The Power Stroke Causes Changes in the Orientation and Mobility of the Termini of Essential Light Chain 1 of Myosin[†]

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ABSTRACT: Binding of ATP to the catalytic domain of myosin induces a local conformational change which is believed to cause a major rotation of an 8.5 nm α-helix that is stabilized by the regulatory and essential light chains. Here we attempt to follow this rotation by measuring the mobility and orientation of a fluorescent probe attached near the C- or N-terminus of essential light chain 1 (LC1). Cysteine 178 of wild-type LC1, or Cys engineered near the N-terminus of mutant LC1, was labeled with tetramethylrhodamine and exchanged into skeletal subfragment-1 (S1) or into striated muscle fibers. In the absence of ATP, the fluorescence anisotropy (r) and the rotational correlation time (ρ) of S1 reconstituted with LC1 labeled near the C-terminus were 0.195 and 66.6 ns, respectively. In the presence of ATP, r and ρ increased to 0.233 and 233 ns, indicating considerable immobilization of the probe. A related parameter indicating the degree of order of cross-bridges in muscle fibers, Δr, was small in rigor fibers (−0.009) and increased in relaxed fibers (0.030). For S1 reconstituted with LC1 labeled near the N-terminus, the steady-state anisotropy was 0.168 in rigor, and increased to 0.223 in relaxed state. In fibers, the difference in rigor was large ($\Delta r = 0.080$), because of binding to the thin filaments, and decreased to 0.037 in relaxed fibers. These results suggest that before the power stroke, in the presence of ATP or its products of hydrolysis, the termini of LC1 are immobilized and ordered, and after the stroke, they become more mobile and partially disordered. The results are consistent with crystallographic structures that show that the level of putative stabilizing interactions of LC1 with the heavy chain of S1 in the transition state is reduced as the regulatory domain rotates to its post-power stroke position.

The power stroke of skeletal muscle myosin is believed to involve the rotation of a long, α-helical regulatory domain (RD)[1] of myosin subfragment-1 (S1) relative to its catalytic domain (CD) (*1*, *2*). Regulatory (RLC) and essential (ELC) light chains stabilize the α-helix in RD, and are important in determining the motile properties of S1 (*3*, *4*). Because light chains reside some distance away from the catalytic domain (*1*) and are not essential for enzymatic activity (*5*, *6*), they offer a convenient location for spectroscopic probes. Until now, only RLC has been used to study orientation of RD in muscle fibers, because the conditions for its exchange into fibers are well-defined (*7*), and the exchange can be carried out under relatively mild conditions (30 min at 30 °C in 20 mM EDTA) compared to the conditions needed to exchange ELC.

In skeletal muscle, the steady-state orientation of RLC changed upon addition of ATP (*8*, *9*), upon rapid length changes (*9*−*11*), and upon sudden generation of ATP (*12*). These changes were consistent with the structural data (*13*), which suggested that the orientation of the RD of S1 carrying MgADP·AlF_4^- (an analogue thought to simulate a pre-power stroke conformation) differed from the orientation found for skeletal S1 in rigor fibers (thought to represent a post-power stroke conformation).

Here we report the effect of the power stroke on the mobility and orientation of essential light chain 1 (LC1) of skeletal S1. To define the changes in LC1, we measured the mobility and the degree of order of its N- and C-termini. We used wild-type LC1 containing Cys at position 178 near the C-terminus and an LC1 mutant (LC1m) in which Cys was engineered near the N-terminus. Cysteine residues were labeled with 5′-iodoacetotetramethylrhodamine (5′-IATR). They were exchanged with the endogenous ELCs of S1, and with the endogenous ELCs of myosin in skeletal muscle

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[1] Abbreviations: S1, subfragment-1 of skeletal myosin; RD, regulatory domain of S1; ELCs, essential light chains; LC1, essential light chain 1; LC3, essential light chain 3; LC1m, LC1 mutant with a single Cys in the N-terminal fusion peptide; 5′-IATR, 5′-iodoacetamidotetramethylrhodamine; TFP, trifluoperazine; HC, heavy chain of S1; A, actin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

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fibers. The mobility of S1 (r) was measured with a conventional fluorometer, and the degree of order in muscle fibers (Δr) was measured with a polarization microscope (*14*, *15*). The anisotropy differential Δr is defined as the difference between the anisotropies of fluorescence measured with the electrical vector of exciting light oriented perpendicular ($r_\perp$) or parallel ($r_{||}$) to the fiber. Provided that the mean angle of the dipole is not near 54.7°, Δr is a model-independent indicator of the degree of order. For a collection of perfectly disordered fluorophores, $\Delta r = 0$. For a collection of fluorophores oriented perfectly along the perpendicular and parallel axes, $\Delta r = 1$ and -1, respectively**.** The values of r measured in a fluorometer and Δr measured in a microscope are related but not equal. The value of r reflects the extent of depolarization of light caused by the rotation of the molecule during the excited-state lifetime of the fluorophore. For S1, it is not equal to 0, due to the relatively slow rotation of the protein compared to the excited-state lifetime. On the other hand, Δr of S1 measured in a microscope is always 0, due to the fact that $r_{||}$ is equal to $r_\perp$. When the dye is completely immobilized and random, r is equal to the intrinsic anisotropy, while $\Delta r = 0$ ($r_\perp = r_{||} = 0.4$).

