Myosin-Induced Changes in F-Actin: Fluorescence Probing of Subdomain 2 by Dansyl Ethylenediamine Attached to Gln-41

Eldar Kim*, Carl J. Miller*, Masao Motoki‡, Katsuya Seguro‡, Andras Muhlrad*, and Emil Reisler*
*Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, California 90095, and ‡Food Research and Development Laboratories, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki-ku, Kawasaki, Kanagawa 210, Japan

ABSTRACT Actin labeled at Gln-41 with dansyl ethylenediamine (DED) via transglutaminase reaction was used for monitoring the interaction of myosin subfragment 1 (S1) with the His-40-Gly-42 site in the 38–52 loop on F-actin. Proteolytic digestions of F-actin with subtilisin and trypsin, and acto-S1 ATPase measurements on heat-treated F-actin revealed that the labeling of Gln-41 had a stabilizing effect on subdomain 2 and the actin filaments. DED on Gln-41 had no effect on the values of K_m and V_{max} of the acto-S1 ATPase and the sliding velocities of actin filaments in the in vitro motility assays. This suggests either that S1 does not bind to the 40–42 site on actin or that such binding is not functionally important. The binding of monoclonal antidansyl IgG to DED-F-actin did not affect acto-S1 binding in the absence of nucleotides, indicating that the 40–42 site does not contribute much to rigor acto-S1 binding. Myosin-induced changes in subdomain 2 on actin were manifested through an increase in the fluorescence of DED-F-actin, a decrease in the accessibility of the probe to collisional quenchers, and a partial displacement of antidansyl IgG from actin by S1. It is proposed that these changes in the 38–52 loop on actin originate from S1 binding to other myosin recognition sites on actin.

INTRODUCTION

Biochemical and structural studies have suggested that actin filaments can exist in different conformations depending on the metal ions, nucleotides, and proteins bound to them (Egelman and Orlova, 1995). Strong evidence implicates the DNaseI-binding loop on actin i.e., residues 38–52 in subdomain 2, in transitions between conformationally distinct states (Tirion et al., 1995; Schutt et al., 1993). In particular, the work of Orlova and Egelman (1993) showed that subdomain 2 and the 38–52 loop were rotated in F-Mg^{2+}-actin to a larger radial distance from the filament axis than in F-Ca^{2+}-actin and suggested that this rotation was responsible for the greater structural flexibility of F-Mg^{2+}-actin filaments. The same authors also demonstrated that the filament structure was considerably stabilized by the binding of a phosphate analog, BeF_x, to F-MgADP-actin (Orlova and Egelman, 1992). BeF_x shifts the position of subdomain 2 in actin to a lower radius, similar to that proposed in the Holmes et al. (1990) model of F-actin structure. Importantly, biochemical studies also showed the stabilization of F-MgADP-actin filaments by BeF_x (Combeau and Carlier, 1988), confirmed the analog-induced changes in subdomain 2, and revealed a strong cooperativity of these transitions (Muhlrad et al., 1994).

Large internal structural cooperativity of F-actin has been also detected upon the binding of phalloidin (Drewes and Faulstich, 1993). This cooperativity was elegantly demonstrated in a recent electron microscopy analysis of actin complexes with gelsolin and phalloidin, and was linked to conformational states of actin's C-terminus and subdomain 2 (Orlova et al., 1995). The subdomain 2-related structural cooperativity of actin is of potential interest in actomyosin interactions for several reasons. First, the structural model of the actomyosin complex obtained by docking the atomic structure of myosin subfragment 1 (S1) (Rayment et al., 1993a) to the model of F-actin structure (Holmes et al., 1990) predicts the binding of S1 to several sites on actin, among them residues 40–42 in the 38–52 loop (Rayment et al., 1993b; Schroder et al., 1993). Second, the binding of S1 and heavy meromyosin to actin has been reported to cause cooperative changes of actin-bound probes (Oosawa et al., 1972), to influence the conformation of the 38–52 loop (Fievez and Carlier, 1993), and to increase the radial coordinate of Gln-41 by 4–5Å (Kasprzak et al., 1988). Third, subtilisin cleavage in the 38–52 loop results in a large decrease in the in vitro motility of actin filaments (Schwyter et al., 1990). These interconnections, as well as the cooperativity involved in the regulation of actomyosin interactions (Butters et al., 1993), and the yet to be determined role of structural changes in actin in actomyosin-based motility merit closer scrutiny of the myosin binding to the 38–52 loop on actin.

In this work, we used a fluorescence probe, dansyl ethylenediamine (DED), attached to Gln-41 via transglutaminase reaction to show myosin-induced changes in the 38–52 loop on F-actin. Previous work demonstrated that this label is a sensitive probe of conformational changes in the 38–52 loop on G-actin (Kim et al., 1995).

Received for publication 20 October 1995 and in final form 14 December 1995.

Address reprint requests to Dr. Emil Reisler, Department of Chemistry and Biochemistry, University of California, 405 Hilgard Avenue, Los Angeles, CA 90095-1569. Tel.: 310-825-2668; Fax: 310-206-7286; E-mail: reisler@ewald.mbi.ucla.edu.

The permanent address of Dr. Muhlrad is: Department of Oral Biology, Hadassah School of Dental Medicine, Hebrew University, Jerusalem, 91010 Israel.

0006-3495/96/03/1439/08 $2.00

MATERIALS AND METHODS

Reagents

ATP, ADP, α-chymotrypsin, trypsin, and subtilisin Carlsberg were purchased from Sigma Chemical Co. (St. Louis, MO). Bradford reagent was obtained from Bio-Rad (Richmond, CA). DED was from Molecular Probes (Eugene, OR). *N*-(4-azido-2-nitrophenyl)-putrescine (ANP) was a generous gift from Dr. G. Hegyi.

