Interaction and Polymerization of the G-Actin–Myosin Head Complex: Effect of DNase I†

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ABSTRACT: The properties of polymerization and interaction of the G-actin–myosin S1 complexes (formed with either the S1(A1) or the S1(A2) isoform) have been studied by light-scattering and fluorescence measurements in the absence and in the presence of DNase I. In the absence of DNase I, the G-actin–S1(A1) and G-actin–S1(A2) complexes were found to be characterized by different limiting concentrations (l.c.), defined as the complex concentrations above which the polymerization occurs spontaneously within 20 h at 20 °C in a "no salt" buffer (l.c. = 0.42 and 8.8 μM for G-actin–S1(A1) and G-actin–S1(A2), respectively). The occurrence of a limiting concentration for either complex together with the kinetic properties of the polymerization led us to conclude that the G-actin–S1 polymerization occurs via a nucleation–elongation process. Fluorescence titrations and proteolysis experiments revealed that G-actin interacts with S1 with a 1:1 stoichiometry (independently of the presence of ATP) with dissociation constants, in the absence of nucleotide, of 20 and 50 nM for the G-actin–S1(A1) and G-actin–S1(A2) complexes, respectively. In the presence of at least a 1.5-fold excess of DNase I, the polymerization of the G-actin–S1 complexes was blocked even at high protein concentration or in the presence of salts. In addition, the affinity of either S1 isoform to actin was reduced 4–5-fold by DNase I, while the stoichiometry of the G-actin–S1 complexes was not changed. However, since the dissociation constants remain in the submicromolar range, we could demonstrate the existence of ternary DNase I–G-actin–S1 complexes stable under polymerizing conditions. Finally, the study of the effect of nucleotides and of various salts on the G-actin–S1 interaction further showed significant differences between the G-actin–S1 and F-actin–S1 interactions.

Muscle contraction as well as a large number of nonmuscle motile processes are based on the cyclic interaction between myosin subfragment 1 (S1)[1] and filamentous actin. Since the mechanochemical transduction events linked to these processes imply a coupling between the ATP hydrolysis by myosin and the interaction between actin and myosin, a large number of studies have been devoted to the understanding of the molecular basis of these events [Huxley & Kress, 1985; for reviews see also, Cooke (1986), Vibert and Cohen (1988), and Botts et al. (1989)]. Nevertheless, these studies had to overcome the difficulties which reside in using the filamentous form of the proteins at physiological ionic strength. Moreover, even under nonphysiological conditions, i.e., at very low ionic strength, S1 had been known for a long time to induce polymerization of monomeric actin (Yagi et al., 1965; Cooke & Morales, 1971; Detmers et al., 1981; Miller et al., 1988). As a consequence, it was believed until recently that a complex of monomeric actin (G-actin) with S1 could be obtained only after chemical modification of actin in order to reduce as much as possible the S1-induced polymerization of actin (Miki, 1989; Bettache et al., 1989, 1990; Miki & Hozumi, 1991). Chaussepied and Kasprzak (1989) found, however, that skeletal S1 isoforms, S1(A1) and S1(A2), behave differently in their capabilities in polymerizing G-actin under no-salt conditions, leading to the purification of a stable G-actin–S1(A2) complex at relatively low protein concentration. Subsequently, Chen and Reisler (1991) confirmed, by analytical ultracentrifugation, the formation of a stoichiometric G-actin–S1 complex in a low-salt buffer, while another group (Valentin-Ranc et al., 1991) proposed, on the basis of fluorescence experiments, the association of two G-actin molecules with one molecule of S1 in the absence of ATP.

The knowledge of the exact actin/S1 stoichiometry in the monomeric complex is of particular importance for at least two reasons. Firstly, it is necessary to understanding the kinetic intermediates in the S1-induced polymerization of actin. Secondly, and most importantly, it is essential to comparing the structure of the actin–S1 complex in its monomeric and filamentous forms. An extensive comparison between these two forms of the complex will indeed be helpful to understanding the activation process of the Mg^{2+}-ATPase of S1 occurring with F-actin (Offer et al., 1972; Estes & Gershman, 1978). Such comparative studies have already been performed on particular sites of the actin–S1 interface using anti-peptide or antibodies (Chaussepied & Kasprzak, 1989b; DasGupta et al., 1990a) and proteolytic and chemical cross-linking approaches (Bettache et al., 1992; Chen et al., 1992; Combeau et al., 1992; Fievez & Carlier, 1993). Nevertheless, none of these could definitively relate the difference in Mg^{2+}-ATPase

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[1] Abbreviations: S1, myosin subfragment 1; S1(A1), S1 isoenzyme with alkaline light chain 1; S1(A2), S1 isoenzyme with alkaline light chain 2; G-, F-actin, monomeric and filamentous actin; pyr-actin, pyrenyl-labeled actin; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; HEPES, *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid.

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activation with a change of a particular subsite on the actin–S1 interface. However, the fact that S1 could interact with two actin monomers on the actin filament (Mornet et al., 1981; Amos et al., 1982; Milligan et al., 1990; Andreev & Borejdo, 1992) led to the hypothesis that the Mg^{2+}-ATPase activation could be due to the contact of S1 with a second actin monomer. Since G-actin does not activate S1 ATPase activity, such an idea would be supported by a 1:1 stoichiometry in the G-actin–S1 complex.

Due to the uncertainties about the composition and the properties of the monomeric actin–S1 complex, the primary goal of this study was to reinvestigate the characteristics of the interaction between G-actin and the two S1 isoenzymes, S1(A1) and S1(A2), by using fluorescence titrations and limited proteolysis experiments. However, we found during preliminary work that when used at high concentrations, both S1 isoforms were in fact capable of polymerizing actin. Therefore the characterization of the G-actin–S1 interaction was also performed in the presence of DNase I, which was found to stabilize the actin–S1 complexes in their monomeric forms, thus allowing the study of the complex under polymerizing conditions. These results were reported previously in a preliminary form (Chaussepied, 1991).

MATERIALS AND METHODS

Reagents. α-Chymotrypsin was from Worthington Biochemicals. ATP, ADP, soybean trypsin inhibitor, and DNase I were from Boehringer Mannheim. Hydroxyapatite, DTT, and PMSF were from Serva. SP-trisacryl M was purchased from IBF. Sephacryl S200 and PD10 columns were obtained from Pharmacia. *N*-(1-pyrenyl)iodoacetamide was from Molecular Probes. All other chemicals were of the highest analytical grade.

Preparation of Proteins. Unless otherwise indicated, all protein preparations were performed at 4 °C. Myosin was prepared from rabbit skeletal white muscle according to Offer et al. (1972). S1 was obtained by chymotryptic cleavage of myosin essentially as described by Weeds and Taylor (1975) with the following modifications. After the chymotryptic cleavage of 1–1.5 g of myosin (enzyme/substrate weight ratio of 1/150 for 20 min at 25 °C, in 10 mM KH_2PO_4, 120 mM KCl, 1 mM EDTA, and 0.1 mM NaN_3, pH 7.0), insoluble material was removed by ultracentrifugation at 150000*g* for 1 h. The supernatant was then filtered through a Millipore membrane (pore size 0.45 μm) and desalted on a Sephacryl S200 column (5 × 60 cm) equilibrated with buffer A: 10 mM MOPS, 0.4 mM DTT, and 0.1 mM NaN_3, pH 7.0. S1-containing fractions were then applied to an SP-trisacryl M column (4 × 3 cm) equilibrated with buffer A in order to separate the S1(A1) and S1(A2) isoenzymes (Trayer & Trayer, 1988). Elution of S1(A2) and S1(A1) was achieved by a step gradient with 40 and 90 mM NaCl, respectively. Proteins were then concentrated in an Amicon cell (PM30 membrane) until the protein concentration reached 70–90 μM. After extensive dialysis against buffer A (3 changes), the proteins were ultracentrifuged at 150000*g* for 90 min, filtered through a Millipore filter (pore size 0.22 μm), and kept on ice for a maximum of 4–5 days. This procedure allowed purification of the S1 isoforms within 48 h.