In the absence of ATP, LC1 of S1 had a small anisotropy and rotational correlation time, indicating high mobility of the C-terminus. Both of these parameters increased significantly in the presence of ATP, suggesting considerable immobilization of the C-terminus. No changes were seen when S1 was labeled at Cys707, or when ADP or PP_i was used instead of ATP. Consistent with these results, relaxed fibers gave a larger Δr than rigor fibers. When the LC1 mutant was labeled at the N-terminus, the mobility also decreased upon addition of ATP. These findings for ELC are compatible with a change in orientation of the light chain domain during the power stroke.

MATERIALS AND METHODS

Chemicals. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), nucleotides, and antibodies against the heavy chain of S1 were from Sigma (St. Louis, MO). 5′-Iodoacetamidotetramethylrhodamine (5′-IATR), 5′-iodoacetamidofluorescein (5′-IAF), and Alexa dye (Alexa 488 maleimide) were from Molecular Probes (Eugene, OR).

Solutions. The rigor solution was 50 mM KCl, 4 mM $MgCl_2$, 10 mM Tris-HCl buffer (pH 7.5). The relaxing solution was 50 mM KCl, 4 mM $MgCl_2$, 2 mM EGTA, 4 mM ATP, 10 mM Tris-HCl buffer (pH 7.5). The EDTA-rigor solution was 50 mM KCl, 2 mM EDTA, 10 mM Tris-HCl buffer (pH 7.5). The EGTA-rigor solution was 50 mM KCl, 4 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris-HCl buffer (pH 7.5). The Ca^{2+}-rigor solution was 50 mM KCl, 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tris-HCl buffer (pH 7.5). The glycerinating solution was 80 mM potassium acetate, 2 mM $MgSO_4$, 2 mM ATP, 5 mM EGTA, 0.2 mg/mL PMSF, 2 mM β-mercaptoethanol, 10 mM Tris-acetate (pH 7.5), and 50% glycerol. The low concentrations of $MgCl_2$ and KCl were used to prevent the formation of actin filament bundles (*16*, *17*). Light scattering measurements did not detect any bundle formation in buffer solutions.

Preparation of Myosin, Myosin Fragments, and Actin. Skeletal myosin, S1 isoforms, and rabbit actin were prepared as described elsewhere (*18*−*20*). The concentrations of proteins were measured using the following values for the extinction coefficients ($A^{1\%}$ at 280 nm): 5.6 for myosin, 7.5 for S1, 6.3 for G-actin (measured at 290 nm), 2.3 for LC1, and 1.9 for LC3. The quality of myosin and isoforms of S1 was checked by measuring ATPase activities and the purity of proteins by SDS−PAGE.

Preparation of Light Chains. The pQE60 vector and *Escherichia coli* M15[pREP4] cells (QIAGEN) were used for the cloning and expression of LC1. The human fast skeletal muscle ELC was subcloned into the pQE60 vector using DNA polymerase chain reaction with the 3′-primer containing a tag of six histidines. The presence of the His tag at the C-terminus of ELC was confirmed by DNA sequencing. The expressed recombinant proteins were purified on the Ni−NTA−agarose columns (QIAGEN). For the experiments using S1, LC1 was prepared from rabbit skeletal myosin by the method described in ref *21*. RLC containing a single cysteine at position 73 (*9*) was prepared by expressing the pT7-7(RLC) construct in BL21(DE3)pLysS cells. One liter cultures were grown for 16−18 h at 37 °C in an enriched medium containing 2% bactotryptone, 1% yeast extract, 0.5% glycerol, 50 mM potassium phosphate (pH 7.2), 100 μg/mL ampicillin, and 20 μg/mL chloramphenicol. Cells were collected at 8000*g* for 10 min, and the RLC was isolated as described in ref *22*.

Preparation of the Cys Mutant and Isolation of LC1m. The cysteine mutant of LC1 was prepared from the full-length clone provided by J. Robbins (Children's Hospital, Cincinnati, OH) in which the endogenous cysteine at position 178 was changed to alanine (C178A). The pT7-7 expression vector was modified to encode cysteine in the fusion peptide and is termed the Cys vector. The C178A cDNA was cloned into the *Eco*RI site of this vector to produce LC1 with an N-terminal tag containing cysteine (A**C**GI) followed by the native LC sequence starting at Pro2 instead of Ala. The sequence was A**C**GI-PKKDVKKPAAAAAPAPAPAPAPA-PAPAKPKEPAIDLKSIKIEFSKEQQDD.

DNA was sequenced (USB Sequenase, version 2.0) to verify the changes made in the cDNA before expressing the protein. Protein expression was in BL21(DE3) as described for the regulatory light chain (*22*). LC1m was isolated as follows. Cells from 1 L of culture were lysed in 50 mL of lysis buffer [25 mM Tris (pH 8.0), 50 mM glucose, and 10 mM EDTA] containing 0.2 mg/mL lysozyme at room temperature (rt) for 30 min. $MgCl_2$ and DNase were added to final concentrations of 20 mM and 100 mg/mL, respectively, and the mixture was incubated for an additional 30 min. Solid guanidine-HCl (6−8 M) and DTT (5 mM) were added, and the mixture was stirred at room temperature for 1 h. This was followed by centrifugation at 45 000 rpm for 30 min in a Beckman Ti-70 rotor. The supernatant was dialyzed against 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, and 1 mM DTT to renature LC1m. The denatured bacterial proteins were removed by centrifugation as described above. The clarified supernatant was dialyzed against 10 mM sodium phosphate (pH 7.2), 1 mM EDTA, 1 mM DTT, and 3 mM NaN_3 and chromatographed on a Bio-Gel HPT column (1.5 cm × 20 cm) using a gradient of 10 to 250 mM sodium phosphate. The fractions were checked by SDS−PAGE and the LC1m-containing fractions pooled and dialyzed against 10 mM imidazole (pH 7.0), 20 mM NaCl,

1 mM EDTA, 1 mM DTT, and 3 mM NaN_3. The protein was clarified, applied to a DEAE-Sephacel column (1.5 cm × 20 cm), and eluted with a linear gradient of 20 to 250 mM NaCl. Finally, the fractions were pooled and stored at −20 °C.

