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The Role of the Bound Nucleotide in the Polymerization of Actin†

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ABSTRACT: Three nucleotides, ATP, ADP, and an unsplittable analog of ATP (adenylyl imidodiphosphate (AMPPNP)), were bound to monomeric actin, and their effects on the rate and extent of the actin polymerization were studied. The kinetics of polymerization, assayed by the change in OD_{232}, followed a simple exponential curve. The rates of polymerization were equal for bound ATP and AMPPNP; both of which were three to five times faster than the rate for ADP. The concentration of actin monomers in apparent equilibrium with the polymer, G(∞), was determined. Values of G(∞) in 100 m*M* KCl were found for different nucleotides to be: G·ATP(∞) = 0.7 μM, G·AMPPNP(∞) = 0.8 μM, and G·ADP(∞) = 3.4 μM. The equilibrium constant of the polymerization is given by $K = [G(\infty)]^{-1}$ when no nucleotide is split. The polymerization of actin·ATP is more complex due to the splitting of the nucleotide and our data require that this polymerization involves more than one step. The kinetic parameters for the polymerization of actin·ATP can be explained by a simple scheme in which the nucleotide dephosphorylation occurs in a step following the polymerization step. The conclusions are: (1) the binding of ATP to actin monomer promotes polymerization slightly more than the binding of ADP, (2) actin bound ATP provides less than 4 kJ/mol of free energy to promote polymerization, and (3) the dephosphorylation of the nucleotide is not coupled to polymerization.

The actin polymer forms the backbone of the thin filaments found in a muscle fiber, and it is also found in the cytoplasm of most, if not all, eucaryotic cells. The interaction of myosin with an actin polymer produces mechanical work at the expense of the energy of ATP. The actin filaments are formed by a helical array of monomers, which can be dissociated by lowering the ionic strength. The polymerization–depolymerization cycle of actin is thought to play no role in the contraction of muscle cells, but is hypothesized to play a major role in the assembly of contractile structures in the cytoplasm of nonmuscle cells. This paper investigates the extent to which the polymerization of actin is controlled by a nucleotide which is bound to each actin monomer. The biochemistry of muscle actin has been reviewed by Oosawa and Kasai (1971) and that of cytoplasmic actin has been reviewed by Pollard (1973).

The actin monomer (G-actin) binds one nucleotide and one divalent cation. In G-actin, the bound nucleotide can be either a di- or triphosphate, and rapidly exchanges with unbound nucleotides in the medium. The nucleotide of the actin polymer (F-actin) exchanges with external nucleotides at a rate which is many orders of magnitude smaller than the rate for G-actin. When G-actin with bound ATP is polymerized to F-actin, the ATP is dephosphorylated, and the nucleotide found in the polymer, or in the thin filament of a muscle, is ADP. Although the dephosphorylation of the bound nucleotide accompanies polymerization it is not a requirement for it since it has been shown that G-actin·ADP (Hayashi and Rosenbluth, 1960) or G-actin with no nucleotide (Barany et al., 1966) can also polymerize.

No function has been quantitatively demonstrated for the nucleotide of actin. It has been implicated as playing a role in promoting or regulating polymerization. The evidence is twofold: the dephosphorylation occurs during polymerization, and G-actin·ATP polymerizes faster than G-actin ADP. Both polymerize faster than G-actin with no bound nucleotides (Hayashi and Rosenbluth, 1960). However, these studies have not explored quantitatively the coupling between the bound nucleotide and the polymerization. Other investigators have speculated that the bound nucleotide may play a role in the energy transducing reactions with myosin. These speculations have found little experimental justification. The only known requirement for the bound nucleotide is to maintain G-actin in a native state. The presence of bound nucleotides on proteins associated with other energy transduction systems—mitochondria (Harris et al., 1973), chloroplasts (Yamamoto et al., 1972), and tubulin (Berry and Shelanski, 1972)—prompts the speculation that bound nucleotides do play an important role in energy transduction. The possibility exists that the actin nucleotide may also have an important but as yet un-

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recognized function in one of the diverse interactions of the protein to which it is bound.

The ATP analog, adenylyl imidodiphosphate (AMPPNP),[1] has a structure similar to ATP with the exception that the oxygen bridge between the terminal phosphates is replaced by an NH group. The bond angles and lengths of AMPPNP resemble those of ATP (Yount et al., 1971a,b). A previous paper investigated the polymerization of actin AMPPNP (Cooke and Murdoch, 1973). We have shown that AMPPNP binds to G-actin with an affinity constant slightly weaker than that of ADP. We have also shown that G-actin·AMPPNP can polymerize and that the AMPPNP becomes incorporated into the actin polymer without being split. G-actin·AMPPNP was found to polymerize with a rate similar to G-actin·ATP. All previous investigations of the role of the actin nucleotide have investigated only its effect on the rate of polymerization. Studies of the extent of polymerization, however, allow one to determine equilibrium constants and thus to evaluate quantitatively the energetics of the nucleotide participation. In the present paper we have measured both the rate and extent of polymerization and have shown that both parameters can be fit to simple kinetic models. Using these models it is shown that the bound ATP provides little free energy, less than 1 kcal/mol, to promote the actin polymerization.

