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Effect of intramolecular cross-linking between glutamine-41 and lysine-50 on actin structure and function

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Abstract

Subdomain 2 of actin is a dynamic segment of the molecule. The cross-linking of Gln-41 on subdomain 2 to Cys-374 on an adjacent monomer in F-actin inhibits actomyosin motility and force generation (Kim *et al.*, 1998; *Biochemistry* 37, 17,801–17,809). To shed light on this effect, additional modifications of the Gln-41 site on actin were carried out. Both intact G-actin and G-actin cleaved by subtilisin between Met-47 and Gly-48 in the DNase 1 binding loop of subdomain 2 were treated with bacterial transglutaminase. According to the results of Edman degradation, transglutaminase introduced an intramolecular zero-length cross-linking between Gln-41 and Lys-50 in both intact and subtilisin cleaved actins. This cross-linking perturbs G-actin structure as shown by the inhibition of subtilisin and tryptic cleavage in subdomain 2, an allosteric inhibition of tryptic cleavage at the *C*-terminus and decrease of modification rate of Cys-374. The cross-linking increases while the subtilisin cleavage dramatically decreases the thermostability of F-actin. The Mg- and S1-induced polymerizations of both intact and subtilisin cleaved actins were only slightly influenced by the cross-linking. The activation of S1 ATPase by actin and the sliding speeds of actin filaments in the *in vitro* motility assays were essentially unchanged by the cross-linking. Thus, although intramolecular cross-linking between Gln-41 and Lys-50 perturbs the structure of the actin monomer, it has only a small effect on actin polymerization and its interaction with myosin. These results suggest that the new cross-linking does not alter the intermonomer interface in F-actin and that changes in actomyosin motility reported for the Gln-41–Cys-374 intrastrand cross-linked actin are not due to decreased flexibility of loop 38–52 but to constrains introduced into the F-actin structure and/or to perturbations at the actin's *C*-terminus.

Introduction

The interaction of myosin with actin filaments (F-actin), coupled to ATP hydrolysis, is the molecular basis of the myosin motor action in muscle contraction and cell motility. Although myosin heads, called S1, are the molecular motors, the dynamic nature of actin filaments suggests that they also have an active role in these processes and are not just passive partners of myosin.

Electron microscopy studies of Orlova and Egelman (1992) revealed that the equilibration of actin filaments among different conformational states can be linked to the dynamic properties of subdomain 2, the DNase 1 binding loop 38–52 in it, and the *C*- terminus of actin. In the monomeric actin these two structural elements have been considered to be dynamic on the basis of crystallographic data on gelsolin- (McLaughlin *et al.*, 1993) and profilin-G-actin (Schutt *et al.*, 1993) complexes, normal mode analysis (Tirion and Ben Avraham, 1993). The changes in the proteolysis (Strzelecka-Golaszewska *et al.*, 1993) and fluorescence of reporter groups (Frieden *et al.*, 1980) at these sites depend on the nucleotide and divalent cation state of G-actin. Both structural (Orlova and Egelman, 1992) and solution studies (Strzelecka-Golaszewska *et al.*, 1996; Muhlrad *et al.*, 1994) have provided evidence for the mobility of the DNase 1 loop in F-actin and its intermolecular coupling to the *C*-terminus region (Kim and Reisler, 1996). These observations raise intriguing questions about the role of loop 38–52 and its dynamics in actin function.

The importance of loop 38–52 to F-actin structure is indicated by its location at the intermolecular interface in structural models of F-actin (Holmes *et al.*, 1990; Lorenz *et al.*, 1993; Chick *et al.*, 1996) and by changes in actin polymerization due to modifications of His-40 (Hegyi *et al.*, 1974) and Gln-41 (Kim *et al.*, 1995), and

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to proteolytic cleavage of this loop (Schwyter *et al.*, 1989; Khaitlina *et al.*, 1993). Direct binding of myosin to residues 40–42 on F-actin has been proposed in structural models of acto-S1 (Rayment *et al.*, 1993). However, such a contact between S1 and F-actin is either unlikely or unimportant because monoclonal antidansyl antibodies do not inhibit S1 binding to F-actin labeled at Gln-41 with a dansyl probe (Kim *et al.*, 1996). Also, while transglutaminase cross-links Gln-41 on G-actin to loop 636–642 on S1, it fails to promote such a reaction between S1 and F-actin (Eligula *et al.*, 1998). F-actin cross-linking to S1 via Lys-50 in the DNase 1 loop was achieved, however, with glutaraldehyde (Bonafe *et al.*, 1994) and maleimidobenzoic acid-*N*-hydroxysuccinimide ester (Bertrand *et al.*, 1994). These results indicate that only the outer part of loop 38–52 is accessible to myosin. Irrespective of the myosin binding to loop 38–52, its integrity and freedom of motion appear critical to actomyosin function. Subtilisin cleavage of this loop leads to strong inhibition of actin sliding in the *in vitro* motility assays (Schwyter *et al.*, 1990). The immobilization of this loop, via intrastrand cross-linking by *N*-(4-azido-2-nitrophenyl) putrescine (ANP) between Gln-41 and Cys-374 (Hegyi *et al.*, 1998) on adjacent actins, has even more interesting effect on actin. Such a cross-linking uncouples actomyosin motility from other aspects of actomyosin interactions; it inhibits the motility without a parallel change in acto-S1 ATPase or the binding of S1 to actin (Kim *et al.*, 1998). This result implies that dynamic transitions at the interface between adjacent actins are an essential element of the contractile process.

Actomyosin motility was shown to be inhibited by other actin cross-linking reactions as well (Prochniewicz and Yanagida, 1990). However, because the ANP cross-linking is localized to specific residues on actin, i.e. Gln-41 and Cys-374, it opens the way for further clarification of the role of loop 38–52 in actomyosin function. The first question we wished to address was which motions or changes within this loop, or between the loop and the adjacent actin, are important to the actomyosin motor.

