Effect of ADP on Binding of Skeletal S1 to F-Actin

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Received July 14, 1998; Revised Manuscript Received October 23, 1998

ABSTRACT: The proximity of skeletal myosin subfragment-1 (S1) to actin, and its orientation with respect to thin filaments of single muscle fibers, were compared in the presence and in the absence of ADP. The proximity was assessed by the efficiency of carbodiimide-induced cross-linking and the orientation by polarization of fluorescence of probes attached to the essential light chains. ADP made no difference in proximity or orientation when the molar ratio of S1 to actin was low or high. However, at the intermediate ratios, ADP made a significant difference. Strong dissociating agents, AMP-PNP and PP_i, made significant differences at all ratios. To explain this behavior, it is unnecessary to invoke the ADP-induced "swinging" of the tail of S1. Rather, it is simply explained by the "two-state" model which we proposed earlier, in which S1 binds to one or to two actin protomers, depending on the saturation of the filaments with S1s. The dissociation induced by the ADP shifts the equilibrium between the two bound states. At high and low degrees of saturation, ADP is unable to significantly decrease the amount of S1 bound to F-actin. However, at intermediate saturation levels, ADP causes significantly more S1s to bind to two actins. These results suggest that the ADP-induced changes seen at the intermediate molar ratios are due to the dissociation-induced reorientation of S1.

It has been suggested that the conformational change associated with the power stroke of a cross-bridge occurs in the "tail" region of myosin subfragment-1 (S1)[1] (*1*). Support for this hypothesis has been obtained from the electric birefringence experiments (*2*) which showed that changes in the hydrodynamic radius of S1 upon hydrolysis of ATP could be explained by the changes occurring in the tail region. Small-angle synchrotron X-ray scattering experiments showed that conformational change occurred in the tail of S1 during the hydrolysis of ATP (*3*) and upon the binding of different ATP analogues (*4*). Similarly, recent studies have suggested that structural (*5*) and chemical (*6*) differences between the complexes of S1·Mg·ADP carrying different phosphate analogues occur near the COOH terminus of the motor domain. Also, the light chain region of myosin is capable of motions independent of the catalytic domain (*7*). In view of these findings, it is reasonable to expect that part of the muscle power stroke arises from the rotational motion of the tail.

Recently, such rotational motion has been observed in the regulatory light chain of smooth S1 and brush border myosin I bound to skeletal actin (*8, 9*). The rotation was caused by the desorption of ADP. However, it has been reported that ADP had no effect on the orientation of S1 from skeletal muscle (*10, 11*). Since the mode of binding of skeletal myosin head depends on the molar ratio S1:actin (*12*−*14*), we hoped to shed light on this apparent discrepancy by studying the effect of ADP at different ratios. We have taken advantage of the fact that the rotational motion is detectable as a change in the proximity and the orientation of S1 bound to F-actin. The proximity was assessed by the ability of carbodiimide to form a zero-length cross-link between S1 and actin, and the orientation was measured by the polarization of fluorescence of probes attached to the light chains of S1. The control experiments confirmed that ADP had an effect (albeit small) on smooth S1, and that it had no effect on skeletal S1 at concentrations which saturate thin filaments. However, at the intermediate ratios of S1 to actin, ADP made *a significant difference* in the cross-linking and orientation of skeletal S1. Strong dissociating agents, AMP-PNP and PP_i, also made a significant difference at any molar ratio.

To explain this behavior, it is unnecessary to invoke the ADP-induced "swinging" of the tail of S1. Instead, it can be explained by a "two-state model" in which skeletal S1 binds to one actin protomer (state 1) when the actin filament is saturated with S1s and binds in a different orientation to two protomers (state 2) when the filament is unsaturated (*12*−*14*). Nucleotides increase the degree of dissociation of the S1 bound in state 1 but do not effect the rate of transition from state 1 to state 2. At high and low S1:actin ratios, ADP is unable to significantly decrease the amount of S1 bound to actin. However, at intermediate ratios, even a weak dissociation caused by ADP is sufficient to cause a significant increase in the amount of S1 bound to the two actins. Strong dissociating agents cause significant change in the amount of S1 bound to one or to two actins at any molar ratio.

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[1] Abbreviations: S1, myosin subfragment-1; ELC, essential light chains; A, actin; HC, heavy chain of S1; LC1, essential light chain 1; LC3, essential light chain 2; RLC, regulatory light chain; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; MR, S1:actin molar ratio; AP_5A, P^1,P^5-diadenosine-5′-pentaphosphate; 5′-IATR, 5′-(iodoacetamido)rhodamine.

