADP-bound monomers.³⁸ This leads us to suggest that the hinge helix may not be the sole mediator of cross-cleft signaling, and is supported by the nature of the inter-domain shifts observed in the co-crystallized AP-actin·CD structure.

Materials and Methods

Expression and purification of AP-actin

A non-polymerizable mutant construct of the *Drosophila* 5C cytoplasmic actin with the point mutations A204E/P243K (AP-actin) has been previously characterized.⁸ Note that *Drosophila* 5C actin is >98% identical with human γ -cytoplasmic actin. The numbering system used here corresponds to that of skeletal muscle actin. Infection of Sf9 cells with recombinant baculovirus encoding the AP-actin construct and purification of AP-actin from the cells has also been described.⁸

Effect of CD on the rate of hydrolysis of ATP bound to AP-actin

Unbound ATP was removed from AP-actin·CaATP or AP-actin·MgATP in G-buffer (5 mM Tris-HCl, pH 8.2, 0.2 mM NaATP, 0.2 mM CaCl₂, 0.5 mM DTT, 0.1 mM sodium azide, 1 μ g/ml leupeptin) by addition of 10% by volume of a 50% slurry of Dowex AG-1 $\times$ 8. The suspension was mixed for 1 min on ice, and spun 20 sec at 10,000 $\times$ g. The Dowex treatment was repeated two more times. The final supernatant was spun 20 min at 300,000 $\times$ g. The AP-actin·CaATP (4 mg/ml) was converted to AP-actin·MgATP by addition of 0.2 mM MgCl₂ and 0.5 mM EGTA for 10 min on ice. For the time course of ATP hydrolysis, AP-actin (50 μ l at a concentration of 4 mg/ml or $\sim$ 100 μ M) was incubated with 100 μ M CD for varying times on ice. All samples including zero time had 2% ethanol. Ethanol was used to solubilize CD and prepare a stock solution. For the dependence of ATP hydrolysis on CD concentration, varying concentrations of CD were added to 50 μ l samples of 4 mg/ml AP-actin, and incubated for 10 min on ice prior to stopping the reaction. The reaction was stopped by addition of 50 μ l of 10% perchloric acid to precipitate the protein and liberate nucleotide. After 5 min on ice, 20 μ l of 4 M potassium acetate in 10 M KOH was added to neutralize the solution, and the sample was spun for 10 min at 12,000 $\times$ g followed by 10 min at 300,000 $\times$ g. The supernatant was diluted to 200 μ l with water and loaded on a 1 ml Mono Q HR5/5 column (Pharmacia) equilibrated with 5 mM triethylammonium bicarbonate pH 8.5 (Sigma-Aldrich) using an AKTA-FPLC system (GE Healthcare). The nucleotides were eluted with a 10 ml gradient of 5 mM to 0.5 M triethylammonium bicarbonate pH 8.5, followed by an additional 4 ml of 0.5 M triethylammonium bicarbonate pH 8.5.

Crystallization and Diffraction Data Collection of CD-soaked actin

Crystals of AP-actin were first obtained by vapor diffusion at 4°C. The protein at 10–11 mg/ml in 5 mM Tris-HCl, pH 8.2, 0.2 mM NaATP, 0.2 mM CaCl₂, 0.5 mM DTT, 0.1 mM sodium azide, 1 µg/ml leupeptin, was mixed with an equal volume of reservoir buffer composed of 25% 2-methyl-2,4-pentanediol (MPD), 100 mM NaAcetate, pH 4.8, 100 mM NaCl, and 40 mM CaCl₂. Crystals arose and attained their full sizes in roughly 7–10 days. Diffraction quality crystals were obtained after several rounds of microseeding. For the microseeding experiments, 7 mg/ml protein was mixed with an equal volume of reservoir buffer composed of 12.5–16.5% 2-methyl-2,4-pentanediol, 100 mM NaAcetate, pH 4.8–5.2, 100 mM NaCl and 20–40 mM CaCl₂. These crystals attained useful sizes within 4–5 days of microseeding. One mM CD, dissolved in a solution containing 90% MPD and 10% ethanol was added to the actin crystals in the mother liquor, and left to soak for 4 days prior to cryofreezing and data collection. Final concentrations were 167 µM CD and 1.7% ethanol. Reference crystals were treated similarly except for the omission of CD, in order to obtain a reference dataset that was isomorphous with the CD-soaked crystals.

