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THE BOUND NUCLEOTIDE OF ACTIN

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The extent of actin polymerization has been studied for samples in which the bound nucleotide of the actin was ATP, ADP, or an analog of ATP that was not split (AMPPNP). The equilibrium constants for the addition of a monomer to a polymer end were determined from the concentration of monomer coexisting with the polymer. An analysis of these results concludes that the bound ATP on G-actin provides little energy to promote the polymerization of the actin. AMPPNP was incorporated into F-actin and the interaction of F-actin · AMPPNP with myosin was studied. F-actin · AMPPNP activated the ATPase of myosin to the same extent as did F-actin · ADP. However, the rate of superprecipitation was slower in the case of F-actin · AMPPNP than in the control.

INTRODUCTION

In muscle fibers actin exists as a long, double-stranded helix (known as F-actin) which forms the backbone of the thin filaments. In vitro, at low ionic strength, the actin filament can be depolymerized into its globular subunits (G-actin) which are repolymerized by addition of salt. Each actin monomer binds one nucleotide, which can be either ATP or ADP in G-actin, but is always found to be ADP in F-actin. Thus when G-actin · ATP is polymerized, a nucleotide dephosphorylation accompanies the polymerization. The presence of such a reaction, which releases useful energy, has attracted much attention, yet to date the role of the actin nucleotide remains unknown. For a recent review of the biochemistry of actin, see Oosawa and Kasai (1).

The nucleotide of G-actin is exchangeable, while the nucleotide of F-actin exchanges only very slowly with unbound nucleotides. Several investigators have examined the exchange of the bound nucleotides of living and glycerinated muscle fibers and have concluded that they are not exchanged during the interaction of actin with myosin that produces force (2). Although G-actin denatures in the absence of a bound nucleotide, F-actin, which is relatively free of nucleotides, can be prepared and is stable. Nucleotide-deficient F-actin can activate myosin ATPase and can participate in superprecipitation (3). From these experiments, it has been concluded that the bound nucleotide is not required for polymer formation or for the interaction with myosin. The only known function of the bound nucleotide is to maintain the native structure of G-actin. However, if this is the only role of the bound nucleotide it prompts the question: why does the nucleotide

Abbreviation: AMPPNP, adenylyl imidodiphosphate.

remain bound to the actin polymer where it is not required for stability, and why is it split during polymerization?

Tightly bound nucleotides, which resemble that of F-actin in that they do not exchange with nucleotides in the medium, are also found on a number of other proteins. All of these proteins are associated with some aspect of the transduction of energy. Examples of such proteins are the F_1 protein complex of mitochondria and a protein component of chloroplasts (4, 5). ADP bound to chloroplasts has been shown to be phosphorylated in situ during the synthesis of ATP following photon capture. The phosphorylation of the bound nucleotide preceded that of ADP in the solution (5). Another example is that of tubulin. Each tubulin dimer binds 2 moles of guanosine nucleotides, one in an exchangeable site and the other in a nonexchangeable site (6). Upon polymerization, all of the bound nucleotides become nonexchangeable. It has been shown that the tightly bound nucleotide on the tubulin dimer can be transphosphorylated by nucleotides added to the medium (6).

The presence of in situ phosphorylations, which occur in other systems, suggests that the possibility of such a reaction should be investigated in actin. To date no in situ phosphorylation of the actin nucleotide has been shown to occur. Perry et al. have shown that the bound nucleotide of myofibrils is not a substrate for the creatine kinase-creatine phosphate system (7). Strohman has shown that the nucleotide of F-actin is not available to this kinase (8). Studies done on G-actin are complicated by artefacts of exchange. The negative results of these experiments do not provide a definitive answer to the question of in situ phosphorylations of the actin nucleotide. During the cycle of an actin-myosin interaction, a phosphate could be passed to the γ position of the bound nucleotide. The phosphate could possibly originate from ATP in the medium or from ATP bound to myosin. Such a transphosphorylation could be done transiently so that it could be hard to detect. Instead of attempting to detect an in situ phosphorylation, the γ position of the actin nucleotide can be blocked by incorporation of an unsplittable analog of ATP. If an in situ phosphorylation of the bound nucleotide is necessary during some interaction of actin with another protein, then the incorporation of adenylyl imidodiphosphate (AMPPNP) into the actin polymer will prevent the phosphorylation and should inhibit any reaction in which it is required. The following paper reports some experiments in which AMPPNP is used to investigate the role of the actin nucleotide in polymerization and in the interactions of actin with myosin and the relaxing proteins.