10.1021/bi981664c CCC: $15.00
Published on Web 12/03/1998

MATERIALS AND METHODS

Chemicals. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), P^1,P^5-diadenosine-5′-pentaphosphate (AP_5A), and nucleotides were from Sigma (St. Louis, MO). 5′-(Iodoacetamido)rhodamine (5′-IATR) was from Molecular Probes (Eugene, OR). 6′-(Iodoacetamido)rhodamine (6′-IATR) was a gift from S. Lowey (University of Vermont, Burlington, VT).

Solutions. The rigor solution contained 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 ADP solution had the same composition as rigor but contained in addition 100 μM AP_5A to inhibit the adenylate kinase and 2 mM ADP.

Proteins. Myosin was prepared from the back muscles of rabbit using the method of Tonomura et al. (*15*). Skeletal S1 and S1 isoforms were prepared as described previously (*16*). Skeletal F-actin was prepared as described in ref *17*. Skeletal ELCs were a gift from R. Takashi. Smooth S1 and smooth regulatory light chains (RLC) were gifts from S. Lowey (University of Vermont). To minimize the digestion of HC of S1 by papain, the digestion of myosin was carried out in the presence of actin. The concentrations of proteins were measured using the following values for the extinction coefficients ($A^{1\%}$ at 280 nm): myosin, 5.0 (*15*); S1, 7.5 (*18*) [using a molecular mass of 120 kDa for S1 and 111 kDa for S1 (LC3)]; G-actin, 6.3 (measured at 290 nm) (*19*); F-actin, 6.7 (measured at 290 nm) (including correction for light scattering, A. Oplatka, personal communication); LC1, 2.3 (*20*); and LC3, 1.9 (*21*). The purity of the proteins was checked by SDS−PAGE.

Cross-Linking Reaction. S1 and F-actin were mixed at different molar ratios and incubated for 0.5−1 h at room temperature; appropriate amounts of EDC were then added. The reactions were stopped by adding an equal volume of electrophoresis sample solution (4% SDS, 24% glycerol, 100 mM Tris, 4% mercaptoethanol, and 0.02% bromphenol blue). Unless otherwise indicated, all cross-linking experiments were carried out in solutions containing 0.2 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 7.5). The low concentrations of $MgCl_2$ and KCl were used to prevent the formation of actin filament bundles (*12*, *22*). The light scattering measurements did not detect any bundle formation in this buffer solution.

Tricine Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Gel electrophoresis was carried out according to Schagger and Jagow (*23*) using 8% polyacrylamide gels. After electrophoresis and staining, the slab was dried using a Novex Gel Dryer Kit (Novex Co., San Diego, CA). Dry gels were scanned in a transmission mode (transmission arm 01719) on a SM3+ Howtek scanner (Hudson, NH). Scanning was controlled by a Sun SPARC Classic XT workstation running PDI (Huntington Station, NY) Discovery series software. The relative intensity of the various bands was measured by the Image Pro Plus program (Media Cybernetics, Silver Spring, MD) as described in ref *24*. The calibration of the scanner was achieved using Kodak Stepped Density Filters.

Labeling and Exchange of RLC into Smooth S1. Isolated smooth RLC was labeled at Cys-108 by incubation with a 5-fold molar excess of IATR for 4 h in 50 mM KCl, 2 mM EDTA, and 10 mM phosphate buffer at pH 7.0 and 5 °C. The free dye was removed by passing the light chains through Sephadex-50 column and by dialyzing against a rigor solution. The labeled light chains were exchanged with the native RLC of smooth S1 as described in ref *25*.

Labeling and Exchange of LC1 and LC3 into Skeletal S1. The procedure was to label isolated ELC at Cys-178 with IATR, remove the free dye, exchange it into S1, and separate free light chains from exchanged S1 on DE-52. The essential light chains were prepared according to ref *21*. The isolated light chains were labeled by incubation with a 5-fold molar excess of IATR for 4 h in 50 mM KCl, 2 mM EDTA, and 10 mM phosphate buffer at pH 7.0 and 5 °C. The free dye was removed by passing the light chains through a Sephadex-50 column and dialyzing against the rigor solution. The labeled light chains were exchanged with light chains of S1 as described in ref *26*. To separate free light chains from exchanged S1, the sample was passed through a DE-52 column. The concentration of labeled S1 was calculated from the absorption of rhodamine at 280 ($A^{1\%} = 18\,750$) and 555 nm ($A^{1\%} = 75\,000$) with the equation $c_{S1}(\text{mg/mL}) = (A_{280} - A_{555}/4)/0.75$ and the concentration of the dye with the equation $c_{IATR}(\text{mM}) = A_{555} \times 10^3/75.0$. Typically, 30−40% of the S1 was labeled.

Irrigation of Fibers with S1. The I-bands were saturated or not saturated with S1 by incubating a fiber with 2 or 0.1 μM labeled S1, respectively. The incubation was carried out in the dark for 1 h at room temperature. Excess S1 was removed by washing with the rigor solution, and the measurements were begun within 5 min of washing (to minimize the increase in the background due to the gradual dissociation of S1).

