Two Different Rigor Complexes of Myosin Subfragment 1 and Actin†

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ABSTRACT: Our previous titration and cross-linking experiments showed that myosin subfragment 1 (S1) can bind to one or two monomers in F-actin [Andreev, O. A., & Borejdo, J. (1991) *Biochem. Biophys. Res. Commun. 177*, 350–356; (1992a) *J. Muscle Res. Cell Motil. 13*, 523–533; (1992b) *Biochem. Biophys. Res. Commun. 188*, 94–101]. In the present work we used a sedimentation method to extend these studies to equilibrium binding and a stopped flow method to investigate its kinetics. Both equilibrium and kinetic data indicated the existence of two different rigor complexes. On the basis of these data we developed a model which suggested that binding of S1 to F-actin occurred in two steps: (i) initial rapid binding to one monomer of F-actin, A + M ↔ A·M and (ii) a consequent slow binding to a neighboring monomer, A·M + A ↔ A·M·A, where A stands for actin and M for myosin subfragment 1. The second reaction can proceed only if the neighboring actin site is unoccupied. The model fit the equilibrium and kinetic binding data with equilibrium constants $K_1 = 6 \times 10^6$ M^{-1} and $K_2 = 4$ and kinetic constants $k_{+1} = 10.5 \times 10^6$ M^{-1} s^{-1}, $k_{-1} = 1.75$ s^{-1}, $k_{+2} = 0.8$ s^{-1}, and $k_{-2} = 0.2$ s^{-1}, where the subscripts refer to the reactions i and ii. These results corroborate our hypothesis that myosin head can make two types of complexes with F-actin and support our speculation that during a power stroke in contracting muscle a myosin head may first bind to one and then to two actins.

Muscle contraction is caused by cyclic interactions of myosin heads with actin filaments driven by hydrolysis of ATP. Myosin head and F-actin form a stable (rigor) complex in solution containing no ATP. It is believed that this complex is an intermediate in the cyclic ATPase activity of S1[1] [e.g., Hibberd and Trentham (1986)]. Even though S1 may spend only a small fraction of total cycle time in the rigor complex (Uyeda *et al.*, 1990), this intermediate is of special significance because it is believed to be the end of the force-generating power stroke. For this reason a significant effort has been invested in characterizing the structural and biochemical properties of the complex. A number of studies have shown that maximal saturation of F-actin by S1 or HMM was one head per one actin (Young, 1967; Margossian & Lowey, 1978; White & Taylor, 1976; Borejdo & Assulin, 1980; Lehrer & Ishii, 1988). This conclusion was reached by analyzing data obtained at high degrees of saturation of actin filaments with myosin heads. At the same time, however, several studies indicated that the interaction of myosin heads with F-actin was different at low and high degrees of saturation. Thus, Tawada (1969) observed a biphasic change of the birefringence of F-actin oriented by flow at different degrees of saturation by HMM. Changes of anisotropy and intensity of fluorescence of ϵ-ADP incorporated in F-actin instead of ADP showed similar dependence on concentration of bound HMM (Miki *et al.*, 1976). Greene (1982) obtained the nonlinear Scatchard plot which reflected many measurements at low molar ratio (MR) of the binding of HMM to actin in the presence of ADP. This result suggested that the association constant depended on the molar ratio of HMM/actin. Szczesna *et al.* (1987) presented the Scatchard plots of sedimentation data, where the stoichiometries of binding of phosphorylated and dephosphorylated HMM to actin were 0.35 and 0.24, respectively. Titrations of F-actin by S1 monitored by turbidity, anisotropy of fluorescence of labeled S1, or intensity of fluorescence of pyrene attached to Cys 374 in actin revealed the existence of two different rigor complexes which corresponded to association of one S1 with one or two monomers in F-actin (Andreev & Borejdo, 1991, 1992a,b). Titrations of G-actin by S1 showed that S1 could form a complex with two G-actins (Valentin-Ranc *et al.*, 1991). Mornet *et al.* (1981) found that F-actin protected the junction between 50 and 20 kDa of bound S1 from tryptic digestion at MR < 0.5 and that it did not protect it at MR > 1. Yamamoto (1990) has shown that the interface between actin and S1 was different when the molar ratio S1/actin was 1:1 and 1:2. Mornet *et al.* (1981) suggested that S1 can be cross-linked by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) to two monomers in F-actin, producing a complex with an apparent molecular mass of 175–185 kDa. However, later it was shown that 175–185-kDa peptides corresponded to the complexes of one S1 with one actin (Sutoh, 1983; Greene, 1984; Heaphy & Tregear, 1984; Chen *et al.*, 1985). Recently, Andreev and Borejdo (1992a) have shown that the product of cross-linking of S1 to F-actin with apparent molecular mass of 265 kDa contained one S1 and two actin molecules. The production of the 265-kDa cross-linked complex strongly depended on the molar ratio of S1 to actin.

The difference in orientation of bound S1 in partially and fully decorated actin filaments could be seen in electron microscopy pictures (Craig *et al.*, 1980). However, only 3-D

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[1] Abbreviations: S1, myosin subfragment 1; HMM, heavy meromyosin; MR, molar ratio of S1 to actin; pyrene, *N*-(1-pyrenyl)iodoacetamide; pyr-F-actin, F-actin labeled by pyrene.

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reconstruction can provide accurate information about the structure of acto–S1 complexes. Unfortunately, a reconstruction of partially decorated actin filaments cannot be done. The reconstruction of fully decorated actin filaments showed that each myosin head in addition to a major contact with one actin had a second contact with an adjacent monomer in the same strand of actin filament (Milligan *et al.*, 1990). In view of our results, it is possible that in partially decorated actin filaments the second contact is stronger and that the structure of rigor complex deduced from reconstruction of fully saturated actin filaments does not correspond to that present in rigor muscle, where actin is always in excess.

In the present work we show that both equilibrium and kinetic data indicate that two different rigor complexes can be formed. We propose the model which assumes that S1 first binds to one monomer of F-actin forming the A·M rigor complex (one S1 per one actin). Providing the neighboring actin monomer is vacant, the model assumes that the A·M rigor complex undergoes a slow conformational change to bind to a second actin, forming A·M·A rigor complex (one S1 per two actins). Analysis of equilibrium data revealed that the total association constant of the second complex was 4 times larger than that of the first rigor complex. We suggest that the second rigor complex (A·M·A) corresponds to a nucleotide-free intermediate at the end of a power stroke.

