Published in final edited form as:

J Mol Biol. 2010 January 22; 395(3): 544–557. doi:10.1016/j.jmb.2009.11.001.

F-actin structure destabilization and DNase-I binding loop fluctuations:

MUTATIONAL CROSS-LINKING AND ELECTRON MICROSCOPY ANALYSIS OF THE LOOP STATES AND EFFECTS ON F-ACTIN

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Abstract

The conformational dynamics of filamentous actin (F-actin) is essential for the regulation and functions of cellular actin networks. The main contribution to F-actin dynamics and its multiple conformational states arises from the mobility and flexibility of the DNase-I binding loop (D-loop, residues 40-50) on subdomain 2. Therefore, we explored the structural constraints on D-loop plasticity at the F-actin interprotomer space by probing its dynamic interactions with the hydrophobic loop (H-loop), the C-terminus, and W-loop via mutational disulfide cross-linking. To this end, residues of the D-loop were mutated to cysteines on yeast actin with a C374A background. These mutants showed no major changes compared to the wild type (WT) actin in their polymerization and nucleotide exchange properties. Copper catalyzed disulfide cross-linking was investigated in equimolar co-polymers of cysteine mutants from the D-loop with either WT (C374) actin or mutants S265C/C374A (on the H-loop) or F169C/C374A (on the W-loop). Remarkably, all tested residues of the D-loop could be cross-linked to residues 374, 265, and 169 by disulfide bonds, demonstrating the plasticity of the interprotomer region. However, each cross-link resulted in different effects on the filament structure as detected by electron microscopy (EM) and light scattering measurements. Disulfide cross-linking in the longitudinal orientation produced mostly no visible changes in the filament morphology, whereas the cross-linking of D-loop residues >45 to H-loop, in the lateral direction, resulted in filament disruption and the presence of amorphous aggregates in the EM images. Similar aggregation was also observed upon cross-linking the residues of the D-loop (>41) to residue 169. The effects of disulfide cross-links on F-actin stability were only partially accounted for by the simulations of current F-actin models. Thus, our results present evidence for the high level of conformational plasticity in the interprotomer space and document the link between D-loop interactions and F-actin stability.

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Keywords

actin; cross-linking; molecular dynamics simulations

INTRODUCTION

Actin is a major component of the cytoskeleton in eukaryotic cells, capable of self-assembly into filaments and higher order structures which participate in numerous cellular processes. Cellular functions of actin are intricately regulated by actin binding proteins and filament dynamics. Thus, an in depth understanding of actin based cellular events requires the comprehension of the structure and conformational dynamics of globular (G-) and filamentous (F-) actin.

The atomic structure of G-actin suggests considerable mobility and flexibility within the molecule¹ while the normal mode analysis of G-actin ascribes this mobility to the movement of subdomains and several dynamic elements, including the DNase-I binding loop (D-loop, residues 39-50), the hydrophobic loop (H-loop, residues 264-273), the C-terminus (including residues 374 and 375), and the W-loop (165-172)². Consistent with this, the D-loop and the C-terminus are disordered in most crystal structures of G-actin³⁻⁶. However, crystallographic studies demonstrated also that the D-loop can adopt specific conformational states. For example, the D-loop was seen as a beta-strand in actin complexed to DNase-I¹, while it formed a short alpha-helix in the crystal of ADP TMR-bound actin⁷ and was unstructured in ATP TMR-bound actin^{3; 8}, indicating the conformational plasticity inherent in this structural loop.

In contrast to over 30 crystal structures reported for G-actin alone and in complex with its binding partners, the information on F-actin structure derives from models built from fiber diffraction data and electron microscopy (EM) reconstructions. The first and most frequently used actin model, the “Holmes model”, was built to 8 Å resolution by fitting the atomic structure of G-actin complexed with DNase-I to the X-ray fibre-diffraction data obtained from oriented F-actin gels in the presence of phalloidin^{1; 9}. The main filament contacts were suggested to be between subdomain 3 of the upper protomer and subdomain 4 of the lower protomer, supported by additional subdomain 1 and 2 contacts involving W-loop, C-terminus and the D-loop residues 40-45. In addition, the two filament strands were hypothesized to be stabilized by the insertion of the H-loop from a lateral protomer into a hydrophobic pocket at the interface between two neighboring protomers in the opposite strand. Although the H-loop hypothesis is consistent with results from several experimental studies (for review see 10), the loop was also shown to distribute among different states and orientations^{11; 12}. This loop dynamics appears essential for filament stability since the locking of the H-loop to the protein backbone by cross-linking in a yeast actin mutant leads to filament destruction^{11; 13}.

The original refinements of the Holmes model preserved its main features while introducing changes in the location and orientation of the D-loop and the C-terminus of actin^{14; 15}. In 2003, Holmes et al. revised the actin filament model by using TMR-ADP G-actin structure with a helical D-loop¹⁶. In this newer model, the helical D-loop is positioned to interact with the W-loop in a protomer above it in a way resembling the binding of WH2 domain containing proteins to actin¹⁷.

The Holmes models and their subsequent refinements were built with the assumption that the monomer structure is invariant upon its incorporation in the filament. This assumption was partially supported by the recent highest resolution Oda model¹⁸. It is revealed that the

G- to F-actin transition involves a simple relative rotation of the two major domains by about 20 degrees to have a flat actin molecule in the filament. The second feature of this model is that the D-loop adopts an extended open loop conformation, positioned along the hydrophobic cleft between subdomains 1 and 3.

