Myosin Subfragment 1 Inhibits Dissociation of Nucleotide and Calcium from G-actin*

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The dissociation rates of 1, N°-ethenoadenosine 5'-triphosphate (ATP) and of Ca²⁺ from G-actin and its complex with myosin subfragment 1 (S1) were measured by recording a large decrease in the fluorescence intensity of the dissociating nucleotide. Under the experimental conditions employed, the binary G-acto-S1A2 complex does not polymerize (Chausseped, P., and Kasprzak, A. A. (1989) Nature 342, 950–953). The released nucleotide was hydrolyzed either by alkaline phosphatase or by apyrase; to trap Ca²⁺, EDTA was used. From the anisotropy of N-iodoacetyl-N°-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS)-actin, it was established that during the dissociation of ATP, the G-acto-S1 complex remained stable and the equilibrium of the system was unaltered. The reactions followed first order kinetics. The dissociation rate constant, k₉ for ATP decreased from 5.5 × 10⁻⁴ s⁻¹ for free G-actin to 1 × 10⁻⁴ s⁻¹ for G-acto-S1A2; for Ca²⁺, k₉ was also similarly reduced from 2.8 × 10⁻⁴ s⁻¹ to 4 × 10⁻⁵ s⁻¹. Two proteolytically derived actin variants were also examined. For free subtilisin-cleaved actin, k₉ for ATP was elevated 2-fold but was almost unchanged for Ca²⁺. In the complex of the cleaved G-actin with S1A2, k₉ for both ATP and for Ca²⁺ were reduced. The removal of the last 3 amino acids from actin produced a derivative whose behavior in binding to S1, as well as in the kinetics of ATP and Ca²⁺ dissociation, was undistinguishable from the unmodified protein.

Since myosin was proposed to be the major element of force generation and energy transduction system in muscle and non-muscle cells, a large effort has been made to characterize conformational changes occurring in the myosin head. Much less is understood how actin is involved in these processes. Nevertheless, several previous investigations have demonstrated that binding of subfragment 1 (S1) to the filament influences the conformation of actin. Electron paramagnetic resonance probes attached to Cys-374 seemed to report that the rotational flexibility of actin is restricted when myosin head interacted with the filament (Thomas et al., 1979; Mosakowska et al., 1988). Although this notion has been questioned recently by experiments that indicated the observed restriction could be due to an S1-induced bundling of the actin filaments (Ostap et al., 1992), other pieces of evidence show that the conformational change of actin in the filament is altered by S1. Knight and Offer (1980) were unable to form an interprotomer actin cross-link in the presence of S1; such a cross-link could readily be formed in its absence. Others found it difficult or impossible to depolymerize covalent acto-S1 complexes even using agents so effective as 0.6 M KI or 4.7 M NH₄Cl, demonstrating that actin-actin interactions are strengthened by S1 binding (Rouayrenc et al., 1985). In a number of laboratories fluorescence labels specifically conjugated to actin sensed the interaction of S1 with the filament. However, insights into the structural dynamics of actin gained from these results are modest, since it is difficult to link changes in the fluorescence intensity to any specific conformational rearrangements of proteins. Nor could it be inferred from these data whether the probe was directly perturbed by S1 or the effect was indirect.

Some more defined S1-induced motions in the actin molecule were detected by fluorescence resonance energy transfer. Using this technique, Miki et al. (1987) observed a significant reduction of the distance between Cys-374 and Lys-61 upon binding of S1 to F-actin. Kasprzak et al. (1988) measured the radial coordinates of Cys-374, Gln-41, and the nucleotide in the filament. In the presence of S1 the radial coordinate of Gln-41 increased by 4–5 Å; there was no effect of S1 on the radial positions of the other two residues. From these data one can conclude that it is unlikely that a large "in place" rotation of the protomer takes place when the myosin head binds to it. Such motion is also rather difficult to envisage due to numerous contacts made by each actin protomer with its neighbors (Holmes et al., 1990). However, it is entirely possible that the actin molecule undergoes considerable structural alterations during its interaction with S1. Such changes are of great significance as they may provide a means for communicating along the filament the events associated with ATP hydrolysis.