MATERIALS AND METHODS

Materials. ATP, phalloidin, and chymotrypsin were from Sigma (St. Louis, MO). Sephadex G-50 gels were from Pharmacia (Piscataway, NJ). Bradford protein assay was purchased from Bio-Rad (Richmond, CA).

Proteins. Myosin was prepared from rabbit skeletal muscle according to the method of Tonomura *et al.* (1966). S1 was obtained by chymotryptic digestion of myosin according to the procedure of Weeds and Taylor (1975), and actin was prepared according to the method of Spudich and Watt (1971). To remove free ATP, G-actin was passed through a Sephadex G-50 column equilibrated with a buffer containing 0.2 mM $CaCl_2$, 0.1 mM NaN_3, and 10 mM Tris-HCl, pH 7.5, and was polymerized for 2 h at room temperature by the addition of 50 mM KCl, 2 mM $MgCl_2$, and 1.2 M excess of phalloidin. The concentrations of proteins were measured by absorbance, using for S1 $A^{1\%}(280) = 7.5$, for G-actin $A^{1\%}(290) = 6.3$, and for F-actin $A^{1\%}(290) = 6.7$.

Labeling of F-Actin with Pyrene. Actin was labeled by pyrene as described by Criddle *et al.* (1985). To remove free dye and nucleotide, the labeled G-actin was passed through the Sephadex G-50 column and polymerized under the same conditions as above for unlabeled actin. Typical degree of labeling was 85–95%.

Sedimentation. The samples, in 200 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.2 mM DTT, all contained 0.4 μM F-actin and increasing concentrations of S1 (0.01–2 μM). Samples were preincubated for 60 min at 15 °C and centrifuged at 131000*g* for 90 min at 15 °C in a Beckman (Fullerton, CA) LS-65B centrifuge using an SW41 rotor. Concentration of proteins in supernatant was measured by the Bradford method (Bradford, 1976) using a standard Bio-Rad protein assay. The error of measurements was about 0.25 μg/mL, which corresponds to 0.002 or 0.006 μM for measurements of S1 or actin concentrations, respectively. The data were plotted in Scatchard form and the errors were calculated as

$$M_B = M_T - M_F;$$

$$\Delta M_B = \Delta M_T + \Delta M_F \approx 0.004\ \mu M;\ \Delta A \approx 0.006\ \mu M$$

$$\theta = \frac{M_B}{A};\ \Delta\theta = \Delta M_B \frac{\partial\theta}{\partial M_B} + \Delta A \frac{\partial\theta}{\partial A} = \Delta M_B\left(\frac{1}{A}\right) + \Delta A\left(\frac{\theta}{A}\right)$$

$$\Delta\left(\frac{\theta}{M_F}\right) = \Delta\theta\left(\frac{1}{M_F}\right) + \Delta M_F\left(\frac{\theta}{M_F^2}\right);$$

$$\text{if } M_f \to 0,\ \Delta\left(\frac{\theta}{M_F}\right) \to \infty$$

where A, M_T, M_B, and M_F are concentrations of actin and of total, bound, and free S1, respectively. The θ/M_F was plotted vs θ. The fraction of F-actin that remained in the supernatant in the absence of S1 was less than 5%. The concentration of S1 in supernatant (M_{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 (M_F) was determined as $M_F = M_{SUP}/0.95$. The fraction of inactive S1, i.e., S1 that was unable to bind to F-actin, was estimated by measuring the S1 concentration in the supernatant after centrifugation of sample containing 0.4 μM S1 and 12 μM F-actin at 50 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.2 mM DTT at 23 °C. All experiments were done with S1 preparations that contained less than 1% of inactive S1.

Stopped Flow Measurements. The rapid mixing experiments were performed in an SLM-500C spectrofluorometer equipped with stopped flow attachment. All experiments were done in solution containing 50 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM DTT, 0.1 mM NaN_3, and 10 mM Tris-HCl, pH 7.5, at 20 °C. Pyrene fluorescence was excited at 368 nm (slit 5 nm) and recorded at 410 nm (slit 20 nm). Data were analyzed by Sigma Plot 4.1 program (Jandel Scientific, San Rafael, CA).

RESULTS

S1 Binding Measured by Sedimentation. Our previous results showed that the affinity of S1 to F-actin at 50 mM KCl, pH 7.5, and 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, 1992a). This made quantitative determination of the affinity constants impossible. In the present sedimentation experiments we have therefore studied the equilibrium binding of S1 to F-actin under conditions where binding was weaker (at 200 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM DTT mM, and 15 °C); 0.01–2 μM S1 was added to 0.4 μM F-actin. To avoid depolymerization, F-actin was stabilized by phalloidin. It was necessary to remove free ATP and ADP, because they reduce the affinity of S1 to F-actin, especially at low [S1] when some ATP could have been left unhydrolyzed. In a typical experiment 12 samples were prepared; 10 samples contained 2 mL of acto–S1 and 2 control samples contained either 0.4 μM F-actin or 0.4 μM S1. Samples were centrifuged, and the concentration of protein in the supernatant was measured as described under Materials and Methods. The sedimentation data obtained from four protein preparations is presented in Scatchard form in Figure 1.