METHODS

Actin and relaxing proteins were prepared by the method of Spudich and Watt (9). Myosin was prepared by the method of Tonomura et al. (10). Myosin subfragment 1 was prepared by the method of Cooke (11). Superprecipitation was monitored in a Bausch and Lomb dual beam spectrometer, which was modified to permit stirring of the sample. The temperature was unregulated at 23°C. Turbidity was measured at 340 nm in 1-cm cuvettes. ATPase was monitored in a temperature-controlled pH stat (Radiometer).

RESULTS

Analog Binding

Several lines of experimental results have shown that the analog AMPPNP binds to

the nucleotide site of actin. Experiments involving competitive binding with a spin label analog of ATP have shown that AMPPNP binds to the actin site with an affinity which is about four to five times weaker than that of ADP (12, 13). More direct experiments using radioactive AMPPNP have confirmed this result (12). It was shown, both by radioactive tracers and by isolation and chromatography on polyetheleneimine cellulose, that the AMPPNP bound to the actin monomer is incorporated into the actin polymer upon polymerization. Since the analog binds to the nucleotide site of both G- and F-actin, it provides a useful tool for the study of the properties of the bound nucleotide.

Polymerization

The kinetics of the polymerization of actin have been studied under conditions where the bound nucleotide was ATP, ADP, or AMPPNP (12). G-actin · ATP polymerizes about three to five times faster than G-actin · ADP, and G-actin · AMPPNP polymerizes with a rate that is similar to that of G-actin · ATP. The rates of polymerization do not contain much information on the energetics of polymerization. Information on the free energy change which drives the polymerization, as is shown later, is related to the extent of polymerization. Previous workers have studied the extent of polymerization of G-actin ATP. They found that very little polymer was formed below a critical concentration of actin and that all actin in excess of that concentration is formed into polymer (14, 15). This is the behavior to be expected for many types of polymerization mechanisms. We call this critical concentration $[G(\infty)]$. Figure 1 shows the amount of polymer formed as a function of the actin concentration for the three different nucleotides. The intercepts on the abscissa give the values of $G(\infty)$.

Actomyosin Binding and ATPase

Since the binding of AMPPNP to actin does not inhibit the process of polymerization, F-actin · AMPPNP was prepared and its interactions with myosin and with the relaxing proteins were studied. F-actin· AMPPNP was prepared by methods previously outlined (12). In a typical preparation, up to 80–90% of the actin sites contain AMPPNP with the remaining sites occupied by ADP. In 0.6 M KCl, actin and myosin bind to each other in a complex that results in a large increase in viscosity. The presence of ATP dissociates the complex and drops the viscosity to the sum of the viscosities of the two proteins. Both the increased viscosity of the actomyosin complex and the decrease upon addition of ATP were unaffected by the incorporation of AMPPNP into the F-actin polymer. Thus, the actin nucleotide does not appear to be involved in the binding of myosin to actin.

At low ionic strength (50 mM KCl) the ATPase of the actomyosin complex is higher than that of myosin alone. The ATPase of both actomyosin and actosubfragment 1 were studied in 50 mM KCl, 3 mM $MgCl_2$, and 2 mM ATP over a wide range of protein concentrations. In all of these studies the steady state ATPase was not affected by the incorporation of AMPPNP into the actin polymer. When relaxing proteins were added to the actin, the ATPase in the presence of EGTA dropped to about one-tenth the value in the absence of the relaxing proteins and the addition of Ca^{++} relieved this inhibition. The relaxing proteins were capable of regulating the ATPase of the complex of