Preparations of proteins

Actin and myosin from rabbit back muscle were prepared according to Spudich and Watt (1971) and Godfrey and Harrington (1970), respectively. S1 was prepared as described by Weeds and Pope (1977). Microbial transglutaminase was obtained and purified as previously described (Huang et al., 1992). Monoclonal antidansyl antibodies (IgG) were a generous gift from Dr. S. Morrison.

Labeling of actin

The labeling of Gln-41 on G-CaATP-actin (2.0 mg/ml) with DED (100 μM) and ANP (100 μM; in dark) was carried out in the presence of 0.4 mM ATP, 0.2 mM $CaCl_2$, 5.0 mM Tris-HCl at pH 7.7, and 0.5 unit/ml microbial transglutaminase for 2 h at 23°C. Excess DED was removed from the labeled actin on Sephadex G-50 spin columns equilibrated with G-actin buffer (0.2 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM β-mercaptoethanol, and 5.0 mM Tris-HCl at pH 7.7). Excess ANP was removed from the labeled actin after its polymerization by 2.0 mM $MgCl_2$ for 30 min at 23°C (in the presence of 0.4 mM EGTA) and pelleting by ultracentrifugation at 40,000 rpm for 2 h. The actin pellet was rehomogenized in F-actin buffer free of β-mercaptoethanol to a final concentration of 1.0 mg/ml. The amounts of DED and ANP bound to actin were determined by using their respective extinction coefficients $\epsilon_{334} = 4.8 \times 10^3\ M^{-1}\ cm^{-1}$ and $\epsilon_{470} = 5.4 \times 10^3\ M^{-1}\ cm^{-1}$. The concentration of the modified actin was determined by Bradford assay. The stoichiometry of G-actin labeling with DED and ANP ranged between 0.90 and 1.0 label per actin.

Photocrosslinking of ANP-F-actin

ANP-F-actin (12.0 μM) was photocrosslinked in the absence and presence of S1 (12.0 μM) as described by Hegyi et al. (1992). At different time points the cross-linking was stopped in reaction aliquots by 1.0 mM β-mercaptoethanol. S1 was removed from the photocrosslinked samples by ultracentrifugation of the actin and S1 mixtures preincubated with 0.4 M NaCl, 1 mM $MgCl_2$, 1 mM ADP, 5 mM NaF, and 0.2 mM $BeCl_2$. The pelleted actin was analyzed by SDS PAGE.

Fluorescence measurements

All fluorescence measurements were carried out in a Spex Fluorolog spectrofluorometer (Spex Industries, Inc., Edison, NJ) at 25°C. DED emission was monitored after excitation at $\lambda = 334$ nm. The accessibility of DED to a collisional quencher was examined by titrating the labeled F-actin with acrylamide at 25°C. The titration data were analyzed in terms of Stern-Volmer plots to yield the Stern-Volmer constants.

Proteolytic digestions

Proteolytic digestions of F-actin (1.0 mg/ml) by subtilisin (0.04 mg/ml) were carried out in F-actin buffer (0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM EGTA, 2.0 mM $MgCl_2$ and 5.0 mM Tris-HCl at pH 7.7) at 25°C. The reaction was stopped with 5.0 mM PMSF, and the rate of actin cleavage was determined by densitometric analysis of reaction aliquots displayed on SDS PAGE. The disappearance of the 42-kDa actin band on SDS gels (due to actin cleavage) followed a first order process and yielded a rate constant for the digestion reaction. Tryptic digestions of F-actin were done as described by Muhlrad et al. (1994).

Acto-S1 ATPase activity measurements

Actin-activated MgATPase activity of S1 was determined under steady-state conditions at 25°C by a colorimetric assay in a solution containing 0.3 μM S1, F-actin and F-DED-actin between 0 and 40 μM, 3.0 mM ATP, 3.0 mM $MgCl_2$, 5.0 mM KCl, and 10 mM imidazole, pH 7.0. ATPase activities were calculated as micromoles of P_i released per micromole of S1/s and fitted directly to the Michaelis-Menten equation to obtain K_m and V_{max} values by using Sigma Plot 4.1

Thermostability experiments

The thermostabilities of DED-labeled and unlabeled actins were compared by assaying their function, i.e., the activation of S1-ATPase activity, after heat treatment at 60°C. F-actin solutions (18 μM) were incubated at 60°C in the presence of 0.2 mM ATP, 2.0 mM $MgCl_2$, 0.2 mM β-mercaptoethanol, and 5 mM EPPS at pH 7.5. Aliquots of actin were removed at different times from the 60°C bath, placed on ice, and then used in acto-S1 ATPase measurements. ATPase measurements were carried out as described above; the concentrations of S1 and F-actin in these assays were 0.3 and 9.0 μM, respectively.

Binding experiments

The molar ratios of antidansyl IgG and S1 bound to DED-F-actin (in F-actin buffer, at 25°C) were determined by pelleting the mixtures of these proteins in a Beckman airfuge for 20 min, then performing an SDS PAGE analysis of the pelleted samples (Adams and Reisler, 1993). Free IgG and S1 were not pelleted under these conditions. The proteins (at 1.0-μM concentration) were preincubated with each other for 20 min before the ultracentrifugation.

In vitro motility assays

The motility assays were performed at 25°C as previously described (Muhlrad et al., 1994).

RESULTS

Structural properties of DED-F-actin

The use of DED-Gln-41-labeled actin for examining the effects of S1 on the environment of the probe in F-actin has been stimulated by the high sensitivity of this probe to nucleotide and metal ion-dependent changes in the loop 38–52 on G-actin (Kim et al., 1995). In G-actin the modification of Gln-41 inhibited the rate of subtilisin cleavage of this loop by 60%, but had only minor, if any, effect on tryptic cleavage at Lys-61 and Lys-68 (Kim et al., 1995). Thus, it appeared that the perturbation of the G-actin structure by DED on Gln-41 was either small and localized to the 38–52 loop or that the probe altered somewhat the subtilisin docking to G-actin.

