P. M., and Meienhofer, J. (1972), *J. Biol. Chem. 247*, 2266.
Houlihan, C. M., Scott, J. M., Boyle, P. H., and Weir, D. G. (1972), *Gut 13*, 189.
Iwai, K., Juttner, P. M., and Toennies, G. (1964), *J. Biol. Chem. 239*, 2365.
Kisliuk, R. L., Gaumont, Y., Krumdieck, C. L., and Baugh, C. M. (1971), *Pharmacologist 13*, 208.
Krumdieck, C. L., and Baugh, C. M. (1969), *Biochemistry 8*, 1568.
Krumdieck, C. L., and Baugh, C. M. (1970), *Anal. Biochem. 35*, 123.
Leslie, G. I. and Rowe, P. B. (1972), *Biochemistry 11*, 1696.
Levy, C. C. and Goldman, P. (1967), *J. Biol. Chem. 242*, 2933.
Meienhofer, J., and Jacobs, P. M. (1970), *J. Org. Chem. 35*, 4137.
Plante, L. T., Crawford, E. J., and Friedkin, M. (1967), *J. Biol. Chem. 242*, 1466.
Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., and Hultquist, M. E. (1949), *J. Amer. Chem. Soc. 71*, 1753.
Shin, Y. S., Buehring, K. U., and Stokstad, E. L. R. (1972), *J. Biol. Chem. 247*, 7266.
Tamura, T., Buehring, K. U., and Stokstad, E. L. R. (1972), *Proc. Soc. Exp. Biol. Med. 141*, 1022.

Interaction of Actin with Analogs of Adenosine Triphosphate†

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ABSTRACT: We have studied the binding to actin of an analog of adenosine triphosphate, adenylyl imidodiphosphate (AMP-PNP), in which a NH replaces the terminal bridge oxygen. This analog will bind to a number of ATPases without being hydrolyzed. The ability of AMP-PNP to bind to G-actin was assayed by three methods: competitive binding with a spin-labeled nucleotide, protection against denaturation, and direct isolation and characterization by chromatography of the nucleotide bound to polymerized actin. All three methods indicated that AMP-PNP will bind to actin, and that the affinity constant was severalfold weaker than that of ADP. G-actin which contains bound AMP-PNP can polymerize to F-actin without the nucleotide dephosphorylation which commonly occurs during polymerization. G-actin·AMP-PNP polymerizes at the same rate as G-actin·ATP, and both polymerize faster than G-actin·ADP. Sonication of the F-actin polymer showed that the stability of its structure was not altered when the bound ADP was replaced with AMP-PNP. The above results suggest that the energy of dephosphorylation, liberated during the polymerization of actin, is not used to facilitate the polymerization.

Actin is a globular protein of molecular weight 45,000 which is capable of polymerizing into a double-stranded polymer. This polymer forms the backbone of the thin filaments in a muscle fiber, and its interaction with myosin in the presence of ATP is known to produce the force of contraction. The actin monomer, known as G-actin, binds one nucleotide and one divalent cation. In G-actin the nucleotide can be either a di- or triphosphate and rapidly exchanges with unbound nucleotides in the medium. The nucleotide of F-actin exchanges with external nucleotides at a rate which is many orders of magnitude smaller than the rate seen in the case of 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 always ADP. Although it has been the center of a number of studies (for a recent review, see Oosawa and Kasai, 1971), the only known role of the actin nucleotide is to stabilize the structure of G-actin. G-actin, but not F-actin, will denature quickly if the nucleotide is removed.

When G-actin·ATP polymerizes, the dephosphorylation of the ATP should provide about 7 kcal/mol of useful energy. There have been two popular theories about the actin nucleotide and the fate of the energy of dephosphorylation. One theory contends that the nucleotide is used during muscle contraction, while the other holds that it is used in the regulation of the polymerization process, possibly to stabilize the polymer. The contraction hypothesis rests largely on the data of Szent-Gyorgyi and Prior (1966) who showed that some exchange of the F-actin nucleotide occurred when actin interacted with myosin in the presence of ATP to superprecipitate. However, the significance of this result has been questioned by other workers who claim that the nucleotide exchange is not related to superprecipitation (Moos *et al.*, 1967). It has also been shown that nucleotide-free actin can interact with myosin, both in the activation of myosin ATPase and in superprecipitation (Barany *et al.*, 1966). The second theory, which holds that the nucleotide is present to regulate or promote polymerization, is very attractive since the nucleotide dephosphorylation occurs during polymerization. Although the nucleotide is not necessary for polymerization as shown by the fact that nucleotide-free G-actin can form a polymer (Barany *et al.*, 1966), G-actin·ATP polymerizes faster than G-actin·ADP (Hayashi and Rosenbluth, 1960). This result has been interpreted as evidence that the energy of the ATP dephosphorylation is used to facilitate the polymerization.

