Interaction of Myosin with F-Actin: Time-Dependent Changes at the Interface Are Not Slow

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ABSTRACT The kinetics of formation of the actin-myosin complex have been reinvestigated on the minute and second time scales in sedimentation and chemical cross-linking experiments. With the sedimentation method, we found that the binding of the skeletal muscle myosin motor domain (S1) to actin filament always saturates at one S1 bound to one actin monomer (or two S1 per actin dimer), whether S1 was added slowly (17 min between additions) or rapidly (10 s between additions) to an excess of F-actin. The carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, EDC)-induced cross-linking of the actin-S1 complex was performed on the subsecond time scale by a new approach that combines a two-step cross-linking protocol with the rapid flow-quench technique. The results showed that the time courses of S1 cross-linking to either of the two actin monomers are identical: they are not dependent on the actin/S1 ratio in the 0.3–20-s time range. The overall data rule out a mechanism by which myosin rolls from one to the other actin monomer on the second or minute time scales. Rather, they suggest that more subtle changes occur at the actomyosin interface during the ATP cycle.

INTRODUCTION

Mechanical force is generated in muscle and nonmuscle actomyosin-based movements by the cyclic interaction of the myosin motor domain (subfragment1 or S1) with filamentous actin (F-actin) under the control of ATP hydrolysis (Huxley, 1969; Lymn and Taylor, 1971). After ATP hydrolysis, S1 first binds weakly to F-actin without large force output (Schoenberg, 1988). Force is generated when the weakly bound acto-S1 complex undergoes an isomerization triggered by the release of the ATPase products, inorganic phosphate, and then ADP (Hibberd et al., 1985), to form a stronger (or rigor) interface (for a review see Cooke, 1986). This rigor or post-power stroke complex is very stable and has been extensively studied, in contrast to the other intermediate complexes.

The exact stoichiometry of this complex has been a subject of debate for a long time. There is experimental evidence for a stoichiometric complex that is formed by either one S1 bound to one actin monomer or two molecules of S1 bound to two actin monomers. X-ray diffraction (Holmes et al., 1990; Amos et al., 1982) and electron microscopy experiments on F-actin decorated with S1 (for a review see Milligan, 1996) suggest that each S1 makes several contacts with two adjacent actin monomers. More precisely, these images reveal one main actin monomer interacting via its subdomain 1 and a second actin monomer, located on the same long-pitch helix toward the barbed end of the actin filament, making contacts via both of its subdomains, 1 and 2. Chemical cross-linking experiments performed both in solution and in myofibrils confirmed the interaction (or cross-linking) of one S1 with two actin monomers when actin is in excess over S1 (Andreev and Borejdo, 1992a; Andreeva et al., 1993; Herrmann et al., 1993; Bonafe and Chaussepied, 1995; Van Dijk et al., 1998). The cross-linking sites were found to involve the N-terminal 1–12 segment (on subdomain 1) of two adjacent actin monomers (Bonafe and Chaussepied, 1995; Andreev and Borejdo, 1997) and two loop structures, loop 2 (residues 626–647) and loop 3 (residues 565–579) of skeletal muscle myosin (Chaussepied and Van Dijk, 1999). The degree of saturation of the thin filament by myosin S1 was found to alter the actin-induced protection of myosin against proteolytic degradation (Mornet et al., 1981; Yamamoto, 1990), as well as the kinetic parameters of the ATP binding process to the actomyosin complex (Tesi et al., 1990).

Andreev and Borejdo proposed that one S1 binds first to one (the main) and then to a second actin monomer: the interaction with the second monomer could be associated with a change in orientation of the myosin motor domain relative to the axis of the thin filament and therefore could be linked to the power stroke (Fig. 1; Andreev et al., 1993a, 1998; Andreev and Borejdo, 1995). This proposal was supported by sedimentation, fluorescence (in both steady-state and stopped-flow methods), and cross-linking experiments, which suggested that S1 binding to the second monomer depends both on the degree of saturation of the thin filaments and on the time course of the formation of the complex (on the second or minute time scale) (Andreev and Borejdo, 1991, 1992; Andreev et al., 1993b).

This model remains controversial for several reasons. First, the two-step binding process was observed by fluorescence and light scattering (or turbidity) experiments that were performed at very low concentrations, where actin depolymerization may occur, as pointed out by Carlier et al. (1991). However, these experiments were repeated in the

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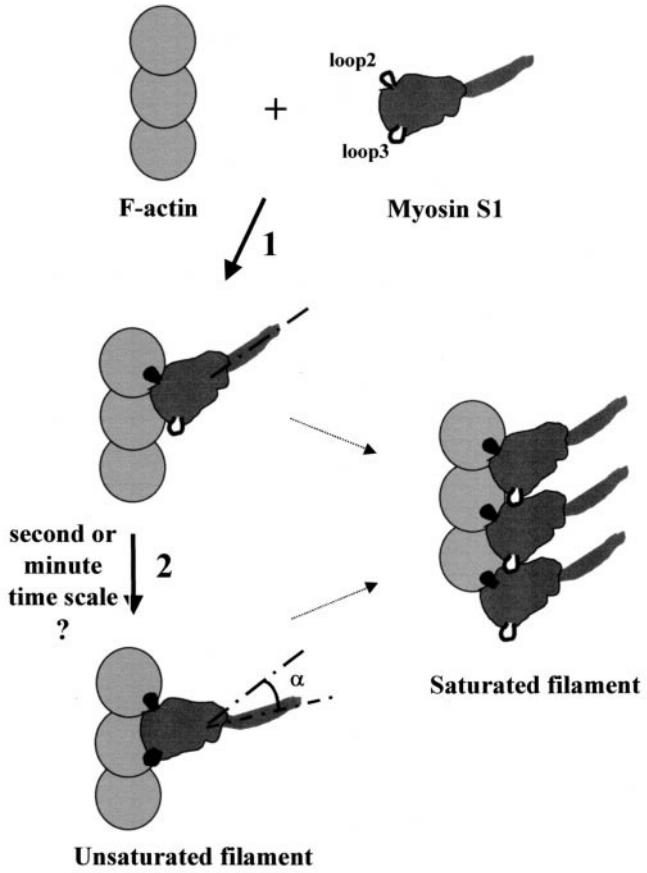

FIGURE 1 Schematic diagram of the two-step model of myosin S1 binding to F-actin as proposed by Borejdo et al. (Andreev and Borejdo, 1992, 1995; Andreeva et al., 1993). In the model S1 binding to the second actin monomer takes place on a seconds or minutes time scale, and the orientations of S1 in saturated and unsaturated filaments are different.

presence of phalloidin, which stabilizes actin in its filamentous form (Andreev and Borejdo, 1992a). Second, the two-step binding process revealed by the fluorescence stopped-flow method with actin in excess (Andreeva et al., 1993) has not been confirmed (Criddle et al., 1985; Geeves, 1989; Taylor, 1991; Blanchoin et al., 1996). Note that a three-step binding process has been used extensively to describe the formation of the actomyosin complex regardless of the degree of saturation of F-actin by S1, based on pressure relaxation studies (Geeves and Conibear, 1995). Third, the model supposes a time dependence of the isomerization step on the seconds or even the minutes time scale, which is much too long to be related to the mechanochemical cycle of the actomyosin complex, which lasts only a few tens of milliseconds (Spudich, 1994).