Diffraction data from CD-soaked and reference crystals were collected using a MAR345 image plate mounted on a Rigaku RUH3R rotating anode X-ray generator at a temperature of 100K. The crystals diffracted to a resolution of 2.5 Å and belong to the space group C2. Data from two different CD-soaked crystals were processed and merged using DENZO and SCALEPACK, respectively.³⁹ Data collection and processing statistics are given in Table 1.

Crystallization and Diffraction Data Collection of co-crystallized actin and CD

Microseeds of AP-actin-CaATP were added to equilibrated drops containing an equimolar solution of AP-actin-CaATP and CD. Conditions for microseeding were identical to those described above. However, precipitation of the protein stalled the growth of microseeds after a few hours. We thus adopted a two-step protocol involving both microseeding and macroseeding to get crystals of the complex. Microcrystals obtained after 6–8 hours of growth of the microseeds were used to seed fresh, equilibrated drops of AP-actin-CaATP-CD. After 6–8 hours of growth, these co-crystals were transferred again to fresh solutions of the protein-ligand complex. The macroseeding process was repeated three times. After the final round of growth, crystals were scooped and frozen in liquid nitrogen.

Most of the co-crystals made by the above method had very high mosaicities. Nevertheless, a single crystal was used to collect data at the home source described above at 100K. Diffraction data were collected to 2.8 Å using a fine slice of 0.3° per frame. A total of 200° of data were collected. The crystal belonged to the space group C2 but had slightly different cell dimensions from the ligand-soaked crystals and was not isomorphous with the latter. The same crystal was subsequently used to collect data at the Advanced Light Source (Argonne National Laboratory, Illinois) where it diffracted to 1.8 Å. Using a ϕ rotation angle of 1° per frame, a total of 110° of data were collected at 100K. The two sets of diffraction data were processed and merged using HKL2000.³⁹ Data collection and processing statistics are given in Table 1.

Structure Determination and Refinement

The structure of CD-bound actin from the soaked method was determined by molecular replacement using the program EPMR,⁴⁰ with the protein portion of the solved structure of ATP-bound AP-actin (PDB Id: 2HF4) as the initial model. All atomic B-factors were reset to 10 Å² and the structure was refined with CNS⁴¹ using data in the 15–2.5 Å resolution range. Manual rebuilding of the model was carried out using the molecular graphics programs COOT⁴² and vuSette zc (M.A.R), based on simulated-annealed omit maps. A single molecule of ATP and two calcium ions were incorporated into the model after several iterations of model building and refinement. Water molecules were incorporated into the model if they gave rise

to peaks exceeding 3σ in Fo-Fc density maps and if they satisfied hydrogen-bonding criteria. The bound cytochalasin D molecule was clearly revealed by an isomorphous difference fourier map calculated using observed diffraction amplitudes from the CD-containing crystals and the reference isomorph, and phases from the AP-actin model before any model for the CD has been built (and thus entirely free of any possible model bias for CD, Figure 2C). Details of the method are given in Rould.¹⁰ Guided by this isomorphous difference map and residual Fourier maps, an atomic model for CD was built in the final stages of refinement. The final refined model had good stereochemistry with 89.8% of the residues in the most favored regions of the Ramachandran plot with none in the disallowed or generously allowed regions (Table 1). For comparison purposes, a molecular model for the isomorphous reference crystal was similarly refined, with final statistics listed in Table 1.

The structure of co-crystallized CD-bound actin was determined by molecular replacement using EPMR⁴⁰ with the protein portion of the solved structure of AP-actin-CaATP-CD from soaking as the initial model. Atomic B-factors were reset to $10\text{\AA}^2$ and the structure was refined with CNS⁴¹ using data between 50 and $1.8\text{\AA}$ resolution. Manual rebuilding of the model was carried out with COOT⁴² using simulated-annealed omit maps. A single molecule of ATP and two calcium ions were incorporated into the model after several iterations of model building and refinement. Water molecules were incorporated into the model if their peaks exceeded 3σ in Fo-Fc electron density maps, and they satisfied hydrogen-bonding criteria. Cytochalasin D, bound at the same site as in the structure determined by soaking in ligand, was clearly visible in unbiased residual electron density maps at 3σ . Cytochalasin D was added to the model after several rounds of model refinement once the R-free was ~ 32 . The D-loop (residues 40–49) was clearly visible in unbiased residual as well as 2Fo-Fc maps. A model for the D-loop was built manually into Fo-Fc maps as the final step of refinement. The final model has good stereochemistry with 90.5% of the residues in the most favored regions of the Ramachandran plot (Table 1).