The abbreviations used are: S1, myosin subfragment 1; ATP, 1,N°-ethenoadenosine 5'-triphosphate; S1A1 and S1A2, isoenzymes of myosin subfragment 1 carrying alkali light chain A1 or A2, respectively; MOPS, 3-(N-morpholino)propanesulfonic acid; DTE, dithioerythritol; Quin-2, 2-[2-bis(carboxymethyl)amino-5-methylphenoxethyl]methyl]-6-methoxy-8-hydroxyethylamino-5-methylphenoxymethyl]-6-methoxy-8-hydroxyethylamino-5-methylphenoxymethyl]laminooquinoline; 1,5-IAEDANS, N-iodoacetyl-N°-(5-sulfo-1-naphthyl)ethylenediamine; NBD, 4-chloro-7-nitrobenz-2-oxa-1,3-diazole; pPDM, N,N'-p-phenylenediamide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

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S1 binding and/or ATP hydrolysis. Propagation of conformational changes along the filament is required to explain a more active role of this protein in motility (Pröchniewicz and Yanagida, 1990; Ando, 1988; DasGupta and Reisler, 1992) and is necessary to account for a cooperative behavior of the unregulated filament induced by certain actin-binding protein fragments (Bertegi et al., 1990).

The availability of the G-acto-S1 complexes in the stable form (Chaussee and Kasprzak, 1989a) and the emergence of the atomic structure of actin (Kabsch et al., 1990) have opened a new way of readdressing the question of the molecular dynamics of the actin molecule in the presence of S1, using a simplified model system. Before trying to interpret the data obtained in such a system, it is important to recall the main similarities and differences between the G-acto-S1 and the F-acto-S1 complexes. The basic feature of the actomyosin interface, i.e. the nucleotide-dependent binding of myosin head to actin is present in both systems. Thus it is expected that the G-acto-S1 interface should exhibit extensive similarity to the one found in the F-acto-S1 complex. For both complexes ATP is a more effective dissociating agent than ADP. On the other hand, G-actin, in either non-covalent or in an EDC-cross-linked covalent complex with S1, is a poor activator of MgATPase of myosin head (Offer et al., 1972; Estes and Gershman, 1978; Combeau et al., 1992). However, when the covalent complex is isolated and polymerized, the MgATPase of S1 is almost entirely restored (Forné and Chaussee, 1992). Since after cross-linking with a zero-length cross-linker, S1 can change its interface with actin only to a very limited degree, this experiment indicates that in both G- and F-form the interactions take place in the same region(s) of the protein(s) but the filamentous (or oligomeric) structure of actin is required to support the MgATPase activity. Both electrostatic and hydrophobic contacts stabilize the F-acto-S1 interactions. The presence of hydrophobic interactions in the G-acto-S1 complexes has not been demonstrated. Although the main EDC cross-linking region of actin to S1 is identical in both proteins, some differences in their interactions in the C-terminal actin region were revealed by pPDM cross-linking of S1 to G-actin (Combeau et al., 1992). In addition, one should mention the lack of EDC cross-linking of an unpolymerizable actin analog (Bettache et al., 1989) to the 50-kDa fragment of S1 (Houmi, 1992). Furthermore, the formation of the G-acto-S1 complex requires a contact of the N-terminal segment of actin with S1 (Chaussee and Kasprzak, 1989b); this contact is energetically important in the F-acto-S1 complex but not absolutely essential (Miller et al., 1987; Mejean et al., 1987).

In summary, there is considerable resemblance between the G-acto- and the F-acto-S1 interfaces. Some new interactions present in the F-form may be created by the proximity of other actin protofilaments in the filament. In addition, polymerization may induce structural changes in the protomer. Such changes are expected to be subtle because it is believed that, with the exception of stretches of the polypeptide chain in subdomain 2, the structure of the protomer is quite similar to that of the monomeric actin (Holmes et al., 1990; Holmes and Kabsch, 1991). In this study evidence is shown that S1 binding affects the nucleotide binding region of actin, partially inhibiting the dissociation of both the nucleotide and calcium. A preliminary report of this finding has appeared (Kasprowski, 1995).

