ATP INDUCES MICROSECOND ROTATIONAL MOTIONS OF MYOSIN HEADS CROSSLINKED TO ACTIN

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ABSTRACT We have used saturation transfer electron paramagnetic resonance (ST-EPR) to study the effect of ATP on the rotational dynamics of spin-labeled myosin heads crosslinked to actin (XLAS1). We have previously shown that ATP induces microsecond rotational motions in activated myofibrils or muscle fibers, but the possibility remained that the motion occurred only in the detached phase of the cross-bridge cycle. The addition of ATP to the crosslinked preparation has been shown to be a model system for active cross-bridges, presumably providing an opportunity to measure the motion in the attached state, without interference from unattached heads. In the absence of ATP, XLAS1 had very little microsecond rotational mobility, yielding a spectrum identical to that observed for uncrosslinked acto-S1. This suggests that all of the labeled S1 forms normal rigor complexes when crosslinked to actin. The addition of 5 mM ATP greatly increased the microsecond rotational mobility of XLAS1, and the effects were reversed upon depletion of ATP. The most plausible explanation for these results is that myosin heads undergo microsecond rotational motion while attached actively to actin during steady state ATPase activity. These results have important implications for the interpretation of spectroscopic data obtained during muscle contraction.

INTRODUCTION

A central goal in muscle research has been to correlate biochemical states with physical conformations of the acto-myosin ATPase system. A powerful approach to this problem has been the use of spectroscopic probes attached to myosin heads. Saturation transfer electron paramagnetic resonance (ST-EPR) has been used to show that spin-labeled myosin heads are rotationally mobile on the microsecond time scale in purified myosin filaments or relaxed myofibrils, but that attachment of myosin heads to actin in the absence of ATP greatly decreases this microsecond mobility (1, 2). Since force is produced when myosin heads are attached to actin (forming cross-bridges) under activating conditions, the most crucial questions concern motions (particularly rotations) that occur in the attached phase of the ATPase cycle. However, spectroscopic measurements made on a heterogeneous cycling system of attached and detached heads cannot unambiguously distinguish motions occurring in the attached phase from those occurring in the detached phase. For example, ST-EPR showed previously that spin-labeled heads are nearly as mobile in activated myofibrils as in relaxed myofibrils (1), indicating either that most of the heads are detached from actin or that actin-attached heads are mobile under activating conditions.

A means of overcoming this ambiguity is suggested by the recently developed procedure for covalently crosslinking myosin heads (in the form of the chymotryptic fragment subfragment 1, designated S1) to actin, resulting in an ATPase activity very near V_{max}, the activity observed for acto-S1 in the limit of infinite actin concentration (3). This suggests that crosslinking produces an active preparation that permits the study of attached states without interfering signals from detached states. Electron microscopy of negatively stained preparations revealed that the characteristic arrowheads of acto-S1 were formed in crosslinked acto-S1 (XLAS1) (4), but were less clearly visible in the presence of ATP, suggesting a change in the conformation of the XLAS1 complex (4). It is essential to extend this observation to a measurement that does not involve staining or fixation and can quantitate the time scale of rotational dynamics occurring in this system in response to ATP. Spectroscopic probe measurements can satisfy these criteria.

METHODS

S1 was prepared by the procedure of Eads et al. (8), except that the chymotryptic digestion time was 5 min. S1 was labeled with MSL (4-maleimido-TEMPO) at 4°C in a solution containing 108 μM S1, 195 μM MSL, 60 mM KCL, 6 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 25 mM MOPS, and pH 7.0. The MSL was added last, and the solution was then dialyzed immediately and exhaustively against the same buffer without MSL. Previous studies have shown that the spin label is attached specifically to SH_1, a reactive thiol residue on S1, and that it is rigidly bound with respect to S1, so that it reports the overall rotational motion of S1 (1). S1 was crosslinked to actin using a modified procedure of Mornet et al. (3). EDC (15 mM) was added to 5.7 ml of a 2.5-mg/ml solution of actin in 0.1 M MES, pH 6.2, 22°C. After 1 min, S1 was added in an equal