Materials and Methods

Actin was prepared from an acetone powder by the method of Spudich and Watt (1971). Protein was determined by optical density at 280 or 290 nm using extinction coefficients of 1.10 (1.18) and 0.63 (0.67), respectively, for G-actin (F-actin). All calculations assumed a molecular weight of 43K for G-actin. AMPPNP and [^{3}H]AMPPNP were purchased from ICN Laboratory. All nucleotides used ran as a single spot when chromatographed on Brinkman cellulose polyethyleneimine (MN 300) using a medium containing 1.0 *M* LiCl and 10 m*M* Tris (pH 9.0). Chromatography of [^{3}H]AMPPNP showed that approximately 85% of the counts ran as a single peak with the same R_f value as AMPPNP. The remaining counts ran ahead of the main peak and probably represent hydrolytic products of AMPPNP. Buffers used were the following: buffer 1, 5 m*M* Tris (pH 8.0)–0.2 m*M* dithiothreitol– 0.1 m*M* $MgCl_2$; buffer 2, buffer 1 with 0.2 m*M* ATP; buffer 3, buffer 1 plus 0.1 *M* KCl.

The polymerization of actin was followed by the increase in optical density at 232 nm. Higashi and Oosawa (1965) showed that there was a peak in the G-actin − F-actin difference spectrum at this wavelength. The change in OD_{232} is due to a local structural change occurring in the individual actin monomers upon polymerization, and it should be proportional to the number of actin–actin bonds formed. However, a second mechanism also influences the OD_{232} since the formation of long actin polymers causes an increase in turbidity. The increase in turbidity was monitored at 310 nm and extrapolated to 232 nm using the relationship that OD (turbidity) $\propto$ (wavelength)$^{-3.4}$ found by Higashi and Oosawa (1965). It was found that about 20% of the increase in OD_{232} was due to an increase in turbidity. Thus this assay system is sensitive both to the number of actin–actin bonds and to the formation of long polymers, where ideally one wants to be sensitive only to the number of bonds. However, this system is still superior to previous methods used (viscosity or flow birefringence) which are only sensitive to the formation of long polymers, and which tend to mechanically stress the filaments causing filament fractures. The polymerization was followed in 1-cm cuvets using a Cary 1650 spectrophotometer with a slit width of 3 mm (band pass = 3–4 nm). The temperature was regulated at 25°.

The extent of [^{3}H]AMPPNP incorporation into F-actin was assayed as follows. Excess ATP was removed from actin by homogenization of an F-actin pellet into buffer 1, polymerization, and sedimentation by centrifugation. This actin was resuspended by homogenization in buffer 1 at approximately 1 mg/ml and passed through a short, 2 cm, column of Dowex 1-X10 to further remove excess nucleotides. Varying amounts of AMPPNP were then added to the actin and polymerization was initiated within 1 min by addition of 0.1 *M* KCl and 2 m*M* $MgCl_2$. All steps prior to polymerization were carried out at approximately 0°. A rapid rise in the viscosity of the solution showed that polymerization had occurred. The polymer was then spun. The resulting pellets were washed in buffer 3 for 10 min and resuspended again in buffer 1. The amount of nucleotide on the actin was determined by OD_{258} following precipitation of the protein with 10% perchloric acid. At this point the stoichiometry of nucleotide to actin monomer varied from 1.0 to 1.2. The slight excess of nucleotides could be removed by passage again through a Dowex column. This reduced the stoichiometry to between 0.9 and 1.1, but did not greatly affect the results. The amount of [^{3}H]AMPPNP was determined and the percent of bound analog calculated.

An analysis of the data for the binding of AMPPNP to G-actin is complicated by the presence of ADP which is competing for the same site. The equilibrium concentrations of the various species in the mixture of G-actin, ADP, and AMPPNP are determined by five equations. Two equations define the binding constants for the two nucleotides and three equations express the conservation of the three molecular species, ADP, AMPPNP, and actin. Assuming that all ADP in the mixture comes in with the actin, and assuming that the binding constant for ADP to the G-actin site is 10^6 M^{-1} (West, 1970), one can derive a cubic equation giving the percent analog bound as a function of total analog concentration divided by the total actin concentration. Binding curves were generated for various values of the AMPPNP binding constant, and the best fit of the theoretical to observed incorporation was found for an AMPPNP binding constant of 2×10^5 M^{-1}, given by the solid line in Figure 1.