To investigate the role of the actin nucleotide further we have used an analog of ATP in which an NH grouping re-

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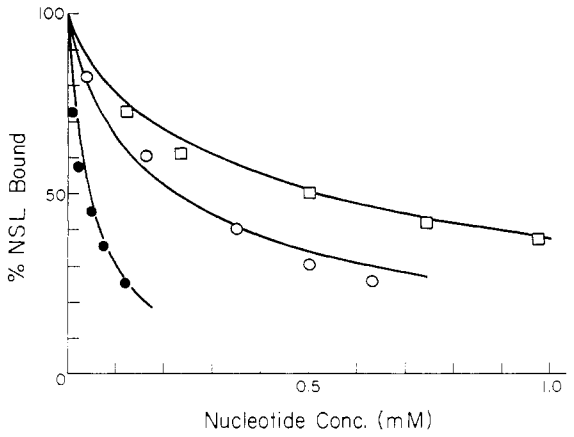

FIGURE 1: The amount of NSL-TP (a spin-labeled analog of ATP) that is bound to G-actin in competition with an added nucleotide is shown as a function of the concentration of the added nucleotide. The amount of bound NSL-TP was determined by peak heights in the electron paramagnetic resonance spectrum. The inital G-actin concentration was 7.3×10^{-5} M (assuming the mol wt = 45,000), the initial NSL-TP concentration was 8.2×10^{-5} M of which 7.2×10^{-5} M was bound to the actin. Aliquots of 1 mM ATP (●), 2 mM ADP (○), or 5 mM AMP-PNP (□) were added to give the final concentrations shown. The solid lines were calculated, assuming simple competitive binding and were fit by eye by varying the ratio of the binding constants: $K_{\text{nucleotide}}/K_{\text{NSL-TP}}$.

places the bridge oxygen between the β,γ-phosphates (Yount *et al.*, 1971a). This analog (AMP-PNP)[1] has a structure similar to ATP; however, it cannot be hydrolyzed by most ATPases (Yount *et al.*, 1971b). Thus the analog can be used to simulate and hold a state in an enzyme system in which ATP has bound to its site but has not been hydrolyzed.

Using this analog we have produced a F-actin polymer which contains a bound β, γ-imido nucleotide triphosphate, and we have found that it has the same stability toward sonication as F-actin containing ADP. In addition, G-actin·AMP-PNP polymerizes at the same rate as G-actin·ATP. These results suggest that dephosphorylation of the actin nucleotide plays no active role in the polymerization process.

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). The nucleotide spin label was prepared by the method of Cooke and Duke (1971). AMP-PNP was purchased from ICN; AMP-CPP and AMP-PCP were purchased from Pabst Laboratory; AMP-PNH_2 was isolated by DEAE chromatography as a by-product of the procedure which is commonly used for the synthesis of AMP-PNP (Yount *et al.*, 1971a). 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 mM Tris (pH 9.0). Electron paramagnetic resonance (epr) spectra were taken on a Varian E-3 using a 1 G modulation amplitude at a power of 100 mW. Viscosity was measured in an Ostwald viscometer with a buffer flow time of 70 sec. Sonication was done in a Bronwill Biosonik at 25°.

Results

The binding constants of various nucleotides were determined by a method involving the competitive binding of a spin-label analog of ATP. In this analog the 6 amino group of the adenosine ring has been replaced by a thiol linkage to a nitroxide spin label (Cooke and Duke, 1971). These workers have shown that this nucleotide spin label (NSL-TP) binds to G-actin, that the actin polymerization is unhindered by the NSL-TP and that F-actin containing NSL-DP is capable of superprecipitation. The stoichiometry of the binding is one NSL-TP per monomer with concommitant displacement of the normally bound ADP or ATP. The NSL-TP exchanges rapidly with an external nucleotide when bound to G-actin and exchanges only very slowly after the actin has been polymerized. NSL-TP bound to G-actin is hydrolyzed upon polymerization. The electron spin resonance spectrum of the NSL in solution shows the three sharp lines which are characteristic of nitroxide spin labels with fast rotational relaxation times. When the NSL-TP binds to G-actin the spectrum is broadened to one characteristic of an immobilized spin label, *i.e.*, with a rotational relaxation time slower than 10^{-8} sec. Polymerization causes no change in the spectrum of the bound NSL.

