The hydrolysis of ATP that accompanies actin polymerization is essentially irreversible

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The hydrolysis of ATP that accompanies the polymerization of actin occurs on the F-actin subsequent to the addition of the G-ATP-actin subunit to the elongating filament. We now show that this ATP hydrolysis is essentially irreversible. Thus, a large decrease in free energy occurs at the cleavage step, F-ATP-actin → F-ADP-P_i-actin.

1. INTRODUCTION

The hydrolysis of the tightly bound ATP that accompanies actin polymerization [1] occurs subsequent to the addition of ATP-actin subunits to the filaments [2–4] through two distinct, sequential steps [5]: cleavage of F-ATP-actin to F-ADP-P_i-actin followed by the slow release of P_i to form F-ADP-actin. P_i binds to F-ADP-actin as $H_2PO_4^-$ with a stoichiometry of 1 mol/mol F-actin subunit and a K_d of ~0.5 mM [6]. Subunits of ADP-P_i-actin dissociate from filament ends at a much slower rate than ADP-actin subunits [6], and this difference is the basis for the lower critical concentration of F-ADP-actin in buffers containing P_i [6–8].

From these and other observations, the following picture of the polymerization of Mg-ATP-actin [6,9] emerges (review [10]): ATP-actin monomers self-associate into small oligomers that elongate at both ends. The transient F-ADP-P_i-actin filament formed following ATP cleavage is very stable, and P_i release results in a large destabilization of the polymer. At steady state in the presence of ATP, a stable cap of terminal ADP-P_i-actin subunits

maintains the stability (low critical concentration) of the filament. As a consequence of ATP hydrolysis, the critical concentrations at the two ends of the filament are different, even in the presence of saturating concentrations of P_i. A remaining uncertainty about this reaction scheme, the reversibility of the ATP-cleavage reaction on F-actin, is addressed in this paper by three different experimental approaches: (i) intermediate exchange: [18]O exchange between actin-bound [γ-[18]O_3]ATP and H_2O during actin polymerization; (ii) incorporation of [32]P_i into ATP in the presence of F-actin at steady state; and (iii) medium exchange; [18]O exchange between [[18]O_4]P_i and H_2O catalyzed by F-actin at steady state. All three approaches lead to the conclusion that cleavage of ATP on F-actin is very poorly, if at all, reversible, indicating that a large free energy change is associated with the hydrolysis of F-actin-bound ATP.

2. MATERIALS AND METHODS

2.1. Reagents

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described [11], to less than 0.002% ^{32}P impurities. [^{18}O$_4$]P$_i$ (98% enriched) was prepared according to Hackney et al. [12]. [γ-^{18}O$_3$]ATP was synthesized by a modification of the method of Von der Saal et al. [13] using 2 mol P$_i$/mol ADP.

2.2. Actin

Actin was prepared from rabbit skeletal muscle [14,15] and isolated as G-actin (50–60 μM) by a final chromatography step using Sephadex G-200 in buffer G (5 mM Tris-Cl, pH 7.8, 0.2 mM dithiothreitol, 0.1 mM CaCl$_2$, 0.2 mM ATP, 0.01% NaN$_3$). Ca-G-actin was converted to Mg-G-actin by incubation for 3 min at 20°C in the presence of buffer G containing 0.2 mM EGTA and 50 μM MgCl$_2$. Ca- or Mg-G-ATP-actin was polymerized to steady state by addition of 1–2 mM MgCl$_2$, 0.1 M KCl, or both to buffer G. Phosphate, when present, was added from a 500 mM stock solution at the desired pH. For studies with F-actin in ADP, unbound ATP was removed from the solution of G-ATP-actin by Dowes-1X8 [16], the G-ATP-actin was polymerized by addition of MgCl$_2$ to 1 mM, and any residual ATP was consumed by repeated sonication [4]. The solution was then supplemented with 0.2 mM ADP and 10 μM diadenosine pentaphosphate, to prevent synthesis of ATP through myokinase catalysis.

2.3. Intermediate exchange

G-ATP-actin (40–50 μM, 1:1 complex), separated from unbound ATP by treatment with Dowex-1, was incubated with 200 μM [γ-^{18}O$_3$]ATP for 1 h on ice to ensure complete equilibration of labeled ATP which, typically, should result in 0.8 mol fraction of [γ-^{18}O$_3$]ATP. The solution was again treated with Dowex-1 to remove all free ATP. The Mg- or Ca-G-actin (25 μM) was then polymerized by adding KCl to 100 mM and incubation at 25°C for 60 min, a period long enough to ensure complete hydrolysis of ATP on the F-actin and release of P$_i$ into the medium. The reaction was stopped by addition of HCl to 0.15 N. The P$_i$ was isolated and converted to its trimethyl derivative [12] and the percentages of species with 0–4 atoms of ^{16}O determined by gas chromatography/ mass spectrometry on a Finegan model 4021 apparatus using chemical ionization. Three determinations were averaged for each sample. These analyses were performed by Mr Robert Smith, Mass Spectrometry Center, Department of Biochemistry, University of West Virginia. The results were analyzed by comparison to the mass distribution of P$_i$ produced from the same [γ-^{18}O$_3$]ATP by the Ca^{2+}-ATPase activity of skeletal muscle myosin subfragment 1 [17].