In this study we designed new experimental protocols for sedimentation and cross-linking experiments that allowed a more precise analysis of the actin-S1 to actin$_2$-S1 isomerization and of its dependence upon both protein concentrations and time. The sedimentation experiments were performed at relatively higher protein concentrations with the two types of skeletal muscle myosin S1 isoforms, S1(A1) and S1(A2) (carrying the alkali light chain A1 or A2), because it was proposed that the isoforms exhibit different behaviors (Andreev and Borejdo, 1995; Andreev et al., 1999). The pattern of the cross-linking reaction could be studied on the subsecond time scale with an original approach that combined the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-induced two-step cross-linking protocol and the rapid flow-quench method. The data obtained do not support a two-step binding for S1 on actin filament on the second or minute time scale.

MATERIALS AND METHODS

Materials

Phalloidin, EDC, and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. *N*-(1-Pyrenyl)iodoacetamide and α-chymotrypsin were from Molecular Probes and Worthington Biochemicals, respectively.

Protein preparations

Rabbit skeletal muscle myosin was prepared as described by Offer et al. (1973). S1 was obtained by chymotryptic digestion of myosin filaments (Weeds and Taylor, 1975). The isoforms S1(A1) and S1(A2) were separated by ion exchange chromatography as described previously (Lheureux et al., 1993). They were subjected to ultracentrifugation at 400,000 × *g* for 15 min before each experiment to remove traces of aggregated material. Rabbit skeletal G-actin and F-actin were prepared according to the method of Eisenberg and Kielley (1974), as detailed in Lheureux et al. (1993). Protein concentrations were determined spectrophotometrically, using extinction coefficients of $A^{1\%}_{280\ nm} = 5.7\ cm^{-1}$ for myosin, 7.5 cm^{-1} for S1, and 11 cm^{-1} for actin. The molecular masses used were 500, 115, and 42 kDa, for myosin, S1, and actin, respectively.

F-actin was pyrenyl-labeled essentially as described by Cooper et al. (1983). F-actin was incubated at room temperature, in the dark, for 16 h with a threefold molar excess of *N*-(1-pyrenyl)iodoacetamide. After ultracentrifugation at 70,000 × *g* for 1 h, depolymerization of the pelleted material in buffer G (5 mM HEPES, 0.1 mM ATP, 0.1 mM $CaCl_2$, pH 8.0) followed by a second ultracentrifugation, pyr-G-actin (supernatant) was passed through a Sephacryl S-200 column equilibrated with buffer G. Pyrenyl-labeled actin (~95% labeled) was mixed with unlabeled G-actin to obtain a 30% labeled actin preparation and filtered through a Millipore filter (pore size 0.22 μm). Polymerization was achieved with 2.5 mM $MgCl_2$ and 120 mM KCl for 40 min at 30°C. The extent of labeling was determined using a molar extinction coefficient of $E_{344\ nm}$ = 22,000 $M^{-1}.cm^{-1}$ for the pyrene moiety (Kouyama and Mihashi, 1981) and the Bradford method for the actin concentration (Bradford, 1976).

Sedimentation assay

Aliquots of S1(A1) or S1(A2) (final concentration 0.4–7 μM) were added at room temperature to 2.5 μM F-actin (stabilized by 5 μM phalloidin) in a final volume of 3 ml of buffer A (30 mM 3-(*N*-morpholino)propanesulfonic acid, 2.5 mM $MgCl_2$, pH 7.0) for unmodified or EDC-treated actin (alternatively in the same buffer adjusted to pH 8.3). For fast titrations, 200-μl aliquots were withdrawn 10 s after each S1 addition. The entire series was completed within 2 min. All of the fractions were centrifuged at once for 15 min at 400,000 × *g* (Beckman TL100 ultracentrifuge). For slow titrations, the incubation time was 17 min between S1 additions, and

each 200-μl aliquot withdrawn just before each addition was centrifuged separately. The concentration of the free S1 that remained in the supernatants was measured by its K^+-ATPase activity. The amount of actin in the supernatant never exceeded 5% of the total actin, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

K^+-ATPase activity measurements

The K^+-ATPase activities were determined by measuring inorganic P_i release, using the colorimetric method of Panusz et al. (1970), modified as follows: 150 μl of S1-containing solution was added to 150 μl of K^+-ATPase buffer (50 mM Tris, 5 mM EDTA, 1 M KCl, and 2 mM DTE, pH 8.0) and preincubated at 30°C for 10 min. The ATPase reaction was initiated by adding 4 mM ATP. After 15 min, the reaction was stopped by the addition of 150 μl trichloroacetic acid (15% w/v), and the precipitated proteins were spun down for 2 min at 10,000 rpm (bench centrifuge). A 250-μl volume of the supernatant was mixed with an equal volume of ammonium molybdate (2.5% stock solution in 5 N H_2SO_4) and two volumes of H_2O. The colorimetric reaction was started by the addition of 30 μl ascorbic acid (1%). Concentrations of inorganic P_i were determined from the optical density at 660 nm after 11 min of incubation at 20°C.

Two-step cross-linking experiment

In the fist step, 25 μM F-actin in buffer A supplemented with 120 mM NaCl was activated at 20°C by 50 mM NHS and 20 mM EDC (freshly dissolved in the buffer). After 15 min, the activation was stopped by the addition of 50 mM 2-mercaptoethanol. The condensation step of EDC-activated F-actin with S1 was performed at 20°C in a rapid flow-quench apparatus (Barman and Travers, 1985) that allows rapid mixing of equal volumes of EDC-activated actin (25 μM) and S1 (5 or 37.5 μM) in 100 mM HEPES, 120 mM NaCl (pH 9.0). The final mixture pH was 8.3. The reaction mixtures were aged for 0.3–20 s and then quenched in SDS (3%). The reaction products were analyzed by SDS-PAGE. The amount of each cross-linked product formed during the cross-linking time course was evaluated from gel scanning at 595 nm with a Shimadzu CS 930 high-resolution gel scanner.

SDS-PAGE

Gel electrophoresis was performed as described by Laemmli (1970), with 4–18% gradient acrylamide gels. Gels were stained with Coomassie blue.

Stopped-flow experiments

The kinetics of the interaction of S1 with F-actin or EDC/NHS-treated F-actin were monitored in the stopped-flow apparatus (SF-61; Hi-Tech Scientific) at 20°C.

The effect of actin modification by EDC/NHS was monitored by light scattering at 90° to the incident light at a wavelength of 400 nm. S1 (37.5 μM) was mixed with F-actin or EDC/NHS-modified F-actin (25 μM) at 20°C under the same buffer conditions as those used during the cross-linking reaction. F-actin was preincubated with NHS in the absence or in the presence of EDC for 15 min at 20°C. Actin modification was stopped by 50 mM 2-mercaptoethanol, and the mixture was immediately poured into the syringe of the stopped-flow apparatus. The transients shown are the averages of four to seven shots.

The effect of SDS on the F-actin-S1 complex was monitored by following the changes in light scattering (at 400 nm as described above), in pyrene fluorescence (excitation wavelength 365 nm, with a KV399 Schott filter on the emission beam) and in tryptophan fluorescence (exciting wavelength 290 nm, with a 320-nm filter for the emitted light). S1 (37.5 μM) and F-actin (or pyr-F-actin) (25 μM) alone or together were mixed with 3% SDS at 20°C under buffer conditions identical to those used in the rapid flow-quench apparatus. The transients shown are the averages of ~5–10 shots, and the data were analyzed using the software GraphPad Prism.

