Orientation of Cross-Bridges in Skeletal Muscle Measured with a Hydrophobic Probe

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ABSTRACT *Cis*-parinaric acid (PA) binds to a hydrophobic pocket formed between the heavy chain of myosin subfragment-1 (S1) and the 41-residue N-terminal of essential light chain 1 (A1). The binding is strong ($K_a = 5.6 \times 10^7\ M^{-1}$) and rigid (polarization = 0.334). PA does not bind to myofibrils in which A1 has been extracted or replaced with alkali light chain 2 (A2). As in the case of S1 labeled with other probes, polarization of fluorescence of S1-PA added to myofibrils depended on fractional saturation of actin filament with S1, i.e., on whether the filaments were fully or partially saturated with myosin heads. Because fluorescence quantum yield of PA is enhanced manyfold upon binding, and because PA binds weakly to myofibrillar structures other then A1, the dye is a convenient probe of cross-bridge orientation in native muscle fibers. The polarization of a fiber irrigated with PA was equal to the polarization of S1-PA added to fibers at nonsaturating concentration. Cross-linking of S1 added to fibers at nonsaturating concentration showed that each S1 bound to two actin monomers of a thin filament. These results suggest that in rigor rabbit psoas muscle fiber each myosin cross-bridge binds to two actins.

INTRODUCTION

We have recently suggested that muscle contraction occurred as a result of transition of myosin head from the state in which it binds through the primary site (residues 626–647) to one actin, to a state in which it binds through both the primary and the secondary sites (presumably residues 567–578) to two adjacent actins (Andreev and Borejdo, 1992). This suggestion was based on an observation that in solution, myosin subfragment 1 (S1) could form a bond with either one or two actin protomers of F-actin and that the conformation of S1 was different when it formed either bond (Andreev et al., 1993a; Xiao et al., 1995). We have shown that the type of bond depended on the fractional saturation of F-actin (Andreev et al., 1993b).

Previous work has been carried out using S1 labeled at the heavy chain (HC Cys-707; Andreev et al., 1993a) or the light chain (ELC Cys-177; Xiao et al., 1996). Cys-707 of HC resides in the functionally important region (Rayment et al., 1993), and labeling may interfere with rigor binding. The exchange of essential light chains (A1 + A2) (ELC) requires the use of harsh conditions, which may disrupt normal packing of the heads on the surface of thick filaments (Ling et al., 1996) and make measurements on native fibers impossible. To sidestep these difficulties we used parinaric acid (PA) labeling. PA attaches specifically to alkali light chain 1 (A1) (see below), i.e., to a part of a molecule that is not involved in ATPase. It has a low fluorescence quantum yield when it is free (Sklar et al., 1975), which makes it possible to use it in native fibers. We took advantage of these properties to compare the orientation of S1 bound to thin filaments through the one and two sites with the orientation of cross-bridges in muscle. In agreement with earlier results, we found that the orientation of cross-bridges was the same as the orientation of S1 bound through two sites. These results suggest that in rigor rabbit psoas muscle fiber, each myosin cross-bridge binds to two actins.

Received for publication 11 November 1996 and in final form 31 January 1997.

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0006-3495/97/05/2268/07 $2.00

MATERIALS AND METHODS

Chemicals

Cis-parinaric acid (PA) was from Molecular Probes (Eugene, OR). It was freshly prepared before each experiment by dissolving it in ethanol at a concentration of 3 mM. All solutions containing PA contained an equimolar concentration of butylated hydroxytoluene and were purged with nitrogen. The concentration of PA was determined from absorbance at 318 nm ($= 74{,}000\ M^{-1}\ cm^{-1}$). 1-Ethyl-3-[3-(dimethyl-amino)-propyl]-carbodiimide (EDC), tricine, and all of the solvents were purchased from Sigma (St. Louis, MO). Wide-range molecular weight markers (catalog no. M 4038 were from Pharmacia Piscataway, NJ),

Solutions

Fibers were glycerinated in a relaxing solution containing 50 mM K-acetate, 2 mM $MgSO_4$, 2 mM ATP, 5 mM EGTA, 0.2 mg/ml phenylmethylsulfonyl fluoride, 2 mM β-mercaptoethanol, 10 mM Tris-acetate (pH 7.5), and 50% glycerol. Rigor solution contained 50 mM K-acetate, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM Tris-acetate (pH 7.5), and 10 mM dithiothreitol (DTT); the relaxing solution contained 50 mM K-acetate, 2 mM $MgCl_2$, 2 mM ATP, 2 mM EGTA, and 10 mM Tris-acetate (pH 7.5). EDTA-rigor solution contained 80 mM K-acetate, 5 mM EDTA, and 10 mM Tris-acetate (pH 7.5). Hasselbach-Schneider solution (0.47 M KCl, 0.1 M PO_4 buffer (pH 6.4), 10 mM PO_4-pyrophospate, 5 mM $MgCl_2$) was used to remove myosin from myofibrils.

Proteins

Myosin was prepared from back muscles of rabbit by the method of Tonomura et al. (1966). S1 was obtained by a chymotryptic digestion of

myosin according to the method of Weeds and Taylor (1975). S1 was separated into alkali light chain 1 (A1) and alkali light chain 2 (A2) isoforms on a diethylaminoethyl column as described by Weeds and Taylor (1975). The concentrations of proteins were measured using the following values of the extinction coefficients: S1 – $A^{1\%}(280) = 7.5$ (using molecular mass of 120 kDa for S1(A1) and 111 kDa for S1(A2)), G-actin – $A^{1\%}(290) = 6.3$, F-actin – $A^{1\%}(290) = 6.7$. The quality of proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of labeled S1

S1 in rigor solution was labeled with an equimolar concentration of PA for 5 min. Free dye was removed with a Sephadex-50 column. The concentration of S1 and labeled S1 was calculated from the absorption at 280 and 318 nm.

