Rotational dynamics of spin-labeled F-actin during activation of myosin S1 ATPase using caged ATP

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ABSTRACT The most probable source of force generation in muscle fibers is the rotation of the myosin head when bound to actin. This laboratory has demonstrated that ATP induces microsecond rotational motions of spin-labeled myosin heads bound to actin (Berger, C. L., E. C. Svensson, and D. D. Thomas. 1989. *Proc. Natl. Acad. Sci. USA.* 86:8753–8757). Our goal is to determine whether the observed ATP-induced rotational motions of actin-bound heads are accompanied by changes in actin rotational motions. We have used saturation transfer electron paramagnetic resonance (ST-EPR) and laser-induced photolysis of caged ATP to monitor changes in the microsecond rotational dynamics of spin-labeled F-actin in the presence of myosin subfragment-1 (S1). A maleimide spin label was attached selectively to cys-374 on actin. In the absence of ATP (with or without caged ATP), the ST-EPR spectrum (corresponding to an effective rotational time of ~150 μs) was essentially the same as observed for the same spin label bound to cys-707 (SH_1) on S1, indicating that S1 is rigidly bound to actin in rigor. At normal ionic strength (μ = 186 mM), a decrease in ST-EPR intensity (increase in microsecond F-actin mobility) was clearly indicated upon photolysis of 1 mM caged ATP with a 50-ms, 351-nm laser pulse. This increase in mobility is due to the complete dissociation of S1 from the actin filament. At low ionic strength (μ = 36 mM), when about half the S1 heads remain bound during ATP hydrolysis, no change in the actin mobility was detected, despite much faster motions of labeled S1 bound to actin. Therefore, we conclude that the active interaction of S1, actin, and ATP induces rotation of myosin heads relative to actin, but does not affect the microsecond rotational motion of actin itself, as detected at cys-374 of actin.

INTRODUCTION

Most models of muscle contraction consider the myosin head as the active component in force generation. Huxley (1969) proposed that the most probable source of force generation in muscle fibers is the rotation of the myosin head when bound to actin. Huxley and Simmons (1971) proposed that the myosin head undergoes millisecond and sub-millisecond rotations while bound to actin. Electron paramagnetic resonance (EPR; Cooke et al., 1982; Barnett and Thomas, 1989) and phosphorescence (Stein et al., 1990) experiments on labeled myosin heads in skinned muscle fibers have shown that most myosin heads are undergoing large-amplitude submillisecond rotations during isometric contraction. X-Ray diffraction experiments on active muscle indicate that most cross-bridges are in close proximity to actin during contraction (Haselgrove and Huxley, 1973), and stiffness measurements suggest that most (60–80%) cross-bridges are attached to actin in contraction during the spectroscopic studies (Barnett and Thomas, 1989; Stein et al., 1990). However, lacking a direct measurement of myosin head binding in fibers, these spectroscopic measurements on fibers cannot directly correlate rotationally mobile heads with actin-attached heads.

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This uncertainty of head binding can be overcome by performing experiments on actin and S1 in solution, where centrifugation experiments can be performed under spectroscopy conditions to provide a direct and quantitative measure of the fraction of S1 bound to actin. Using saturation transfer EPR (ST-EPR), flash photolysis of caged ATP, and centrifuge binding experiments on actin and spin-labeled myosin heads (S1) in solution, Berger et al. (1989) obtained directly the EPR signal due to actin-bound S1 during steady-state ATPase activity, and concluded that ATP induces microsecond rotational motion of actin-attached S1 heads in solution. The most straightforward interpretation of this result is that the S1 head, or at least the labeled part of the S1 head, rotates relative to the actin filament. An alternative interpretation (as pointed out by Huxley, 1974) is that some or all of the ATP-induced microsecond motions of the myosin head originate with motions of actin itself. This rotation could be due to a change in actin filament flexibility or to an active force-generating movement of actin when myosin and ATP are bound. Thomas et al. (1979) used ST-EPR to show that spin-labeled F-actin is rotationally dynamic on the microsecond time scale, and that actin's rotational motions are restricted by the binding of S1. Other results suggest that actin flexibility is crucial for proper actin-S1 function

0006-3495/91/06/1235/07 $2.00

(Prochniewicz-Nakayama and Yanagida, 1982; Prochniewicz and Yanagida, 1990).

