Binding of S1(A1) and S1(A2) to F-Actin†

M. Xiao,‡ A. Tartakowski,§ O. A. Andreev,‡ and J. Borejdo*,‡

Baylor Research Institute, Baylor University Medical Center, 3812 Elm Street, Dallas, Texas 75226, and Department of Oral Biology, Hebrew University−Hadassah School of Dental Medicine, POB 1172 Jerusalem 91-010, Israel

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ABSTRACT: The binding curve of myosin subfragment-1 (S1) to F-actin is not a simple hyperbola: at high concentrations of S1 the binding curve can be transformed into a linear plot ("normal" binding), but at small concentrations of S1 the binding complications deform the binding curve and produce nonlinear transforms ("anomalous" binding) [Andreev, O. A., & Borejdo, J. (1992) *J. Muscle Res. Cell Motil. 13*, 523−533]. This anomalous behavior may result either from the heterogeneity of S1 in regard to light chain isoforms or from the cooperativity between S1's. To distinguish between these possibilities we measured the affinity and the orientation of S1(A1) and S1(A2) with respect to F-actin. Affinity was measured *in vitro* by ultracentrifugation in the presence of F-actin, and orientation was measured *in vivo* by a combination of polarization of fluorescence and linear dichroism. We found that both the affinity and the orientation depended on the relative concentration of S1 isomer and actin: when S1 was in excess or was equimolar with actin (filament saturated with S1), each isomer bound F-actin with an affinity of 2×10^6 M^{-1} and was oriented approximately perpendicularly to the muscle axis. When actin was in excess (filament unsaturated with S1), each isomer bound F-actin with an affinity of 1.2×10^7 M^{-1} and was oriented more parallel to the muscle axis. S1(A1) and S1(A2) labeled on the light chain had different polarizations when bound to unsaturated filaments but had the same polarizations when bound to saturated filaments. These results excluded heterogeneity as a reason for anomalous binding and suggested that binding occurred with negative cooperativity. We think that the negative cooperativity occurs when saturation of actin filaments with heads leads to the lack of vacant adjacent sites on a filament and a consequent prevention of S1 binding to two actin protomers.

Muscle contraction is thought to be caused by ATP-driven interactions of myosin heads (S1) with actin (A). It is believed that in the power stroke the contact area between the cross-bridges and actin monomers is changed, and so the orientation of a cross-bridge relative to the actin filament axis changes (Huxley, H. E., 1969; Huxley, A. F., & Simmons, 1971). Recently, we proposed that at the beginning of the power stroke the cross-bridge binds to one actin protomer and that at the end of the power stroke the cross-bridge binds to two protomers (Andreev & Borejdo, 1991, 1992). This suggestion was based on the facts that S1 binds to F-actin anomalously (i.e., the binding reaction was more complicated than the formation a simple equilibrium complex $A + S1 \leftrightarrow A{\cdot}S1$) (Andreev *et al*., 1993a) and that the orientation of S1 was different depending on whether S1 was in excess of actin or not (Andreev *et al*., 1993c). Similarly, cross-linking (Andreev *et al*., 1993b) and digestibility (Xiao *et al*., 1995) experiments suggested that the conformation of S1 varied depending on the level of saturation. Kinetic experiments revealed rapid transition between the two types of complexes (Andreev *et al*., 1993a, 1995).

An anomalous binding curve could have at least three origins. (1) Isoform heterogeneity of S1 (S1 consists of two homogenous fractions of light chain isoforms): each fraction could bind simply to a single actin but with different affinity. (2) Positive cooperativity: S1 initially binds to F-actin in a simple manner, but bound S1's enhance binding of the remaining molecules. (3) Negative cooperativity: S1 initially binds simply, but bound S1's inhibit binding of the remaining molecules. We show here that each isomer binds F-actin anomalously (i.e., like unfractionated S1). When the molar ratio S1:actin is high, each isomer binds to one actin monomer within F-actin with an affinity of 2×10^6 M^{-1}, and when the molar ratio is low, each isomer binds to two monomers with an affinity of 1.2×10^7 M^{-1}. The polarization data shows that each S1 isomer binds differently to a saturated than to an unsaturated actin filament. These findings excluded isomer heterogeneity and positive cooperativity as causes of anomalous binding. We concluded that S1 binds to actin with negative cooperativity. We speculate that the negative cooperativity occurs when saturation of actin filaments with heads leads to the lack of vacant adjacent sites on a filament and a consequent prevention of S1 binding to two actin protomers.

In this work we used S1 labeled at its essential light chain. Previous studies showing orientational differences between the two complexes were done with S1 which had been labeled at SH_1 (Cys-707). This is a convenient location, because thiol 707 reacts specifically and rapidly with a

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* To whom correspondence should be addressed.

‡ Baylor University Medical Center.

§ Hebrew University−Hadassah School of Dental Medicine.

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[1] Abbreviations: S1, myosin subfragment-1; A, actin; A1, A2, essential light chains 1 or 2; S1(A1), S1(A2), S1 isomers carrying light chains A1 or A2; S1(A1R), S1(A2R), S1 isomers carrying light chains A1 or A2 which have been labeled with rhodamine; S1R(A1), S1R-(A2), S1 isomers which have been labeled at the heavy chain with rhodamine; S1R(A1), S1R(A2), S1 isomers which have been labeled at the heavy chain with rhodamine; IATR, 5-(iodoacetamido)tetramethylrhodamine; ELC, essential light chains; HC, heavy chain of S1; MR, molar ratio S1 to actin.