RESULTS

Titration of F-actin with S1

While previous works provided strong evidences for a 1:1 actin-to-S1 stoichiometry in the rigor complex (White and Taylor, 1976; Greene and Eisenberg, 1980; Sutoh, 1983), Andreev and Borejdo (1991) found that if sufficient time is left between S1 additions during titration experiments, the actin-S1 complex can saturate at a 2:1 molar ratio. Here we reinvestigated this finding under similar buffer and ionic strength conditions (except that we used higher protein concentrations), using a sedimentation procedure instead of light scattering or fluorescence. S1(A1) or S1(A2) was added stepwise, either every 10 s or every 17 min, to phalloidin-stabilized actin filaments. After each addition, actin-S1 complexes were removed by ultracentrifugation as a pellet. The amount of free S1 remaining in the supernatant was quantified from its K^+-ATPase activity, a method that allows determination of S1 concentrations to a precision of 5 nM. Fig. 2 shows very tight binding to F-actin regardless of the S1 isoform or the time spent between two S1 additions. From the dependence, we estimate the association for the actin-S1 complex to be higher than 10^8 M^{-1}, as expected under the very low ionic strength conditions used (see below). The data obtained for all of the conditions used agreed with a titration curve saturating at a stoichiometric actin:S1 ratio (Fig. 2, *solid line*) and not at a 2:1 molar ratio (Fig. 2, *dash-dotted line*).

Kinetics of S1 cross-linking to F-actin

The kinetics of S1 cross-linking to F-actin were studied using a two-step cross-linking reaction and the rapid flow-

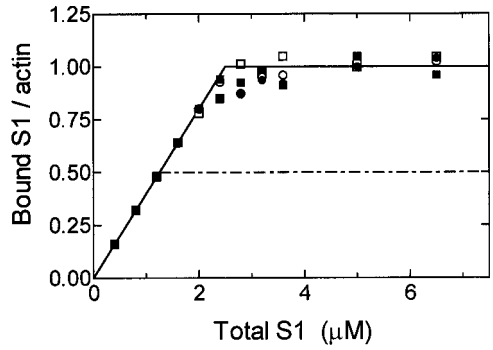

FIGURE 2 Isotherms of binding of S1 isoenzymes to native F-actin. S1(A1) (○, ●) or S1(A2) (□, ■) was added stepwise every 10 s (○, □) or 17 min (●, ■) to a fixed (2.5 μM) F-actin concentration. Bound S1 was obtained by estimating the amount of S1 present in the pellet of sedimentation experiments as described in Materials and Methods.

quench method. During the first step, which was performed outside the rapid flow-quench apparatus, F-actin carboxyl groups were activated by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in the presence of NHS. To ensure a high yield of actin activation, we carried out this first step at pH 7.0 for 15 min at 20°C. The activation step was terminated by the addition of 2-mercaptoethanol, which reacts with excess EDC. The subsequent condensation reaction of active carboxylates in actin with S1 amino groups was performed in the rapid flow-quench apparatus, using SDS to quench the reaction at various reaction times. The nature of the actin-S1 cross-linked products was then analyzed in the quenched reaction mixture by gel electrophoresis.

The efficiency of the EDC-induced cross-linking reaction is affected strongly by a side reaction between water and the activated carboxylic groups that competes with the condensation reaction. This deactivation process was reduced in two ways. First, an excess of NHS, which stabilizes the *O*-acylisourea derivative by the transient formation of active *N*-succinimidyl esters (Grabarek and Gergely, 1990), was present during the activation step. Second, the condensation step was carried out at a final pH of 8.3 (obtained by rapidly mixing F-actin in 30 mM 3-(*N*-morpholino)propanesulfonic acid at pH 7.0 with S1 in 100 mM HEPES, pH 9.0) to increase the deprotonation of the amino groups.

Before the analysis of the patterns of the cross-linking reactions, we first validated the experimental protocol by measuring the effect of the EDC reaction on the actin-S1 binding parameters as well as the effectiveness of SDS as a dissociation/denaturation agent compared with the kinetics of formation of the actin-S1 complex.

Fig. 3 *A* shows comparative isotherms for binding of S1 to native or EDC-modified actin obtained by a "fast" sedimentation experiment. The dissociation constants derived from these experiments remained almost unchanged (within a factor of 2) whether the sedimentation assay was performed with modified or unmodified actin at pH 7.0 or pH 8.3, i.e., the pH used during the condensation step. The lack of a significant effect of the carboxyl modification of actin on S1 binding was shown further by following the formation of the actin-S1 complex by light scattering in a stopped-flow apparatus (Fig. 3 *B*). The traces obtained for unmodified and modified actin were very similar, and each could be fitted reasonably well with a single-exponential of rate constant of 24.5 s^{-1}.

The condensation step was studied in the time range 0.3–20 s in a rapid flow-quench apparatus with 1.5% (final) SDS as the quenching agent. But first we checked on the efficiency of SDS as quencher. The rapidity of SDS quenching was investigated by stopped flow, using three methods (Fig. 4). First, the decrease in light scattering observed when the F-actin-S1 complex was mixed with SDS could be fitted using a single-exponential decay with a half-life of 0.084 s (Fig. 4 *A*). This half-life was longer than the 0.062 s or 0.040 s obtained when actin or S1 alone was mixed with SDS. The longer half-life for the F-actin-S1 complex could

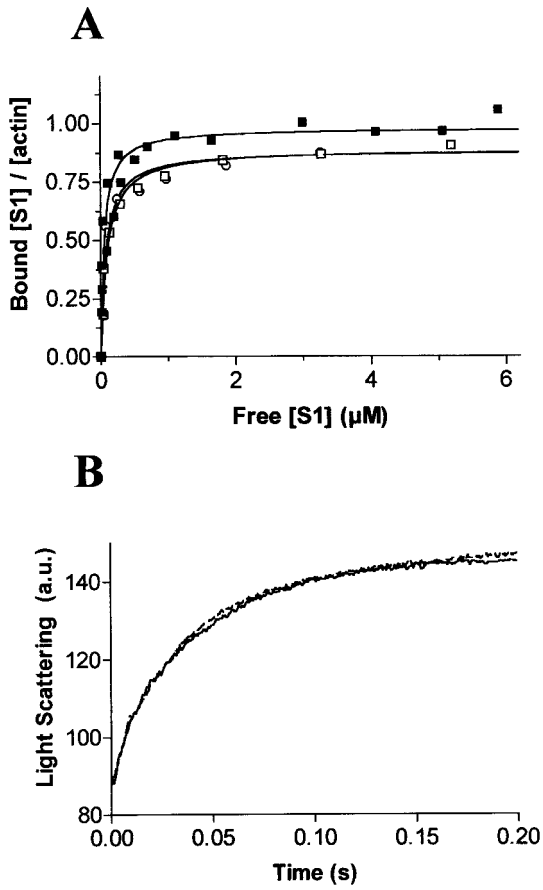

FIGURE 3 Effect of EDC/NHS on the formation of the F-actin-S1 complex. (*A*) Isotherms of binding of S1 to native or EDC/NHS-modified F-actin. S1 was added to native F-actin at pH 7.0 (■) and to EDC/NHS-activated F-actin at pH 7.0 (□) or pH 8.3 (○). Free and bound S1 concentrations were determined by a sedimentation assay (see Materials and Methods). Binding curves were computed by assuming one binding site per actin. The dissociation constants obtained are 50 nM for native actin and 93 and 77 nM for EDC-activated actin at pH 7.0 and 8.3, respectively. (*B*) Light scattering stopped-flow experiment. S1 (37.5 μM) was mixed with 25 μM F-actin (– – –) or EDC/NHS-treated F-actin (——) as described in Materials and Methods.

