The rate constant for ATP hydrolysis by polymerized actin

Thomas D. Pollard and Alan G. Weeds*

MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England

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Polymerized actin hydrolyzes bound ATP in a reaction that depends on the concentration of polymerized ATP-actin, not on the rate of incorporation of ATP-actin into the polymer. The apparent first order rate constant is about 0.07 s^{-1} at 21°C in 50 mM KCl with 1 mM $MgCl_2$ or $CaCl_2$.

Actin *ATPase* *Polymerization*

1. INTRODUCTION

During the polymerization of actin the ATP bound to the protein is hydrolyzed, releasing one inorganic phosphate for each molecule incorporated into filaments (review [1]). ADP remains bound to the actin molecules in the filament and does not exchange with ATP in the medium. Usually the time course of phosphate release follows the time course of polymerization rather closely (see, e.g., [2]), suggesting that hydrolysis is closely coupled to incorporation of actin into filaments. Most models for the mechanism of polymerization have assumed that hydrolysis occurs on the terminal subunit [1,3]. On the other hand, three different experiments suggest that ATP hydrolysis actually occurs at some time after an ATP-actin monomer binds to a filament: (i) In 0.1 M KCl without added divalent cations the time course of ATP hydrolysis lags behind polymerization for both muscle and *Dictyostelium* actin [4]. (ii) At steady-state in 0.5 mM $MgCl_2$ the rate of subunit exchange at the ends of actin filaments is higher than the rate of ATP hydrolysis [2]. (iii) The rate constant for the dissociation of ADP-actin from the ends of filaments is larger than that calculated from the elongation rate of ATP-actin, suggesting together with other data that ATP-actin occupies both ends of an actively growing filament [5]. Similarly for microtubules, the hydrolysis of GTP bound to tubulin can lag behind assembly [6], and steady-state subunit exchange can exceed the rate of GTP hydrolysis [7].

On theoretical grounds the hydrolysis of ATP by polymerized actin was thought to be a first order reaction [8]. Given the large second order rate constant (10^7 $M^{-1} \cdot s^{-1}$) [9] for elongation of actin filaments, it was possible, in principle, to select conditions where ATP hydrolysis is cleanly separated from polymerization, providing that the ATPase rate constant was < 1 s^{-1}. This has proven to be true. Even at relatively low concentrations of nuclei and actin monomers, there is a lag of phosphate release relative to polymerization that is compatible with a first order reaction mechanism where the ATPase rate depends solely on the concentration of polymerized actin with bound ATP.

2. MATERIALS AND METHODS

2.1. *Materials*

Chemicals were obtained from the following sources: BDH (imidazole, sodium azide); Calbiochem (dithiothreitol, ATP); Molecular Probes (pyrene iodoacetamide); and Sigma (EGTA). Sephadex G-150 was from Pharmacia Fine Chemicals. Radioactive ATP was from Amersham International plc.

* To whom correspondence should be addressed

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2.2. *Preparation of actin*

Rabbit muscle actin was prepared [10] and 10% labeled with pyrene-iodoacetamide [11] by reaction of 2 mol of the dye per mol polymerized actin for 16 h at 4°C. After depolymerization by dialysis against buffer G (2 mM imidazole, pH 7.5, 0.5 mM dithiothreitol, 0.2 mM $CaCl_2$, 0.2 mM ATP, 1 mM NaN_3) the labeled and unlabeled actins were recombined, clarified by centrifugation and further purified by gel filtration on Sephadex G-150 in buffer G. The samples of monomers used in these experiments had a concentration of 50 μM actin and 5 μM covalently bound pyrene. A 50 μM solution of labeled monomer in buffer G was equilibrated with [γ-^{32}P]ATP by adding 4 × 10^6 cpm·ml^{-1} of radioactive ATP (3 Ci·μmol^{-1}) and incubating for at least 24 h at 4°C.