In the present study, we have used ST-EPR and laser-induced photolysis of caged ATP to determine whether the ATP-induced rotational motions of the actin-bound S1 heads are accompanied by changes in the microsecond rotational motions of actin. Flash photolysis of P^3-1-(2-nitrophenyl)ethyl ester of ATP (caged ATP), a photoactivatible analogue of ATP, was required because of the high ATPase activity of the preparation (McCray et al., 1980). As in Berger et al. (1989), we have used ST-EPR because of its optimal sensitivity to rotations in the microsecond time range, (Thomas, 1987) and low ionic strength conditions to maximize S1 binding to actin. However, the spin label was covalently attached to cys-374 of actin rather than to the myosin head, allowing us to monitor the rotational dynamics of actin during the active interaction of actin, S1, and ATP.

METHODS

Solutions

Solutions used in the present study were the same as used by Berger et al. (1989). Low ionic strength conditions (μ = 36 mM) are defined as 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA (pH 7.0) plus either 5 mM magnesium nucleotide (ATP or caged ATP) or 25 mM potassium propionate. Normal ionic strength conditions (μ = 186 mM) were identical to low ionic strength conditions, except for the addition of 150 mM potassium propionate. All preparations were done at 0–4°C, and all EPR and binding measurements were done at 25°C.

Preparations

F-actin was prepared as described by Pardee and Spudich (1982). Chymotryptic S1 was prepared as described by Eads et al. (1984), except that the chymotryptic digestion time was 10 min. F-actin was spin-labeled at cys-374 with 4-maleimido-2,2,6,6,-tetramethyl-1-piperidinyloxy (MSL; Aldrich Chemical Co., Milwaukee, WI) to the extent of 0.96 ± 0.03 label bound per actin monomer (Thomas et al., 1979). The spin concentration was determined by double integration of the digitized conventional EPR spectrum (Squier and Thomas, 1986). Caged ATP (adenosine-5′-triphosphate, p^3-1-[2-nitrophenyl]-ethyl ester) was obtained from Calbiochem (San Diego, CA), and ATP (adenosine-5′-triphosphate) was obtained from Sigma Chemical Co. (St. Louis, MO).

Assays

Protein concentrations were determined spectrophotometrically with extinction coefficients: 0.63 mg ml^{-1} cm^{-1} at 290 nm for G-actin (Mossakowska et al., 1988), 0.74 mg ml^{-1} cm^{-1} at 280 nm for S1 (Margossian and Lowey, 1982). ATPase activities were measured under low ionic strength conditions at 25°C by determining the rate of release of inorganic phosphate (P_i). The reaction was initiated by the addition of 5 mM MgATP, and aliquots were quenched at timed intervals in an acidic P_i-determining solution (Lanzetta et al., 1979) and then assayed by measuring the absorbance at 660 nm. The fraction of S1 bound to MSL-actin during ATP hydrolysis was determined by centrifugation binding experiments (Chalovich and Eisenberg, 1982; Berger et al., 1989), except that the S1 concentration used was 0.5 μM. The amount of caged ATP photolyzed in an experiment was determined by measuring the amount of inorganic phosphate produced as a result of ATP hydrolysis by the acto-S1 ATPase (Lanzetta et al., 1979).

EPR spectroscopy

Steady-state EPR spectra were obtained (Squier and Thomas, 1986) with a Bruker ESP 300 spectrometer (Bruker Instruments, Billerica, MA). Transient ST-EPR spectra were obtained at a single field position (L'') 10 Gauss up-field from the first peak of the ST-EPR spectrum (L). The L'' intensity parameter is not sensitive to minor errors in either phase or H_1, but is very sensitive to changes in microsecond rotational motion (Squier and Thomas, 1986). The samples were contained in a 25-μl well on a TPX cover plate which was attached to a quartz flat cell (WG-807; Wilmad, Buena, NJ). The temperature of the samples were maintained at 25.0 ± 0.5°C by using a variable temperature controller (ER4111; Bruker Instruments). Caged ATP was photolyzed during EPR experiments by using a 50-ms burst (100 Hz) at 351 nm from a XeF excimer laser (LPX200i, Lambda Physik, Acton, MA). The light was introduced directly into the optical port of a TE_{102} EPR cavity (ER4102 ST; Bruker Instruments). The TPX sample well was the same size as the optical port on the EPR cavity, so the entire sample was uniformly exposed. Light energy incident on the sample was ~ 150 mJ/cm^2 for a single 100-Hz pulse.