F-actin was prepared from acetone powder of rabbit skeletal white muscle according to Spudich and Watt (1971). After ultracentrifugation at 150000*g* for 1 h, F-actin was resuspended in buffer G_{100} (2 mM HEPES, 0.1 mM ATP, 0.1 mM $CaCl_2$, 0.2 mM DTT, and 0.1 mM NaN_3, pH 8.0), depolymerized into G-actin by sonication (3 × 1 min with a Microson ultrasonic cell disruptor, at 50 W), and dialyzed against buffer G_{100}. After centrifugation at 150000*g* for 90 min, the G-actin present in the supernatant was further purified by Sephacryl S200 gel chromatography (2.5 × 120-cm column) equilibrated with buffer G containing either 100 or 5 μM ATP, as described in the text. In every case, G-actin was filtered through a Millipore membrane (pore size 0.22 μm), kept on ice at a concentration lower than 30 μM, and used within 3 days. In the text, G buffers will be designated according to their content of ATP, i.e., buffers G_0, G_5, and G_{100} contained no ATP, 5 μM ATP, and 100 μM ATP, respectively. As expected from the high affinity of the nucleotide for actin [for review, see Carlier (1991)], actin structure was not affected by the presence of the substoichiometric concentration of external ATP (generally 5 μM external ATP in 20–30 μM actin solution), as it fully polymerized and could activate the Mg^{2+}-ATPase activity of S1 to the same extent as actin purified with 100 μM ATP (not shown). F-actin was obtained by polymerization of G-actin with 100 mM KCl and 2.5 mM $MgCl_2$ at 30 °C for 2 h and sedimentation at 150000*g* for 1 h. F-actin was kept on ice for less than a week in buffer F: 2 mM HEPES, 0.1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 100 mM KCl, 0.2 mM DTT, and 0.1 mM NaN_3, pH 8.0.

Pyrenyl-labeled G-actin (pyr-G-actin) was prepared according to Cooper et al. (1983). After depolymerization, pyr-G-actin was mixed with native G-actin to obtain 30–90% labeled actin. The protein mixture was then passed through a Sephacryl S200 column equilibrated with buffer G as described above. The extent of labeling was determined by using a molar extinction coefficient of E_{344nm} = 22 000 M^{-1} cm^{-1} for the pyrene–protein complex (Kouyama & Mihashi, 1981).

Commercial DNase I from bovine pancreas was purified by hydroxyapatite chromatography (Price et al., 1969) as follows: 100 mg of DNase I dissolved in 10 mL of 20 mM KH_2PO_4 and 2.0 mM PMSF, pH 6.8, was passed through a 30-mL hydroxyapatite column (2.5 × 15 cm) equilibrated with 20 mM KH_2PO_4, 0.1 mM PMSF, and 0.1 mM NaN_3, pH 6.8. Elution was carried out by a linear gradient from 20 to 300 mM KH_2PO_4, pH 6.8 (2 × 150 mL). DNase I-containing fractions were pooled and dialyzed against 10 mM MOPS, 0.2 mM $CaCl_2$, 0.2 mM DTT, and 0.1 mM NaN_3, pH 7.0, ultracentrifuged at 150000*g* for 90 min, filtered through 0.22-μm-pore-size Millipore membrane, and kept at 4 °C.

Protein concentrations were determined spectrophotometrically assuming $A^{1\%}_{280nm}$ = 7.5 cm^{-1} for S1, $A^{1\%}_{280nm}$ = 12.3 cm^{-1} for DNase I, and $A^{1\%}_{280nm}$ = 11 cm^{-1} or $A^{1\%}_{290nm}$ = 6.3 cm^{-1} for F-actin or G-actin, respectively. The molecular masses assumed were 112, 105, 31, and 42 kDa for S1(A1), S1(A2), DNase I, and actin, respectively. The concentration of labeled actin was measured by the Bradford assay (Bradford, 1976) with native actin as a standard.

Determination of the Limiting Concentration. The critical concentration of pyr-G-actin and the limiting concentrations of the pyr-G-actin–S1 complexes were measured by quantifying the fluorescence increase of the pyrenyl moiety (at λ_{exc} = 366 nm and λ_{em} = 387 nm; Valentin-Ranc et al., 1991) induced by the polymer formation. Fluorescence measurements were performed before and after 20 h of incubation at 20 °C of various concentrations of G-actin in the presence of 2 mM $MgCl_2$ or mixed with a 1.2 M excess of S1(A1) or S1(A2) in buffer G containing a stoichiometric amount of nucleotide to actin.

Titration and Binding Experiments. The interaction between pyr-G-actin and S1(A1) or S1(A2) was studied in the absence and in the presence of DNase I at low ATP

concentration in G_0 buffer at 20 °C using the increase of fluorescence intensity of the pyrenyl chromophore induced by S1 binding (λ_{exc} = 366 nm, λ_{em} = 387 nm).

During titration experiments, increasing concentrations of S1 isoenzymes (0–10 μM) were added consecutively to 1 mL of solution containing 2 μM pyr-actin (60% labeled) in buffer G_0 with a final concentration of 0.5 μM ATP (brought from actin stock solution), and fluorescence intensity was recorded after a stable fluorescence value (reading) was reached (at the most within 2 min). Maximum dilution of the samples induced by S1 additions was less than 10%. Titration data were analyzed on an IBM-compatible 386SX computer according to the model proposed by Valentin-Ranc et al. (1991), using a computer program initially written by A. Kasprzak. According to the model, S1 and G-actin interact with two independent sites. Computed parameters used to draw the curves in Figure 5 are the dissociation constants of the two binding sites (K_{d1} and K_{d2}) and the maximum relative fluorescence (F_m/F_o). K_{d1}, K_{d2}, and F_m/F_o were 6 nM, 6.5 μM, and 3.13 (without DNase I) or 23 nM, 6.4 μM, and 3.12 (with DNase I) for S1(A1) and 42 nM, 5.9 μM, and 3.27 (without DNase I) or 47 nM, 5.2 μM, and 3.27 (with DNase I) for S1(A2).

In order to determine more accurately the affinity constants of the G-actin–S1 complexes, binding experiments were performed under conditions identical to those used in titration experiments except that lower actin concentrations were used (see the caption of Figure 7 for more details).

Binding experiments were also performed in the presence of various concentrations of salts. Under the nonpolymerizing conditions used, i.e., in the presence of DNase I, none of the salts ($MgCl_2$, $CaCl_2$, KCl, or KCH_3COO) affected the fluorescence of pyr-G-actin alone. Therefore, the decrease in the fluorescence of the DNase I–pyr-G-actin–S1 complexes observed upon addition of salt was related to the dissociation of the actin–S1 interaction. The percentage of bound actin was then calculated from the equation $B = B_0F/F_0$, where F and F_0 are the fluorescence values before and after addition of salt and B_0 is the percentage of actin bound to S1 under the initial conditions. B_0 values were calculated from the concentrations of actin and S1 and the corresponding affinity constant (Table I). B_0 was found to be 96.0 and 91.5% for G-actin–S1(A1) and G-actin–S1(A2), respectively.