Measuring Polarization of Fluorescence. The polarization of fibers was measured as described previously (*27*). The birefringent crystal split the emitted light into ordinary and extraordinary rays. The different polarization quantities are defined as

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

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

where subscripts before the symbol I indicate the direction of the polarization of the exciting light ($\perp$ and $||$ for perpendicular and parallel polarization with respect to the axis of filaments, respectively) and the subscripts after the symbol I indicate the direction of the polarization of the emitted light. The correction factors $C_{||}$ ($={}_{||}I_{||}/{}_{||}I_{\perp}$) and $C_{\perp}$ ($={}_{\perp}I_{\perp}/{}_{\perp}I_{||}$) measure the transmission efficiency of the dichroic mirror. For the rhodamine mirror (Zeiss FT 580), $C_{\perp}$ and $C_{||}$ were 1.93 and 0.98, respectively. Intensities were corrected for photobleaching as described elsewhere (*28*).

Control Experiment: The Effect of ADP on Orientation of Smooth S1. To determine whether the previously reported effect of ADP on the orientation of smooth S1 (*8*, *10*) could be reproduced here, myofibrils were irrigated with smooth S1 carrying labeled RLC and placed on a rotating stage of a polarization microscope. The stage was rotated until a single myofibril was oriented horizontally (in the laboratory frame of reference). The mean parallel polarization was 0.350 ± 0.006 in rigor and 0.343 ± 0.008 in the presence of ADP. The mean perpendicular polarization was 0.151 ± 0.005 in rigor and 0.198 ± 0.007 in the presence of ADP. The

difference in $P_{||}$ was marginally significant ($p = 0.518$, $t = 0.676$), but the difference in $P_{\perp}$ was highly significant ($p = 0.002$, $t = -5.01$). The data were fitted to a Gaussian model (*29*), and the angles were estimated by the method of Xiao and Borejdo (*28*). Θ, the average polar angle of the transition dipole of 6-IATR with respect to the axis of the actin filament, was $22 \pm 1°$ for the myofibrils in rigor and $24 \pm 1°$ for the myofibrils in the presence of ADP. δ, the average standard deviation of Θ (characteristic of the degree of disorder of the dipoles), was $45 \pm 1°$ for the myofibrils in rigor and $47 \pm 1°$ for the myofibrils in the presence of ADP. Thus, these results show that ADP has a small but statistically significant effect on the polar angle and on the degree of order of the rhodamine excitation/emission dipole attached to RLC of smooth S1. The same results were obtained with a different dye (5′-isomer of IATR) and with a different muscle preparation (the single muscle fibers, data not shown).

All subsequent results refer to skeletal S1.

RESULTS

Effect of ADP on Cross-Linking. Skeletal S1 and actin were mixed at different ratios. The results of cross-linking are shown in Figure 1A. At a low S1:actin ratio, the following major bands are visible (in order of decreasing molecular masses): 235, 210, 185, 160, 150, 120, 95, 66, 43, and 24 kDa. In agreement with earlier work, the formation of the 235, 210, 185, 160, and 66 kDa complexes was inhibited at high molar ratios (*13*). The comparison of Coomassie blue staining (Figure 1A), Western blots using antibodies against LC1 (Figure 1B), and the fluorescence patterns obtained with fluorescent actin (Figure 1C) and fluorescent S1 (not shown) suggests that the bands correspond to the following complexes: 235 kDa, HC + A + A + LC1; 210 kDa, HC + A + A; 185 kDa, HC + A + LC1; 160 kDa, HC + A (cross-linked through a site on a 50 kDa tryptic fragment); 150 kDa, HC + A (cross-linked through a site on a 20 kDa tryptic fragment); 120 kDa, HC + LC1; 66 kDa, A + LC1; 95 kDa, HC; 43 kDa, A; and 24 kDa, LC1.

The effect of ADP on the formation of adducts at different molar ratios of S1(LC1):actin was examined in three experiments using different protein preparations. Qualitatively, ADP had the same effect in each experiment. Quantitatively, the least accurate measurement was that of the intensity of the 210 kDa band at a molar ratio of 1. Even in this case, however, the ratio R of the intensity in rigor to the intensity in the presence of ADP could be determined with 6% accuracy (the average value of R was 0.73 ± 0.04). The 95% confidence interval was 0.17. The same experiment was carried out with S1(LC3). In this case, the bands corresponding to HC + A + A + LC1 (235 kDa), HC + A + LC1 (185 kDa), and A + LC1 (66 kDa) were absent. The 95% confidence interval was 0.19. Therefore, all R values smaller than 0.83 or larger than 1.17 [for S1(LC1)] and smaller than 0.81 or larger than 1.19 [for S1(LC3)] were considered significant. The data are summarized in Table 1. Deviations from an R of 1 that are statistically significant are bold and underlined.