Digitized EPR spectra were analyzed on IBM-compatible computers using a program developed by Robert L. H. Bennett. All ST-EPR spectra were normalized by dividing by the double integral of the low-power (H_1 = 0.032 G) conventional EPR spectrum. This parameter is independent of rotational motion and corrects for any variation in the concentration of spin labels between the samples (Squier and Thomas, 1986).

RESULTS

Characterization of spin-labeled actin

Double integration of EPR spectra showed that MSL-actin contained 0.96 ± 0.03 spin labels per actin monomer. The reaction of MSL with F-actin under these conditions has been shown to be quite specific for cys-374 (Thomas et al., 1979), so virtually every actin monomer in the MSL-actin preparation is specifically labeled. Therefore, any measured properties (e.g., S1 binding) are representative of the spin-labeled actin. Denatured or incompletely polymerized actin exhibits a weakly immobilized component in the conventional EPR spectrum (Thomas et al., 1979). This component represented <2% of the spin label in the experimental spectra (Fig. 1, *left*). This result not only implies that the labeled actin is virtually all in its native polymerized state, but also ensures that the ST-EPR spectra are reliable indicators of actin's microsecond rotational motion (Thomas et al., 1979; Squier and Thomas, 1986).

The fraction of actin-attached S1 heads in the presence of saturating ATP was determined by the use of centrifuge binding experiments (Chalovich and Eisen-

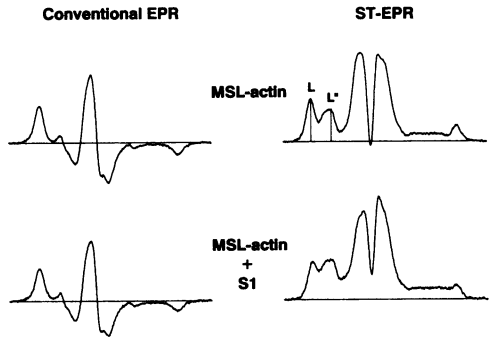

FIGURE 1 Effect of S1 on conventional and ST-EPR spectra at low ionic strength (μ = 36 mM). (*Top*) 200 μM MSL-actin (No S1). (*Bottom*) 200 μM MSL-actin + 40 μM S1.

berg, 1982; Berger et al., 1989). To determine whether the spin labeling of actin alters the affinity of S1 for actin during ATP hydrolysis, samples with labeled and unlabeled actin were simultaneously centrifuged in the presence of ATP. The fraction bound was determined, and a binding constant was calculated (K_B). There was always less than a 5% difference in the fraction of S1 bound between samples containing labeled and unlabeled actin, and the resulting value of K_B at low ionic strength was in good agreement with that previously measured for unlabeled actin and MSL-S1: $K_B = 7.4 \pm 0.4 \times 10^3\ M^{-1}$ (Berger et al., 1989). Using this value of K_B, the fraction of S1 heads attached to actin, at low ionic strength in the presence of 5 mM ATP under EPR conditions (100 μM S1, 200 μM MSL-actin, 25°C), was calculated to be 52 ± 2%. There was no significant binding in the presence of saturating ATP at normal ionic strength.

Effect of S1 on EPR spectra of spin-labeled actin

It has been demonstrated that the binding of S1 to MSL-actin restricts the microsecond rotational motion of actin filaments (Thomas et al., 1979). Fig. 1 illustrates the effect of S1 on both the conventional EPR and ST-EPR spectra. S1 does not affect the conventional EPR spectrum, indicating that the label is strongly immobilized (no sub-microsecond motion) in both the presence and absence of S1, so any change in the ST-EPR spectra must be due to a change in microsecond rotational motion (Thomas et al., 1979). S1 does have a significant effect on the ST-EPR spectrum. When S1 is added to actin at a ratio of 0.2 S1/MSL-actin, the L''/L intensity ratio increases from 0.78 ± 0.01 to 1.02 ± 0.02. This corresponds to a greater than twofold increase in the effective rotational correlation time of the MSL-actin, to a final value of ~150 μs (Squier and Thomas, 1986).