Preparation and labeling of muscle fibers

Two to four fibers were dissected from psoas muscle stored at −18°C in glycerinating solution and tied to a plastic stick. Relaxed fibers were washed for half an hour with EDTA-rigor solution, then with a rigor solution at 0°C, and incubated at room temperature with the rigor solution containing 20 μM PA and 20 μM butylated hydroxytoluene for 5 min. Labeled fibers were placed on a quartz microscope slide and washed with the rigor solution. Fibers were freshly labeled before each experiment. Cys-707 was labeled with tetramethylrhodamine-5-iodoacetamide (5′-IATR), as described previously (Xiao et al., 1995).

Preparation and labeling of myofibrils

The bundle of fibers was transferred from a relaxing glycerinating solution to EDTA-rigor, incubated for half an hour in rigor solution, and homogenized on ice in an Omni Mixer (Waterbury, MA) with two bursts of 15 s at a setting of 8. Myofibrils were filtered through two layers of gauze and used immediately after homogenization to prevent proteolysis. The concentration was measured by dissolving myofibrils in 2% SDS and using $A^{1\%}(280) = 7.0$. Labeling was done by incubating 2 mg/ml myofibrils in 0.1 mM PA for 5 min and washing them two or three times by precipitation/resuspension. The extent of labeling was measured by comparing the fluorescence intensity of the native myofibrils with the intensity of myofibrils from which the indigenous myosin had been removed by Hasselbach-Schneider solution (see Results).

Extracting ELCs and exchanging A1

A modified procedure of Sweeney (1995) was used to extract or exchange ELCs: myofibrils were washed with EDTA-rigor and incubated for 40 min at 40°C with extracting (150 mM KCl, 5 mM ATP, 10 mM EDTA, 5 mM DTT, 10 mM imidazole, pH 6.5) or exchanging (150 mM KCl, 5 mM ATP, 10 mM EDTA, 5 mM DTT, 10 mM imidazole, pH 6.5, 200 μM A2) solution. After incubation the temperature was lowered to 4°C, myofibrils were washed with rigor solution, and PAGE was carried out.

Spectroscopic measurements

Intensity and polarization of fluorescence of PA and S1(A1·PA) were measured at 20°C in a SLM 500C spectrofluorometer. The excitation and emission wavelengths were 325 and 410 nm, with the corresponding slits set at 2.5 and 10 nm.

Cross-linking and SDS-PAGE

Myofibrils (4 mg/ml) in rigor solution were irrigated with 0.5 μM S1 and incubated with 50 mM EDC for 30 min at room temperature. The reaction was stopped by adding an equal volume of electrophoresis sample solution (4% SDS, 24% glycerol, 100 mM Tris, 4% β-mercaptoethanol, 0.02% bromphenol blue). Gel electrophoresis was carried out according to the method of Schagger and Jagow (1987). After electrophoresis and staining, the slab was dried using a Novex Gel Dryer Kit (Novex Co., San Diego, CA). The intensity of the bands was measured from scanned images by the Image Pro Plus (Media Cybernetics, Silver Spring, MD) image analysis program.

Measuring polarization of fluorescence

The light source was a He-Cd ion laser (Liconix Model 4240B/UV, Sunnyvale, CA) operating at 325 nm. A polarized light beam from the laser was passed through the Pockels Cell (model 3079FW; Lasermetrics, Englewood, NJ), which was used to change the direction of the exciting light. The Zeiss UV dichroic mirror (model FT395) reflected the light through a Zeiss UD 40× dry long working distance objective (NA = 0.65) into a fiber. The same objective collected the fluorescence. The emitted light was orthogonally polarized by a Wollaston prism, which was mounted before the pair of photomultiplier tubes (model 8850; RCA Co., Cherry Hill, NJ). The photomultiplier pulses were amplified (amplifier model 2A50; Pacific Instruments, Concord, CA), discriminated (discriminator model 620 CL; LeCroy Research, Spring Valley, NY), inverted with a home-built inverter, and counted with a digital counter integrated with a personal computer (counter model CTM-05; MetraByte, Taunton, MA). The same counter provided a square wave signal to drive a Pockels Cell. The measurements were made by applying an amplified pulse generated by the counter (amplified by a wideband amplifier, model GA 21; Lasermetrics) to the Pockels Cell to define the excitation polarization. Photon pulses were collected by photomultipliers during the time that the signal to the Pockels Cell was constant, and were counted with a CTM counter.

If the incident intensities with perpendicular and parallel polarization are ${}_{\perp}I$ and ${}_{\parallel}I$, and the emitted intensities are $I_{\perp}$ and $I_{\parallel}$, then the different polarization quantities are

$$P_{\parallel} = [({}_{\parallel}I_{\parallel})/C_{\parallel} - ({}_{\parallel}I_{\perp})]/[({}_{\parallel}I_{\parallel})/C_{\parallel} + ({}_{\parallel}I_{\perp})] \quad (1)$$

$$P_{\perp} = [({}_{\perp}I_{\perp})/C_{\perp} - ({}_{\perp}I_{\parallel})]/[({}_{\perp}I_{\perp})/C_{\perp} + ({}_{\perp}I_{\parallel})] \quad (2)$$

$$Q_{\parallel} = [({}_{\parallel}I_{\parallel}) - ({}_{\perp}I_{\parallel})]/[({}_{\parallel}I_{\parallel}) + ({}_{\perp}I_{\parallel})], \quad (3)$$

where $C_{\perp} = {}_{\perp}I_{\perp}/{}_{\perp}I_{\parallel}$ and $C_{\parallel} = {}_{\parallel}I_{\parallel}/{}_{\parallel}I_{\perp}$ are the correction factors accounting for differential transmittance of perpendicular and parallel components of emitted light by the dichroic mirror. $Q_{\parallel}$ does not require correction because the intensity of the light impinging on the sample does not depend on the orientation of the polarizer. For PA (Zeiss dichroic mirror FT 395) $C_{\parallel}$ and $C_{\perp}$ were 1.48 and 1.08.