Preparation of Muscle Fibers. Isolated muscle fibers were prepared from glycerinated rabbit psoas muscle bundles by dissecting single fibers in glycerinating solution and attaching the ends of the tautly stretched fiber to the aluminum clips mounted on the microscope slide. Mounted fibers were thoroughly washed with rigor solution and covered with a cover slip.

Labeling of Light Chains. The isolated ELC was labeled by incubation with a 5-fold molar excess of 5′-IATR for 4 h in 50 mM KCl, 2 mM EDTA, 10 mM phosphate buffer (pH 7.0) at 5 °C. The RLC was labeled with 5′-IATR and 5′-IAF at room temperature for 24 h. The free dye was removed by a Sephadex-50 column, and samples were dialyzed against the rigor solution.

Exchange of Light Chains into S1. Fluorescently labeled LC1 or LC3 was exchanged into S1(LC3) as described in ref *21*. S1 was purified on a DE-52 column to separate exchanged S1(LC1) from unexchanged S1(LC3). The concentration of labeled S1 was calculated from the extinction coefficient of rhodamine at 280 ($\epsilon^{1\%} = 18\,750$) and 555 nm ($\epsilon^{1\%} = 75\,000$) with the equation $c_{S1}(\text{mg/mL}) = (A_{280} - A_{555}/4)/0.75$ and the concentration of the dye with the equation $c_{IATR}(\text{mM}) = 10^3 A_{555}/75.0$. Typically, 30−40% of the S1 was labeled.

Exchange of Light Chains into Fibers. The labeled LC1 was exchanged with endogenous light chains of myosin in muscle fibers at 30 °C using the exchange solution described by Sweeney (*23*). We originally carried out the exchange at 37 °C, but control experiments showed that a higher temperature was detrimental to force development; we measured maximum isometric force using a sensitive tension transducer (*24*). Small bundles of fibers were mounted between the force transducer and the rigid arm, and force was measured as described in ref *25*. In three experiments, the incubation in relaxing solution at 37 °C for 20 min (without TFP) caused the tension to decrease on average by 55.5 ± 4.9%. Subsequent incubation with an exchange solution containing 100 μM TFP at 37 °C for 20 min caused no further decrease in the tension (average decrease of 2.5 ± 2.5%). Thus, it seems that TFP does not influence tension, but that the initial decrease is due solely to increasing the temperature to 37 °C. We therefore carried out exchange at a lower temperature (30 °C). Sweeney (*23*) suggested that exchange at low temperatures was feasible, and Malmqvist et al. (*26*) and Matthew et al. (*27*) used lower temperatures to exchange RLC and ELC into smooth muscle myosin. The degree of labeling was estimated by comparing the intensity of fluorescence of fibers that underwent exchange with the intensity of a known concentration of labeled light chains. The concentration of myosin in the fibers was taken to be 120 μM (*28*). Approximately 5% of the cross-bridges contained labeled LC1. We could tolerate this low level of exchange because fluorescence was detected by a highly efficient confocal polarization instrument (*15*).

Although TFP seems to be benign for smooth muscle myosin (*26*, *27*, *29*), its concentration was fixed at a low level, 100 μM, to reduce direct effects of TFP on ELCs (*30*). No CDTA was used. After being washed with EDTA-rigor solution, fibers did not contract in relaxing solution, suggesting that the exchange procedure resulted in only limited extraction of the regulatory proteins. For this reason, the fibers were not irrigated with troponin, troponin C, or RLC. In our hands, the exchange using 100 μM TFP at 30 °C was 2.5 times less efficient than that with 100 μM TFP at 37 °C. TFP (1 mM) at 30 °C was twice as efficient as 100 μM TFP at 37 °C. The RLC was exchanged into fibers as described in ref *31*.

Since the degree of labeling was low, the tension alone is not a sensitive measure of muscle function. We reasoned that temperature and TFP might affect the orientation of the RD without affecting the tension. To test this, we compared the orientation of RLC labeled at position 73 and incorporated into muscle fibers, with and without TFP. When the exchange of RLC was performed at 30 °C, $P_\perp$ was 0.315 ± 0.034 and $P_{||}$ was 0.275 ± 0.025. These values are similar to those reported previously [0.330 and 0.257, respectively (*9*)]. At 37 °C, the values increased to 0.409 ± 0.019 and 0.309 ± 0.016, respectively. Exchange at 37 °C in the presence of 0.1 mM TFP gave statistically the same values as the exchange at 37 °C alone ($P_\perp = 0.402 \pm 0.012$, $P_{||} = 0.298 \pm 0.036$). We conclude that incubation at higher temperatures, not TFP, is responsible for the change in polarization.

Specificity of Labeling. The specificity was estimated by examining the striation pattern of muscle fibers and myofibrils exchanged with ELC. The striation pattern in fibers was visualized by imaging thin fiber sections. Confocal microscopy was used with the confocal pinhole set at 0.87 μm in the object plane (a wide-field microscope showed no striation pattern). Figure 1A shows that only the A-bands of muscle fibers were fluorescent. We confirmed the specificity by examining the fluorescence of isolated myofibrils. Labeled fibers were stretched in relaxing solution by approximately 50%, and homogenized to make myofibrils as described previously (*14*). Fluorescent ELC stained exclusively the A-band (Figure 1B).