Because the purpose of this study is to determine whether actin plays an active role in inducing microsecond rotational motions of S1 during ATP hydrolysis, it is essential for any changes in the ST-EPR spectrum to be due to the active interaction of ATP, actin, and S1, rather than due to changes in the binding stoichiometry. To characterize changes in the dynamics of actin filaments due to the binding of S1 in the absence of ATP, the L''/L intensity ratio was calculated from ST-EPR spectra of 200 μM MSL-actin plus 0–100 μM S1 at both low and normal ionic strengths, to produce titration curves (Fig. 2). The MSL-actin-S1 mixture exhibited a time-dependent immobilization, i.e., the L''/L intensity ratio increased (indicating restriction of rotational motion) with time. Spectra acquired 30 min after mixing indicated less motion than spectra acquired 10 min after mixing. The spectra did not change significantly after 45–60 min, so all ST-EPR spectra were acquired 1 h after the proteins were mixed and loaded into the flat cell. At both low and normal ionic strength, MSL-actin has about the same maximum immobilization, but the ratio of S1/MSL-actin at which this maximum immobilization occurs is significantly different (Fig. 2). At normal ionic strength, the maximum immobilization occurs at ~0.1 S1/MSL-actin, in agreement with previous reports (Thomas et al., 1979; Mossakowska et al., 1988). At low ionic strength, more S1 (0.2 S1/MSL-actin) was required to maximally restrict the rotational motion.

Transient EPR after photolysis of caged ATP

To determine whether actin's microsecond rotational motions change due to the active interaction of MSL-actin, S1, and ATP, it is essential that ATP-induced changes in the fraction of bound heads are not sufficient to change the motion of MSL-actin. This was accomplished by using low ionic strength conditions, with bound S1/MSL-actin ratios that would keep the L''/L intensity ratio constant, i.e., on the plateau of the titration curve (Fig. 2*A*) even in the presence of saturating ATP. The transient ST-EPR experiments were performed at 200 μM MSL-actin and 100 μM S1. Fig. 3 illustrates ATP's effect on MSL-actin mobility. Upon conversion of caged ATP to ATP at low ionic strength (Fig. 3*A*), the bound S1/MSL-actin ratio decreases from 0.50 to 0.26, remaining on the plateau of the titration curve (Fig. 2*A*). However, the L'' intensity does not change significantly upon the generation of ATP, indicating that the microsecond rotational motion does not change. Under the conditions of Fig. 3*A* (low ionic strength, 25°C, 200 μM MSL-actin, 100 μM S1),