Similar proteolytic digestion experiments on DED-labeled F-actin revealed an increase in filament stability due to Gln-41 modification. Subtilisin digestions of modified and unmodified F-Mg^{2+}-actin yielded the same products

but at almost fivefold different rates (Fig. 1 *a*). The ~80% decrease in the rate constant of DED-F-actin cleavage, relative to that of F-actin cleavage (from 0.19±0.01 min^{-1} to 0.04±0.01 min^{-1}, Fig. 1 *a*), is almost twofold greater than the inhibition of subtilisin cleavage in G-actin. This suggests DED-related changes in the 38–52 loop environment in F-actin. Digestions of copolymers of labeled and unlabeled F-actin with subtilisin for 10 min revealed little change in the rates of their cleavage with up to 35% DED-actin in the filaments (Fig. 1 *b*). This is in contrast to the strongly positive cooperativity of subdomain 2 stabilization by BeF_x (Muhlrad et al., 1994) and phalloidin (Drewes and Faulstich, 1993), and suggests a smaller effect of DED on filament structure.

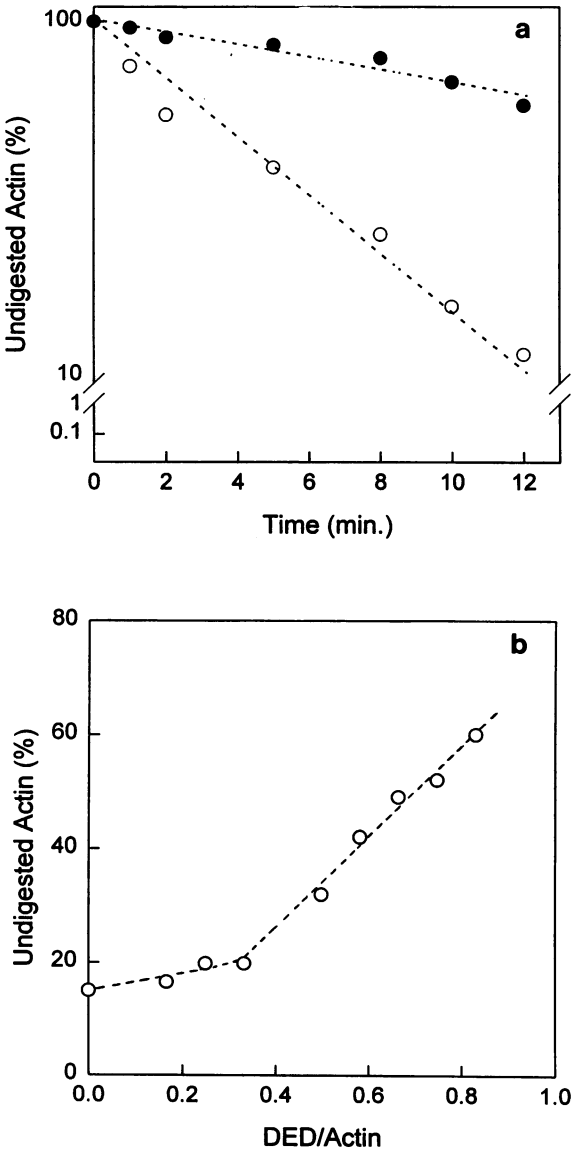

FIGURE 1 Subtilisin cleavage of F-actin (○) and DED-F-actin (●). Actin (1.0 mg/ml) was digested with subtilisin (0.04 mg/ml) in F-actin buffer (0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM EGTA, 2.0 mM $MgCl_2$, and 4.0 mM Tris-HCl pH 7.7) at 25°C. *a*) Semilogarithmic plot of the time course of actin digestion by subtilisin. Reaction aliquots were analyzed by densitometry of the 42-kDa band of uncleaved actin on SDS PAGE. The first order rate constants were 0.19±0.01 min^{-1} and 0.04±0.01 min^{-1} for the cleavage of F-actin and DED-F-actin, respectively. *b*) Percentage of undigested actin in copolymers of unlabeled and DED-labeled actin after a 10 min cleavage with subtilisin.

Tryptic digestions of DED-F-actin confirmed this conclusion. Unlabeled and labeled F-actin yielded the same tryptic products with first order rate constants of 0.18±0.03 min^{-1} and 0.11±0.01 min^{-1}, respectively (results not shown). Because such differences in the rates of tryptic cleavage were not detected for G-actin (Kim et al., 1995), the F-actin digestions support the idea of subdomain 2 stabilization in F-Mg^{2+}-actin by DED on Gln-41. The stabilization of F-actin by DED was confirmed also by monitoring the inhibition of actin's ability to activate S1-ATPase activity because of heat treatment at 60°C. The thermal "inactivation" of F-actin followed a first order process (Fig. 2) and occurred at a ninefold slower rate in DED-F-actin (k_{DED} = 0.034 min^{-1}) than in F-actin (k_F = 0.31 min^{-1}).

Functional properties of DED-F-actin

Earlier study on the labeling of Gln-41 on actin by dansyl cadaverine reported that this modification had no effect on acto-S1-ATPase activities of uncrosslinked and carbodiimide cross-linked acto-S1 (Takashi, 1988). Because a shorter, DED probe is attached to Gln-41 in this work, the acto-S1-ATPase activities were remeasured over a range of labeled and unlabeled actin concentrations (up to 40 μM). At equal actin concentrations the rates of ATP hydrolysis by acto-S1