The electron spin resonance spectrum of the bound NSL shows a broad peak at low field which has very little overlap with a sharp peak of the free NSL which occurs at a slightly higher magnetic field. Thus the amplitudes of these two peaks can be used to quantify the amount of bound or free NSL in a sample containing actin and other nucleotides. Although the spectrum which is observed is the first derivative of the absorption, a peak height is still proportional to the concentration of the species responsible for that peak if no change in peak shape occurs, and this condition is fulfilled in the above case.

The NSL-TP was incorporated into G-actin by homogenizing an F-actin pellet in a solution of 0.1 mM NSL-TP, 0.1 mM $MgCl_2$, and 5 mM Tris (pH 8.0) (final actin concentration, 2–3 mg/ml). Excess nucleotides were next removed by passage through a Dowex 1 column. The resulting solution of G-actin was shown to contain one NSL-TP per actin monomer within experimental accuracy (10%) by the peak amplitude in the electron spin resonance spectrum, and by the optical density at 282 nm following protein precipitation with perchloric acid.

Aliquots of various nucleotides (1–5 mM in 0.1 mM $MgCl_2$ and 5 mM Tris (pH 8.0)) were added to samples of G-actin·NSL-TP or G-actin·NSL-DP (usually around 2 mg/ml), and the amount of NSL exchanged by the added nucleotide was estimated from the electron spin resonance spectrum. The amount of NSL displaced as a function of the concentration of the added nucleotide is shown in Figure 1 for several nucleotides. Addition of ATP causes a greater displacement of the NSL than does an equal concentration of added ADP. This is to be expected because the affinity of the nucleotide site for ATP is approximately 30–100 times greater than for ADP (Seidel, 1967). The addition of AMP-PNP caused less NSL displacement than did addition of ADP indicating that the affinity of actin for AMP-PNP is weaker than for ADP. Ad-

[1] The following abbreviations have been used: AMP-PNP, adenylyl imidodiphosphate; AMP-PNH_2, adenylyl phosphoramidate; AMP-CPP, α,β-methyleneadenosine triphosphate; AMP-PCP, adenylyl-methylene diphosphate; NSL, 6-mercapto-*N*-(1-oxyl-2,2,6-tetramethyl-4-piperidinyl)acetamido, α,β-D-ribofuranosylpurine 5′-triphosphate (or -diphosphate), when the state of the phosphates have been determined this is signified by NSL-TP for the triphosphate form or NSL-DP for the diphosphate form.

TABLE I: Binding Constants for Various Nucleotides.[a]

Nucleotide	$K_{nucleotide}/K_{NSL\text{-}TP}$
ATP	>2
ADP	0.25
AMP-PNH_2	0.20
AMP-PNP	0.08
AMP-CPP	0.04
AMP-PCP	0.04
AMP	$\sim 10^{-4}$
	$K_{nucleotide}/K_{NSL\text{-}DP}$
ATP	>5
ADP	0.9
AMP-PNP	0.2
GTP	0.1

[a] The binding constants of G-actin for various nucleotides are listed relative to the binding constant for either NSL-TP or NSL-DP. The values listed are obtained by competitive binding of the nucleotide with the appropriate form of the nucleotide spin label, as shown in Figure 1.

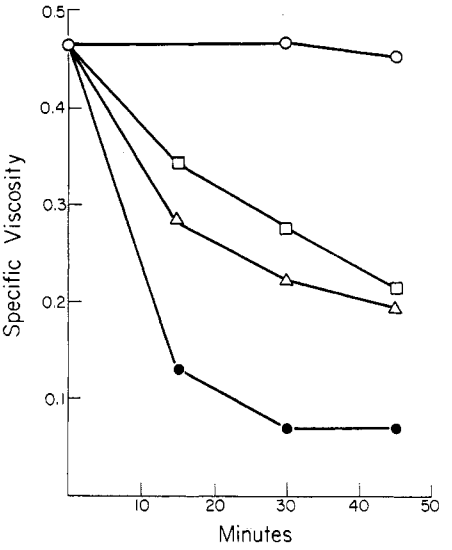

FIGURE 2: The denaturation of G-actin at 25° in the presence of various nucleotides was determined by measuring its ability to polymerize upon addition of salt. The specific viscosity of the polymer, measured 15 min after addition of 0.1 M KCl, is shown as a function of the time at which the salt was added. The G-actin (0.46 mg/ml) was incubated with: 0.1 mM $MgCl_2$ and 5 mM Tris (pH 8.0) (●); the above + 0.2 mM ATP (○); the above + 0.25 mM ADP, (△); the above + 1 mM AMP-PNP (□).

dition of 10 mM AMP caused only 30% NSL displacement, corresponding to the extremely weak affinity of actin for AMP.