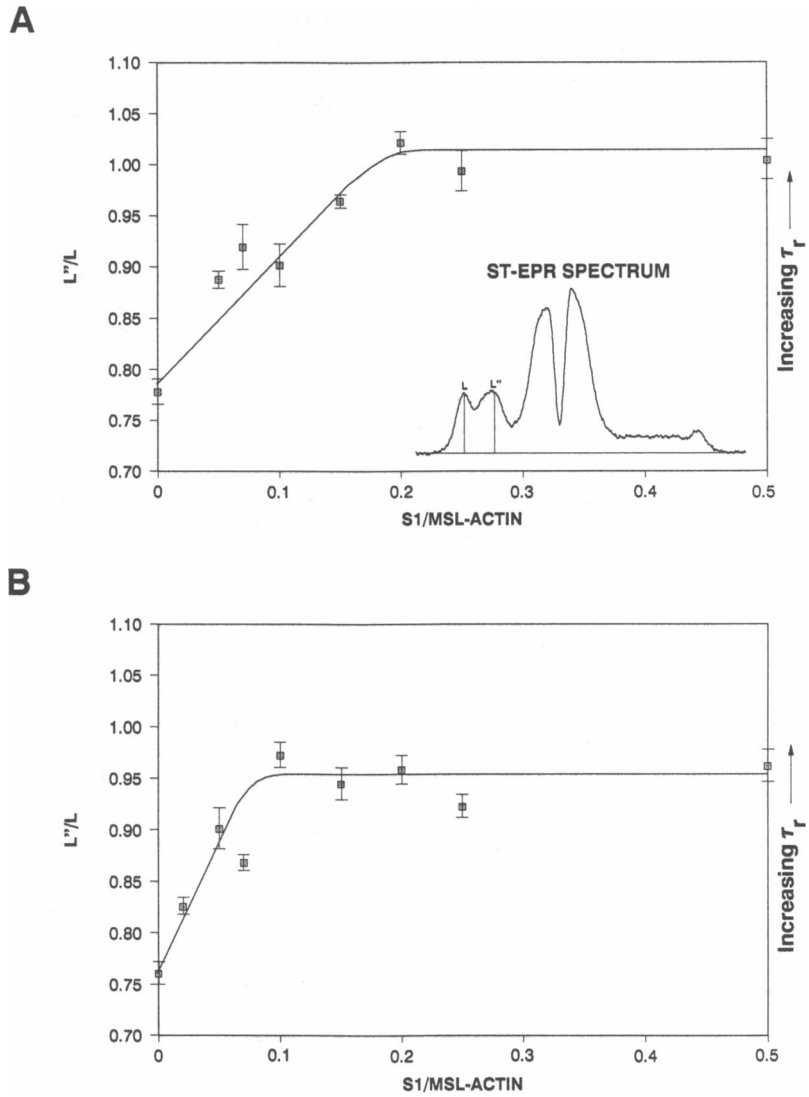

FIGURE 2 Effect of S1 addition on the ST-EPR intensity of MSL-actin at (*A*) low ionic strength (μ = 36 mM), and (*B*) normal ionic strength (μ = 186 mM). The *L* and *L"* intensities are defined in the inset. The actin concentration for each point was 200 μM and the S1 concentrations ranged from 0 to 100 μM. Each point represents the mean of 3–5 preparations and the error bars illustrate the SEM.

the ATPase rate is 16.1 ± 1.5 s^{-1}, so the 1.5 mM ATP produced by the laser pulse is sufficient to saturate the myosin active sites in the sample for 0.87 ± 0.06 s. This time interval is much greater than the instrumental response time (set by the acquisition computer to be 0.16 s), so any increased mobility during the steady state of the ATPase reaction would have been detected. Experiments were also performed at 4°C (data not shown). At this temperature, the ATP lasts for more than 1 min at low ionic strength. These experiments produced the same results as 25°C, i.e., no detectable change in the microsecond rotational motion. When ATP is generated in the system at normal ionic strength (Fig. 3 *B*), the intensity of *L"* decreases within 0.15 s to the signal level corresponding to free MSL-actin, verifying that the instrumental response time is much shorter than 0.87 s (the time required for ATP depletion at low ionic strength, Fig. 3 *A*). This increase in MSL-actin rotational motion can be explained by the complete dissociation of the S1 from the actin filament, thus achieving the signal level of the free MSL-actin spectrum (Fig. 3 *B*, *dotted spectrum*). Control experiments with MSL-actin minus S1 show no changes upon generation of ATP (Fig. 3 *C*). The data in Fig. 3 constitute direct evidence that MSL-actin does not undergo a significant change in microsecond rotational motion during the active interac-