Displaced Solvent-Accessible Surface Calculations

Solvent-accessible surface areas were calculated for both free actin and CD and the actin-CD complexes using the program AreaIMol which is part of the CCP4 suite of programs.⁴³ The change in free energy of solvation associated with CD-actin binding was estimated based on the linear dependence of the free energy of solvation on the total displaced solvent accessible surface area upon ligand binding.⁴⁴ Atomic solvation parameters used for estimating the change in solvation free energy were the same as those determined by Eisenberg and McLachlan.⁴⁴

Analysis of ligand-induced shifts and inter-domain rotational angles

The program ESCET¹³ was used to assess the significance of all backbone coordinate shifts, including small shifts induced by ligand binding. Concerted domain-level shifts were also detected using the program DYNDOM.^{12; 45}

Accession Codes

The PDB codes are 3EKS (co-crystallized structure of AP-actin + cytochalasin D), 3EKU (AP-actin + cytochalasin D determined by the soak method) and 3EL2 (AP-actin control for 3EKU).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AP-actin	an expressed non-polymerizable actin containing the mutations A204E/P243K
CD	cytochalasin D
D-loop	residues in subdomain 2 of actin that comprise part of the DNase I binding site
N-MVF	N-methylvinyl formamide
WASP	Wiskott-Aldrich syndrome protein
MPD	2-methyl-2,4-pentanediol

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Figure 1.

Cytochalasin D stimulates ATP hydrolysis by AP-actin. (A) The time course of CaATP hydrolysis or (B) MgATP hydrolysis was followed as a function of time on ice in the absence (open blue circles) or the presence (filled red circles) of 100 μ M CD. The filled triangles in (A) were data obtained in the presence of 100 μ M cytochalasin B. (C) Effect of CD concentration on the amount of MgATP hydrolyzed after 10 min on ice.

Figure 2.

Structure of CD bound to actin. (A) Ribbon diagram showing AP-actin bound to CD as determined by soaking protein crystals in ligand solution. The four subdomains of actin are labeled. CD, in space-filling representation (orange), binds between subdomains 1 and 3. ATP, in stick representation (orange), binds between subdomains 2 and 4. Two Ca^{2+} ions, one at the primary divalent cation binding site in the vicinity of the β - and γ -phosphates, and the other close to the hydrophobic plug between subdomains 3 and 4, are shown as yellow spheres. (B) Ribbon diagram showing AP-actin bound to CD as determined by co-crystallization. Color-coding as well as ionic and molecular representations are identical to those in A. (C) Portion of an isomorphous difference map between CD-bound and native actin ($F_{\text{obs,actin,CD}} - F_{\text{obs,actin}}$) showing positive difference density (in green) contoured at 2.2σ . This map was calculated using diffraction data from the ligand-soaked crystal and its corresponding native isomorphous data. The map shows unambiguous positive electron density (in green) for CD. The map shown was calculated using a model before CD was ever built in. (D) Portion of an unbiased $F_o - F_c$ map at 2.0σ calculated using diffraction data collected from a crystal obtained by co-crystallization of actin with CD. Figures C and D clearly demonstrate the presence of CD in the cleft between subdomains 1 and 3. All figures were rendered using PyMOL (DeLano Scientific).

Figure 3.