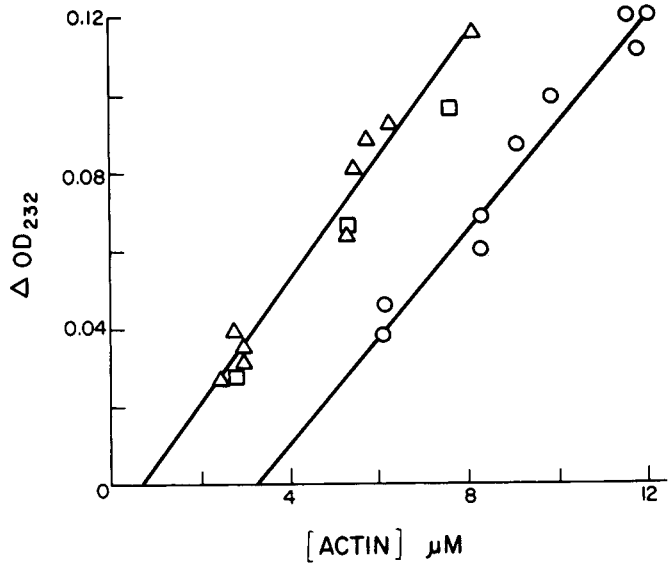

Fig. 1. The amount of polymer formed is shown as a function of the total actin concentration. The polymer formation was measured by the change in OD_{232}. The actin was initially in 0.1 mM $MgCl_2$, 0.2 mM DTT, and 5 mM Tris, pH 8.0, plus: 0.4 mM ADP, (○); 0.4 mM ATP, (△); or 0.4 mM AMPPNP, (□). Polymerization was initiated by addition of KCl to 100 mM (12).

myosin and F-actin • AMPPNP to the same extent as the control. Thus, the ATPase of the actomyosin complex and the regulation of this ATPase by the relaxing proteins does not require the participation of the bound nucleotide of actin.

Superprecipitation

Addition of ATP to an actomyosin gel which has been precipitated at low ionic strength (50 mM KCl) results in a shrinking of the gel and a rise in the turbidity. This reaction, known as superprecipitation, was studied as a function of the amount of AMPPNP incorporated into the F-actin polymer. The amount of analog incorporation was determined using radioactive analog by the methods outlined by Cooke (12). It was found that the extent of superprecipitation was independent of the incorporation of analog into the actin polymer. However, as the incorporation of analog increased, the rate of superprecipitation decreased, as is shown in Fig. 2. Extrapolation of the data shown in Fig. 2 indicates that if F-actin had all of its sites occupied by AMPPNP, it would have a low, and possibly a zero, rate of superprecipitation with myosin. The abscissa of Fig. 2 gives the percent analog incorporation measured before the addition of ATP to produce the superprecipitation. There is some exchange between the bound nucleotide of the actin and ATP in the medium, which has been studied previously (16). This exchange reaction, which decreases the amount of AMPPNP incorporation, can be minimized by using deoxy ATP to induce superprecipitation. Deoxy ATP binds only weakly to the actin nucleotide site. The reduction of the velocity of superprecipitation was slightly greater when deoxy ATP