In this work we describe a bacterial transglutaminase catalyzed zero-length intramolecular cross-linking between Gln-41 and Lys-50 in the DNase 1 binding loop of subdomain 2 both in intact and subtilisin cleaved actin. Proteolytic digestions and chemical modification of G-actin revealed a perturbation in G-actin structure by this cross-linking. On the other hand, the cross-linking affected neither actin polymerization nor the interaction of F-actin with myosin, including the *in vitro* motility of actin. These results suggest that the new intramolecular cross-linking does not perturb the intermonomer interface in F-actin. Moreover, our results also indicate that the changes in actomyosin motility observed earlier for the Gln-41–Cys-374 cross-linked F-actin (Kim *et al.*, 1998) are due to constrains introduced into F-actin structure and/or restrictions of the *C*-terminus mobility.

Materials and methods

Reagents

Adenosine triphosphate (ATP), 1,N^6-ethenoadenosine triphosphate (ε-ATP), dithioerythritol (DTE), dithiothreitol (DTT), subtilisin, trypsin, soybean trypsin inhibitor and phenylmethanesulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO). Dansyl ethylenediamine (DED) and N-(iodoacetyl)N′-(5-Sulfo-1-naphtyl) ethylene diamine (IAEDANS) were purchased from Molecular Probes (Eugene, OR). Bacterial transglutaminase was a generous gift from Drs Masao Motoki and Katsuya Seguro (Food Research and Development Laboratories, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210, Japan). All other chemicals were of analytical grade.

Proteins

Myosin and actin were prepared from back and leg muscles of rabbit by the methods of Tonomura *et al.* (1966) and Spudich and Watt (1971), respectively. S1 was obtained by digestion of myosin filaments with chymotrypsin at a 300:1 ratio (by mass) (Weeds and Taylor, 1975). The digestion was stopped using 0.2 mM PMSF. Protein concentrations were obtained by absorbance, using an $A_{280}^{1\%}$ = of 7.5 for S1 and an $A_{290}^{1\%}$ = of 6.3 for actin. Molecular masses were assumed to be 115 and 42 kDa for S1 and actin, respectively.

Preparation of various forms of actin

Actin was stored as CaATP-G-actin in G-actin buffer (0.2 mM $CaCl_2$, 0.1 mM ATP, 0.5 mM β-mercaptoethanol and 2 mM Tris–HCl, pH 7.6. This actin was used in all experiments unless stated otherwise. F-actin was prepared from CaATP-G-actin by polymerization with 0.2 mM EGTA and 2 mM $MgCl_2$. MgATP- and MgADP-G-actin were prepared essentially according to the procedure of Drewes and Faulstich (1991).

Cross-linking reaction

Intact or subtilisin cleaved CaATP-G-actin (50–100 μM) in G-actin buffer (0.2 mM $CaCl_2$, 0.1 mM ATP and 2 mM Tris–HCl, pH 7.6) was routinely incubated with 0.2 unit/ml bacterial transglutaminase overnight at 0°C and used within 24 h. The extent of cross-linking of subtilisin cleaved actin was determined by densitometric analysis of the Coomassie blue-stained protein bands on sodium dodelyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). For quantitation of the cross-linking of intact actin the transglutaminase treated actin samples were digested at 500:1 (w/w) ratio with subtilisin for 40–50 min at 25°C, run on SDS–PAGE, and then analyzed by densitometry as described above. The fraction of apparently uncleaved actin band at the end of digestion gives the extent of cross-linking,

which with intact and subtilisin cleaved actin was between 55 and 75%, and 80 and 100%, respectively.

Sequencing

Transglutaminase treated CaATP-G-actin was digested by subtilisin as described above, then run on SDS–PAGE and blotted to PDGF membrane. The blotted samples were sequenced with an Applied Biosystem Protein Sequencer (model 471 A) employing an Edman degradation sequencer program (Hunkapiller *et al.*, 1983).

Limited proteolysis

1. Tryptic digestion of CaATP-G-actin (12 μM) was performed in G-buffer at 16:1 (w/w) actin to trypsin ratio, at 25°C, for various time periods. Soybean trypsin inhibitor was added at 2:1 (w/w) ratio to trypsin to quench the reaction. The digested actin was analyzed by SDS–PAGE.
2. Tryptic digestion at the *C*-terminus of actin was carried out on AEDANS-Cys-374-CaATP-G-actin (12 μM) in G-buffer, at 16:1 (w/w) actin to trypsin ratio, at 25°C, in a cuvette of a PTI spectrofluorometer (Photon Technology Industries Co., South Brunswick, NJ). The digestion was monitored via changes in fluorescence intensity at 460 nm. Excitation wavelength was set at 340 nm.
3. Subtilisin digestion of CaATP-G-actin (12 μm) in G-buffer was carried out at 1000:1 or 500:1 actin to subtilisin (w/w) ratio, at 25°C, for various time intervals. PMSF (1.0 mM) was added to terminate the reaction. The digested actin was analysed by SDS–PAGE.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate gel electrophoresis was carried out according to Mornet *et al.* (1981) on either 7–18% polyacrylamide gradient or on 10% linear slab gels. Molecular masses of protein bands were estimated by comparing their electrophoretic mobility to that of markers of known molecular masses.

Labeling actin Gln-41 by dansyl ethylenediamine (DED)

Dansyl ethylenediamine labeling of actin was done essentially by the method of Kim *et al.* (1995). Briefly, G-actin (50 μM) was incubated with 100 μM DED and 0.4 unit/ml bacterial transglutaminase in G-actin buffer. The reaction was carried out overnight at 0°C and was stopped by removing excess DED on a Sephadex spin column equilibrated with G-buffer.

Labeling of Cys-374 of actin by IAEDANS

N-(iodoacetyl)N′-(5-Sulfo-1-naphtyl) ethylene diamine (120 μM) was added to CaATP-G-actin (60 μM) in 0.2 mM $CaCl_2$, 0.1 mM ATP and 2 mM Tris–HCl, pH 8.0, and incubated overnight at 0°C. The reaction was terminated by adding 1.0 mM DTE.