Hydrogen bonding interactions between actin and CD. (A) There are six hydrogen bonds between the protein and ligand. Five of these are mediated by the isoindolone core and substituents on its rings, suggesting a key role for this moiety in stabilizing the actin-CD interaction. One hydrogen bond involves the macrocyclic ring. All but one hydrogen bond (Tyr143) involves the actin main-chain. This figure was made using the structure of the soaked CD-bound complex; nearly identical interactions are observed in the co-crystal structure. (B) Schematic showing molecular structure of CD and hydrogen bonding interactions with amino acid residue chemical groups. CD possesses an isoindolone core (pink and yellow) carrying numerous substitutions, including a benzyl group (green) at C3, and this core is fused to a large

11-membered macrocycle (blue). Five of the six hydrogen bonds involve the amide group of the lactam ring (pink) and the –OH at C7 suggesting a crucial role for these functional groups and their overall arrangement in CD-actin binding. Hydrogen bond distances are provided in Ångströms with the first number corresponding to the distance observed in the ligand-soaked complex structure and the second number to the distance in the co-crystallized complex.

Figure 4.

Interactions of CD with actin. Stereoview of the hydrophobic cleft between subdomains 1 and 3, with CD (orange) bound close to the back half of the cleft. Amino acid residue side-chains that interact with CD are shown as sticks. This figure was made using the structure of the soaked CD-bound complex; nearly identical interactions are observed in the co-crystal structure.

Figure 5.

Comparison of actin from the CD-bound co-crystal structure with that of an AP-actin-ATP structure (PDB Id: 2HF4)⁹ (A) Superposition of the structure of AP-actin from the co-crystallized CD bound form (red), on the structure of ATP-bound AP-actin (2HF4)⁹ (black). Subdomains 1–4 are indicated. Only alpha-carbon atoms from the large domain (subdomains 3 and 4, residues 139–338) were used for this superposition. The RMSD for this superposition is 0.41Å, whereas the RMSD for an overall superposition is 1.07Å, indicating that subdomains 3 and 4 move as a single rigid body upon binding CD. (B) Superposition of the same structures using the alpha-carbon atoms of the small domain (subdomains 1 and 2, residues 5–137 and 339–374). The RMSD for this superposition is 0.43Å, indicating that subdomains 1 and 2 also behave as a single rigid body upon binding CD. Color-coding is the same as in Figure 5A. (C) A 90° rotation of the structure in Figure 5A around a vertical axis, indicating that the small domain twists relative to the large domain upon binding CD. (D) A comparison of the structure of actin from the CD-bound co-crystal structure with the AP-actin-ATP structure (PDB Id:

2HF4)⁹ using a difference distance matrix generated by the program ESCET.¹³ The upper right half of the plot shows absolute difference distances between the two structures in Ångströms, while in the lower left half of the plot, the same difference distances are error-scaled. A contraction in the AP-actin-ATP structure is shown in blue while an expansion is shown in red. Color gradients represent the magnitudes of the changes. The colored stripe at the base of the matrix represents subdomain boundaries in actin. Subdomains 1, 2, 3 and 4 are represented by green, yellow, orange and black respectively. The red arrows along the margins of the plot mark the position of the D-loop in the AP-actin sequence (since this segment is missing in one of the two structures compared, it is omitted from the plot).

Figure 6.

Stabilization of the D-loop by crystal packing interactions. (A) Crystal packing interactions involving the D-loop seen in the co-crystallized AP-actin-ATP-CD structure. The D-loop interacts with 3 neighboring symmetry-related molecules (shown in cyan, magenta and yellow). Cytochalasin D molecules, bound to the four actin monomers, are represented as sticks in orange. Crystal packing interactions between the two longitudinally placed monomers (in blue and cyan) are reminiscent of the interactions seen between actin monomers along the long axis in actin filament models. (B) Enlarged view of the interactions between the D-loop and residues from neighboring symmetry-related actin molecules. The D-loop is shown in stick representation (atom-based coloring with carbon atoms in blue, nitrogen atoms in indigo,

oxygen atoms in red and sulfur atoms in yellow), and interacting residues from neighboring molecules are shown as spheres and labelled. Also shown is unbiased residual electron density (Fo-Fc) for the D-loop (residues 39–49) as well as CD (orange) bound to a symmetry-related actin molecule (cyan). The residual map was calculated using a model preceding the building of the D-loop or the CD molecule. Gln49 of the D-loop was modeled as an alanine as the side-chain of this residue appears disordered. (C) Stereoview of the interactions between D-loop residues and those of the longitudinally placed symmetry-related monomer. Color-coding is the same as in Figures 6A–B. There are two hydrogen bonds between the D-loop residues and the residues of the symmetry-related monomer, including a water-mediated bond. The side-chain Tyr169 stacks on the His40 side-chain of the D-loop. The aromatic phenyl ring of CD in turn partially stacks on the Tyr169 ring. A minor conformer of the His40 side-chain forms a hydrogen bond with the symmetry-related CD molecule, but it was not modeled.