Tricine Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis and Western Blots. Gel electrophoresis was carried out according to the method of Schagger and von Jagow (*32*) using 8% polyacrylamide gels. After electrophoresis, the peptides were electroblotted onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were incubated for 1 h with a blocking solution, then with the primary monoclonal antibodies for 1 h, and finally with the horseradish peroxidase-conjugated secondary antibodies (ECL, Amersham) for 1 h. Luminescence was detected with X-ray film.

Anisotropy of S1. Steady-state anisotropy (r) was measured using an SLM 500C spectrofluorometer (Spectronic Instruments, Rochester, NY). 5′-IATR was excited at 530 nm, and r was measured at 580 nm, with the excitation and emission slits set at 5 and 10 nm, respectively. Excited-state lifetimes (τ) and rotational correlation times (ρ) were measured on an ISS-K2 digital multifrequency fluorometer (ISS Inc., Urbana, IL) using 510 nm excitation.

Anisotropy of Muscle Fibers. The anisotropy of rigor fibers was measured as described previously (*33*) using an argon ion laser operating at 514.5 nm (Spectra Physics, model 164) or a green He−Ne laser operating at 543 nm (Melles-Griot,

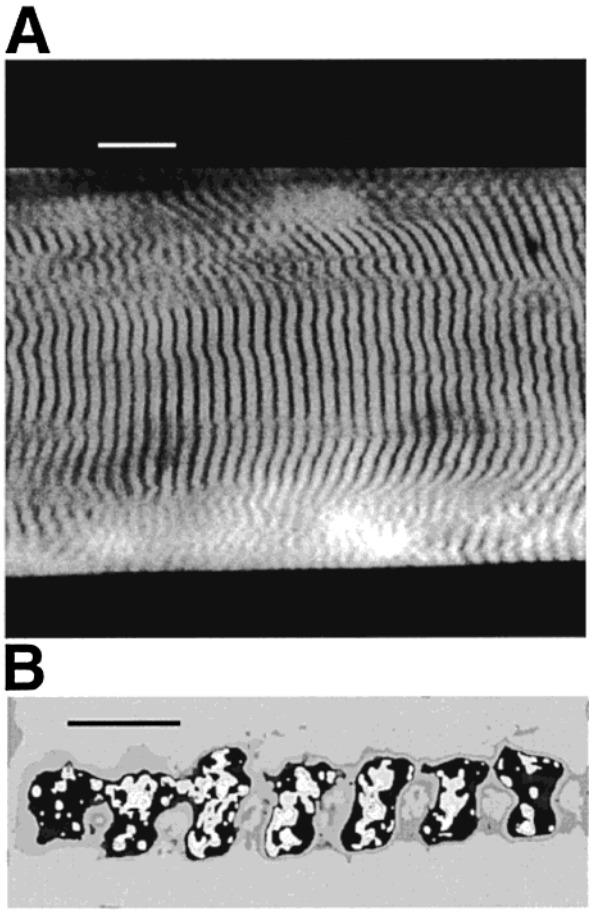

FIGURE 1: Fibers (A) and myofibrils (B) exchanged with ELC. (A) Fibers in relaxing solution (bar, 10 μm). (B) Stretched myofibrils in rigor (bar, 5 μm; sarcomere length, 3.4 μm). Phase (black) and fluorescent (white) images are superimposed. Images were obtained using a 40× (NA = 1.2) objective and a Zeiss LSM 410 confocal microscope.

model 05-LGP-193) as an excitation source. Before relaxing solution was applied, fibers were washed with the EDTA-rigor solution. Otherwise, fibers contracted due to the insensitivity of the regulatory system at low ATP concentrations (*34*). The objective was 10× (Toyoda, NA = 0.25). The convergence of the exciting beam was eliminated by using confocal illumination (*15*). A birefringent crystal split the emitted light into ordinary and extraordinary rays. The anisotropies are

$$r_{||} = ({}_{||}I_{||}/C_{||} - {}_{||}I_{\perp})/({}_{||}I_{||}/C_{||} + 2{}_{||}I_{\perp})$$

$$r_{\perp} = ({}_{\perp}I_{\perp}/C_{\perp} - {}_{\perp}I_{||})/({}_{\perp}I_{\perp}/C_{\perp} + 2{}_{\perp}I_{||})$$

where left and right subscripts indicate the direction of the polarization of the exciting and emitted light, respectively, with respect to the fiber axis and *C*'s are correction factors. The correction factors were as follows: $C_{||} = {}_{||}I^{R}{}_{||}/{}_{||}I^{R}{}_{\perp} = 0.70$ and $C_{\perp} = {}_{\perp}I^{R}{}_{\perp}/{}_{\perp}I^{R}{}_{||} = 1.54$, where I^{R} is the fluorescence of a 5 μM solution of 5-IATR in dimethylformamide. Anisotropy is related to polarization of fluorescence by the relation $P = 3r/(2 + r)$.

Segmental Mobility of ELC. By measuring the dependence of the anisotropy on the viscosity of the solvent (Perrin plot), we were able to estimate the "wobble" angle, α, through which the fluorophore rotates during the lifetime of the excited state (*35*). If it is assumed that rhodamine binds rigidly to the light chain, this angle depends only on the segmental motion of the peptide. If $\alpha = 0°$, there is no wobble; if $\alpha = \pi/2$, the dye wobbles freely. Half-angles ($\alpha/2$) of wild-type LC1 and of mutant LC1m were 14.5° and 22.4°, respectively, showing that the N-terminus was more mobile than the C-terminus.