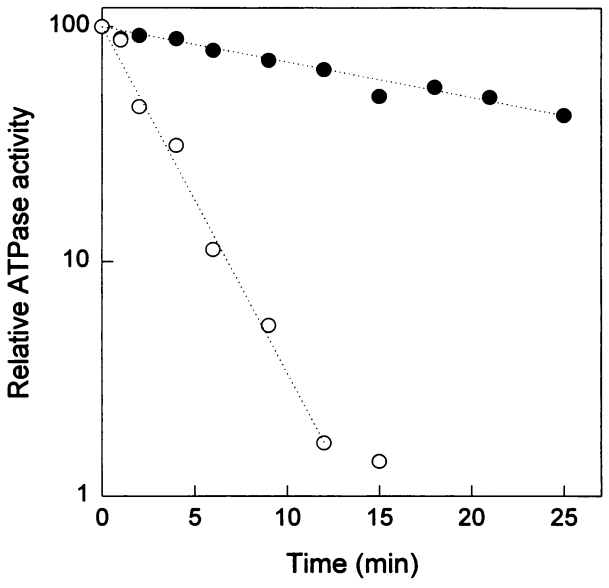

FIGURE 2 Semilogarithmic plot of the S1 ATPase activation by actin as a function of incubation time of F-actin (○) and DED-F-actin (●) at 60°C. Actin concentration during the heating was 18 μM, the concentrations of S1 and actin in the ATPase assays (at 25°C) were 0.3 and 9.0 μM, respectively. The first order rates of ATPase activation loss obtained from the slopes of the straight lines were 0.31 min^{-1} and 0.034 min^{-1} for F-actin and DED-F-actin, respectively.

TABLE 1 Kinetic parameters of acto-S1 ATPase and the in vitro motility of actin filaments

Actin	V_{max} (s^{-1})	K_m (μM)	V_f (μm/s)
Unmodified	13.6 ± 0.4	21.6 ± 1.5	4.08 ± 0.36
DED-actin	14.0 ± 0.5	26.0 ± 5.0	3.88 ± 0.45

V_{max} and K_m values of acto-S1 ATPase and the mean sliding velocities of actin filaments (V_f) were determined at 25°C. V_f was measured on three different actin preparations; in each case the motion of at least 50 filaments was monitored.

were indistinguishable between the DED-F-actin and F-actin systems. Accordingly, the K_m and V_{max} values obtained from fitting the kinetic data to the Michaelis-Menten equation were virtually the same for the unlabeled and labeled F-actin (Table 1).

Similarly, in vitro motility assays carried out with F-actin and DED-F-actin did not reveal any difference between these proteins. Both actins moved at similar mean velocities (Table 1) and with similar distribution of velocities (not shown) over a lawn of heavy meromyosin adsorbed to a nitrocellulose-coated coverslip. Thus, in agreement with Takashi's (1988) conclusion, the labeling of Gln-41 does not have any effect on the functional interactions of actin with S1.

S1-induced changes in the 40–42 site on F-actin

The binding of S1 to DED-F-Mg^{2+}-actin increased the fluorescence emission of the probe by ~20% (at λ_{max} = 526 nm) without any detectable band shift (Fig. 3). Quenching titrations with acrylamide (Fig. 4) yielded the Stern-

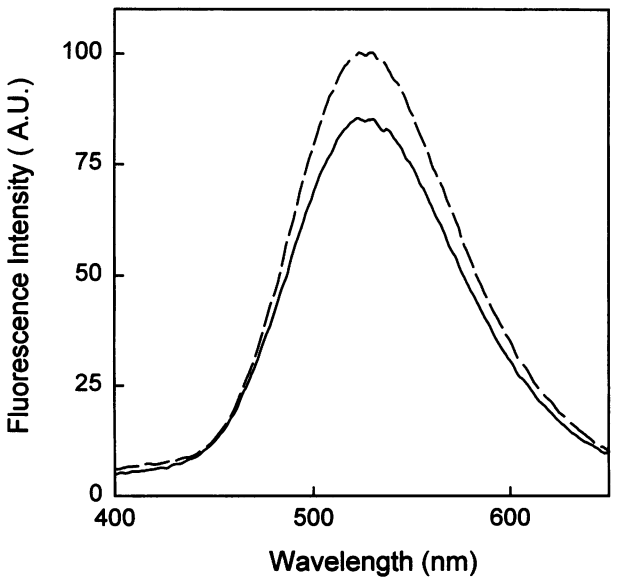

FIGURE 3 Emission spectra of DED-F-actin in the presence (*dashed spectrum*) and absence (*solid spectrum*) of S1. The emission was recorded at 3.0-μM concentrations of actin and S1 in F-actin buffer with the excitation wavelength set at 334 nm. Fluorescence intensities are reported in arbitrary units (A.U.).

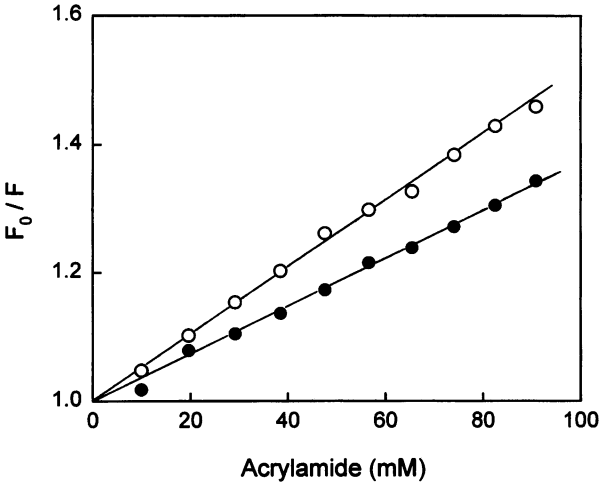

FIGURE 4 Stern-Volmer plots for the quenching of DED-F-actin fluorescence by acrylamide in the presence (●) and absence (○) of S1. Actin and S1 concentrations were 3.0 μM. F_o and F correspond to the fluorescence of DED-F-actin in the absence and presence of acrylamide, respectively. The Stern Volmer constants, K_{sv}, obtained from the slopes of the straight lines were 5.1±0.1 M^{-1} and 3.8±0.1 M^{-1} in the absence and presence of S1, respectively.

Volmer constants for DED-F-actin in the absence and presence of S1 of 5.1±0.1 M^{-1} and 3.8±0.1 M^{-1}, respectively. This indicates that the accessibility of DED on Gln-41 to collisional quenchers is smaller on the S1-bound F-actin than free F-actin.