Assuming that the binding of each nucleotide is described by

$$K_i = [N_i \cdot A]/[N_i][A]$$

where N_i = ith nucleotide and K_i its binding constant; and assuming that the free actin concentration [A] is negligible one can calculate the curves expected in Figure 1.

The solid lines in Figure 1 were fitted by eye using the ratio of K_i/K_{NSL} as the only independent variable. Table I summarizes the values of K_i/K_{NSL} which were found to give the closest fit to the data. This method is not sensitive when the binding constant of the added nucleotide is equivalent to or exceeds that of the nucleotide spin label, so that the values for the relative binding constant of ATP shown in Table I are only lower limits. The binding of ADP is found to be three times stronger than AMP-PNP when measured by competition with a NSL-TP and 4.5 times stronger when measured by competition with a NSL-DP; these two values are probably equal within experimental error. The important point is that AMP-PNP binds to G-actin, but it binds more weakly than does ADP. Since ADP binds from 30 to 100 times more weakly than ATP (see Oosawa and Kasai, 1971), AMP-PNP binds with an affinity that is approximately 100–200 times weaker than that of ATP. The other analogs, AMP-PCP and AMP-CPP, bind even more weakly than AMP-PNP. The binding constant for AMP-PNH_2, on the other hand, closely resembles that of ADP. The pH dependence of the binding was not systematically studied, however, it was determined that the binding of the AMP-PNP was several fold weaker at pH 7.0 and became stronger as the pH was increased up to pH 8.5 (higher pH's were not studied). This behavior is consistent with the data of Yount *et al.* (1971a) who found that the pK of the terminal phosphate was 7.9.

When G-actin loses its nucleotide it quickly denatures to a form which is incapable of rebinding nucleotide or of polymerizing (Asakura, 1961). Figure 2 shows the loss of polymerizability as a function of time for G-actin in the presence of ATP, ADP, and AMP-PNP. The ATP excess, coupled with its strong binding constant, protects the actin completely; while the smaller binding constants of either ADP or of AMP-PNP allow a slow denaturation to occur. Assuming that the binding constant of AMP-PNP for actin is four times weaker than that of ADP, the concentration of AMP-PNP was adjusted to give roughly equal amounts of free actin in the ADP and AMP-PNP samples in Figure 2. As shown this resulted in approximately equal denaturation rates for these two samples. When no nucleotide is added to the sample, the denaturation rate is very rapid as is also seen in Figure 2.

G-actin can be obtained with either a diphosphate or triphosphate as the bound nucleotide. However, the nucleotide of F-actin has previously always been found to be a diphosphate. To answer the question of whether the dephosphorylation of the G-actin bound ATP was obligatory upon polymerization, G-actin·AMP-PNP was polymerized by addition of salt. The extent of polymerization of actin·AMP-PNP was checked by viscosity and sedimentation. ATP, ADP, and AMP-PNP (all 0.5 mM) were added respectively to three samples of G-actin in 0.1 mM MgCl, 0.1 mM dithiothreitol, and 5 mM Tris (pH 8.0). Polymerization was quickly induced by addition of 0.1 M KCl, and its extent was checked after a 0.5-hr incubation at room temperature. The viscosities of the three polymerized samples were identical within ±2%, showing that the extent of actin polymerization was the same for the three different bound nucleotides. The samples were next spun at 78,000g for 3 hr and the absorbance at 280 nm of the supernatant was checked to determine the amount of unpolymerizable actin left in solution. All three samples showed less than 5% actin remaining unpolymerized. Thus the presence of the AMP-PNP on the G-actin did not hinder the extent of polymerization.