Table 1: Steady-State Anisotropies of S1(LC1) and S1(LC1m)[a]

sample	*r*	change (%)
S1(LC1)	0.195 ± 0.001	–
S1(LC1) and A	0.216 ± 0.009	10.7
S1(LC1) and ATP	0.233 ± 0.002	19.5
S1(LC1), A, and ATP	0.233 ± 0.001	19.5
S1(LC1) and ADP	0.198 ± 0.001	0.0
S1(LC1m)	0.168 ± 0.002	–
S1(LC1m) and A	0.273 ± 0.002	62.5
S1(LC1m) and ATP	0.212 ± 0.001	26.2
S1(LC1m), A, and ATP	0.223 ± 0.001	32.7
S1(LC1m), ATP, and A	0.224 ± 0.001	33.3
S1(LC1m) and ADP	0.176 ± 0.001	4.0
S1(LC1m), ADP, and A	0.198 ± 0.003	17.8

[a] Actin (A) was added in a 4-fold molar excess over S1. ATP was added to the final concentration of 2 mM. LC1 and LC1m are labeled near the C- and N-termini, respectively.

RESULTS

C-Terminus. ATP had a similar effect on the mobility of the C-terminus of LC1 whether the light chain was incorporated into S1 or into muscle fibers. The steady-state anisotropies of S1(LC1) are listed in Table 1. The average *r* in the absence of ATP was 0.195 ± 0.001. This is larger than the average *r* of free LC1 (0.166). Addition of a 4-fold molar excess of F-actin caused only a small increase in *r*, although binding to F-actin causes significant immobilization of the HC (*36*). This suggests that the interaction of the HC with actin, and the direct interaction of the LC1 with actin through the N-terminus, only weakly inhibits the motion of the C-terminus. Addition of ATP dramatically increased *r*. No such increase was seen with ADP or PP_i (not shown). Addition of ATP to acto-S1 increased *r* beyond the anisotropy characteristic of rigor acto-S1 complex, to the extent characteristic of S1 and ATP. The anisotropy of free LC1 was unchanged in the presence of either actin or ATP (data not shown). These results suggest that in the absence of ATP, the C-terminus of LC1 has considerable freedom of motion, and that it becomes strongly immobilized when ATP binds to the active site of S1.

Time-resolved measurements are summarized in Table 2. Anisotropy decays were obtained from the frequency response of the polarized emission as previously described (*37*, *38*). The data were fitted to the biexponential decay $r(t) = r_o[g_1 \exp(-t/\rho_1) + g_2 \exp(-t/\rho_2)]$, where r_o is the limiting anisotropy in the absence of rotational motions, ρ is the rotational correlation time, and g_i's are the associated amplitudes. Our results showed that the two rotational correlation times were different for LC1 alone, S1(LC1), and for S1(LC1) in the presence of ATP. The rotational correlation time was the highest in the presence of ATP, indicating a significant decrease in the mobility of the label on Cys178 of LC1. Addition of ATP did not change τ_1 or τ_2, but increased ρ. This suggests that immobilization was not due to a change in the environment of the probe. The lifetimes

Table 2: Lifetimes (τ), Rotational Correlation Times (ρ), and Their Relative Contributions (α and g, respectively) of LC1, S1(LC1), and S1(LC1) and ATP[a]

sample	τ_1 (ns)	τ_2 (ns)	α_1 (%)	α_2 (%)	ρ_1 (ns)	ρ_2 (ns)	g_1 (%)	g_2 (%)
LC1	2.91	0.66	91	9	11.73	0.54	55	45
S1(LC1)	2.95	0.55	90	10	66.61	0.68	77	23
S1(LC1) and ATP	3.07	0.69	85	15	314.85	1.44	74	26

[a] Lifetimes were calculated from a fit to a biexponential intensity decay $I(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2)$. Rotational correlation times were calculated from a fit to a biexponential anisotropy decay $r(t) = r_o[g_1 \exp(-t/\rho_1) + g_2 \exp(-t/\rho_2)]$. A fit to a single-exponential decay was clearly worse than a fit to a biexponential decay; the values of χ^2 were similar to the ones reported previously (*38*). Actin was added in a 4-fold molar excess over S1; the ATP concentration was 2 mM. The values of χ^2 for ρ and τ ranged from 2 to 4.

Table 3: Anisotropies of Muscle Fibers Containing LC1 (labeled near the C-terminus) and LC1m (labeled near the N-terminus)

sample, state	$r_{\|\|}$	$r_\perp$	Δr ($=r_\perp - r_{\|\|}$)
LC1, rigor	0.183 ± 0.018	0.174 ± 0.024	-0.009
LC1, relax	0.143 ± 0.012	0.173 ± 0.014	0.030
LC1m, rigor	0.184 ± 0.002	0.264 ± 0.001	0.080
LC1m, relax	0.186 ± 0.002	0.223 ± 0.002	0.037

and correlation times of free LC1 were unchanged in the presence of either actin or ATP (not shown). This confirms the results of steady-state measurements suggesting that the motion of the C-terminus of LC1 is strongly inhibited in the presence of ATP. Cheung et al. (*38*) also reported two rotational correlation times (288.7 and 9.5 ns) for 1,5-IAEDANS attached to Cys707 of S1. Consistent with earlier work (*39*), ATP had a negligible effect on the mobility of the fluorescent dyes attached to Cys707 of S1 (data not shown).