be related to the dissociation of the complex. This hypothesis was supported by following the change in pyrene fluorescence of pyrene-labeled F-actin (Fig. 4 *B*). In the absence of S1 the pyrene fluorescence decreased slowly upon mixing with SDS, with a half-life of 3.6 s, while in its presence there was a first rapid increase followed by a slow decrease, with a half-life of 3.3 s, similar to that observed without S1 (Fig. 4 *B, inset*). We propose that the rapid increase in fluorescence describes the dissociation of the actin-S1 complex for three reasons. First it occurs only when S1 is present (Fig. 4); second, dissociation of the complex under nondenaturing conditions (by ATP, for example) always results in an increase in pyrene fluorescence (Kouyama and Mihashi, 1981); and third, the half-life of this phase (0.081 s) is in the same range as that obtained for

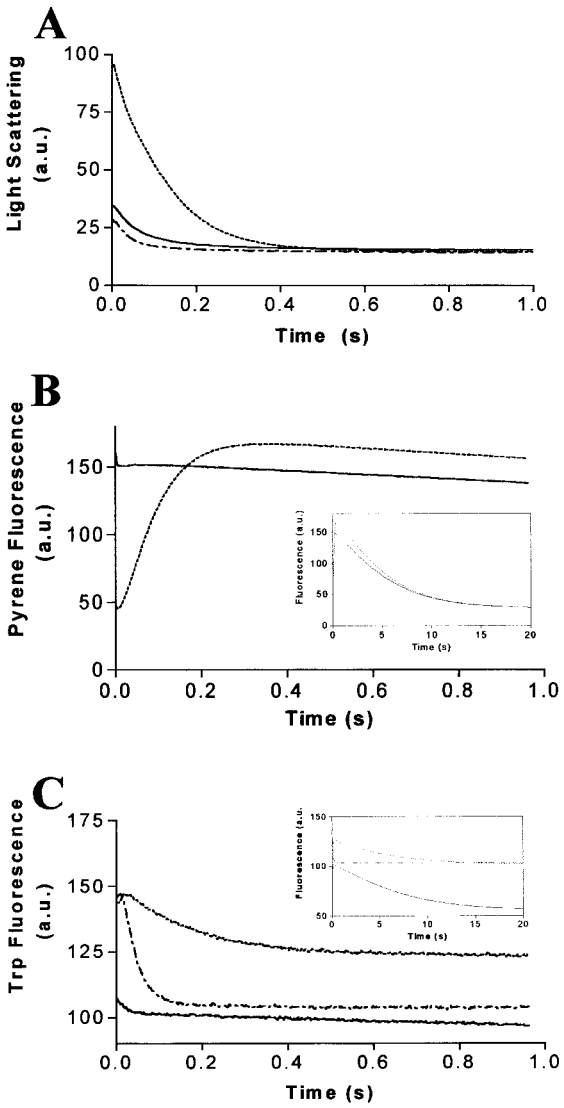

FIGURE 4 Stopped-flow transients of SDS-induced dissociation/denaturation of the F-actin-S1 complex by light scattering (*A*), pyrene fluorescence (*B*), or tryptophan fluorescence (*C*). In *A* and *C*, F-actin plus S1 (·····) or F-actin alone (——) or S1 alone (– · –) were mixed with 3% SDS. In *B*, pyr-F-actin plus S1 or pyr-F-actin alone was mixed with 3% SDS. The final reaction mixture concentrations were 37.5 μM S1 and F-actin or 25 μM pyr-F-actin. Experiments were performed as described in Materials and Methods.

the decrease in light scattering (0.085 s; see above). The slow decrease in fluorescence seems to be due to the depolymerization/denaturation of actin itself, as it occurs in both experiments. This last conclusion is strengthened by the observation that such a slow phase is also obtained by the SDS effect on the tryptophan fluorescence of F-actin, both in the absence and in the presence of S1, but not on S1 alone (Fig. 4 *C, inset*). The fluorescence of S1 tryptophan residues rapidly decreases (with a half-life of 0.031 s) and reaches a value that is stable for up to 20 s, indicating a rapid change in the tryptophan environment (due to a local denaturation) that is faster than the S1 dissociation from actin filaments. From these experiments we conclude that in the time range of interest (0.3 s and longer), 1.5% SDS is an efficient quenching agent of the condensation step.

Fig. 5 shows gel electrophoretic patterns obtained from the cross-linking experiments carried out with either an excess of actin (Fig. 5 *A*) or an excess of S1 (Fig. 5 *B*). Under all conditions tested so far, EDC reaction with the actin-S1 complex gives rise to three main well-characterized cross-linked products of apparent masses of 165, 175, and 265 kDa (Fig. *5, lines C3*). As depicted in Fig. 6, these three cross-linked products have been identified as resulting from the covalent linkage between the N-terminal segment 1–7 of two adjacent actin monomers and either myosin loop 2 (165 kDa), myosin loop 3 (175 kDa), or both loops 2 and 3 (265 kDa) (Sutoh, 1982, 1983; Andreev et al., 1993b; Bonafe and Chaussepied, 1995; Van Dijk et al., 1999a,b). Therefore, the stoichiometry is 1:1 actin:S1 for the 165-kDa and 175-kDa products and 2:1 for the 265-kDa product.

Both the qualitative (Fig. 5) and quantitative (Fig. 7) analyses of the cross-linking time course revealed that the first product generated, regardless of the actin:S1 ratio, is none of the products mentioned above but is, instead, a 200-kDa species. This band was always formed in very low amounts, and its concentration did not seem to increase during the cross-linking reaction. Note that the 200-kDa band was previously found to contain one actin bound to one S1 (Combeau et al., 1992). However, its low yield did not allow a more precise identification of the cross-linking sites involved. We note that it was the only cross-linked product generated in the presence of ATP and ATPγS that is under conditions where no specific complex is formed (Van Dijk et al., 1998). Therefore, we think that the 200-kDa species does not correspond to any specific interaction at the actin-S1 interface.

As can be seen in Fig. 5, the 165-kDa and 175-kDa adducts increased with time right from 0.3 s. The kinetics of formation of these two covalent complexes were very similar and independent of the actin:S1 ratio (Figs. 5, *A–C*, and 7, *A–C*). However, the 175-kDa product was reduced by more than half when the F-actin was saturated with S1, in accord with Andreev and Borejdo (1995).

The 265-kDa product, which contains two actin molecules cross-linked to loops 2 and 3 of the same S1 molecule, was generated last but only when the actin was in excess over S1 (Figs. 5, *A* and *B*, and 7, *A* and *B*). Interestingly, although S1 can be cross-linked via both of its loops, 2 and 3 (although to a low extent), in the presence of an excess of S1 (Figs. 5 *B* and 7 *B*), there is no 265-kDa product generated (see above). The presence of (some) 175-kDa product obtained with an excess of S1 was not sufficient to generate the 265-kDa product. Note that in both cases (excess of actin or excess of S1), a higher-molecular-mass band was also generated, the nature of which is unknown (Fig. 5).