Table 1 shows that ADP had no effect on the formation of the intensity of an adduct indicative of 1:1 binding (150 kDa) at all MR, and on the adducts that are indicative of 1:2 binding (210, 160, and 66 kDa) at a molar ratio of 0.25.

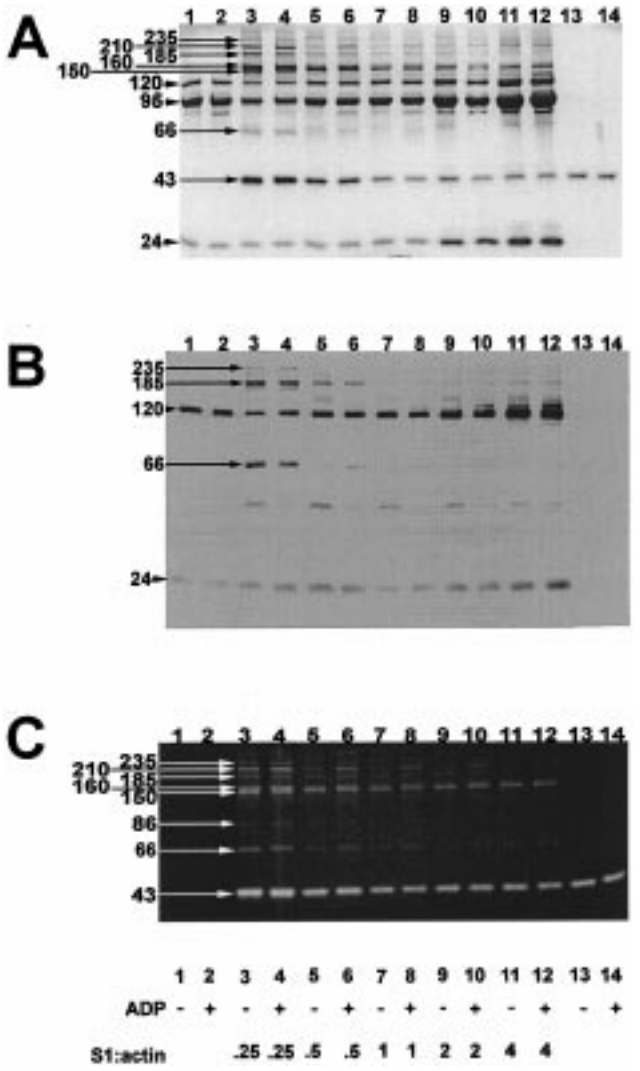

FIGURE 1: Effect of ADP on cross-linking of skeletal S1 with F-actin at different molar ratios. Cross-linking was achieved with 75 mM EDC for 40 min at room temperature in 50 mM KCl, 0.5 mM $MgCl_2$, and 10 mM Tris-HCl buffer at pH 7.5 and 23 °C. (A) Tricine SDS−polyacrylamide (8%) gel stained with Coomassie blue. (B) Western blot of the same gel using antibodies against LC1. (C) Fluoresence picture of the same gel, with actin fluorescently labeled at Gln-41 with dansyl cadaverine-rhodamine according to Takashi (1988). All lanes contain EDC: lane 1, S1(LC1); lane 2, same as lane 1 and 2 mM ADP; lane 3, 8 μM actin and 2 μM S1; lane 4, same as lane 3 and 2 mM ADP; lane 5, 4 μM actin and 2 μM S1; lane 6, same as lane 5 and 2 mM ADP; lane 7, 4 μM actin and 4 μM S1; lane 8, same as lane 7 and 2 mM ADP; lane 9, 4 μM actin and 8 μM S1; lane 10, same as lane 9 and 2 mM ADP; lane 11, 2 μM actin and 8 μM S1; lane 12, same as lane 11 and 2 mM ADP; lane 13, 2 μM actin; and lane 14, same as lane 13 and 2 mM ADP. The ADP concentration was 2 mM, and the sample contained 1 mg/mL hexokinase and 100 mM glucose.

However, ADP significantly increased the formation of 210 and 160 kDa adducts when the cross-linking was carried out at a ratio of 0.5 (lanes 5 and 6). Similarly, ADP significantly increased the extent of formation of 210 and 66 kDa adducts when the cross-linking was carried out at a ratio of 1 (lanes 7 and 8) and 2 (lanes 9 and 10). The significance of these results will be dealt with in the Discussion.