The fact that the slope of the Scatchard curve is large when the molar ratio of S1 to actin is low and that it is small when

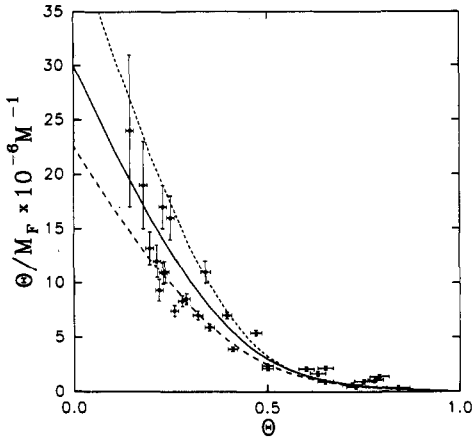

FIGURE 1: Scatchard plot of binding of S1 to F-actin obtained from sedimentation experiments of four different protein preparations as described under Materials and Methods. Conditions: 0.4 μM F-actin, 0.48 μM phalloidin, 200 mM KCl, 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM DTT, 15 °C. The solid curve is the theoretical curve with $K_1 = 6 \times 10^6$ M^{-1}, $K_2 = 4$; broken curve with $K_1 = 5 \times 10^6$ M^{-1}, $K_2 = 3.5$; and dashed curve with $K_1 = 6.5 \times 10^6$ M^{-1}, $K_2 = 4.5$.

the molar ratio is high suggests that binding of S1 to F-actin is anticooperative. Under Discussion we present a theory to explain such binding in which anticooperativity is brought about by two-step binding. The theory assumes that S1 binds first to one actin monomer in a thin filament with equilibrium constant K_1 and rate constants k_1 and k_{-1} and that it then isomerizes to bind to two actin monomers with equilibrium constant K_2 and rate constants k_2 and k_{-2}. A solid line is the curve calculated from eq 1.12 (Discussion) assuming $K_1 = 6 \times 10^6$ M^{-1} and $K_2 = 4$. Almost all experimental points were contained in the area restricted by two curves: one (broken curve) corresponded to constants $K_1 = 5 \times 10^6$ M^{-1}, $K_2 = 3.5$, and another (dashed curve) to $K_1 = 6.5 \times 10^6$ M^{-1}, $K_2 = 4.5$. The average constants at these conditions were determined as $K_1 = (6 \pm 1) \times 10^6$ M^{-1} and $K_2 = 4 \pm 0.5$. The total equilibrium constant of adsorption of S1 in state 2 is $K_1K_2 = 24 \times 10^6$ M^{-1}.

Kinetics of Binding. S1 and pyrene-labeled F-actin (pyr-F-actin) were rapidly (1–2 ms) mixed in the stopped flow apparatus. The quenching of pyrene fluorescence is proportional to the degree of occupation of binding sites of actin (Kouyama & Mihashi, 1981). To examine a possibility that quenching of pyrene fluorescence arose from factors other than S1 binding (such as photobleaching or actin depolymerization), pyr-F-actin was mixed with buffer solution containing no S1. The records of fluorescence signal (Figure 2A,C,E, traces 1) indicated that fluorescence intensity was stable at the conditions employed in our experiments. Depolymerization of pyr-F-actin was examined in sedimentation experiments by measuring the concentration of actin in the supernatant after centrifugation as described under Materials and Methods. Even at the lowest actin concentration, the fraction of nonpolymerized actin was less than 5%. This is consistent with our earlier results showing that under our experimental conditions depolymerization of F-actin stabilized by phalloidin did not occur (Andreev & Borejdo, 1992b). In subsequent binding experiments, all records of fluorescence changes were normalized with respect to pyr-F-actin alone.

The experimental records of quenching of fluorescence of pyr-F-actin by S1 at different molar ratios of S1/actin are presented in Figure 2A,C,E, traces 2 and 3. When S1 was in excess, the fluorescence intensity decreased rapidly and reached a plateau within 200 ms after mixing (Figure 2A, trace 2). In contrast, when pyr-F-actin was in excess, two distinct phases of signal change—fast and slow—were observed (Figure 2C, traces 2 and 3, and Figure 2E, trace 2). In Figure 2E the process was completed within 10 s after mixing. Figure 2C shows that, in contrast to the rate of the fast process, the rate of the slow process did not depend on total protein concentration. The slow process decayed with the average rate constant of 0.43 s^{-1} (Figure 3, trace 1).

The two-state model (see Discussion) provides a satisfactory fit to the experimental data and predicts the time dependence of the fractional number of myosin heads in states 1 and 2. The fit was constructed as follows: The fluorescence signal (F) is comprised by contributions by actins in three different states: free actin (f_0) and actin occupied by S1 in state 1 (f_1) or in state 2 (f_2).

$$F = f_0A_F + f_1A_1 + f_2A_2 \qquad \text{(i)}$$

The substitution $A_F = A - A_1 - A_2 = A(1 - m_1 - 2m_2)$ and $f_0A = F_0$ (where A is the total amount of actins and m_1 and m_2 are the fractions of S1 in states 1 and 2) gives

$$F/F_0 = 1 - (1 - f_1/f_0)m_1 - 2(1 - f_2/f_0)m_2 \qquad \text{(ii)}$$

It was found that 81% of the fluorescence was quenched at full saturation of pyr-F-actin by S1, therefore $f_1 = 0.19f_0$. Assuming that $f_2 \approx f_1$, eq ii takes the form

$$F/F_0 = 1 - 0.81(m_1 + 2m_2) \qquad \text{(iii)}$$

The functions $m_1(t)$ and $m_2(t)$ can be obtained from numerical solution of the set of eq 1.8–1.9 given under Discussion. The equilibrium constants were found from sedimentation experiments. The characteristic times (τ_1 and τ_2) of fast and slow processes were found by fitting the experimental data by two-exponential functions. The approximate kinetic constants were calculated according to eq 1.16 and 1.18. The kinetic constants further were adjusted by fitting the experimental data by curves obtained from numerical solutions of the eq 1.8–1.9. We found that all experimental data could be fitted well using the same set of constants, $K_1 = 6 \times 10^6$ M^{-1}, $K_2 = 4$, $k_{+1} = 10.5 \times 10^6$ M^{-1} s^{-1}, $k_{-1} = 1.75$ s^{-1}, $k_{+2} = 0.8$ s^{-1}, and $k_{-2} = 0.2$ s^{-1}. Figure 2A,C,E solid lines show the fit together with the experimental data. The time dependence of fractional numbers of S1 in states 1 and 2 is shown in Figure 2B,D,F.