Chromatography on polyethylenimine was used to make a direct check of the nature of the bound nucleotide of F-actin

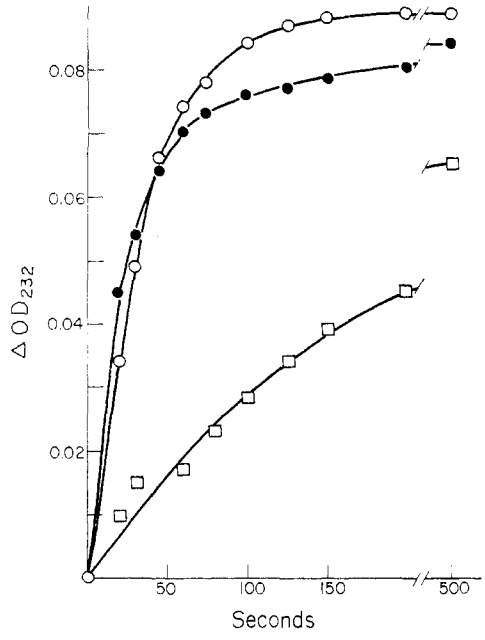

FIGURE 3: The rate of G-actin polymerization is shown for G-actin having bound ADP (□); bound ATP (○); and bound AMP-PNP (●). The solution contained 0.7 mg/ml of actin, 0.1 mM $MgCl_2$, 0.1 mM dithiothreitol, 5 mM Tris (pH 8.0), and 0.5 mM ADP or AMP-PNP. The sample with bound ATP was obtained by addition of 0.2 mM ATP to the AMP-PNP sample. Polymerization was followed by the change in optical density which occurs at 232 nm. 15 mM KCl and 0.5 mM $MgCl_2$ were added at time zero.

polymerized from G-actin AMP-PNP. G-actin (1.5 mg/ml) was incubated in 2.5 mM AMP-PNP, 0.1 mM $MgCl_2$, and 5 mM Tris (pH 8.0) for several minutes on ice, polymerized by addition of 100 mM KCl, diluted fivefold with 100 mM KCl, and sedimented by centrifugation. The F-actin pellets were homogenized in NH_4OH (pH 12) to release the nucleotide and adjusted to pH 9 with acetic acid to precipitate the denatured protein. The nucleotide content of the actin sample was checked by determining the nucleotide content from the optical density at 258 nm. The ratio of nucleotide to actin monomer was found to be 0.9. The sample was then concentrated on a vacuum evaporator and chromatographed. The results given in Table II show that good separation of AMP-PNP from ATP, ADP, or AMP-PNH_2 can be achieved. The nucleotide isolated from F-actin ran as a single spot with an R_F value identical with that of AMP-PNP. In addition, a mixture of this nucleotide and AMP-PNP ran as a single spot. If the concentration of AMP-PNP in the G-actin medium was lower a second spot appeared which had a R_F value of 0.23. Although ADP and AMP-PNH_2 cannot be distinguished from one another, this second nucleotide is assumed to be ADP. Assuming that the ADP concentration in the reaction mixture was one ADP per actin monomer and assuming that the binding constant of ADP is four times that of AMP-PNP, one can calculate the amount of AMP-PNP expected to be bound to the actin. Although the estimation of nucleotide content by spot intensity is qualitative at best, the results obtained are in rough agreement with those expected. In summary, the results of the polyethyleneimine chromatography show that: (a) AMP-PNP is incorporated into F-actin, (b) that it is not hydrolyzed and, (c) that the amount of binding is in rough agreement with the amount expected from the NSL exchange experiments.

The fact that G-actin·ADP polymerizes more slowly than

TABLE II: R_F Values of Nucleotides.[a]

Nucleotide	R_F[b]
ATP	0.04
ADP	0.21
AMP	0.46
AMP-PNP	0.14
AMP-PNH_2	0.24
Isolated from actin	0.14

[a] Nucleotides were chromatographed on cellulose polyethylenimine. The R_F values are shown for various nucleotides and for nucleotide isolated from F-actin which had been polymerized from G-actin·AMP-PNP. [b] All values ±0.02.

G-actin·ATP has led to the speculation that the energy of dephosphorylation, released during polymerization, may be used to facilitate the polymerization. If this conclusion were true one would expect that G-actin·AMP-PNP would polymerize more slowly than G-actin·ATP, since no energy from dephosphorylation is available in the former case. The rate of polymerization was followed by the method of Higashi and Oosawa (1965), who showed that a change in the optical density of actin occurs at 232 nm when the actin polymerizes. The increase in OD_{232} as a function of time following addition of salt to G-actin·ADP, G-actin·ATP, and G-actin·AMP-PNP is shown in Figure 3. G-actin·ADP, G-actin·AMP-PNP, and G-actin·ATP were obtained by homogenizing a F-actin pellet in 5 mM Tris (pH 8.0), 0.1 mM $MgCl_2$, 0.1 mM dithiothreitol, and 0.5 mM nucleotide. G-actin·ATP which was obtained by addition of 0.2 mM ATP to either of the other samples gave results identical with those obtained by addition of the ATP alone. The data of Figure 3 show that the rate of polymerization of G-actin is the same whether the bound nucleotide is ATP or AMP-PNP, and that both these nucleotides induce a faster rate than does ADP. Thus the faster rate of polymerization seen with G-actin·ATP is not a result of energy which is derived from the splitting of the ATP.