Effect of AMP-PNP and PP_i on Cross-Linking. As a control, we examined the effect of agents that have stronger

Table 1: Ratio of the Intensities of the Various Bands in the S1−Actin Cross-Linking Experiment in the Presence and Absence of ADP

band	adduct	*R* (rigor:ADP ratio) 0.25 MR	0.5 MR	1 MR	2 MR	4 MR
	Data from Cross-Linking of S1(LC1) with Actin					
235	HC + A + A + LC1	0.96	NM[a]	NM	NM	NM
210	HC + A + A	0.88	**0.70**[b]	**0.73**	**0.80**	1.02
185	HC + A + LC1	1.12	1.00	NM	NM	NM
160	HC + A	1.02	**0.82**	NM	NM	NM
150	HC + A	1.06	1.09	0.97	1.12	0.96
120	HC + LC1	0.95	1.01	1.00	0.90	1.02
95	HC	1.00	1.01	1.04	**0.78**	0.99
66	A + LC1	0.97	0.84	**0.71**	NM	NM
43	A	1.00	1.06	0.99	1.00	1.00
24	LC1	0.96	0.99	1.05	0.83	1.02
	Data from Cross-Linking S1(LC3) with Actin					
210	HC + A + A	1.02	**0.81**	**0.77**	0.83	1.12

[a] NM indicates that the intensity was to weak to be measured. This is because the formation of the 235, 185, 160, and 66 kDa bands is inhibited at high S1:actin molar ratios (see the text). [b] Statistically significant deviations from 1 are bold and underlined.

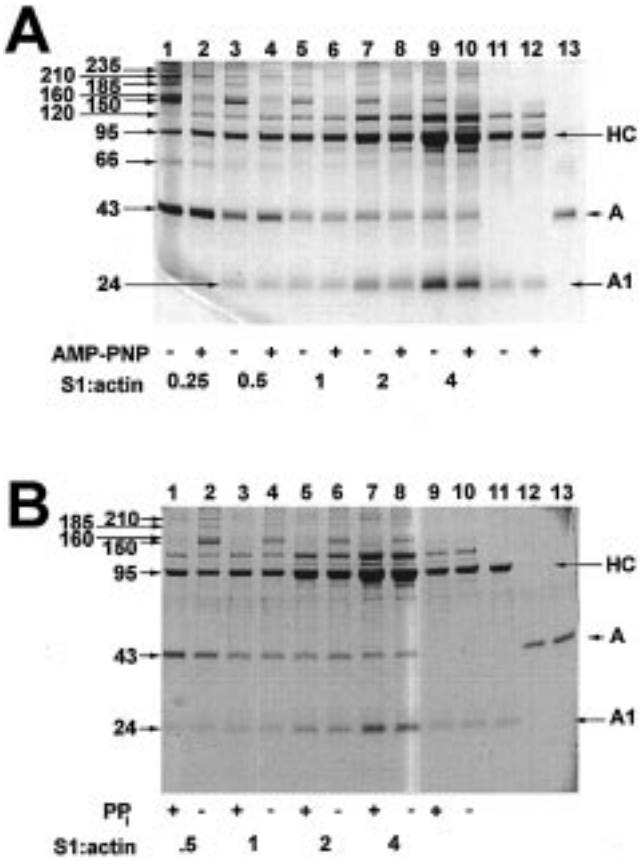

FIGURE 2: Effect of AMP-PNP (A) and PP_i (B) on cross-linking of F-actin with skeletal S1 at different molar ratios. Conditions were identical to those described in the legend of Figure 1. The assignment of bands is based on the assumption that the bands are equivalent to those seen in the ADP experiments. The molecular masses, in kilodaltons, of the cross-linked adducts are at the left. AMP-PNP and PP_i were either absent (−) or present (+). The S1:actin molar ratios are indicated at the bottom. Abbreviations: HC, heavy chain of S1; A, actin; A1, essential light chain 3.

dissociating action than ADP, AMP-PNP and PP_i (*30*−*32*). The pattern of cross-linking is shown in Figure 2. The assignment of bands is based on the assumption that the bands are equivalent to those seen in the ADP experiments. The intensity of an adduct indicative of 1:1 binding (150 kDa) decreased at all ratios. The intensities of adducts indicative of 1:2 binding (210, 160, and 66 kDa) were more complex. At a high ratio, the intensity of the 210 kDa adduct was by 14% larger in the presence of AMP-PNP (Figure 2A, lane 10) than in its absence (Figure 2A, lane 9), and its intensity was by 11% larger in the presence of PP_i (Figure 2B, lane 7) than in its absence (Figure 2B, lane 8). Conversely, at a low molar ratio, the intensity of the 210 kDa adduct was by 55% smaller in the presence of AMP-PNP (Figure 2A, lane 2) than in its absence (Figure 2A, lane 1), and its intensity was by 31% smaller in the presence of PP_i (Figure 2B, lane 1) than in its absence (Figure 2B, lane 2). At intermediate ratios, the intensities of 210 and 160 kDa adducts decreased. The significance of these findings will be dealt with in the Discussion.