Results

It was previously shown that AMPPNP could bind to G-actin and that the G-actin could then be polymerized to F-actin with incorporation of the analog into the F-actin structure (Cooke and Murdoch, 1973). The binding studies were done indirectly via competitive binding with a spin-label analog of ATP. A recent report that actin·AMPPNP does not polymerize (Brehme et al., 1973) prompts a reexamination of this question. Using [^{3}H]AMPPNP we confirmed our original observations. The apparent discrepancy between these results is discussed later. The incorporation of [^{3}H]AMPPNP into F-actin is shown in Figure 1 plotted as a function of the ratio of analog to actin in the G-actin solution prior to polymerization. The results of Figure 1 show that analog has bound to the G-actin and has been in-

[1] Abbreviation used is: AMPPNP, adenylyl imidodiphosphate.

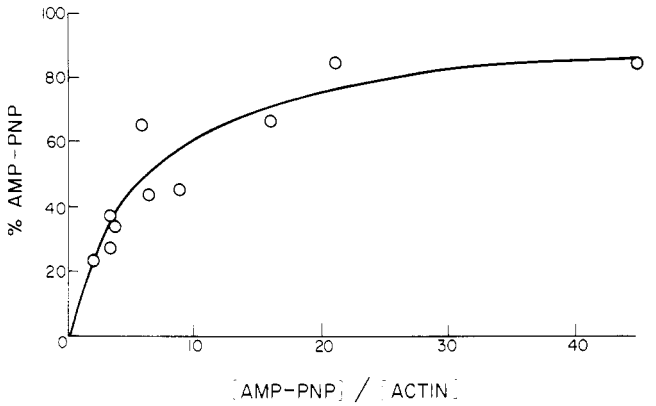

FIGURE 1: The incorporation of AMPPNP into F-actin is shown. [^{3}H]AMPPNP was added to a solution of G-actin (approximately 1 mg/ml) in buffer 1. The actin was polymerized and sedimented to remove excess nucleotides and the incorporation of [^{3}H]AMPPNP into F-actin was determined. The percent of actin monomers containing AMPPNP is plotted as a function of the ratio of AMPPNP to G-actin in the solution prior to polymerization.

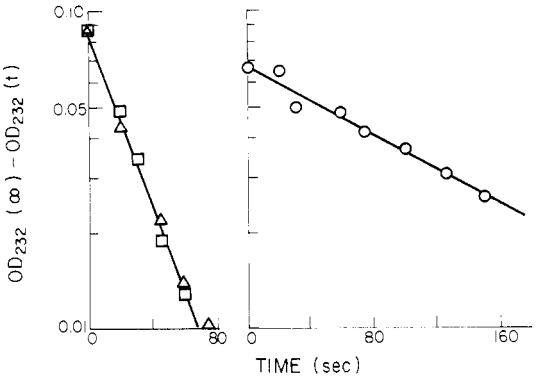

FIGURE 2: The decay of actin monomer concentration is shown as a function of time following addition of KCl (to 15 mM) and $MgCl_2$ (to 0.5 mM). The solution contained 0.7 mg/ml of actin in buffer 1 plus 0.5 mM ADP (○); 0.5 mM AMPPNP (□); or 0.5 mM ATP (△). A 5-mm cuvet was used.

corporated into the F-actin polymer. Experiments were done to confirm that the analog incorporation shown in Figure 1 is incorporation into a site on F-actin which is not exchangeable. Resuspension of the labeled pellet in buffer 3 and subsequent dialysis overnight removed only about 10% of the counts. Resuspension of the labeled pellet in buffer 3 and resedimentation showed that 85–95% of the counts spun down again with the actin. The binding constant of [^{3}H]AMPPNP was calculated from the data of Figure 1 to be $2 \times 10^5\ M^{-1}$. This result agrees well with the value of $2.5 \times 10^5\ M^{-1}$ previously obtained from competitive binding studies.

At low ionic strength actin depolymerizes into monomers. Addition of KCl causes a rapid polymerization which can be measured as an increase in viscosity, flow birefringence, or optical density at 232 nm. About half the actin is polymerized at 15 mM KCl and a maximum polymerization is attained at 100 mM KCl. Actin·ATP polymerizes faster than actin·ADP, although the extent of polymerization has only been determined for actin·ATP (Kasai et al., 1962). Since the extent of polymerization gives information on the equilibrium constant we have measured both the extents and rates of the actin polymerization as a function of the type of bound nucleotide.