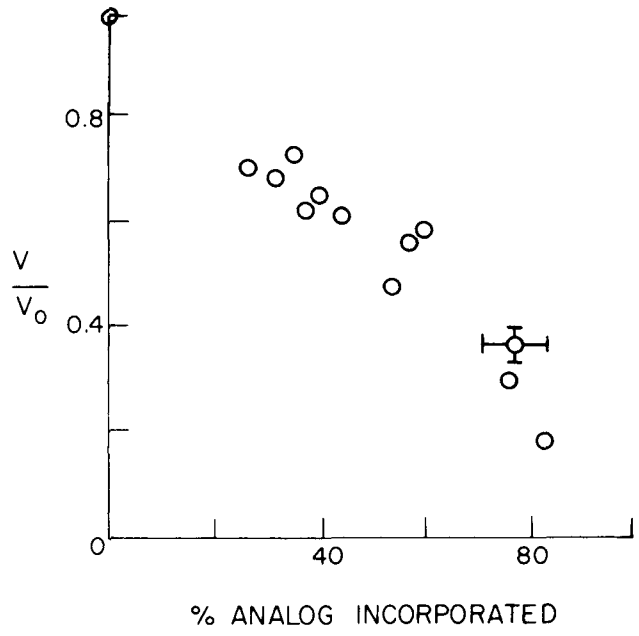

Fig. 2. The relative velocity of superprecipitation (where the velocity is taken as the inverse of the time required for the turbidity change to reach half maximum) is shown as a function of the percent of nucleotide sites on the F-actin polymer that are occupied by AMPPNP. The superprecipitation of an actomyosin solution was measured by the increase in turbidity at 340 nm. The solution contained 50 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.3 mM DTT, and 20 mM TES, pH 7.0. ATP was added to a final concentration of 0.3 mM. The protein concentrations were myosin 0.18 mg/ ml, actin 0.09 mg/ml.

was used than when ATP was used. Thus, the rates of superprecipitation shown in Fig. 2 have probably been increased by approximately 10% due to nucleotide exchange.

The reduction of the rate of superprecipitation by incorporation of analog into the actin polymer might be due to denaturation of the actin caused by analog incorporation. In order to determine whether the incorporation of AMPPNP into actin caused a denaturation of the actin, we have reincorporated ADP, replacing the AMPPNP. With AMPPNP incorporated into F-actin (~80%), the velocity of superprecipitation was 0.35 that with ADP incorporated. Both experimental and control samples were depolymerized by dialysis against a solution containing ATP and then repolymerized. The analog was completely replaced by ADP during this process. In the superprecipitation assay both actin samples now showed fast velocities, equal within experimental error (± 10%). This experiment shows that no permanent denaturation of the actin has occurred, which could explain the slower rate of superprecipitation seen with the incorporation of analog into the F-actin.

The effect of AMPPNP incorporation on the velocity of superprecipitation is reminiscent of the experiment of Tokiwa and Morales (17), who showed that limited reaction of myosin with an affinity label affected the rate of superprecipitation but not the extent. The extent was affected only after the great majority of the myosin active sites

had been killed by the label. We have further investigated these phenomena. Myosin filaments were subjected to papain (0.01 mg/ml) digestion for varying lengths of time and their ability to superprecipitate was then assayed. Digestion first slowed the rate of the superprecipitation and finally (30 min) affected the extent. Gel electrophoresis of the digested myosin in 20 mM PP_i (no SDS) could resolve native myosin, one-headed myosin, and myosin rods. At points when the rate of superprecipitation was affected, some of the myosin heads had been cleaved off. After 30 min of digestion, when the extent was also affected, little native myosin was seen. The conclusion of this experiment is that modification of the superprecipitation reaction by removal of some of the myosin heads reduces the rate of the reaction, and that most of the heads must be removed in order to observe an effect on the extent.

DISCUSSION

Polymerization

The mechanisms and kinetics of polymerization reactions have been extensively studied. Kasai et al. (14) were the first to study the kinetics of the actin polymerization, and Kasai (15) constructed a kinetic model from these results. Cooke (12) has extended the analysis to include the effect of the splitting of ATP which occurs during the polymerization. The pertinent result of these treatments is that the equilibrium constant K for the addition of a monomer to a polymer end is inversely proportional to the concentration of monomer which is in equilibrium with the polymer:

$$K = k_+/k_- = 1/[G(\infty)]$$

where K has the units of 1/mol, k_+ and k_- are the forward and reverse rate constants for the addition of a monomer to a polymer end, and $[G(\infty)]$ is the monomer concentration in equilibrium with the polymer. This result is exact for the case in which no nucleotide is split. The polymerization of G-actin · ATP is more complex and probably involves a nucleotide splitting step which follows the monomer addition step. A kinetic scheme, which incorporates these complications and explains the data for the polymerization of G-actin · ATP, has been presented by Cooke (12).

