Comparative Studies of the Monomeric and Filamentous Actin−Myosin Head Complexes†

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ABSTRACT: The functional and structural properties of the monomeric and filamentous actin−myosin head (S1) complexes were compared under strictly controlled conditions which avoid the S1-induced polymerization of monomeric actin. Under these conditions, monomeric (G) and filamentous (F) actin were found to activate S1 Mg^{2+}-ATPase by 3- and 120-fold, respectively, in the presence of a 5-fold excess of actin over S1. Using the change in fluorescence intensity of pyrene-G-actin induced by S1 binding in the presence of various nucleotide analogues, we discovered that the ternary G-actin−S1−AMPPNP complex could not be formed. Moreover, the association constants of G-actin to S1−ADP or of ADP to the G-actin−S1 complex were at least an order of magnitude lower than in the filamentous state. Such a low affinity between G-actin and the S1−nucleotide intermediates can reasonably explain the lack of ATPase activation by the monomeric complex. Analysis of the G-actin−S1 interface by chemical cross-linking and limited proteolytic experiments showed that, in the monomeric complex, S1 interacted almost exclusively by its positively charged segment 636−642 with the patch of negative residues located on the actin flexible loops 1−7, 20−28, and 90−100. Moreover, the variation in the cross-linking pattern and in the proteolytic susceptibility of S1 segment 636−642 demonstrated that this electrostatic interface was different in the monomeric and the filamentous complexes. Taken together, the results suggested that the G-actin−S1 interaction encompasses only a small fraction of the strong as well as of the weak F-actin−S1 interface. The monomeric complex would in fact resemble more the collision complex which takes place early in the F-actin−S1 interaction.

The hydrolysis of ATP[1] by myosin provides the chemical energy needed for muscle contraction and the actin-based motility processes. Whether the myosin ATPase activity is tightly coupled with the production of mechanical forces has not been fully demonstrated (Howard, 1994; Ishijiama et al., 1994; Finer et al., 1994). However, it is now well admitted that there is an intimate relationship between the actin and the ATP binding sites on myosin [Eisenberg et al., 1968; Highsmith, 1976; for a review, see Cooke (1986)]. But how does ATP binding to myosin weaken actin interaction, and how can actin binding activate up to 500-fold the turnover of the myosin ATPase activity?

A rather simple molecular mechanism based on the three-dimensional structure of myosin head (Subfragment-1 or S1) together with the reconstruction of the filamentous actin−S1 complex obtained from electron microscopy data (Milligan et al., 1990; Rayment et al., 1993; Schröder et al., 1993) was recently proposed. Thus, it appears that S1 interacts mainly with one actin subunit and with a so-called "secondary" interacting site of a second subunit below, on the same long-pitch helix of the actin filament. Rayment et al. (1993) proposed a model in which the molecular communication between the actin and the ATP binding sites would reside in the opening and the closing of a narrow cleft in S1 structure which separates the upper and the lower domains of the central 50-kDa segment of S1 and which encompasses most of the actin binding sites. ATP binding to S1 would open the cleft and consequently would weaken the actin−S1 interaction. On the other hand, the cleft's progressive closing would be associated with the isomerization of the acto-S1 interaction from the weak to the strong binding states. Such isomerization has been linked to the acceleration of the ATPase rate as well as the force generation process (Geeves, 1991; Geeves & Conibear, 1995). Rayment's model suggests that the interface between S1 and a single actin monomer contains all the information necessary for the activation of the myosin ATPase activity. The straightforward implication of this result is that the activation of S1 ATPase should not be dependent on the oligomeric state of actin. However, it has been known for a long time that monomeric actin activates myosin ATPase activity only poorly (Tawada & Oosawa, 1969; Offer et al., 1972; Estes & Gershman, 1978).

This lack of activation could be explained by a lower affinity between G-actin and S1 in key steps of the actomyosin ATPase kinetic scheme (Offer et al., 1972). In order to explain the F-actin activated myosin ATPase, it

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[1] Abbreviations: S1(A1), S1 isoenzyme with alkaline light chain 1; S1(A2), S1 isoenzyme with alkaline light chain 2; G- and F-actin, monomeric and filamentous actin; AMP, ADP, and ATP, adenosine mono-, di-, and triphosphate; AMPPNP, adenyl-5′-yl imidophosphate; DMS, dimethyl suberimidate; EDC, 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide; EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GA, glutaraldehyde; NHS, *N*-hydroxysuccinimide; PAGE, polyacrylamide gel electrophoresis; pPDM, *N,N′*-paraphenylenedimaleimide; SDS, sodium dodecyl sulfate.

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seems today essential to identify these steps and to understand how they are related with the variation of the actin−S1 interface between the monomeric and the oligomeric complexes.

In the monomeric state, actin forms a stoichiometric complex with skeletal myosin head as shown by fluorescence intensity or anisotropy measurements (Chaussepied & Kasprzak, 1989a; Lheureux et al., 1993; Kasprzak, 1993, 1994), analytical centrifugation (Chen & Reisler, 1991), and limited proteolysis experiments (Lheureux et al., 1993). The presence of a 2:1 G-actin−S1 complex was also reported by Valentin-Ranc et al. (1991), but this result has not been confirmed by any other laboratories so far. Under zero ionic strength conditions and in the presence of trace amounts of nucleotide, the dissociation constant (K_d) for the monomeric complex varied from 20 to 100 nM depending on the S1 isoform, S1(A1) or S1(A2), employed (Lheureux et al., 1993) and on the metal (Mg^{2+} or Ca^{2+}) bound to actin (Kasprzak, 1993, 1994). On the other hand, the K_d for the filamentous complex, which cannot be directly determined under such a low ionic strength, is believed to be in the nanomolar range or lower (Geeves et al., 1984).

In contrast to the filamentous complex, the G-actin−S1 interaction is very sensitive to the ionic strength and is apparently composed of electrostatic linkages (Lheureux et al., 1993). Therefore, the hydrophobic components of the F-actin−S1 interface (Rayment et al., 1993) are likely to be absent or seriously impaired in the monomeric complex. Moreover, chemical cross-linking experiments and the use of anti-peptide or specific antibodies have revealed that in both complexes the actin N-terminal segment 1−7 interacted with the connecting segment between the 50- and 20-kDa tryptic fragments of S1 (segment 636−642 of the skeletal S1 heavy chain; Chaussepied & Kasprzak, 1989b; DasGupta et al., 1990; Combeau et al., 1992). Finally, the binding of S1 to actin subdomain 2, first suggested by electron microscopy image reconstruction (Milligan et al., 1990; Rayment et al., 1993; Schröder et al., 1993), was recently confirmed experimentally in the filamentous complex by cross-linking experiments (Bonafé et al., 1994). In the monomeric complex such a contact was only indirectly suggested by a limited proteolytic approach (Chen et al., 1992; Fievez & Carlier, 1993) and by the fact that S1 strongly reduced the rate of nucleotide exchange and hydrolysis in actin (Kasprzak, 1993, 1994). In addition, it was recently proposed that a chemically modified G-actin could be cross-linked to S1 at residue Lys50 of its subdomain 2 (Bertrand et al., 1994). However, it was not clear whether this modified actin interacted with S1 as a monomer or as short oligomers (Arata, 1991).