Figure 2 shows the data of Cooke and Murdoch (1973)

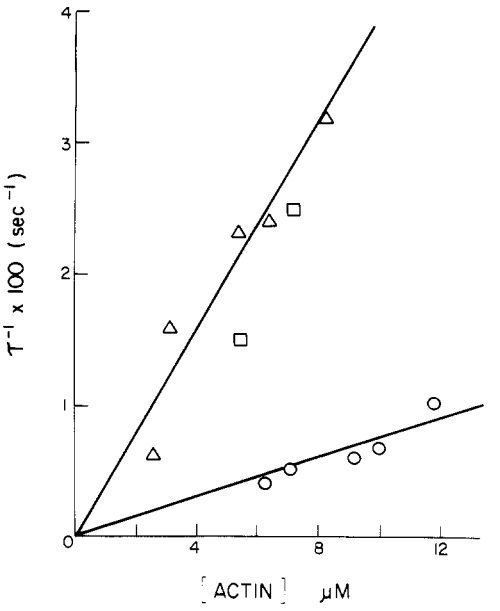

FIGURE 3: The rate of polymerization is shown as a function of the actin concentration. The exponential decay of actin monomer is defined by eq 4 where the rate of decay, τ^{-1}, is equal to $k_+[F]$. Actin was initially in buffer 1 plus: 0.4 mM ADP (○); 0.4 mM ATP (△); or 0.4 mM AMPPNP (□). Polymerization was initiated by addition of 100 mM KCl.

replotted on a semilogarithmic graph. Following addition of salt to a G-actin solution a rapid rise in OD_{232} occurs which reaches a steady-state value in a few minutes. The difference between the OD_{232} at a time t and the steady-state value of OD_{232} which is attained after the polymerization is complete is plotted in Figure 2. The decay of the monomer concentration follows a single exponential which can be expressed in the form: $\exp(-t/\tau)$, where τ is a constant with the dimensions of time. All samples studied showed single exponential forms with the two following exceptions. (1) Mixtures of G-actin·AMPPNP and G-actin·ADP showed two exponentials each with a value of τ appropriate for one of the nucleotides. (2) At low concentrations of G-actin ADP, 5–8 μM, a slow rise in OD followed the initial exponential rise. The nonexponential change in OD represented between 5 and 10% of the total change and may be due to a slower formation of longer polymers which give rise to turbidity. The rate of the reaction is expressed by τ^{-1}. Both G-actin·ATP and G-actin·AMPPNP have a rate which is five times faster than the rate for G-actin·ADP, see Figure 2. The rate increased linearly with protein concentration for all types of bound nucleotides as shown in Figure 3.

Previous workers have studied the 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. This is the behavior to be expected for many types of polymerization mechanisms. We call this critical concentration G(∞). Figure 4 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(∞). Table I summarizes values of G(∞) and τ for two different KCl concentrations. At KCl concentrations less than 30 mM the value of G(∞) rises abruptly and the value of τ becomes long.

In all cases where τ or G(∞) are compared for different bound nucleotides the following procedure was used. Excess

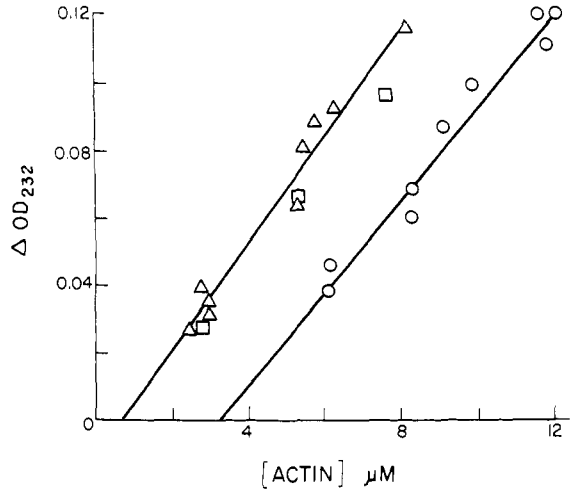

FIGURE 4: The amount of polymer formed is shown as a function of the actin concentration. The polymer formation was measured by the change in OD_{232}. The actin was initially in buffer 1 plus: 0.4 m*M* ADP (○); 0.4 m*M* ATP (△); or 0.4 m*M* AMPPNP (□). Polymerization was initiated by addition of 100 m*M* KCl.