DISCUSSION

Equilibrium Binding of S1 to F-Actin Studied by Sedimentation. The use of an ultracentrifuge is a standard technique to measure binding of S1 to F-actin (Young, 1967; Margossian & Lowey, 1978; Marston & Weber, 1975). The technique assumes that bound S1 cosediments with F-actin and free S1 stays in a supernatant. However, it is necessary to make correction for the fraction of actin that remains in the supernatant and the fraction of free S1 that does sediment in the absence of F-actin. The corrected data can be analyzed by the Scatchard method. In the case of independent binding of monovalent S1 molecules the Scatchard plot must be linear. In the case of cooperative binding, the Scatchard plot must be bell-shaped. However, our experimental data could not be fitted either by linear or by bell-shaped Scatchard plot. We therefore suggest S1 binds to F-actin in anticooperative fashion. We propose that S1 can bind to two actin monomers in F-actin. Anticooperativity results naturally from this fact: S1 is sterically prevented from binding to actin which has a neighbor already occupied by S1. Our previous results indicated that S1 was able to bind to one or two monomers in F-actin (Andreev

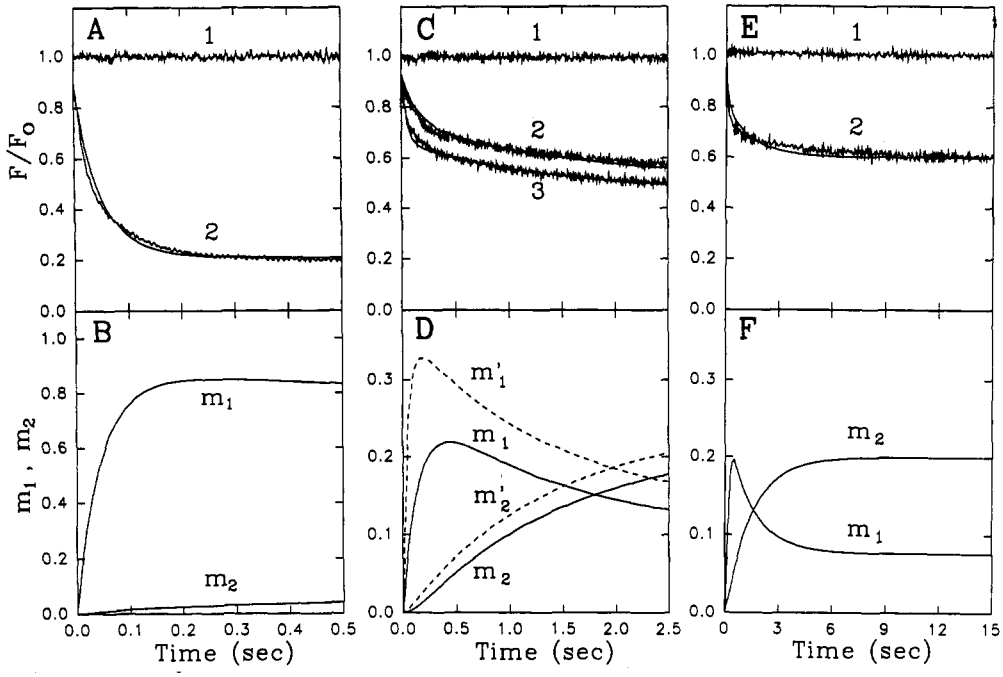

FIGURE 2: Kinetics of binding of S1 to pyr-F-actin. (A, C, and E) Time course of the quenching of fluorescence of pyr-F-actin by binding of S1. The fitting curves (solid lines) were obtained from numerical solution of eqs 1.8 and 1.9 using the protein concentrations indicated below and the constants $K_1 = 6 \times 10^6$ M^{-1}, $K_2 = 4$ and $k_{+1} = 10.5 \times 10^6$ M^{-1} s^{-1}, $k_{-1} = 1.75$ s^{-1}, $k_{+2} = 0.8$ s^{-1}, and $k_{-2} = 0.2$ s^{-1}. All experiments were done in solution containing 50 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM DTT, 0.1 mM NaN_3, and 10 mM Tris-HCl, pH 7.5, at 20 °C. The concentrations of pyr-F-actin (stabilized by phalloidin) and S1 were (A) 0.5 μM actin, no S1 (trace 1, average of 4 records) or 2 μM S1 (trace 2, average of 11 records); (C) 0.5 μM actin, no S1 (trace 1, average of 3 records) or 0.2 μM S1 (trace 2, average of 5 records), and 2.5 μM actin, 1 μM S1 (trace 3, average of 5 records); (E) 1 μM actin, no S1 (trace 1, average of 3 records) or 0.3 μM S1 (trace 2, average of 3 records). (B, D, and F) Time dependence of fractional numbers of bound S1 in state 1 (m_1) and state 2 (m_2) predicted by the model for the experimental data presented in (A), (C), and (E), respectively. (D) m_1 and m_2; m_1' and m_2' correspond to the experimental data presented by traces 2 and 3, respectively.

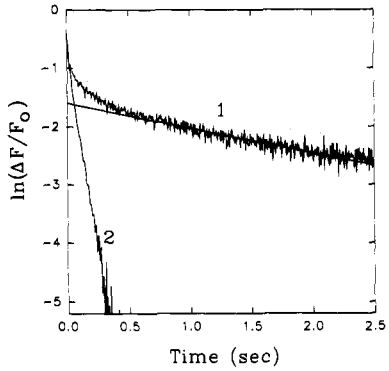

FIGURE 3: Logarithmic presentation of the change of fluorescence of pyr-F-actin induced by S1 binding. $\Delta F = F - F_{min}$, where F_{min} corresponds to the equilibrium level when fluorescence change is completed. Trace 1 corresponds to the experimental data presented in Figure 2E (trace 2, 1 μM pyr-F-actin and 0.3 μM S1); the straight line corresponds to the function $(-1.6 - 0.43t)$. Trace 2 corresponds to the experimental data presented in Figure 2A (trace 2, 0.5 μM pyr-F-actin and 2 μM S1).

& Borejdo, 1991, 1992a,b). The model suggested below incorporates these findings. We consider here a model in which S1 first binds to one and, consequently, to two actin monomers. To rule out an obvious alternative mechanism, we consider also an adsorption of two types of S1, each of which can bind to a single actin monomer with different adsorption constant. Predictions of both models will be checked against experimental data.