### MATERIALS AND METHODS

**Proteins—Myosin subfragment 1 (S1)** was prepared from rabbit skeletal muscle myosin by the method of Weeds and Taylor (1975). The isoenzymes S1A1 and S1A2 were separated by step elution from an SP-Trisacyl-agarose column (IBP Biotechnics, Villevoule-la-Garenne, France) with, respectively, 40 and 80 mM NaCl in 10 mM MOPS (Sigma), 0.4 mM DTE (Serva), pH 7.0, followed by (NH4)2SO4 precipitation and extensive dialysis against 10 mM MOPS, 0.4 mM DTE buffer. Only S1A2 isoenzyme was used, since in low ionic strength buffers it forms a stable complex with G-actin that does not polymerize (Chaussee and Kasprzak, 1989a). Rabbit muscle actin was purified from acetone powder by the method of Spudich and Watt (1971), followed by gel filtration (MacLean-Fletcher and Pollard, 1980) on Sephacryl S-200 (Pharmacia) using a 2.2 x 125-cm column that had been equilibrated with buffer G1 (2 mM Hepes, 0.1 mM CaCl2, 0.2 mM DTE, 0.1 mM NaN3, 5 mM ATP (Boehringer), pH 7.4). The proteins were used within 1 week, and for most experiments within 3 days. Actin was labeled with N-(1-pyrenyl)iodoacetamide (Molecular Probes) according to Cooper et al. (1983). Modification of actin with 1.5-IAEDANS (Sigma) was done as described by Kasprzak et al. (1988). Actin cleaved at Met-47/Gly-48, referred to as "subtilisin-cleaved actin," was prepared essentially as described by Schwyetz et al. (1988), followed by dialysis against G1 buffer to remove phenylmethylsulfonfyl fluoride. The "truncated actin," i.e. actin that is devoid of the last 3 amino acid residues, was obtained by the limited proteolysis of Mg-G-actin with trypsin at a weight ratio of 1:50 for 1 h, followed by addition of soybean trypsin inhibitor (Mossakowska et al., 1992). The protein was then subjected to one polymerization-depolymerization cycle and finally purified by gel filtration chromatography on Sephacryl S-200. In a separate experiment the truncated actin was prepared starting from NBD-labeled protein (Sigma) covalently modifies Lys-373 (Detmers et al., 1981). It was shown by gel electrophoresis that this lysine residue, the presence of which was monitored by the NBD fluorescence, had been lost during proteolysis. The initial rate of polymerization for the modified actin was lower than the unmodified protein. KCl-monomer was obtained by adding 0.1 M KCl to ATP-actin at concentration of 2 μM.

**Spectroscopic Measurements—** All spectroscopic measurements were performed in buffer G0 containing 2 mM Hepes, 0.1 mM CaCl2, 0.2 mM DTE, 0.1 mM NaN3, final pH in the cuvette 7.35-7.4 at 20 °C. Before experiments, the proteins were centrifuged for 1 h at 45,000 rpm and passed through 0.2-μm filters (Sartorius).

**Exchange of ATP by iATP—** Exchange of ATP by iATP in G-actin was done by adding 0.2 mM iATP (Sigma) to a solution of actin in buffer G0, and incubating the mixture overnight. The 1:1 ATP-G-actin complex was prepared by removing the excess of the nucleotide with AG 1-X8 resin as described by Mulhern et al. (1975). The resin (Bio-Rad) was recycled and prewashed as recommended by Kuehl and Gergely (1969).

**Kinetic Fluorescence Measurements—** For binding experiments of S1 to pyrene-labeled actin the procedure of Valentin-Rasc et al. (1991) was folowed. For anisotropy titrations of 1.5-IAEDANS-actin with S1, the experimental procedure was the same as reported previously (Chaussee and Kasprzak, 1989a), except that the polarization accessory of a Perkin-Elmer LS50 fluorometer and ATP-free buffer were used. For both of the above titrations, aliquots of concentrated S1A2 were added sequentially to the solution of labeled actin for anisotropy measurements of ATP dissociating from the nucleotide-binding site of actin. The fluorescence intensity of ATP bound to actin (Fo) and the fluorescence intensity of ATP free in solution (F0) were measured at 410 nm. Fo/F0 = 1.40, which was monitored by the fluorescence anisotropy of a large excess of ATP (1 mM), which completely displaced actin-bound ATP. The progress of the reaction was recorded using λex = 340 nm and λem = 410 nm.
RESULTS