The differences noted among the various actin models in the conformation and location of dynamic elements, such as the D-loop, can be viewed as reflecting the dynamic nature of the filament. In fact, a recent high resolution cryo EM map of actin scruiin bundle assigns numerous conformational states to the filament¹². In that study, subdomain 2 displayed angular disorder resulting in an average 10 degree rotation of subunits from their ideal positions, in agreement with prior EM reconstructions of F-actin^{19; 20}. EM studies also revealed that variable tilt²¹ and twist²² form spontaneously in pure filaments of actin. These dynamic motions can be modulated by actin-binding proteins such as cofilin, which was shown to shift the mean twist of the protomers by 5 degrees by stabilizing one of the pre-existing states²³.

Actin models and experimental evidence suggest that the dynamic loops of monomeric actin form important interprotomer contacts within the filament²⁴ and probably determine its conformational states. In this context, the D-loop is of particular interest since it represents the most flexible region on actin, while its modifications or proteolytic cleavage directly affect actin polymerization²⁵⁻²⁸. Therefore, we investigated the plasticity of the D-loop (residues, 40-50) by probing its dynamic interprotomer interactions with the H-loop, the C-terminus, and the W-loop via disulfide cross-linking within co-polymers formed with cysteine mutants from each element (Fig. 1). The characterization of these mutants revealed no major changes compared to the WT actin. Strikingly, all residues of the D-loop (mutated to cysteine) could be cross-linked to the C-terminus (residue 374), the H-loop (residue 265), and the W-loop (residue 169) by disulfide bonds, demonstrating the plasticity of the interprotomer region. However, differences in the cross-linking rates and extents reveal the link between filament stability and specific D-loop states. Taken together with our light scattering and EM observations, these results show the extensive conformational plasticity of the interprotomer interface, which allows also for the presence of intrinsically destabilized states of the filament.

RESULTS

The goal of this study has been to demonstrate the plasticity of D-loop and the inherent dynamics of the actin filament resulting from the interactions between the D-loop, C-terminus, the W-loop, and the H-loop by using copper-catalyzed disulfide cross-linking. To this end, we have generated single cysteine mutants of the D-loop (residues 40-50) and residue 169 by mutating the yeast actin (*Saccharomyces cerevisiae*) gene along with the mutation of native cysteine 374 to an alanine. Therefore, each mutant has a single reactive cysteine that is available for disulfide cross-linking. All actin mutations, except for M44C/C374A, were found to produce viable yeast colonies and the properties of the resulting actins were characterized. To simplify the notation, all actin mutants will be referred to in this manuscript using a single letter code, e.g., “C44” instead of M44C/C374A.

Functional characterization of mutants

To test the functional integrity of actin mutants, we carried out nucleotide exchange and polymerization experiments. The exchange of fluorescent ϵ -ATP with excess ATP can be used to detect mutation effects on the nucleotide binding cleft. The C374A mutant exchanged ϵ -ATP at rates similar to the WT actin, indicating that the differences observed for various mutants can be attributed to the introduction of new cysteines (Fig. 2a). Mutants C48, C49 exchanged ϵ -ATP at rates similar to WT, whereas the C47-C45 and C43-C40

exhibited up to two fold slower rates as compared to the WT protein (Fig. 2a). Since these mutations are not in the cleft-forming residues, the relatively small changes observed are of allosteric nature.

Next, we monitored the polymerization of mutant actins via the increase in light scattering, to detect if the mutations affect the nucleation and elongation behavior of actin. All actins, but the C40 and C50 mutants, polymerized at similar rates, indicating little if any change in the nucleation or elongation steps (Fig. 2b). Modification of histidine 40, which is suggested to participate in extensive interprotomer contacts⁹ was shown previously to inhibit actin polymerization²⁵. Hence, it is not surprising that the cysteine replacement of this residue inhibits actin polymerization. The slow nucleation phase observed for C50 might be due to the loss of undefined ionic interactions with the replacement of the native K50 (Fig. 2b). In both cases, the negative effect of mutations can be compensated by forming copolymers with WT, C265, or C169, which polymerize at rates only slightly slower to the other copolymers (see Fig. 4a and 6b for C50).

Intermolecular cross-linking in F-actin and its structural consequences

Disulfide (zero length) and dibromobimane (4.4 Å in average) cross-linkings between residues 41-265 and 41-374 were demonstrated previously²⁴. This work extends the disulfide cross-linking to all residues of D-loop between 40 and 50 (except for residue 44 as mentioned above). These residues were cross-linked to the C-terminus residue 374, H-loop residue 265, and W-loop residue 169 in equimolar-polymers of D-loop mutants with either WT (C374) actin or mutants C265 or C169, thus allowing for a maximum possible cross-linking yield of 50% (materials and methods). In control experiments, we exposed filaments of WT actin or single reactive cysteine carrying mutants to the same oxidizing conditions as used for the co-polymers, and did not detect any cross-linked dimers (data not shown). Also, the monomers of WT and mutants did not form cross-linked dimers under reducing conditions. This agrees with previous results with WT, C41, and C265 and shows that the disulfide bonds are formed specifically between the reactive cysteines in the co-polymer²⁴.