In this study, we have further characterized the interaction of S1 with monomeric actin. Preliminary experiments confirmed that G-actin could not activate the S1 ATPase activity even with a large excess of actin over S1. In order to understand this lack of activation, we then studied the interaction between S1 and G-actin in the presence of nucleotides using the change in fluorescence intensity of pyrenyl-actin induced by S1 binding. The data pointed to a very unlikely formation of ternary G-actin−S1−nucleotide intermediates under the ATPase conditions. This profound difference with the F-actin−S1 complex was finally correlated with structural dissimilarities in the actin−S1 interface using cross-linking and limited proteolysis approaches.

MATERIALS AND METHODS

Reagents. Trypsin, papain, EDC, NHS, and pPDM were purchased from Sigma. DMS and α-chymotrypsin were from Pierce and Worthington Biochemicals, respectively. ATP, ADP, AMPPNP, soybean trypsin inhibitor, DNase I, and endoproteinase Arg-C were from Boehringer Mannheim. EEDQ was obtained from Aldrich Chemical Co. Glutaraldehyde (25% aqueous solution) and hydroxyapatite were from Serva. S-200 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. Myosin was isolated from rabbit back and leg muscles according to Offer et al. (1973). S1 was prepared by chymotryptic digestion of myosin filaments (Weeds & Taylor, 1975). Both isoforms S1(A1) and S1(A2) were purified as previously described (Lheureux et al., 1993). Rabbit skeletal F-actin was prepared as described by Eisenberg and Kielley (1974). G-actin was obtained by depolymerization of F-actin (2 mg/mL) in buffer G (2 mM HEPES, 0.1 mM ATP, 0.1 mM $CaCl_2$, and 0.1 mM NaN_3, pH 8.0). The solution was sonicated three times for 1 min with 1-min intervals in a Microson cell disrupter (Model XL 2005, at 20 MHz). After 48 h of dialysis against buffer G, the protein was isolated by centrifugation at 150000*g* for 1 h at 4 °C and purified over Sephacryl S-200 equilibrated in buffer G_5, i.e., buffer G containing only 5 μM ATP. G-actin was kept on ice and used within 3−4 days.

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% labeled actin. The protein was then passed through a Sephacryl S-200 column equilibrated with buffer G_5 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 as described by Price et al. (1969) and further modified as described by Lheureux et al. (1993).

Protein concentrations were determined spectrophotometrically with extinction coefficients of $A^{1\%}_{280nm} = 7.5\ cm^{-1}$ for S1, 11 cm^{-1} for actin, and 12.3 cm^{-1} for DNase I. The molecular masses used were 105, 42, and 31 kDa for S1-(A2), actin, and DNase I, respectively.

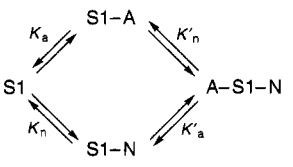
Proteolytic Digestions. Limited proteolysis was carried out at 20 °C in buffer G_5 on 10 μM S1(A2) alone or mixed with 10 μM G-actin (with or without 2 mM $MgCl_2$) in the presence or in the absence of 15 μM DNase I. The digestions were accomplished by trypsin at a trypsin/S1 mass ratio of 1:100 for 30 min, by endoproteinase Arg-C (20 units of protease/mg of protein) for 45 min, or by papain at a papain/S1 mass ratio of 1:100 for 30 min. The proteolysis reactions were stopped by incubating an aliquot of each reaction mixture with 3 vol of Laemmli's boiling solution (50% glycerol, 5% 2-mercaptoethanol, 1.5% SDS, and 50 mM HEPES, pH 8.0; Laemmli, 1970). The results of the proteolysis were visualized by gel electrophoresis.

Cross-Linking Reactions. The covalent cross-linking reactions between actin and S1 were conducted at 10 °C for the G-actin−S1(A2) complex in the absence of DNase I and at 25 °C in the presence of DNase I or for the F-actin−S1

complex. The EDC-induced cross-links were performed in a two-step reaction as described (Schgal & Vijay, 1994; Grabarek & Gergely, 1990) with the following modifications. First, 20 μM G-actin in buffer G_5 (supplemented by 10 mM MOPS, pH 7.0) was activated by 2.5 mM EDC and NHS. After 5 min of reaction, the unreacted reagent was quenched by the addition of 10 mM 2-mercaptoethanol. Second, EDC-modified actin was incubated for 30 min with S1(A2) (at final actin and S1 concentrations of 10 μM) in the absence or the presence of 15 μM DNase I or 2 mM $MgCl_2$ (both added prior to S1). The reaction was terminated by the addition of Laemmli's solution. The reactions promoted by DMS (11-Å span), GA (9-Å span), and EEDQ (0-Å span) were performed in a one-step reaction as follows. Ten micromolar S1(A2) and 10 μM G-actin were mixed in the absence or in the presence of 15 μM DNase I or 2 mM $MgCl_2$ in buffer G_5 supplemented by 10 mM TEA (pH 8.5), 10 mM HEPES (pH 8.0), and 10 mM MOPS (pH 7.0), and the reactions were induced by 2.5 mM DMS, 0.5 mM GA and 0.5 mM EEDQ, respectively. Cross-linking reactions were stopped by the addition of Laemmli's boiling solution after 15, 30, and 45 min, respectively, and the samples were analyzed by SDS−PAGE.

Nucleotide Effects. The dissociative effects of the nucleotide analogues (AMP, ADP, and AMPPNP) on the G-actin−S1 complexes were evaluated at 20 °C by monitoring the decrease in the pyrenyl fluorescence bound to actin upon the dissociation of the complexes. This approach was possible only because the probe was shown not to alter the actomyosin binding and dissociation rates (Criddle et al., 1985). Nucleotides were added to 1.5 mL of solution containing buffer G_0, i.e., buffer G without nucleotide, with either 1 μM (30% labeled) pyr-G-actin and 1 μM S1 isoforms in the absence of DNase I or 2 μM pyr-G-actin and 4 μM S1 isoforms in the presence of 4 μM DNase I. After each subsequent addition of nucleotide, the fluorescence values were recorded and expressed as relative fluorescence (F_r) equal to the fluorescence value divided by the value obtained in the absence of nucleotide.

The data were then analyzed according to the scheme proposed by Botts and Morales (1953) and futher developped by Highsmith (1976) and Greene and Eisenberg (1980) which shows all the possible complexes (and their association constants) formed between S1, actin (A), and the nucleotide (N).