In muscle, Δr was measured from five fibers exchanged with fluorescent LC1. Since in rigor $r_\perp \approx r_{||}$, a simple model cannot represent the distribution of labels.[2] The model-independent Δr values are summarized in Table 3. The fact that for rigor fibers Δr was small, and not statistically different from 0 (the value of t and level of significance of the t distribution were 0.736 and 0.595, respectively), suggests that the C-terminus of LC1 is disordered and therefore mobile. This mobility is consistent with the results obtained from S1 and actin in solution, and is present even though the N-terminus of LC1 and the catalytic domain of S1 are bound to actin. In relaxation fibers, Δr was statistically different from 0, indicating some order with preferred orientation perpendicular to the fiber axis ($r_{||} < r_\perp$).

N-Terminus. ATP had similar effects on the mobility of the N-terminus of LC1 when it was incorporated either in S1 or in muscle fibers. The average r of S1(LC1m) in the absence of ATP was 0.168 ± 0.002 (Table 1). Addition of excess F-actin caused a large increase in r due to the binding of the N-terminus to actin (*42*−*47*). This binding has been shown to take place through the N-terminal lysines (*23*, *47*) and the N-terminal α-amino group (*47*). However, addition of ATP to S1(LC1m) alone also caused a significant increase in r (from 0.168 to 0.212). Addition of ATP to acto-S1 decreased r to the extent characteristic of S1 and ATP. ADP and PP_i caused no changes. The average r of free LC1m was unchanged in the presence of either actin or ATP (data not shown). These results suggest that the N-terminus is immobilized by actin in the absence of ATP, and remains partially immobilized in the presence of ATP. The question arises whether this partial immobilization is due to the N-terminus binding to actin even in the presence of ATP. We show below that this is not the case.

In muscle, Δr was measured from 10 different fibers exchanged with fluorescent LC1m. The average Δr in rigor fibers was significantly different from 0 (Table 3). Since in muscle fibers actin is in molar excess over myosin [the ratio ranges from 3.2 (*48*) to 1.8 (*49*)], the order is due to the fact that the N-terminus of LC1 is bound to thin filaments. The anisotropy of relaxed fibers was also significantly different from 0, i.e., showed some order. Control experiments (see below) showed that this anisotropy was not due to the fact that ATP was unable to dissociate LC1 from F-actin. Instead, hydrolysis of ATP seems to be necessary for immobilization of the N-terminus. These results suggest that the motions of the N-terminus of LC1 are restricted in rigor fibers and remain restricted in a relaxed muscle.

Does LC1 Bind to Actin in the Presence of ATP? It is possible that adding ATP to rigor muscle dissociates only the HC, but S1 remains tethered to actin filaments through ionic interactions with LC1. The N-terminus would then be immobilized by actin and not by ATP. To determine whether the actin−LC1 bond is retained in the presence of ATP, we have carried out the following control experiments:

(i) It Is Impossible To Cross-Link LC1 to F-Actin in the Presence of ATP. The LC1−actin complex migrates at 66 kDa and is produced only when actin is in molar excess over S1 (*46*). To see whether this complex is produced in the presence of ATP, a 4-fold molar excess of actin was preactivated with EDC and mixed with S1(LC1) and S1(LC3). Coomassie Blue staining (lane 2, Figure 2A) showed 66 (A + LC1), 210 (A + A + HC), and 150/160 kDa (A + HC) bands characteristic of cross-linking in rigor fibers (*50*). No 66 kDa complex was obtained in the presence of ATP. Western blots using antibodies against HC (B) show that HC does not form a complex with actin.

(ii) S1(LC1) and S1(LC3) Dissociate from Thin Filaments with a Similar Half-Time. Fluorescent ELCs were incorporated into S1, which was then diffused into myofibrils in rigor solution. Rigor was gently replaced with relaxing solution and the fluorescence intensity of the myofibrils measured every 30 s. ATP-induced dissociation of S1(LC1) and S1(LC3) was similar (Figure 3). This supports the view that ATP is able to break the actin−LC1 bond.

(iii) Electron Microscopy Shows That ATP Fully Dissociates Acto-S1(LC1) and Acto-S1(LC3). S1(LC1) and S1(LC3) were used to decorate the phalloidin−actin complex. A 4-fold excess of actin over S1 resulted in sparse decoration, but negatively stained specimens clearly showed that ATP caused complete dissociation of both isoforms (data not shown).

DISCUSSION

Our anisotropy measurements suggest that the power stroke may be associated with changes in the mobility and

[2] Consider a simple Gaussian model (*40*, *41*). Θ is the polar angle that a long axis of S1 makes with the vertical. Dipoles are distributed with a Gaussian probability around the mean value Θ_o with a standard deviation δ; i.e., the probability of dipole orientation at any angle Θ is given by $r(\Theta) = \exp[-(\Theta - \Theta_o)^2/2\delta^2]$. The angles Θ and δ cannot be estimated for LC1 in rigor by the method described in ref *33*.