Measurement of the Rates of Dissociation of cATP and Ca\(^{2+}\) — Since ATP and ADP dissociate the G-acto-S1 complex and, in addition, ATP is hydrolyzed by S1, the conventional methods of nucleotide exchange could not be used. Instead, the fluorescent nucleotide analog, 1,N\(^{6}\)-etheno-ATP (cATP), which remained in equilibrium with actin-bound cATP, was hydrolyzed by an excess of alkaline phosphatase (Purich and McNeal, 1978) or apryrase, leading to a mass-action driven dissociation of the protein-bound nucleotide. The progress of the dissociation was followed fluorometrically by recording a large decrease of the cATP fluorescence (~83%), upon transferring the etheno base of the nucleotide from its actin-binding site to the solution. To ascertain that the method indeed measures the rate of the nucleotide dissociation (\(k_d\)), it was first established that alkaline phosphatase is in excess in the reaction mixture. To show this, additions of that enzyme at concentrations 5 and 10 times higher than the initial concentration were used without change in the apparent dissociation rate of cATP. The equilibrium between G-actin and S1 is reached within 5 s (Valentin-Ranc et al., 1991), so theoretically, measuring the initial reaction rate should provide the true values of \(k_d\). Nonetheless, in the first few minutes after addition of phosphatase, nucleotide-free (and presumably denatured) actin is produced. Its behavior, binding properties, and the tendency to aggregate are unknown. Furthermore, alkaline phosphatase itself could interfere with the binding of S1 to G-actin. Therefore, in parallel to the dissociation of cATP effected by alkaline phosphatase, I measured the fluorescence anisotropy of 1,5-IAEDANS in an cATP-1,5-IAEDANS-actin-S1A2 complex. Although the progress of the reaction was substantial (Fig. 1), no change in the fluorescence anisotropy of 1,5-IAEDANS attached to actin was observed. For the experiments shown in Fig. 1, the average values of the fluorescence anisotropy of cATP-1,5-IAEDANS-actin (\(\lambda_{ex} = 380 \text{ nm}, \lambda_{em} = 467 \text{ nm}\)), were 0.146 ± 0.006 in the absence of S1, 0.187 ± 0.006 in the presence of 1.2 \(\mu\)M of S1A2, and 0.248 ± 0.007 in the presence of 9.7 \(\mu\)M of S1A2. These findings demonstrate that in spite of the nucleotide removal from actin in the G-acto-S1 complex, the protein remained bound to S1 and the G-acto-S1 equilibrium in the system was not shifted. From the semilogarithmic plot in Fig. 1, it is evident that the reaction of cATP dissociation was following first order kinetics rather well. Fig. 1 illustrates also the striking effect of S1 on the rate of cATP dissociation.

When EDTA was used instead of alkaline phosphatase, the complexation of calcium led to immediate nucleotide dissociation, as the removal of the metal ions from G-actin lowers the affinity of ATP 10\(^{4}\)-fold (Valentin-Ranc and Carlier, 1991). Thus the method measures, in fact, the rate of calcium dissociation (Nowak et al., 1988). Additionally, in this case polarization measurements were performed (data not shown), which led to the conclusion that during the time necessary to perform the measurements (~1 min) the G-acto-S1 complex remained intact.

Binding Isotherms — To obtain interpretable kinetic data concerning the dissociation of either nucleotide or Ca\(^{2+}\) from the G-acto-S1 complex, it is necessary to know the parameters for the interaction of G-actin with S1A2: the number of binding sites and the binding constant. I have followed the binding of S1A2 to G-actin by three methods: (a) the enhancement of pyrenyl fluorescence (the pyrene moiety was conjugated with Cys-374 of actin; Chen and Reisler (1991)), (b) the increase of the anisotropy of Cys-374-bound 1,5-IAEDANS (Chaussepied and Kasprzak, 1989a), and (c) the increase of the anisotropy of actin-bound cATP, for the first time reported here. The last method is of particular value, since it does not require chemical modification of the protein and can be performed on actin in which the C-terminal residues have been proteolytically removed (see below).

All three methods produced nearly identical results, as shown in Fig. 2. In this figure, the dashed lines are computed assuming one binding site and \(K_d = 0.08 \mu\)M. Small deviations from the computed line in the low concentration region are seen and indicate that there exists a second weak binding site on S1 for actin, but its affinity is so low that it cannot be neglected. These data qualitatively agree but are quantitatively at variance with the results of Valentin-Ranc et al. (1991), who postulated that the second binding site on S1 also has a high affinity for actin. I emphasize that the results chosen for the presentation are the most typical examples taken from a large body of data. In a considerable number of experiments, no deviations were seen; with other protein preparations, the deviations were somewhat higher than the ones shown.