To characterize the proposed motion of the 38–52 loop due to S1 binding to actin (Kasprzak et al., 1988), we examined the time course of actin photocrosslinking from Gln-41 to the adjacent monomer, within the same filament strand. This reaction, described by Hegyi et al. (1992) for the *N*-(4-azidobenzoyl)-putrescine attached to Gln-41 (via transglutaminase), was carried out with *N*-(4-azido-2-nitrophenyl)-putrescine (ANP), a more reactive analog of the original reagent. The UV irradiation of the ANP-labeled actin was carried out in the presence and absence of S1. Aliquots of both reactions were processed identically, including the steps of S1 (or mock S1) removal (see Materials and Methods). SDS PAGE analysis of the isolated (pelleted) actin revealed identical cross-linked products in both reactions, including monomers, dimers, trimers, and higher order species, but did not identify any cross-linked acto-S1. The rate of cross-linking was monitored by following the time-dependent disappearance on SDS PAGE of the original 42-kDa actin band (Fig. 5). The similar rates of F-actin cross-linking in the presence and absence of S1 suggest that the mean distance between Gln-41 and the next actin does not change significantly upon S1 binding to actin.

The effect of S1 on the 38–52 loop on F-actin can be also probed by using monoclonal antidansyl IgG. The binding of IgG to DED-F-actin causes about threefold increase in fluorescence and a blue shift in λ_{max} from 526 to 507 nm (Fig. 6). Surprisingly, the binding of antidansyl IgG to DED-F-actin is rather strong, as indicated by fluorescence

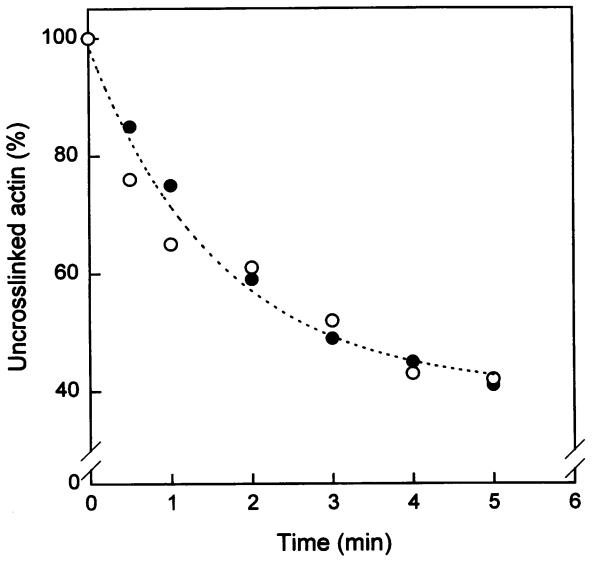

FIGURE 5 Photocrosslinking of ANP-labeled actin in the presence (●) and absence (○) of S1. ANP-F-actin (12 μM) and S1 (12 μM), when present, were prepared, photocrosslinked, and separated for SDS PAGE analysis of the cross-linked product as described in Materials and Methods. The percentage of uncrosslinked actin was obtained from densitometric analysis of actin bands on SDS gels.

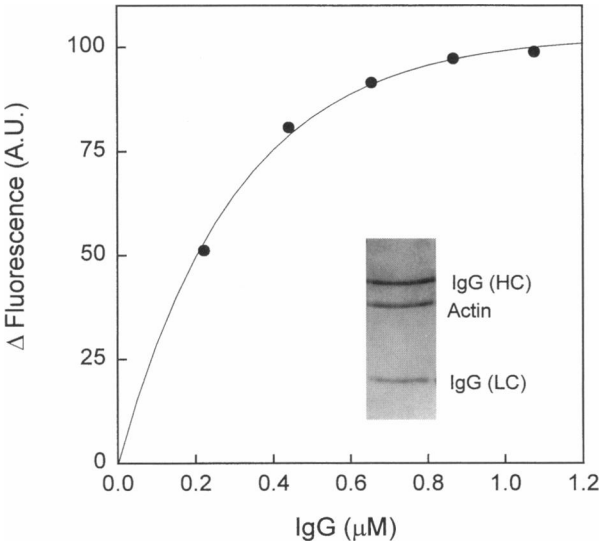

FIGURE 7 Increase in DED-F-actin fluorescence in the presence of monoclonal antidansyl IgG. The titration of DED-F-actin (1.0 μM) in F-actin buffer was recorded with excitation and emission wavelengths set at 334 and 520 nm, respectively. *Inset*: Representative SDS PAGE pattern of a pelleted 1.1:1.0 IgG and actin mixture. The molar ratio of IgG bound to actin in the pellet was 1:1. HC and LC correspond to the heavy and light chains of IgG.

titration of actin with the antibody (Fig. 7). The binding saturates at equimolar ratios of IgG to actin and the data fit well to a binding curve corresponding to K_d = 0.1 μM. The pelleting and SDS PAGE analysis of the complex formed between 1.1 μM IgG and 1.0 μM actin verified the 1:1 molar ratio of IgG bound to actin (Fig. 7, *inset*). Clearly the dansyl moiety is not buried in F-actin; it is accessible to IgG, and the binding of Ig to the probe does not depolymerize actin. Yet, as expected, the environments of DED on G- and F-actins are different. IgG causes a twofold greater fluorescence increase of DED-G than of DED-F-actin (data not shown). Moreover, the fluorescence change is completed within mixing time (.20 s) in G-actin whereas ~100 s are required for this process in F-actin (at 1:1 IgG:actin). These observations are consistent with transitions of loop 38–52 between two or more conformational states in F-actin (Egelman and Orlova, 1995) that expose to different degrees the DED moiety on Gln-41 to IgG.