molar ratio to actin and allowed to react for 14 min. The reaction was quenched by adding 1 vol of 10 mM imidazole (pH 7.0) and lowering the temperature to 4°C. 5 mM MgATP was added to dissociate uncrosslinked S1, and the sample was sedimented at 150,000 *g* for 60 min. The pellet was resuspended in 20 ml of G-actin buffer (0.2 mM $CaCl_2$, 1 mM NaN_3, 1 mM EPPS, pH 8.5), using a hand-held Teflon/glass homogenizer, and the MgATP washing procedure was repeated. SDS-gel electrophoresis of this sample showed that <5% of the S1 was uncross-linked, which could not have a significant effect on the ST-EPR spectra.

EPR samples were contained in a standard quartz flat cell (Wilmad Glass Co., Buena, NJ), and both conventional and ST-EPR spectra were recorded and analyzed as described previously, using an IBM Instruments/Bruker ER200D spectrometer (5). The samples were maintained at a temperature of 20° ± 0.2°, using a variable temperature controller (model ER 4111 VT; IBM Instruments Inc., Danbury, CT). The effective rotational correlation time was defined as the correlation time of a MSL-hemoglobin sample having the same value of the ST-EPR spectral parameter C'/C (5).

RESULTS

As shown in Table 1, MSL-S1 has twice the activity of unlabeled S1 and is activated ~20-fold by crosslinking to actin, resulting in an activity ~20% as great as obtained with unlabeled XLAS1. Despite this partial inhibition, the activity of the labeled S1 crosslinked to actin is comparable to the maximal actin-activated ATPase activity previously reported for SH_1-blocked HMM in solution (6).

The left side of Fig. 1 shows the ST-EPR spectra of XLAS1, along with spectra from uncrosslinked AS1 and S1 alone, all in the absence of ATP. The conventional EPR spectra (not shown) of these samples are all characteristic of strongly immobilized spin labels, i.e., probes that are not undergoing nanosecond rotational motions relative to the protein (1). Thus the ST-EPR spectra can be analyzed in terms of microsecond rotational motions (1, 5), which decrease the intensity of the ST-EPR spectrum, particularly in the center of the spectrum (5). In the absence of ATP, XLAS1 and AS1 yield identical spectra, indicating that their rotational mobilities are identical, and suggesting that the actin-S1 interaction in the absence of ATP is unperturbed by crosslinking. The high intensity in the center of these spectra implies little or no microsecond mobility, confirming our previous finding that the actin-S1 complex is rigid in the absence of ATP. In contrast, the spectrum of S1 alone shows sharply decreased intensity, particularly in the center, due to the rapid rotational motion of S1 tumbling freely in solution.

TABLE I
ATPASE ACTIVITIES

	S1	XLAS1
Unlabeled	0.048 ± 0.002	9.35 ± 0.34
Labeled	0.087 ± 0.001	2.73 ± 0.08
Unlabeled (corrected)	0.043 ± 0.007	10.1 ± 1.4
Labeled (corrected)	0.092 ± 0.009	1.90 ± 0.24

ATPase activities (per seconds) of S1 and XLAS1. The solutions contained 5 mM ATP, 6 mM $MgCl_2$, 150 mM KCl, 20 mM MOPS, 1 mM EGTA, pH 7.0, 20°C. The preparation of unlabeled XLAS1 actually contained 10% labeled S1 during crosslinking, so that the concentration of S1 could be determined from the intensity of the conventional EPR spectrum, using labeled S1 as a standard. The fraction of S1 molecules labeled in the original MSL-S1 sample was determined to be 0.90 ± 0.09 from the K^+/EDTA ATPase activity (1). Using this information, the values in the bottom two rows have been corrected for the small contaminations of labeled S1 in the unlabeled sample and of unlabeled S1 in the labeled sample. Thus these corrected values reflect directly the activities of the unlabeled or labeled S1 when crosslinked to actin.