Spectral Measurements. Fluorescence and light-scattering measurements were carried out on a Kontron SFM 25 spectrofluorimeter, with a cell thermostated by a circulating water bath. The data were transferred on line to an IBM-XT microcomputer for further analysis. Prior to measurements, all samples and buffers were passed through 0.22-μm Millipore filters. Pyrenyl fluorescence was monitored at 386 nm with the excitation wavelength at 366 nm. Light-scattering intensity was measured at 90° with unpolarized light at 600 nm (Cooper and Pollard, 1982).

Data Analysis. Limiting concentrations and binding data were analyzed by using the ENZFITTER nonlinear regression program (Biosoft-Elsevier, Cambridge, UK).

Gel Electrophoresis. The purity of the proteins was verified by 3–18% polyacrylamide gradient gel electrophoresis in the presence of SDS as described by Laemmli (1970). The gels stained with Coomassie blue were scanned with a Shimadzu CS 930 high-resolution gel scanner.

RESULTS

Polymerization Properties of the G-Actin–S1 Complex

At High Concentration, S1(A2) Is Able To Induce G-Actin Polymerization. Chaussepied and Kasprzak (1989a) found that, in contrast to S1(A1), S1(A2) at protein concentrations of 11–13 μM could form a complex with G-actin that was stable for more than 2 h at 20 °C. To test whether this property of S1(A2) was dependent on the experimental conditions (temperature, protein concentrations, etc.), we examined the stability of the G-actin–S1(A2) complex at higher temperature and protein concentrations. Figure 1 depicts a typical time course of the fluorescence changes of pyrenyl-G-actin upon addition of either S1(A1) or S1(A2). The fluorescence of the pyrenyl moiety attached to Cys374 of G-actin is a good reporter for S1 interaction and for the polymerization of the actin–S1 complex (DasGupta et al., 1990). Addition of 22 μM S1(A2) to 20 μM G-actin at 25 °C in buffer G induced an instantaneous increase of the fluorescence intensity corresponding to the formation of the G-actin–S1(A2) complex followed by a short stationary phase and a further slow increase of the fluorescence. The complete polymerization of the complex (corresponding to the plateau) was confirmed by the absence of monomeric actin in the supernatant after centrifugation at 150000g for 90 min (data not shown). The addition of the same amount of S1(A1) induced the polymerization of G-actin, though the rate of polymerization was more rapid than with S1(A2). Interestingly, the jump of fluorescence promoted by S1(A1) interaction was of a higher amplitude than that of S1(A2) and was followed by a rapid decrease of the fluorescence prior to the onset of polymerization. These later time-dependent changes in fluorescence, which result from structural rearrangement around Cys374, were already described for mixed S1 isoforms by Miller et al. (1988) and DasGupta et al. (1990). However, the present experiments revealed that these changes are more prononced for the S1(A1) isoform. Moreover, the relative extent of the fluorescence increase was dependent on the concentration of the actin–S1 complex (data not shown).

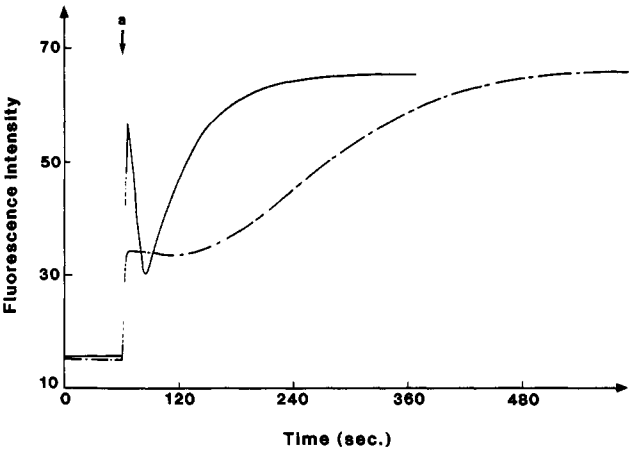

FIGURE 1: Time course of pyrenyl fluorescence during the polymerization of G-actin induced by S1(A1) and S1(A2). At the arrow 22 μM S1(A1) (—) or S1(A2) (- - -) was mixed with 20 μM pyr-G-actin (30% labeled) in buffer G_{20} at 25 °C.

G-Actin–S1(A1) and G-Actin–S1(A2) Complexes Have Different Limiting Concentrations. On the basis of the apparent concentration dependence on the rate of polymerization of the G-actin–S1(A2) complex, it was necessary to determine the concentration above which the G-actin–S1 complexes could polymerize in the absence of salt. Since the polymerization kinetics of actin alone and in the presence of S1 may proceed through different mechanisms, we used the term "limiting concentration" (l.c.) to define this protein concentration usually denoted as "critical concentration" for the salt-induced polymerization of G-actin.

The limiting concentrations were measured using the method first described by Valentin-Ranc et al. (1991) in which the amount of oligomeric complex is measured from the increase

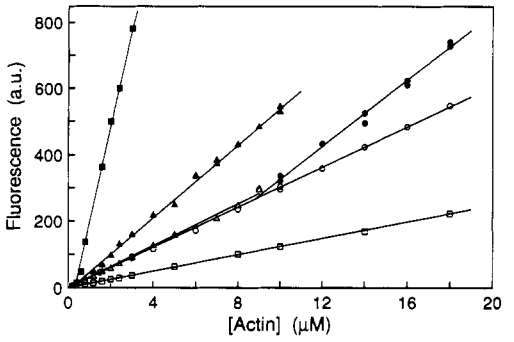

FIGURE 2: Determination of the limiting concentrations of actin in the absence and in the presence of S1 isoenzymes. Fluorescence intensities were measured at various concentrations of pyr-G-actin alone (□, ■) or pyr-G-actin–S1(A1) (△, ▲), and pyr-G-actin–S1(A2) (○, ●) complexes before (open symbols) and after (filled symbols) 20 h at 20 °C. In the case of the G-actin–S1 complexes, two different sets of experiments were performed with two different protein preparations. The fluorescence intensity was recorded as described under Materials and Methods.

of fluorescence of pyr-actin during polymerization. In the experiments described in Figure 2, various concentrations of G-actin were mixed with a 1.2-fold molar excess of either S1(A1) or S1(A2) in buffer G containing stoichiometric amount of ATP over actin. The relative increase of pyrenyl fluorescence was measured just after G-actin and S1 were mixed and 20 h later. These values are in good agreement with the 3–3.5-fold increase due to the formation of monomeric G-actin–S1 complexes and the 5–6-fold increase due to the formation of the decorated filaments (Figure 1 and Valentin-Ranc et al., 1991). Values of the limiting concentrations were obtained from the intersection of the linear regression lines computed for the oligomeric and monomeric species. Limiting concentration values were found to be 0.42 and 8.8 μM for the actin–S1(A1) and actin–S1(A2) complexes, respectively. The control experiment performed with actin alone but in the presence of 2 mM $MgCl_2$ gave a critical concentration of 0.35 μM, in good agreement with previous values obtained in similar concentrations of salt (Gordon et al., 1976). The limiting concentration value of 8.8 μM for the G-actin–S1(A2) complex is not in contradiction with the data reported previously (Chaussepied & Kasprzak, 1989a) which did not reveal polymerization of the complex at 11 μM after 2 h of incubation since the limiting concentration experiment described herein was performed after 20 h of incubation. However, these values of limiting concentration are significantly higher than those of 0.1 and 1.5 μM for G-actin–S1(A1) and G-actin–S1(A2) complexes obtained under very similar conditions by Valentin-Ranc et al. (1991). These differences can be accounted for neither by the extent of actin labeling nor by the nucleotide concentration in the solution since a mixture of 8 μM unlabeled G-actin and 10 μM S1(A2) in the presence of 100 μM ATP or stoichiometric amounts of ATP over actin did not polymerize after 20 h at 20 °C, as shown by cosedimentation. The reasons for this discrepency could be the use by Valentin-Ranc et al. (1991) of Dowex-treated actin to remove external nucleotide, although this treatment is not known to alter actin properties. The presence of traces of salt or protein aggregates could also explain the lower values of limiting concentrations they have obtained. Another possible explanation is the presence of contaminant S1(A1) in the preparations of S1(A2) (as indicated in the paper of Valentin-Ranc et al., 1991) since less than 10% of S1(A1) is sufficient to induce the polymerization of the entire preparation of S1(A2) (Chen & Reisler, 1991; P. Chaussepied, unpublished results) and therefore dramatically alter the values of limiting concentrations.

