

# Stabilization of Actin Filaments by ATP and Inorganic Phosphate

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Both inorganic orthophosphate and ATP stabilize actin filaments. This is reflected by a reduced nucleotide exchange and by a protection against filament breakdown by SDS or KI. When the filament-stabilizing effect of ATP was maximal, only about 15% of the actin subunits of the filament had bound one molecule of the nucleotide offered in the stabilizing solution.

## Introduction

Actin is a protein of the motile and cytoskeletal apparatus of all eukaryotic cells. In muscle cells it is the main constituent of the thin filaments. At low ion concentrations actin is monomeric (G-actin), upon raising ion concentration (to 50–100 mM KCl and/or 1–2 mM MgCl<sub>2</sub>), actin assembles into long actin filaments. Conversely, after lowering ion concentration F-actin can depolymerize into G-actin (see [1] for a general review of actin properties). In non-muscle cells actin can, despite a constant high ion concentration, undergo frequent changes between the monomeric and the polymeric state. These changes are controlled by specific actin-binding proteins in a way which is only poorly understood [2]. It is this dynamic behaviour of actin in non-muscle cells which has stimulated a most vivid research on actin polymerization.

One of the most fascinating aspects of actin polymerization is the hydrolysis of G-actin-bound ATP during the course of polymerization (the current view on the mechanism of the ATP hydrolysis associated with actin-polymerization is reviewed by Korn *et al.* [3]). The state of the actin-bound nucleotide quite obviously influences the stability of the actin

filament. At least the monomer concentration, which, during steady state, coexists with the filaments ("critical concentration") is lower in the presence of ATP than of in the presence of ADP suggesting that the apparent affinity of actin subunits for the filament ends is higher for ATP-actin than for ADP-actin. Since inorganic phosphate is generated during ATP hydrolysis, it can be expected that Pi might also influence filament stability. That phosphate compounds like ATP or Pi stabilize actin filaments has already been shown by Nonomura *et al.* [4]. Wendel and Dancker [5] observed that actin depolymerization is slower in phosphate buffer than in Tris-HCl-buffer. Rickard and Sheterline [6], Wanger and Wegner [7], Korn *et al.* [3], Carlier and Pantaloni [8], observed that the relatively high critical concentration of ADP-actin is lowered by Pi and assume that this is due to a generation of an ADP-Pi-actin state. The present paper addresses this problem by showing that phosphate compounds not only stabilize actin filaments against structure-disturbing compounds like iodide (I<sup>-</sup>) and sodium dodecyl sulfate (SDS) but also reduce the accessibility of nucleotide binding sites of the filament.

## Methods

Actin was prepared after Spudich and Watt [9] as described [5]. The coupling of N-(3-pyren)maleimide (from Serva, Heidelberg, F.R.G.) to Cys 374 of actin was essentially as described by Kouyama and Mihashi [10] (*cf.* [5]). If desired, actin was freed from unbound ATP by passing it through Dowex 1 × 8, 200–400 mesh (from Serva, Heidelberg, F.R.G.).

**Abbreviations:** AMP-PNP, adenosine-5'-[ $\beta,\gamma$ -imido]triphosphate; Pi, inorganic phosphate; PPi, inorganic diphosphate (pyrophosphate); SDS, sodium dodecyl sulfate.

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### Measurement of polymerization and depolymerization

G-Actin was mixed with 10% of pyrene-labelled actin. Polymerization or depolymerization (in the presence of sodium dodecylsulfate) was measured by following fluorescence intensity in a Shimadzu RF-520 dual beam fluorescence spectrophotometer (see [5]) at 22 °C. Excitation and emission wavelength were 365 nm and 410 nm, respectively. Alternatively, polymerization was measured in the same instrument by monitoring light scattering intensity with both monochromators at 400 nm.

### Viscosity measurements

Viscosity was measured in an Ostwald type viscosimeter with a spirally formed capillary (outflow time of water as 20 s) or an Ostwald viscosimeter with a straight capillary (outflow time of water was 80 s).

### Formation of "paracrystals"

Actin aggregation at 50 mM  $\text{MgCl}_2$  was monitored by the increase in turbidity at 400 nm.