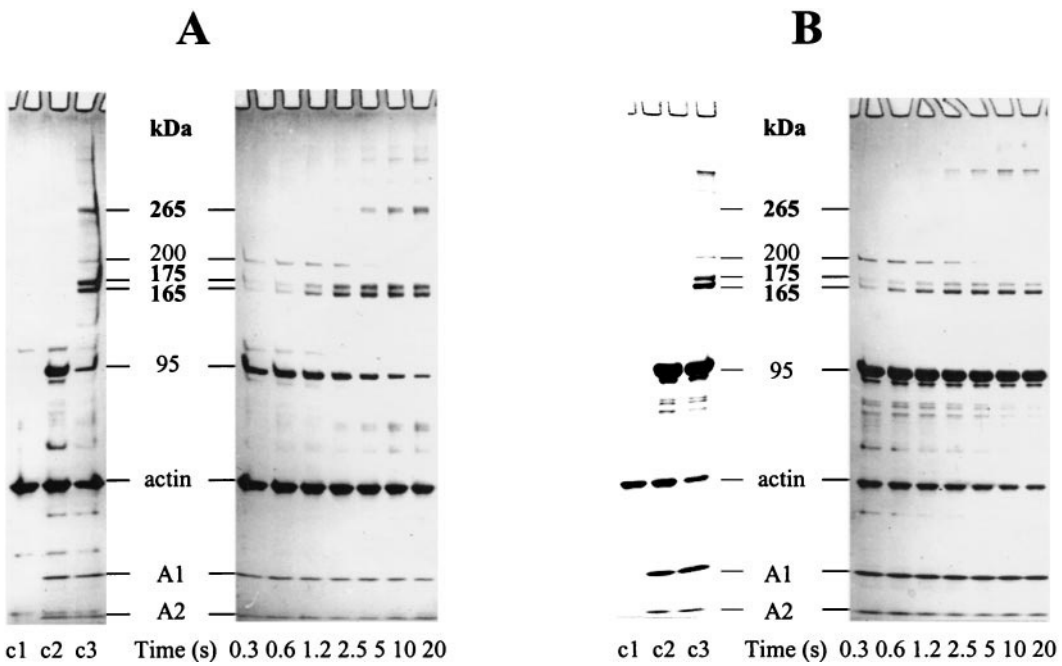

FIGURE 5 SDS-PAGE of the cross-linking reaction on the actin-S1 complex. The cross-linking was performed in a two-step reaction in the rapid-flow quench apparatus as described in Materials and Methods, with an actin:S1 molar ratio of 5:1 (*A*) or 2:3 (*B*). Control samples of EDC/NHS-modified actin alone (c1), mixed with S1 directly in the boiling denaturing Laemmli solution (c2), or mixed with S1 for 15 min at 20°C before denaturation (c3), are also shown.

Under these cross-linking conditions, the amounts of the 165-kDa and 175-kDa products increased up to maximum and stable plateaus, reached after ~5 s. Under more stringent cross-linking conditions (for example, in a one-step process with higher EDC concentration), however, the amounts of these two products decrease, with a concomitant formation of higher-molecular-mass products (Bonafe and Chaussepied, 1995).

The most important result of these experiments is that regardless of the final amount of cross-linked product generated, the kinetics of formation of the 165-kDa and 175-kDa products are very similar (Fig. 7 *C*). Therefore, cross-linking of loop 2 and loop 3 of S1 to actin occurs at the same rates.

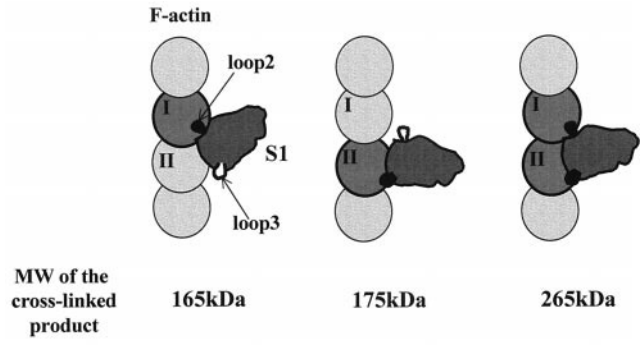

FIGURE 6 Schematic drawing of the EDC/NHS-induced cross-linking reaction, showing the molecular masses of the cross-linked products and the location of the cross-linking site on myosin loop 2 and/or loop 3 and on subsite 1 of the two adjacent actin monomers (I and II).

DISCUSSION

Our results demonstrate that on the seconds and minutes time scales, the kinetics of S1 binding to F-actin are not dependent on the degree of saturation of the actin filament by S1.

Stoichiometry of myosin binding to actin filaments

Our sedimentation experiments clearly show that the stoichiometry of the rigor complex saturates with one mole of S1 per actin monomer, regardless of the S1 isoform and the time left for the formation of the actin-myosin interface. These results disagree with the 2:1 actin:S1 complex obtained in previous binding experiments (Andreev and Borejdo, 1992b; Andreev and Borejdo, 1991), in which the interaction with the second monomer was strongly time dependent in the minutes time scale. Our experimental protocol is different in two ways from those employed previously. First, we used higher protein concentrations to facilitate the formation of the putative $(actin)_2$-S1 complex. Second, we carried out sedimentation experiments, which have the property—in contrast to the light scattering and fluorescence measurements used previously—of being in-

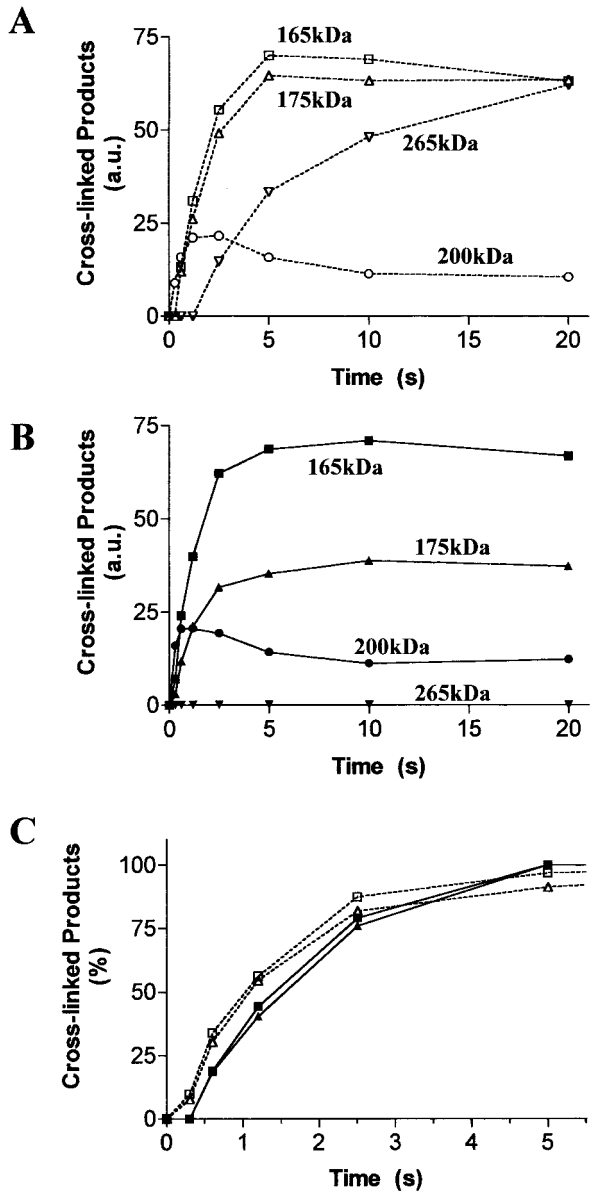

FIGURE 7 Quantitative analysis of the cross-linking reaction. SDS-PAGE gels as in Fig. 6 were scanned, and the relative amounts of the 165-kDa (□, ■), 175-kDa (△, ▲), 200-kDa (○, ●), and 265-kDa (▽, ▼) products were plotted versus time for the experiments performed at the 5:1 (*A*) or the 2:3 (*B*) actin:S1 ratio. For easier comparison, the data obtained for the 165-kDa and the 175-kDa products are shown as percentages of the final plateaus (*C*). Note the enlarged time scale in *C*.

sensitive to the modifications of actin or S1 structure that are unrelated to the formation of the complex. The "poor sensitivity" of the sedimentation experiment allowed us to measure only the amount of F-actin-bound S1. For example, it is well known that S1 binding to F-actin strongly promotes filament bundles that are characterized by an increased light scattering value and are generated on a time scale of minutes (Ando and Scales, 1985). The presence of such actin bundles was not eliminated experimentally in the previous experiments; they are a possible explanation for the discrepancy between our results and those obtained by the Borejdo group.