Figure 7.

Comparison of the contacts between longitudinally placed actin monomers in current models of the actin filament and packing contacts between actin monomers in the crystal. (A) Arrangement of actin monomers along the long axis in current models of the filament.²⁰ (B) Longitudinally placed actin monomers in the crystal showing a similar arrangement to that seen in the actin filament. (C) View generated by the application of a 50° rotation along the vertical-axis and a 10° rotation along the horizontal-axis to the structure in Figure 7A. (D) Application of identical rotations along to the structure in Figure 7B generates this view. Figures 7C and 7D highlight the differences in interactions between the filament model and crystal packing, which are a consequence of increased inter-domain twisting as well as an

increased twist angle between monomers observed in the filament model. It is noteworthy that the D-loop forms a bridge between subdomains 1 and 3 of the adjacent monomer in the crystal while penetrating the hydrophobic cleft between subdomains 1 and 3 in the filament.

Figure 8.

Representation of two actin monomers showing contacts along the long axis of the actin filament. The structural model of the filament shown here (actin monomers in blue and green) was proposed by Holmes.²⁰ The binding of CD (orange) between subdomains 1 and 3 would compete with the formation of the inter-monomer contacts necessary for filament growth at the barbed end.

Figure 9.

A comparison of the barbed end targeting compounds CD, bistramide A and jaspisamide A as they would bind to the hydrophobic cleft. (A) 'Back' view of the actin molecule (spaghetti representation in blue). CD (orange) targets the back half of the cleft between sub-domains 1 and 3. (B) 'Front' view of the actin molecule. The macrocyclic ring of jaspisamide A (green) binds to a hydrophobic patch on the front face of actin bordering the cleft. (C) View of the hydrophobic cleft. The binding regions of jaspisamide A and CD on actin barely overlap. Bistramide A (cyan) spans the length of the cleft. Apart from the present structure, this figure is based on the crystal structures of jaspisamide A (PDB Id: 1QZ6) and bistramide A (PDB Id: 2FXU) complexed with an actin monomer.

Figure 10.

(A) Stick representation of CD (orange), jaspisamide A (green) and bistramide A (cyan) showing regions of overlap when bound to actin. The orientation is nearly identical to that in Figure 9C. (B) Enlarged view of the overlapping regions among CD, bistramide A and jaspisamide A. Ligands are represented as thick sticks whereas interacting segments of actin are represented as thinner sticks. Water molecules are represented as spheres. Hydrogen bonding interactions that are conserved between actin and the ligands are represented by dotted lines. While three of the four interactions are conserved in all three complexes, one water-mediated interaction is observed only in the CD- and bistramide A bound structures (see Table 2). Although the jaspisamide A tail does not penetrate deep into the actin cleft, water mediates two of its three hydrogen bonds. The structure of the AP-actin-CD complex determined by soaking was used in the structure comparisons above. Nearly identical interactions are observed in the co-crystal structure of the AP-actin-CD complex.