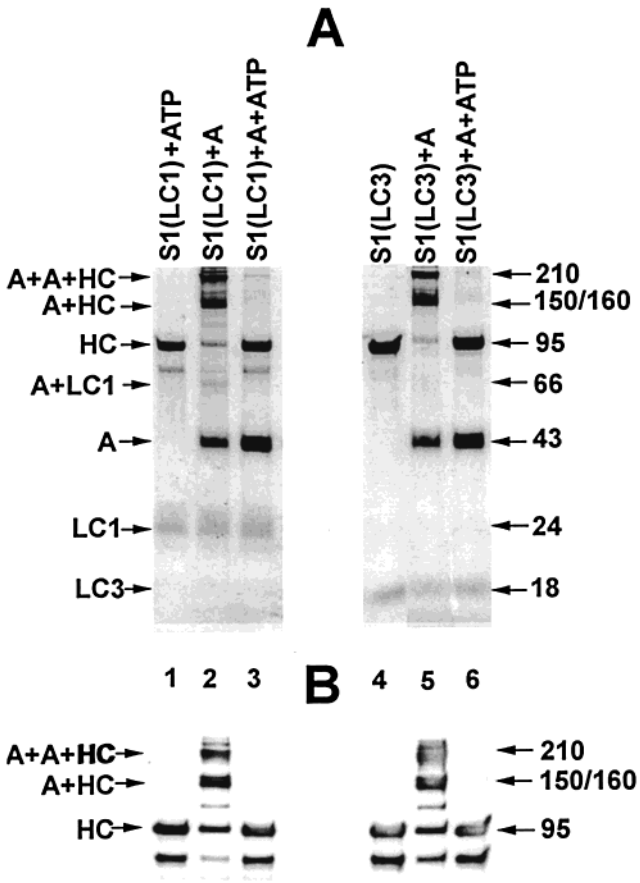

FIGURE 2: Effect of ATP on cross-linking of isoforms of S1 with actin. Actin was preactivated with 20 mM NHS and 10 mM EDC at 4 °C. The reaction was stopped with 0.1 M β-mercaptoethanol after 20 min. Aliquots of preactivated actin were added to S1 or to S1 containing 5 mM MgATP and the mixtures allowed to equilibrate for 30 min at 4 °C. The disappearance of ATP was monitored by a change in the turbidity of the acto-S1 complex (*54*). (A) Gels stained with Coomassie Blue showing that ATP dissociates LC1 from actin: lane 1, 1 μM S1(LC1) and 5 mM ATP; lane 2, 1 μM S1(LC1) and 4 μM actin; lane 3, 1 μM S1(LC1), 4 μM actin, and 5 mM ATP; lane 4, 1 μM S1(LC3) and 5 mM ATP; lane 5, 1 μM S1(LC3) and 4 μM actin; and lane 6, 1 μM S1(LC3), 4 μM actin, and 5 mM ATP. (B) Western blots stained with antibodies against the myosin heavy chain. This has been done to eliminate the possibility that the light chain composition affects dissociation of the heavy chain. Lane assignments are as for panel A.

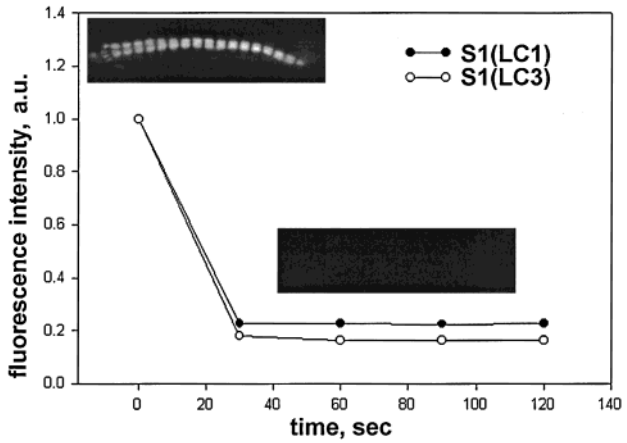

FIGURE 3: Dissociation of S1(LC1) (●) and S1(LC3) (○) from myofibrils. To avoid photobleaching, ELCs were labeled with the maleimide derivative of Alexa dye by incubation with a 5-fold molar excess of the dye at 0 °C for 3 h, followed by dialysis and passage through a Sephadex G-50 column. Exchange with native ELCs of S1 was as described in Materials and Methods. Labeling of Cys707 of the HC was not feasible, because maleimide impairs sensitivity to dissociation by ATP (*55*). Myofibrils (0.5 mg/mL) were incubated with 1 μM S1 for $^1/_2$ h at room temperature and centrifuged for 3 min at 3000 rpm in a desktop centrifuge at 4 °C to remove free S1. Myofibrils in rigor solution were placed on the stage of a fluorescent microscope (Zeiss Photomicroscope III), illuminated with a 488 nm beam from an Ar ion laser, and after the rigor solution was rapidly replaced with the relaxing solution, observations were made every 30 s using fluorescein filters. The rate of replacement of solutions was not controlled. Insets are fluorescent images of a myofibril before (top left) and after (bottom right) the rigor solution was replaced with the relaxing solution. The magnification is 412.5×.

the degree of order of the termini of LC1. Before the power stroke (S1 with ATP, relaxation in fibers), the C-terminus of LC1 is more immobilized and ordered than after the power stroke (S1 without ATP, rigor in fibers). The N-terminus is also immobilized and partially ordered before the power stroke, but remains in this state after the stroke, presumably due to binding to actin.