### Binding of ATP to actin

A solution of [ $^{14}\text{C}$ ]ATP was diluted with "cold" ATP to such an extent that 1 nmol gave about 2000 cpm in a Tri-Carb-Packard-Scintillation counter, 4 ml assays were prepared with 2 mg/ml F-actin, 2 mM Tris-HCl pH 8.0, 1 mM  $\text{MgCl}_2$ , 0.3 M KI and variable concentrations of ATP. After 60 min incubation the assays were centrifuged for 2 h at 40,000 rpm in a Beckman 50 Ti rotor. The volume of the actin pellet was deduced from the weight difference of the empty and the pellet-containing centrifuge tubes. The protein content of the pellet was determined by subtracting from the total amount of added actin the amount of protein which was in the supernatant (determined by the method of Bradford [11]). After weighing the pellet was dissolved in 1 ml concentrated formic acid and was counted in 20 ml Rotiszint<sup>R</sup> (Roth, Karlsruhe, F.R.G.). From the total amount of pellet ATP the amount of free ATP that was included in the pellet volume (*i.e.* pellet volume  $\times$  supernatant) concentration was subtracted. For the accompanying viscosity measurements identical parallel assays were prepared.

### Protein concentration

Actin concentration was determined by using an extinction coefficient (at 290 nm) for 1 mg/ml solution of 0.62.

## Results

### The stabilizing action of ATP

During polymerization G-actin-ATP is converted to F-actin-ADP, the ADP of which exchanges with free nucleotides only very slowly (Martonosi *et al.* [12], Dancker and Fischer [13]). The stability of the filament should be independent of free nucleotides since the nucleotide binding sites are "occluded" in the filament. However, as can be seen from Fig. 1, added ATP has a pronounced effect on the stability of actin filaments. In these experiments we used KI to perturb filament structure. Actin, in the absence of free ATP, had under these conditions a very low viscosity, but with rising ATP concentration the filament-disturbing effect of 0.3 M KI disappeared. Since the effective ATP concentration range was roughly stoichiometric to actin (which was 45  $\mu\text{M}$ ), one might suspect that every actin subunit will bind one molecule of ATP. In fact, ATP binding closely paralleled the increase in viscosity. Binding however was by far substoichiometric: only about 3  $\mu\text{mol}$  of ATP were bound per mg of actin or about 1 molecule of ATP per 7 actin subunits. This low amount of ATP bound to the filaments is not due to the presence of the iodide ion: in the absence of KI ATP binding to F-actin was not higher (Table I). Table I further demonstrates the influence of the ATP analogue AMP-PNP on ATP-binding. This analogue can be expected to be a competitor of ATP. Table I shows an experiment in which AMP-PNP reduced ATP binding only when added before ATP but not when added after ATP. This suggests that, when a free binding site is filled with either ATP or AMP-PNP, it is no longer accessible to the competitor, in other words: binding of the ligand "closes" the binding site. Still another phenomenon, which is influenced by ATP, is reflected in Fig. 1: at higher  $\text{Mg}^{2+}$  concentrations (above 10 mM) actin filaments form sheets of parallelly aligned filaments ("paracrystals") (Hanson [14], Strzelecka-Golaszewska *et al.* [15]) which lead to a turbidity increase and eventually to precipitation of actin. In Fig. 1 turbidity increase is plotted against ATP concentration. The observation