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variety of labels [e.g., Nihei *et al*. (1974)]. However, the recent 3D structure of S1 (Rayment *et al*., 1993) suggests, in support of an earlier clue (Cooke, 1986), that the large orientational change in S1 occurs in the "tail" region of the molecule. This site is distant from the "globular" part of the molecule where the enzymatic activity is known to occur. The structure shows that the residue 707 is at the functionally important interface between the two proteolytic domains (so called 20- and 50-kDa tryptic domains). During the contractile cycle the helix connecting Cys-707 with the other reactive cysteine (Cys-697, SH_2) may melt, causing a shift of SH_1 relative to SH_2 (Dalbey *et al*., 1983; Botts *et al*., 1989). The essential light chains, on the other hand, reside some distance away from the catalytic part of the molecule (Holt & Lowey, 1975; Rayment *et al*., 1993) and are not necessary for the enzymatic activity (Wagner & Giniger, 1981; Sivaramakrishnan & Burke, 1982), although they might contribute to the stability of the myosin heavy chain. In contrast to the regulatory LC, the single thiol of the ELC's can be specifically modified with fluorescent reagents (Marsh & Lowey, 1975) and can be readily exchanged with the native light chains of S1 (Wagner & Weeds, 1977). All of our earlier work has been carried out using tryptic S1 (which has ELC's but no regulatory light chain). Unfortunately, the polarizations of S1 labeled at the ELC are small, making it impossible to calculate the angle between the dipole and the axis of the fiber. To estimate this angle we took advantage of the fact that the polarizations were large when S1 was labeled at Cys-707.

We also show that at low, but not at high, degrees of saturation of actin filaments with S1, the orientation of A1 is different than the orientation of A2.

MATERIALS AND METHODS

Materials. ATP, phalloidin, and chymotrypsin were from Sigma (St. Louis, MO). 5-(Iodoacetamido)tetramethylrhodamine (IATR, lot 2841) was from Molecular Probes (Eugene, OR). The absorption spectrum of IATR (not shown) had a small shoulder at 530 nm and high absorption coefficient at 555 nm (75 000 M^{-1}), suggesting that the dye (lot 2841, Molecular Probes) was composed predominantly of 5′-isomer of tetramethylrhodamine (Ajtai *at al*., 1992). All other chemicals were of analytical grade.

Proteins. Myosin was prepared from rabbit skeletal muscle using the method of Tonomura *et al*. (1966). Actin and myosin subfragment-1 were prepared from rabbit skeletal muscle according to Spudich and Watt (1971) and Weeds and Taylor (1975), respectively. S1 was separated into A1 and A2 isoforms on a cation exchange HPLC WCX-ZORBAK column (DuPont) or on a DEAE column according to 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 SDS−PAGE.

Solutions. It was necessary to use relatively high ionic strength because our previous results showed that the affinity of S1 to F-actin in 50 mM KCl, pH 7.5, 23 °C, in the absence of ATP was so high that more than 95% of 0.4 μM S1 was bound to 1 μM F-actin (Andreev & Borejdo, 1992). This made the quantitative determination of the affinity constants at low [KCl] impossible. For this reason, the solution used for the sedimentation experiments contained 200 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.4 mM β-mercaptoethanol. The fibers were glycerinated in a relaxing solution containing 50 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. Polarizations were measured in a solution containing 50 mM potassium acetate, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM TRIS-acetate, pH 7.5, and 10 mM DTT. The relaxing solution contained 50 mM potassium acetate, 2 mM $MgCl_2$, 2 mM EGTA, and 10 mM TRIS-acetate, pH 7.5. The EDTA-rigor solution contained 80 mM potassium acetate, 5 mM EDTA, 10 mM TRIS-acetate, pH 7.5.

Preparation of Muscle Fibers. The psoas muscle was stored at −18 °C in glycerinating solution. To prevent contraction, which often results when relaxed fibers are transferred to a rigor solution containing Mg (because muscle was Ca^{2+}-insensitive when [ATP] became small; Weber & Murray, 1973), the bundle of 20−50 fibers was first transferred to a large volume of EDTA-rigor solution for $^1/_2$ h. Before each experiment, muscle was extensively washed with rigor solution, and single fibers were dissected from the bundle, placed on a quartz microscope slide, and washed with rigor solution.

Preparation of Labeled S1. The procedure was as follows: label isolated ELC's → remove free dye → exchange into S1 → separate on DE-52 → run SDS−PAGE. The essential light chains were prepared according to Holt and Lowey (1975). Isolated light chains were labeled by incubation with 5 M excess of IATR for 4 h in a solution containing 50 mM KCl, 2 mM EDTA, 10 mM phosphate buffer, pH 7.0, at 5 °C. The free dye was removed by passing the light chains through Sephadex-50 column and by dialysis against rigor solution. The labeled light chains were exchanged with light chains of S1 as described by Wagner and Weeds (1976). In order to separate S1(A1R) and S1(A1) [or S1(A2R) and S1(A2)] from the other species remaining after the exchange, the sample was passed through the DE-52 column. Cys-707 (in the heavy chain) was labeled by the incubation of 22−30 μM S1 in rigor solution with 1.5 M excess of the dye for 6−12 h. The free dye was removed as above. The concentration of rhodamine-labeled S1 was calculated from the absorption at 280 and 555 nm as c_{S1} (mg/mL) = $(A_{280} - A_{555}/4)/0.75$ and the concentration of the dye as c_{IATR} (μM) = $A_{555} \times 10^3/75.0$. Typically, 30%−40% of S1 was labeled at the light chain and 80%−100% at the heavy chain.

Assessment of Labeling. The specificity of labeling was assessed by measuring the intensity of the various bands on SDS−PAGE. The labeling of essential light chains could have been unspecific because any free dye remaining after exchange could have migrated to the heavy chain. That this was not the case is shown in Figure 1: the figure shows Coomassie Blue and fluorescent patterns of fractions released from the DE-52 column after applying S1 which has been exchanged with fluorescent A1 (top) and with fluorescent A2 (middle). Intensity of the low molecular weight material migrating below A2 was taken as measure of the free dye. Intensities were measured with the Image Pro Plus (Silver Spring, MD) image-processing program. The analysis revealed that in 100% of S1 exchanged with A1R the label