Although the above results have shown that an F-actin·AMP-PNP polymer can be formed, they do not exclude the possibility that this polymer may differ in structure or stability from a polymer containing ADP. To determine the stability of the two types of polymers we used the well-known lability of F-actin to sonication (Asakura *et al.*, 1963). The nucleotide of F-actin, which normally is unexchangeable, becomes exchangeable during sonication, probably due both to breaks in the polymer and to changes in the actin–actin bonds. The rate of nucleotide exchange during sonication was followed by determining the incorporation of the NSL-TP into F-actin polymers.

F-actin was polymerized from G-actin in 1 mM AMP-PNP or ATP, sedimented, resuspended, and dialyzed overnight against 50 mM KCl, 0.5 mM MgCl, 0.1 mM dithiothreitol, and 5 mM Tris (pH 8.0). NSL-TP (5×10^{-5} M) was added and the samples were sonicated. The incorporation of the NSL could be followed by the increase in the low-field peak in the electron paramagnetic resonance spectrum. As shown in Figure 4, the amount of NSL-DP which is bound to the actin increases with increasing length of sonication but does not depend on the nucleotide which was originally incorporated into the polymer. This result shows that the structural lability of the actin polymer to the mechanical forces of sonication does not

depend on whether the bound nucleotide is a diphosphate or a β,γ-imidotriphosphate.

Discussion

When using an analog one must be careful to recognize that the properties of the analog may perturb the system in some unexpected and undesirable way. The specific question of interest here is whether the AMP-PNP is representative of ATP in all ways, with the exception that it cannot be split. This question cannot be completely resolved but some pertinent information is given below.

The properties of AMP-PNP have been studied by Yount *et al.* (1971a). The angles and bond lengths of the PNP bond are very close to those of the POP bond. It was also found that AMP-PNP is capable of binding both Mg^{2+} and Ca^{2+} but with affinities about twice those of ATP. One difference found between ATP and AMP-PNP is that the ionization of the terminal phosphate occurs at 7.1 in the former and 7.9 in the latter. Because of this difference, these studies were performed at pH 8.0, where the majority of the AMP-PNP will carry the same charge as ATP.

A number of enzymatic studies have been made using AMP-PNP. It has been shown that it binds to myosin with an affinity constant close to the K_m of the myosin ATPase and that it is not cleaved (Yount *et al.*, 1971b). AMP-PNP will also dissociate actomyosin in 0.6 M KCl (Yount *et al.*, 1971b). Glycerinated muscle fibers can be relaxed with AMP-PNP in a way similar to ATP (dos Remedios *et al.*, 1972). AMP-PNP is cleaved by some enzymes which are not specific for the β,γ-phosphate linkage, *i.e.*, by snake venom phosphodiesterase. *E. coli* alkaline phosphatase is the only enzyme found which cleaves the β,γ bond of AMP-PNP (Yount *et al.*, 1971a). However, AMP-PNP has been found not to replace ATP on phosphoenolpyruvate synthetase, an enzyme which attacks the α,β-phosphate link of ATP (Berman and Cohn, 1970). In general, the studies which have been made indicate that AMP-PNP will bind to ATP sites and that it cannot be cleaved by enzymes which hydrolyze only the β,γ-phosphate linkage.

The available evidence indicates that AMP-PNP binds to G-actin at the nucleotide site. When actin was incubated with AMP-PNP, analysis of the nucleotide content of the resulting F-actin showed that within experimental error there was one nucleotide per actin monomer, and PEI chromatography showed that this nucleotide was AMP-PNP. Thus the AMP-PNP has displaced the ADP or ATP which was originally on the G-actin, and has bound with the expected stoichiometry.