Despite the fact that polarization of fluorescence was independent of the instance of measurement (see Results), polarization was measured only once from the same spot on a fiber. Each spot was exposed for no more than 15 s. The measurements were taken from four spots.

RESULTS

Binding of PA to S1

The quantum yield of PA increases approximately 13-fold when the probe binds to unfractionated S1 (Borejdo, 1983). This enhancement of fluorescence was used to measure the affinity and stoichiometry of binding of PA to the isoforms of S1. Fig. 1 *A* shows the titration of PA with S1 and its isoforms. The concentration of PA was fixed at 2.6 μM. It

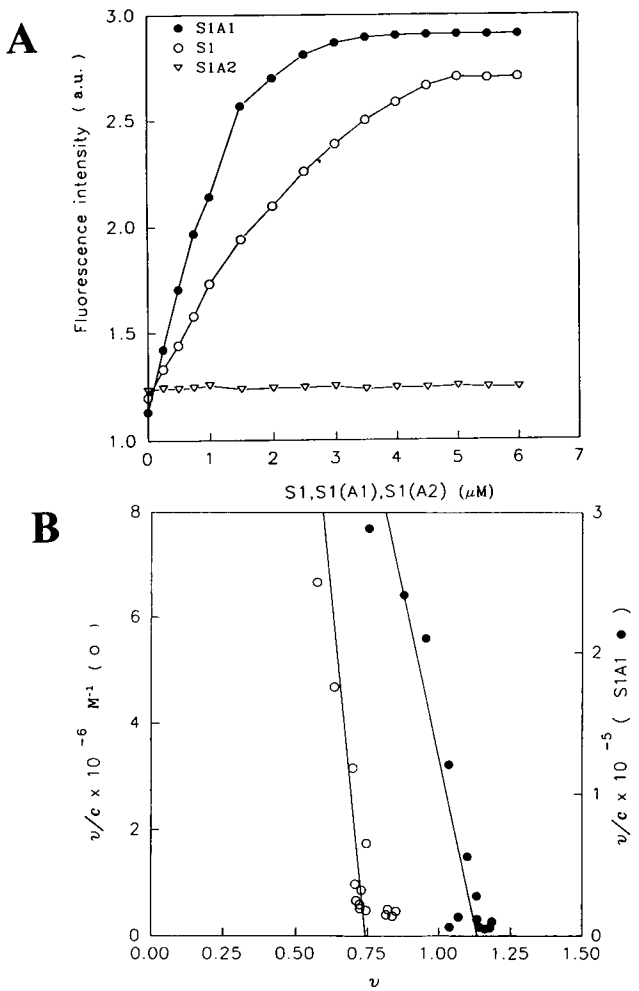

FIGURE 1 (*A*) Titration of PA with isoforms of S1 to show that PA binds specifically to the essential light chain 1. The concentration of PA fixed at 2.6 μM. λ_{ex} = 325 nm, λ_{em} = 395 nm. (*B*) Scatchard plots of binding of S1 (○) and of S1(A1) (●) to PA. ν is the fraction of S1 isoforms bound.

can be seen that the fluorescence of PA bound to S1(A1) saturates at around 2.6 μM (*filled circles*), whereas the fluorescence from PA bound to unfractionated S1 saturates only above 5 μM (*open circles*). PA does not bind to S1(A2) at all (*triangles*). The polarization of fluorescence of S1(A1) was independent of the molar ratio PA/S1(A1) (data not shown). The data of Fig. 1 *A* are plotted in Scatchard form in Fig. 1 *B*. The affinity of binding of PA to S1(A1) is $5.6 \times 10^7\ M^{-1}$, and the stoichiometry is 1.1.

The polarization of fluorescence of solution of S1(A1)-PA was 0.334 (Table 1), showing that PA was better immobilized on the surface of A1 than 5′-IATR on the surface of the regulatory light chain of chicken gizzard exchanged into skeletal S1 (Ling et al., 1996). The absolute value of polarization increased when F-actin was added (Table 1), reflecting the fact that F-actin was immobile on the scale of the fluorescence lifetime of PA. Under the conditions of the experiment (excess of actin over S1), A1 on S1 is interacting with actin filament (Prince et al., 1981; Andreev and Borejdo, 1995). The addition of ADP to acto-S1 decreased absolute polarization only slightly, suggesting that, in contrast to probes bound to SH_1 (Borejdo et al., 1982; Tanner et al., 1992), absorption/emission dipoles did not undergo a gross change in orientation. PA bound weakly to isolated A1 and to F-actin (data not shown). We conclude, in agreement with Borejdo (1983), that PA binds to a hydrophobic pocket formed between the heavy chain of S1 and the 41-residue N-terminal "difference piece" of A1.

TABLE 1 Polarization of fluorescence of solutions of S1 (A1) labeled with PA

Sample	*P*	Intensity (%)
S1 (A1 · PA)*	0.334	100
S1 (A1 · PA) + F-actin#	0.375	92
S1 (A1 · PA) + F-actin + ADP§	0.338	96

*1 μM S1 (A1) + 1 μM PA.
#1 μM S1 (A1 · PA), 4 μM actin.
§1 μM S1 (A1 · PA), 4 μM actin, 2 mM ADP, 1 mg/ml hexokinase, 100 mM glucose.