Table 1
Data collection, processing and refinement statistics

<i>Data collection and processing</i>			
	AP-actinCaATP + CD (co- crystal)	AP-actin·CaATP + CD (soak)	AP-actin·CaATP (reference crystal)
Resolution	50-1.8 Å (1.85-1.8) Å	15-2.5 Å (2.59-2.5) Å	15-2.5 Å (2.59-2.5) Å
R _{merge} ^a	12.5% (36.3)%	10.8% (27.8%) ^b	9.3% (26.8%)
#Unique reflections	31036	13808	13523
Completeness	81.3% (54.8%)	97.9% (85.8%)	95.8% (77.7%)
Mean redundancy	3.1	6.3	3.4
<I>/<σ>	12.6 (1.89)	16.1 (3.2)	12.8 (3.2)
R _{iso} on I (/on F) ^c			37.3%/24.1%
<i>Refinement</i>			
# Protein atoms	2900	2820	2822
# ATP atoms	31	31	31
# Calcium ions	2	2	2
# Solvent atoms	271	249	196
# CD atoms	37	37	-
R _{cryst} ^d	23.7%	17.7%	20.3%
R _{free} ^e	28.4%	22.8%	26.2%
Estimated coordinate error ^f	0.35 Å	0.32 Å	0.39 Å
<i>Deviations from ideal stereochemistry</i>			
RMSD bonds	0.0073 Å	0.0059 Å	0.0064 Å
RMSD angles	1.27°	1.22°	1.28°
RMSD B-factors ^g	1.48 Å ² /2.1 Å ²	1.3 Å ² /1.9 Å ²	1.2 Å ² /1.8 Å ²
Average B-factors ^h	36.1 Å ² /44.2 Å ² /28.0 Å ² /29.2 Å ²	26.8 Å ² /33.8 Å ² /17.3 Å ² /26.5 Å ²	31.5 Å ² /41.5 Å ² /30.1 Å ²
<i>Ramachandran plot analysisⁱ</i>			
Core region	90.5%	89.8%	90.5%
Allowed region	9.5%	10.2%	9.5%
Generously allowed region	0	0	0
Disallowed region	0	0	0

^a $R_{merge} = \sum (|I_i - \langle I \rangle|) / \sum (I)$, where $\sum$ is over all reflections measured more than once, and $\langle I \rangle$ is the mean intensity of all measured observations equivalent to reflection I_i

^b Values in parentheses are for the highest resolution shell (2.59-2.5 Å)

^c R_{ISO} on $I = \Sigma(|I_{CD} - I_{NATIVE}|) / \Sigma 0.5(I_{CD} + I_{NATIVE})$, where Σ is over all reflections for which intensities from both the AP-actin-CD and native AP-actin crystals are measured. R_{ISO} on F is analogous to R_{ISO} on I , substituting observed amplitudes for intensities

^d $R_{cryst} = \Sigma(|F_{obs} - F_{calc}|) / \Sigma(F_{obs})$, where Σ is over all reflections used in refinement, and F_{obs} are the observed diffraction amplitudes, and F_{calc} are their corresponding calculated amplitudes from inverse Fourier transformation of the model

^e 9.3% and 8.9% of the reflections were set aside from the derivative and native isomorphous datasets respectively for calculating R_{free}

^f Estimated coordinate error as determined by a Luzzati plot⁴⁶ using cross-validated reflections

^g RMSD in B-factor for bonded main chain atoms and side-chain atoms

^h Average B-factors for protein/solvent/nucleotide and Ca^{2+} , and ligand atoms

ⁱ Ramachandran plot analysis is from PROCHECK⁴⁷

Table 2

Equivalent atoms in different structures and their hydrogen-bonded interactions with actin

Actin interacting group	Cytochalasin D	Bistramide (2FXU)	Jaspisamide A (1QZ6)
-CO (Val134)	Wat 8/O7	Wat 1103/O4	-
-NH (Ile136)	O7	O4	Wat 20/O13
-OH (Tyr143)	N2	N2	N1
-NH (Ala170)	O1	O3	Wat 18/O13

In addition to these common interactions, CD also has hydrogen bonds with the backbone carbonyl groups of Gly168 and Ala170.

Table 3

Inter-domain rotation angles determined using the program DYNDOM

Pairs of structures compared ^a		Inter-domain rotation angle between structure pairs (°)
actin-CD co-crystallized	2HF4 ^b	5.7
actin-CD co-crystallized	actin-CD ligand-soaked	3.8
actin-CD co-crystallized	actin EtOH-soaked control	5.8
actin-CD ligand-soaked	actin EtOH-soaked control	2.2
actin-CD ligand-soaked	2HF4 ^b	3.6
actin EtOH-soaked control	2HF4 ^b	No dynamic domains

^a Subdomain 2 was omitted from the above analysis since it is structurally highly variable. Therefore, the inter-domain angles reported above are those between the large domain and subdomain 1.

^b PDB ID of the structure of AP-actin-ATP⁹