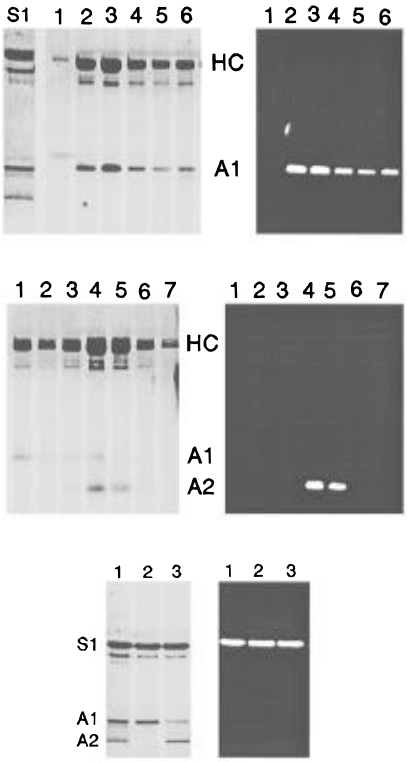

FIGURE 1: Labeling S1 isomers with IATR at the ELC (top and middle) or at the heavy chain (bottom). Top: S1 labeled at ELC, DE-52 elution profile of S1 exchanged with fluorescent A1. Coomassie Blue (left) and fluorescence (right). Samples shown in lanes 2 and 3 have been used in the experiments. Middle: S1 labeled at ELC, DE-52 elution profile of S1 exchanged with fluorescent A2. Coomassie Blue (left) and fluorescence (right). Samples shown in lanes 4 and 5 have been used in experiments. Bottom: S1 labeled at the heavy chain, Coomassie Blue (left), fluorescence (right). 1, unfractionated S1; 2, 7.2 μM S1(A1); 3, 6.4 μM S1(A2). Abbreviations: S1, heavy chain of S1; A1, light chain 1; A2, light chain 2; ELC, essential light chains. 15% gradient gels.

remained attached to A1 (i.e., no detectable amount of the heavy chain of S1 was labeled). Similarly, in 100% of S1 exchanged with A2R the label remained at A2. Fractions in lanes 2 and 3 for S1(A1) and in lanes 4 and 5 for S1(A2) have been used in polarization experiments. Heavy chain labeling was also very specific: Figure 1 (bottom) shows Coomassie Blue and fluorescent patterns of unfractionated S1 (lane 1), S1(A1) (lane 2), and S1(A2) (lane 3) labeled with IATR. The analysis (bottom) revealed that for S1(A1) 96.9% and 3.1% of fluorescence originated from the heavy and light chain, respectively. For S1(A2) 99.3% and 0.7% of fluorescence originated from the heavy and light chains, respectively. Free dye was not detectable in either isomer.

Measuring Polarization of Fluorescence and Dichroism. The apparatus to measure the polarization of fluorescence and dichroism was essentially the same as described earlier (Borejdo *et al*., 1982) except that the 496 nm line of the argon laser was used to excite fluorescence to increase Stoke's shift. The emitted light was passed through sheet polarizers and measured by the photomultiplier. The microscope objective was 25× Zeiss UD 40 (NA = 0.45). To minimize photobleaching, all of the solutions contained 10 mM DTT and each set of orthogonal intensities was measured from a different spot on the fiber (each spot was exposed for no more than 5 s, during which time the intensity did not change).

To correct for the fact that the dichroic mirror transmits horizontally polarized fluorescent light more efficiently than vertically polarized light, we measured the correction factors $C_h = {}_hI_h/{}_hI_v$ and $C_v = {}_vI_v/{}_vI_h$ as described earlier (Andreev *et al*., 1993c). Here I is the intensity of the fluorescent light, the direction of the excitation polarization is indicated by a subscript v or h before a symbol, and the direction of emission polarization is indicated by a subscript after a symbol. The intensity components were also corrected for the fluorescence of the background (bck). The different polarization quantities were defined as follows:

$$P_h = [({}_hI_h - {}_hbck_h)/C_h - ({}_hI_v - {}_hbck_v)]/[({}_hI_h - {}_hbck_h)/C_h + ({}_hI_v - {}_hbck_v)] \quad (1)$$

$$P_v = [({}_vI_v - {}_vbck_v)/C_v - ({}_vI_h - {}_vbck_h)]/[({}_vI_v - {}_vbck_v)/C_v + ({}_vI_h - {}_vbck_h)] \quad (2)$$

$$Q_h = [({}_hI_h - {}_hbck_h) - ({}_vI_h - {}_vbck_h)]/[({}_hI_h - {}_hbck_h) + ({}_vI_h - {}_vbck_h)] \quad (3)$$

$$R_h = ({}_hI - {}_hbck)/({}_vI - {}_vbck) \quad (4)$$

where R_h is the dichroic ratio and ${}_hI$ and ${}_vI$ are the amounts of light absorbed by the fiber when it is illuminated with horizontally and vertically polarized light, respectively. Q_h does not require correction because the intensity of the light impinging on the sample does not depend on the orientation of the polarizer. The emission polarization is the same for both intensities. The dichroism of a 5 μM solution of IATR was 1.02, and therefore R_h was not corrected. For rhodamine dye C_h and C_v were 1.61 and 0.82, respectively. All measurements were done at room temperature (~23 °C).

Sedimentation. A 0.5 μM solution of F-actin and increasing concentrations of S1 (0.01−2 μM) were in the high ionic strength rigor solution. Samples were preincubated for 1 h at 15 °C and centrifuged at 70 000 rpm (213 000*g*) for 15 min at 15 °C in a Beckman TL-100 centrifuge (Fullerton, CA). The concentration of proteins in supernatant was measured by Bradford method (1976) using the standard Bio-Rad protein assay. The error of measurement was about 0.25 μg/mL, which corresponds to 0.002 or 0.006 μM for measurements of S1 or actin concentrations, respectively. The fraction of F-actin which remained in the supernatant in the absence of S1 was less than 5%. The concentration of S1 in supernatant ($S1_{SUP}$) was found by subtracting the contribution of actin from the total concentration of proteins in the supernatant. The fraction of free S1 which precipitated under our ionic conditions in the absence of F-actin was estimated as 5%. The concentration of free S1 was determined as $S1_F = S1_{SUP}/0.95$. The fraction of inactive S1, i.e., S1 which was unable to bind to F-actin, was estimated by measuring S1 concentration in supernatant after centrifugation of the sample which contained 0.5 μM S1 and 12 μM F-actin in a solution that contained 50 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.4 mM β-mercaptoethanol (βME) at 23 °C. All experiments were done with S1 preparations which contained less than 1% of inactive S1.