Disulfide cross-linking of D-loop mutants with the C-terminus

The CuSO₄ (20 μM) catalyzed disulfide cross-linking of D-loop mutants with C374 reached a plateau of 41.6 ± 3.0 % cross-linked dimers within 30 seconds or less (representative copolymers are shown in Fig. 3a). Under these conditions, the F-actin stabilizing toxin phalloidin²⁹ did not appear to affect the rate and extent (41.4 ± 2.3 % dimers) of the cross-linking reaction (Fig. 3a). However, with increased kinetic resolution of these reactions achieved at low CuSO₄ concentrations (5.0 μM), the rate of cross-linking C41, C43, and C50 to C374 was about 30-50% faster in the presence of phalloidin consistent with previous results for C41 (Fig. 3b)³⁰.

Earlier reports have shown that the cross-links introduced either by disulfide bonds or chemical reagents can alter the filament morphology and may result in filament destruction^{11; 13}. Thus, we checked this possibility by both light scattering and electron microscopy observations. As shown in figure 4a the disulfide cross-linking to C374 had either no effect (with C41, C45, C47) or caused only a slight decrease in light scattering (with C43 and C50). As confirmed by EM, all control co-polymers exhibited the regular morphology of yeast actin filaments and the disulfide cross-linked filaments appeared normal for C41, C45, and C47 (data not shown). The oxidized filaments of C43 had also regular morphology, indicating that the decrease in light scattering by cross-linking might be due to depolymerization from the filament ends with no detectable effect on their filament structure (Fig. 4b). For C50, the oxidized filaments appeared shorter and kinked, and in many cases were also severed or fragmented (Fig. 4b). Overall, these observations demonstrate that the

residues of the D-loop are highly dynamic, with each one of them residing, at least transiently, in close proximity to the C-terminus. These dynamic motions produce multiple conformational states, all of which are accommodated within the filament structure, including those that - if locked - destabilize the filament.

Disulfide cross-linking of D-loop mutants with the H-loop residue C265

In contrast to similar disulfide cross-linking rates of D-loop cysteine mutants to C374 (at 20 μM Cu^{2+}), the C-terminal region of the D-loop cross-links slower than its N-terminal residues to C265 on the H-loop (Fig. 3a and 3c). These differences in cross-linking rates can be detected by monitoring the amount of dimers formed in 30 seconds. As shown in figure 3c, residues 41, 43, and 45 cross-link equally well and fast to C265, whereas the rate and extent of the reaction declines from residue 47 through 50. When the same experiments are performed in the presence of phalloidin, the decrease in cross-linking rates is more pronounced, yielding only a small percentage of dimers for the C48, C49, and C50 co-polymers with C265 actin.

Light scattering measurements shed additional light on the above cross-linking experiments. The light scattering decreases upon CuSO_4 addition to each co-polymer tested, except for C41-265, with the decrease becoming progressively larger as the residue involved is closer to C50 (Fig. 5a). Electron microscopy of oxidized filaments confirms that the cross-links became more disruptive as they are closer to C50 (Fig. 5b). Even for the 43-265 co-polymers, which showed similar change in the light scattering upon cross-linking to the 43-374 co-polymers, the former (lateral) cross-link resulted in more kinked and shorter filaments. Starting with residue 45, through 50, all tested cross-linked co-polymers with C265 yielded amorphous aggregates when viewed by EM (Fig. 5b). These aggregates can be “rescued” to form regular filaments with the addition of phalloidin, indicating that their conformations are not drastically different from the native and stable F-actin conformations. However, for the co-polymers showing the greatest decrease in light scattering upon cross-linking, the filaments recovered with phalloidin are shorter than control actin filaments.

Overall, the trend we have observed for the disulfide cross-linking rates most likely reflects the average proximities and/or the frequency at which D-loop residues approach the H-loop. According to this interpretation, the C-terminal part of the D-loop approaches the H-loop at a lower frequency compared to its N-terminal region, and such conformations are inherently destabilizing to the filament structure when locked by disulfide cross-linking. Consistent with this interpretation, the cross-linking of C45 to C265 with a bi-functional reagent such as MTS-6, which has a 9.6 Å long (mean length) flexible linker region, does not destroy the filament (Fig. 3a and 5b). Therefore, the average range of dynamic fluctuations between residue C45 and C265 appears to be ~ 10 Å.

Disulfide cross-linking of D-loop mutants with the W-loop residue 169

All D-loop mutants could be cross-linked to Cys169 with high efficiency, yielding 36.5 $\pm$ 2.2 % dimers within 30 seconds of the reaction and reaching a plateau (43.5 $\pm$ 4.2 %) in 1 minute. Phalloidin had a marginal, if any, effect on the rate of these cross-links, yielding 39.0 $\pm$ 1.6% dimers at 30 sec, and up to 41.7 $\pm$ 2.4 % with longer incubation times (representative 41-169, 43-169, 45-169, 47-169, and 50-169 cross-links are shown in Fig. 6a). Only mutant C50, showed a slightly slower rate of cross-linking and formed 28.2 $\pm$ 1.7 % and 31.1 $\pm$ 1.9% dimers at 30 seconds with or without phalloidin, respectively. This is likely to result from a longer average distance between C50 and C169.