Binding of PA to myofibrils

The following experiment (Fig. 2) was carried out to determine whether in myofibrils, as in isolated S1, PA bound to A1. A1 of myofibrils (*lanes 2* and *3*) was extracted or exchanged with A2, as described in Materials and Methods. Extraction removed ELCs only partially, resulting in the myofibrils shown in lane 5. Exchange removed more A1 and regulatory light chain, resulting in the profile shown in lanes 7 and 8. Comparison of extraction (*lanes 5* and *3*) and exchange (*lanes 8* and *3*) showed that the intensity of the bands corresponding to myosin heavy chain decreased 1.44 and 1.45 times. The intensity of the A1 band decreased by a factor of 3.2 during the extraction and 5.7 during the exchange. At the same time, extraction and exchange resulted in 3.3-fold and 6.3-fold decreases, respectively, in the fluorescence intensity of PA (Fig. 2, *bottom, ratio of slopes*). Thus there was a good correlation between the removal of A1 and a decrease in fluorescence intensity, suggesting that A1 is necessary for PA binding to myofibrils.

Polarization of fibers irrigated with S1-PA

The excitation was at 325 nm. No exciter filter was used. The dichroic mirror was FT 395; the emission filters were an LP 397 and a bandpass filter with a maximum at 420 nm and a half-width at half-height of 110 nm. A single (unlabeled) fiber was placed horizontally on a stage of a microscope (i.e., its long axis was at 45° to the plane of the dichroic mirror). When excited with parallel polarized light, PA bleached rapidly ($\tau_{1/2} \approx 1.5$ s), but the rate of photobleaching was the same for both emission components. The same rate of photobleaching means that the polarization of fluorescence is independent of the instance of the measure-

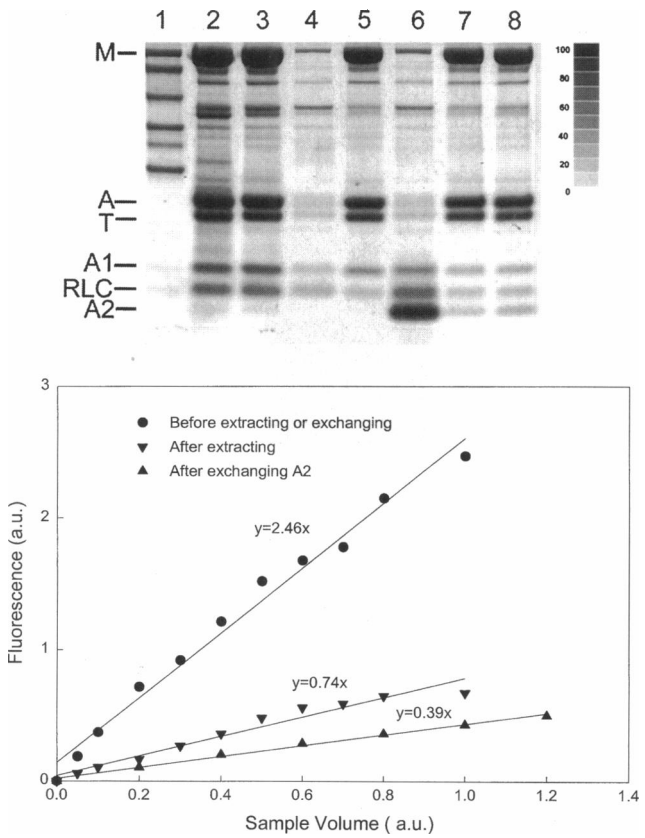

FIGURE 2 Extraction and exchange of A1 of myofibrillar myosin, showing that A1 is neccesary for PA to bind to myofibrils. (*Top*) SDS-PAGE of myofibrils in which ELC's were extracted or exchanged. *Lane 1:* Molecular mass markers (in order of decreasing molecular mass): 212, 170, 116, 76, and 53 kDa; *lane 2:* control myofibrils (3 mg/ml); *lane 3:* myofibrils after washing with EDTA-rigor; *lane 4:* supernatant after extracting ELCs and spinning myofibrils for 2 min in a desktop centrifuge; *lane 5:* myofibrils after extracting ELCs; *lane 6:* supernatant after exchanging A1 with A2 and spinning myofibrils for 2 min in a desktop centrifuge; *lane 7:* myofibrils after exchanging A1 with A2; *lane 8:* myofibrils after exchanging A1 with A2 and washing with rigor solution. Tricine buffer. All lanes: 30 μl/sample. (*Bottom*) Fluorescence of myofibrils before (●) and after (▼) extraction and after exchange (▲).

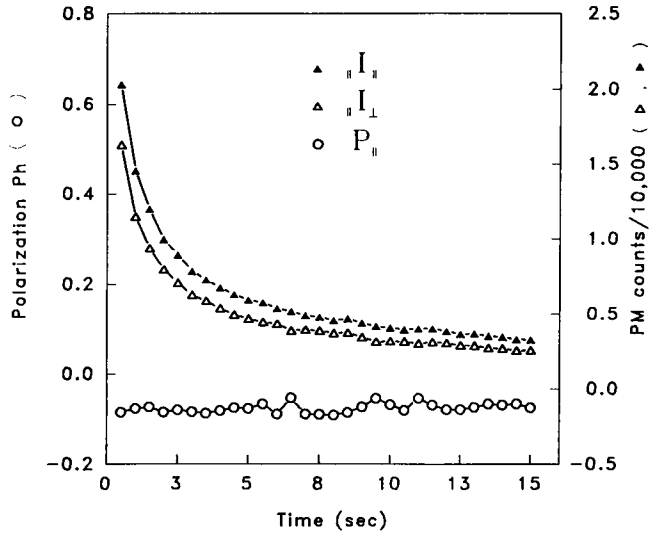

FIGURE 3 The time course of polarized intensities (△, ▲) and of polarization of fluorescence (○) showing that in spite of photobleaching the polarization of fluorescence is approximately constant during the first 15 s after the beginning of illumination. Muscle fiber illuminated with parallel polarized light. △, $_{\parallel}I_{\perp}$; ▲, $_{\parallel}I_{\parallel}$; ○, $P_{\parallel}$.