Since ATP binds to G-actin without hydrolysis and is also easily exchangeable, it is thought that the binding does not involve any intermediates. In support of this conclusion, the optical rotatory dispersion spectrum of G-actin·ATP and G-actin·ADP are superimposable indicating that little change in the actin conformation occurs upon changing ATP to ADP (Nagy and Jencks, 1963). If in fact the interaction of actin and ATP involves only a simple binding without attendant conformation changes in either the actin or nucleotide it is surprising that AMP-PNP binds so weakly. Three methods, the NSL exchange, the protection against denaturation, and the analysis of the nucleotide content on polyethyleneimine plates, all indicate that AMP-PNP binds with an affinity which is less than that of ADP and thus is about 100–200 times weaker than ATP. Other analogs with substituted phosphate linkages which do not hydrolyze (AMP-PCP) or hydrolyze slowly (AMP-CPP) bind even less strongly. This is an indication that the binding of ATP to G-actin requires a phosphate configuration which these analogs cannot assume easily. However, once bound to the actin the AMP-PNP is capable of inducing the same rate of polymerization as ATP, indicating that with respect to this process the AMP-PNP does bind in a manner similar to ATP. The weak binding of AMP-PNP remains unexplained but may indicate that the binding of ATP to G-actin involves an interaction more complex than simple binding.

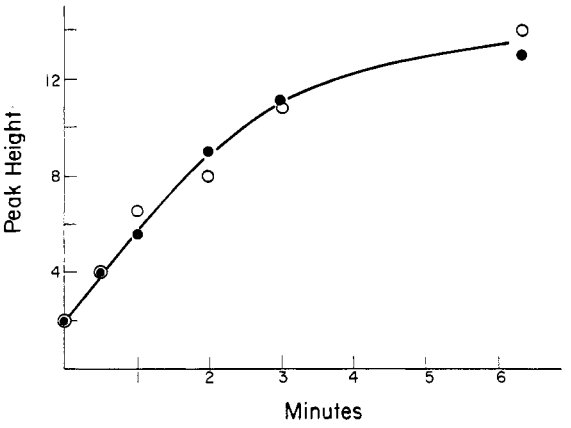

FIGURE 4: The stability of the F-actin polymer during sonication was measured by the rate of incorporation of NSL-TP into the polymer. The solution contained 50 mM KCl, 0.5 mM $MgCl_2$, 5×10^{-5} M NSL-TP, 5 mM Tris (pH 8.0), and 1 mg/ml of either F-actin with bound ADP (●) or F-actin with bound AMP-PNP (○). The exchange of the bound nucleotide with the external NSL-TP was followed by the increase in the height of the low-field peak in the electron paramagnetic resonance spectrum of the NSL-DP. This peak height is plotted as a function of the length of time the polymer had been sonicated.

When G-actin is polymerized the bound ATP is dephosphorylated releasing about 7 kcal/mol of potentially useful energy. One use to which this energy may be put has been suggested by studies which show that G-actin·ATP polymerizes more rapidly than does G-actin·ADP. Thus the energy of dephosphorylation could be used simply to stabilize the actin polymer. This stabilization could play a part in the construction of an actin polymer in a growing muscle fiber, or it could be part of a repair mechanism which would operate to "reconnect" breaks in the actin polymer occuring during the normal use of a muscle. This latter role would explain the slow exchange of actin nucleotide which occurs in a working fiber. However, the present results indicate that the energy of dephosphorylation plays no role in the actin polymerization, since the kinetics of actin polymerization are unchanged by substitution of an ATP analog which is not dephosphorylated. It should be noted that this conclusion is independent of the questions raised earlier concerning the properties of the analog. The faster polymerization of G-actin·ATP over G-actin·ADP may be due to a passive role of the additional negative charge of ATP.

None of the properties of the actin polymer which were studied were changed by substitution of AMP-PNP for ADP. The viscosity of F-actin·AMP-PNP was found to be the same as that of F-actin·ADP. The change in OD_{232} was also found to be approximately the same for the samples polymerized from G-actin·ATP and G-actin·AMP-PNP. The small difference in the total OD change seen in Figure 3 can probably be attributed to the slower polymerization of some G-actin·ADP in the G-actin·AMP-PNP sample. In addition, the sonication data have shown that the stability of the polymer is also not

changed by this substitution. Thus the stability of the polymer to mechanical stress is not enhanced by the energy of dephosphorylation. This conclusion is not surprising since the literature contains no results which imply that F-actin obtained from G-actin·ADP is different from that obtained from G-actin·ATP. However, the sonication data also show that the stability of the polymer is unchanged when the nucleotide remains unhydrolyzed.