RESULTS

Sedimentation. In order to avoid having the concentration of S1 in the supernatant too low for reliable measurements,

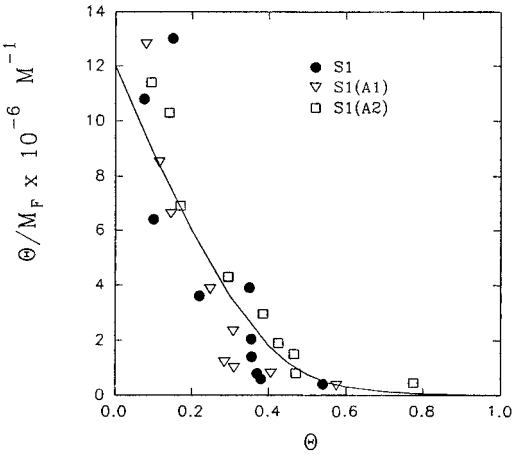

FIGURE 2: Scatchard plot of binding of S1 to F-actin obtained from sedimentation experiments as described in Materials and Methods in a solution containing 0.5 μM F-actin, 0.5 μM phalloidin, 200 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.4 mM β-ME, at 15 °C. Stock solutions were S1 = 15.0 μM, S1(A1) = 8.9 μM, S1(A2) = 10.2 μM, and F-actin = 29.5 μM.

we have studied binding of S1 to F-actin under conditions where binding was weak (15 °C, 200 mM KCl). To avoid depolymerization, F-actin was stabilized by adding equimolar phalloidin to a stock solution of F-actin (29.5 μM) and incubating it overnight. To remove the free nucleotides, which may reduce the affinity of S1 to F-actin, F-actin was dialyzed overnight against rigor buffer. In a typical experiment 15 samples were prepared: 13 samples contained 0.01−2 μM S1 and 0.5 μM F-actin, and two control samples contained either 0.5 μM F-actin or 0.5 μM S1. Samples were centrifuged, and the concentration of protein in the supernatant was measured as described in Materials and Methods. The fraction bound, Θ, was defined as $S1_B/A_T$, where $S1_B$ and A_T were concentrations of bound S1 and total actin, respectively. $S1_F$, concentration of free S1, was calculated from A_T and the molar ratio (MR) $S1_T/A_T$ as $S1_F = A_T(MR - \Theta)$. It follows that $\Theta/S1_F = \Theta/[A_T(MR - \Theta)]$. The data was plotted in Scatchard form, i.e., $\Theta/S1_F$ was plotted versus Θ (in graphs, the concentration of $S1_F$ is denoted by M_F). The data are presented in Figure 2. The Scatchard plot is nonlinear and concave downward. Most of the experimental points are grouped along two straight lines: a line connecting abscissa = 0.5 and ordinate = 14 and a second line at abscissa = 1 and ordinate = 1.5. In three experiments using different protein preparations, no systematic differences between either isomer or between either isomer and unfractionated S1 could be seen.

Difference in the Orientation between High and Low Concentration of Isomers. Unfractionated S1 binds to thin filaments of muscle at an orientation that depends on the concentration (Andreev *et al*., 1993c; Borejdo & Andreev, 1994). The same is true of both S1(A1R) and S1(A2R). To measure the orientation, a single (unlabeled) fiber was irrigated with a high concentration (2 μM) of isomers for 1 h at room temperature in the dark. Excess S1 was removed by extensive washing with rigor solution, and polarization of fluorescence and linear dichroism measurements were begun within 5 min of washing to minimize the increase in the background due to gradual dissociation of S1. To measure the polarizations and dichroism of fibers irrigated with the low concentration of S1(A1R) or S1(A2R), an

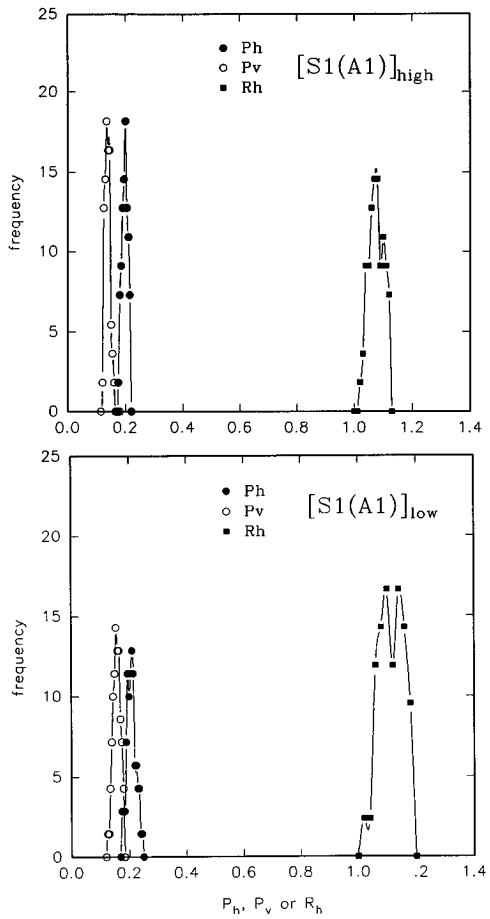

FIGURE 3: Histogram of polarizations and of linear dichroism from muscle fiber irrigated with S1(A1R) (label on A1). Top: Muscle irrigated with high (2 μM) concentration of S1(A1R). Bottom: Muscle irrigated with low (0.1 μM) concentration of S1(A1R). To make histograms look smooth, the data points were running-averaged (window size = 3). Solid circles, P_h; solid squares, R_h; open circles, P_v.