ment. To measure polarization, we first determined the time course of photobleaching (Fig. 3). Polarizations were taken as averages of polarizations obtained between 2 and 5 s after the beginning of illumination. The rate of photobleaching was the same when the fiber was illuminated with perpendicularly polarized light (data not shown).

The *I*-bands were saturated with S1 by incubating a fiber with a high (2 μM) concentration of labeled S1. Incubation was carried out in the dark for 1 hr at room temperature. Excess S1 was removed by washing with the rigor solution, and measurements were begun within 5 min of washing (to minimize the increase in background due to gradual dissociation of S1). The results of 14 experiments on four different fibers are plotted in histogram form in Fig. 4 *A* and are summarized in Table 2. To measure polarizations from fiber in which the *I*-bands were unsaturated with S1, a fiber was incubated with the low (0.1 μM) concentration of S1. Incubation was carried out overnight at 4°C in the dark. Excess S1 was removed as before. The results of 14 experiments obtained from four different fibers are plotted in Figs. 4 *B* and 6 *B* and are summarized in Table 2. The statistical analysis of the differences of the means showed that the difference was highly significant statistically ("highly significant" means $p < 10^{-6}$). The probabilities that $P_{\parallel}$ and $P_{\perp}$ of saturated and unsaturated fibers were the same were 2.6×10^{-16} and 1.6×10^{-16}, respectively.

It can be demonstrated directly that added S1 bound to two actin monomers: HC was labeled with 5′-IATR at Cys-707, added at 0.5 μM to 4 mg/ml myofibrils (actin in excess), and cross-linked with EDC for 30 min at room temperature. The result is shown in Fig. 5. A comparison of the resulting cross-linking pattern (*lane 3*) with that of acto-S1 (*lane 1*) shows that the two patterns are virtually identical. In particular, the 210-kDa band, which is characteristic of the actin-actin-S1 adduct (Andreev and Borejdo, 1992), is present, suggesting that in myofibrils, as in solution, S1 binds to two actins.

Polarization of native fibers

To measure polarizations of muscle containing labeled cross-bridges, a single fiber was labeled just before the experiment by adding to it 1 mM PA for 3 min and washing out the excess of the dye with rigor solution. The histogram of polarizations obtained in 14 experiments obtained from four different fibers is plotted in Fig. 6 *B* (summary in Table 2). The comparison with fiber irrigated with unsaturated S1 (shown again in Fig. 6 *A*) shows that polarizations are the same. The difference in mean polarizations was not statistically significant (probabilities that $P_{\parallel}$ and $P_{\perp}$ of unsatur-

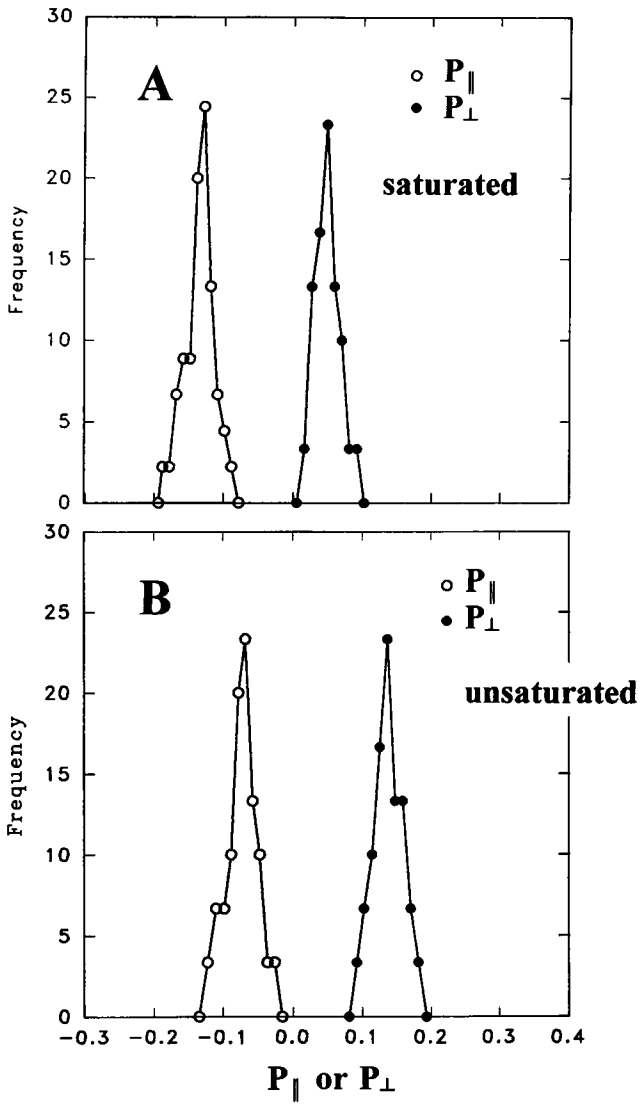

FIGURE 4 Comparison of polarizations of a single fiber irrigated with saturating (*A*) and nonsaturating (*B*) concentrations of S1(A1·PA). ●, $P_\perp$; ○, $P_\parallel$.