Kinetics of labeling of Cys-374 with CPM

CPM (0.5 μM) was added to 5 μM cross-linked and control G- or F-actin in 0.2 mM $CaCl_2$, 0.1 mM ATP and 2 mM Tris–HCl, pH 7.8, in the case of F-actin 2 mM $MgCl_2$ was also present. The time course of the reaction was followed by monitoring the fluorescence change at 10°C in a thermostated cell of PTI spectrofluorometer at excitation and emission wavelength of 390 and 460 nm, respectively.

$1,N^6$-ethenoadenosine triphosphate–adenosine triphosphate (ε-ATP–ATP) exchange

CaATP-G-actin (100 μM) was filtered through a Sephadex G-50 spin column equilibrated with 100 μM $MgCl_2$ and 5 mM Tris–HCl, pH 7.8. $1,N^6$-ethenoadenosine triphosphate was added at a 1.5-fold molar excess over filtered actin and the sample was incubated on ice for 1 h. Immediately before the measurements, actin was diluted at 3.0 μM. The sample was transferred to a thermostated spectrofluorometer cell (25°C) and the change in fluorescence was recorded after addition of 200 μM ATP. Excitation and emission wavelengths were set at 340 and 410 nm, respectively.

Actin-activated S1 ATPase

Actin-activated S1 ATPase activity (micromoles of phosphate/micromole of S1/s) was calculated from the inorganic phosphate produced, as measured according to Fiske and Subbarow (1925). The reaction was performed at 25°C on 1-ml aliquots taken at various time intervals. Incubation times were chosen so that no more than 15% of the ATP was hydrolyzed. The assay contained 0.1 μM S1 and between 2.5 and 40 μM F-actin in 2 mM $MgCl_2$, 20 mM HEPES buffer, pH 7.4 and 2 mM ATP. The ATPase data were fitted to the Michaelis–Menten equation to obtain the values for K_M and V_{max}.

Actin polymerization

Polymerization of 10 μM CaATP-G-actin in 0.1 mM $CaCl_2$, 0.4 mM ATP and 5 mM Tris–HCl pH 7.8 was initiated by adding 2.0 mM $MgCl_2$ or 10 μM S1. The polymerization reaction was followed by measuring the light scattering at 25°C in a PTI spectrofluorometer. Both excitation and emission wavelengths were set at 360 nm. Actin polymerization was tested also by sedimentation. In this method, the polymerized mixture was sedimented at 75,000 rpm for 45 min in a Beckman TL-100 ultracentrifuge and the resulting pellet and supernatant were analyzed by SDS–PAGE.

In vitro *actin motility assays*

These assays were performed at 25°C as described previously (Miller *et al*., 1996). Heavy meromyosin at a concentration of 300 μg/ml was adsorbed to the nitrocellulose-coated coverslips. Adenosine triphosphate-desensitized HMM was removed from the stock solution by pelleting HMM in the presence of actin and ATP. Assay solution was composed of 25 mM MOPS, pH 7.4,25 mM KCl, 2.0 mM $MgCl_2$, 2.0 mM EGTA, 5 mM DTT, 1.0 mM ATP and the glucose oxidase–catalase system to slow the photobleaching. Methylcellulose (0.4%) was present in all solutions. Actin filaments were labelled by rhodamine phalloidin as described by Miller *et al*. (1996). Determination of sliding speeds was made by Expertvision system (Motion Analysis, Santa Rosa CA).

Thermostability of actin

Thermostabilities of intramolecularly cross-linked and untreated actins were studied by assaying their ability to activate S1-ATPase activity, after heat treatment at 60°C, essentially as described by Kim *et al*. (1996). Briefly, F-actin solutions (50 μM) were incubated in F-actin buffer at 60°C. Aliquots were taken at different time intervals, cooled on ice, and then used for acto-S1 ATPase assays as described above. The concentrations of S1 and actin in these assays were 0.3 μM and between 9.0 and 20 μM, respectively.

Results

Cross-linking of actin and the mapping of cross-linked sites

CaATP-G-actin treated by bacterial transglutaminase appeared to be at least partially resistant to proteolysis by subtilisin (Figure 1). Because subtilisin cleaves actin between Met-47 and Gly-48, the apparent resistance to subtilisin cleavage indicates that an intramolecular bond has been formed between regions 1–47 and 48–374 of actin. This conclusion was substantiated by the finding that fragments 1–47 and 48–374 resulting from subtilisin cleavage of G-actin were joined by a covalent cross-linking bond following a treatment with transglutaminase. This is shown in Figure 1C, in which the 35 kDa band (representing the subtilisin-produced *C*-terminal 48–374 fragment of actin) essentially disappears and the intact actin band reappears again after the transglutaminase treatment, indicating the cross-linking of the two fragments. The intramolecular cross-linking of F-actin by transglutaminase was also attempted. However, no evidence of such cross-linking in F-actin was obtained from its subtilisin cleavage, which was not inhibited by the transglutaminase treatment (data not shown).

Because Gln-41 is the only glutamine residue on actin that reacts with primary amines in the transglutaminase catalyzed reaction (Takashi, 1988; Hegyi *et al*., 1992; Kim *et al*., 1995, 1996; Eligula *et al*., 1998; Hegyi *et al*., 1998), this residue is assumed to be one of the cross-linking sites on G-actin. This assumption was confirmed by the observed competition (data not shown) between intramolecular cross-linking and the specific labeling of Gln-41 on actin by DED (Kim *et al*., 1995).

The second cross-linking site was determined by the *N*-terminal sequencing of two G-actin preparations: (i), first treated by transglutaminase and then digested by subtilisin and (ii), first digested by subtilisin and then treated by transglutaminase. Control sample, untreated by transglutaminase but cleaved by subtilisin, was also sequenced as described in 'Materials and methods'. Because the *N*-terminus of actin is blocked, the sequences are expected to start at Gly-48, which is the first residue after the site of the subtilisin cleavage. The following *N*-terminal sequence was obtained for the two actins treated with transglutaminase: Gly-Gln-*X*-Asp-Ser-Tyr-Val-Gly-Asp. The same sequence was obtained from the untreated control sample, except that Lys was detected instead of the unknown *X*. This sequence corresponds to residues 48–56 on actin with *X* identified as Lys-50. The fact that Lys-50 is missing from the cross-linked samples identifies this residue as the second cross-linking site in both the cleaved and uncleaved actin.