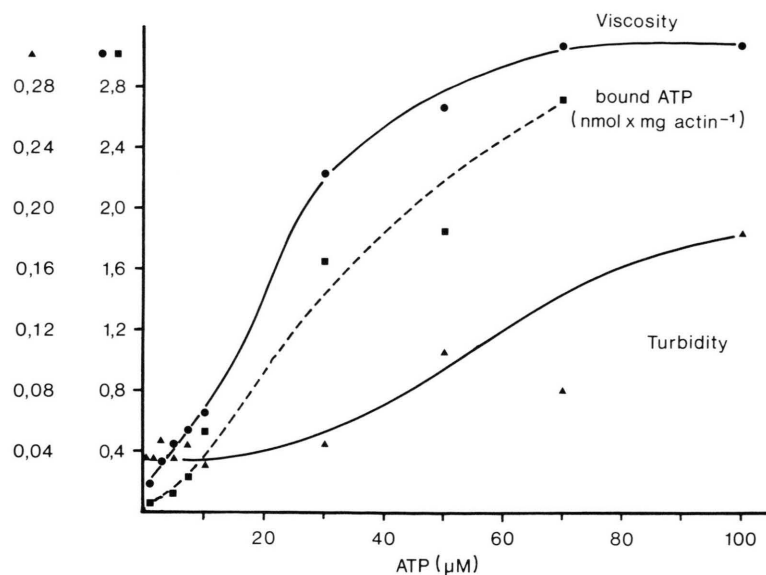


Fig. 1. Suppression by ATP of the destabilizing action of KI on actin filaments. — Conditions: 2 mg/ml F-actin (from Dowex-treated actin), 1 mM (for ATP binding and viscosity) or 50 mM (for turbidity measurements)  $\text{MgCl}_2$ , 5 mM Tris-HCl, pH 8.0, 0.3 M KI and ATP concentrations as indicated at the abscissa.  $[^{14}\text{C}]\text{ATP}$  was used for ATP binding. Each assay was prepared twice: one for measuring ATP binding, the other one for viscosimetry. Actin was added as the last component. After 60 min incubation the assays were either centrifuged or transferred to the viscosimeter. At the end of the viscosity measurement  $\text{MgCl}_2$  was added to a final concentration of 50 mM and turbidity was measured after 15 min. Viscosity is presented as specific viscosity  $\eta_{\text{spec}} = (t/t_0) - 1$  where  $t_0$  and  $t$  are the outflow times of the solvent without and with actin, respectively, turbidity is measured as optical density (extinction).

that turbidity appeared in the presence of KI only with increasing ATP concentration, means that KI inhibits paracrystal formation and that this inhibition can be counteracted by ATP.

#### The influence of inorganic phosphates

As has already been shown by Nonomura *et al.* [4] not only ATP but also inorganic orthophosphate (Pi) favours the formation of straight filaments (*cf.* also Rickard and Sheterline [18]).

The stabilization by phosphates against KI is shown in Fig. 2: whereas the initial viscosity (without

KI) was slightly higher in the presence of phosphate compounds, only inorganic orthophosphate (Pi) markedly reduced the structure-disturbing effect of KI. The dependence on the concentration of phosphate compounds of the effects seen in Fig. 2A are shown in Fig. 2B. Here it can be seen that the effects were nearly maximal at about 10 mM.

The polymer state of actin can also be probed by investigating the exchange of actin-bound nucleotide against free nucleotide (see [13]). The nucleotide of G-actin is readily exchangeable with that of the medium whereas the exchange of F-actin-bound nu-

Table I. Influence of the ATP analogue AMP-PNP on ATP binding to F-actin. — ATP binding was measured as described in the Method section. AMP-PNP was added either 30 min before or after the addition of ATP. Actin was 5 mg/ml.

	no AMP-PNP	1 mM AMP-PNP added prior to the addition of 0.1 mM ATP	1 mM AMP-PNP added after the addition of 0.1 mM ATP
bound ATP (mol/mol actin)	0.1	0.03	0.10