Assuming a negligible effect of nucleotide binding to S1 on the fluorescence of the G-actin−S1 complexes , we could estimate the fraction of S1 which is bound to actin (A−S1 and N−S1−A) and free (S1 and S1-N). The concentration of bound S1 ($[S1]_b$) was calculated with the equation

$$[S1]_b = F_r[\text{G-actin}-S1]_i \quad (1)$$

were $[\text{G-actin}-S1]_i$ is the initial [actin−S1] in the absence of nucleotide computed from the known [actin], [S1] and the corresponding affinity constants in the absence of nucleotide (Lheureux et al., 1993; Table 1). $[\text{G-actin}-S1]_i$ was found to be 0.81 and 0.88 μM in the absence of DNase I and 1.83 and 1.92 μM in the presence of DNase I for the G-actin−S1(A2) and the G-actin−S1(A1) complexes, respectively.

The deduced concentrations of bound S1, free S1 ($[S1]_f$), and free actin ($[actin]_f$) were then used to calculate the apparent association constants, K_{app}:

$$K_{app} = [S1]_b/([S1]_f[actin]_f) \quad (2)$$

Upon simple algebraic manipulation and substitution of the association constants in the reaction scheme, eq 2 yields the following equation:

$$K_{app} = K_a(1 + K'_n[N])/(1 + K_n[N]) \quad (3)$$

with K_a known for each type of G-actin−S1 complex used (Table1). K'_n and K_n were computed by nonlinear least-squares analysis of the plots $K_{app} = f([N])$ using the Marquardt−Levenberg algorithm implemented in the program MARQ-IT written by Dr. A. A. Kasprzak. The remaining constant K'_a was then obtained using the equation

$$K'_a = K_aK_n'/K_n \quad (4)$$

Polyacrylamide Gel Electrophoresis. SDS−PAGE was carried out in 3−18% polyacrylamide gradient gel electrophoresis in the presence of 0.1% SDS as described by Laemmli (1970).

Fluorescence Measurements. Fluorescence measurements were carried out on a Kontron SFM 25 spectrofluorimeter, with a cell thermostated by a circulating water bath. 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.

ATPase Measurements. The Mg^{2+}-ATPase activities of the monomeric and filamentous actin−S1 complexes were determined by following the ATP-induced change in the fluorescence signal of the pyrenyl moiety attached to actin. The activities were measured at 20 °C in 10 mM Hepes, 0.1 mM $CaCl_2$, and 0.12 mM EGTA, pH 7.6. F-actin was obtained by polymerization of G-actin in the presence of 2 mM $MgCl_2$. Prior to the experiment, F-actin was ultracentrifugated and kept in the ATPase buffer (with 1 mM $MgCl_2$) at a concentration higher than 120 μM. Activities were calculated as described by Mandelkov and Mandelkov (1973) and Bachouchi et al. (1986) who measured the ATPase activities of S1 by means of the intrinsic fluorescence of tryptophan. It was recently reported that the pyrenyl labeling of actin reduced the actin-activated ATPase activity by about 40% (Crosbie et al., 1994). Such an effect should not alter our results since we compared the S1 ATPase activation induced by the same actin preparation in the polymerized or unpolymerized form. Moreover, the actin used was only partly (30%) labeled, thus reducing the effect of labeling on the ATPase to 12%. The Mg-ATPase activities of S1 (in the absence of actin) were determined as described by Bachouchi et al. (1986).

RESULTS

Functional Differences between the Monomeric and the Filamentous Actin−S1 Complexes. We previously described

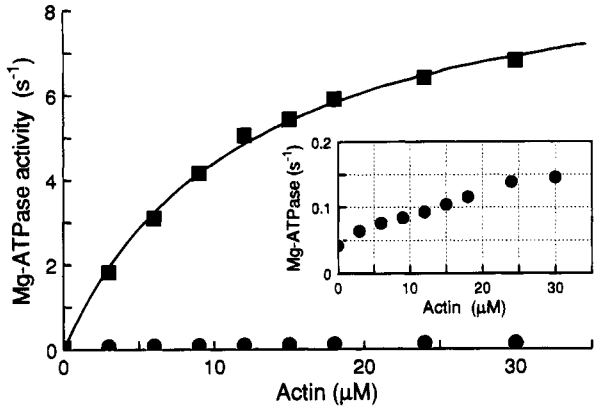


FIGURE 1: Dependence of the S1 Mg^{2+}-ATPase on F-actin (■) or G-actin (●) concentration. The ATPase activities were measured as described under Materials and Methods. Conditions: S1 was at 6 μM, and Mg^{2+}-ATP initial concentrations were 500 and 50 μM for F-actin and G-actin, respectively. The initial ATPase activity of 0.045 s^{-1}, which was independent of the nucleotide concentration, was subtracted from all the values obtained. The line was computed according to the Michaelis–Menten equation with V_{max} and K_m values of 9.7 s^{-1} and 12 μM, respectively. Inset: Uncorrected data obtained with G-actin plotted with an enlarged ordinate scale.

the experimental conditions necessary for stabililizing the actin–S1 complex in its monomeric form (Lheureux et al., 1993). These conditions included the preferential use of the S1(A2) isoform at concentrations lower than 8 μM and the absence of salt together with a low ionic strength buffer. When the S1 Mg^{2+}-ATPase activity was measured under such conditions [with S1(A2) at a concentration of 6 μM in 10 mM Hepes buffer], in the presence of increasing amounts of actin (up to 30 μM), we observed an activation of the S1 ATPase of maximally 3-fold when actin was in its monomeric state (Figure 1). The monomeric state of the complexes was revealed by the absence of pelleted material during ultracentrifugation (at 380000*g* for 20 min) of the samples at the end of the ATPase assays. Conversely, the activation induced by filamentous actin under identical conditions reached 120-fold with a specific activity of 6.8 s^{-1} (Figure 1). The maximum velocity extrapolated for infinite F-actin concentration was 9.7 s^{-1} with a K_m of 12 μM, in good agreement with the values of 4.2 s^{-1} and 4.3 μM, repectively, obtained in an experiment also performed at low ionic strength but in the presence of 5 mM KCl and at lower pH (pH 7.0) and temperature (T = 15 °C) (Offer et al., 1972). It was not possible to compute the small increase of the ATPase activity observed with G-actin since no plateau was approached at the highest actin concentration used.

The most obvious reason for this lack of activation could be a weak binding of G-actin to one or several of the S1–nucleotide intermediates formed during the acto-S1 ATPase activity [for a review, see Geeves et al. (1984)]. In order to determine which of these complexes is affected by the oligomeric state of actin, we analyzed the interaction between G-actin and S1 by measuring the pyrenyl fluorescence attached to actin in the presence of S1 and increasing concentrations of Mg^{2+}-AMPPNP (as an analogue of the triphosphorylated nucleotides), Mg^{2+}-ADP, and Mg^{2+}-AMP (as control). In the presence of trace amounts of nucleotide, S1 binding increases the fluorescence of pyr-G-actin by a factor of 3 independently of the S1 isoform or of the presence of DNase I in the solution (Lheureux et al., 1993).