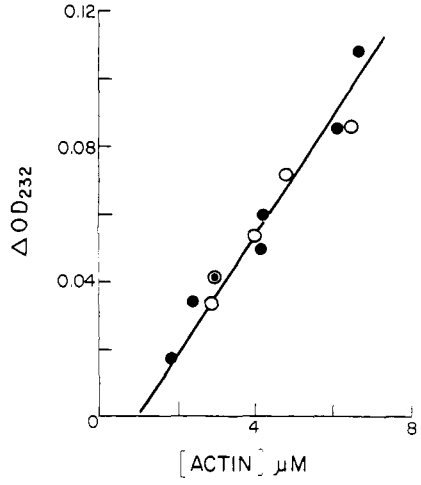

FIGURE 5: The amount of polymer formed is shown as a function of the actin concentration. The actin was in buffer 1 and 0.5 m*M* ATP. Polymerization was initiated by addition of 30 m*M* KCl (●), or 11 m*M* K_2HPO_4 (pH 8) (○).

nucleotides were removed from the actin by homogenization of a pellet into buffer 1, polymerization, and sedimentation. The resulting pellet was again resuspended by homogenization in buffer 1. Various nucleotides were then quickly added to aliquots of this solution.

When G-actin·ATP is polymerized the products are F-actin and P_i. If the release of P_i is coupled to the polymerization process the equilibrium concentration of monomer, $G(\infty)$, would depend on the concentration of P_i since addition of products should drive a reaction back toward the reactants. To determine the exact dependence of $G(\infty)$ on the concentration of P_i, G-actin·ATP was polymerized with KCl and with K_2HPO_4 (pH 8). At this pH the ionic strength of a 1 *M* solution of the latter (*86*% K_2HPO_4 and *14*% KH_2PO_4) was calculated to be 2.7 *M*. The actin was polymerized in two solutions of equal ionic strength. If PO_4^{2-} has no specific influence on the equilibrium of the actin polymerization (i.e., related to the fact that it is a product of the reaction) one expects that in equal ionic strengths of KCl or K_2HPO_4/KH_2PO_4 actin would have equal values of $G(\infty)$. Figure 5 shows that this is the case, and thus that PO_4^{2-} is not a direct product of the addition of a monomer to a polymer end. Most probably it is a product of a subsequent step which does not influence the monomer-polymer equilibrium.

In the absence of a bound nucleotide G-actin quickly denatures (for a detailed discussion, see Oosawa and Kasai, 1971). ATP binds with high affinity to G-actin and completely protects the actin for up to 8 hr as judged by the parameters of polymerization. ADP and AMPPNP bind more weakly to G-actin, and both the rate and extent of polymerization decrease as a function of time for samples of G-actin incubated with these nucleotides. All polymerizations shown here are done with G-actin incubated with 0.4 m*M* nucleotides in buffer 1 at 0°. Under these conditions actin incubated with AMPPNP lost the ability to polymerize in approximately 30 min. A similar rate of denaturation was seen for G-actin incubated with 0.1 m*M* ADP. Since ADP binds to G-actin four to five times more strongly than AMPPNP one expects that the same amount of nucleotide free actin would be present in the two above samples. Thus one can conclude that the loss of polymerizability depends only on the amount of bound nucleotide and not on the nature of the nucleotide. Raising either the nucleotide or the Mg^{2+} concentration provided better protection for the actin. Addition of ATP to G-actin which had lost its polymerizability could restore the extent of polymerization, without, however, restoring the rate, which remained about an order of magnitude slower than expected. This shows that the denaturation process is complex, probably involving more than one step, and that the early steps are reversible. Although most of the actin monomers possess bound nucleotides at the above concentrations, the small fraction of nucleotide free actin can undergo conformational changes which can, with time, affect the entire population. These results reconcile the apparent differences between this paper and the findings of Brehme et al. (1973). These workers reported that G-actin lost its ability to polymerize following dialysis against a solution of AMPPNP and that polymerizability could be restored by the addition of ATP. Their conclusion, that G-actin·AMPPNP cannot polymerize, is clearly not valid if one looks at this process rapidly before the process of denaturation begins.

Table I: The Rate and Extent of Actin Polymerization.

	KCl (m*M*)	τ^{-1} (sec^{-1})[a]	$[G(\infty)]^{-1}$ (M^{-1})[b]
Actin·ATP	100	2.8×10^{-2}	1.2×10^{6}
Actin·AMPPNP	100	2.5×10^{-2}	1.0×10^{6}
Actin·ADP	100	5.4×10^{-3}	3×10^{5}
Actin·ATP	37	9.5×10^{-3}	7.7×10^{5}
Actin·AMPPNP	37	8.3×10^{-3}	6.0×10^{5}
Actin·ADP	37	2.4×10^{-3}	1.9×10^{5}

[a] τ is the time constant for the exponential decay of G-actin concentration following addition of KCl to 0.3 mg/ml of G-actin in buffer 1. [b] $[G(\infty)]$ is the G-actin concentration remaining in solution when the polymerization has reached steady state.