The nucleotide and divalent cation state of G-actin affects the extent of intramolecular cross-linking, which was found to decrease in the order MgADP- > MgATP- > CaATP-G-actin. The extent of cross-linking in the above states were 68.6, 64.5 and 51.5%, respectively.

Perturbation of actin structure by intramolecular cross-linking

The effect of cross-linking on actin structure was studied by the method of limited proteolysis by trypsin and subtilisin. Trypsin cleaves G-actin in subdomain 2, at Arg-62 and Lys-68 (Jacobson and Rosenbusch, 1976), and near the *C*-terminus, at Arg-372 and Lys-373 (Mornet and Ue, 1984). The tryptic cleavage at subdomain 2 of cross-linked and control actin was compared by following the appearance of the 33 kDa *C*-terminal fragment on SDS–PAGE (Figure 2). The results indicate that the intramolecular cross-linking perturbs actin structure since it strongly inhibits the tryptic cleavage of subdomain 2. The effect of cross-linking on the subtilisin cleavage between Met-47 and Gly-48 was studied by the *N*-terminal Edman sequencing. According to the sequencing data (not shown), the cleavage at this site was inhibited by between 45 and 65% in the cross-linked actin relative to that in control actin, which again indicates the perturbation of G-actin structure. The tryptic cleavage near the *C*-terminus was examined on CaATP-G-actin labeled with IAEDANS on Cys-374. The digestion in this case was followed by monitoring the decrease in the fluorescence intensity of the IAEDANS probe (Figure 3). The fluorescence decrease data

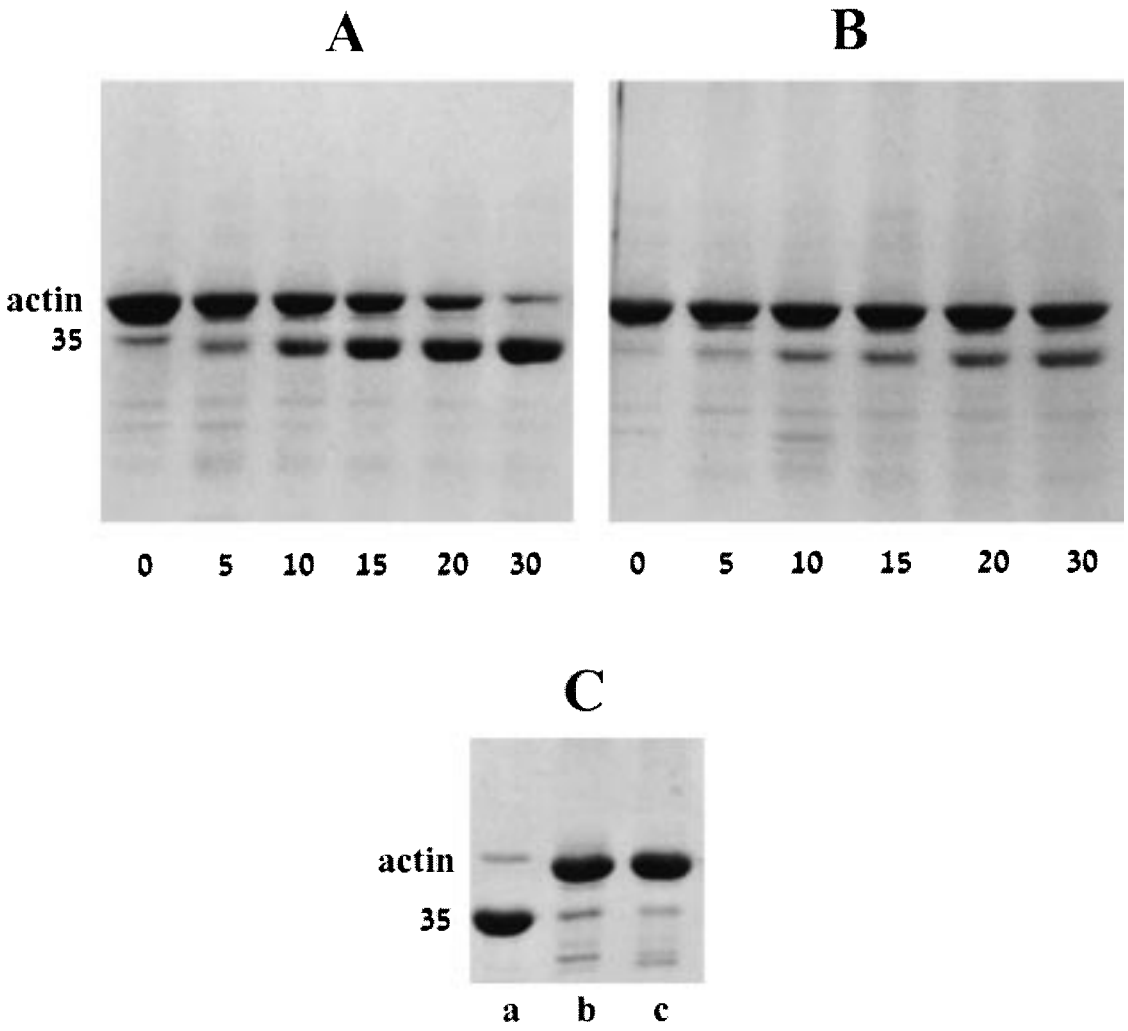

Fig. 1. Subtilisin digestion of CaATP-G actin treated with transglutaminase and transglutaminase treatment of CaATP-G-actin cleaved by subtilisin. Untreated (A) and transglutaminase treated (B) CaATP-G-actin were digested with subtilisin for various time intervals and analyzed by SDS–PAGE and densitometry as described in 'Materials and methods'. According to such an analysis, 72% of actin is cross-linked. Digestion times (min) are given under each lane. (C), CaATP-G-actin first cleaved by subtilisin for 30 min and then treated with transglutaminase (94% of the cleaved actin is cross-linked). Symbols under panel C: (a), subtilisin cleaved actin; (b), subtilisin cleaved actin treated with transglutaminase; (c), uncleaved and untreated actin. Molecular mass or protein name (actin) is given next to each band.