Two-State Adsorption of Myosin Subfragment 1 on F-Actin: General Equations. Let us suppose that actin filament is a long single row of A monomers and that each S1 molecule can bind to this filament in two possible states. In state 1 it occupies one actin monomer. We visualize this

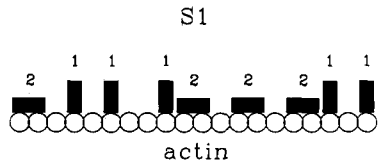

FIGURE 4: Schematic diagram of the two-state binding of myosin subfragmental 1 to F-actin. S1 can bind to one actin monomer in the vertical position (state 1) or to two actin monomers in the horizontal position (state 2).

state as a "vertical" position of S1 molecule. In state 2 it occupies two actin monomers and is in a "horizontal" position. Figure 4 illustrates this idea in a schematic form. Adsorption of S1 proceeds as two consecutive and reversible reactions

$$A + M \leftrightarrow A{\cdot}M \quad (1.1)$$

$$A{\cdot}M + A \leftrightarrow A{\cdot}M{\cdot}A \quad (1.2)$$

We assume that S1 first adsorbs from solution (to any free actin binding site) in state 1 (A·M). After initial binding, providing that the neighboring binding site is free, S1 changes conformation and binds an additional actin monomer, i.e., enters state 2 (A·M·A). The kinetic constants of these reactions are k_{+1}, k_{-1}, and and k_{+2}, k_{-2}, respectively.

Let A_F be the concentration of unoccupied actins, M_F the concentration of free S1 in the solution, M_1 the amount of bound S1 in state 1, M_2 the amount of bound S1 in state 2, M total amount of S1, $K_1 = k_{+1}/k_{-1}$ an equilibrium adsorption constant for state 1 (dimension M^{-1}), and $K_2 = k_{+2}/k_{-2}$ an equilibrium constant of transition from state 1 to state 2 (dimensionless). Let us first consider the adsorption step A + M ↔ A·M, i.e., the adsorption of free S1 in state 1. The

rate of this reaction is

$$v_1 = k_{+1}M_F A_F - k_{-1}M_1 \quad (1.3)$$

where $A_F = A - M_1 - 2M_2$ and $M_F = M - M_1 - M_2$. The rate of transition from state 1 to state 2 is given by

$$v_2 = k_{+2}M_1 P_F - k_{-2}M_2 \quad (1.4)$$

where P_F is the probability that the site next to a given singly bound S1 is free. Any free actin or any acto–S1 complex can be the nearest neighbor to a singly bound S1. The number of ways for free actin to be in this place is equal to a number of free actins, A_F. The number of ways of all occupations (by free actin or by acto–S1) of this place is equal to the sum of free actins and acto–S1 complexes, i.e., $A_F + M_1 + M_2$. So the probability P_F is

$$P_F = A_F/(A_F + M_1 + M_2) = (A - M_1 - 2M_2)/(A - M_2) \quad (1.5)$$

The change of the number of myosin molecules in different states with time is determined by

$$dM_1/dt = v_1 - v_2 \quad (1.6)$$

$$dM_2/dt = v_2 \quad (1.7)$$

For further analysis it is convenient to introduce relative concentrations of adsorbed S1 molecules on actin filament, $m_1 = M_1/A$, $m_2 = M_2/A$, $\Theta = m_1 + m_2$, and to normalize the concentration of free S1 in solution, $m_F = K_1 M_F$. Then the set of adsorption equations that determine the change of the number of myosin molecules in states 1 and 2 is

$$\frac{dm_1}{dt} = k_{-1}m_F(1 - m_1 - 2m_2) - k_{-1}m_1 - \frac{k_{+2}m_1(1 - m_1 - 2m_2)}{(1 - m_2)} + k_{-2}m_2 \quad (1.8)$$

$$\frac{dm_2}{dt} = \frac{k_{+2}m_1(1 - m_1 - 2m_2)}{(1 - m_2)} - k_{-2}m_2 \quad (1.9)$$

Equilibrium. In equilibrium eqs 1.8–1.9 simplify to

$$m_F(1 - m_1 - 2m_2) = m_1 \quad (1.10)$$

$$K_2 m_1(1 - m_1 - 2m_2) = m_2(1 - m_2) \quad (1.11)$$

All of the variables and parameters in this set of adsorption equations are dimensionless. The objective of the further analysis is to determine the adsorption constants K_1 and K_2 from the set of experimental data. The equilibrium binding data are usually analyzed by the Scatchard method which plots the ratio $M_B/M_F A$ vs the ratio M_B/A (or Θ/M_F vs Θ, $\Theta = m_1 + m_2$). In a simple case of one-state adsorption this plot is linear and the intercept as $M_B/M_F A$ axis gives the adsorption constant, K_1. In the case of two-state adsorption, two adsorption constants should be found. Solving the set of eqs 1.10–1.11 for Θ/m_F, one obtains

$$\frac{\Theta}{m_F} = \frac{1 - 2\Theta + 2\Theta^2 + K_2(1 - 2\Theta)^2}{2(1 - \Theta)} \pm \left\{\left[\frac{1 - 2\Theta + 2\Theta^2 + K_2(1 - 2\Theta)^2}{2(1 - \Theta)}\right]^2 - \Theta^2\right\}^{1/2} \quad (1.12)$$

The choice of sign depends on the value of Θ. It is "plus" if $0 \le \Theta < 0.5$ and "minus" if $0.5 \le \Theta < 1$. The equilibrium problem in a similar system where a bivalent myosin fragment was HMM molecule was solved by Peller (1975) and Hill (1978) using different formal presentation.