2.3. *Polymerization and phosphate assays*

Nucleated polymerization was carried out at 20.8°C by mixing, in order, water, buffer G, 10× polymerization buffer (usually 500 mM KCl, 10 mM EGTA, 10 mM $MgCl_2$, 100 mM imidazole, pH 7), 50 μM polymerized, unlabeled actin, and 50 μM actin monomers labeled with both pyrene and [γ-^{32}P]ATP to give final concentrations of 10 μM monomers, 10 μM filaments, 50 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.1 mM ATP, 0.25 mM dithiothreitol and 10 mM imidazole, pH 7 (KCl–Mg–EGTA buffer). The sample was vortexed gently for about 0.5 s and 0.35 ml were transferred with minimal shearing to a thermostated fluorimeter cuvette. Actin polymer concentration was measured by fluorescence enhancement [11] using a Perkin-Elmer Model MPF-3 spectrofluorimeter with excitation at 365 nm and emission at 384 nm. Data collection was usually initiated within 8 s of the addition of the monomers to the sample. Spontaneous polymerization of actin monomers was carried out in the same way except that the polymerized actin was omitted. In the KCl–Mg–EGTA buffer the critical concentration for polymerization was 0.1 μM by both steady-state and elongation rate methods [12]. In the KCl–Ca buffer (as above, but with 1 mM $CaCl_2$ substituted for the $MgCl_2$) the critical concentration was 0.3 μM. The steady-state ATPase activity of G-actin monomers was measured in buffer G and of polymerized actin in KCl–$MgCl_2$–EGTA, both without added unlabeled ATP. The reactions were terminated by pipetting 50-μl samples from the reaction mixture into 50 μl of 4 N H_2SO_4–20 mM silicotungstic acid–4% ammonium molybdate and vortex mixing the sample for 2 s. The phosphomolybdate complex was extracted into 1 ml of 65:35 xylene:isobutanol by vortexing, followed by low-speed centrifugation to separate the phases [13]. A sample of 0.8 ml of the organic phase was counted in 4 ml of Aquasol (Beckman Instruments) in an LS7000 liquid scintillation spectrophotometer (Beckman Instruments).

3. RESULTS

The spontaneous polymerization of actin monomers in a KCl–Mg–EGTA buffer follows a sigmoidal time course with the hydrolysis of bound ATP lagging slightly behind (fig.1). This suggested that ATP hydrolysis might occur subsequent to the incorporation of actin monomers into filaments.

With a high concentration of added nuclei, the time course of polymerization is hyperbolic, while the release of $^{32}P_i$ from bound ATP is sigmoidal and clearly lags behind polymerization (fig.2). Identical results were obtained in 50 mM KCl with 1.1 mM $CaCl_2$. We varied the concentrations of nuclei and monomers to change the speed and amplitude of the reactions and observed the same relation between polymerization and ATP hydrolysis (table 1).

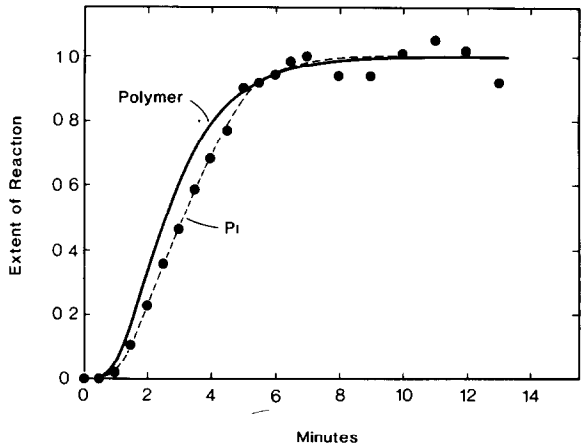

Fig.1. ATP hydrolysis during the spontaneous polymerization of 10 μM actin in KCl–$MgCl_2$–EGTA. Polymer concentration (——) $^{32}P_i$ released from [^{32}P]ATP bound to the actin monomers (●---●).