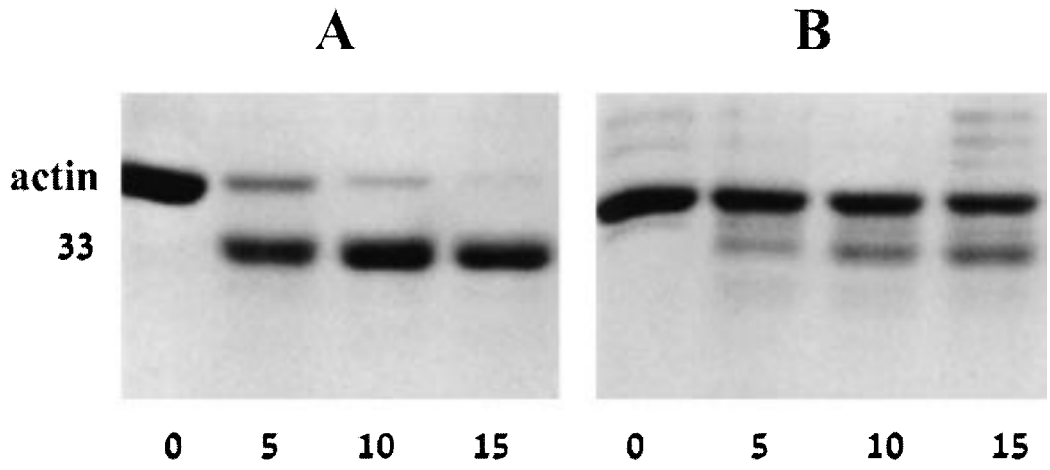

Fig. 2. Tryptic digestion of transglutaminase treated CaATP-G-actin. For conditions of transglutaminase treatment and tryptic digestion see 'Materials and methods'. (A), Untreated actin; (B), transglutaminase treated actin (68% cross-linked). Molecular mass or protein name (actin) is given next to each band. Digestion times (min) are given under each lane.

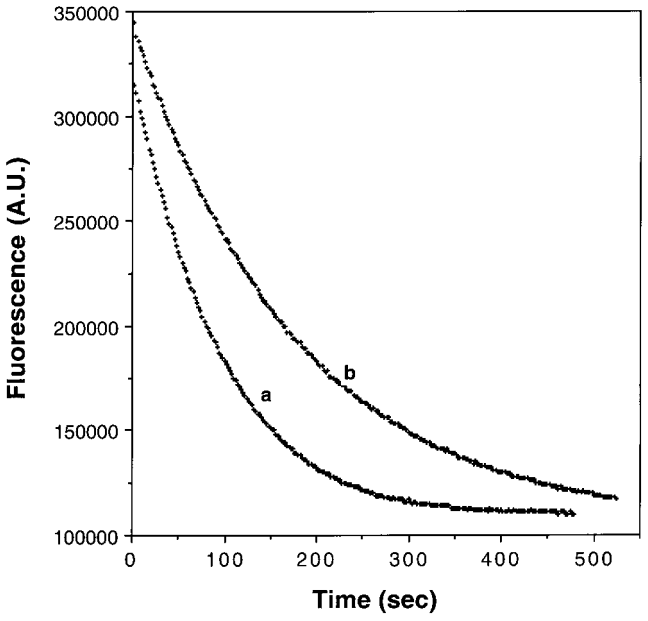

Fig. 3. Tryptic digestion of transglutaminase treated CaATP-G-actin labeled with IAEDANS at Cys-374. Tryptic digestion at the *C*-terminus was followed by monitoring the decrease in AEDANS fluorescence intensity at 460 nm. Excitation wavelength was set at 340 nm. Transglutaminase untreated (a) and treated (73% cross-linked) (b) CaATP-G-actin. For conditions of Cys-374 modification and tryptic digestion of actin see 'Materials and methods'.

fitted single exponential curves and yielded the apparent first order rate constants for the digestion of cross-linked and control actin of 6.3×10^{-3} and 10.1×10^{-3} s^{-1}, respectively. Thus, the intramolecular cross-linking of Gln-41 and Lys-50 significantly inhibited the proteolysis also at the distant *C*-terminal site revealing allosteric changes in actin.

The effect of cross-linking of actin on the specific modification of Cys-374 by CPM (Muhlrad *et al.*, 1994) was monitored by recording the fluorescence increase during the course of reaction (Figure 4). The fluorescence increase was fitted to a single exponential from which the first order rate constant of modification were calculated. The cross-linking significantly decreased both the rate and the extent of modification of G- and F-actins. The rate constants of control and cross-linked G-actin and F-actin were 0.085, 0.058, 0.071 and 0.037 s^{-1}, respectively. The finding that both in G- and F-actin the intramolecular cross-linking at subdomain 2 influences the modification at the *C*-terminus again indicates an existing communication between these two distant sites.

Nucleotide exchange (ε-ATP–ATP) on G-actin was virtually unchanged by the intramolecular cross-linking and the resulting changes in subdomain 2 and *C*-terminus. The exchange was monitored via a decrease in the fluorescence intensity of ε-ATP. Single exponential plots of fluorescence intensity vs. time yielded the same rate constants for the exchange of ε-ATP in control and cross-linked actin: 2.57×10^{-3} and 2.55×10^{-3} s^{-1}, respectively (data not shown).