unlabeled fiber was incubated with 0.1 μM of labeled S1 overnight at 4 °C in the dark. The excess S1 was removed as before. The excitation was at 496 nm using a Zeiss rhodamine dichroic cube (Zeiss filters: excitation, BP 546/12; dichroic mirror, FT 580; emission, LP 590). The results of 12 experiments on four different fibers irrigated with a high concentration of S1(A1R) are plotted in histogram form in Figure 3A and are summarized in Table 1. The results of 12 experiments on four different fibers irrigated with a low concentration of S1(A1R) are plotted in Figure 3B and are summarized in Table 1. All three components of the polarization as well as the linear dichroism of muscle irrigated with low concentrations of S1(A1R) were statistically significantly different from polarizations and dichroism of muscle irrigated with high concentrations of S1(A1R). According to the *t*-test, the differences in R_h, P_h, and P_v were significant at the 0.06 level of confidence. Q_h was not plotted in Figure 3 or 4 and was not statistically analyzed because it was not an independent variable: when the angle between absorption and emission dipoles is small (as is the case with rhodamine; Andreev *et al*., 1995), it can be shown that $Q_h \approx P_h$.

The results of 11 experiments on four different fibers irrigated with the high concentration of S1(A2R) are plotted in Figure 4A and are summarized in Table 1. The results of 12 experiments on four different fibers irrigated with low

Table 1: Linear Dichroism and Polarization of Fluorescence of Muscle Fibers Loaded with High (2.0 μM) and Low (0.1 μM) Concentration of S1 Carrying Rhodamine-Labeled Essential Light Chains

irrigation with[a]	R_h	P_h	P_v	Q_h
2.0 μM S1(A1R)	1.070 ± 0.007	0.196 ± 0.003	0.137 ± 0.004	0.221 ± 0.004
2.0 μM S1(A2R)	1.054 ± 0.004	0.199 ± 0.004	0.194 ± 0.003	0.235 ± 0.007
0.1 μM S1(A1R)	1.107 ± 0.010	0.206 ± 0.004	0.154 ± 0.003	0.237 ± 0.008
0.1 μM S1(A2R)	1.158 ± 0.009	0.266 ± 0.006	0.273 ± 0.007	0.335 ± 0.026

[a] mean ± SE of 11 or 12 experiments.

Table 2: Linear Dichroism and Polarization of Fluorescence of Muscle Fibers Loaded with High (2.0 μM) and Low (0.1 μM) Concentration of S1 Labeled at the Heavy Chain (at Cys-707) with Rhodamine

irrigation with[a]	R_h	P_h	P_v	Q_h
2.0 μM S1R(A1)	0.294 ± 0.012	−0.149 ± 0.009	0.673 ± 0.001	−0.120 ± 0.014
2.0 μM S1R(A2)	0.283 ± 0.013	−0.231 ± 0.007	0.734 ± 0.009	−0.159 ± 0.008
0.1 μM S1R(A1)	0.402 ± 0.014	−0.091 ± 0.007	0.579 ± 0.012	−0.033 ± 0.001
0.1 μM S1R(A2)	0.491 ± 0.013	0.018 ± 0.002	0.543 ± 0.021	0.059 ± 0.007

[a] mean ± SE of 15 experiments.

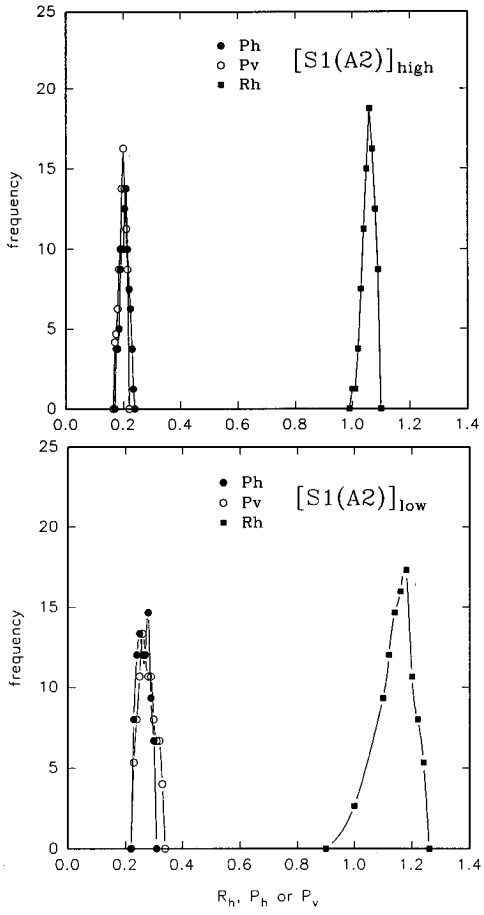

FIGURE 4: Histogram of polarizations and of linear dichroism from muscle fiber irrigated with S1(A2) (label on A2). Top: Muscle irrigated with high (2 μM) concentration of S1(A2R). Bottom: Muscle irrigated with low (0.1 μM) concentration of S1(A2R). Smoothing and symbols as in Figure 3.

concentration of S1(A2R) are plotted in Figure 4B and are summarized in Table 1. Similarly to S1(A1), the differences in polarization and dichroism were statistically highly significant. According to the *t*-test, the differences in R_h, P_h, and P_v were significant at the 0.0006 level of confidence.

Difference in the Polarization of Fluorescence between S1(A1R) and S1(A2R). Table 1 shows an interesting fact: At low but not at high concentrations, S1(A1R) had significantly different polarization of fluorescence than S1-(A2R). Comparison of polarization and dichroism data shows that the differences in the mean values of P_v, P_h, R_h, and Q_h for fibers irrigated with the low concentrations of S1 were statistically significant at the 0.001 level of confidence. In contrast, the differences in the mean values of P_h, R_h, and Q_h for fibers irrigated with the high concentration of S1 were not, with the exception of P_v, statistically significant at the 0.05 level of confidence.