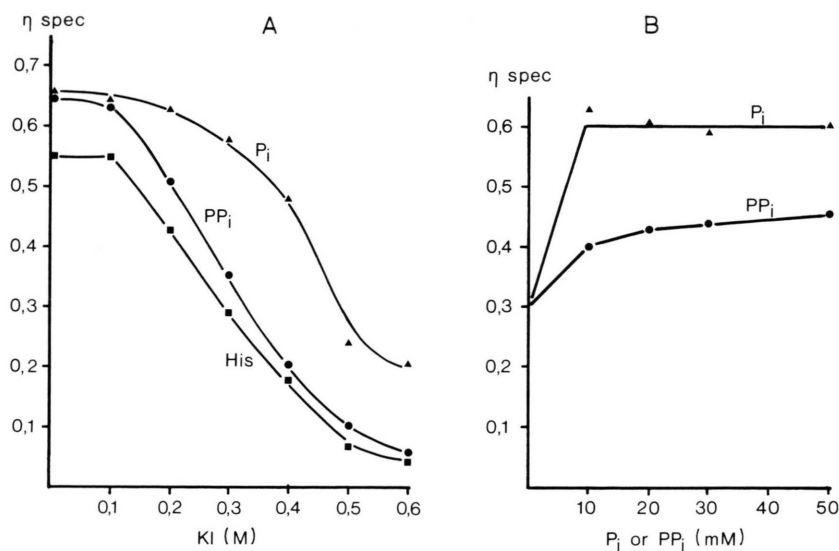


Fig. 2. Influence of phosphates on the specific viscosity of F-actin in the presence of KI. — 1 mg/ml F-actin (from Dowex-treated actin) was in 1 mM  $\text{MgCl}_2$  and the conditions specified below. A: Actin in different buffers, pH 7.0 (histidine, potassium orthophosphate or sodium pyrophosphate, 10 mM each), was treated with the KI concentrations indicated at the abscissa. B: The viscosity of F-actin in 5 mM histidine, pH 7.0, 0.3 M KI was measured in the presence of the concentrations of either potassium phosphate or sodium pyrophosphate indicated at the abscissa.

cleotide is very slow [12, 13]. The slow nucleotide exchange of the filaments quite obviously reflects the stability of actin filaments. Fig. 3 shows that the slow nucleotide exchange was much slower in 10 mM  $P_i$  than in 10 mM Tris-HCl.

In order to see if there is any correlation between stabilization and polymerization we compared the kinetics of polymerization in different buffers. Fig. 4 shows that polymerization was slower in phosphate than in histidine. This is probably due to the fact that

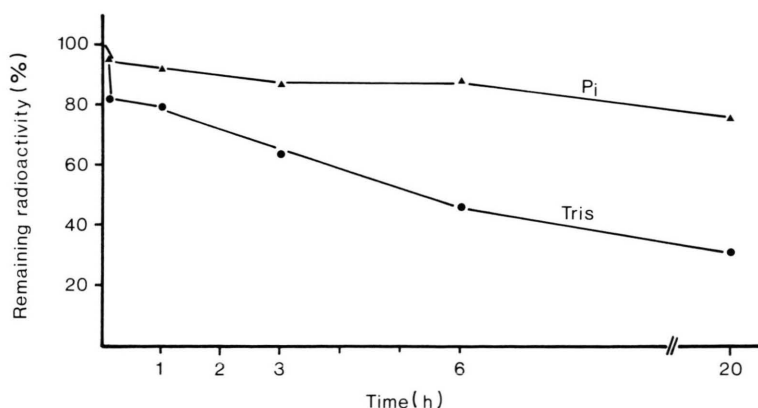


Fig. 3. Influence of inorganic orthophosphate on nucleotide exchange of F-actin. — G-actin was incubated with 0.1 mM  $[^{14}\text{C}]$ ATP, passed through a Dowex column to remove unbound radioactive ATP and then polymerized with 2 mM  $\text{MgCl}_2$ . After polymerization F-actin was incubated with 2 mM non-radioactive ATP either in 5 mM Tris-HCl, pH 8.0 or 10 mM phosphate, pH 8.0. At the time points indicated in the curves 4 ml aliquots were removed, brought to 0.1 M KCl and centrifuged for 2 h at 40000 rpm in a Beckman 50 Ti rotor and the radioactivity of the pellets was determined. The remaining radioactivity in the pellet is proportional to the amount of ADP (derived from ATP during polymerization) which was not displaced by the chasing ATP.