A more important question concerning these experiments is whether the values of fluorescence obtained after 20 h represent a "true" steady state reached by the system or whether they reflect only an intermediate state of an extremely slow polymerization process, as suggested by Valentin-Ranc and Carlier (1992). This question is difficult to resolve experimentally since after 30 h of incubation at 20 °C in buffer G, S1 and to some extent G-actin tend to denature, thus rendering any reliable measurement impossible. By analogy with similar experiments performed on actin alone, however, one may expect that the equilibrium was reached after 20 h of incubation.

DNase I Stabilizes the G-Actin–S1 Complexes in Their Monomeric States. The values of limiting concentrations determined previously imply that studies on the G-actin–S1 complexes can be performed only under restricted protein concentrations. In order to extend these studies to experimental conditions using higher protein concentrations or salts, we tried to prevent polymerization with the G-actin sequestering protein DNase I. In the experiment illustrated in Figure 3, we studied by light scattering and fluorescence the effect of DNase I on the polymerization of actin induced by S1(A1) or by S1(A2) and $MgCl_2$.

Light-scattering measurements clearly showed that DNase I inhibited the polymerization of actin induced by S1(A1) as well as that of the G-actin–S1(A2) complex induced by 2 mM $MgCl_2$ (Figure 3A). In accord with these results, DNase I totally abolished the increase of fluorescence of the pyr-G-actin due to the polymerization of the G-actin–S1(A1) and G-actin–S1(A2) complexes (Figure 3B,C). On the other hand, the interaction between G-actin and S1(A1) or S1(A2) did not seem strongly weakened by DNase I (Figure 3B,C). It is interesting to note that DNase I abolished the initial jump in fluorescence that we attributed to the effect of the A1 light chain on the actin structure. The addition of $MgCl_2$ to the DNase I–G-actin–S1 mixture induced a rapid decrease of the fluorescence corresponding to the dissociation of the actin–S1 interaction since the fluorescence of pyr-actin remained unchanged in the presence of DNase I without as well as with $MgCl_2$ (not shown).

This lack of polymerization in the presence of DNase I was further confirmed for both S1 isoforms after incubation of the protein mixtures for 20 h at 20 °C and analysis by cosedimentation and PAGE of the monomeric species remaining in the supernatant (data not shown). One should note, however, that a complete inhibition of the polymerization of the G-actin–S1(A1) complex needed at least a 2.5-fold molar excess of DNase I, while 1.5-fold excess was sufficient to block the S1(A2) complex in its monomeric form. The high DNase I excess needed for S1(A1) can be explained by a decrease in the affinity of DNase I when S1(A1) binds to G-actin.

These experiments showed that a concentration of DNase I from 1.5- to 2.5-fold higher than G-actin was sufficient to prevent polymerization of G-actin induced by S1. However, before the effect of DNaseI on the binding parameters of the G-actin–S1 complex was studied, it was of interest to determine whether the G-actin–S1 interaction was affected by DNase I in a concentration dependent manner. Figure 4 illustrates the effect of increasing DNase I concentrations on the fraction of S1 bound to actin measured by the fluorescence changes of pyr-G-actin. In the absence, as in the presence, of nucleotide, DNase I was found to dissociate partly the G-actin–S1(A1) as well as the G-actin–S1(A2) complexes. The dissociative effect saturated in all cases at about 1.5 mol of DNase I per actin monomer. Interestingly, this result agrees

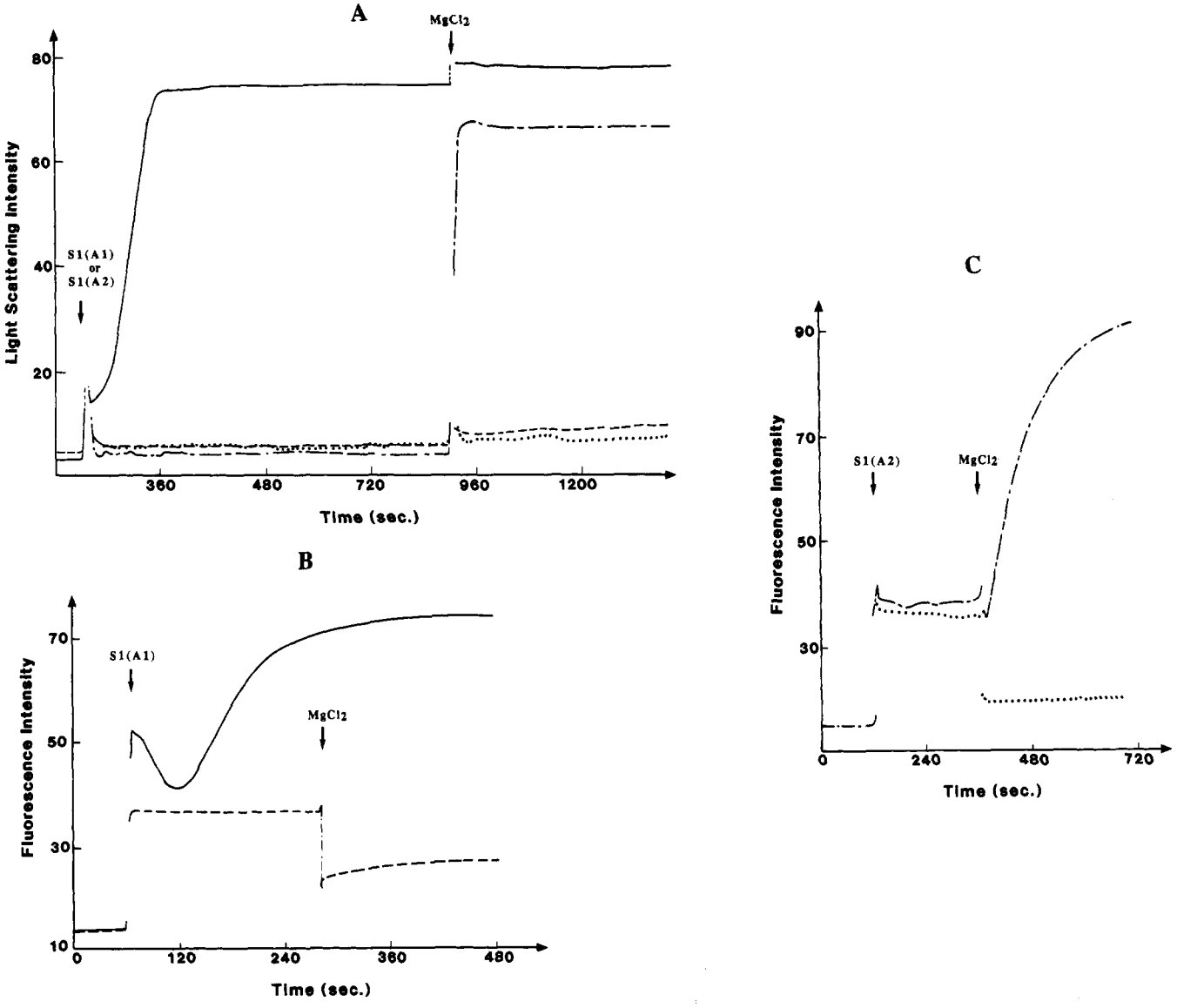

FIGURE 3: DNase I effect on the polymerization of the G-actin–S1 complexes. Polymerization of the G-actin–S1 complexes was monitored by following the changes of light scattering (A) or pyrenyl fluorescence (B, C) as described under Materials and Methods. Conditions: 10 μM pyr-G-actin alone (—, - - -) or mixed with 15 μM DNase I (- - -, · · ·) was incubated in buffer G_{100} at 25 °C. At the times indicated, 12 μM S1(A1) (—, · - ·) or S1(A2) (– – –, · · ·) and 2 mM $MgCl_2$ were added.

quite well with the amount of DNase I needed to block the actin–S1(A2) in its monomeric form (see above). In addition, this result confirms the idea that S1(A2) binding to G-actin does not significantly perturb the binding of DNase I to G-actin since it was also found not to alter G-actin capabilities to inhibit DNase I activity (Chen et al., 1992).