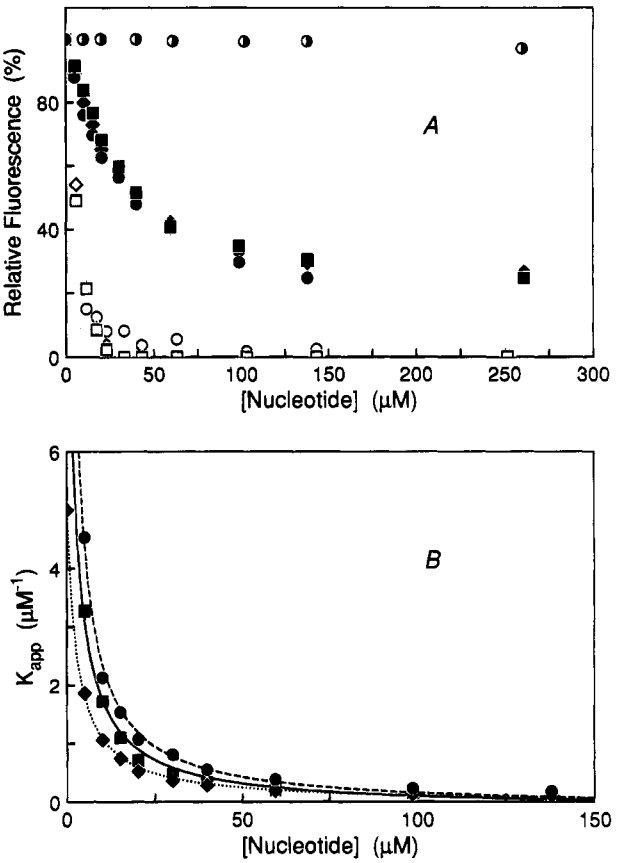


FIGURE 2: Nucleotide effects on the G-actin–S1 interaction. Increasing amounts of nucleotide analogue were added to 1.5 mL of G_0 buffer containing either 1 μM pyr-G-actin and 1 μM S1(A2) (●, ○, ◑) or 2 μM pyr-G-actin, 4 μM DNase I, and 4 μM S1(A2) (◆, ◇) or S1(A1) (■, □), and the fluorescence intensities were recorded as described under Materials and Methods. (A) Relative fluorescence intensities as a function of nucleotide concentration. (B) Evolution of K_{app} as a function of nucleotide concentration. The values of relative fluorescence and K_{app} were determined from the fluorescence intensities as described under Materials and Methods. The nucleotides used were Mg^{2+}-ADP (closed symbols), Mg^{2+}-AMPPNP (open symbols), and Mg^{2+}-AMP (◑).

Table 1: Formation of Ternary Actin–S1–Mg^{2+}-ADP Complexes

association constant ($μM^{-1}$)	S1(A1)		S1(A2)	
	−DNase I	+DNase I	−DNase I	+DNase I
K_a[a]	65.1	11.1	23.3	5.0
K_n[b]	nd	0.49	0.84	0.35
K'_n[b]	nd	<0.001	<0.001	<0.001
K'_a[c]	nd	<0.02	<0.03	<0.01

[a] K_a was obtained from Lheureux et al. (1993). [b] K'_n and K_n were computed by fitting the data of Figure 2B as described under Materials and Methods. [c] K'_a was determined from K'_nK_a/K_n.

Figure 2A summarizes the dissociative effect of the three nucleotides on the monomeric complex. The effect of Mg^{2+}-AMP was negligible even at a concentration of 1.5 mM. In contrast Mg^{2+}-ADP and Mg^{2+}-AMPPNP had strong dissociative effects, with a much more pronounced effect of Mg^{2+}-AMPPNP since most of the complexes were dissociated at less than 50 μM nucleotide. It is interesting to note that neither the type of S1 isoform nor the presence of DNase I altered the extent of dissociation observed with either nucleotide. Note also that the experiment could be performed with S1(A1) isoform only in the presence of DNase I which protected the complex from its polymerization at the employed protein concentration (Lheureux et al., 1993).

The data obtained with AMPPNP could not be fitted according to the four-states model proposed by Botts and Morales (1953) and described under Materials and Methods, due to the strong dissociation effect of this nucleotide analogue. Therefore, the binding of AMPPNP and of G-actin to S1 seemed to take place in an almost exclusive fashion so that the ternary G-actin−S1−AMPPNP complex was very unlikely to exist. This conclusion could eventually be extented to other ADP·P_i analogues since a stable S1−ADP·vanadate complex could not interact with G-actin at protein concentrations as high as 25 μM (data not shown).

In contrast, the ternary G-actin−S1−ADP complex could be formed since the binding data obtained with Mg^{2+}-ADP could be reasonably fitted according to the same model (Figure 2B). Of the binding constants generated by the fitting process and listed in Table 1, it is interesting to note that the values of K_n (binding constant of nucleotide for S1) of 0.4−0.8 μM fell within the range of the values determined in previous work [Highsmith, 1976; Greene & Eisenberg, 1980; for a review, see Geeves et al. (1984)]. On the other hand, the binding constants K'_n and K'_a characteristic of the formation of the ternary actin−S1−ADP complex were very low, at least 500−800-fold lower than K_n and K_a, respectively. Although it was not possible to measure these binding constants with the filamentous complex under such low ionic strength conditions, the data were in great contrast with those obtained on the filamentous complex under slightly different ionic strength and temperature (Highsmith, 1976; Greene & Eisenberg, 1980). For example, with K_a and K_n values of 2 and 0.3 μM, very close to those obtained in this work with the DNase I−G-actin−S1(A2) complex (Table 1), K'_n and K'_a values were about 0.01 and 0.2 μM for the F-actin−S1 complex, at least 1 order of magnitude higher than those obtained for the monomeric complex (Highsmith, 1976; Table 1). The difference in temperature or in ionic strength between the two experiments was unlikely to account for these differences since the values obtained with the filamentous complex were not very sensitive to these two parameters (Green & Eisenberg, 1980).

Our experiments were performed with Mg^{2+}−nucleotide complexes, while the metal bound to the high-affinity binding site of actin was Ca^{2+}. However, no exchange of nucleotide from actin was expected since the rate of nucleotide exchange is known to be either dramatically reduced by S1 (Kasprzak, 1993, 1994) or almost totally blocked by DNase I binding to monomeric actin (Mannherz et al., 1980). On the other hand, the studies performed on the F-actin−S1 complexes were achieved with Mg^{2+}-containing actin (Highsmith, 1976; Greene & Eisenberg, 1980). Moreover, it was shown that actin structure is sensitive to the nature of the metal bound to the active site (Kasprzak, 1994; Orlova & Egelman, 1993; Strzelecka-Golaszewska et al., 1993; Estes et al., 1992). However, the fact that we did not find any difference in the activation of S1 ATPase induced by Ca^{2+}- and Mg^{2+}-containing F-actin (data not shown) validated our comparison between Ca^{2+}-G-actin and Mg^{2+}-F-actin.