Discussion

The mechanisms and kinetics of polymerization reactions have been extensively studied (for a good introduction see Tanford, 1961). Kasai et al. (1962) were the first to study the kinetics of the actin polymerization and Kasai (1969) constructed a kinetic model from these results. These work-

ers determined G(∞) and followed the velocity of the reaction for the polymerization of G-actin·ATP. Their values of G(∞) agree well with those determined here for G-actin ATP. They also observed that the polymerization followed a sigmoidal curve; the reaction starts slowly, accelerates, and then decelerates as it approaches equilibrium. The initial stage of the polymerization was thought to represent the slow formation of nuclei, on to which single monomers could then be added. In contrast to the polymerizations described by Kasai the polymerizations described in this paper follow a single exponential. This is predictable behavior for a polymerization which begins with a fixed number of nuclei whose number does not change during the course of the reaction. The differences between these results and those of the previous workers may be due to different methods of sample preparation. The previous workers attempted to eliminate actin oligomers via 48-hr dialysis and sometimes addition of urea. The time course of the polymerization then observed was very slow, of the order of 20 min, compared to those reported here, which with comparable actin and KCl concentrations would be complete in a few seconds. A small number of either actin oligomers or other proteins in our preparations may act as nuclei, explaining the difference in results. We cannot rule out the possibility that trace amounts of other proteins, such as α-actinin, may also act as nuclei.

The nature of these nuclei does not influence the kinetic analysis developed below. The number of nuclei, however, is important since the number of polymer ends and therefore the rate of the reaction will be proportional to the number of nuclei. Although we cannot determine the exact number of nuclei, we can still obtain valuable information on rate constants if we know that the concentration of nuclei is proportional to the concentration of protein and that the proportionality is independent of conditions and protein concentration. The following lines of evidence indicate that this proportionality remains a constant in our systems. The kinetics, which follow single exponentials, show that during a polymerization the number of nuclei has not changed. The rate of the reaction depends linearly on the protein concentration (as shown in Figure 3) which is to be expected if the number of nuclei is proportional to the amount of protein independently of concentration. Addition of ATP a few seconds before polymerization of a G-actin·ADP (or AMPPNP) sample gave kinetics appropriate for the polymerization of G-actin·ATP. This shows that the number of nuclei in the G-actin·ADP sample had not decreased due to the presence of ADP, for if it had, the rapid substitution of ATP for the bound ADP would have given slower kinetics due to the fact that the formation of nuclei is slow (Kasai et al., 1962). The above results show, in agreement with the conclusions of Kasai (1969), that the formation or destruction of nuclei is slow compared to the duration of our experiments. These conditions allow us to obtain information on rate constants which cannot be obtained from the more complex system used by previous workers.

A kinetic scheme for the polymerization of actin in the absence of ATP dephosphorylation can be easily written down. The polymerization rate depends linearly on the protein concentration indicating that the reaction involves the addition of single actin monomers to the polymer end; this reaction can be written:

$$G + F_n \underset{k_-}{\overset{k_+}{\rightleftharpoons}} F_{n+1} \qquad (1)$$

where G represents actin monomer and F_n and F_{n+1} represent polymers of length n and $n + 1$. The kinetics of this reaction is described by

$$dG/dt = -k_+[G]\sum_{n=1}^{m-1}[F_n] + k_-\sum_{n=2}^{m}[F_n] \qquad (2)$$

where the summation is now over all polymer lengths from 1 to some maximum length m. We assume that the two summations are equal: $\Sigma_{n=1}^{m-1}[F_n] = \Sigma_{n=2}^{m}[F_n] = [F]$, where [F] is the approximate concentration of polymer ends. In other words we are dealing with very long polymers, and addition of one more monomer does not change the reactivity of the polymer end. Setting dG/dt equal to zero at equilibrium, one obtains from eq 2

$$K = k_+/k_- = 1/[G(\infty)] \qquad (3)$$

where $[G(\infty)]$ is the monomer concentration at $t = \infty$ when the polymerization reaction has attained equilibrium, and K is by definition the equilibrium constant. Using eq 3 the solution to eq 2 can be found:

$$G(t) - G(\infty) = (G(0) - G(\infty))e^{-k_+[F]t} \qquad (4)$$

where G(0) is the initial actin concentration. The time constant for the exponential decay of monomer is given by $\tau^{-1} = k_+[F]$.

Equation 4 predicts that τ^{-1} should depend linearly on [F] and therefore should depend linearly on protein concentration if the number of nuclei is a fixed fraction of the total amount of protein. Figure 3 shows that τ^{-1} does depend linearly on the actin concentration. This is in contrast to the work of Kasai et al. (1962) who found that the initial velocity of their polymerization, which required the formation of nuclei, depended on the third to fourth power of the actin concentration.