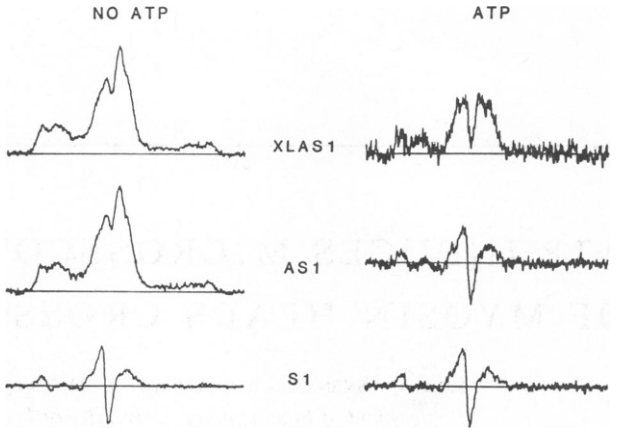

FIGURE 1. ST-EPR spectra of MSL-S1 crosslinked to actin (*top row*), in the presence of 2 mol actin/mol S1 (*center row*), and free in solution (*bottom row*), in the absence (*left*) and presence (*right*) of 5 mM ATP and 20 mg/ml creatine phosphokinase. All solutions contained 6 mM MgC12, 50 mM creatine phosphate, 1 mM EGTA, 20 mM MOPS, pH 7.0 at 20°C. The S1 concentration was approximately 85 μm in all cases. Spectra on the right were recorded during the steady state of ATP hydrolysis.

Upon the addition of ATP to XLAS1, the spectral intensity decreases considerably (Fig. 1, *top right*), implying increased microsecond rotational mobility. An even greater increase in mobility is induced by ATP in uncrosslinked AS1 (Fig. 1, *center right*), producing a spectrum identical to that of S1 alone (which is unaffected by ATP, Fig. 1, *bottom right*), presumably because ATP dissociates S1 from actin. When the ATP is completely hydrolyzed, both the acto-S1 and XLAS1 spectra return to their original shapes, indicating little or no microsecond motion (data not shown, but spectra are identical to those at the left side of Fig. 1).

DISCUSSION

We will first consider the spectroscopic implications of these results, independent of their biophysical interpretations. The effective rotational correlations times characterizing the motions of the spin-labeled heads are given in Table II, determined by the method of Squier and Thomas (5). Thus ATP decreases the effective correlation time (increases the rotational mobility) of S1 in XLAS1 by more than a factor of 10. The detailed interpretation of these effective correlation times, in terms of the rates and amplitudes of motion present in the system, will require the additional resolution attainable from conventional EPR on

TABLE II
EFFECTIVE CORRELATION TIMES
FROM ST-EPR SPECTRA

	No ATP	5 mM ATP
S1	0.14 ± 0.04	0.18 ± 0.06
Acto-S1	90 ± 18	0.09 ± 0.13
XLAS1	85 ± 11	7 ± 4

Effective correlation times (in microseconds) were determined by comparing experimental spectra with hemoglobin reference spectra, using the C'/C parameter, which characterizes the shape of the center of the spectrum (5).

an oriented sample (7), or from transient phosphorescence anisotropy (8) (both being applied to XLAS1 currently in this laboratory). Regardless of the details of the data interpretation, it is clear that ATP induces microsecond rotational mobility in XLAS1 (Fig. 1, *top row*) that is intermediate between that of free and bound S1 in the absence of ATP (Fig. 1, *second row*), and that is similar to that observed in activated myofibrils (1).