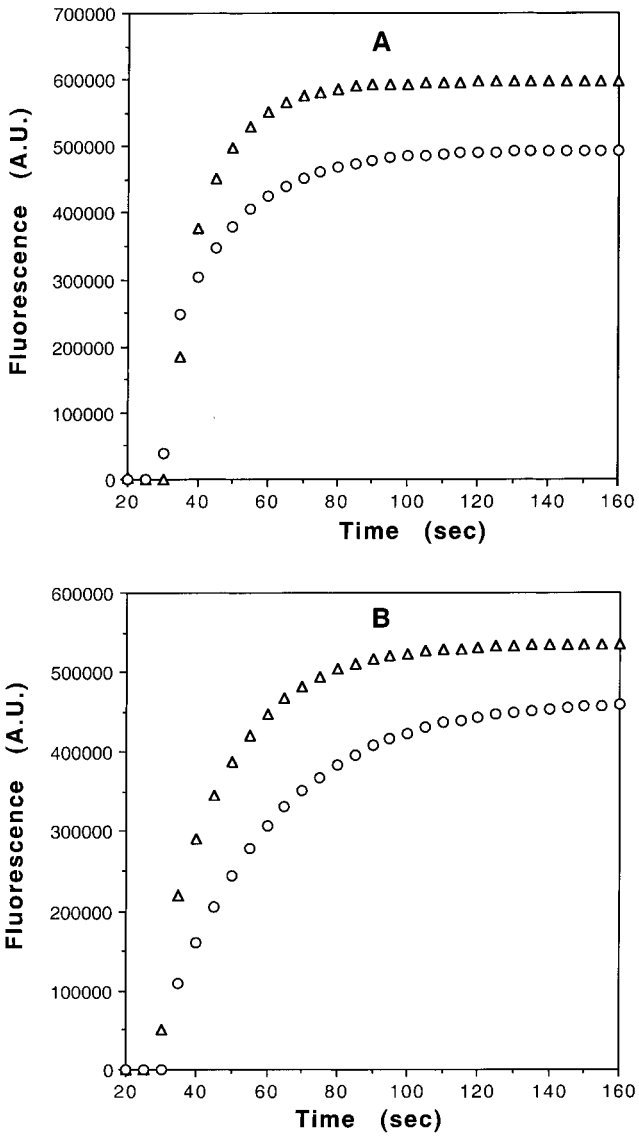

Fig. 4. Effect of cross-linking on the modification of Cys-374 by CPM. To control and 69% cross-linked G-actin (A) and F-actin (B) CPM was added and the increase in fluorescence intensity was monitored as described in 'Materials and methods'. Symbols: (Δ), control; (○), cross-linked actin.

The cross-linking of Gln-41 to Lys-50 was accompanied by a stabilization of F-actin structure. The thermostabilities of untreated, cross-linked, subtilisin cleaved and cross-linked, and subtilisin cleaved F-actins were assessed by measuring their ability to activate S1 ATPase after incubation at 60°C (Figure 5). (Cross-linking and subtilisin cleavage were carried out on CaATP-G-actin, which was then polymerized to F-actin.) The results show that cross-linking of intact actin considerably improved while the subtilisin cleavage dramatically decreased the thermostability of F-actin. The indicates that the DNase 1 binding loop has a significant influence on the stability of actin. It should be mentioned, however, that the cross-linking of subtilisin cleaved actin does not affect its thermostability.

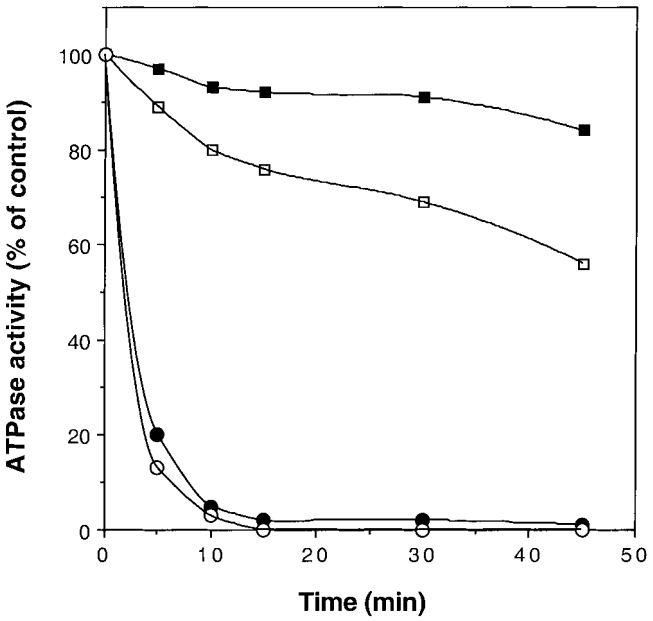

Fig. 5. Effect of cross-linking and subtilisin cleavage on the thermostability of F-actin. CaATP-G-actin was treated with transglutaminase, cleaved by subtilisin and polymerized as described in 'Materials and methods'. After polymerization, F-actin (50 μM) were incubated at 60°C. Aliquots were taken at different time intervals, cooled on ice and then used for measuring actin-activation of S1 ATPase. Symbols: (□), actin; (■), cross-linked actin (74% cross-linked); (○), subtilisin cleaved actin; and (●), cross-linked subtilisin cleaved actin (92% cross-linked).