Equations 2–4 cover the case in which no nucleotide is split during the polymerization. The data are shown in Table I. The equilibrium constant $[G(\infty)]^{-1}$ is roughly three to four times greater when the bound nucleotide is AMPPNP. The value of τ^{-1} and therefore the value of k_+ is also three to four times greater for AMPPNP. Using these values in eq 3 we obtain the result that k_- is similar for the two nucleotides. Thus the actin polymer with bound analog depolymerizes at the same rate as that with bound ADP. Attempts to measure the depolymerization rate directly by dilution of a concentrated F-actin solution into low ionic strength did not succeed. The F-actin in the concentrated solution forms into very long polymers which means that there are few polymer ends, giving rise to a slow rate of depolymerization. The measured rate had a time constant of the order of 10^{+3} sec. Mechanical agitation, homogenization, sonication, or even rapid pipetting increased the depolymerization rate supposedly by breaking up the polymers. Sonication of actin polymers has previously shown indirectly that the depolymerization rates are the same for F-actin having ADP and AMPPNP (Cooke and Murdoch, 1973).

The model for polymerization presented above is not applicable to the polymerization of G-actin·ATP. This polymerization does not reach an equilibrium since ATP is constantly being split. Although the rate of splitting is small in a solution of polymerized actin, this splitting is very important since it is a part of the depolymerization and repolymerization steps which determine the equilibrium constant of the polymerization. The reaction proceeds to a steady state and the steady-state value of the monomer concentration can be measured and is given in Table I. The additional reaction of nucleotide dephosphorylation necessitates a

more complex reaction scheme. Since ATP is being split, the reaction can only be considered to have reached a steady state, which is not a true equilibrium. This has consequences for the theoretical analysis employed. These complexities were not taken into account by Kasai (1969). If one assumes that the dephosphorylation is part of the reaction mechanism of the addition of a monomer to a polymer end the reaction would be:

$$G\cdot ATP + F_n \rightleftharpoons F_{n+1} + P_i \quad (5)$$

where F_n and F_{n+1} represent polymers having n and $n + 1$ monomers. The equilibrium constant for this reaction obtained analogously to eq 3 is given by

$$K = [P_i]/[G\cdot ATP] \quad (6)$$

The above equation gives the equilibrium constant for the addition of a monomer to the polymer end. The depolymerization step, i.e., the subtraction of a monomer from the polymer end, does not involve a rephosphorylation and thus would follow the kinetic parameters determined by eq 2–4. Equations 2–4 do not depend on the concentration of P_i. Since Figure 5 shows that the value of $G(\infty)$ is independent of P_i we conclude that reaction 5 is not valid.

The above argument suggests that the dephosphorylation is not coupled to the polymerization. Thus the simplest reaction scheme to explain the polymerization of G-actin·ATP would entail a polymerization step followed by a dephosphorylation step:

$$F_n + G\cdot ATP \overset{1}{\rightleftharpoons} F_n\cdot G\cdot ATP \overset{2}{\rightleftharpoons} F_n\cdot G\cdot ADP + P_i$$
$$F_n + G\cdot ADP \overset{3}{\rightleftharpoons} F_n\cdot G\cdot ADP \quad (7)$$

In the presence of excess ATP any G-actin·ADP produced by depolymerization is quickly returned to G-actin·ATP by nucleotide exchange so that the following reaction must also be added to the above scheme:

$$ATP + G\cdot ADP \overset{4}{\rightleftharpoons} G\cdot ATP + ADP \quad (8)$$

The kinetics of reactions 7 and 8 can be analyzed in the same fashion as in the simpler case in which no nucleotide was split if two assumptions are made. One, it is assumed that the values of k_{+1} and k_{-1} can be approximated by the values of k_+ and k_- found for the polymerization of actin·AMPPNP. In other words, in the step which does not involve the splitting of ATP, G-actin·ATP is mimicked by G-actin·AMPPNP. This assumption has some experimental justification since the rate of polymerization of G-actin·ATP is equal to that of G-actin·AMPPNP, implying that the forward rate constant for addition of a monomer to a polymer end is the same for the two bound nucleotides. Secondly, we assume that [G·ADP] ≪ [G·ATP]. If this assumption were not valid the kinetics of G-actin·ATP would not follow a single exponential. The rate equations for dG·ATP/dt and dG·ADP/dt, analogous to eq 4, can be written

$$dG\cdot ATP/dt = -k_{+1}[F][G\cdot ATP] + k_{-1}[F\cdot G\cdot ATP] + k_{+4}[ATP][G\cdot ADP] - k_{-4}[ADP][G\cdot ATP] \quad (9)$$

$$dG\cdot ADP/dt = -k_{+3}[F][G\cdot ADP] + k_{-3}[F\cdot G\cdot ADP] - k_{+4}[ATP][G\cdot ADP] + k_{-4}[ADP][G\cdot ATP]$$