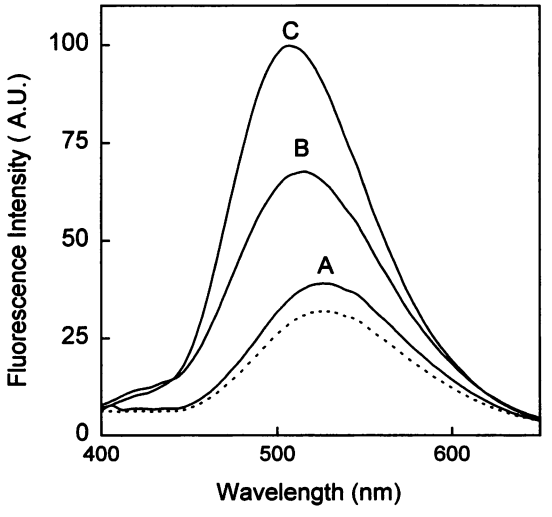

FIGURE 6 Emission spectra of DED-F-actin. The spectra correspond to DED-F-actin (*dashed curve*), and actin in the presence of: S1 (*curve A*, λ_{max} = 526 nm), S1 and monoclonal antidansyl IgG (*curve B*, λ_{max} = 514 nm), and IgG (*curve C*, λ_{max} = 507 nm). All protein concentrations were 1.0 μM and the excitation wavelength was set at 334 nm.

The binding of S1 to DED-F-actin-IgG complex causes partial dissociation of the antibody (Figs. 7 and 8). The fluorescence intensity changes, λ_{max} shift, and the pelleting of equimolar mixtures of F-actin, IgG, and S1 reveal that the stoichiometry of these proteins in the ternary complex is 1:0.5:1, respectively (Figs. 6 and 8, *inset*). Notably, whereas S1 displaces ~50% of the bound IgG, the latter has no significant effect on the equilibrium binding of S1 to F-actin (Fig. 8, *inset*). The release of IgG from actin can be monitored via a time-dependent decrease in DED fluorescence (Fig. 8). The relatively slow rate of this process suggests some reorganization of the IgG-bound DED-F-actin upon binding of S1. The fact that a considerable fraction of the actin filament (≈50%) can bind both S1 and antidansyl IgG excludes the 40–42 residues on actin from a class of sites that are essential for the binding of myosin.

DISCUSSION

The main goal of this work was to examine the suggested binding of residues Asn-552 to His-558 on S1 to residues

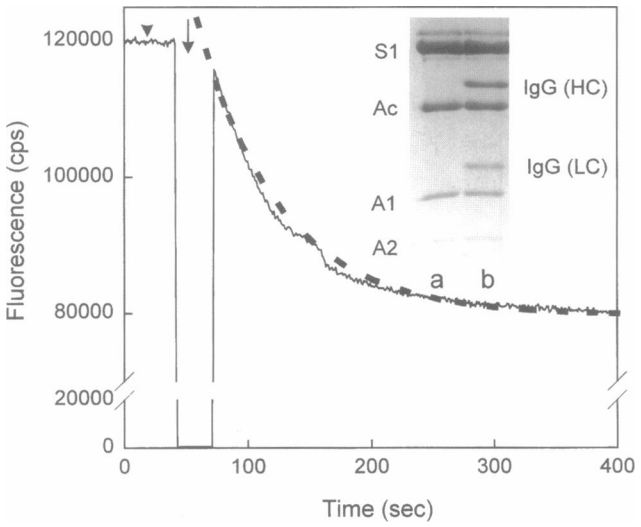

FIGURE 8 Displacement of IgG from DED-F-actin by S1 as reported by a decrease in fluorescence intensity. F-actin, antidansyl IgG, and S1 concentrations were 1.0 μM. Arrowhead shows the fluorescence of DED-F-actin-IgG complex (1:1), the addition of S1 to this complex is indicated by an arrow. Dashed line represents a single exponential fit to the fluorescence decay curve and yields the first order off rate of IgG from actin k_{off} = $1.49 \pm 0.06 \times 10^{-2}$ s^{-1}. *Inset*: SDS PAGE pattern of the pelleted DED-F-acto-S1 (*lane a*) and DED-F-acto-S1-IgG complexes (*lane b*). The pelleting was done after a 20-min preincubation of the protein mixtures. All proteins were at the concentration of 1.0 μM. The molar ratio of S1 bound to actin was 1:1 in both lanes, and that of IgG bound to actin was 0.5:1 (in *lane b*). HC and LC correspond to the heavy and light chains of IgG, respectively. A1 and A2 correspond to the myosin light chains; Ac denotes actin.

40–42 on actin (Rayment et al., 1993b) by using the DED probe attached to Gln-41. To this end the effect of DED on the properties of F-actin was examined in some detail.

Structural and functional properties of DED-F-actin

The environment of subdomain 2 in F-actin and DED-F-actin was compared in subtilisin and tryptic digestion experiments. Because the inhibition of proteolysis was greater in DED-F-actin than in DED-G-actin and the thermal "inactivation" of F-actin was much faster than that of DED-F-actin, our results indicate a stabilization of F-actin structure by DED. The connection between subdomain 2 conformation and filament stability has been established by Orlova and Egelman (1992, 1993).

The effect of DED on subdomain 2 and filament structure, although readily detected, is smaller than that produced by the P_i analog, BeF_x (Combeau and Carlier, 1988; Orlova and Egelman, 1992; Muhlrad et al., 1994). In fact, DED-stabilization of subdomain 2 was hardly different from that observed in G-actin (Kim et al., 1995) in filaments containing up to 40% DED monomers. The effect of DED on F-actin is consistent with the models of F-actin structure (Lorenz et al., 1993; Tirion et al., 1995). According to these models, residues 39–42 on one actin and residues between Tyr-166 and Tyr-169 on the adjacent actin are in close proximity to each other, allowing for the formation of hydrogen bonds between them. DED on Gln-41 may contribute to intermonomer contacts by interacting with the tyrosine residues on the adjacent actin. In this scenario filament stabilization would result from enhanced interactions of subdomain 2 with the adjacent actin.