Calculating the Dipolar Angle: S1 Labeled at HC. Knowledge of the angle of the transition dipole of the dye relative to the muscle axis allows one to estimate the amount of conformational change involved in binding of high and low concentrations of S1. But small values of polarization and dichroism of S1 labeled at the ELC make it impossible to calculate this angle. To estimate it, we took advantage of the fact that the polarizations of S1(A1) and S1(A2) were large when the heavy chain was labeled at Cys-707 (suggesting that the globular part of S1 was immobile). The results of 15 experiments on five different fibers irrigated with high and low concentrations of S1 in which the heavy chain was labeled [S1R(A1) and S1R(A2)] are shown in Table 2. The differences in R_h, P_h, and P_v when HC was labeled were highly statistically significant at a level of confidence better than 4.9×10^{-5}. Similarly to S1(A1), the differences in polarization and dichroism in 15 experiments on five different fibers irrigated with high and low concentrations of S1R(A2) were statistically highly significant at a level of confidence better than 4.5×10^{-9}.

Using data for fibers irrigated with S1 labeled at the HC it is possible to calculate the angles that isomers of S1 make with the axis of muscle fiber. Let Θ define the polar angle that a long axis of S1 makes with the vertical. Following Wilson and Mendelson (1983) we assume that the chromophores are distributed with a Gaussian probability around the mean value of Θ with a standard deviation δ, i.e., that the probability of the dipole orientation at any angle Θ is given by a Gaussian function $\rho(\Theta) = \exp[-(\Theta - \Theta_{A1})^2/2\delta^2]$. Then it can be shown that for muscle in which thin filaments are saturated with isomers (see Appendix) $\Theta_{A1} = 72 \pm 1°$ and $\Theta_{A2} = 73 \pm 1°$. For muscle in which thin filaments are unsaturated, $\Theta_{A1} = 65 \pm 1°$ and $\Theta_{A2} = 65 \pm 1°$ (Table 3). Of course the absolute value of the angles may be different for S1 labeled at the ELC's.

Table 3: Orientation of the Transition Dipole of Rhodamine Incorporated into HC of S1(A1) or S1(A2)

irrigation with	Θ (deg)	δ (deg)
2.0 μM S1R(A1)[a]	72 ± 1[b]	23 ± 2
2.0 μM S1R(A2)[c]	73 ± 1	24 ± 1
0.1 μM S1R(A1)[d]	65 ± 1	23 ± 2
0.1 μM S1R(A2)[e]	65 ± 1	24 ± 1

[a] $\beta = 57°$. [b] All angles are with respect to the long muscle axis. [c] $\beta = 73°$. [d] $\beta = 57°$. [e] $\beta = 73°$.

DISCUSSION

Scatchard plots that are concave downward can result either from the non-homogeneity of the sample or from the cooperativity of binding. The data presented here suggests that non-homogeneity cannot be responsible for the anomaly. It is possible to demonstrate the same point theoretically: Let the equilibrium binding constant of the first fraction be C_1 and the equilibrium binding constant of the second fraction be C_2 [under our ionic conditions ([KCl] = 200 mM) both constants should be similar (Wagner *et al*., 1979; Chalovich *et al*., 1984), but in principle nonlinear Scatchard plots could have resulted from even a small difference]. We can write

$$C_1 = \frac{S1_B(1)}{A_F S1_F(1)}$$

$$C_2 = \frac{S1_B(2)}{A_F S1_F(2)}$$

$$S1_B(1) + S1_F(1) = S1_B(2) + S1_F(2) = {}^1/_2(S1_B + S1_F)$$

where $S1_B(1)$, $S1_B(2)$, $S1_F(1)$, and $S1_F(2)$ are the concentrations of bound and free fractions 1 and 2 of S1, respectively. A_F is the concentration of free actin. If each S1 can bind just one actin, then $A_F = A_T - M_B$. Solving the above set of equations for $S1_B/(S1_F A_T)$ as a function of Θ gives curves shown in Figure 5. Curve 1 is for $C_1 = 5 \times 10^6$ M^{-1}, and curve 2 is for $C_2 = 30 \times 10^6$ M^{-1}. Curve 3 is the Scatchard plot for the binding of the equimolar mixture of the two isomers (similar result is obtained if the mixture is not equimolar). All calculations were done for $A_T = 0.4$ μM. The Scatchard curve is concave upward (the second derivative of the Scatchard equation is negative) and is clearly different from the observation. If $C_1 = C_2 = C$, then the equations above reduce to a standard Scatchard equation $\Theta/S1_F = C(1 - \Theta)$.

Negative Cooperativity of Binding. If binding is not normal, then it must be cooperative: i.e., the binding of S1 to a single actin monomer must influence the binding of the subsequent S1's. This cooperativity cannot be of the positive type because in such a process the binding isotherms (θ vs $[S1_F]$) are sigmoidal, i.e., Scatchard plots ($\Theta/S1_F$ vs Θ) must be concave upward. This suggests that S1 binds F-actin with the negative cooperativity. This conclusion is consistent with the classical interpretation of a concave downward Scatchard plot being indicative of negative cooperativity (Cantor & Schimmel, 1980). Recent electron microscopic data supports this view (Funatsu *et al*., 1993).

There are two possible mechanisms by which the negative cooperativity could operate: (1) S1 could bind to a single actin monomer, and this could indirectly (through actin filament) reduce the affinity of the binding of different S1 to the neighboring monomer, and (2) S1 could bind to two actin monomers, and this could directly prevent the binding of another S1 to the neighboring actin. Binding to two monomers would occur only at low molar ratios of S1 to actin. Recent experimental evidence does not reveal any negative cooperativity in unregulated F-actin (Orlova *et al*., 1995), and therefore the first mechanism is unlikely to operate. On the other hand, direct evidence for the second mechanism exists: if S1 is indeed able to bind to two actins at low molar ratios, then its orientation should depend on the concentration. The data presented here shows that this is indeed the case for either isomer. Moreover, S1 could be cross-linked to two actin monomers by a zero-length cross-linker [1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide, EDC] to give a product containing two actins and one S1, this product was observed only at low molar ratios of S1 to actin (Andreev & Borejdo, 1992; Andreev *et al*., 1993b; Bonafe & Chaussepied, 1995).