According to the Holmes (2003) and Oda models of F-actin, C169 is within 5-10 Å from D-loop residues. In the more recent Oda model ¹⁸, C43, C44, C45, C46, and C47 are predicted

to be close to C169. Therefore, we did not expect to see filament destruction upon disulfide formation for these residues. However, all co-polymers showed some (slight to major) decrease in light scattering upon CuSO_4 addition (Fig. 6b). As indicated by light scattering decrease, the cross-linked co-polymers of C43-C169, C45-C169 and C50-C169 did not appear in EM images as filaments, but mainly as amorphous aggregates (Fig. 6d). Similar, but larger aggregates were observed for C47-C169 (Fig. 6f), consistent with the light scattering results (Fig. 6b). All aggregates could be transformed into filaments by phalloidin as shown in figure 6e for the restored C45-C169 filament. For C41-C169 co-polymer, which shows the least decrease in light scattering upon cross-linking, the filaments appeared irregular and shortened (Fig. 6c). These results show that the D-loop flexibility or plasticity is needed for filament stability as its cross-linking to the W-loop, which has more restricted motions compared to the C-terminus, destroys the filament.

Molecular dynamics simulations of F-actin models

Using the coordinates from Oda and Holmes actin filament models, we performed 30 ns molecular dynamics simulations on eight protomer F-actin structures we constructed for each model 9¹⁸. In Holmes model, we replaced the existing monomer structure with the ADP TMR-bound actin where the D-loop is in the form of a short alpha-helix ⁷. Specifically, we have looked at the C α -C α distances between the residues of the D-loop and residues 169, 265, and 374 to test if the models explain the observed cross-linking effects. In addition, we considered also the distances between the same residues in the 13-protomer actin scruin bundle model (Cong model), in which all the protomers were depicted to have their unique orientation and conformation for dynamic regions like H-loop and D-loop ¹². Overall, the analysis of average C α -C α distances between the D-loop and targeted residues revealed that no single model provides a comprehensive description for the observed effects of disulfide cross-links summarized in Table 1. In other words, they each offered partial description for our cross-linking results.

Firstly, neither Oda or Holmes models predict the disulfide cross-links between C-terminal residue 374 and all D-loop residues since average C α -C α distances are at least 9 Å, i.e., too far for disulfide bond formation (Fig. 7a). A few cross-links are expected to occur to C374 (in Fig. 7a from 43-45 in Oda model and 46, 47 in Holmes model), but other cross-links could disrupt the filament. Yet, each residue of the D-loop can be cross-linked to 374, and, except for 43-374 and 50-374, these cross-links do not impair the filament structure.

Secondly, Oda and Holmes models simulations and Cong model predict distances >10 Å between H-loop residue 265 and the D-loop residues (Fig. 7b). The minimum distances obtained from both models predict that residues 40-42 can be cross-linked to 265 (C α -C α ~5Å), but for the rest of the D-loop the minimum distances are beyond the disulfide cross-linking range. Interestingly, Oda model shows a distinct trend, with distances increasing from 10 Å (41-265) to 20 Å (between 45-265 to 50-265). This trend coincides with the cross-links to the H-loop disrupting the filament starting from residue 43. Taken together, our analysis indicates that the models would predict only part of the disulfide cross-links and/or their effects, pointing to more extensive dynamic rearrangements in the interprotomer space than previously thought.

Finally, the inter-residue distances between W-loop residue 169 and the D-loop residues obtained from simulations are the only set of data displaying discrete differences between Oda and Holmes models of F-actin (Fig. 7c). The Holmes model simulations give an average distance of 15 Å between residue 169 and D-loop, whereas the Oda model simulations result in less than 10 Å distances (with a minimum of ~5Å) from residue 169 to 40 through 45. Thus, our D-loop-C169 disulfide cross-linking data appear more consistent with the values of the Oda model. However, such cross-links destroy the filament structure

as shown by light scattering and EM experiments (Fig. 6b and 6c), and these destabilizing effects can be rationalized better by the much higher average distances observed in Holmes model simulations. For example, the average distance of C45 to C169 is 10 Å (while facing each other) and 17 Å (while facing away) in Oda and Holmes models, respectively. Overall, C169-D-loop cross-linkings and simulation results suggest that in the filament the D-loop might be in dynamic equilibrium between an ordered (2003 Holmes model)¹⁶ and a disordered extended state (Oda model), and reveal that blocking this conformational exchange by cross-linking destroys the filament integrity.

DISCUSSION

The goal of our work was to explore the constraints on D-loop plasticity at the interprotomer space as determined by its interactions with other dynamic elements of actin, including the C-terminus, the W-loop, and the H-loop. The participation of these structural loops in the interprotomer contacts in actin filament was predicted by the models of F-actin^{9; 14; 15}, and solution evidence confirmed such interactions²⁴. However, the solution results rested either on the use of single residue mutant in the D-loop, C41 in yeast actin^{11; 24; 27; 31}, or on proteolytic digestions as a tool to examine the environment of the N- or C-terminal part of the D-loop^{26; 28}. Thus, a comprehensive analysis of the motions and interactions of the entire loop across the interprotomer space had yet to be pursued. The combined disulfide cross-linking, light scattering and EM studies presented here offer such an analysis.

Structural states of F-actin include intrinsically destabilizing transient conformations

Actin filament was demonstrated to have multiple structural states depending on the bound nucleotide, divalent cations, and binding partners by EM, limited proteolysis, fluorescence spectroscopy, and, radiolytic protein footprinting^{28; 32-38}. Former work suggests that these states might arise from the dynamic interactions of subdomain 2 with its longitudinal and/or lateral contact sites in the filament. The disulfide cross-linking results of the present work add credence to this view and demonstrate that the interplay between the D-loop in subdomain 2 and other dynamic elements of F-actin, including the C-terminus, the H-loop, and the W-loop, is more extensive than predicted by the simulations of F-actin models^{9; 12; 16; 18}. The fact that each residue of the D-loop could be cross-linked to C374, C265, and C169 clearly reflects the plasticity and mobility of the interface between actin protomers. However, as we have shown by EM and light scattering measurements, some of the multiple states created by dynamic rearrangements of these structural loops are intrinsically destabilizing the filament, as evidenced by filament disruption upon trapping these states via disulfide cross-linking.