Structural Differences between the Monomeric and Filamentous Actin−S1 Complexes. The low affinity observed between G-actin and the S1−nucleotide complexes could be explained by a loss in some of the actin−S1 contact sites. An obvious candidate would be the hydrophobic contacts which are strongly impaired in the monomeric complex (Lheureux et al., 1993). We looked for further differences in the actin−S1 interaction using cross-linking and protection against limited proteolysis experiments.

Chemical Cross-Linking Experiments. In order to reduce as much at possible the cross-linking-induced polymerization of the G-actin−S1 complexes, we performed these experiments only with S1(A2) either at low temperature (T = 10 °C) or at 25 °C but in the presence of DNase I.

We first employed two zero-length cross-linking reagents, EDC and EEDQ, which generated on the F-actin−S1 complex two main covalent acto-S1 adducts with apparent masses of 165 and 175 kDa (Figure 3, lanes a and b). These two covalent adducts corresponded to the formation of isopeptidic bonds between the actin N-terminal segment 1−7 and the segment 636−642 or the tryptic 50-kDa fragment of the S1 heavy chain, respectively (Mornet et al., 1981a; Sutoh, 1983; Bertrand et al., 1988). In contrast, only the 165-kDa product was obtained when the reaction was performed on the G-actin−S1(A2) complex, regardless of the presence of DNase I (Figure 3, lanes e and f). Note that the loss of the 175-kDa cross-linked product was not due to a decrease in the cross-linking yield since in the case of the EDC reaction neither a lower temperature (10 instead of 25 °C) nor the presence of DNase I altered the amount of the 165-kDa product (Figure 3A). Moreover, the EDC reaction was performed in a two-step reaction, so both G-actin and F-actin were activated at the same time in the G- form prior to addition of S1(A2) with or without $MgCl_2$. This implied that the same actin carboxylates were activated during the first activation step so that the loss of the 175-kDa product was only due to a difference of interaction in the monomeric and the filamentous complexes. Identical results were obtained when the EDC reaction was performed between S1 and G-actin but under uncontrolled conditions (Yamamoto, 1993) or G-actin whose polymerization properties had been hindered by extensive (Hozumi, 1992) or specific (Combeau et al., 1992) chemical modification. In contrast to our data, however, Combeau et al. (1992) reported that EDC cross-linking between unmodified G-actin and S1 generated a doublet band with apparent masses of 165−170 kDa. This difference could be accounted for by the fact that these authors apparently worked with a complex formed of 2 mol of G-actin bound to 1 mol of S1, different from the stoichiometric complex used in this study.

EDC reaction on the monomeric complex generated an additional cross-linked product with a mass of 200 kDa (Figure 3A). This band was previously found to be composed of 1 mol of actin coss-linked to 1 mol of S1 heavy chain (on its tryptic 50-kDa fragment; Combeau et al., 1992). It is interesting to note that the EEDQ reaction produced a band of a similar mass identified as a cross-link between the S1 heavy chain and actin segment 40−113 (more probably at residues Glu99 and Glu100) (Figure 3B; Bertrand et al., 1988). This band was generated with the filamentous as well as the monomeric complexes. Because EDC and EEDQ are two carboxylate-directed reagents, and because Glu100 was also found to be modified by EDC (Elzinga, 1987), we propose that the two EDC- and EEDQ-induced 200-kDa adducts corresponded to a cross-link between S1 and the actin residues Glu99 and Glu100.

One could note that when the EEDQ reaction was performed in the presence of DNase I, the 200-kDa product migrated as a doublet of 195−200 kDa. This showed that DNase I bound to G-actin induced a slight shift in the actin−

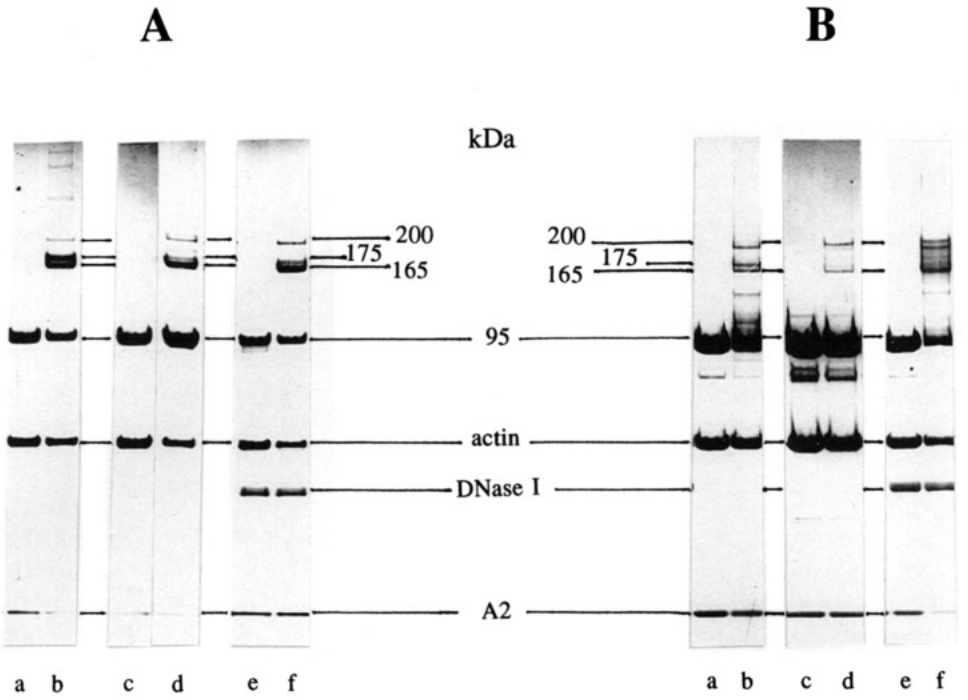


FIGURE 3: Comparative EDC (A) and EEDQ (B) cross-linking experiments on different acto-S1 complexes. S1(A2) was cross-linked with F-actin (T = 25 °C; lanes a and b) and G-actin without (T = 10 °C; lanes c and d) or with DNase I (T = 25 °C; lanes e and f). The cross-linking reactions were carried out as specified under Materials and Methods. Aliquots were withdrawn and analyzed by SDS−PAGE at the beginning (lanes a, c, and e) and at the end (lanes b, d, and f) of the cross-linking reaction.