Taken together, these data demonstrate the existence of a stable ternary DNase I–G-actin–S1 complex useful to study of the G-actin–S1 interaction at high protein concentrations or in the presence of polymerizing agents such as $MgCl_2$ (see below).

Interaction Properties of the G-Actin–S1 Complex

The interaction between G-actin and S1 isoforms was mainly followed by monitoring the fluorescence of pyr-actin as described previously. We first determined the two parameters describing the interaction between G-actin and the S1 isoforms: the number of binding sites and the corresponding binding constants.

The G-Actin–S1 Complex Is Composed of 1 mol of Actin and 1 mol of S1 in the Absence as Well as in the Presence of DNase I. The number of binding sites was obtained from titration experiments in which the relative pyr-actin fluorescence was plotted versus increasing S1(A1) and S1(A2) concentrations. Under our experimental conditions (low actin concentration, experiment completed within 30 min) no polymerization of the complex was observed. For this reason, the increase of fluorescence corresponded only to the amount of complex reaching saturation when all the actin was complexed by S1. The data presented in Figure 5 describe the formation of a 1:1 complex between G-actin and S1(A1) or S1(A2) isoforms in the absence or in the presence of a saturating amount of DNase I. The slight deviation from the theoretical line describing a 1:1 stoichiometry with infinitely high affinity suggests the presence of a second weaker actin binding site on S1. Nonetheless, these results are in profound disagreement with those obtained by Valentin-Ranc et al. (1991) under identical conditions except for the presence, in our case, of trace ATP; the final ATP concentration was 25% that of actin and was negligible compared to the concentration of the actin–S1 complex. For comparison, data obtained by these authors were plotted using the binding constants and the two-sites model described in their paper. By computing

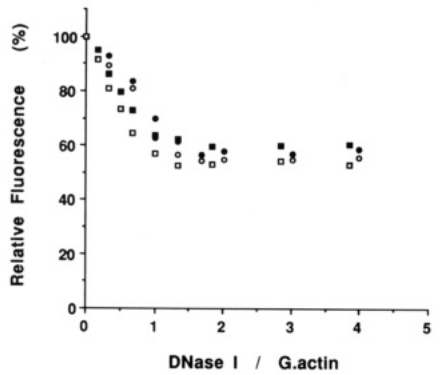

FIGURE 4: DNase I effect on the formation of pyr-G-actin–S1 complexes. Increasing amounts of DNase I were added to 1.5 mL of buffer G_0 containing a mixture of 0.5 μM pyr-G-actin and 1 μM S1(A1) (closed symbols) or S1(A2) (open symbols) in the absence (○, ●) and in the presence (□, ■) of 0.1 mM ATP, respectively (T = 20 °C). Fluorescence intensities of pyr-G-actin alone (F_o) or with S1 isoenzymes in the absence (F_s) and in the presence of various amounts of DNase I (F) were recorded as described under Materials and Methods. Relative fluorescence was calculated from $(F/F_o - 1)/(F_s/F_o - 1) \times 100$.

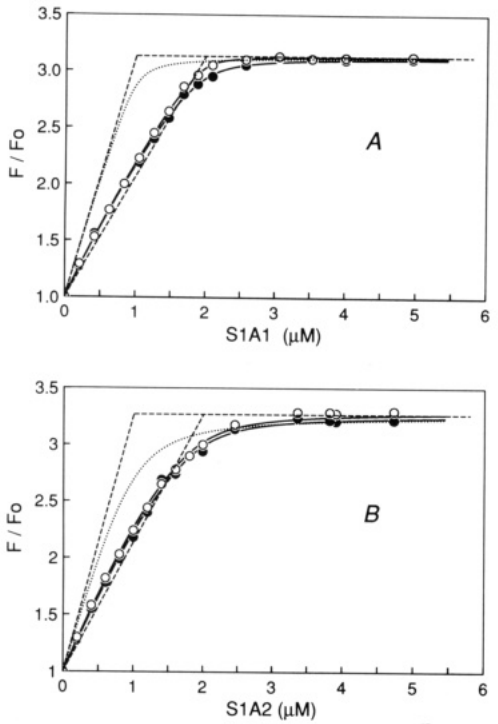

FIGURE 5: Fluorescence titration of pyr-G-actin with S1 isoenzymes. The effect of S1(A1) (A) or S1(A2) (B) on pyr-G-actin fluorescence was measured at 20 °C in buffer G_0 as described under Materials and Methods. Increasing amounts of S1 isoenzymes were added to 1-mL solutions containing 2 μM pyr-G-actin in the absence (○) or in the presence (●) of 4 μM DNase I. Fluorescence intensities were recorded in the absence (F_o) and in the presence (F) of S1 isoenzymes. Continuous lines (—) are theoretical curves calculated according to the model of Valentin-Ranc et al. (1991), assuming two dependent binding sites of G-actin for S1 isoenzymes with the parameters described under Materials and Methods. Dotted lines were obtained by assuming the parameters of Valentin-Ranc et al. (1991) (see text for explanation). Theoretical titration curves expected for high-affinity 2:1 or 1:1 complexes of G-actin and S1 are also drawn (- - -).

our data assuming their model, we found that the dissociation constant for the second binding site would be in the range 5–7 μM, approximately 100 times higher than that obtained by Valentin-Ranc et al. (1991). In addition, though these data represent the most typical examples, the deviation from the theoretical 1:1 stoichiometry line was not systematic. The difference in stoichiometry observed between our two laboratories is not yet understood. Among the possible explanations