TABLE 2 The polarization of fluorescence of single muscle fibers labeled with PA and of fibers irrigated with saturating and nonsaturating concentrations of S1-PA

Add	$P_\parallel$	$P_\perp$
0.1 μM S1 (A1)*	−0.061 ± 0.008	+0.131 ± 0.004
2.0 μM S1 (A1)*	−0.127 ± 0.006	+0.041 ± 0.006
Fiber in rigor#	−0.075 ± 0.013	+0.118 ± 0.005

*S1 (A1) was labeled with equimolar concentration of PA, polarization was measured in rigor solution.
#Fibers were labeled with 1 mM PA.

ated and native fibers were the same were 6.7×10^{-2} and 2.9×10^{-2}, respectively). On the other hand, the difference in polarizations for fibers irrigated with saturating concentrations of S1(A1)-PA and native fibers was statistically significant (probabilities that $P_\parallel$ and $P_\perp$ of the two sets of

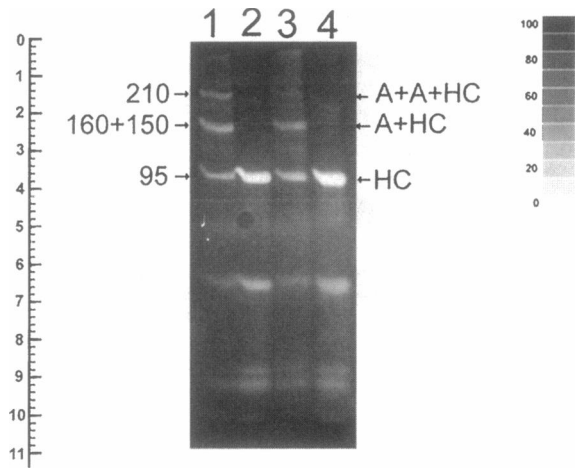

FIGURE 5 Cross-linking of S1 to thin filaments of myofibrils, showing that S1 cross-links identically to myofibrillar thin filaments and F-actin. S1 (0.5 μM) labeled with 5′-IATR was incubated with 4 mg/ml myofibrils in the presence (*lane 3*) and absence (*lane 4*) of 50 mM EDC. Control experiment: 0.5 μM S1(5′-IATR) was incubated with 4 μM F-actin in the presence (*lane 1*) and absence (*lane 2*) of 50 mM EDC.

measurements were the same were 1.3×10^{-12} and 2.3×10^{-17}, respectively).

DISCUSSION

Earlier studies were conducted with rhodamine probe bound to Cys-707 of the HC of S1 (Andreev and Borejdo, 1992; Andreev et al., 1993a,b; Xiao et al., 1995), with rhodamine bound to Cys-177 of the ELC (Xiao et al., 1995, 1996) and with 9-anthroylnitrile bound to the Ser-180 of the active site of S1 (Andreev et al., 1995). The labeling involved extensive washings of the muscle fiber to remove unreacted probe or the light chains. This procedure resulted in imperfect labeling because unreacted dye or light chains could not be completely washed out. Moreover, light-chain exchange necessitates raising the temperature and exposing fibers to extracting media containing EDTA, which may disrupt normal packing of the heads on surface of the thick filaments (Ling et al., 1996). Labeling with PA sidesteps these difficulties because labeling simply involves adding PA to fibers and washing the excess with physiological solution. Furthermore, PA monitors movement in a part of the molecule that is believed to undergo large orientational change (Holt and Lowey, 1975; Rayment et al., 1993), has relatively high polarization (Borejdo, 1983), binds weakly to myofibrillar structures other then myosin (see above), and does not change the ATPase activity of S1 (Borejdo, 1983), and the orientation of the dipole is insensitive to the nucleotides (see above). The disadvantage of PA is that it binds noncovalently, which raises the possibility of dye migration. We think that the extent of migration is minimal, because if significant redistribution were to occur, the value of polarization would depend on the time that the measurement was taken. This would decrease the accuracy of measurements,