Close proximity of the D-loop's C-terminal to the H-loop creates destabilizing modes in F-actin

A well known example of actins' structural states is the twist of 5° (to 162°) induced by the actin severing protein, cofilin²³. This variable twist was also found to be inherent to pure actin filaments, suggesting that cofilin acts by stabilizing this pre-existing conformer of the filament²². This 162° state might be highly relevant to the states corresponding to our disulfide cross-links between D-loop residues and H-loop residue 265. Our data shows that these cross-links impair filament stability, starting from residue 43, as shown by EM images of severed and kinked filaments. As the residue number in D-loop approaches 50, the cross-links cause complete filament disruption, yielding small aggregates which can be restored into short filaments by phalloidin. This rescue indicates that the conformations of the cross-linked actins can still be filament compatible given additional stabilizing and/or bridging factors. Since the rate and extent of disulfide cross-linking can be viewed as a qualitative reflection of the real average proximities of the targeted thiol residues, our results suggests

that the C-terminus of the D-loop approaches the H-loop at a lower frequency than its N-terminus, with the latter proximity appearing necessary for the filament stability.

Intriguingly, the destabilized filament conformers created by motion/proximity of the C-terminal region of D-loop to the H-loop may be “used” by severing proteins such as cofilin. Moreover, some pathogens may also capitalize on these states for cell invasion. For example, *Vibrio cholerae* MARTX_{VC} toxin induced cross-linking of two actin molecules between D-loop residue 50 and H-loop residue 270^{39; 40} impairs actin polymerization by locking these subunits in a conformation incompatible for filament elongation. Since, residue 270 is close to residue 265 used in this work, it is attractive to speculate that the toxin traps actin in an unstable, transient oligomeric form present at the early stages of actin oligomerization, similar to the states we have identified in this study.

Phalloidin stabilization of filaments has different effects on cross-linking rates

Filament stabilization by phalloidin, most likely through the restriction of the breathing motions in F-actin 29, resulted in different effects on the rate of formation of longitudinal and lateral disulfide cross-links. In the longitudinal cross-links of C-terminus and D-loops, phalloidin increased the cross-linking rates by 30-50 percent, most likely by eliminating large amplitude fluctuations of these elements and thus, increasing the probability of their cross-linking. In the lateral H-loop and D-loop cross-links, phalloidin inhibited the formation of cross-links from D-loop residue 47 and beyond to residue 265. This inhibitory effect is also compatible with reduced amplitude of loop fluctuations, i.e., the elimination of destabilizing states which bring residues 48-50 close to residue 265. In line with this argument, incubation of C50-C265 co-polymers with BeFx, an ADP-Pi analog known to reduce the amplitude of subdomain 2 motions³³, also inhibited the formation of 50-265 cross-linked dimers (data not shown). Notably, higher frequency states detected by 43-265 and 45-265 cross-links, which are unaffected by phalloidin, demonstrate the existence of these mildly destabilizing states also in the presence of filament stabilizing factors.

The conformational plasticity of the D-loop is required for F-actin stability

Because the W-loop has more restricted motions and flexibility than the C-terminus and H-loop, the disulfide cross-linking data provide in this case a stronger set of constraints on the conformation and position of the D-loop residues. The D-loop can be cross-linked to C169 from every position tested with similar efficiency, demonstrating that it might assume a position in the hydrophobic cleft -between subdomains 1 and subdomain 3- as proposed by Dominguez¹⁷. It was further hypothesized in that study that the D-loop might bind in this cleft in a helical conformation similar to that observed for the WH2-domain containing proteins. Our experiments show that locking C169 to any residue tested in the D-loop, except for C41, disrupts the filament structure and results in actin aggregation. Surprisingly, this is also true for the C45-C169 cross-link, which is modeled to face C169 within an average distance of 10 Å (and minimum of 6.5 Å) in our Oda model simulations, allowing for a possible disulfide bond formation between these residues¹⁸. The same pair of residues was 17 Å in average, and 9.6 Å at a minimum, in Holmes model simulations -with the D-loop in a helical conformation- suggesting that a disulfide crosslink between them would impair the filament stability¹⁶. Thus, our data strongly suggests conformational fluctuations bringing D-loop residues close to C169 are destabilizing the filament. This is evidenced by filament aggregation through trapping these D-loop-C169 states by disulfide cross-linking. Dynamic rearrangements and/or the conformational exchange of the D-loop are essential for the dissipation of these destabilizing filament modes and filament stability. It is an intriguing possibility, yet to be tested, that the Holmes and Oda models might represent ordered and disordered conformers of the D-loop in the filament. An alternative possibility is that the

cross-linking of D-loop and C169 may preclude some conformational states of the hydrophobic loop within the interprotomer space from the opposite strand 9; 41.