The Effect of S1A2 on the Dissociation Rate of cATP and of Calcium from Native G-actin — The rate of cATP dissociation, \(k_{\text{d(cATP)}}\), obtained from the measurements of the initial reaction rates, for free G-actin was found to be 5.5 \(\times\) \(10^{-4}\) s\(^{-1}\) (Table I), in good agreement with the previous studies of Kuehl and Gergely (1969), who obtained 3 \(\times\) \(10^{-4}\) s\(^{-1}\) at pH 7, and of Nowak et al. (1988) (5 \(\times\) \(10^{-4}\) s\(^{-1}\) at pH 7.2). In the presence of increasing concentration of S1A2, a progressive reduction of the apparent \(k_{\text{d(cATP)}}\) was seen (Fig. 3A). The dependence of \(k_{\text{d(cATP)}}\) on S1 concentration followed closely the binding isotherm in Fig. 2; in both figures the dashed lines were computed assuming the same binding parameters (given in the preceding section). Thus it seems clear that formation of the binary G-acto-S1A2 complex is the only cause of the reduced \(k_d\) in this system. As described above, addition of EDTA to G-actin or to G-acto-S1 results in the release of both nucleotide and calcium, allowing for measurement of \(k_d\) for Ca\(^{2+}\). The rate of calcium dissociation from actin were 2 orders of magnitude higher than for the nucleotide. For free actin a value of 2.8 \(\times\) \(10^{-2}\) s\(^{-1}\) was obtained (Table I), which is identical to the value of 2.8 \(\times\) \(10^{-2}\) s\(^{-1}\) measured with Quin-2 at slightly higher pH of 7.6 (Estes et al., 1987), similar to Frieden and Patane (1988) (~3 \(\times\) \(10^{-2}\) s\(^{-1}\)), and to Zimmerle
FIG. 2. Binding isotherms of S1A2 to G-actin obtained by measuring (A) fluorescence enhancement of pyrenyl fluorescence, (B) increase in fluorescence anisotropy of 1,5-IAEDANS conjugated to G-actin, and (C) increase in fluorescence anisotropy of actin-bound cATP. In all panels, [actin] = 2.5 μM. In A and B the nucleotide bound to actin was ATP; in C, cATP. The dashed lines were computed assuming one binding site and $K_d = 0.08 μM$.

**TABLE I**

The effect of S1A2 on the rate constants for dissociation of εATP bound to G-actin, to proteolytically modified G-actin, and to KCl-monomer

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dissociation effected by</th>
<th>$k_d$ $s^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native G-actin</td>
<td>Alkaline phosphatase $^b$</td>
<td>5.5 x 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>EDTA $^b$</td>
<td>2.8 x 10^{-2}</td>
</tr>
<tr>
<td>Subtilisin-cleaved</td>
<td>Alkaline phosphatase</td>
<td>11 x 10^{-4}</td>
</tr>
<tr>
<td>G-actin</td>
<td>EDTA</td>
<td>3.3 x 10^{-2}</td>
</tr>
<tr>
<td>Truncated G-actin</td>
<td>Alkaline phosphatase</td>
<td>5.0 x 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>2.5 x 10^{-2}</td>
</tr>
<tr>
<td>KCl-monomer</td>
<td>Alkaline phosphatase</td>
<td>29 x 10^{-4}</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>7.2 x 10^{-3}</td>
</tr>
</tbody>
</table>

$^a$ At saturating concentration.

$^b$ Or apyrase.

$^c$ 0.2 mM EDTA added to G-buffer containing 0.1 mM Ca$^{2+}$.

$^d$ ND, not determined; in 100 mM KCl G-acto-S1A2 complexes polymerize rapidly.

The Effect of S1A2 on $k_d$ for Proteolytically Modified Actin—
In the search for the region of actin that is essential for the inhibition of the nucleotide and calcium dissociation by S1, I used two actin variants obtained by limited proteolysis. In the subtilisin-cleaved actin the peptide bond between residues 47 and 48 is broken. However, the two pieces remain associated and the modified protein retains most of its properties (filament formation, activation of the S1 ATPase, etc.), with the exception of failing to support efficiently the sliding motion over myosin (Schwyter et al. 1990). In the absence of S1, the dissociation rate of εATP for subtilisin-cleaved actin was found to be approximately twice as high as for the unmodified protein (Chen et al., 1992); the rate of Ca$^{2+}$ dissociation was not, however, significantly affected (Table I). In the binary complex of the cleaved actin with S1A2, its behavior closely resembled that of the native protein as it underwent the reduction of the $k_d$ for both εATP and Ca$^{2+}$ (Table I).