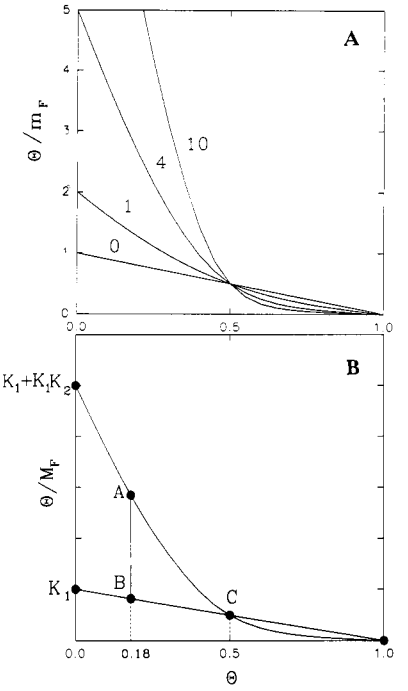

FIGURE 5: (A) Scatchard plots of binding of S1 to F-actin predicted by two-state model (eq 1.6). $K_2 = 0$ (one-state binding) and $K_2 = 1, 4, 10$. (B) Approximate method of determination of constants K_1 and K_2. The straight line corresponds to the basic Scatchard plot ($K_1 > 0$), the convex curve to the two-state binding Scatchard plot ($K_1 > 0$, $K_2 > 0$). It can be shown that the distance AB $\approx {}^1/_2 K_1 K_2$. In an experiment the points A and C can be found as average experimental points at $\Theta = 0.18$ and 0.5, respectively. Point B is given by the intercept of vertical line at $\Theta = 0.18$ with straight line (basic Scatchard plot) drawn through point C and point $\Theta = 1.0$ on the abscissa.

The plot of the function 1.12 is presented in Figure 5a for values of constant K_2 ranging from 0 to 10. If $K_2 = 0$, there is no second adsorption state, and the standard linear Scatchard plot is recovered (the straight line in Figure 5A). As expected, the intercept on the ordinate is 1; if Θ/M_F is plotted instead of Θ/m_F, it is K_1. If K_2 is finite, the Scatchard plot is curved and the intercept at ordinate is $K_2 + 1$, or $K_1(K_2 + 1)$ in dimensional units. All of the curves of this family cross at the point with coordinates (0.5, 0.5) or (0.5, $K_1/2$). Therefore, this unique point helps to determine the adsorption constant K_1. The second adsorption constant K_2 in principle could be determined by intercept at the ordinate, but because of the scatter of experimental points at low-level adsorption and because of the obvious absence of points at zero adsorption, the extrapolation procedure becomes uncertain. For this reason we developed another, more accurate method. The nature of the function 1.12 is such that if one draws a vertical line at the point $\Theta = 0.18$ (Figure 5B), then the intercept between the given curve and the basic Scatchard line is fairly close to $K_2/2$ (or $K_1 K_2/2$ in dimensional units). The value of K_2 found by this method differs from the exact value by less than 5% at low K_2, and this difference quickly decreases at higher K_2. Therefore, the accuracy of this method is quite satisfactory. This procedure was applied to the present data (Figure 1), and we ended up with the constants $K_1 = 6 \times 10^6$ M^{-1} and $K_2 = 4$.

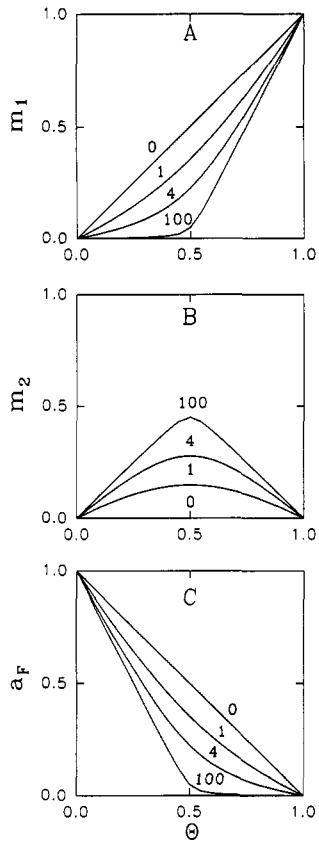

FIGURE 6: Dependence of fractional concentration of bound S1 on Θ and K_2 (K_2 = 0, 1, 4, 100). (A) S1 in state 1, (m_1, 1:1 complexes); (B) S1 in state 2 (m_2, 1:2 complexes); (C) ratio of free actins to total number of actins, A_F/A.

The relative concentrations of m_1 and m_2 are

$$m_1 = \Theta - \frac{1}{2} + \frac{1}{2}\left[\frac{1 + K_2(1-2\Theta)^2}{K_2 + 1}\right]^{1/2} \quad (1.13)$$

$$m_2 = \frac{1}{2} - \frac{1}{2}\left[\frac{1 + K_2(1-2\Theta)^2}{K_2 + 1}\right]^{1/2} \quad (1.14)$$

Parts A and B of Figure 6 present m_1 and m_2 for different values of the constant K_2. When $K_2 = 0$, the population of vertical state 1 is equal to the total population (the population of horizontal state 2 is zero). If $K_2 > 1$, then the population of state 2 is initially growing faster with increasing Θ from 0 to 0.5 than the population of state 1. This is because the adsorption energy of the state 2 is higher than that of state 1. But after the total adsorption reaches the point Θ = 0.5, the population of state 2 passes through the maximum and begins to decline. At the maximum adsorption this population goes to zero. The factor that determines such a behavior is the entropy of the system. With the increasing concentration of S1 in the solution it becomes more favorable to supplant one molecule adsorbed in state 2 with two molecules in state 1.

Kinetics. The kinetics of binding of S1 to F-actin is determined by the set of eq 1.8–1.9 with initial conditions $m_1(0) = 0$ and $m_2(0) = 0$. The solutions of the equations can be obtained numerically, but it would be helpful to have an estimate of the kinetic constants of the process. The equilibrium constants (K_1 and K_2) can be obtained from sedimentation experiments. It is reasonable to assume that the first reaction—adsorption of S1 from solution to state 1—is a fast process in comparison with the second reaction; therefore, the concentration of free S1 is $m_F = K_1A(M/A - m_1)$, and the concentration m_1 at the first stage changes approximately as follows:

$$m_1(t) \approx \frac{K_1 M}{[K_1(M + A) + 1]}[1 - \exp(-t/\tau_1)] \quad (1.15)$$

$$\frac{1}{\tau_1} \approx k_{-1}[K_1(M + A) + 1] \quad (1.16)$$

The inverse value of characteristic time ($1/\tau_1$) depends linearly on the total concentration of proteins ($M + A$). The characteristic time τ_1 can be determined by fitting the beginning part of the experimental curve by exponential function, and consequently the constants k_{+1} and k_{-1} can be calculated. The estimation of the kinetics constants k_{+2} and k_{-2} can be easily done for the case when $M \ll A$. If the initial binding can be treated as a rapid equilibrium with respect to the second step ($k_{-1} \gg k_{+2}$) and then a population in state 2, m_2 is changed with time as

$$m_2(t) \approx \frac{K_1 K_2 M}{1 + K_1 A(1 + K_2)}[1 - \exp(-t/\tau_2)] \quad (1.17)$$

$$\frac{1}{\tau_2} \approx \frac{K_1 A}{1 + K_1 A} k_{+2} + k_{-2} \quad (1.18)$$

The characteristic time τ_2 of a transition of S1 from state 1 to state 2 is rather insensitive to actin concentration when $K_1A \gg 1$. If we know the equilibrium constants K_1 and K_2 and can find the characteristic times τ_1 and τ_2 from experimental data, then we can estimate all kinetic constants k_{+1}, k_{-1}, k_{+2}, and k_{-2}. These values can be adjusted from the final curve fitting.