The interplay between the dynamic loops of F-actin is functionally important

The ability of F-actin to undergo conformational rearrangements is likely to play an important role in the regulation of actin mediated functions in the cell¹⁰. In line with this view, the longitudinal cross-link between residue 41 on the D-loop and residue 374 on the C-terminus inhibits the actomyosin force generation in the absence of morphological changes in the cross-linked filament structures^{42; 43}. Therefore, these dynamic fluctuations are required for the function of actin; possibly for actin to interact with its many binding partners in eukaryotic cells. Our work, along with previous studies, demonstrates that the highly conserved dynamic loops of actin are essential for these conformational rearrangements^{11; 13; 30; 35; 41; 44; 45}. Interestingly, the unusual properties of the *Leishmania* actin^{46; 47} were attributed to sequence alterations of the H-loop or D-loop. Therefore, it is likely that different organisms tailor these structural elements for proper dynamic functioning of their actin networks.

Conformational plasticity of actin filaments

It has been recognized that actin isoforms can have different structural and dynamic properties from each other. For example, the yeast actin filaments are reported to be slightly more flexible compared to the skeletal actin as evidenced by fluorescence⁴⁸, limited proteolysis⁴⁹, and three dimensional EM reconstructions⁵⁰. This raises the possibility that the observed inconsistency between our cross-linking results (yeast actin-based) and the current actin models (skeletal actin-based) might be a result of isoform specificity. To address this concern, we have performed molecular dynamics simulations of yeast actin filaments using the Oda model as a starting point. Molecular dynamics simulations of skeletal and yeast F-actin are remarkably similar and show only minor differences in the distances between cross-linked residues (Fig. 7a, b, and c; black and blue symbols). Therefore, the conformational flexibility demonstrated by our data, is unlikely to be isoform specific and is probably a common feature of actin filament dynamics.

In conclusion, our study presents unambiguous evidence for a high level of conformational plasticity in the interprotomer interface in F-actin formed with the participation of dynamic loops including the D-loop, the H-loop, the W-loop, and the C-terminus. The disulfide cross-linking of the D-loop to each of the given loops, reveals the coexistence of destabilizing and stabilizing states of the filament which may lead to multiple binding surfaces needed for filament functions. The fact that the structural effects of disulfide cross-links can only be partially described by the dynamic modeling simulations of current actin filament models implies that these dynamic rearrangements in the subunit-subunit interface are more extensive than predicted by these models. Therefore, our cross-linking results and the cross-linking effects on filament integrity provide structural constraints that can be used to describe and model the dynamic modes of actin filaments. .

MATERIALS and METHODS

Cloning and Mutagenesis

The QuikChange Site-Directed Mutagenesis kit from Stratagene was used for the preparation of actin mutants. Yeast actin (*Saccharomyces cerevisiae*) mutations were generated on pRS314 containing TRP1 marker and C374A actin gene under control of the native actin promoter. Then, yeast haploid cells with mutated actins were generated with the resulting plasmids as described previously⁵¹. Preparation of mutants Q41C/C374S, S265C/C374A, and, K50C/C374A are described elsewhere^{24; 40; 51}. Forward primers used for

cloning and site-directed mutagenesis of actin genes are given in Table 2. Reverse primers are complementary to the forward primers given.

Purification of actin mutants

Yeast actin was purified as described earlier²⁴. The protein was eluted from a DNase I affinity column with buffer A (10 mM Tris (pH 7.8), 1 mM DTT, 0.2 mM ATP, and 0.2 mM CaCl₂) containing 20-25% sucrose and 50% formamide, and was then applied directly to a DEAE column. Actin was eluted from the DEAE column in the same buffer containing 350 mM KCl. Purified G-actin was dialyzed and stored in buffer B (10 mM HEPES (pH ~7.6), 0.2 mM CaCl₂, 0.2 mM ATP, 1.0 mM DTT) on ice.

Actin cross-linking and SDS-PAGE analysis

Immediately prior to cross-linking, DTT was removed from actin mutants (20 μ M) by passing them through Sephadex G-50 spin columns equilibrated with buffer B. The hetero-mixture of actins was prepared by mixing equal molar amounts of mutant actins to yield a combined final concentration of 10 μ M. This hetero G-actin mixture was then polymerized with 3.0 mM MgCl₂ at 22°C, for 20 minutes. Copper or MTS reagents were added at a 1:2 actin:cross-linking catalyst/reagent mole ratio and aliquots were removed at the desired time points. The reactions were stopped by the addition of 2.0 mM N-ethylmaleimide and SDS electrophoresis sample buffer without reducing reagents. The cross-linking products were run on non-reducing 10% SDS-PAGEs and visualized by Coomassie staining. The density values for protein bands were calculated by Scion Image (Alpha 4.0.3.2), and plots were generated by Sigma Plot 11 (Systat Software) or Excel 2007 (Microsoft Office).

Light scattering Assays

Actin polymerization, disulfide cross-linking and disulfide reduction were monitored via light scattering measurements with the PTI fluorometer set at 325 nm for the excitation and emission wavelengths. Actin polymerization was induced by adding 3.0 mM MgCl₂ to protein solution at 5.0 μ M for standard polymerization assays and at 10 μ M for experiments with co-polymer cross-linking experiments. 20 μ M CuSO₄ was added to catalyze disulfide bond formation in co-polymers, and once the plateau was reached 10mM DTT or TCEP was added to reduce the disulfide bonds.

Nucleotide exchange

The ATP exchange in G-actin (1-2 μ M) was followed through the fluorescence of ϵ -ATP. The release of ϵ -ATP from actin in buffer B (containing 5 μ M ϵ -ATP) was initiated with the addition of 100 μ M ATP. The subsequent decrease in the fluorescence of released ϵ -ATP was monitored in a PTI fluorometer with the excitation and emission wavelengths set at 350 nm and 410 nm, respectively. The rates of exchange were obtained by fitting the fluorescence decay data to single exponential expression.