Note that our 1:1 stoichiometry does not disagree with the reconstruction of electron microscopy images, which showed that each S1 binds to two actin monomers, but also that each actin monomer interacts with two S1, making a macroscopic $(actin)_2$-$(S1)_2$ complex (Rayment et al., 1993; Schroder et al., 1993).

Another noteworthy result is the lack of significant differences in actin binding between the two skeletal muscle myosin S1 isoforms, S1(A1) and S1(A2), although the specific interaction of the N-terminus of the A1 light chain with actin is now well documented at low ionic strength (Prince et al., 1981; Sutoh, 1982; Yamamoto and Sekine, 1983; Trayer et al., 1987; Boey et al., 1992; Lowey et al., 1993; Timson and Trayer, 1997). However, in the previous works it was proposed that the A1 light chain interacts with subdomain 1 of the lower actin monomer (Timson et al., 1998), that this interaction is favored when actin is in excess over S1 (Andreev and Borejdo, 1995), and that the overall structure of the resulting actin-S1(A1) is different from that of the actin-S1(A2) complex (Nikolaeva et al., 1994). One plausible explanation for this is that S1 binding to the adjacent actin monomer displaces the N-terminal part of the A1 light chain from its interaction with the lower monomer. This is reasonable, considering the relative strengths and salt sensitivities of the S1 heavy chain and light chain binding to actin (Winstanley et al., 1979).

Kinetics of S1 cross-linking to F-actin

The binding of F-actin to S1 occurs on a time scale of tens of milliseconds, as shown by fluorescence and light scattering studies. With an apparent association rate of 24.5 s^{-1} observed under our experimental conditions, more than 95% of the complex is already formed before the first sampling, i.e., after 0.3 s of cross-linking. Because actin carboxyl groups are activated by EDC before S1 addition, the limiting step of the cross-linking must be mainly due to the condensation step, that is, to the lifetime of the contacts between the carboxyls of actin subdomain 1 and S1 loops 2 and 3. Another important result of this rapid flow-quench experiment is that SDS-induced dissociation of the actin-S1 complex, which corresponds to the termination of the cross-linking reaction, occurs with a half-life of 80 ms. This time is short enough to make the first time point of 0.3 s meaningful.

Interestingly, the EDC-induced activation of actin carboxylates did not seem to modify significantly the properties of actin binding to S1, as judged by equilibrium or rapid kinetics experiments. This is surprising, as these carboxylates are thought to participate actively in the formation of the actomyosin interface (Miller et al., 1995). One reason

could be that among the four to six carboxylate residues accessible for activation, only a few are actually modified (Elzinga, 1986; Yamamoto, 1989; Bertrand et al., 1989).

The rates of formation of the 165-kDa and the 175-kDa products, which correspond, respectively, to S1 loop 2 and loop 3 cross-linked to the N-terminus 1–7 segment of actin, are similar. The strength of loop 2 and loop 3 binding to their respective actin monomers is therefore very comparable in the rigor actin-myosin complex, regardless of the degree of saturation of F-actin by S1. A possible piece of evidence against this conclusion is the fact that the observed equal kinetic rates of 165 kDa and 175 kDa formation are coincidental. This idea supposes a difference in the microenvironment of the two cross-linking sites. We feel that this is unlikely for the following reasons. First, very similar cross-linking patterns, i.e., with equal kinetic rates for the two $actin_1$-S1 products, were obtained under very different conditions resulting in very different time courses (this study; Chen et al., 1985; Andreev and Borejdo, 1992b; Andreeva et al., 1993; Bonafe et al., 1995; Van Dijk et al., 1998). Second, if myosin binds first to loop 2 and second to loop 3 (Borejdo's proposal), then the coincidental explanation implies that actin cross-linking to loop 3 takes place preferentially, and linking to loop 2 happens comparatively less frequently. Unfortunately, there is no way to measure precisely the difference in reactivity between these two loci. However, we note that loop 2 contains almost twice as many positively charged residues as loop 3. So by this criterion alone one would expect loop 2 to cross-link more favorably with the negatively charged N-terminus of actin. Interestingly, the two loops are unstructured in all of the 3-D structures of S1 so far available and therefore seem to belong to very flexible loop structures protruding from the surface of S1. As a consequence, one would expect a high degree of solvation, which would minimize any difference in their microenvironment. One should also note that these two loop structures are equally accessible to peptide antibodies (Cheung and Reisler, 1992; Blotnick et al., 1995).

With an excess of actin, the amounts of 165-kDa and 175-kDa products increase at similar rates and reach the same final level. Under these conditions, S1 binding is not affected by neighboring S1, and the two loops have the same probability of being cross-linked. With an excess of S1, i.e., when the filament is fully saturated with S1 (Fig. 1), only half of the amount of the 175-kDa product is formed, showing that neighboring myosin S1 interferes and reduces the contacts between the lower actin subdomain 1 and S1 loop 3. The cross-linking of loop 3 is therefore sensitive to the actin/S1 ratio. Interestingly, actin cross-linking to loop 2 was also sensitive to the actin/S1 ratio, although not in a quantitative manner; rather, it was sensitive to the nature of the exact residue involved in the covalent linkage (Yamamoto, 1990).

The 265-kDa product, which corresponds to the cross-link of loops 2 and 3 of a single S1 to the N-terminus of two adjacent actin monomers, appears only when actin is in excess over S1 (Andreev and Borejdo, 1992b; Andreev et al., 1995; Bonafe and Chaussepied, 1995). It has been proposed that that the formation of the 265-kDa product is correlated directly with the presence of the 175-kDa product, because without cross-linking of S1 loop 3 to the lower actin monomer, the doubly cross-linked product does not occur (Andreev and Borejdo, 1995). Our data show a less straightforward correlation, because with the fully saturated complex, in which the 265-kDa band is not generated, there is still ~50% of the 175-kDa product generated. A microheterogeneity in the cross-linked residues of loop 2 or loop 3 may explain this result.