These results support the idea that binding of ATP to the active site on the catalytic domain has a significant effect on the mobility of the essential light chain. This occurs even though the active site and the regulatory domain are separated by a large distance (*1*) (the distance between the α-carbons of Cys178 on LC1 and Trp131 in the active site is 36.9 Å). In a related observation, made more than 20 years ago, it was found that S1 containing a fluorescein-labeled LC1 exhibited a 6−10% decrease in fluorescent intensity upon addition of ATP (*51*). Further examination of this effect by pre-steady-state kinetics showed that the decrease could be accounted for mainly by the ATP hydrolysis step in the kinetic cycle; the rate of fluorescence change of the fluorophore attached to Cys178 was equal to the rate of change of the intrinsic tryptophan fluorescence in the heavy chain (*52*). It was known from fluorescence resonance energy transfer studies that this information had to be transmitted by some mechanism over a large distance (*51*), but no atomic structures were yet available to provide structural insights.

A comparison of the structure of skeletal muscle S1 (*1*) with the atomic structure of the smooth muscle catalytic domain complexed with ELC (*13*) supported the hypothesis that a swing of the lever arm is responsible for force generation during the contractile cycle. In the pre-power stroke conformation, presumably represented by the smooth muscle subfragment with a transition-state analogue at the active site, the C-terminal domain of ELC is located near the variable 25/50 kDa loop of the catalytic domain (*13*), whereas in the post-power stroke conformation of nucleotide-free skeletal S1, it is found near the N-terminal domain of the heavy chain (*1*) (see Figure 4). The simplest explanation for an increased level of immobilization of ELC during ATP hydrolysis is that the C-terminal lobe containing Cys178 near several Glu residues is stabilized by electrostatic interactions with Lys residues in the 25/50 kDa loop (*13*). The high thermal factor, 76 Å^2, for the region including Cys178 compared with the average thermal factor, 38 Å^2, for all atoms of the skeletal S1 heavy chain, suggests a highly flexible chain for ELC in the absence of nucleotide (*1*).

Although crystallography can provide a rationalization for the changes in mobility for the C-terminus of ELC, the

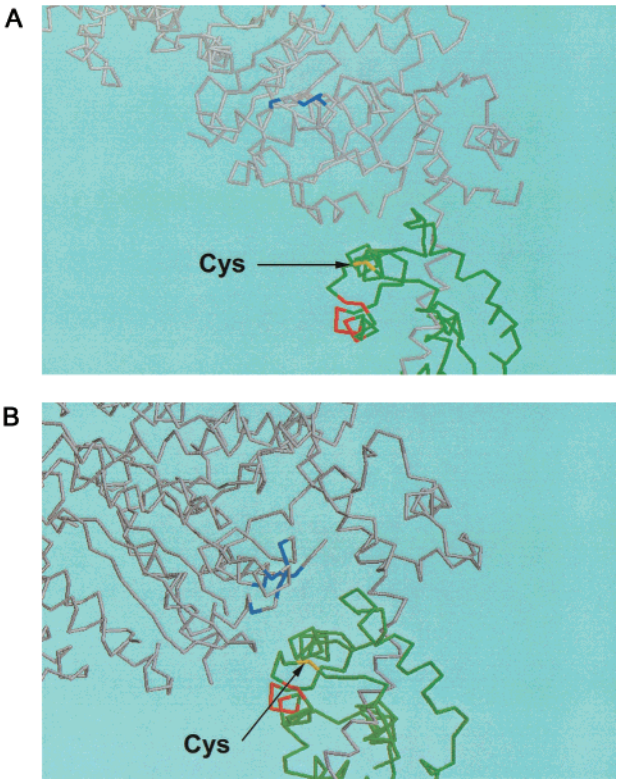

FIGURE 4: Proposed interactions between the C-terminus of LC1 and the heavy chain: white, HC; and green, ELC. (A) Structure of chicken skeletal S1 in rigor (PDB entry 2MYS). Acidic residues on ELC [numbers refer to skeletal muscle LC1 (E162, E163, E164, E167, E173, and D174)] are in red. Cys178 is in yellow. Basic residues in the vicinity of the 25/50 kDa junction (R144, K146, K147, and R148) are in blue (lysine residues in the 25/50 loop are disordered and therefore not visible). (B) Structure of chicken gizzard S1 in the presence of ADP·BeF_x (PDB entry 1BR4). Acidic residues on ELC [numbers refer to smooth muscle LC17 (E120, E121, E122, E124, and E125)] are in red. The homologous Cys is in yellow. Basic residues in the vicinity of the 25/50 kDa junction (K142, K144, K145, and R146) are in blue (lysine residues in the 25/50 loop are disordered).

N-terminal extension of ELC has not been visualized in any crystal structure. The biochemical and biophysical evidence provides strong support for the belief that the N-terminus of LC1 interacts with actin in the actomyosin complex, despite a distance of more than 60 Å from the regulatory domain to the nearest actin (reviewed in ref *47*). In the anisotropy results reported here, we observed the largest increase in anisotropy upon addition of actin, consistent with the literature. However, when ATP was added to the complex, with the demonstrated complete dissociation of myosin from actin, considerable immobilization remained in the N-terminus of LC1. One is led to the tentative conclusion that the Pro- and Lys-rich extension may be stabilized by weak interactions with regions of the catalytic domain, perhaps including the N-terminal domain of the heavy chain which has a topology similar to that of the Src-homology 3 (SH3) domain (*1*). Although there is no direct evidence for such interactions, there are physiological data showing that the well-known effect of LC1 and LC3 isoforms on shortening velocity depends markedly on the myosin heavy chain composition of the fiber type (*53*). Thus, a variation in LC1 content has a much greater impact on the velocity of IIB fibers than IIA or IIX fibers. In the absence of other evidence, the results presented here may provide the first indication that stabilizing interactions between the N-terminus of LC1 and the catalytic domain of myosin do in fact occur.

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