Effect of ADP on Orientation of S1. The orientation of S1 diffused into single muscle fiber was assessed by polarization of fluorescence of rhodamine probe attached to the single thiol (Cys-178) of LC1 or LC3. Cys-178 was modified with 5′-IATR, and fluorescent ELC were exchanged with the native light chains of chymotryptic S1 as described in Materials and Methods. In agreement with Holt and Lowey (*19*), the modification of Cys-177 had little effect on the enzymatic activity of S1. The saturation or nonsaturation of the I-bands of a single muscle fiber was achieved by irrigating muscle with 2.0 or 0.1 μM S1, respectively. The polarization of fluorescence was measured as described in ref *27*. Four measurements were obtained from different regions of each fiber (except fiber 4 from which three measurements were obtained). Immediately after the polarization in rigor was measured, 2 mM ADP was added. No contraction of fibers was detected. The value of every polarization in rigor was subtracted from the value of every polarization in ADP, giving 16 measurements from each fiber (nine from fiber 4). The differences between the parallel and perpendicular polarizations for S1(LC1) are shown in panels A and C of Figure 3, respectively. The mean difference is close to zero, and the differential is random. A similar plot for S1(LC3) is shown in panels B and D of Figure 3. Addition of ADP had no statistically significant effect on average values of $P_{||}$ for S1(LC1) ($t = -1.78$, $p = 8.83 \times 10^{-2}$) and for S1(LC3) ($t = -2.03$, $p = 5.44 \times 10^{-2}$). Similarly, ADP had no effect on avearge values of $P_{\perp}$ for S1(LC1) ($t = -1.98$, $p = 6.00 \times 10^{-2}$) and for S1(LC3) ($t = -3.72$, $p = 1.26 \times 10^{-3}$). Similar results were obtained from fibers which were irrigated with nonsaturating concentrations of S1 (not shown).

DISCUSSION

Cross-linking with a zero-length reagent EDC is a sensitive method for assessing changes in proximity (*33*). Even a small shift in the position of the amino relative to the carboxyl groups of the two moieties is expected to modify the rate of the reaction. Similarly, the polarization of fluorescence is a sensitive probe of orientation (*34*). Control experiments showed that ADP had a small effect on the orientation of smooth S1. This result is in general agreement with earlier work (*8*, *10*) except that the rotation reported here is smaller. It is unlikely that the small size can be explained by the fact that the rotation occurs mostly in the azimuthal plane (*35*),