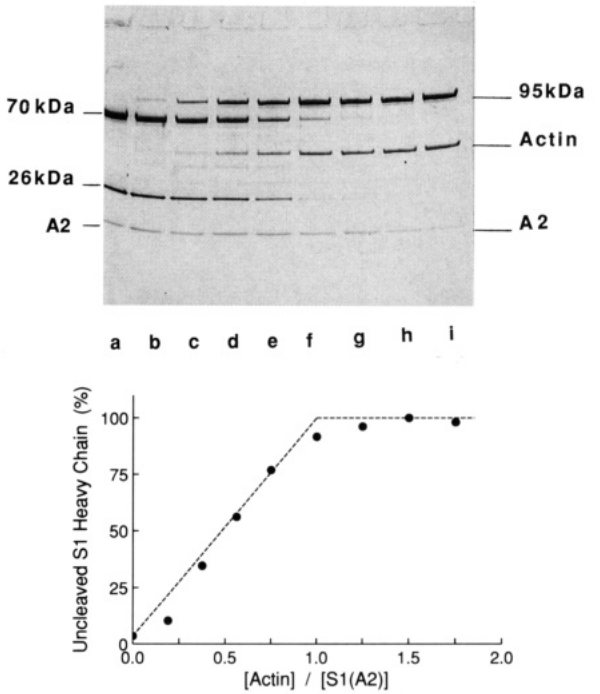

FIGURE 6: Proteolytic titration of G-actin with S1(A2) isoenzyme. S1(A2) (8 μM) was treated by papain (1/100 mass ratio) in buffer G_0 at 25 °C for 45 min in the presence of increasing G-actin concentrations. The proteolysis was stopped by boiling an aliquot of each reaction mixture in the PAGE denaturing solution. The amount of uncleaved S1 heavy chain (95 kDa) was measured by densitometric analysis of the gel (A, top) and plotted vs the [actin]/[S1(A2)] ratio (B, bottom). The letters a, b, c, d, e, f, g, h, and i in panel A stand for 0, 1.5, 3, 4.5, 6, 8, 10, 12, and 14 μM G-actin in the reaction mixture, respectively. The dashed line in panel B represents the theoretical curve for a high-affinity 1:1 G-actin–S1 complex.

tested, neither the extent of the chymotryptic cleavage of myosin used during S1 preparation (E/S ratios of 1/50 to 1/250 were tested) nor the use of old S1 gave us a clear reason for the discrepency. One should note, however, that a more pronounced deviation from the 1:1 stoichiometry was usually observed when S1 was not thouroughly and extensively centrifuged before titration experiments or when old pyr-G-actin preparations were used. In fact, the presence of some actin or S1 aggregates (partly induced by the very low ionic strength of the buffer G) could explain the small deviation that we observe.

The 1:1 stoichiometry of the G-actin–S1 complexes was independently confirmed by a proteolytic digestion approach. It was possible to estimate the amount of S1 bound to G-actin by taking advantage of the fact that G-actin protects the S1 heavy chain against papain digestion. This approach presented two advantages over the fluorescence experiments: it utilized unmodified proteins, and it avoided optical measurements sensitive to protein aggregates even when aggregates were present in minute amounts. In the experiment described Figure 6, we used only the S1(A2) isoform in order to perform the proteolysis at the highest protein concentration (stable for at least 50 min at 25 °C) suitable for the formation of the largest fraction of $(actin)_2$–S1 complex. Under our conditions, papain treatment of S1 in the absence of G-actin resulted in the cleavage of more than 96% of the S1 heavy chain, producing the NH_2-terminal 26-kDa and COOH-terminal, 70-kDa fragments (Applegate & Reisler, 1983). Addition of increasing amounts of G-actin progressively protected the S1 heavy chain from papain degradation. The plot of the concentration of the protected S1 heavy chain band (95-kDa band) vs the [actin]/[S1] ratio shows that the concentration of protected S1 follows the theoretical line corresponding to

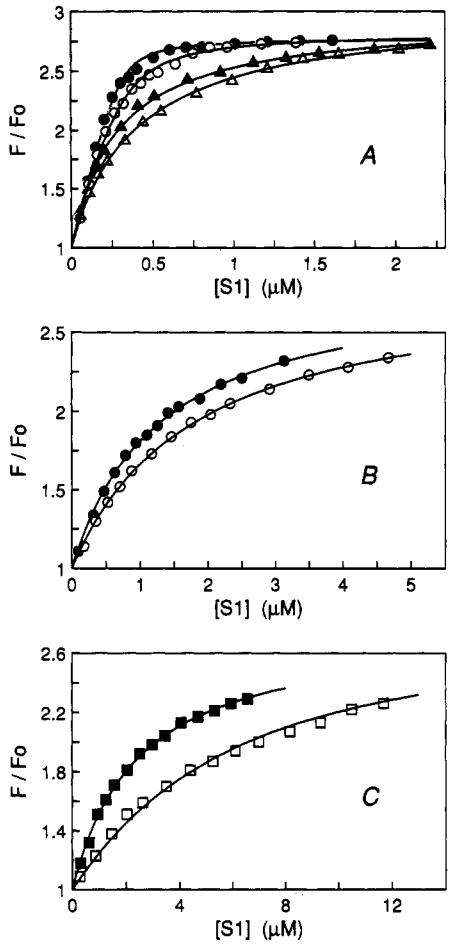

FIGURE 7: Binding isotherms of S1 isoenzymes to pyr-G-actin in the absence and in the presence of DNase I. S1(A1) (filled symbols) or S1(A2) (open symbols) aliquots were added to solutions containing (A) 0.3 μM pyr-G-actin and 0.1 μM ATP without (○, ●) or with (△, ▲) 1 μM DNase I, (B) 1 μM pyr-G-actin and 100 μM ADP, or (C) 2 μM pyr-G-actin, 100 μM ADP, and 4 μM DNase I in buffer G_0. Binding curves were computed by assuming 1 binding site and using the binding constants and maximum fluorescence enhancement values reported in Table I.

the formation of a 1:1 actin–S1 complex. Since the presence of $(actin)_2$–S1 complex should have shifted the total protection of S1 heavy chain toward a higher [actin]/[S1] ratio, these data argue again very strongly for the 1:1 stoichiometry of the complex.

DNase I Affects the Dissociation Constants of the G-Actin–S1 Complexes. In order to determine the affinity constants of the G-actin–S1 complexes and their sensitivity to DNase I, we obtained binding isotherms at lower protein concentrations. The amount of bound protein was estimated by monitoring the enhancement of pyr-actin fluorescence upon addition of S1(A1) or S1(A2) under various conditions (with or without DNase I, in the absence or the presence of 0.1 mM ADP; Figure 7). The experimental data were fitted by assuming the binding of one S1 per actin monomer, and the corresponding dissociation constants are summarized in Table I. Four remarks can be made from this set of data.

Firstly, the maximum effect of S1 on the pyr-G-actin fluorescence was not significantly dependent on the presence of DNase I and nucleotide. Secondly, S1(A1) bound to G-actin with an affinity 2–3 times higher than S1(A2). This result, also observed by Valentin-Ranc et al. (1991) (though with the two binding sites model), was not dependent on the presence of nucleotide or DNase I. Thirdly, DNase I decreased the binding constant for the G-actin–S1(A1) and the G-actin–S1(A2) complexes by factors of 5 and 4 in the absence of

Table I: Dissociation Constants and Maximum Fluorescence Enhancements for G-Actin–S1 Complexes in the Absence and in the Presence of DNase I

	conditions		binding parameters[a]	
isoenzyme	ADP (μM)	DNase I	K_d (μM)	F_m/F_o
S1(A1)	0	–	0.02	2.8
	0	+	0.1	2.8
	100	–	0.5	2.9
	100	+	1.7	2.9
S1(A2)	0	–	0.05	2.8
	0	+	0.2	2.9
	100	–	1.1	2.9
	100	+	4.7	2.9

[a] Dissociation constants (K_d) and maximum fluorescence enhancements (F_m/F_o) were obtained from the binding experiments described in Figure 7.

nucleotide and by factors of 3.4 and 4.3 in the presence of 0.1 mM ADP, respectively, which is in part in good agreement with the results obtained with the G-actin–S1(A2) complex by Chen et al. (1992). Fourthly, ADP had a dramatic dissociating effect on the monomeric complexes since 100 μM ADP increased the dissociation constants about 20 times in the absence as well as in the presence of DNase I. One should note that, due to the high Ca^{2+}-ATPase activity of S1 in buffer G (2 s^{-1} at 20 °C; Valentin-Ranc et al., 1991), ADP rather than ATP was used in these experiments. Therefore, the dissociating effect observed was due to the binding of Ca^{2+}-ADP to S1.