The second type of actin derivative was the truncated actin containing amino acids 1-372. It has been used previously to grow crystals of the DNase I-actin complex for which the three-dimensional structure was solved by x-ray crystallography (Kabsch et al., 1990). The interest in this protein stems from the fact that some parts of S1 are very close to the C terminus of actin, since in the G-acto-S1 complex a pPDM cross-link can be made between Cys-374 and S1 (Combeau et al., 1992). In order to find out whether the removal of the last
3 residues has changed the affinity of the protein to S1, I performed a polarization titration of the 1:1 αATP-truncated actin complex (Fig. 4). Although a more comprehensive study is required to characterize the interaction between the modified protein and S1, one must conclude from Fig. 4 that the affinity of this actin was not substantially lowered by the modification. This is in accord with O'Donoghue et al. (1992) who found that removal of the last 2 residues of actin did not alter $K_m$ for activation of S1 Mg$^{2+}$-ATPase by filamentous actin. The data in Fig. 4 and in Table 1 demonstrate that the truncated actin was undistinguishable from the native protein in respect to its ability to have its dissociation rate constants for αATP and for Ca$^{2+}$ inhibited by S1A2. Thus, despite their close proximity to S1 (Combeau et al., 1992), the C-terminal residues of actin are not a major part of the G-acto-S1 interface.

The Rate of αATP and Ca$^{2+}$ Dissociation from KCl-monomer—At subcritical actin concentration, the presence of 0.1 M KCl leads to a new actin species referred to as “KCl-monomer” (Pardee and Spudich, 1982), “F-monomer” (Rich and Estes, 1981), or “G-actin” (Rouyarenc and Travers, 1981). The conformation of actin in KCl-monomer is believed to be intermediate between G- and F-actin. On the other hand, the G-acto-S1 complex has a more pronounced propensity to polymerize so that monovalent salts at concentration < 5 mM are usually sufficient to initiate the assembly of the G-acto-S1 complexes into decorated filaments. This and other considerations have led to the suggestion that in the G-acto-S1 complex, actin exists in a “pre-F” state (Chen et al., 1992). As shown in Table 1, for KCl-monomer, the values of $k_{d}$ for αATP and Ca$^{2+}$ are significantly higher than for free G-actin. These values cannot, however, be used directly for comparison, since they have to be corrected for the known effect of ionic strength on the rate of nucleotide exchange in G-actin (Kuehl and Gergely, 1969; Nishida, 1985). Using the data of Nishida et al. (1985), the rate of αATP exchange is 2.5-fold higher as the ionic strength of the solution is raised from I < 5 mM to I = 100 mM. Even with this correction, $k_{d}(\text{ATP})$ in KCl-monomer is still higher than for free G-actin. On the other hand, assuming that the same correction applies for the Ca$^{2+}$ dissociation, the increase of $k_{d}(\text{Ca}^{2+})$ in KCl-monomer can be entirely accounted for by the ionic strength effect. Nevertheless, one has to conclude that in contrast to the acto-S1 complexes, in KCl-monomer the dissociation of αATP and Ca$^{2+}$ is not inhibited.

**Fig. 4.** Binding isotherm (●) and dependence on the S1A2 concentration of $k_d$ for αATP dissociation (●) from truncated actin. The binding curve was obtained from anisotropy titration of actin-bound αATP. Actin concentration was 2.5 μM for ●, and 2.2 μM for ○. The lines were simulated assuming $K_m = 0.68$ μM and one binding site.

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### DISCUSSION

For the first time it has been demonstrated that the interaction of S1 with G-actin alters the structure of the nucleotide and the calcium binding regions of the protein. The effect appears to be indirect because S1 itself is not known to block the entrance to the nucleotide cleft of actin. Harvey et al. (1977) and Miki and Wahl (1984) reported that binding of S1 and heavy meromyosin to F-actin shortened the fluorescence lifetime of actin-bound αADP. Whether these observations are pertinent to our present results and whether the shortening of the lifetime and the inhibition of the nucleotide dissociation originate from the same structural change in both F- and G-actin remain to be seen. Inferencers concerning the topography of the cleft in F-actin made from the kinetics of nucleotide exchange are misleading. Nucleotides exchange extremely slowly in F-actin (for review, see Korn (1982)), but this is due to steric blocking of the cleft by another actin protomer. On the other hand, experiments with dimeric actin obtained by pPDM cross-linking indicated that contact with another protomer produced structural changes in both actin molecules, which dramatically slowed the exchange process (Mockrin and Korn, 1981). Steric hindrance of the cleft is not expected to take place in the pPDM-actin dimer; however, some deformation of the molecules involved due to cross-linking cannot be excluded.