S1 interface resulting in a cross-linking reaction between two vicinal residues on actin or the S1 heavy chain or both. Moreover, the cross-linking yield was higher with the monomeric (in the presence of DNase I) than with the filamentous complex though the reaction was carried out under identical conditions in both cases. This result may be explained either by a difference in the carboxylate reactivity or by a difference in S1 binding to the two forms of actin. Finally, EDC was also reported to generate an actin−S1 covalent product with an apparent mass of 265 kDa when the reaction was performed on unsaturated filaments (Andreev & Borejdo, 1992; Bonafé & Chaussepied, 1995). The fact that such a product was not obtained with fully saturated filaments or with the monomeric complexes confirmed that it corresponded to S1 doubly cross-linked to two molecules of actin.

Two amino group-directed cross-linkers, DMS and GA, of 11 and 9 Å span were also applied on the actin−S1 complexes (Figure 4). However, due to their slow reactivities at low temperature, they were used with the monomeric complex only at 25 °C in the presence of DNase I.

The DMS-induced reaction on the monomeric complex yielded only one product with a mass of 185 kDa, while the filamentous saturated complex produced two covalent acto-S1 adducts with masses of 180−185 kDa (corresponding to the cross-links of actin with the tryptic 50-kDa fragment of S1) and a dimer of two S1 heavy chains which migrated as a doublet band with a mass of 240 kDa (Figure 4A; Labbé et al., 1982). These results revealed that one of the actin cross-link with the tryptic 50-kDa fragment of S1 was absent in the monomeric complex. They also confirmed that the molecules of S1 had to be bound to adjacent actin monomers in the filament in order to form cross-linked S1 dimers (Labbé et al., 1982).

Between the two cross-linked products with masses of 165 and 200 kDa generated by GA with the filamentous complex

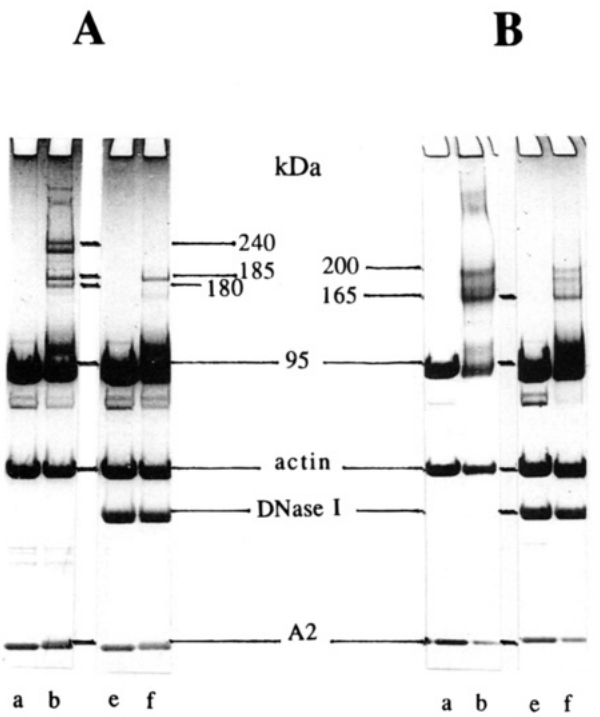


FIGURE 4: SDS−PAGE analysis of the DMS (A) and GA (B) cross-linking reactions on different actin−S1 complexes. Reactions were performed either with F-actin−S1 complex (lanes a and b) or with the DNase I−G-actin−S1(A2) complex (lanes e and f). The reactions were carried out as described under Materials and Methods. Other details were as in Figure 3.

(Bertrand et al., 1988; Bonafé et al., 1994), only the 165-kDa product (corresponding to the cross-linking of actin residue Arg28 to S1 segment 636−642) was present with the monomeric complex (Figure 4B). Because the 200-kDa product was the result of a cross-link of Lys50 of actin subdomain 2 to the 50-kDa segment of S1 (Bonafé et al., 1994), this result demonstrated that S1 could not be cross-

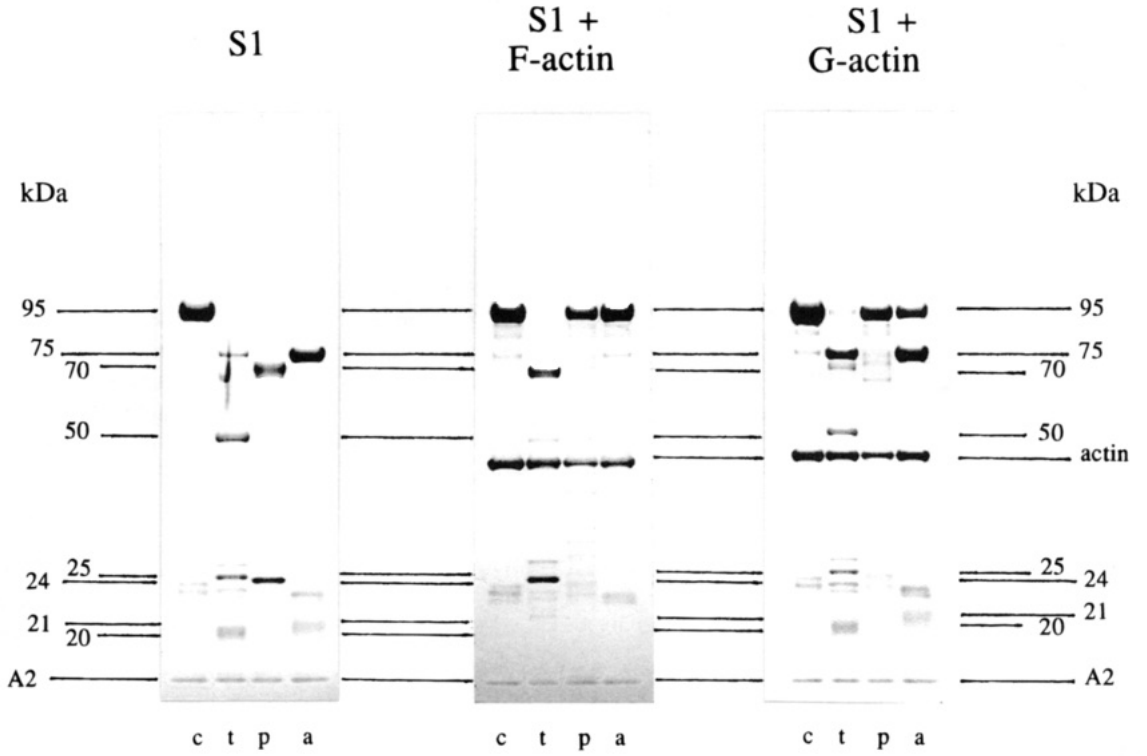


FIGURE 5: Electrophoretic analysis of the limited proteolysis of various actin−S1(A2) complexes. Proteolysis of 10 μM S1(A2) alone and mixed with an equimolar amount of F-actin or G-actin was performed with trypsin (t), papain (p), and Arg-C protease (a) as described under Materials and Methods; lane c, control sample before proteolysis reactions.

linked, and probably could not bind, to actin subdomain 2 in the monomeric complex. Note that the lack of S1 cross-linking to Lys50 was not due to the binding of DNase I to the same subdomain 2 since DNase I interacts with residues Arg39, Gln41, Val43, and Val45 which are located at a region distinct from Lys50 (Kabsch et al., 1990). Moreover, a very similar cross-linking pattern, although with a lower yield, was obtained by a two-step reaction in which G-actin was activated first by GA in the absence of DNase I, which was added in a second step with S1(A2) (data not shown).