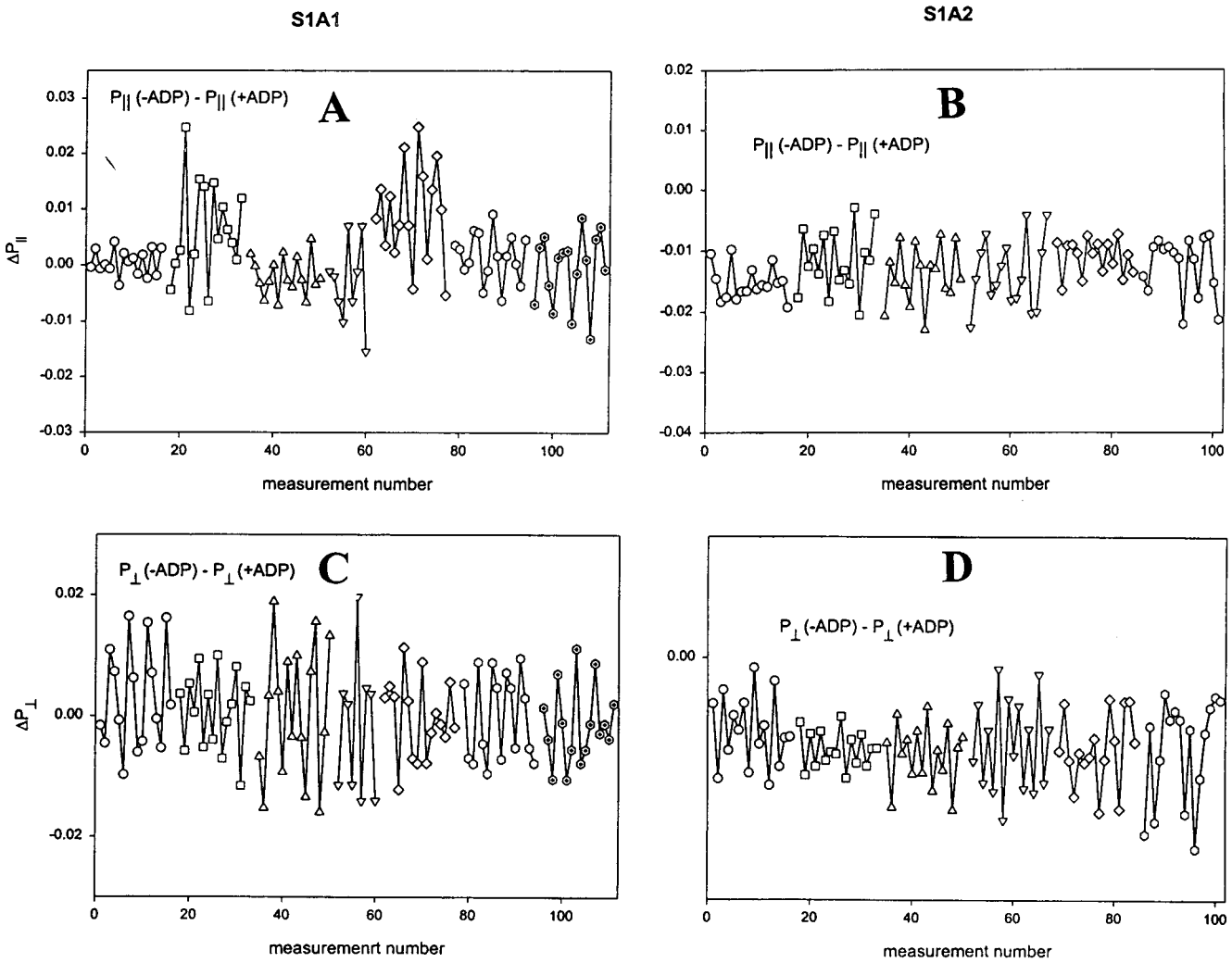

FIGURE 3: Difference in polarization of fluorescence of single muscle fibers in rigor and in the presence of 2 mM ADP. Fibers were irrigated with 2 μM skeletal S1 labeled with 5′-IATR at LC1 (=A1) (A and C) or LC3 (=A2) (B and D). (A and B) $\Delta P_{||}$. (C and D) $\Delta P_{\perp}$. Symbols indicate different fibers.

because the same results were obtained with the 6′-isomer of IATR (data not shown).

The main finding of this paper is that ADP had no effect on skeletal S1 when actin was fully or sparsely decorated with S1, but that there was a significant effect at intermediate levels of saturation. This finding can be simply explained by a two-state model which we proposed previously (*12*−*14*). In this model, the heavy chain of S1 binds to one actin protomer (state 1) when the actin filament is saturated with heads and binds in a different orientation to two protomers (state 2) when it is unsaturated. Let us suppose that the actin filament is a long single row of "A" monomers and that the S1 molecule can bind to this filament in two possible states. In state 1, it binds to one actin monomer (schematically visualized as a "vertical" position), and in state 2, it binds to two actin monomers (schematically visualized as a "horizontal" position, Figure 4). Adsorption of S1 proceeds as two consecutive, reversible reactions

$$A + M \leftrightarrow A{\cdot}M$$

$$A{\cdot}M \{+ A\} \leftrightarrow A{\cdot}M{\cdot}A$$

The first reaction is a "classical" second-order reaction where S1 adsorbs to F-actin from the solution. The second reaction is an isomerization where S1 changes the conformation and now binds to two actins. Braces are used to emphasize the fact that S1 now attaches to the second actin. In this reaction, the rate constants do not depend on the absolute concentration of actin. They depend only on the probability that the actin monomer, the neighbor of the point of attachment of S1, is unoccupied (*14*).

We assume that S1 first adsorbs from solution (to any free actin binding site) in state 1 (A·M). After initial binding, S1 changes orientation and, provided that the neighboring binding site is free, binds an additional actin monomer (A·M·A). The kinetic constants of these reactions are k_{+1} and k_{-1}, and k_{+2} and k_{-2}, respectively. K_1 ($=k_{+1}/k_{-1}$) is the equilibrium adsorption constant for state 1 (units of M^{-1}). K_2 ($=k_{+2}/k_{-2}$) is the equilibrium constant of transition from state 1 to state 2 (unitless). The model makes the following predictions. At high S1:actin molar ratios, most S1s are in state 1. A moderate decrease in the equilibrium constant K_1 with no concurrent change in the constant K_2 causes only a small decrease in the number of S1s in this state. Consequently, the number of S1s in state 2 is not significantly changed. At low degrees of saturation, most S1s are bound