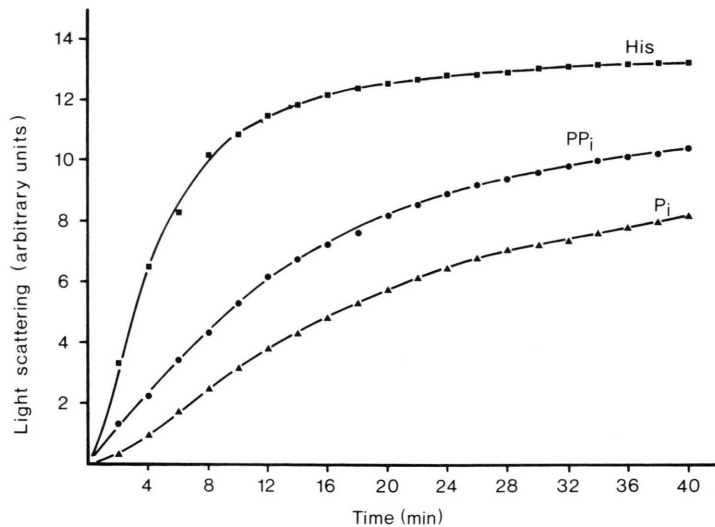


Fig. 4. Polymerization of actin in the presence of phosphate compounds. — 0.6 mg/ml G-actin were polymerized in 2 mM  $\text{MgCl}_2$ , 20  $\mu\text{M}$  ATP and 10 mM of the buffers (pH 7.0) indicated. Polymerization was monitored by light scattering.

10 mM phosphate lowers free Mg concentration from 1 mM to about 0.3 mM. Pyrophosphate, however, lowers free Mg concentration even more, but nevertheless polymerization was faster in pyrophosphate than in phosphate. Taken together these experiments show that the stabilizing action of phosphate cannot be explained in terms of an elevated polymerization velocity. Thus, the elevated filament stability in the presence of structure-perturbing agents should be due to a lower breakdown velocity. In order to address this question we used the ionic detergent sodium dodecyl sulfate (SDS) rather than KI because the latter interferes (by formation of schlieren or by fluorescence quenching) with the optical methods commonly used to probe the filamentous state of actin. For the experiment shown in Fig. 5 actin was polymerized overnight at 1 mM  $\text{MgCl}_2$  and at 10 mM of different buffers. As in the experiment of Fig. 4 there was a small difference in the intensity parameter (in this case fluorescence) of the polymerized actin being highest in histidine and lowest in phosphate. Immediately after the addition of 3 mM SDS there was a fast drop in fluorescence followed by a slower decrease. As can be seen the decrease was fastest in histidine and slowest in phosphate. After 4 h the same final values were attained by all three probes. This result is consistent with the observations of Wendel and Dancker [5], Rickard

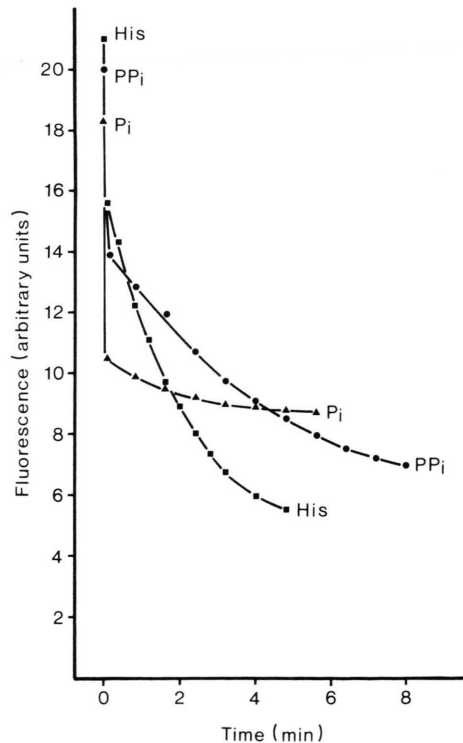


Fig. 5. Disintegration by SDS of F-actin in the presence of phosphates. — 0.5 mg/ml of pyrene-F-actin were dissolved in 10 mM histidine, potassium phosphate or sodium pyrophosphate, pH 7.0 each. Disintegration was initiated by the addition of SDS to a final concentration of 3 mM.



and Sheterline [6] who found that after dilution actin depolymerized more slowly in the presence of phosphate than in its absence. These observations were interpreted as being due to a decrease in the rate constant  $k_-$  of depolymerization.