We mentioned before that a similar problem of binding of bivalent myosin fragments was considered by Peller (1975) and Hill (1978). With the same equilibrium constants (K_1 for state 1 and K_1K_2 for state 2), the Scatchard plots generated by our model and models by Peller and Hill were identical. However, the models by Peller and Hill describe only equilibrium binding, while the model presented here describes also the kinetics of binding.

Alternative Model: Binding to F-Actin of Two Different Monovalent Myosin Fragments. In the present work we used chymotryptic S1 which usually contains equimolar amounts of S1(A1) and S1(A2) isomers. One could expect that the presence of two different S1 species may affect the shape of the Scatchard plot. However, we show below that an alternative model of binding to F-actin of an equimolar mixture of two monovalent S1 species with different association constants gave a Scatchard plot that was completely different from the observed one. Moreover, our preliminary results (not shown) indicated that S1(A1) and S1(A2) isomers bound to F-actin in a similar way as unfractionated S1; i.e., the Scatchard plot was nonlinear and convex. Let the adsorption constant of S1(A1) isomer be C_1 and the adsorption constant of S1(A2) isomer be C_2. In the case of an equimolar mixture of these two species, the Scatchard equation is

$$\frac{\Theta}{M_F} = \frac{(1-\Theta)[C_1 + C_2 + 2AC_1C_2(1-\Theta)]}{2 + A(1-\Theta)(C_1 + C_2)} \quad (1.19)$$

where $\Theta = M_B/A$, M_B and M_F are the total concentrations of bound and free S1, and A is the total concentration of actin.

The second derivative of this function is

$$\frac{-4A(C_1 - C_2)^2}{[2 + A(1-\theta)(C_1 + C_2)]^3} \quad (1.20)$$

which is obviously negative, and hence the Scatchard plot is given by a concave curve. It means that a model with two populations of monovalent myosin fragments, contrary to the two-state model, does not fit experimental data and must be ruled out.

The affinity constant and stoichiometry have been measured earlier in sedimentation experiments (Margossian & Lowey, 1978; Marston & Weber, 1975; Greene & Eisenberg, 1980). The question then arises, why were the two binding processes not seen earlier? We suggest six possible reasons: 1. Measurement of free S1 in the supernatant, after sedimentation, is inaccurate, especially at low MR. Therefore, most data points were obtained by measuring binding at high MRs [for example, Margossian and Lowey (1978) measured only two points at which MR was less than 0.5]. To obtain firm evidence of a departure from simple hyperbolic binding, many measurements at low MR are required. 2. Most of the measurements of dissociation constants were carried out at actin concentration higher than 1 μM. Since the dissociation constant can only be reliably measured when the protein concentration is of the same order of magnitude as the dissociation constant, submicromolar dissociation constants could not be detected. Marston and Weber (1975) were the first to use low concentrations of F-actin and S1. They measured the dissociation constant as about 0.07 μM at 0.14 M ionic strength, pH 7.0, and 25 °C. However, their data were not plotted in Scatchard form, where the two binding states could perhaps have been revealed. 3. Depolymerization of F-actin can affect the accuracy of measurements at low F-actin concentrations, as clearly demonstrated by Marston and Weber (1975). To avoid depolymerization, we carried out all experiments in the presence of phalloidin. 4. A small fraction of S1 precipitates even in the absence of F-actin. It is necessary to correct for this extra S1 by estimating, at each MR, the amount of S1 in the pellet. 5. It was usually postulated that the amount of bound actin is equal to the amount of bound S1; i.e., an association constant was taken as $[\text{complex}]/S1_F A_F = S1_B/S1_F(A - S1_B)$. But if the binding is not a simple Michaelis process, it is not true that $A_F = A - S1_B$. 6. Traces of ATP or ADP may be sufficient to abolish high-affinity binding. It is necessary to remove free ATP and ADP from the stock solution of F-actin.

Some of the values for the binding constant of S1 to actin reported more recently were $K_a = 10^6$ M^{-1} (4 °C, 0.15 KCl; Highsmith *et al.*, 1976), $K_a = 3 \times 10^6$ M^{-1} (20 °C, 0.10 KCl; Margossian & Lowey, 1978), $K_a = 10^7$ M^{-1} (25 °C, 0.10 KCl; Marston & Weber, 1975), and $K_a = 4 \times 10^7$ M^{-1} (20 °C, 0.155 ionic strength; Taylor, 1991). The saturation values were one myosin head per actin. These values were obtained by measuring binding mostly at high MRs. This is close to K_1 reported here (which also reflects affinity and stoichiometry prevailing at high MRs), considering the differences in ionic conditions, temperature, and presence of phalloidin.