The binding of antidansyl IgG to DED-F-Mg^{2+}-actin can be viewed as showing that subdomain 2 on DED-F-actin can still move out radially and thus, expose the probe to the antibody. Alternatively, DED may never be completely buried in the F-actin structure. The binding of IgG to DED-F-actin neither depolymerizes actin nor leads to any significant cross-linking of the filaments. The first conclusion is supported by the results of actin pelleting in the presence of IgG, whereas the second observation is based on light scattering measurements of DED-F-actin and IgG mixtures. The antibodies increased the light scattering from F-actin solutions somewhat less than S1 (if added at equal molar ratios). These results indicate that the IgG does not have a major effect on F-actin structure.

The fact that K_m and V_{max} values of the acto-S1 ATPase activity and the in vitro motility of actin are unchanged by Gln-41 labeling is in agreement with earlier observations on dansyl cadaverine-modified actin (Takashi, 1988). These findings indicate that the Gln-41 probes do not significantly change the actin filaments, as suggested above, and that such changes are inconsequential to acto-S1 interactions. More importantly, functional assays on Gln-41-labeled actin suggest that either the 40–42 site is not directly bound to S1 or that such binding has little functional significance.

The binding of S1 to F-actin changes the conformation of the 38–52 loop

The conclusion about the 40–42 site is supported by the binding of up to 0.5 IgG/actin in a 1:1 complex of actin and S1 and the observation that IgG has little or no effect on equilibrium acto-S1 binding under rigor conditions. Thus, if S1 binds to the 40–42 site on actin, such a binding does not contribute much to the overall affinity of S1 for actin. The partial displacement of IgG from actin by S1 may result from both steric crowding and S1-driven, time-dependent transitions in the 38–52 loop. It is possible that the partial nature of this effect is the consequence of equilibrium between different conformational states of acto-S1. The crowding factor, if indeed significant, is not without some steric specificity, perhaps related to the proposed proximity of S1 to Lys-50 on actin (Bertrand et al., 1994). In earlier studies S1 binding to actin did not displace the antibodies to sequence 18–28 (Adams and Reisler, 1993), and caused only a limited release of antibodies to residues 1–7 on actin (DasGupta and Reisler, 1991). Both epitopes are components of the acto-S1 interface in the presence of ATP (Miller et al., 1995, Rayment et al., 1993b; Schroder et al., 1993) and are not essential for rigor acto-S1 binding.

The possibility that the release of antidansyl IgG from DED-F-actin is affected by S1 driven changes in subdomain 2 is supported by several lines of evidence. First, our fluorescence and cross-linking experiments with DED-actin and ANP-actin show that S1 alters the environment of Gln-41 but not its mean distance from the next actin. Second, Kasprzak et al. (1988) detected a change in the radial position of the Gln-41 probe upon S1 binding to actin. Third, S1 protects subdomain 2 in F-actin from proteolytic digestions (Fievez and Carlier, 1993). In principle, the above changes may be caused by direct binding of S1 to the 40–42 site, a binding to an adjacent site, or via allosteric changes due to the binding of S1 at other sites. The last possibility is favored because of the above discussed indications that S1 does not bind to the 40–42 site on actin and because of the proteolytic studies of Turk and Muhlrad (1995). These authors have shown that the protection of subdomain 2 from α-chymotrypsin by S1 is highly cooperative and that substoichiometric amounts of S1 strongly inhibit the cleavage of F-actin. This observation suggests that the protection of subdomain 2 from proteolysis is achieved via allosteric changes that propagate within the filament, and does not involve an obligatory S1 binding to the protease-protected regions. It is also pertinent that changes in the DED-actin fluorescence were observed upon binding of IgG against residues 18–28 on actin (Adams and Reisler, 1994) i.e., the site involved in S1 binding. This strengthens the possibility of signal transmission to the 40–42 site from other sites on actin but does not completely rule out the possibility of S1 binding to a site adjacent to residues 40–42.

In conclusion, the results of this work show that the environment of loop 38–52 on actin changes upon S1 binding to F-actin. This is manifested in fluorescence changes in DED-F-actin, changes in the accessibility of the probe to collisional quenchers, and in the displacement of antidansyl IgG from actin by S1. From functional assays on DED-F-actin and the binding of IgG and S1 to F-actin it is concluded that S1 either does not bind to residues 40–42 or that such binding is of little significance to acto-S1 interactions. Consequently, it is proposed that S1-induced changes in the 38–52 loop originate from S1 binding to other myosin recognition sites on actin. As suggested before (Egelman and Orlova, 1995), such changes may be an important element of the motile function of actin.

We thank Dr. G. Hegyi for the generous gift of ANP.

This work was supported by grant MCB 9206739 from NSF and grant AR 22031 from USPHS.

REFERENCES

Adams, S. B., and E. Reisler. 1993. Role of sequence 18–29 on actin in actomyosin interactions. *Biochemistry.* 32:5051–5056.

Adams, S. B., and E. Reisler. 1994. Sequence 18–29 on actin: antibody and spectroscopic probing of conformational changes. *Biochemistry.* 33: 14426–14433.

Bertrand, R., J. Derancourt, and R. Kassab. 1994. The covalent maleimidobenzoyl-actin-myosin head complex. Crosslinking of the 50-kDa heavy chain region to actin subdomain-2. *FEBS Lett.* 345:113–119.

Butters, C. A., K. A. Willadsen, and L. S. Tobacman. 1993. Cooperative interactions between the adjacent troponin-tropomyosin complexes may be transmitted through the actin filament. *J. Biol. Chem.* 268: 15565–15570.

Combeau, C., and M.-F. Carlier. 1988. Probing the mechanism of ATP hydrolysis in F-actin using vanadate and the structural analogs of phosphate BeF_3^- and ALF_4^-. *J. Biol. Chem.* 263:17429–17436.