Although actin cleaved at Met-47/Gly-48 hardly supports the movement of its filaments over myosin in vitro (Schwyter et al., 1990), and exhibits a higher value of $k_d$ for ATP, the “nicked” protein responded to S1 binding in a fashion similar to native actin. Thus the reason for the inefficient sliding in vitro was not the inability to undergo structural change in the vicinity of the nucleotide cleft. The results obtained for KCl-monomer are more difficult to correlate with the data presented above. Acrylamide quenching experiments have indicated that the formation of the G-acto-S1 complex did not change the solvent accessibility for native G-actin. In contrast, for KCl-monomer the quenching rate constant was higher, which may indicate that KCl-monomer possesses a nucleotide cleft more open than G-actin. The question concerning the occurrence of a “pre-F” actin state in G-acto-S1 complexes cannot be answered conclusively, since structural differences have been detected in the state of actin in KCl-monomer and in the G-acto-S1 complex. Therefore, if they both represent a “pre-F” actin state, either there are two distinct pathways to polymerize actin, or, alternatively, the conformation of the protein in KCl-monomer and the G-acto-S1 complexes may correspond to different steps of a common polymerization pathway.

Finally, I shall consider a plausible molecular mechanism for explaining the inhibition of nucleotide and calcium dissociation from G-actin by S1. For both native and modified protein, the presence of S1A2 reduces $k_{d}(\text{ATP})$ and $k_{d}(\text{Ca}^{2+})$ by approximately the same factor (Table 1) because binding of the nucleotide and calcium occur in an interdependent fashion. The nucleotide in the cleft bridges the two (large) domains of actin, hence stabilizing the protein. The tightly bound calcium ion is situated at the bottom of the cleft and forms bonds with the phosphate moiety of ATP and with the protein (Kabsch et al., 1999). The adenine base is surrounded by a hydrophobic niche between subdomains 3 and 4. Besides the calcium ion, the phosphate groups of the nucleotide are held by Ser-14, Gly-15, and Leu-16 of subdomain 1, and by Asp-132, Gly-133, Val-134, and Gly-302 of subdomain 4. SI, by binding to residues 1-7, 18-28, and/or 95-113 of subdomain
1 could rearrange the spatial position of segment 14–16, hence altering the nucleotide-protein interactions. It is conceivable that such a rearrangement could be produced for a motion of the entire "small" domain of actin (see below). In agreement with this notion, a 2-fold decrease in the rate of nucleotide dissociation was observed when an antibody directed against segment 18–29 of actin bound to it (Adams and Reisler, 1993). Nonetheless, other mechanisms can also explain the data; S1 may induce a movement of segment 61–69 located at the mouth of the cleft to create an obstruction for the dissociating nucleotide.

This hypothesis is in accord with the reported effect of Lys-61 modification on S1 binding (Miki et al., 1987); Barden and Phillips, 1990) and the recently reported protection against proteolytic cleavage in segment 61–69, afforded by S1 (Chen et al. 1992). It is noteworthy that the two above mechanisms are non-exclusive and may operate at the same time. In the third mechanism, the nucleotide and calcium dissociate from actin while the protein momentarily swings its two (large) domains apart, partially exposing the cleft and weakening the bonds of the two ligands with the protein. The occurrence of such short-lived "open" conformation of actin was postulated from the similarity of the three-dimensional structure of protein to that of hexokinase for which an opening of the molecule is well documented (Holmes and Kabsch, 1991). That kind of motion is also expected from the normal mode analysis of the G-actin molecule (Tirion et al., 1993). S1, by interacting with actin, and especially with its two "CONNECT" segments (Bork et al., 1992), could make the transitions to the open conformation less frequent or decrease the time that the protein spends in this conformation. Presently, the available data do not allow for an unequivocal selection of the mechanism of nucleotide and calcium dissociation.

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