In conclusion, our work does not support a slow (seconds to minutes time scale) isomerization of actin-S1 to $actin_2$-S1 as proposed by Borejdo et al. (Andreev and Borejdo, 1991, 1992; Andreev et al., 1993b). Of course, this does not contradict a difference in the so-called secondary binding subsites at the interface (between the lower actin monomer and S1 loop 3 that we confirm with the cross-linking experiments) or a change in S1 orientation, depending on the degree of saturation of the thin filament. But does the actin-S1 to $actin_2$-S1 transition have any significance with respect to the power stroke? Recent mutagenesis work showed that adding positively charged residues to loop 3 of the *Dictyostelium discoideum* myosin motor domain enhances its cross-linking yield to the lower actin monomer but does not significantly increase either its rate of association to F-actin or its actin-activated ATPase activity (Van Dijk et al., 1999b). These results could be explained by different F-actin interactions with nonmuscle *D. discoideum* and skeletal muscle myosin. On the other hand, Andreev et al. (1993b), using a fluorescence stopped-flow method, proposed that an isomerization could indeed take place in the subsecond time scale with an excess of F-actin, i.e., when the secondary binding site is fully accessible. Unfortunately, these results could not be confirmed by others who found, using the same method, a two-step binding process only when S1 is in excess over F-actin (Blanchoin et al., 1996). Therefore this issue remains, pending rapid kinetic experiments involving myosin S1 with mutated loop 3.

The cross-linking data obtained in the presence of nucleotide analogs that mimic the $ADP{\cdot}P_i$ intermediate states suggest that S1 loop 2 and loop 3 interact simultaneously with the two neighbor actin monomers in the weak binding states (Van Dijk et al., 1998). This result supports the idea that the $actin_2$-S1 complex is formed at the beginning of the actomyosin ATPase cycle in the so-called weak binding collision, and the formation of the A-state complexes, which further undergo conformational rearrangement during the formation of the force generating rigor complex. However, the finding that the binding of myosin loop 3 to actin in the secondary subsite is specific to the skeletal muscle myosin isoforms still remains a puzzle if this binding does not lead

to any differences in the catalytic activity of the actomyosin complex.

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REFERENCES

Amos, L. A., H. E. Huxley, K. C. Holmes, R. S. Goody, and K. A. Taylor. 1982. Structural evidence that myosin heads may interact with two sites on F-actin. *Nature.* 299:467–469.

Ando, T., and D. Scales. 1985. Skeletal muscle myosin subfragment-1 induces bundle formation by actin filaments. *J. Biol. Chem.* 260: 2321–2327.

Andreev, O. A., A. L. Andreeva, and J. Borejdo. 1993a. Polarization of fluorescently labeled myosin subfragment-1 fully or partially decorating muscle fibers and myofibrils. *Biophys. J.* 65:1027–1038.

Andreev, O. A., A. L. Andreeva, V. S. Markin, and J. Borejdo. 1993b. Two different rigor complexes of myosin subfragment 1 and actin. *Biochemistry* 32:12046–12053.

Andreev, O. A., and J. Borejdo. 1991. The myosin head can bind two actin monomers. *Biochem. Biophys. Res. Commun.* 177:350–356.

Andreev, O. A., and J. Borejdo. 1992a. Binding of myosin subfragment-1 to F-actin. *Biochem. Biophys. Res. Commun.* 188:94–101.

Andreev, O. A., and J. Borejdo. 1992b. Two different acto-S1 complexes. *J. Muscle Res. Cell Motil.* 13:523–533.

Andreev, O. A., and J. Borejdo. 1995. Binding of heavy-chain and essential light-chain 1 of S1 to actin depends on the degree of saturation of F-actin filaments with S1. *Biochemistry.* 34:14829–14833.

Andreev, O. A., and J. Borejdo. 1997. Interaction of the heavy and light chains of cardiac myosin subfragment-1 with F-actin. *Circ. Res.* 81: 688–693.

Andreev, O. A., L. D. Saraswat, S. Lowey, C. Slaughter, and J. Borejdo. 1999. Interaction of the N-terminus of chicken skeletal essential light chain 1 with F-actin. *Biochemistry.* 38:2480–2485.

Andreev, O. A., R. Takashi, and J. Borejdo. 1995. Fluorescence polarization study of the rigor complexes formed at different degrees of saturation of actin filaments with myosin subfragment-1. *J. Muscle Res. Cell Motil.* 16:353–367.

Andreev, O. A., D. S. Ushakov, and J. Borejdo. 1998. Effect of ADP on binding of skeletal S1 to F-actin. *Biochemistry.* 37:17836–17842.

Andreeva, A. L., O. A. Andreev, and J. Borejdo. 1993. Structure of the 265-kilodalton complex formed upon EDC cross-linking of subfragment 1 to F-actin. *Biochemistry.* 32:13956–13960.

Barman, T. E., and F. Travers. 1985. The rapid-flow-quench method in the study of fast reactions in biochemistry: extension to subzero conditions. *Methods Biochem. Anal.* 31:1–59.

Bertrand, R., P. Chaussepied, E. Audemard, and R. Kassab. 1989. Functional characterization of skeletal F-actin labeled on the NH_2-terminal segment of residues 1–28. *Eur. J. Biochem.* 181:747–754.

Blanchoin, L., D. Didry, M. F. Carlier, and D. Pantaloni. 1996. Kinetics of association of myosin subfragment-1 to unlabeled and pyrenyl-labeled actin. *J. Biol. Chem.* 271:12380–12386.

Blotnick, E., C. Miller, U. Groschel-Stewart, and A. Muhlrad. 1995. Immunochemical probing of the functional role of the 238–246 and 567–574 sequences of myosin heavy chain. *Eur. J. Biochem.* 232: 235–240.

Boey, W., A. W. Everett, J. Sleep, J. Kendrick-Jones, and R. C. dos Remedios. 1992. Uncoupling of actin-activated myosin ATPase activity from actin binding by a monoclonal antibody directed against the N-terminus of myosin light chain 1. *Biochemistry.* 31:4090–4095.

Bonafe, N., and P. Chaussepied. 1995. A single myosin head can be cross-linked to the N termini of two adjacent actin monomers. *Biophys. J.* 68:35S–43S.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Carlier, M. F., D. Didry, and C. Valentin-Ranc. 1991. Interaction between chromium GTP and tubulin. Stereochemistry of GTP binding, GTP hydrolysis, and microtubule stabilization. *J. Biol. Chem.* 266: 12361–12368.

Chaussepied, P., and J. Van Dijk. 1999. Role of charges in actomyosin interactions. *In* Molecular Interactions of Actin. Springer-Verlag, Berlin and New York.

Chen, T., D. Applegate, and E. Reisler. 1985. Cross-linking of actin to myosin subfragment 1 in the presence of nucleotides. *Biochemistry.* 24:5620–5625.

Cheung, P., and E. Reisler. 1992. Synthetic peptide of the sequence 632–642 on myosin subfragment 1 inhibits actomyosin ATPase activity. *Biochem. Biophys. Res. Commun.* 189:1143–1149.

Combeau, C., D. Didry, and M. F. Carlier. 1992. Interaction between G-actin and myosin subfragment-1 probed by covalent cross-linking. *J. Biol. Chem.* 267:14038–14046.

Cooke, R. 1986. The mechanism of muscle contraction. *CRC Crit. Rev. Biochem.* 21:53–118.

Cooper, J. A., S. B. Walker, and T. D. Pollard. 1983. Pyrene actin: documentation of the validity of a sensitive assay for actin polymerization. *J. Muscle Res. Cell Motil.* 4:253–262.

Criddle, A. H., M. A. Geeves, and T. Jeffries. 1985. The use of actin labelled with *N*-(1-pyrenyl)iodoacetamide to study the interaction of actin with myosin subfragments and troponin/tropomyosin. *Biochem. J.* 232:343–349.