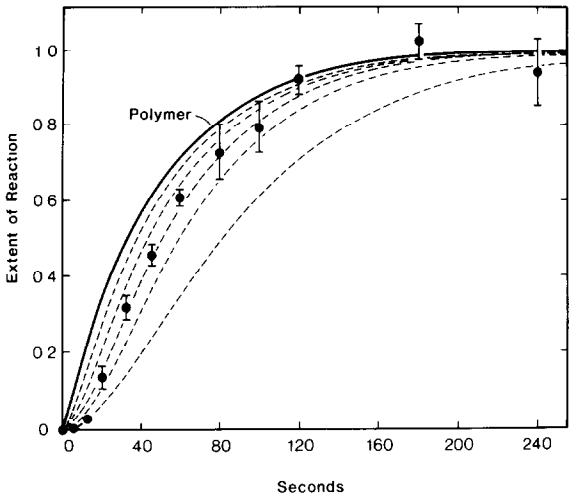

Fig.2. ATP hydrolysis during the nucleated polymerization of actin in KCl–$MgCl_2$–EGTA. Conditions: 10 μM actin monomers, 10 μM polymerized actin. Time course of polymerization (——) calculated from the average k_{app} (= 0.0212 s^{-1}) from 2 separate samples and of $^{32}P_i$ release from [^{32}P]ATP bound to the actin monomers (●). Each point is the mean (± SD) of 4 separate experiments one day. The dashed lines are theoretical plots of the time course of ATP hydrolysis calculated from eq.3 in the text. The values of k_{ATPase} are, from left to right, 0.2, 0.1, 0.06, 0.04 and 0.02 s^{-1}. The data fit the curve with k_{ATPase} = 0.06 better than the other curves. Here and in other experiments the greatest scatter in the values of $^{32}P_i$ were at the end of the polymerization reaction, presumably due to errors in pipetting viscous samples.

For comparison with these transient state experiments, the rate of ATP hydrolysis at steady-state by polymerized 10 μM actin in KCl–Mg–EGTA was 5 × 10^{-10} M·s^{-1} or 5 × 10^{-3} s^{-1} per actin monomer. In buffer G, 10 μM actin had a steady-state ATPase activity of about 10^{-10} M·s^{-1} or 10^{-5} s^{-1} per actin monomer.

These data were analyzed using a simple model for the polymerization and hydrolysis reactions and testing how well it fits the data. With added nuclei, elongation predominates over nucleation, so that the rate of polymerization can be expressed as:

$$dCp/dt = k_+[N]\cdot[A] - k_-[N] \simeq k_+[N]\cdot[A] = k_{app}[A] \quad (1)$$

where Cp is the concentration of polymerized actin, [N] is the number concentration of filaments, [A] is the actin monomer concentration and k_+ and k_- are the association and dissociation rate constants. The apparent rate constant (k_{app}) can be calculated from semi-logarithmic plots (table 1). In our experiments the dissociation reaction, (k_-[N]), can be omitted because the initial association rate per filament (100 s^{-1}) is much larger than the dissociation rate (1 s^{-1}). For modeling ATP hydrolysis, we assumed that: (i) the reaction is first order with respect to the concentration of polymerized ATP-actin; (ii) only polymerized actin splits ATP during the time of these experiments; (iii) the reaction is irreversible; and (iv) there is no

Table 1

Data summary

Experiment	Buffer conditions	A_o (μM)	A_p (μM)	k_{app} (s^{-1})	[N] (nM)	k_{ATPase} (s^{-1})
20-1	KME	10	10	0.0164	1.6	0.04, 0.04, 0.08
24-1	KME	10	10	0.0247	2.5	0.07, 0.07
		5	10	0.0239	2.4	0.07, 0.15
		10	20	0.0409	4.1	0.04, 0.04
27-1	KME	10	10	0.0212	2.1	0.06, 0.06, 0.06, 0.06
	KCl–$CaCl_2$	10	10	0.0212	2.1	0.08, 0.08

A_o is the initial actin monomer concentration. A_p is the initial concentration of polymerized actin. $k_{app} = k_+[N]$ where k_+ is the association rate constant for elongation (10^7 $M^{-1}\cdot s^{-1}$) and [N] is the number concentration of filaments. k_{ATPase} is the first order rate constant for ATP hydrolysis, as defined by eq.3, that gave the best fit for each set of data. KME is KCl–$MgCl_2$–EGTA buffer

exchange of nucleotide bound to the actin molecules in the time of these experiments. Hence:

$$dP_i/dt = k_{ATPase}[A_P^T] \quad (2)$$

where P_i is the concentration of inorganic phosphate released from ATP bound to actin molecules, $[A_P^T]$ the concentration of polymerized ATP-actin and k_{ATPase} the first order rate constant. The time course of phosphate release during a first order nucleated polymerization is:

$$dP_i/dt = k_{ATPase}[A_o](1 - e^{-k_{app}t}) - [P_i(t)] \quad (3)$$

where $[A_o]$ is the initial concentration of actin monomers, and $[P_i(t)]$ the concentration of inorganic phosphate at each time point.