Finally, the oligomeric state of actin bound to S1 did not affect the DMS reaction on the S1 heavy chain which generated an internally cross-linked S1 with a mass slightly higher than 95 kDa (Figure 4B, left panel; Labbé et al., 1982). In contrast, GA seemed to react more efficiently with S1 bound to G-actin than to F-actin since an internally cross-linked S1, migrating slower than the 95-kDa heavy chain, was produced mainly with the G-actin−S1 complex (Figure 4B, right panel).

Proteolytic Experiments. The actin-induced protection against the limited proteolysis of S1 was studied using three different proteases—trypsin, papain, and Arg-C protease—which cut the S1 heavy chain within the two connecting segments named loop 1 and loop 2 by Spudich (1994) and located at approximately 25 and 75 kDa from the N-terminal part of the S1 heavy chain primary sequence (Figure 6A). Since the proteolytic reactions usually lasted for less than an hour at 20 °C, the addition of DNase I was not necessary to prevent the polymerization of the monomeric complexes, even at a protein concentration of 10 μM (Chaussepied & Kasprzak, 1989a). As shown in Figure 5 and in agreement with previously published data, F-actin protected differently the two connecting regions, according to the protease used. For example, papain cleavage at loop 1 was totally protected by F-actin, thus inhibiting the formation of the 70- and 24-kDa fragments (Figure 5; Applegate & Reisler, 1983), while trypsin degradation of the same loop was not protected since the 25-kDa fragment was not produced in the presence of F-actin (Figure 5; Mornet et al., 1981b). On the other hand, loop 2 was always protected by F-actin either from trypsin degradation, generating a 70-kDa fragment, or from Arg-C protease, leaving uncleaved the S1 heavy chain (Figure 5; Mornet et al., 1981b; Yamamoto, 1991).

The results obtained with the monomeric complex were quite different except that, as in the filamentous complex, papain cleavage was totally protected (Figure 5; Lheureux et al., 1993). Since S1 and G-actin were employed in a stoichiometric amount during the proteolysis experiments, the full protection of S1 confirmed that S1 and G-actin formed a tight 1:1 complex (Lheureux et al., 1993). Trypsin proteolysis of the G-actin−S1 complex produced a significant amount of 75-kDa fragment, arguing for a protection of the tryptic cleavage of loop 1. However, this protection was only partial since a nonnegligible amount of 25-kDa fragment was also produced. It is interesting to note that identical results were obtained when the trypsin proteolysis was performed on a complex between S1 and a chemically modified G-actin (Bettache et al., 1990; Hozumi, 1992). In addition, trypsin treatment of the monomeric complex yielded the 20- and 50-kDa fragments revealing that loop 2 was not protected by G-actin. The lower protection of loop 2 in the monomeric complex was confirmed with Arg-C protease, which partially cleaved the S1 heavy chain, in the 75- and 21-kDa fragments. In all cases S1 binding to G-actin protected actin from degradation as recently reported (Chen et al., 1992; Fievez & Carlier, 1993).

Figure 6 summarizes the overall results of cross-linking and limited proteolysis experiments characterizing the monomeric and the filamentous actin−S1 complexes.

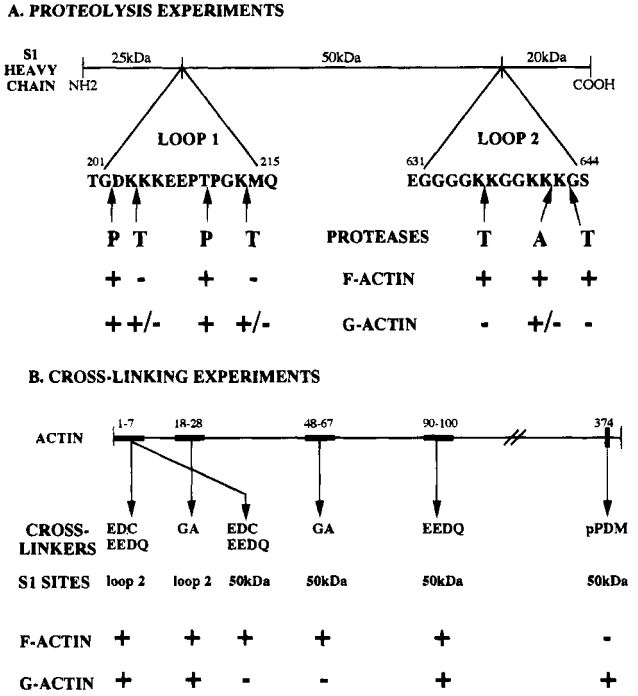


FIGURE 6: (A) Summary of the proteolytic sites on S1 and their level of protection (+) or unprotection (−) during their interaction with F- and G-actin. (B) Summary of the cross-linking sites on S1 and on actin in its monomeric (G-actin) or filamentous (F-actin) form. The presence (+) or the absence (−) of cross-linking and the type of cross-linking reagent used are indicated. Cross-linking between the 50-kDa fragment of S1 and actin residue Cys374 was previously described by Combeau et al. (1992).

DISCUSSION

In this work we have brought experimental evidence which explains why filamentous but not monomeric actin activates the myosin ATPase activity.

Our functional studies first confirmed the absence of significant activation of the S1 ATPase even at relatively high concentrations of monomeric actin. The very low but significant 3-fold activation observed with the monomeric complex could be accounted for by two reasons: either (1) the G-actin−S1 interface contains all the information needed for the activation to take place, but the affinity between G-actin and S1 or the S1−nucleotide complex is so low that much higher protein concentrations (than those authorized) are necessary to get a full activation or (2) the actin−S1 contacts necessary for the ATPase activation are missing in the monomeric complex, so that the small activation observed is due to the presence of a small fraction (less than 2.5%) of actin oligomers unavoidable at high actin concentrations.

Studies on the binding of G-actin to S1 in the presence of nucleotide revealed that, in contrast to F-actin, G-actin is very unlikely to form a stable ternary G-actin−S1−nucleotide complex. Even the actin−S1−ADP complex, known to belong to the strong binding complexes in the filamentous state [for a review, see Geeves et al. (1984)], would need very high protein concentrations in order to take place in the monomeric state. In fact, the formation of these ternary complexes is necessary for actin to accelerate the release of the ATPase products from the S1 active site. So their absence would be sufficient to explain the lack of ATPase activation by G-actin. In other words, the actin−S1 contacts necessary for S1 ATPase activation would be missing in the monomeric complex under our experimental conditions, and the small 3-fold activation observed would be due to the presence of a small fraction of oligomers, as we show in the accompanying paper (Lheureux & Chaussepied, 1995).