Now we turn to the biophysical interpretation of these results. There are two principal models that can explain the ATP-induced increase in rotational motion in XLAS1. (*a*) The labeled heads are predominantly in a physical state analogous to an actin-bound state that occurs during the ATPase cycle in uncrosslinked acto-S1 (and presumably, in a contracting muscle fiber), or (*b*) the heads are predominantly in a physical state analogous to detached S1, but are held ("tethered") in close proximity to actin by the crosslinks. Most of the biochemical data favors model *a*. The labeled XLAS1 has a Mg^{2+}-ATPase activity (Table I) comparable to the V_{max} measured for SH_1-blocked acto-HMM in solution (6), which suggests that the S1 in labeled XLAS1 spends most of its time interacting actively with actin, as previously argued for unlabeled XLAS1 (3, 4). A study of the initial P_i burst of XLAS1 concluded that 75% of the S1 is in the AM · ADP · P_i state during steady state ATPase activity at 170 mM ionic strength (9). This suggests that the predominant state present during steady state ATPase activity is the same for XLAS1 as for uncrosslinked acto-S1 at infinite actin concentration, a proposed "weakly attached state" (9). The partial ATPase inhibition caused by SH_1-blocking (Table I) could indicate a change in the distribution of states present during steady state ATPase activity. However, previous actin-activated ATPase studies on SH_1-blocked myosin and its subfragments indicated that, despite this inhibition, the same state should be predominant as in the absence of modification. Thus, during steady state ATPase activity, the predominant state in our labeled XLAS1 preparation is probably similar to the predominant attached state of the acto-S1. Therefore, the most likely interpretation of our data is that microsecond rotational motions occur within the predominant actin-attached cross-bridge state during the ATPase cycle.

The present results do not rigorously rule out model *b*, and further tests of this model are called for. For example, EPR measurements would be helpful if performed on uncrosslinked acto-S1 at very high actin concentrations, under conditions where most of the S1 is bound to actin even without crosslinking. However, the latter condition would only be satisfied at low ionic strength, a condition not presently accessible to EPR because of the need for high concentrations of ATP and CP to maintain the ATPase reaction during the EPR scan. If model *b* does turn out to be correct, and S1 spends a substantial portion of its time detached from actin during the ATPase cycle in XLAS1, these EPR results will require the reevaluation of much of the work on XLAS1, since they would suggest that the XLAS1 ATPase cycle has a detached phase similar to that in AS1. Since the ATPase activity actually increases with increasing ionic strength (which decreases the acto-S1 affinity) in XLAS1 (9), this would suggest that a certain minimum dissociation phase is optimal for the acto-S1 ATPase cycle.

Because the motion detected is that of the probe, the macromolecular interpretation of these motions depends on other data. Since previous studies indicate that the probe remains rigidly fixed to S1 (1), the simplest interpretation is that the probe reflects the overall motion of S1 relative to actin. Increased mobility of a domain within S1 could also cause the increase in rotational mobility in XLAS1 upon the addition of ATP. Although isolated S1 shows no increase in rotational motion when ATP is added (Fig. 1, *bottom row*), it is still possible that ATP induces some domain mobility when S1 is bound to actin. Although evidence has been presented against this possibility (1), it can only be rigorously ruled out by probe studies at a different site on S1. Such studies will also be needed to show that the detected myosin head mobility is not affected by SH_1 blocking. The increase in rotational mobility seen in XLAS1 when ATP is added could also be due to an increase in the flexibility of the actin filaments (10), independent of any motion of S1 relative to actin. To investigate this possibility, a probe must be attached to actin (10) in XLAS1.

The present finding of ATP-induced microsecond rotational motion of attached heads, as detected by MSL, has important implications for studies of intact myofibrils and fibers (1, 7, 11). In those studies, it was found that most of the MSL-heads in contracting myofibrils or fibers have dynamic disorder (on the microsecond time scale) similar to that of detached heads. Only a small fraction of heads was found to be less mobile, and that fraction was found to be oriented precisely as in rigor, at the end of the power stroke (7). Those results suggested that only a small fraction of heads are actively attached during contraction, and that those attached heads do not change their orientation. The present result indicates that, in addition to the rigorlike heads, it is possible that some of the mobile heads in contracting fibers are attached to actin, as previously

proposed (1, 12). That is, active myosin heads may rotate on actin after all, although it seems likely that they are predominantly in a weakly attached state that precedes force generation (12).

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