Electron Microscopy

The co-polymers (10 μ M) were prepared as described above and divided into two fractions. 1.0 mM DTT was added to the first fraction, which was used as control filaments. The second fraction was incubated with 20 μ M CuSO₄ for 45 minutes. 20 μ M phalloidin was added to a fraction removed from the oxidized sample and incubated for 15 minutes. Eventually, all fractions were diluted to 2.5 μ M final concentrations with the corresponding polymerization buffers, and applied to 400-mesh carbon-coated copper grids coated with formvar films (EM Sciences). After 60 seconds of adsorption, the grids were blotted dry and treated with 1% uranyl acetate for 45 seconds. The grids were examined in a JEOL 1200EX-

II electron microscope operated at 80 kV. The electron microscopy images were analyzed by using IMAGE J software.

Molecular dynamics simulations

We used three models of the actin filament for simulation purposes. The first was the Holmes model 9 where we replaced the existing monomer structure with the newer ADP-actin crystal structure (1J6Z)⁷. The second filament model was the Oda et al. structure (2ZWH)¹⁸. The final structure was a yeast filament structure based on the Oda filament model. Since yeast and muscle actin have 88% identity with no insertions or deletions, we were able to use Modeller 52 to create the homology model. Although a yeast G-actin structure does exist, using the Oda F-actin model as a template was important since the subdomains of the monomer are slightly rearranged in the filament. We constructed eight monomer filaments for each model, solvated the systems with TIP3P water, added neutralizing ions and set the ionic strength to 50 mM. Using the molecular dynamics package NAMD 53, simulations of each filament were performed using the CHARMM27 force field, an NpT ensemble at 1 atm pressure, a temperature of 300 K, a 10 Å cutoff for van der Waals interactions with a switching distance of 8.5 Å, and Particle Mesh Ewald for long range electrostatics. Covalent bonds with hydrogens were held rigid allowing us to take 2 fs time steps. Each system was heated in 50 K steps, equilibrated at 300K for 5 ns, and we performed production runs of 30 ns, saving structures every 50 ps for analysis.

Acknowledgments

We thank Dr. Xiang Zheng for help with the molecular dynamics simulations. This work was supported by grants from USPHS (GM-077190) and NSF (MCB 0316269) to E.R. and GM067246 to D.S.

Abbreviations

G-actin	globular actin
F-actin	filamentous actin
EM	electron microscopy
C169	F169C/C374A
C265	S265C/C374A
C41	Q41C/C374S
C42	G42C/C374A
C43	I43C/C374A
C45	V45C/C374A
C46	G46C/ C374A
C47	M47C/C374A
C48	G48C/C374A
C49	Q49C/C374A
C50	K50C/C374A
WT	wild type
MTS 6	1,6-hexanediyl bis (methanethiosulfonate)
NEM	N-ethyl maleimide

TCEP tris (2-carboxyethyl) phosphine

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Figure 1.

A three protomer Oda¹⁸ model of the actin filament shown with the cross-linked regions highlighted. Residues of the D-loop are colored red, the C-terminal residue C374 is colored blue, the W-loop residue Y169 (mutated to cysteine in this study) is colored blue, and the H-loop residue S265 is colored green (mutated to cysteine in this study). Individual protomers are shown in pink, green, and blue ribbon color.

Figure 2.

Functional characterization of mutant actins. a) Ratios of ϵ -ATP exchange rates of mutant actins to that in WT actin. The ϵ -ATP exchange assays were performed as described in Materials and Methods. The ratios of the calculated rates are presented with the error bars indicating the mean error. For WT actin, the rate of ϵ -ATP exchange was $0.0157 \pm 0.0018 \text{ sec}^{-1}$. b) Actin ($5.0 \mu\text{M}$) polymerization by MgCl_2 (3.0 mM) as monitored by light scattering at 325 nm . The plots were normalized to reveal kinetic differences in the polymerization reactions. For clarity, only representative polymerization data are given: for WT (overlapping with C41, C47) in black, for C49 (overlapping with C48, C45, and C42) in blue, for C46 in green, and C43 in gray. Mutant (C40) in cyan completed its polymerization at 6000 seconds under these conditions.

Figure 3.

Disulfide cross-links between D-loop and C265 and C374. a) SDS-PAGE (non-reducing) of representative disulfide or MTS-6 cross-linked yeast F-actin co-polymers of D-loop mutants with C374 and C265 actins. The cross-linked pairs are indicated above the columns. The (-) and (+) signs indicate the presence or absence of 20 μ M of phalloidin, CuSO₄, or MTS6 in the reaction mixtures. The time points at which the aliquots were removed are indicated in each panel. b) Stabilization of filaments by phalloidin increases the rates of longitudinal cross-linking. The observed rate constants in the presence (white circles) and absence (black circles) of phalloidin for the oxidation of co-polymers C43-C374 (on the right) and C50-C374 (on the left) were calculated by fitting the percentage of monomers to a first order exponential decay equation. The ratios of cross-linking rates in the presence of phalloidin to those in the absence of phalloidin were 1.44 $\pm$ 0.12 and 1.41 $\pm$ 0.14 for C43-C374 and C50-C374 filaments, respectively. c) The percentage of representative cross-linked dimers formed between D-loop and H-loop mutants in 30 seconds. The cross-linking products were analyzed on non-reducing SDS-PAGE after treatment with 2.0 mM NEM. The error bars represent the mean percentage error and the mean values are given inside the bars.