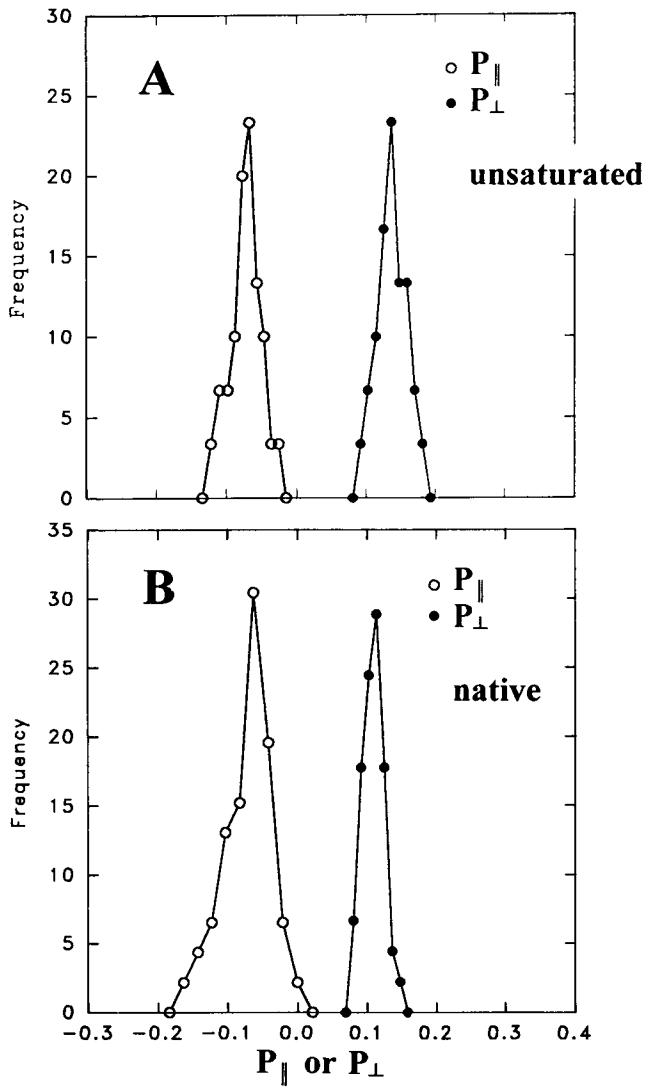

FIGURE 6 Comparison of polarizations of a single fiber in which native myosin has been labeled with PA (*B*) with a fiber irrigated with nonsaturating (*A*) concentrations of S1(A1·PA). ●, $P_\perp$; ○, $P_\parallel$.

but the standard error listed in Table 2 is small. Furthermore, free PA is nearly nonfluorescent, and PA binds strongly to S1(A1) but weakly to other myofibrillar elements. We conclude that PA is a useful probe in studies of orientation of muscle cross-bridges.

Orientation of cross-bridges

Bulk polarization of PA on S1 is large in comparison with A1 ($p = 0.230$, labeled at Cys-177) or PA alone, but small in comparison with S1 labeled at Cys-707 ($p = 0.450$). This suggests that binding of PA to the hydrophobic pocket is relatively rigid, but that PA is not completely immobile on the surface of S1. The facts that the polarization is also small when A1 or A2 is labeled at Cys-177 (Xiao et al., 1996) and that the 41-nucleotide difference piece is not visible in the atomic structure of S1 (Rayment et al., 1993) suggest that the part of A1 to which PA binds is disordered. In fact, the whole light-chain binding region of S1 may be disordered (Ling et al., 1996). Polarizations obtained from fibers are further decreased because of depolarization by microscope optics. Despite this fact, the data can be fitted with a simple "Gaussian" model (Thomas and Cooke, 1980), which assumes that dye dipoles are parallel (the actual angle, calculated from the polarization of PA solutions, is 28°) and oriented with respect to the long axis of actin filaments at a mean polar angle $\Theta°$ with standard deviation δ. The probability that the dipole is at any angle Θ is $\rho(\Theta) = \exp[-(\Theta - \Theta_G)^2/2\delta^2]$. The polarizations $P_\perp$ and $P_\parallel$ are expressed as functions of the averages $S_2 = \langle \sin^2\Theta \rangle$ and $S_4 = \langle \sin^4\Theta \rangle$,

$$P_\perp = (7S_4 - 4S_2)/(4S_2 - S_4)$$

$$P_\parallel = (2 - 5S_2 + 3S_4)/(2 - 3S_2 + S_4).$$

S_2 and S_4 are given, e.g., by Wilson and Mendelson (1983). The full formulas for $P_\perp$ and $P_\parallel$ are symmetrical with respect to Θ and δ (equations are available from the authors upon request). Polarizations are plotted against Θ and δ, using Mathematica 2.1 as described before (Borejdo and Burlacu, 1994), and Θ and δ are estimated from the plots. No solution exists for muscle saturated with S1 (Fig. 7 *A*). For muscle unsaturated with S1 and for native muscle fibers, the solutions are $\Theta \approx 54 \pm 3°$, $\delta \approx 0$–$10°$ and $\Theta \approx 55 \pm 3°$, $\delta \approx 0$–$10°$, respectively, or a symmetrical set $\delta \approx 54 \pm 3°$, $\Theta \approx 0$–$10°$ and $\delta \approx 55 \pm 3°$, $\Theta \approx 0$–$10°$, respectively (Fig. 7, *B* and *C*). We think that the fact that polarizations can be fitted to unsaturated and native fibers but not to saturated muscle reflects less randomness in the former case. In unsaturated or native muscle, A1 on S1 binds to both HC of S1 and to actin (Andreev and Borejdo, 1995), and attachment at two points decreases disorder. In contrast, when actin filament is saturated with S1, A1 binds only to HC of S1.

S1 added at unsaturating concentrations to myofibrils and cross-linked with EDC gave a pattern identical to that of S1 cross-linked with F-actin (Fig. 5). Because S1 mixed with actin at low ratios (S1:actin) forms a 1:2 complex with actin (Andreev and Borejdo, 1991, 1992), the present result shows that S1 added to myofibrils also forms a complex with two actins. The results of cross-linking (Fig. 5) support this conclusion. The fact that the orientation of myosin heads in muscle was similar to the orientation of nonsaturating S1 suggests that the conformation of the two is the same. It follows that myosin heads in rigor muscle also form a 1:2 complex with actin. This result was expected, because thin filaments in skeletal muscle are not saturated with cross-bridges (Morimoto and Harrington, 1974; Squire, 1981). Because the orientation of myosin heads in muscle is different from the orientation of S1 added to muscle at high concentration, we conclude that there is a structural difference between rigor complexes that muscle cross-bridges form with thin filaments and complexes that high concen-