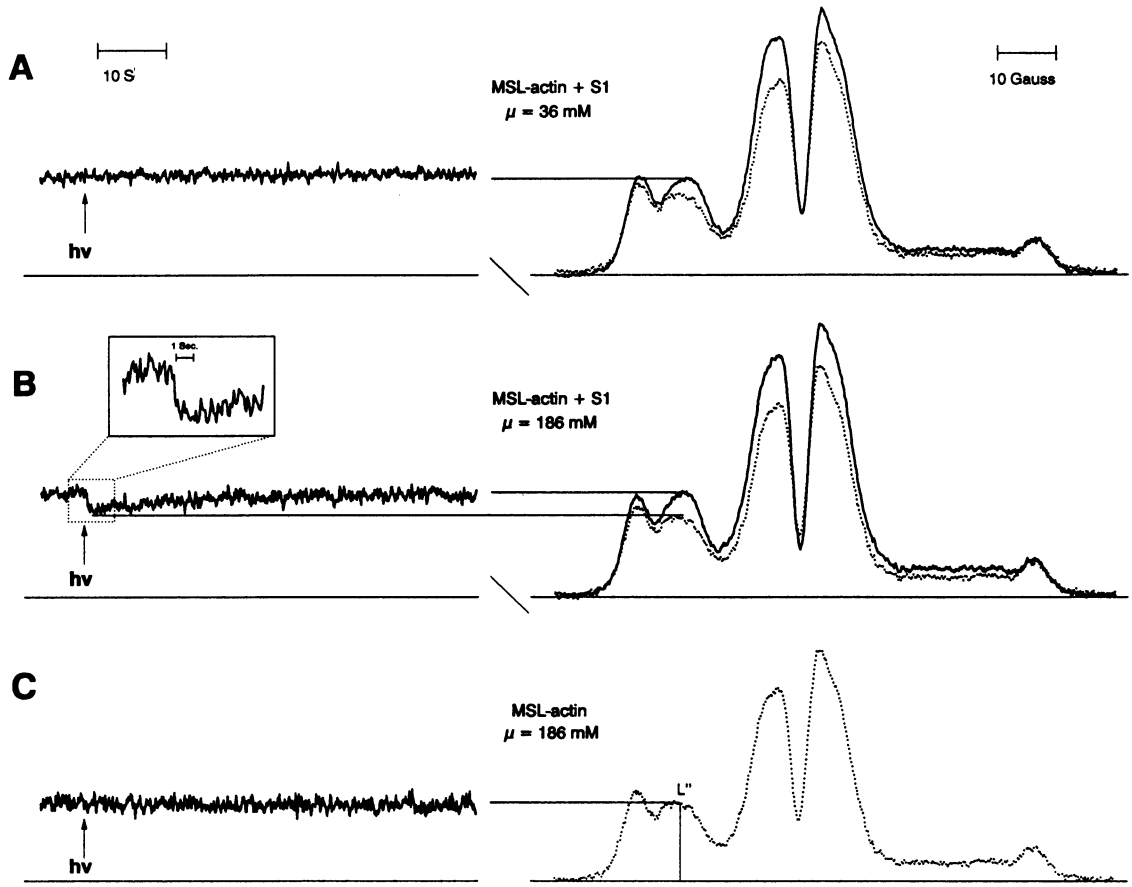

FIGURE 3 Effect of the photolysis of caged ATP on the ST-EPR intensity of MSL-actin and MSL-actin-S1 at low (μ = 36 mM) and normal (μ = 186 mM) ionic strengths. (*Right*) the steady-state ST-EPR spectra of 200 μM MSL-actin plus S1 (*solid spectrum*) and minus S1 (*dotted spectrum*). (*Left*) the spectral intensity at the *L"* field position plotted as a function of time. ATP was released in the sample at the laser pulse (*arrow*). (*A*) 200 μM MSL-actin + 100 μM S1 at low ionic strength. (*B*) 200 μM MSL-actin + 100 μM S1 at normal ionic strength. (*Inset*) expanded time base during transient, showing that instrument response time is 0.15 s. (*C*) 200 μM MSL-actin (no S1) at normal ionic strength.

tion of actin, S1, and ATP, despite the greatly increased motion of actin-bound S1 induced by ATP (Berger et al., 1989).

DISCUSSION

Rotational motion of S1-bound actin in rigor

Thomas et al. (1979) used ST-EPR to demonstrate that MSL-actin undergoes sub-millisecond rotational motions and that these motions are restricted by the binding of S1 heads. They also reported that low ratios of S1/MSL-actin maximally restrict the microsecond rotational motions of actin. The present study confirms the restriction of MSL-actin at low ratios of S1/MSL-actin, and demonstrates further that the immobilization is time dependent and varies with ionic strength (Fig. 2). Thomas et al. (1979) suggested that the restriction of the MSL-actin by S1 is a cooperative effect that is propagated along the actin filament, i.e., the binding of one S1 restricts the rotational motion of 10 actin monomers. However, information from this and other studies provide an alternate explanation for the observed cooperative effect.

The actin motions being detected by ST-EPR are most likely large-scale intrafilament bending or torsional motions (Thomas et al., 1979; Yoshimura et al., 1984; Egelman et al., 1982). However, these intrafilament motions could be restricted by interfilament cross-links. Ando and Scales (1985) and Ando (1987) reported that S1 causes interfilament cross-links, forming actin bundles. This bundle formation may result in the restriction of rotational motion upon the addition of S1. Like the motional restriction detected by ST-EPR, the formation of actin bundles by S1 is a time-dependent process, for

which the maximum rate occurs at low ratios of S1/MSL-actin (Ando and Scales, 1985). Electron micrographs of negatively stained EPR samples used in the present study also show the S1-dependent bundling of MSL-actin filaments (data not shown).

Rotational motion of S1-bound actin in the presence of ATP

Fig. 3 shows the effect of caged ATP photolysis, producing 1–2 mM ATP, on the rotational motion of MSL-actin-S1. At normal ionic strength (Fig. 3 *B*), the *L″* intensity parameter decreased to the level of MSL-actin in the absence of S1, indicating increased mobility. This result is expected because the centrifuge binding experiments indicate that virtually all of the S1 dissociates from the MSL-actin in the presence of saturating ATP. As the ATP was depleted in the transient ST-EPR experiment, the intensity of *L″* increased rapidly to the original level, indicating decreased mobility. As mentioned above, this S1-induced immobilization may be due to bundling of the actin filaments. Although the time-dependent immobilization of actin, produced by mixing S1 with MSL-actin in the absence of ATP, was not complete until 45 min after the proteins were mixed, this immobilization was complete within 1 min after the depletion of ATP. This increased rate of immobilization during the depletion of ATP may arise because the filaments are already in an orientation conducive to bundling.