Figure 4.

Effect of disulfide cross-links between D-loop and C374 on filament stability. a) Light scattering experiments detect the effect of disulfide cross-links between D-loop and C374 on F-actin. Co-polymers of D-loop mutants with C374 were polymerized with 3.0 mM MgCl_2 and then 20 μM CuSO_4 (indicated by star) was added to catalyze disulfide bond formation (observed on SDS-PAGE gels). Upon cross-linking completion, 10 mM TCEP (indicated by arrow) was added to the mixture to reduce the disulfide bonds. For easier comparison of the cross-linking effects, all light scattering data were normalized to 100%. b) Electron micrographs of yeast actin co-polymers before and after disulfide cross-linking. Micrographs of control (polymerized with 3 mM MgCl_2) and disulfide cross-linked filaments are in the first and second columns; phalloidin induced recovery of the cross-linked filaments is shown in the third column. The rows are labeled with the corresponding co-polymer names. The MTS-6 cross-linked C45-C265 co-polymer micrograph is also shown. The scale bar corresponds to 0.1 micron in each image.

Figure 5.

Effect of disulfide cross-links between D-loop and C265 on filament stability. a) Light scattering experiments detect the effect of disulfide cross-links between D-loop and C265 on F-actin. All experiments were performed as described in legend to Fig. 4a. The star and arrow denote the addition of 20 μM CuSO_4 and 10 mM TCEP, respectively. b) Electron micrographs of yeast actin co-polymers before and after disulfide cross-linking. Micrographs of control and disulfide cross-linked filaments are in the first and second columns; phalloidin induced recovery of the cross-linked filaments is shown in the third column. The MTS-6 cross-linked C45-C265 co-polymer micrograph is also shown. The scale bar corresponds to 0.1 micron in each image.

Figure 6.

SDS-PAGE (non-reducing) and the effect of disulfide cross-links between D-loop and C169 on actin filaments. a) SDS-PAGE of disulfide cross-linked for 30 seconds and 10 minutes F-actin (10 μM) co-polymers of D-loop mutants with the C169 mutant in the presence of 20 μM phalloidin. The co-polymers are identified above the columns. b) Light scattering experiments detect the effect of disulfide cross-links between the D-loop and C169 on F-actin. All experiments were performed as described in legend to Figure 4. The star and arrows denote the addition of 20 μM CuSO₄ and 10 mM TCEP, respectively. The right column shows the EM images of oxidized F-actin copolymers of C41-C169 (c), C45-C169 (d), and C47-C169 (f). The EM image of filaments regenerated with the addition of phalloidin to aggregates shown in (d) is given in (e).

Figure 7.

The minimum and mean Ca-Ca distances between the D-loop residues and C374 (a), C265 (b), and C169 (c) based on distances from the Cong actin scruiin bundle model and molecular dynamics simulations of Holmes and Oda (2009) F-actin models. The C374 distance values are missing for Cong model since the C-terminus was not present in that model. The cross-links listed in Table 1 are color coded here according to their classification as having a minor (no change in filament morphology, green box), moderate (causing partial filament destruction, orange box) or severe phenotype (causing filament destruction, red box). “Mean” is the time averaged mean distance among the cross-linked pairs during simulations and “Min” is the minimum distance observed among the cross-linked residues during the simulations.

Table 1

Summary of disulfide cross-linking effects

Cross-linked residues	374	265	169
41	ok	ok	partial filament destruction
43	partial depolymerization	partial filament destruction	filament destruction
45	ok	filament destruction	filament destruction
47	ok	filament destruction	filament destruction
50	partial filament destruction	filament destruction	filament destruction

Ok= Neither a decrease in light scattering was observed nor a difference in filament structure was noted in EM images

Partial depolymerization= Slight decrease in light scattering upon cross-linking was observed, but no difference in filament structure was noted in EM images.

Partial filament destruction= Slight to moderate decrease in light scattering upon cross-linking and kinks and shortening of the filaments observed in EM images.

Filament destruction= Moderate to massive decrease in light scattering upon cross-linking and no filaments are observed in EM images, just oligomers and aggregates.

Table 2

Forward primers used for site-directed mutagenesis

Mutation	Forward primer
H40C	5' CGTCGGTAGACCAAGAT <u>GT</u> CAAGGTATCATGGTCGG 3'
G42C	5' GTAGACCAAGACACCAAT <u>T</u> GTATCATGGTCGGTATG 3'
I43C	5' GACCAAGACACCAAGGT <u>TGT</u> ATGGTCGGTATGGGTC 3'
M44C	5' CCAAGACACCAAGGTATC <u>TGT</u> TCGGTATGGGTCAAAAAG 3'
V45C	5' CAAGACACCAAGGTATCAT <u>TGT</u> TGGTATGGGTCAAAAAGACTC 3'
G46C	5' CCAAGGTATCATGGTC <u>T</u> GTATGGGTCAAAAAG 3'
M47C	5' CAAGGTATCATGGTCGGT <u>TGT</u> GGTCAAAAAGACTCCTAC 3'
G48C	5' GTATCATGGTCGGTATG <u>T</u> GTCAAAAAGACTCCTAC 3'
Q49C	5' CATGGTCGGTATGGGT <u>TGT</u> AAAGACTCCTACGTTGG 3'