Ionic Strength Dependence of the G-Actin–S1 Interaction. As shown in Figure 3, 2 mM $MgCl_2$ was sufficient to strongly diminish the amount of G-actin–S1 complex in the presence of DNase I but also in its absence since dissociation of the complex was always observed prior to its polymerization induced by the salts. The salt-induced dissociation was also observed in the case of the unpolymerizable maleimidobenzoyl-treated G-actin, whose interaction with S1 was almost totally abolished by 100 mM KCl (Bettache et al., 1990). Because DNase I did not seem to alter this salt sensitivity, as it did not change the binding parameters for the actin–S1 interaction, we investigated the effect of various salts on the G-actin–S1 interaction in the presence of DNase I. As presented in Figure 8, divalent as well as monovalent ions are able to dissociate the G-actin–S1 complex independently of the S1 isoform employed. However, while salts such as KCl or KCH_3COO had to be used at concentrations of 20–30 mM to dissociate half of the complex, only 2 mM $MgCl_2$ or $CaCl_2$ was needed to produce the same effect. Since 2 mM $MgCl_2$ or $CaCl_2$ corresponds to an ionic strength of 5 mM, the result obtained with these two salts cannot be accounted for by a simple increase in the ionic strength. The effect of Mg^{2+} could be due to the exchange of highly bound Ca^{2+} for Mg^{2+} on actin. However, this is unlikely because the affinity constant of Mg^{2+} for its high-affinity site is too high (in the range of 10^{-8} M; Gershman et al., 1986) to explain an effect seen in the millimolar range and because DNase I dramatically reduces the exchange process at the high-affinity metal site (Mannherz et al., 1980). Another hypothesis could be that the dissociation of the actin–S1 complexes is due to the specific binding of Mg^{2+} or Ca^{2+} to the so-called low-affinity binding sites on actin, whose dissociation constants are in the millimolar range [Martonosi et al., 1964; see Estes et al. (1992) for a review]. On the other hand, the slight difference in the dissociation efficiency between KCl and KCH_3COO could be explained by a different effect of the anions Cl^- and CH_3COO^-. Taken together, these results indicate very strongly that there is an interaction between G-actin and S1 mostly composed of

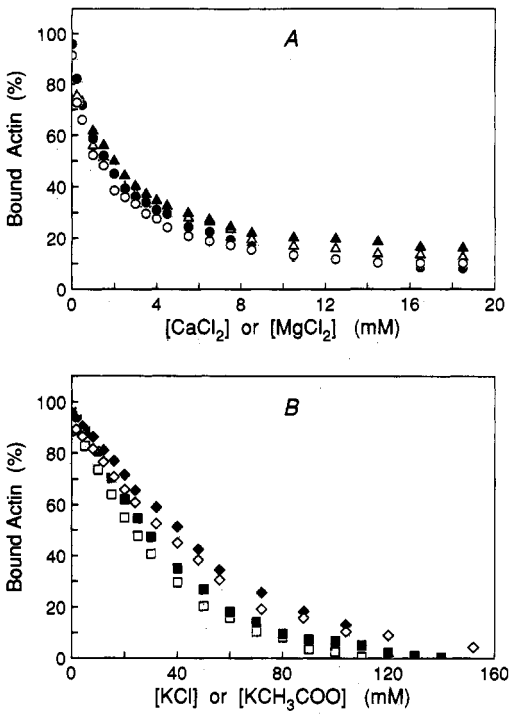

FIGURE 8: Salt-induced dissociation of G-actin–S1 complexes. Increasing amounts of salts were added to 1.5-mL solutions containing 2 μM pyr-G-actin (30% labeled), 4 μM S1(A1) (open symbols) or S1(A2) (closed symbols), and 5 μM DNase I in buffer G_0. The fluorescence intensity was recorded, and the percentage of bound actin was calculated as described under Materials and Methods. Salts used were $MgCl_2$ (○, ●) or $CaCl_2$ (△, ▲) in panel A and KCl (□, ■) or KCH_3COO (◇, ◆) in panel B.

electrostatic bonds. This was further confirmed by the fact that up to 40% ethylene glycol did not have a significant effect on the affinity constants of the G-actin–S1 complexes both in the presence and in the absence of DNase I. Such concentrations of ethylene glycol actually decreased the maximum value of fluorescence of the complex, while they did not induce its polymerization (results not shown). The absence of an effect of ethylene glycol emphasizes the predominance of electrostatic over hydrophobic bonds between G-actin and S1.

DISCUSSION

Interaction between G-Actin and S1 Isoforms. One of the most important results obtained in this work is that monomeric actin interacts with S1 with a 1:1 stoichiometry independent of the S1 isoform used and regardless of the presence of nucleotide in solution. This result obtained by two different but complementary approaches extends the previous work performed in the presence of 0.1 mM nucleotide (Chaussepied & Kasprzak, 1989a; Chen & Reisler, 1990). Moreover, these data have been further confirmed by a recent report of Kasprzak (1993), who presented evidence for the formation of a 1:1 complex between G-actin and S1(A2) by using three independent methods. These data, however, do not exclude the possible interaction between S1 and two actin monomers on filamentous protein, but they provide very strong evidence that $(G\text{-actin})_2$–S1 complexes are very unlikely to exist with monomeric actin.

The binding parameters obtained for the G-actin–S1 complexes revealed a rather strong affinity between the two proteins in "no salt" buffer [with $K_a \approx (2\text{–}5) \times 10^7$ M^{-1}]. Similarly to the filamentous complex, the monomeric complex is strongly sensitive to the nucleotide content of the solution (Chaussepied & Kasprzak, 1989a; Valentin-Ranc et al., 1991). However, we found that the monomeric complex was particularly sensitive to ADP, while the oligomeric form is known to be poorly dissociated by diphosphorylated ligands (Beinfeld & Martonosi, 1975).

Another interesting feature of the interaction between G-actin and S1 resides in its high sensitivity to ionic strength. Consequently, the interaction between actin and S1 seems to involve mainly electrostatic contacts, in contrast to the F-actin–S1 interface, which contains both electrostatic and hydrophobic components (Highsmith, 1990; Kabsch et al., 1990). Interestingly, in the presence of ATP, i.e., when the F-actin–S1 interaction is in the weak binding state, the actin–S1 interface also becomes highly ionic strength dependent (Brenner et al., 1982; Chalovich et al., 1984; Chaussepied et al., 1988; Highsmith & Murphy, 1992). Another common property of the "weak" F-actin–S1 binding state and the G-actin–S1 complex is the importance of the interaction between the positive charges present at the junction of the 50- and the 20-kDa fragments of the S1 heavy chain and the negative charges of the N-terminus of actin (Chaussepied, 1989; Chaussepied & Kasprzak, 1989b; DasGupta et al., 1990b; Yamamoto, 1991). However, further studies are needed to determine whether the monomeric actin–S1 complexes are real analogs of the weak binding state.

The occurrence of hydrophobic components in the actin–S1 interaction during polymerization of the actin–S1 complex confirms previous observations that the interaction between actin and S1 depends upon the oligomeric state of the complex (Chaussepied & Kasprzak, 1989b). These changes imply a restructuring of the actin–S1 interface that could be due either to additional contacts between S1 and the adjacent actin monomer along the long-pitch helix (Milligan et al., 1990) or to a change in the actin structure unmasking new contact sites on the surface of the molecule. Since the interaction of S1 with the adjacent actin monomer seems to involve mainly electrostatic bonds (Holmes & Kabsch, 1991), we would favor changes in the actin structure that could take place in the vicinity of the α-helical structure 338–348 thought to be part of the hydrophobic contacts with S1 (Kabsch et al, 1990). Finally, the appearence of hydrophobic bonds during polymerization of the actin–S1 complex can be related to the involvement of strong and hydrophobic actin–S1 contact sites in the activation of S1 ATPase activity by F-actin (Eto et al., 1990; Keane et al., 1990; Morales, 1992). This conclusion tends to show that the electrostatic interaction between S1 and the second actin monomer would be of minor importance in the activation of S1 ATPase activity.