Since k_- was found above to be the same for bound AMPPNP and ADP we assume that $k_{-1} = k_{-3}$. Setting the sum of dG·ATP/dt and dG·ADP/dt equal to zero one obtains

$$1/[G(\infty)] = k_{+1}/k_{-1} \quad (10)$$

and

$$G(t) - G(\infty) = (G(0) - G(\infty))e^{-k_{+1}[F]t} \quad (11$$

These two equations state that the values of the rate and extent of polymerization of G-actin·ATP are the same as those of G-actin·AMPPNP and are independent of the concentration of P_i. Thus all the values of the parameters observed for the polymerization of G-actin·ATP are explained by this model. The results are independent of P_i because the depolymerization rate does not depend on the nature of the nucleotide bound to the polymer.

Several conclusions can be drawn from this kinetic model. The binding of ATP to G-actin promotes actin polymerization but only slightly. It is the binding not the splitting of the ATP which provides this energy since the binding of the unsplittable analog also promotes the actin polymerization. The amount of free energy provided by either ATP or AMPPNP additional to that provided by ADP can be calculated from the ratio of the equilibrium constants for the polymerizations of steps 1 and 3 in reaction 7. This ratio is between 3 and 5 and thus represents less than 4 kJ/mol of free energy. Since ATP has about 30 kJ/mol of 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. These results support the hypothesis (Morales and Botts, 1952; Boyer et al., 1973) that the binding of ATP not the splitting provides energy for protein interactions. However, since the amount of energy provided in this case is very small a rigorous test of the hypothesis has not been made.

It has recently been shown that a reactive ATP analog can bind to G-actin, which can then be polymerized, but that once polymerized the actin cannot be depolymerized (Bender et al., 1974; Faust et al., 1974). This is a very interesting observation; however, the conclusion drawn from it, i.e., that the ADP bound to the F-actin stabilizes the polymer, conflicts with the results of this paper. We have found that the values of k_+ and of the steady-state monomer concentration are of the same order of magnitude for all the nucleotides. This implies that the depolymerization rates must also be of the same order of magnitude irrespective of whether the polymer was formed from G-actin with bound ATP, ADP, or AMPPNP. Thus, the strong dependence of depolymerization rates on the nature of the bound nucleotide postulated by the above workers is not supported by our observations.

The most pertinent measure of the equilibrium constant for polymerization for the kinetic scheme presented here, as well as for that given by Kasai, is the value of $[G(\infty)]^{-1}$. If more free energy is supplied to drive a polymerization reaction toward completion, it would be reflected by a larger value of K and a smaller value of $[G(\infty)]$. The change in free energy is related to the equilibrium constant for the reaction by: $K = \exp(-\Delta G/RT)$ where RT is approximately equal to 2.5 kJ/mol at room temperature. When G-actin·ADP is polymerized in 100 mM KCl the change in free energy is 32 kJ/mol, calculated from the data of Table I and the above equation. In the case where ATP is split the situation is more complex; however, the value of $G(\infty)$ for this reaction is of the same magnitude as that of G-actin·ADP. If the polymerization of G-actin·ADP were driven by a free

energy change of 35 kJ/mol (an addition of 3 kJ/mol) the reaction would attain the same equilibrium value of monomer as does G-actin·ATP. Thus we conclude that ATP bound to G-actin provides no more than 3 kJ/mol to promote polymerization. This conclusion does not depend on the details of the reaction schemes or on the results obtained using the analog. It should be noted that if the equilibrium constants found above are applicable inside a muscle cell more than 99% of the intracellular actin would be polymerized irrespective of the type of bound nucleotide.

Tightly bound nucleotides have been found on several proteins that are involved in energy transduction. Although the function of these nucleotides remains unknown, their presence in diverse systems suggests that they play some role in the interactions of their associated proteins. Several roles have been proposed for the bound nucleotide of actin. In the absence of a bound nucleotide monomeric actin denatures, so that one role of the nucleotide is to maintain the native structure of G-actin. However, its presence on F-actin is not required for stability prompting the question: if the only role of the nucleotide is to provide stability why does the ADP remain bound to the polymer and why is ATP split during polymerization? The role most often proposed for the bound nucleotide of actin has been that of promoting and regulating the polymerization of the actin. This paper, however, shows that the nucleotide plays little role, energetically speaking, in the polymerization, which reopens the question of why the actin has a bound nucleotide. The answer to this question is important since it may provide insight into one of the interactions of actin. Thus the possibility that the bound nucleotide plays an as yet unrecognized role in the interactions of actin with myosin or with the relaxing proteins should be reexamined.

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