Eisenberg, E., and W. W. Kielley. 1974. Troponin-tropomyosin complex. Column chromatographic separation and activity of the three, active troponin components with and without tropomyosin present. *J. Biol. Chem.* 249:4742–4748.

Elzinga, M. 1986. Carboxyl group reactivity in actin. *In* Methods in Protein Science Analysis. K. E. Walsh, editor. The Human Press. 615–623.

Geeves, M. A. 1989. Dynamic interaction between actin and myosin subfragment 1 in the presence of ADP. *Biochemistry.* 28:5864–5871.

Geeves, M. A., and P. B. Conibear. 1995. The role of three-state docking of myosin S1 with actin in force generation. *Biophys. J.* 68:194S–199S.

Grabarek, Z., and J. Gergely. 1990. Zero-length crosslinking procedure with the use of active esters. *Anal. Biochem.* 185:131–135.

Greene, L. E., and E. Eisenberg. 1980. The binding of heavy meromyosin to F-actin. *J. Biol. Chem.* 255:549–554.

Herrmann, C., J. Sleep, P. Chaussepied, F. Travers, and T. Barman. 1993. A structural and kinetic study on myofibrils prevented from shortening by chemical cross-linking. *Biochemistry.* 32:7255–7263.

Hibberd, M. G., M. R. Webb, Y. E. Goldman, and D. R. Trentham. 1985. Oxygen exchange between phosphate and water accompanies calcium-regulated ATPase activity of skinned fibers from rabbit skeletal muscle. *J. Biol. Chem.* 260:3496–3500.

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

Huxley, H. E. 1969. The mechanism of muscular contraction. *Science.* 164:1356–1366.

Kouyama, T., and K. Mihashi. 1981. Fluorimetry study of *N*-(1-pyrenyl)iodoacetamide-labelled F-actin. Local structural change of actin protomer both on polymerization and on binding of heavy meromyosin. *Eur. J. Biochem.* 114:33–38.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Lheureux, K., T. Forne, and P. Chaussepied. 1993. Interaction and polymerization of the G-actin-myosin head complex: effect of DNase I. *Biochemistry.* 32:10005–10014.

Lowey, S., G. S. Waller, and K. M. Trybus. 1993. Skeletal muscle myosin light chains are essential for physiological speeds of shortening. *Nature.* 365:454–456.

Lymn, R. W., and E. W. Taylor. 1971. Mechanism of adenosine triphosphate hydrolysis by actomyosin. *Biochemistry.* 10:4617–4624.

Miller, C. J., P. Cheung, P. White, and E. Reisler. 1995. Actin's view of actomyosin interface. *Biophys. J.* 68:50S–54S.

Milligan, R. A. 1996. Protein-protein interactions in the rigor actomyosin complex. *Proc. Natl. Acad. Sci. USA.* 93:21–26.

Mornet, D., R. Bertrand, P. Pantel, E. Audemard, and R. Kassab. 1981. Structure of the actin-myosin interface. *Nature.* 292:301–306.

Nikolaeva, O. P., N. L. Golitsina, D. I. Levitsky, L. N. Moiseeva, and B. I. Kurganov. 1994. Kinetics of interaction of myosin subfragment 1 isoforms with F-actin. *Biochem. Mol. Biol. Int.* 33:553–560.

Offer, G., C. Moos, and R. Starr. 1973. A new protein of the thick filaments of vertebrate skeletal myofibrils. Extractions, purification and characterization. *J. Mol. Biol.* 74:653–676.

Panusz, H. T., G. Graczyk, D. Wilmanska, and J. Skarzynski. 1970. Analysis of orthophosphate-pyrophosphate mixtures resulting from weak pyrophosphatase activities. *Anal. Biochem.* 35:494–504.

Prince, H. P., H. R. Trayer, G. D. Henry, I. P. Trayer, D. C. Dalgarno, B. A. Levine, P. D. Cary, and C. Turner. 1981. Proton nuclear-magnetic-resonance spectroscopy of myosin subfragment 1 isoenzymes. *Eur. J. Biochem.* 121:213–219.

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

Schoenberg, M. 1988. The kinetics of weakly and strongly binding cross-bridges: implications for contraction and relaxation. *Adv. Exp. Med. Biol.* 226:189–202.

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* myosin S1. *Nature.* 364:171–174.

Spudich, J. A. 1994. How molecular motors work. *Nature.* 372:515–518.

Sutoh, K. 1982. Identification of myosin-binding sites on the actin sequence. *Biochemistry.* 21:3654–3661.

Sutoh, K. 1983. Mapping of actin-binding sites on the heavy chain of myosin subfragment 1. *Biochemistry.* 22:1579–1585.

Taylor, E. W. 1991. Kinetic studies on the association and dissociation of myosin subfragment 1 and actin. *J. Biol. Chem.* 266:294–302.

Tesi, C., F. Travers, and T. Barman. 1990. Cryoenzymatic studies on actomyosin ATPase. Evidence that the degree of saturation of actin with S1 affects the kinetics of the binding of ATP. *Biochemistry.* 29: 1846–1852.

Timson, D. J., and I. P. Trayer. 1997. The role of the proline-rich region in A1-type myosin essential light chains: implications for information transmission in the actomyosin complex. *FEBS Lett.* 400:31–36.

Timson, D. J., H. R. Trayer, and I. P. Trayer. 1998. The N-terminus of A1-type myosin essential light chains binds actin and modulates myosin motor function. *Eur. J. Biochem.* 255:654–662.

Trayer, I. P., H. R. Trayer, and B. A. Levine. 1987. Evidence that the N-terminal region of A1-light chain of myosin interacts directly with the C-terminal region of actin. A proton magnetic resonance study. *Eur. J. Biochem.* 164:259–266.

Van Dijk, J., C. Fernandez, and P. Chaussepied. 1998. Effect of ATP analogues on the actin-myosin interface. *Biochemistry.* 37:8385–8394.

Van Dijk, J., M. Furch, J. Derancourt, R. Batra, M. L. Knetsch, D. J. Manstein, and P. Chaussepied. 1999a. Differences in the ionic interaction of actin with the motor domains of nonmuscle and muscle myosin II. *Eur. J. Biochem.* 260:672–683.

Van Dijk, J., M. Furch, C. Lafont, D. Manstein, and P. Chaussepied. 1999b. Functional characterization of the secondary actin binding site of myosin II. *Biochemistry.* (in press).

Weeds, A. G., and R. S. Taylor. 1975. Separation of subfragment-1 isoenzymes from rabbit skeletal muscle myosin. *Nature.* 257:54–56.

White, H. D., and E. W. Taylor. 1976. Energetics and mechanism of actomyosin adenosine triphosphatase. *Biochemistry.* 15:5818–5826.

Winstanley, M. A., D. A. Small, and I. P. Trayer. 1979. Differential binding of myosin subfragment one species to immobilized ADP and actin: the influence of the alkali light chains. *Eur. J. Biochem.* 98: 441–446.

Yamamoto, K. 1989. Binding manner of actin to the lysine-rich sequence of myosin subfragment 1 in the presence and absence of ATP. *Biochemistry.* 28:5573–5577.

Yamamoto, K. 1990. Shift of binding site at the interface between actin and myosin. *Biochemistry.* 29:844–848.

Yamamoto, K., and T. Sekine. 1983. Interaction of alkali light chain 1 with actin: effect of ionic strength on the cross-linking of alkali light chain 1 with actin. *J. Biochem. (Tokyo).* 94:2075–2078.