Our structural studies provide important clues to understand the differences between the monomeric and filamentous actin−S1 interfaces. Though the affinity constant for the G-actin−S1 complex is high ($K_a = 20-60\ \mu M^{-1}$) under salt-free conditions, the complex is surprisingly sensitive to the ionic strength but poorly affected by organic solvents (Lheureux et al., 1993). These characteristics strongly suggest that most of the S1 hydrophobic subsites located on F-actin residues Ala144, Ileu341, Ileu345, Leu349, and Phe352 (Rayment et al., 1993) are not accessible, or at least are much less accessible, to S1 in the monomeric form.

Moreover, the cross-linking experiments have shown that the S1 contacts in actin subdomain 2 are absent in the monomeric complex. This result apparently disagrees with the cross-linking between S1 and subdomain 2 of an actin molecule in which polymerization had been impaired by extensive chemical modification (Bertrand et al., 1994). A comparison between this result and ours, however, is not straightforward since this modified actin has a tendency to dimerize even under low-salt conditions (Arata, 1991). On the other hand, our results are in good agreement with the atomic reconstruction of the F-actin−S1 complex in which the binding of S1 to actin subdomain 2 was described on a monomer different from that carrying the main S1 binding sites (Rayment et al., 1993; Schröder et al., 1993). This second monomer is indeed absent in the stoichiometric G-actin−S1 complex.

The lack of S1 binding to G-actin subdomain 2 is also consistent with the formation of the ternary DNase I−G-actin−S1 complex in which DNase I would interact with actin subdomain 2 (Kabsch et al., 1990). The formation of this ternary complex has already been proposed by several groups (Lheureux et al., 1993; Kasprzak, 1993; Chen et al., 1992; Bettache et al., 1990). It is further confirmed in this work by the fact that DNase I did not affect the nucleotide sensitivity of the G-actin−S1 complex and since DNase I did not change most of its cross-linking and proteolytic patterns (data not shown). These data, however, are in total contradiction with a recent report which excludes the formation of such a ternary complex (Blanchoin et al., 1995).

The main G-actin−S1 contact sites seem to reside in the electrostatic bonds formed between the positively charged S1 segment 636−642 (loop 2 in Figure 6A) and the patch of negative residues which belong to the actin flexible loops 1−7, 20−28, and 90−100 (residues 1−4, 24−25, and 99−100, respectively). Each of these actin loops either was cross-linked to the S1 heavy chain in the monomeric (this work) and filamentous complexes (Sutoh, 1983; Bertrand et al., 1988; Bonafé et al., 1994) or was proposed to interact with S1 loop 2 by image reconstruction of the saturated filament (Schröder et al., 1993). Furthermore, this result is in total accordance with the fact that masking S1 segment 636−642 with an antipeptide fully blocks the binding of S1 to G-actin (Chaussepied & Kasprzak, 1989b), while an antibody directed against actin segment 1−7 only partly inhibits the formation of the monomeric complex (DasGupta et al., 1990). On the other hand, one should note that this coulombic contact is not fully identical in the monomeric and filamentous complexes. Indeed, the 175-kDa cross-

linked product promoted by EDC and EEDQ and consisting of actin residues 1−7 attached to the tryptic 50-kDa fragment of S1 disappears in the monomeric form, and the S1 loop 2 was only partially protected against proteolysis in the monomeric complex. Taken together, these results demonstrated that this electrostatic interaction is less tight in the monomeric than in the filamentous complex. In addition, the orientation of the complex seems different in the two forms since the 50-kDa fragment of S1 is close enough (at most 13 Å) to be cross-linked to actin residue Cys374 only in the monomeric form (Combeau et al., 1992; unpublished observation).

Despite the fact that S1 interacts mainly with the negative residues of actin subdomain 1 in the monomeric complex, its binding alters the structure of other loci of the actin molecule. For example, the S1 effect on the actin critical concentration demonstrates that S1 alters the actin−actin contact sites located on actin subdomains 2, 3, and 4 (Lheureux et al., 1993; Holmes et al., 1990). The internal communication between actin subdomain 2 and the S1 binding site located in actin subdomain 1 is further sustained by two separate lines of evidence: (i) DNase I binding to subdomain 2 decreases the affinity of the G-actin−S1 complex (Lheureux et al., 1993) and affects the proteolytic digestion of the C-terminal segment also located in actin subdomain 1 (Crosbie et al., 1994), and (ii) S1 binding to G-actin changes the proteolytic succeptibility of actin subdomain 2 (Chen et al., 1992; Kasprzak, 1993, 1994; Fievez & Carlier, 1993).

Finally, the effect of actin on S1 structure seems to be different in the two complexes. For example, the proteolytic succeptibility of S1 loop 1 (S1 heavy chain segment 204−216) was different in the monomeric and the filamentous forms.

In conclusion, the G-actin−S1 interaction is not a good analogue of the strong F-actin−S1 complex which is formed in the absence of nucleotide. Indeed, the actin−S1 interface is composed mainly of three components in the filamentous form (electrostatic, hydrophobic on the main monomer, and an additional interface on a second monomer), while only the electrostatic component is partially present in the monomeric form. On the other hand, several works have implicated this electrostatic component in the formation of the weak acto-S1 binding state (Chen et al., 1985; Chaussepied, 1989; Bertrand et al., 1989; Yamamoto, 1989; DasGupta & Reisler, 1992; Adams & Reisler, 1993; Miller & Reisler, 1995). We have provided strong experimental evidence that these electrostatic contacts are not sufficient for the formation of the weak interface since the monomeric complex could not bind nucleotides. As a consequence, one can conclude that the G-actin−S1 interaction is not an analogue of the weak binding state [or the A state proposed by Geeves (1991)] which predominates in the F-actin−S1−nucleotide complex.

In fact, the G-actin−S1 complex is characterized by a strong dependence on the ionic strength but apparently only a low dependence on the temperature (Geeves, 1995; Lheureux, 1993; Figure 3). These characteristics are also shared by the short-lived collision complex which takes place in the early time of the formation of the F-actin−S1 interface (Geeves, 1991; Geeves & Conibear, 1995). Therefore, the G-actin−S1 complex could be a good analogue of this unstable complex, also called the pre-weak F-acto-S1 binding state (Holmes, 1995).

The formation of the weak binding state would involve additional residues besides the patch of negative residues located on actin subdomain 1. Whether these additional residues belong to the same or to a different monomer is an important question that will be tentatively resolved in the accompanying paper (Lheureux & Chaussepied, 1995).

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