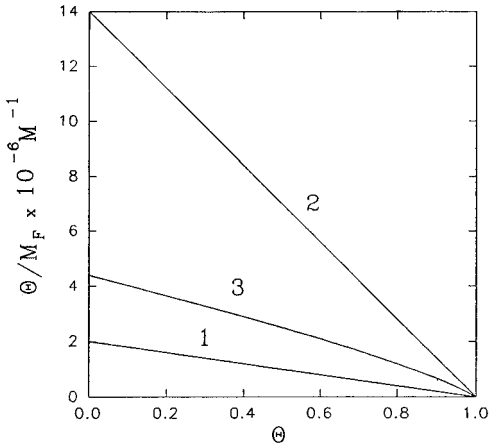

FIGURE 5: Scatchard plots of binding of two isomers of S1 to F-actin. Solution of the simultaneous equations gives the following dependence of Θ/M_F ($S1_F = M_F$) on Θ:

$$\frac{\Theta}{M_F} = \frac{(1 - \Theta)[C_1 + C_2 + 2AC_1C_2(1 - \Theta)]}{2 + A(1 - \Theta)(C_1 + C_2)}$$

Curve 1, both S1(A1) and S1(A2) bind to a single actin monomer with affinity C_1; $\Theta/M_F = C_1(1 - \Theta)$. Curve 2, both S1(A1) and S1(A2) bind to a single actin with affinity C_2; $\Theta/M_F = C_2(1 - \Theta)$. Curve 3, binding of equimolar mixture of S1(A1) and S1(A2) to show that Scatchard plot is very different than that observed here.

To quantify the negative cooperativity and to extract equilibrium constants from the observed concave downward shape of the Scatchard plots, we developed a two-state model of adsorption in which we suggested that S1 binds first, with equilibrium constant K_1, to one monomer in F-actin which is followed by slow binding, with isomerization constant K_2, to a neighboring monomer (Andreev *et al*., 1993a). This model predicts that in Scatchard form

$$\frac{\Theta}{m_F} = \frac{1 - 2\Theta + 2\Theta^2 + K_2(1 - 2\Theta)^2}{2(1 - \Theta)} \pm \sqrt{\left[\frac{1 - 2\Theta + 2\Theta^2 + K_2(1 - 2\Theta)^2}{2(1 - \Theta)}\right]^2 - \Theta^2}$$

where $m_F = K_1 M_F$. The best least-squares fit to the present

data using this equation, for both S1(A1) and S1(A2), was obtained with $K_1 = 2 \times 10^6$ M^{-1} and $K_2 = 6$. This is close to values reported before (Andreev *et al*., 1993a), considering differences in protein preparation.

Difference in the Orientation between High and Low Concentration of Isomers. Polarization measurements show that the orientation of S1(A1) added to muscle at high concentration is different from the orientation of this isomer added at low concentration. The same is true for S1(A2). This confirms the original observation of myofibrils irrigated with unfractionated S1 labeled at Cys-707 (Andreev *et al*., 1993c). It is impossible that the effect was due to attachment of S1 to different parts of a sarcomere at different concentrations because such an effect is known to depend only on Ca^{2+} concentration (Swartz *et al*., 1990) and in the present experiments the rigor solution always contained 0.1 mM Ca^{2+}.

Difference in Polarization of Fluorescence between S1(A1R) and S1(A2R). No difference in polarization was found when actin filaments were fully saturated with S1(A1R) or with S1(A2R). In contrast, in unsaturated filaments polarization of S1(A1R) was different from polarization of S1(A2R). This is not surprising, in view of the recent finding that A1 of S1(A1) could be cross-linked to actin only at a low degree of saturation of actin filaments with S1(A1) (Andreev & Borejdo, 1995). A2 of S1(A2) could not be cross-linked to actin at any degree of saturation. Thus, in saturated filaments neither A1 nor A2 interacts with actin, and therefore their orientation is similar. In contrast, in unsaturated filaments A1 does interact with actin, and this interaction is likely to affect orientation of A1 in comparison with A2.

The differential binding of isomers of S1 in unsaturated filaments is consistent with the fact that the 41-amino acid difference piece in the sequence of A1 enables it to associate with actin. Proton NMR shows immobilization of A1 by actin (Prince *et al*., 1981), and the affinity chromatography of the thrombic digests of A1 (Henry *et al*., 1980), the cross-linking of A1 to actin (Sutoh, 1982), and the immobilization of fluorescent hydrophobic probe bound to A1 by actin (Borejdo, 1983) all suggest direct interaction of A1 with actin. The small magnitude of the difference of polarizations is no doubt due to the fact that the label is located at Cys-177 which is distant from the N-terminus of A1 through which A1 binds to F-actin. Scatchard plots at low molar ratios do not show clear differences between S1(A1) and S1(A2). This is due to the fact that the measurements at low concentrations of S1 are inaccurate (Andreev *et al*., 1993a) and to the weak interaction of A1 with F-actin in conditions under which sedimentation experiments were carried out (200 mM KCl).

APPENDIX

Let Θ define the angle that a fixed line drawn through a cross-bridge (e.g., its long axis) makes with the vertical in the laboratory coordinate frame $X-Y-Z$ (Figure 6, left). Let μ be the angle between the absorption and emission dipoles of the dye residing on a cross-bridge (in the case of HC labeling it is the angle between the averages resulting from stacking of two IATR molecules; Ajtai *et al*., 1992). Following Wilson and Mendelson (1983) we make the transformation to cross-bridge coordinate frame $U-V-W$. Since we are not interested in the absolute orientation of the