The experiments reported so far relate concentrations of phosphate to their effects but they give no information about phosphate binding to actin. The amount of Pi actually bound is difficult to determine since the affinity of Pi for actin filaments is relatively low. Half-maximal effects are in the range of 5 mM (see Fig. 2B, *cf.* also Wanger and Wegner [7]). Carlier and Pantaloni [8] report a dissociation constant of 1.5 mM at pH 7.0. Therefore, when actin is saturated with phosphate the concentration of free phosphate is much higher than that of actin so that a given volume of actin solution (and this holds true also for a F-actin pellet after centrifugation) contains much more free phosphate than actin-bound phosphate. Nonetheless Carlier and Pantaloni [8] report an 1:1 stoichiometry of phosphate binding. However, one cannot see from their paper how they allowed for the high background of free phosphate which must have been included in the pellets after F-actin centrifugation. Note that in the case of ATP we found a much less than stoichiometric binding sufficient for filament stabilization (Fig. 1).

## Discussion

The results of the present study show that ATP and inorganic phosphate markedly stabilize actin filament structure against the perturbing action of KI and SDS. We do not know by which mechanism filaments break down under the influence of the perturbing agents. They may either disaggregate at many points along their whole length or they may depolymerize from their ends. The curves of Fig. 5 remind very much of depolymerization curves (*cf.* [5]), so that, at least in the case of SDS, one is tempted to assume that genuine depolymerization is induced. On the other hand the observation of Nonomura *et al.* [4] that actin filaments are longer and straighter in the presence of phosphates than in their absence is more easily explained by the first mechanism as is the observation that ATP favors  $Mg^{2+}$ -induced filament aggregation in the presence of KI. Depending on which model is valid phosphate would either stabilize actin bonds over the entire filament length or would stabilize only filament ends.

A most remarkable fact is that phosphates (inorganic phosphate or nucleotides) not only stabilize actin-actin-interactions but also make the nucleotide binding sites inaccessible. In other words: binding of phosphate compounds reduced the displacement of bound nucleotides by free nucleotides (Table I and Fig. 3). This can be interpreted in either of two ways:

1. Only in monomeric actin are the nucleotide and phosphate binding sites accessible. For binding and exchange of bound for free nucleotide to occur monomers must first leave the filaments. The dissociation of monomers from the filament ends can be prevented by nucleotide and/or phosphate.

2. For binding and nucleotide exchange to occur some binding sites in the filament must be more accessible than the rest and may be empty. Related with these "open sites" putative "breaking" points of the filament may exist. Filling of these sites may "close" these sites and as a consequence may stabilize the filament, thereby reducing nucleotide exchange. Both models would predict a rather low number of binding sites in accordance with our results (*cf.* however Carlier and Pantaloni [8]) and would further predict that phosphate or ATP would reduce the number of accessible binding sites.

The first of the two just mentioned interpretations is consistent with current thinking on actin polymerization. During polymerization-associated ATP hydrolysis actin-ADP-Pi is a distinct intermediate which is different from both ATP-actin and ADP-actin (Carlier and Pantaloni [16, 8]; Carlier [17]). Since monomers enter the filaments at the filament ends, there are "caps" of ATP- and ADP-Pi-subunits at these ends as opposed to the ADP-subunits of the filament core. There is evidence that these caps stabilize the filament ends to that loss of subunits from the filament is prevented (*cf.* Korn *et al.* [3] for a review). It is tempting to speculate that addition of Pi partially reverses the reaction chain of ATP hydrolysis by generating the ADP-Pi-intermediate [6, 7, 17, 18]. The stabilizing action of ATP could also be accommodated to this model, since ATP could substitute for ADP at the filament ends. But why is there stabilization by Pi even in the presence of ATP (as indicated by the reduced displacement of bound nucleotide by free ATP, *cf.* Fig. 3)? Is it easier to complete a labile ADP cap with Pi than to exchange ADP for ATP? And can we exclude that KI or SDS disaggregate actin filaments at many points, so that interpretation (2) is valid? Or does Pi act quite differ-

ently in a way that cannot be explained by the generation of an ATPase intermediate?

At any rate, the experiments of this paper show that the specific role of Pi (as compared to, *e.g.*, PPi) is far from being completely understood.

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