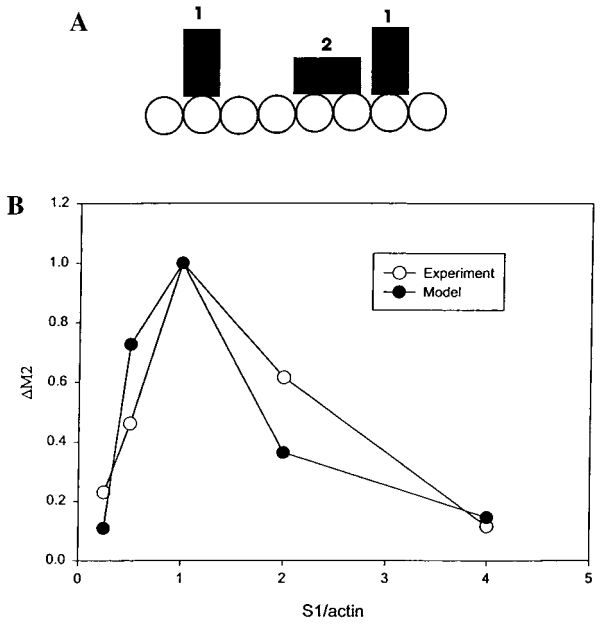

FIGURE 4: (A) Schematic diagram of two-state binding of skeletal S1 to F-actin. S1 can bind to one actin protomer (depicted as a vertical position) or to two actin protomers (depicted as a horizontal position). (B) The amount of S1 bound to two actins in the presence of ADP minus the amount bound in rigor, as a function of a S1:actin molar ratio for the model (●) and experiment (○).

in state 2 (i.e., there are few S1s in state 1). A moderate decrease in the equilibrium constant K_1 causes a small decrease in the number of S1s in already sparsely populated state 1, and consequently only a small increase in the number of S1s in state 2. However, at intermediate levels of saturation, the release of some S1s leads to a situation where many actin sites adjoining the remaining S1s in state 1 become vacant with the result that some S1s in state 1 can now undergo the transition to state 2. Consequently, the fraction of S1s in state 1 remains approximately unchanged, while the fraction of S1s in state 2 is significantly increased.

ADP is an analogue which decreases K_1 by a moderate amount (*30*−*32*, *36*). The predictions of the model and experiment were compared by measuring the extents of production of 160 and 210 kDa complexes (Figure 4). Both are proportional to the number of S1s in state 2 (*14*). The 160 and 210 kDa complexes contain one S1 cross-linked via the second site to one actin and via both sites to two actins, respectively. We have measured the intensities of 160 and 210 kDa bands in the gel and calculated the contribution of S1 in these bands by assuming that the actin uptake of Coomassie Blue was 1.3 times higher than that of S1 (*37*) and that S1 contains 2.21 times more amino acid residues than actin. Thus, the amount of S1 in the 160 kDa band was calculated with the equation $[S1]^{160} = I/(1 + 1.3/2.21)$, and the amount of S1 in the 210 kDa band with the equation $[S1]^{210} = I/(1 + 1.3/2.21 + 1.3/2.21)$, where I is the intensity of the band. The sum of the contributions of S1 in the 160 and 210 kDa bands was taken as a value proportional to the number of S1s in state 2 (Figure 4, open circles). Predictions of the model, assuming that ADP decreases K_1 by a factor of 10, are shown as filled circles.

If an analogue decreases the equilibrium constant K_1 by a large amount (as AMP-PNP and PP_i do; *30*−*32*), the model predicts that the number of S1s in state 1 always decreases. The behavior of S1s in state 2 depends on the S1/actin molar ratio; at high ratios where most S1s are in state 1, there is a significant decrease in the number of S1s in this state. Consequently, many actin sites adjoining the remaining S1s in state 1 become vacant. The result is that the remaining S1s in state 1 are now able to undergo the transition to state 2 and the total number of S1s in state 2 is significantly increased. At low and intermediate degrees of saturation, on the other hand, the large release of S1s leaves only a few S1s in state 1 that are able to undergo the transition to state 2 to replace those S1s which slowly dissociate. The result is that the number of S1s in state 2 decreases.

It is worth pointing out that the well-known effect of ADP that causes the reorientation of the rhodamine dipole bound to Cys-707 of the heavy chain in skeletal muscle fibers (*38*, *39*) may have the same origin as the one reported here. Since the ratio of myosin heads to actin in vertebrate skeletal muscle varies between 1:3.2 (*40*) and 1:1.8 (*41*), it is possible that adding ADP to skeletal muscle causes a mild dissociation and redistribution of the heads between states 1 and 2.

Comparison with Other Work. The fact that when the ratio of S1:actin was large, ADP had no effect on cross-linking is consistent with other work: the addition of ADP had no effect on (i) the EPR signal of skeletal muscle (*10*), (ii) the polarization of fluorescence of myofibrils loaded with excess S1 (*11*), (iii) the distance measured by luminescence energy transfer between Cys-707 of the catalytic domain and Cys-108 of the regulatory domain (*42*), and (iv) the distance between Cys-177 of ELC and various points on the heavy chain (*43*).

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BI981664C