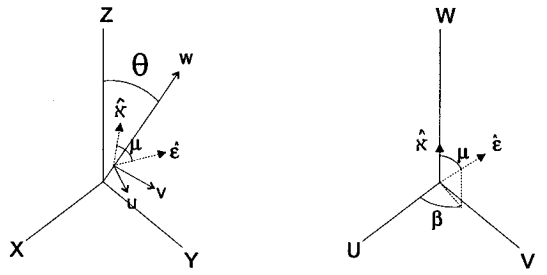

FIGURE 6: Definition of the parameters of the model. The muscle axis lies along the *Z*-axis. The direction of propagation of exciting light is perpendicular to the muscle axis. The direction of observation forms an angle of 360° with direction of propagation of exciting light. Left panel: Θ is the polar angle of cross-bridge. $\aleph$ and ϵ are the absorption and emission angles of the dye, respectively, and μ is the angle between them. Right panel: after transformation to the cross-bridge coordinate frame, μ is the angle of emission dipole and β is the angle between *U* and the projection of μ on the $U-V$ plane. Angles μ and β are assumed to be the same for cross-bridges in both states.

cross-bridge, we set absorption dipole $\aleph$ parallel to the vertical in the molecular coordinate frame. It follows that $\epsilon = \mu$. Let β be an angle between projection of ϵ on the $U-V$ plane and *U*-axis (Figure 6, right). The fluorescent intensity of the horizontal component of fluorescent light emanating from a fiber is a function of Θ, δ, μ, and β

$$ {}_{h}I_{h} = {}_{h}I_{h}(\Theta, \delta, \mu, \beta) \tag{5}$$

and similarly for other orthogonal components. By using the expressions given by Wilson and Mendelson (1983), one can derive explicit expressions for the orthogonal intensities (the full expressions are available from the authors). Since

$$P_h = ({}_{h}I_{h} - {}_{h}I_{v})/({}_{h}I_{h} + {}_{h}I_{v}) \tag{6}$$

$$P_v = ({}_{v}I_{v} - {}_{v}I_{h})/({}_{v}I_{v} + {}_{v}I_{h}) \tag{7}$$

$$Q_h = ({}_{h}I_{h} - {}_{v}I_{h})/({}_{h}I_{h} + {}_{v}I_{h}) \tag{8}$$

$$R_h = 2(1 - \sin(\delta)^2 - \sin(\Theta)^2 + \tag{9}$$

$$(4 \sin(\delta)^2 \sin(\Theta)^2)/3)/$$

$$(\sin(\delta)^2 + \sin(\Theta)^2 -$$

$$(4 \sin(\delta)^2 \sin(\Theta)^2)/3)$$

substitution of expressions for polarization component (such as the explicit form of eq 5) into eqs 6−9 obtains polarizations and dichroism as functions of β, δ, μ, and Θ_2. μ can be calculated from the anisotropy, *r*, of immobilized rhodamine (34%; Xiao *et al*., 1995) as $\mu = \cos^{-1}\mu = [(5r + 1)/3]^{1/2} = 18°$. One obtains thus a set of four equations relating polarization components to the angles β, δ, and Θ.

The object of further analysis is to calculate β, δ, and Θ from the experimental data. We follow here the treatment detailed in Xiao *et al*. (1995). The experiments yield the range of values for P_h, P_v, Q_h, and R_h. We seek a set of angles β, δ, and Θ that are consistent with the set of experimental values of polarization components and of

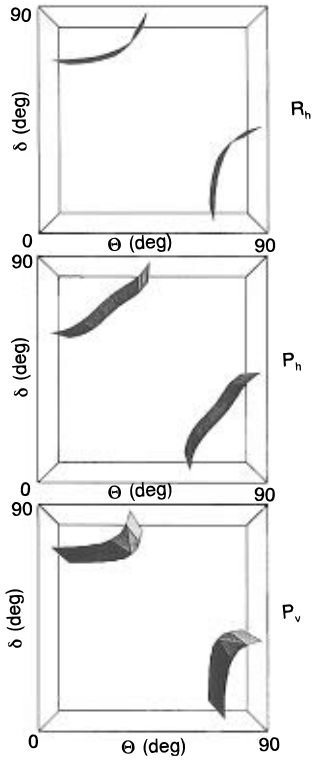

FIGURE 7: The dichroic ratio (top) and polarizations (middle and bottom) for fiber irrigated with 2.0 μM IATR−S1(A1). In these 3D curves the dichroism or polarizations are plotted along *Z*-axis; the angle Θ characterizing the inclination of the cross-bridges is plotted along the *X*-axis, and δ, the angle characterizing disorder, is plotted along the *Y*-axis. Only the values $0.282 < R_h < 0.306$, $-0.158 < P_h < 0.140$, and $0.663 < P_v < 0.683$ are shown. $\beta = 57°$ and $\delta = 23 \pm 2°$. The ribbons overlap at $\Theta = 72°$ and $\delta = 23°$.

dichroism. R_h for a fiber irrigated with a high concentration of rhodamine−S1 is shown in the three-dimensional graph in Figure 7 (top). The experimental range ($0.282 < R_h < 0.306$) is drawn on the *Z*-axis, and Θ and δ are on *X*- and *Y*-axes, respectively. Shaded areas indicate possible values of δ and Θ that are consistent with the measured R_h. Corresponding plots for P_h and P_v are given in Figure 7 (middle and bottom). (As pointed out in the Results, Q_h is not an independent variable because $Q_h \approx P_h$.) The three ribbons for muscle irrigated with high concentration of S1 overlap at the following values of δ and Θ: $\Theta = 72°$, $\delta = 23°$ and $\Theta = 23°$, $\delta = 72°$. The second solution can be rejected, because it implies poor orientation of the dye, whereas in fact heavy chains are quite immobile as indicated by large values of polarizations and small dichroism (Table 1). The fit to the data obtained when fiber was irrigated with a low concentration of S1(A1) gives $\beta = 57°$, $\delta = 23°$, and $\Theta = 65°$ (Table 3). The fit to the data obtained when fiber was irrigated with S1(A2) is given in Table 3.

An attempt was made to fit the data with a more complex model, in which S1's are distributed between two populations corresponding to the orientation of S1 added at high and low concentration (Xiao *et al*., 1995). However, because of low polarizations (high mobility of essential light chain), such a fit could not be made.

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