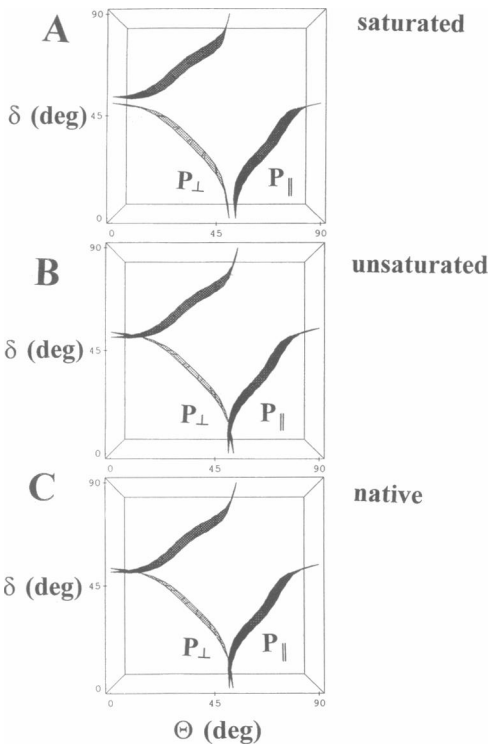

FIGURE 7 The dependence of $P_{\parallel}$ (*black*) and $P_{\perp}$ (*gray*) on δ and Θ for the case of fiber saturated (*A*) and unsaturated (*B*) with S1-PA. (*C*) Data of native muscle fiber. The experimental range of polarization is drawn on the z axis: In *A*, -0.133 to -0.121 for $P_{\parallel}$; $+0.035$ to $+0.047$ for $P_{\perp}$. In *B*, -0.069 to -0.052 for $P_{\parallel}$; $+0.127$ to $+0.135$ for $P_{\perp}$. In *C*, -0.085 to -0.065 for $P_{\parallel}$; $+0.113$ to $+0.123$ for $P_{\perp}$. Θ and δ are on x and y axes, respectively. Shaded areas indicate possible values of δ and Θ consistent with the measured range of $P_{\parallel}$ and $P_{\perp}$. The sought-after solution is the one for which shaded areas overlap.

trations of S1 make with F-actin in solution. Because the 3D reconstructions of acto-S1 were carried out using actin fully saturated with S1 (i.e., S1 binding only through the primary site), deductions from the in vitro experiments may not be fully applicable to the cross-bridges in native muscle. The same conclusion was reached on the basis of proteolytic digestions of myofibrils (Xiao et al., 1995).

REFERENCES

Andreev, O. A., A. L. Andreeva, and J. Borejdo. 1993a. Polarization of fluorescently labeled myosin subfragment-1 fully or partially decorating muscle fibers and myofibrills. *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.* 32:12046–12035.

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. 1992. Two different acto-S1 complexes. *J. Muscle Res. Cell Motil.* 13:523–533.

Andreev, O. A., and J. Borejdo. 1995. Binding of heavy- 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., 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.

Borejdo, J. 1983. Mapping of hydrophobic sites on the surface of myosin and its fragments. *Biochemistry.* 22:1182–1187.

Borejdo, J., O. Assulin, T. Ando, and S. Putnam. 1982. Cross-bridge orientation in skeletal muscle measured by linear dichroism of an extrinsic chromophore. *J. Mol. Biol.* 158:391–414.

Borejdo, J., and S. Burlacu. 1994. Orientation of actin filaments during motion in in vitro motility assay. *Biophys. J.* 66:1319–1327.

Fajer, P. G. 1994. Method for the determination of myosin head orientation from EPR spectra. *Biophys. J.* 66:2039–2050.

Holt, J., and S. Lowey. 1975. An immunological approach to the role of the low molecular weight subunits in myosin. I. Physical-chemical and immunologic characterization of the light chains. *Biochemistry.* 14: 4600–4609.

Huxley, A. F., and R. M. Simmons. 1971. Proposed mechanism of force generation in striated muscle. *Nature.* 233:533–538.

Ling, N., C. Shrimpton, J. Sleep, J. Kendrick-Jones, and M. Irving. 1996. Fluorescent probes of the orientation of myosin regulatory light chains in relaxed, rigor, and contracting muscle. *Biophys. J.* 70:1836–1846.

Morimoto, K., and W. F. Harrington. 1974. Substructure of the thick filament of vertebrate striated muscle. *J. Mol. Biol.* 83:83–97.

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. *Eur. J. Biochem.* 121: 213–219.

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

Sklar, L. A., B. S. Hudson, and R. D. Simoni. 1975. Conjugated polyene fatty acids as membrane probes: preliminary characterization. *Proc. Natl. Acad. Sci. USA.* 72:1649–1653.

Schagger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.

Squire, J. 1981. *In* The Structural Basis of Muscular Contraction. Plenum Press, New York. 543.

Sweeney, H. L. 1995. Function of the N-terminus of the myosin essential light chain of vertebrate striated muscle. *Biophys. J.* 68:112s–119s.

Tanner, J. W., D. D. Thomas, and Y. E. Goldman. 1992. Transients in orientation of a fluorescent cross-bridge probe following photolysis of caged nucleotides in skeletal muscle fibers. *J. Mol. Biol.* 223:185–203.

Thomas, D. D., and R. Cooke. 1980. Orientation of spin-labeled myosin heads in glycerinated muscle fibers. *Biophys. J.* 32:891–905.

Tonomura, Y., P. Appel, and M. F. Morales. 1966. On the molecular weight of myosin. II. *Biochemistry.* 5:515–521.

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

Wilson, M. G. A., and R. A. Mendelson. 1983. A comparison of order and orientation of cross-bridges in rigor and relaxed muscle fibers using fluorescence polarization. *J. Muscle Res. Cell Motil.* 4:671–693.

Xiao, M., O. A. Andreev, and J. Borejdo. 1995. Rigor cross-bridges bind to two actin monomers in thin filaments of rabbit psoas muscle. *J. Mol. Biol.* 248:294–307.

Xiao, M., A. Tartakowski, O. A. Andreev, and J. Borejdo. 1996. Binding of S1(A1) and S1(A2) to F-actin. *Biochemistry.* 35:523–530.