At low ionic strength (Fig. 3 *A*), when greater than half of the S1 heads remain bound to the actin filament in the presence of saturating ATP, the *L″* intensity parameter shows no significant change upon conversion of caged ATP to ATP. While the ATPase rate at low ionic strength was significantly greater than at normal ionic strength, the time constant on the EPR spectrometer was set appropriately to record any changes during the steady state of active ATP hydrolysis, which should last ~1 s under the conditions of Fig. 3 *A*. Under identical solution conditions and protein concentrations, but with the spin label (MSL) on S1 instead of actin, it was reported that the MSL-S1 that is not dissociated from actin undergoes ATP-induced rotational motion with an effective correlation time on the order of 1 μs (Berger et al., 1989). If actin were the source of this increase in rotational motion, a dramatic decrease (~4 times that seen in Fig. 3 *B*) in the *L″* intensity parameter would have been observed in Fig. 3 *A*. It remains possible that motions within actin, not detected at cys-374, are induced by the acto-S1 ATPase cycle, but these are not submillisecond motions of the entire actin monomer.

Recent reports suggest that cys-374 is within the same large domain as the S1 binding site (Kabsch et al., 1990; Milligan et al., 1990). Also, ST-EPR spectra in which myosin heads, actin, or both were spin labeled indicate that the *L″/L* intensity ratios in the absence of ATP are essentially independent of the location of the spin label (Thomas et al., 1979), i.e., the spin-label on myosin heads has the same microsecond rotational motion as the spin label on actin. Therefore, it is likely that the ST-EPR spectra of MSL-actin report the microsecond rotational motions of the region of actin to which S1 binds.

Conclusions

We conclude that the active interaction of ATP and S1 with actin does not induce microsecond rotational motions in the actin filament, as detected by a spin label at cys-374. Therefore, the ATP-induced microsecond rotation of S1 bound to actin (Berger et al., 1989) must correspond to rotation of S1 relative to the actin filament, not to S1 that is rigidly coupled to mobilized actin. Similar microsecond rotations have been observed for spin (Barnett and Thomas, 1989; Fajer et al., 1990*a*) and phosphorescent (Stein et al., 1990) probes attached to myosin heads in contracting muscle fibers. These results are consistent with the hypothesis that in contraction there is a large population of attached cross-bridges that are dynamically disordered (Huxley and Kress, 1985). The results are also consistent with the proposal that a rapid rotation of cross-bridges among several attachment angles could help explain force transients observed in quick-release experiments (Huxley and Simmons, 1971). It remains possible that actin undergoes conformational changes upon the binding of S1 that are necessary for activation of muscle contraction (Prochniewicz-Nakayama and Yanagida, 1982; Prochniewicz and Yanagida, 1990; Ando, 1989; Criddle et al., 1985). These conformational changes might be undetectable in the present studies, because either (*a*) the site at which actin is labeled (cys-374) is not sensitive to these changes, or (*b*) the changes in actin conformation do not occur on the sub-millisecond time scale. To thoroughly investigate these possibilities, (*a*) spin labels should be attached to other sites on actin (e.g., the myosin binding site), or (*b*) conventional EPR on oriented samples (Barnett et al., 1986; Fajer et al., 1990*b*) should be used to detect orientational changes of labeled actin upon active interaction with S1 and ATP.

We thank C. L. Berger, R. A. Stein, and J. E. Mahaney for helpful discussions, R. L. H. Bennett, F. L. Nisswandt, and P. G. Fajer for technical assistance. We thank J. Hodgkinson of E. H. Egelman's laboratory for performing the electron microscopy. This work is based in part on preliminary experiments and suggestions by T. Yanagida.

This work was supported by grants to Dr. Thomas from the National Institutes of Health (NIH) (AR 39754) and the Minnesota Supercom-

puter Institute. Mr. Ostap was supported by a Biophysics Training grant from NIH (GM 08277-03).

Received for publication 13 September 1990 and in final form 19 February 1991.

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