For each experiment, we calculated the average value of k_{app} from the time course of polymerization of 2–4 separate samples and then computed theoretical curves for the time course of ATP hydrolysis over a range of values for k_{ATPase} (fig.2). In all cases the data fit the model very well over the complete time course of polymerization. For example in fig.2, the phosphate data fit best with a k_{ATPase} of 0.06 s^{-1}, and, within experimental error, all of the points fall between the theoretical curves for k_{ATPase} equal to 0.04 and 0.10 s^{-1}. From 15 separate determinations over a range of conditions (table 1) the mean value of k_{ATPase} is 0.07 s^{-1} (SD 0.03 s^{-1}). For comparison, the first order rate constant for GTP hydrolysis by polymerized tubulin is about 0.005 s^{-1} at 37°C [5].

4. DISCUSSION

Our experiments confirm that the hydrolysis of ATP bound to actin molecules is not coupled directly to polymerization. The data are consistent with a simple mechanism of ATP hydrolysis where the reaction is first order in polymerized actin with bound ATP and, at least at the barbed end of the filaments, is independent of the rate of subunit incorporation into the polymer. This rate constant appears to be independent of the number concentration of filaments, although this needs to be tested over a wider range.

Under non-polymerizing conditions, actin monomers have a much lower ATPase activity, about 10^{-5} s^{-1}, so incorporation into filaments increases the ATPase rate by about 7000-fold. We attribute this change in enzymatic activity to conformational changes that accompany the incorporation of actin monomers into filaments. The higher ATPase activity is not due simply to the salt concentrations used to induce polymerization, because the rate of ATP hydrolysis by a polymer sample at steady-state (5×10^{-3} s^{-1} per unpolymerized actin monomer) is an order of magnitude less than the rate of hydrolysis by the actin monomers in the polymerization reaction. Furthermore, as discussed below, much of this steady-state ATPase is probably attributable to the hydrolysis of ATP by actin monomers that bind transiently to the ends of the filaments.

Knowledge of the rates of ATP hydrolysis and polymer elongation allows us to confirm previous arguments [2,4,5] that ATP-actin occupies the barbed end of the filament (and perhaps the pointed end as well) during rapid elongation of the polymer. When the monomer concentration is high, the rate of ATP-actin addition to the barbed end of filaments greatly exceeds the ATPase rate, giving rise to a terminal segment of filament with bound ATP. For example, with 10 μM G-actin, during each second, 100 monomers add to the barbed end of the filament but only 6% of these hydrolyze their ATP. This gives rise to a stretch of several hundred actin subunits that predominantly, but not exclusively, have bound ATP.

At steady-state, most of the actin subunits in a filament have bound ADP, but ATP-actin is still likely to be the major species occupying the barbed end of the filament. Under our conditions at steady-state, the subunit exchange rate at the barbed end (equal to k_-) is about 1 s^{-1}. Since k_{ATPase} is 0.06 s^{-1}, one would expect that for every 16 ATP-actin monomers that bind to the barbed end only 1 will hydrolyze its ATP before dissociating. Qualitatively, this agrees with the analysis of steady-state exchange data in [2].

The steady-state ATPase rate for a sample with 10 μM total actin and 0.1 μM monomer (5×10^{-10} M·s^{-1}) is slightly higher than expected if ATP hydrolysis takes place exclusively on the terminal subunit at the barbed end. With a concentration of barbed ends of about 2 nM in these 10 μM actin filament samples (table 1), the steady-state ATPase should be the product of the concentration of ends and k_{ATPase} (2×10^{-9} M $\times$ 0.06 s^{-1} = 1.2×10^{-10} M·s^{-1}) if ATP hydrolysis takes place only on the terminal subunit. Since the measured

rate is somewhat higher, 1 or more internal subunits at the barbed end may be included in the rapidly exchangeable pool of ATP-subunits and contribute to the steady-state ATPase. Alternatively, the ATPase rate may be higher at the pointed end even though the exchange rate is lower [9].

These new insights into the ATPase activity of polymerized actin molecules raise a number of interesting questions about events at the pointed end of the filament. One expects that the mechanism of ATP hydrolysis might be different there, at least under the conditions where the 2 ends have different critical concentrations.

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