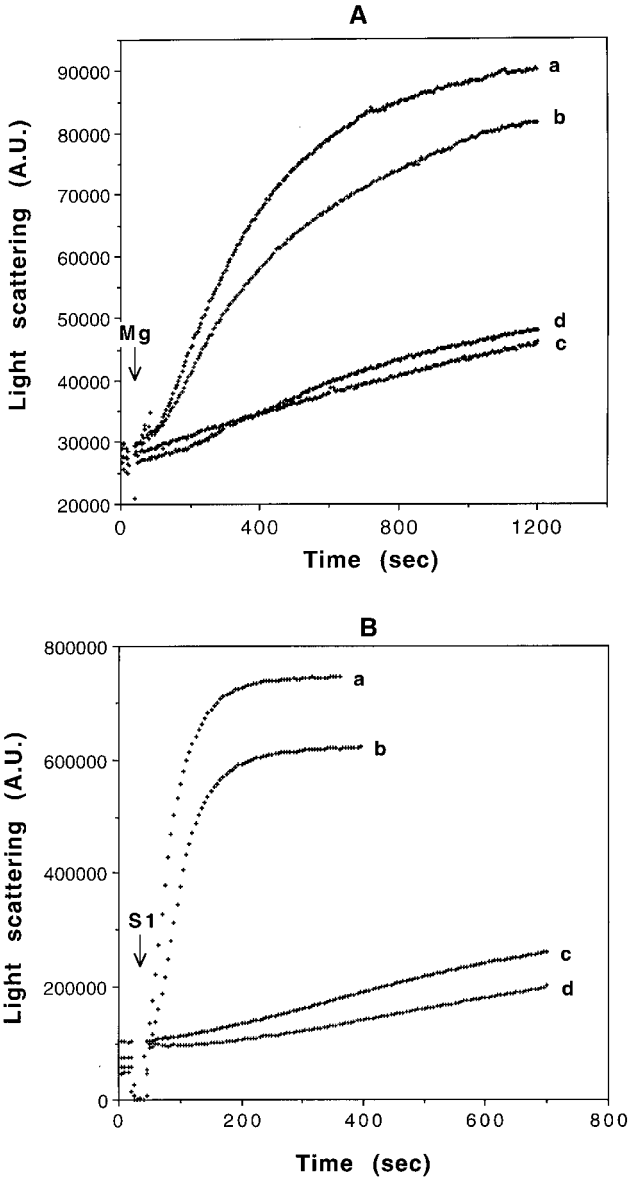

Fig. 6. Effect of intramolecular cross-linking on actin polymerization. CaATP-G-actin (10 μM) was polymerized by addition of 2.0 mM $MgCl_2$ (A) or 10 μM S1 (B). The polymerization was followed by monitoring changes in light scattering at 360 nm as described in 'Materials and methods'. (a), cross-linked actin (65% cross-linked); (b), untreated actin; (c), subtilisin cleaved actin, and (d), subtilisin cleaved and then cross-linked actin (87% cross-linked).

Effect of intramolecular cross-linking on actin function

Because the DNase 1 binding loop is directly involved in intermonomer interactions in F-actin (Holmes *et al.*, 1990; Lorenz *et al.*, 1993; Chick *et al.*, 1996), its was of interest to examine the effect of Gln-41 to Lys-50 cross-linking on the polymerization of actin. The cross-linking of intact actin slightly increased the rate and extent of both Mg- and S1-induced polymerization (Figure 6A and B, respectively). Cross-linking of subtilisin cleaved actin did not improve its polymerization, which is inhibited by the cleavage (Figure 6). The extent of actin polymerization was checked by sedimentation (for details see 'Materials and methods'). All actins were completely pelleted following a 30 min polymerization by 2 mM $MgCl_2$, except for the cleaved and cleaved and cross-linked actin, for which about 20% of the protein remained in the supernatant. However, when the polymerization was carried out in the presence of equimolar phalloidin, all of the cleaved actin was pelleted in such assays. (Data not shown.)

The ability of cross-linked actin to activate the ATPase activity of S1 was determined over a range of actin concentrations. Intramolecular cross-linking of actin has only a minor effect, if any, on the actin activation of S1 ATPase and the kinetic parameters of this reaction are essentially unchanged. The V_{max} and K_M values were 23.9 ± 1.0 s^{-1} and 18.6 ± 2.9 μM for intact actin and 22.9 ± 1.8 s^{-1} and 26.1 ± 0.5 μM for the cross-linked actin. The sliding speed of intact actin filaments in the *in vitro* motility assays did not change as a result of the intramolecular cross-linking (Table 1). It should be noted that neither the activation of S1 ATPase by subtilisin cleaved actin (results not shown) nor the sliding speed of such actin, which are significantly reduced by the cleavage (Schwyter *et al.*, 1989, 1990), are improved to any extent by its intramolecular cross-linking (Table 1).

Discussion

This work was promoted by the striking result of a recent study which described the inhibition of force and motion generation by actomyosin due to intrastrand

Table 1. Effect of cross-linking Gln-41 to Lys-50 in G-actin on the *in vitro* motility of actin filaments

Actin	Sliding speed (m/s)
Uncross-linked	4.5 ± 0.4
Cross-linked	4.4 ± 0.3
Cleaved	2.3 ± 0.5
Cleaved and cross-linked	2.4 ± 0.7

All data are the means (SD) of four independent experiments. The *in vitro* motility assays were carried out as described in 'Materials and methods'. The sliding of between 100 and 200 actin filaments was monitored in all *in vitro* motility assays.

cross-linking of Gln-41 to Cys-374 on adjacent monomers in F-actin (Kim *et al.*, 1998). The uncoupling between the mechanochemical function of actomyosin and its ATPase activity and binding observed for such cross-linked actin sharpens the interest in the role in the crossbridge cycle of dynamic changes in F-actin, and in particular at its intermonomer interfaces. Because of the reported flexibility of loop 38–52 (Orlova and Egelman, 1992; McLaughlin *et al.*, 1993; Schutt *et al.*, 1993; Muhlrad *et al.*, 1994; Strzelecka-Golaszewska *et al.*, 1996), the first question related to the inhibition of the *in vitro* motility of Gln-41–Cys-374 cross-linked actin (Kim *et al.*, 1998) is whether this effect arises from the blocking of internal rearrangements in loop 38–52 on actin (tethered via Gln-41 to Cys-374 on adjacent monomer) or the restriction of motions at the intermonomer interface. The aim of this study was to prepare actin with intramolecularly cross-linked Gln-41 and to explore the effect of such a cross-linking on actin structure and its the mechanochemical properties.

The main result of this work is the finding that transglutaminase produces an intramolecularly cross-linked Ca-G-actin, between Gln-41 and Lys-50 (Figure 7), and that such cross-linking has no effect on the *in vitro* motility of actin filaments and the acto-S1 ATPase. Thus, in contrast to the immobilization of the subdomain 2/subdomain 1 interface in F-actin via intermolecular cross-linking of Gln-41 to Cys-374, the internal immobilization of loop 38–52 does not impede the mechanical function of actomyosin. That such loop cross-linking has little, if any, effect on the intermonomer interface is indicated by similar rates of polymerization of the uncross-linked and cross-linked actins. It follows that the impaired contractile function of Gln-41–Cys-374 cross-linked actin can be attributed to changes in the dynamic properties of the intermonomer interface in F-actin and/or the constraints imposed on actin's *C*-terminus.