Several conclusions can be drawn from these kinetic models. The binding of ATP to G-actin promotes the polymerization of actin slightly more than the binding of ADP. This can be seen in the data on Fig. 1 which show that $[G(\infty)]$ is about three times less for the polymerization of G-actin · ATP than for G-actin · ADP. It is the binding of ATP and not its hydrolysis which provides this energy, since the binding of the unsplittable analog promotes the actin polymerization. The amount of standard free energy provided by AMPPNP (or by ATP) additional to that provided by ADP can be calculated from the ratio of the equilibrium constants for the polymerizations of G-actin · AMPPNP and G-actin · ADP. This ratio is between 3 and 5 and thus represents less than 4 kJ/mol of standard free energy. Since ATP has about 30 kJ/mol of standard free energy additional to that of ADP, this represents only a small fraction of the energy which it could provide to drive the actin polymerization if that were its only function. The conclusion is that the binding of ATP to G-actin plays little role, energetically speaking, in promoting the polymeriza-

tion. Thus one has to look for other interactions which may involve this nucleotide.

Actomyosin Interactions

The results of Tokiwa and Morales (16) and the results of the papain digestion of myosin, reported here, both indicate that the inhibition of some of the actin-myosin interactions inhibits the rate but not the extent of superprecipitation. The extent of superprecipitation was only inhibited after prohibition of most of the actin-myosin interactions. These results do not appear unreasonable if one thinks of the superprecipitation reaction as the result of the sliding of one set of filaments relative to another driven by the actomyosin interaction. As some of the actin-myosin force-generating events are removed, the rate of filament movement would be slowed. However, the movement of filaments would continue until some minimum in the potential energy of the filament array was reached. Thus, as long as there are sufficient native molecules on each individual filament to produce some motion, the extent of superprecipitation would be unaffected.

If the above picture of superprecipitation is assumed to be correct, then the slow rate of superprecipitation seen when using F-actin · AMPPNP may be an indication that AMPPNP incorporation has inhibited the actin-myosin interaction which slides the filaments into the low energy array. The lack of inhibition of the extent of superprecipitation may then be due to an insufficient incorporation of the AMPPNP. This interpretation of the results should be taken as hypothetical, since superprecipitation is a complex reaction whose details are not understood. Although electron micrographs indicate that movement of filaments relative to one another does occur during superprecipitation (18), the nature of the movement and whether it is driven by the same contractile events which occur in a muscle remain unknown.

If AMPPNP incorporation into F-actin inhibits the actin-myosin interaction in the superprecipitation reaction, why does it not inhibit the actomyosin ATPase reaction? The explanation of this discrepancy may be that the in vitro assays of ATPase and superprecipitation may be measuring different aspects of the actomyosin interaction. In an intact muscle, the biochemical events are coupled to the production of mechanical force. When the proteins are extracted and the in vitro assays performed, the extent to which the biochemical events are coupled to mechanical events is unknown. What portion of the force generating cycle is required for the in vitro splitting of ATP or for superprecipitation is also not known. Thus, the use of any in vitro assay to decide whether a modification of the actomyosin system has affected the capability of that system to produce force can lead to ambiguous conclusions. A clear answer to the question of whether the incorporation of AMPPNP into F-actin affects the ability of the actin to participate in the generation of force will have to be decided by the use of systems in which the production of force can be measured.

The conclusions to be drawn from these studies are the following. (1) The bound nucleotide of actin plays little role, energetically speaking, in the polymerization of the actin. (2) The conclusion reached by previous investigators, that modification of the bound nucleotide has no effect on the interaction of actin with myosin, is not valid.[1]

[1] Although it is not discussed by Barany et al. (3), careful inspection of their data reveals that removal of some of the bound nucleotides of actin also slows the rate of superprecipitation.

The modification used here, i.e. AMPPNP incorporation, has some effect on the actin-myosin-ATP interaction that is responsible for superprecipitation. (3) These preliminary experiments suggest that actin-bound ADP may be essential for force-generating interactions of actin with myosin.

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