DasGupta, G., and E. Reisler. 1991. Nucleotide-induced changes in the interaction of myosin subfragment 1 with actin: detection by antibodies against the N-terminal segment of actin. *Biochemistry.* 30:9961–9966.

Drewes, G., and H. Faulstich. 1993. Cooperative effects on filament stability in actin modified at the C-terminus by substitution or truncation. *Eur. J. Biochem.* 212:247–253.

Egelman, E. H., and A. Orlova. 1995. New insights into actin filament dynamics. *Curr. Opin. Struct. Biol.* 5:172–180.

Fievez, S., and M. Carlier. 1993. Conformational changes in subdomain-2 of G-actin upon polymerization into F-actin and upon binding myosin subfragment-1. *FEBS Lett.* 316:186–190.

Godfrey, J. E., and W. F. Harrington. 1970. Self-association in the myosin system at high ionic strength. I. Sensitivity of the interaction to pH and ionic environment. *Biochemistry.* 9:886–893.

Hegyi, G., H. Michel, J. Shabanowitz, D. F. Hunt, N. Chatterjie, G. Healy-Louie, and M. Elzinga. 1992. Gln-41 is intermolecularly crosslinked to Lys-113 in F-actin by *N*-(4-azidobenzoyl)-putrescine. *Protein Sci.* 1:132–144.

Holmes, K. C., D. Popp, W. Gebhard, and W. Kabsch. 1990. Atomic model of the actin filament. *Nature (London).* 347:44–49.

Huang, Y.-P., K. Seguro, M. Motoki, and K. Tawada. 1992. Cross-linking of contractile proteins from skeletal muscle by treatment with microbial transglutaminase. *J. Biochem. (Tokyo).* 112:229–234.

Kasprzak, A. A., R. Takashi, and M. F. Morales. 1988. Orientation of the actin monomer in the F-actin filament: Radial coordinate of glutamine-41 and the effect of myosin subfragment-1 binding on the monomer orientation. *Biochemistry.* 27:4512–4522.

Kim, E., M. Motoki, K. Seguro, A. Muhlrad, and E. Reisler. 1995. Conformational changes in subdomain 2 of G-actin: fluorescence probing by dansyl ethylenediamine attached to Gln-41. *Biophys. J.* 69: 2024–2032.

Lakowicz, J. R. 1983. Principles of fluorescence spectroscopy. Plenum Press, New York.

Lorenz, M., D. Popp, and K. C. Holmes. 1993. Refinement of the F-actin model against x-ray fiber diffraction data by the use of a directed mutation logarithm. *J. Mol. Biol.* 234:826–836.

Miller, C. J., and E. Reisler. 1995. Role of charged amino acid pairs in subdomain-1 of actin in interactions with myosin. *Biochemistry.* 34: 2694–2700.

Muhlrad, A., P. Cheung, B. C. Phan, C. Miller, and E. Reisler. 1994. Dynamic properties of actin: structural changes induced by beryllium fluoride. *J. Biol. Chem.* 269:11852–11858.

Oosawa, F., S. Fujime, S. Ishiwata, K. Mihashi. 1972. Dynamic property of F-actin and thin filament. *Cold Spring Harb. Symp. Quant. Biol.* 37: 277–285.

Orlova, A., and E. H. Egelman. 1992. Structural basis for the destabilization of F-actin by phosphate release following ATP hydrolysis. *J. Mol. Biol.* 227:1043–1053.

Orlova, A., and E. H. Egelman. 1993. A conformational change in the actin subunit can change the flexibility of the actin filament. *J. Mol. Biol.* 232:334–341.

Orlova, A., E. Prochniewicz, and E. H. Egelman. 1995. Structural dynamics of F-actin. II. Cooperativity in structural transitions. *J. Mol. Biol.* 245:598–607.

Owen, C., and D. DeRosier. 1993. A 13C map of the actin-scruin filament from the Limulus acrosomal process. *J. Cell Biol.* 123:337–344.

Rayment, I., W. R. Rypniewski, K. Schmidt-Base, R. Smith, D. R. Tomchick, M. M. Benning, D. A. Winkelman, G. Wesenberg, and H. M. Holden. 1993a. Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science.* 261:50–57.

Rayment, I., H. M. Holden, M. Whittaker, C. B. Yohn, M. Lorenz, K. C. Holmes, and R. A. Milligan. 1993b. Structure of the actin-myosin complex and its implications for muscle contraction. *Science*. 261:58–65.

Schroder, R. R., D. J. Manstein, W. Jahn, H. Holden, I. Rayment, K. C. Holmes, and J. A. Spudich. 1993. Three-dimensional atomic model of F-actin decorated with Dictyostelium S1. *Nature (London)*. 364:171–174.

Schutt, C. E., J. C. Myslik, M. D. Rozycki, N. C. W. Goonesekere, and U. Lindberg. 1993. The structure of crystalline profilin-β-actin. *Nature*. 365:810–816.

Schwyter, D. H., S. J. Kron, Y. Y. Toyoshima, J. A. Spudich, and E. Reisler. 1990. Subtilisin cleavage of actin inhibits in vitro sliding movement of actin filaments over myosin. *J. Cell Biol.* 111:465–470.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. I. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Takashi, R. 1988. A novel actin label: a fluorescent probe at glutamine-41 and its consequences. *Biochemistry*. 27:938–943.

Tirion, M. M., D. Ben-Avraham, M. Lopez, and K. C. Holmes. 1995. Normal modes as refinement parameters for the F-actin model. *Biophys. J.* 68:5–12.

Turk, R., and A. Muhlrad. 1995. Effect of salts and myosin subfragment 1 on the structure of actin. Workshop on "Dynamics of myosin-based motile systems." Abstracts. Alpbach, Austria.

Weeds, A., and B. Pope. 1977. Studies of the chymotryptic digestion of myosin: effects of divalent cations on proteolytic susceptibility. *J. Mol. Biol.* 111:129–157.