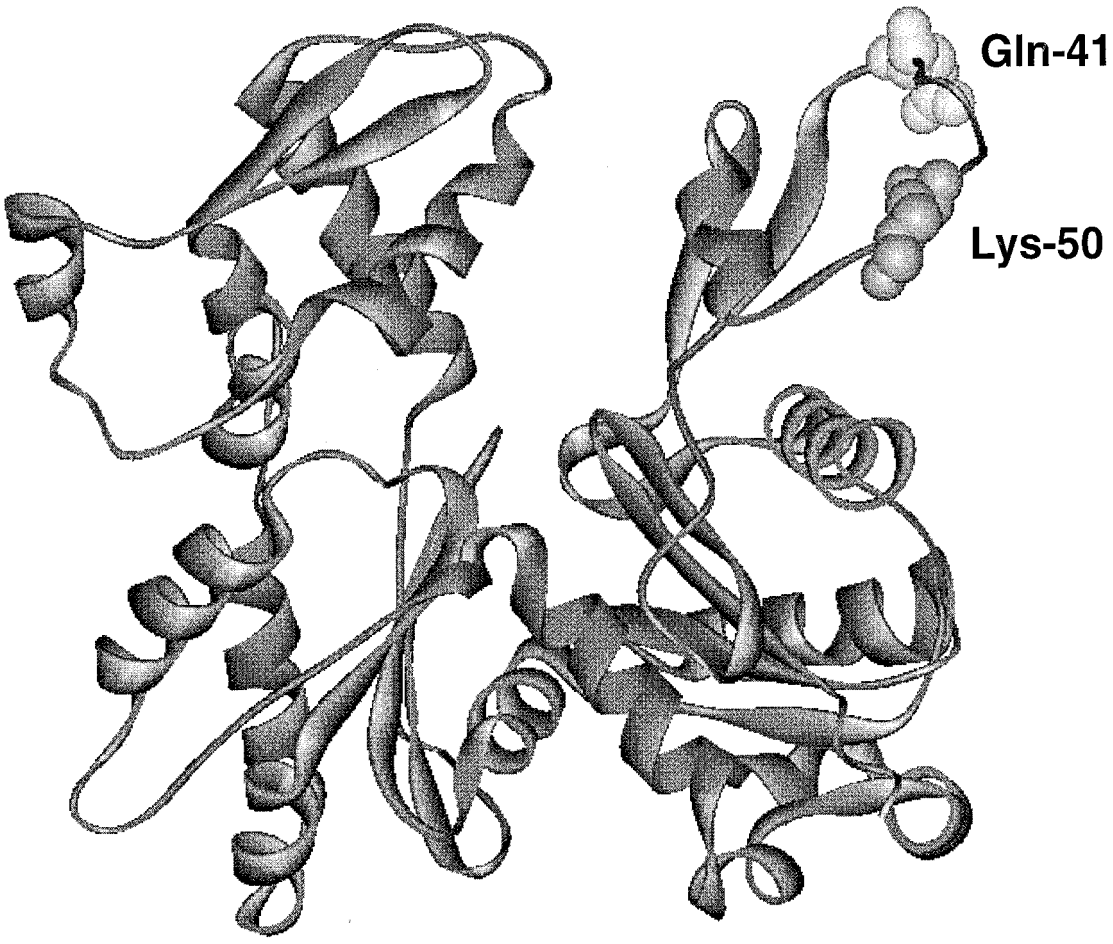

Fig. 7. The location of the cross-linked residues at the DNase I binding loop of subdomain 2 in the atomic structure of G-actin. The atomic structure is according to Kabsch *et al.* (1990).

Despite the functional integrity of Gln-41–Lys-50 cross-linked actin significant short and long range conformational changes are induced in G-actin by such a reaction. It is possible that such changes, which among others decrease the proteolytic accessibility of subdomain 2 and the *C*-terminus and reduce the rate of modification of Cys-374, are perhaps masked or corrected for by actin's polymerization and therefore, do not affect actin-myosin interactions. This assumption is supported by the lack of any improvement in the polymerization, *in vitro* motility, and the activation of S1 ATPase by subtilisin cleaved actin after its cross-linking. Clearly, linking Gln-41 to Lys-50 is not enough to undo the damage caused by the cleavage between Met-47 and Gly-48. In the simplest scenario this indicates that the short segment between Gly-42 and Met-47, which remains unconstrained in the cleaved and cross-linked actin, plays an important role in intermonomer contacts in F-actin. Interestingly, Tirion *et al.* (1995) speculated that in F-actin loop 38–52 might form two β-strands, with Gly-48 situated at a turn. If that were the case, subtilisin cleavage of actin could partially unravel one or both of these β-strands and thus alter intermonomer contacts in F-actin.

The formation of the zero length cross-link between the γ-oxygen of Gln-41 and the ε-amino group of Lys-50, whose distance is 12.86 Å in the atomic structure of G-actin-DNase 1 complex (Kabsch *et al.*, 1990), implies that the structure of the DNase 1 binding loop is highly mobile, which is in accordance with the conclusion of crystallographic studies (McLaughlin *et al.*, 1993) and other findings (Orlova and Egelman, 1992; Muhlrad *et al.*, 1994; Strzelecka-Golaszewska *et al.*, 1993, 1996; Tirion *et al.*, 1995) about the dynamic nature of actin structure. The dependence of the extent of cross-linking on the bound nucleotide and cation state of G-actin further emphasizes the dynamic nature of loop 38–52 in agreement with the results of Strzelecka-Golaszewska *et al.* (1993). Our observations on the increased thermostability of cross-linked and decreased thermostability of the cleaved-F-actin add to the evidence on the role of this loop also in the stabilization of F-actin structure (Khaitlina *et al.*, 1993).

Taken together, the results of this work advance the understanding of the role of subdomain 2 and loop 38–52 in F-actin structure and function. Most importantly, by showing that intraloop cross-linking of Gln-41 to Lys-50 does not uncouple actin motility from acto-S1 ATPase they add credence to the view that such uncoupling can be caused by blocking dynamic rearrangements of the intermonomer interface in F-actin (Kim *et al.*, 1998).

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