It is noteworthy that all the experiments described in this paper were performed with Ca^{2+}-containing G-actin, while F-actin usually contains Mg^{2+} as the tightly bound metal. Since a large body of evidence has revealed differences in the structures of Ca^{2+}- and Mg^{2+}-actin [Strzelecka-Golaszewska et al., 1993; for a review, see Estes et al. (1992)], it would be worthwhile to test the effect of the metal exchange in the G-actin–S1 interaction. Another important parameter which also affects the actin structure and therefore its interaction with S1 is the state of the actin-bound nucleotide (Strzelecka-Golaszewska et al., 1993). Kasprzak (1993) recently showed a dramatic reduction of the nucleotide exchange by S1 binding to G-actin. Therefore, it is very likely that during most of our experiments the nucleotide bound to actin was ATP.

Actin Polymerization Induced by S1. Both S1 isoenzymes are able to induce actin polymerization in the absence of salt, though with different efficiencies, i.e., different limiting concentrations and rates of polymerization for the G-actin–S1(A1) and G-actin–S1(A2) complexes. This finding implies

that the additional 42 residues of the A1 light chain facilitate but are not absolutely required for the actin polymerization induced by S1.

The polymerization of actin induced by S1 can apparently be subdivided into three steps independently of the S1 isoform used. The first step of the polymerization seems to occur during the formation of the G-actin–S1 complexes, during which G-actin undergoes structural alterations as evidenced by specific changes in its proteolytic succeptibility and by the significant reduction of the rate of nucleotide exchange (Chen et al., 1992; Kasprzak, 1993). These alterations would produce a so-called "pre-F"-actin state which could, however, be different from the activated monomeric actin yielded by salts (Kasprzak, 1993). An interesting result of this work is that these alterations appear different with S1(A1) and with S1(A2) as evidenced by the peculiar effect of S1(A1) on the fluorescence of pyr-actin. The absence of an identical effect for S1(A2) suggests that the change in the environment of a Cys374-bound pyrenyl group is not necessary for the polymerization of the complex, though it could be implicated in the difference in the rates of polymerization for G-actin–S1(A1) and G-actin–S1(A2). In addition, the occurence of this structural change in the C-terminus of actin is in good agreement with the possible interaction of the 41 N-terminal amino acids of the A1 light chain with the same region of actin (Sutoh, 1982; Trayer et al., 1987; Milligan et al., 1990). Other evidence for structural alterations in the G-actin structure resides in the decrease of DNase I binding to G-actin in the presence of S1(A1). In fact, the effect of S1(A1) in the environment of Cys374 together with that observed at the top of actin subdomain 2 (DNase I binding site) could at least partly explain its higher efficiency in inducing actin polymerization since these two loci are involved in the actin–actin contacts in the filament (Holmes et al., 1990).

The second step of polymerization starts when the concentration of the protein complexes reaches a critical value (referred to here as the limiting concentration). At this stage, there is no evidence of either dissociation or rearrangement of the stoichiometric G-actin–S1 complexes (Figure 1) or polymer formation as revealed by light-scattering, fluorescence-intensity, and anisotropy measurements (Figures 1 and 3; Chaussepied & Kasprzak, 1989a; Chen & Reisler, 1991). Because this phase has a size dependent on the complex concentration, it is unlikely to represent only an additional activation step. Moreover, we found that the addition of a minute amount of sonicated F-actin–S1 complex (polymerized in the absence of salt), used as nuclei, dramatically reduced the size of this phase (data not shown). Henceforth, this phase can be accounted for by the formation of nuclei—the composition of which has yet to be determined—similarly to the lag phase observed during the salt-induced polymerization.

The last step of polymerization is the elongation step ending with the formation of the 1:1 F-actin–S1 complex obtained for either of the S1 isoforms or in the presence of an excess of actin over S1 (Miller et al., 1988; P. Chaussepied and T. Forné, unpublished observation).

With this simplified scheme of the actin polymerization induced by S1, it appears that the only noticeable difference between S1-induced and salt-induced actin polymerization resides in the absence of equilibrium between the monomeric and the oligomeric forms when F-actin and S1 are mixed in the absence of salt (Korn, 1982; Miller et al., 1988). This property can be related to the dramatic effect of S1 on the stabilization of the actin filament, which was reported to occur at a substoichiometric actin:S1 ratio as low as 1:10 (Thomas et al., 1979) and which was found to be due to the strengthening of the actin–actin contact along the long-pitch helix of the actin filament (Stokes & DeRosier, 1987). This stabilization was not only found to stiffen actin filaments (Ménétret et al., 1991) but also to inhibit the depolymerization of F-actin by DNase I (Hitchcock et al. 1976; P. Chaussepied, unpublished results). The absence of monomer–oligomer equilibrium for the actin–S1 complexes implies that limiting and critical concentrations are not equivalent quantities. However, the presence of a limiting concentration and of a lag phase for the G-actin–S1 polymerization shows that the S1-induced polymerization of actin is not a simple isodesmic process as recently proposed by Valentin-Ranc and Carlier (1992). However, it is difficult to compare their model to ours since, in their case, the polymerization was initiated by $(actin)_2$–S1 complexes (whose existence could not be confirmed by the present study) and since apparently no lag phase was present during their polymerization process.

The study on the effect of divalent cations on the actin–S1 interaction revealed that 2 mM Mg^{2+} or Ca^{2+} was sufficient to decrease by half the amount of complex. Since the binding of millimolar concentrations of these cations to actin induces its polymerization (Martonosi et al., 1964), one could speculate that S1 and divalent cations share in part their binding sites on actin. Such a hypothesis would again strongly favor similar polymerization processes induced either by S1 or by divalent cations.

Finally, the strong dissociating effect of the ionic strength on the interaction between monomeric actin and S1 rules out the possibility that such interaction occurs within cells. Therefore, the only, if any, physiological role of S1 or myosin in the regulation of actin polymerization would be to accelerate the rate of the polymerization by stabilizing actin nuclei as proposed by in vitro experiments (Chaussepied & Kasprzak, 1989b ; Chen & Reisler, 1991).

On the Formation of the Ternary DNase I–G-Actin–S1 Complexes. Though the G-actin–S1 complexes could be kept in a monomeric form at concentrations as high as 8 μM for the G-actin–S1(A2) complex, their sensitivity to buffer conditions such as temperature or ionic strength prompted us to use an actin-sequestering protein to protect them from polymerization. Our data provide strong evidence that DNase I can indeed protect the G-actin–S1 complexes against polymerization.

In addition, the detailed study on the effect of DNase I on the G-actin–S1 interaction revealed that DNase I has a significant dissociating effect on the G-actin–S1 interaction (increasing the dissociation constants by 4–5-fold). However, since even in the presence of DNase I the K_d values of the G-actin–S1 complexes remain in the range of 10^{-7} M, they allow the formation of stable ternary DNase I–G-actin–S1 complexes under the usual experimental conditions.

In contrast to its effect on the G-actin–S1 interaction, however, DNase I was not found to change the sensitivity of the actin–S1 complexes to either nucleotide or salts. While this finding could be useful for the biochemical characterization of the complexes, it indicates also that salts represent a real obstacle for any crystallographic work, which remains the ultimate goal of the studies undertaken on the monomeric actin–S1complexes.

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