Stopped Flow Experiments. The results of rapid-mixing experiments showed that interactions of S1 with F-actin are different at low and high molar ratios of S1 to actin. When S1 was in excess, only the fast phase of a decay of fluorescence signal was observed, in agreement with earlier observations of Criddle *et al.* (1985) and Taylor (1991). The rate constant of fast decay ($1/\tau_1$) depended linearly on total concentration of S1 and actin ($M + A$) and did not depend on their molar ratio. We concluded that fast decay corresponded to the initial binding of S1 to pyr-F-actin or to formation of a 1:1 complex (A·M). We considered the formation of a 1:1 complex as a one-step process, though, according to Taylor (1991), it may include some intermediate states. At molar ratios of less than 0.5 both fast and slow decays of fluorescence were observed. Taylor (1991) observed a similar slow decrease of fluorescence when pyr-F-actin was in excess but attributed it to bleaching of pyrene fluorescence. We examined the possibility of bleaching of pyrene fluorescence and found that at our conditions it was negligible. The other factor that could affect fluorescence intensity is depolymerization of actin. But this possibility has to be ruled out because the fluorescence of pyr-F-actin in the absence of S1 was constant. It is also unlikely that the decrease in fluorescence results from depolymerization of F-actin because S1 enhances polymerization. Moreover, in sedimentation experiments, depolymerization of F-actin stabilized by phalloidin was found to be negligible (Andreev & Borejdo, 1992b). The rate constant of slow decay ($1/\tau_2$) was not dependent on the total concentration of proteins (in the range 1–5 μM), indicating that a conformatio change occurred when an initial binding of S1 reached equilibrium. We concluded that the slow change of fluorescence of pyr-F-actin was associated with the transition from a 1:1 rigor complex to a 1:2 complex (A·M → A·M·A). Figure 2 shows that the two-state binding model provides good fit to the experimental data and gives the time dependence of the fractional numbers of myosin heads in states 1 and 2. The fitting procedure can be improved if more information about a fluorescence of actin occupied by S1 in state 2 would be available.

Relationship to Earlier Work. In earlier work, we monitored binding of S1 to F-actin by measuring turbidity, anisotropy of fluorescence of labeled S1, or quenching of fluorescence of labeled S1, or quenching of fluorescence of pyrene covalently bound to actin (Andreev & Borejdo, 1991, 1992a,b). All of these methods revealed that titration curves were biphasic (i.e., two plateaus were observed: one at MR $\approx$ 0.5 and another at MR > 1.0) when titrations were "slow", i.e., when the time intervals between additions of S1 were long (5–10 min). Titration curves were monophasic (saturation at MR $\approx$ 1.0) when titrations were "fast", i.e., when the time intervals between additions of S1 were short (10–20 s). Carlier and Didry (1992) suggested that this phenomenon could be due to depolymerization of 50% of F-actin, but this explanation should be ruled out because in this case both fast and slow titrations should show a saturation at MR $\approx$ 0.5. Moreover, we showed by sedimentation method that at our conditions depolymerization of F-actin was not significant and that the same results were obtained with F-actin stabilized by phalloidin (Andreev & Borejdo, 1992b). The two-state binding model can explain the difference between fast and slow titrations: during slow titration most of S1 would be in state 2 when MR < 0.5 and addition of new portions of S1 would not induce immediate transition of all bound S1 into state 1. This transition should only proceed gradually. Indeed, when S1 and F-actin were incubated for 1 h at MR = 0.5 and then S1 was added to make MR = 2, the turbidity increased only gradually (over a period of 1 h) to a level corresponding to a 1:1 acto–S1 complex (Andreev & Borejdo, 1992b).

The rigor binding of myosin subfragments was earlier studied by different methods, and there are several reports suggesting nonlinear relation between changes of certain parameters and the amount of bound myosin heads. These changes were biphasic (Tawada, 1969; Miki *et al.*, 1976) or

monotonic (Harvey *et al.*, 1977). All of these results suggested that the conformation of rigor complexes was different at molar ratio of bound myosin heads to actin below 1:3 than at more higher ratios. Our model can provide an explanation of this biphasic behavior. The observed biphasic changes might be related to formation of 1:2 acto–S1 complexes (m_2), which depends biphasically on the molar ratio of bound S1 to actin (Figure 6B). Although the experimental curves shown by these authors were not symmetrical with respect to $\Theta = 0.5$ as the ideal theoretical relationship between m_2 and Θ requires (Figure 6B), it is likely that experimental data show deviation from the ideal equilibrium binding and indicate the contribution of free actins and of 1:1 complexes. Changes observed by Harvey *et al.* (1977), who saw a monotonic decrease in the lifetime of fluorescence of ϵ-ADP incorporated in F-actin upon titration by S1 or HMM which nearly saturated at molar ratio of heads to actins of about 0.5, can likewise be explained by our model: the monotonic response might be related to the amount of free actins, which depends nonlinearly and monotonically on the amount of bound S1 (Figure 6C). Changes in the intensity of fluorescence of pyr-F-actin during slow titration by S1 showed a similar dependence on the amount of bound S1 (Andreev & Borejdo, 1992a). In these experiments fluorescence reached near saturation at a molar ratio of S1 to actin of about 0.5. In summary, different methods are sensitive to different aspects of acto–myosin interaction: some of them sense a formation of 1:2 acto–S1 complexes (m_2), others 1:1 complexes (m_1), while still others sense a fraction of unoccupied actins (a_f). Depending on which method was used to detect interaction, a response could be either biphasic or monotonic.

On the basis of our results, we suggest that during power stroke in contracting muscle each myosin head first binds to one and then to two actins: $A + M{\cdot}ADP{\cdot}P_i \leftrightarrow A{\cdot}(M{\cdot}ADP{\cdot}P_i) \leftrightarrow A{\cdot}M{\cdot}A + ADP + P_i$, where $M{\cdot}ADP{\cdot}P_i$ indicates myosin head carrying products of hydrolysis of ATP. This suggestion is in line with the models proposed by Huxley (1969), Huxley and Simmons (1971), and Huxley and Kress (1985), where myosin head attaches to different sites on actin filament during a power stroke. Huxley and Simmons (1971) estimated that the potential energy difference between stable positions of attachment of cross-bridge was $\approx 4kT$ and that axial shift between thick and thin filaments was about 8 nm. The free energy difference between the two states observed here (rigor, [KCl] = 200 mM) is $kT \ln K_2 \approx 1.4kT$. However, the difference between free energy of $A^*(M{\cdot}ADP{\cdot}P_i)$ and $A{\cdot}M{\cdot}A$ could be higher during muscle contraction. Our model predicts that the size of a power stroke is of the same order as the average size of actin dimer, i.e., $\approx$10 nm, which is in a good agreement with the value measured by Huxley and Simmons (1971).

ADDED IN PROOF

Recently we have shown by polarized fluorescence that orientation of S1 is different in fully and partially decorated thin filaments in striated myofibrils (Andreev *et al.*, 1993).

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