In summary, the present studies show that neither the rate of actin polymerization nor the stability of the resulting polymer is coupled to the dephosphorylation of the actin nucleotide that occurs during the polymerization process. We were unable to detect any difference in the properties of F-actin when the bound ADP was substituted by AMP-PNP. Thus, although the nucleotide dephosphorylation occurs during polymerization, there now seems to be no evidence that it plays any active role in regulating or facilitating the process of the polymerization or in effecting the properties of the resulting polymer. The data do not, unfortunately, point to a possible role for the actin nucleotide, and further studies are underway to investigate the interaction of F-actin·AMP-PNP with myosin.

Acknowledgment

We would like to thank Dr. Ralph Yount for providing some samples of AMP-PNP and for many encouraging and enlightening discussions. We would also like to thank Dr. Manuel Morales for his invaluable help and advice.

References

Asakura, S. (1961), *Arch. Biochem. Biophys. 92*, 140.
Asakura, S., Taniguchi, M., and Oosawa, R. (1963), *Biochim. Biophys. Acta 73*, 140.
Barany, M., Tucci, A. F., and Conover, T. W. (1966), *J. Mol. Biol. 19*, 483.
Berman, K., and Cohn, M. (1970), *J. Biol. Chem. 245*, 5319.
Cooke, R., and Duke, J. (1971), *J. Biol. Chem. 246*, 6360.
dos Remedios, C. G., Yount, R. G., and Morales, M. F. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 2542.
Higashi, S., and Oosawa, F. (1965), *J. Mol. Biol. 12*, 843.
Hayashi, T., and Rosenbluth, R. (1960), *Biol. Bull. 119*, 290.
Moos, C., Eisenberg, E., and Estes, J. E. (1967), *Biochim. Biophys. Acta 147*, 536.
Nagy, B., and Jencks, W. (1963), *Biochemistry 1*, 987.
Oosawa, F., and Kasai, M. (1971), *in* Subunits in Biological Systems, A, Timashoff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 261.
Seidel, D., Chak, D., and Weber, H. (1967), *Biochim. Biophys. Acta 140*, 93.
Spudich, J. A., and Watt, S. (1971), *J. Biol. Chem. 246*, 4866.
Szent-Gyorgyi, A. G., and Prior, G. (1966), *J. Mol. Biol. 15*, 515.
Yount, R. G., Babcock, D., Ballantyne, W., and Ojala, D. (1971a), *Biochemistry 10*, 2484.
Yount, R. G., Babcock, D., and Ojala, D. (1971b), *Biochemistry 10*, 2490.

Kinetics and Thermodynamics of the Formation of Glucose Arsenate. Reaction of Glucose Arsenate with Phosphoglucomutase†

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ABSTRACT: The reaction of glucose and arsenate in aqueous solution at neutral pH and room temperature produces an equilibrium mixture containing small amounts of glucose 6-arsenate and a smaller amount of the 1-arsenate as well. Under these conditions the thermodynamic stability of the 6-arsenate is essentially the same as that of glucose 6-phosphate, although the approach to equilibrium is more rapid by several orders of magnitude: $k_{form} = 1.4 \times 10^{-6}$ M^{-1} sec^{-1}; $k_{hydrol} = 4.2 \times 10^{-4}$ sec^{-1}. K_{form} thus is about 3.4×10^{-3} M. The glucose arsenates are measured by means of their rapid stoichiometric reaction with ^{32}P-labeled phosphoglucomutase to produce glucose [^{32}P]phosphates. The criteria for selection of enzyme systems to assay other arsenate esters *in situ* are discussed.

Arsenate is isosteric and isoelectronic with phosphate (Sisler, 1956) and can enter into enzyme-catalyzed reactions in its place (Braunstein, 1931; Harden, 1932; Warburg and Christian, 1939; Doudoroff *et al.*, 1947; Katz *et al.*, 1948). Although *both* acyl and glycosyl phosphates and arsenates are thermodynamically unstable at neutral pH and room temperature, the arsenates appear to differ from their phosphate analogs in that they hydrolyze much more rapidly. Hence, in enzymatic reactions which involve acyl or glycosyl transfer to inorganic phosphate, substitution of arsenate for phosphate can give rise to abortive hydrolysis products. Transient formation of acyl and glycosyl arsenates in such enzymatic reactions has been demonstrated by ^{18}O tracer studies (Slocum and Varner, 1960) but no information has been available on the rate and thermodynamics of their hydrolysis or formation.

Lagunas and Sols (1968) first proposed that the hydroxymethyl group of compounds such as glucose, fructose, fruc-

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§ National Institutes of Health Career Development awardee.