2.4. Incorporation of ^{32}P$_i$ into ATP

Mg- or Ca-F-actin (22–36 μM, 0.5 ml) was incubated for 20 min to 20 h in buffer G containing 0.1 M KCl and either 50 μM, 1 or 2 mM MgCl$_2$, or 2 mM MgCl$_2$ alone, 0.135–0.25 mM ATP or 0.25 mM ADP, and 20–50 mM ^{32}P$_i$ (approx. 2–8 × 10^7 cpm). Control samples were identical except for the absence of actin. The reaction was stopped by addition of HClO$_4$ to 4% at 0°C. The samples were centrifuged and the supernatant solutions were diluted at least 20-fold and applied to columns containing AG1-X8 (Econo column, BioRad, 5 ml). The columns were washed with 25 ml water, P$_i$ was eluted with 60 mM HCl until the radioactivity was reduced to 50–70 cpm/5 ml, and then ATP was eluted with 1 N HCl [11].

2.5. Medium exchange

Mg-F-actin (50 μM) was incubated with 10 mM [^{18}O$_4$]P$_i$ for 20 h in 50 μl buffer G (pH 7 or 7.8) containing either 2 mM MgCl$_2$ or 2 mM MgCl$_2$ and 0.1 M KCl, and Ca-F-ADP-actin (50 μM) was incubated with [^{18}O$_4$]P$_i$ (10 mM) for 20 h in 50 μl buffer G (pH 7 or 7.8) containing 0.1 M KCl and either ATP or ADP. Control samples contained no actin. The reactions were stopped by addition of Dowex-50-H$^+$ (20 μl of a 50% suspension) which acidified the solution and denatured the actin. The samples were centrifuged and the Dowex beads washed twice in 0.1 ml water. The pooled supernatants were neutralized with 1 M Tris and processed for mass spectrometer measurement of the number of water oxygen atoms exchanged into P$_i$ as in [18]. Three mass spectral measurements were averaged for each sample. The mass distribution of P$_i$ species was corrected for the mass distribution of P$_i$ species in the corresponding control samples to obtain the corrected value of the percent of ^{18}O loss from the [^{18}O$_4$]P$_i$.

3. RESULTS

3.1. Intermediate exchange

The hydrolysis of ATP accompanying actin polymerization proceeds as follows:

$$G\text{-}ATP \underset{k_-}{\overset{k_+}{\rightleftharpoons}} F\text{-}ATP \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} F\text{-}ADP\text{-}P_i \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} F\text{-}ADP + P_i \quad (1)$$

where G and F represent G-actin and F-actin, respectively. Because the phosphoryl group (PO$_3$) is cleaved, the P$_i$ formed from [γ-^{18}O$_3$]ATP must contain at least 1 atom of ^{16}O. Each reversal of the cleavage step will result in the introduction of additional ^{16}O into the P$_i$ (if the actin-bound P$_i$ is free to rotate) and, thus, the extent of incorporation of ^{16}O is a quantitative measure of the ratio k_{21}/k_{23}.

In two experiments, the measured ^{16}O/P ratios for the P$_i$ produced by the action of the myosin subfragment 1 Ca^{2+}-ATPase, which is essentially irreversible, were 1.299 and 1.152 [the theoretical values were 1.2 assuming 0.8 mol fraction of [γ-^{18}O$_3$]ATP (see section 2.3)]. In the same two experiments, the measured ratios for the P$_i$ produced during the polymerization of Mg-actin were 1.399 and 1.156, those for Ca-actin being 1.254 and 1.191, respectively. When these values are normalized to control ratios of 1, the ^{16}O/P ratios for Mg-actin become 1.100 and 1.004, and for Ca-actin 0.955 and 1.039. We do not consider these experimental ratios to be significantly larger than the value of 1, the ratio expected if the hydrolysis of ATP were irreversible.

3.2. Incorporation of $^{32}P_i$ into ATP

As mentioned, the intermediate exchange experiments would fail to detect reversal of the cleavage step if the actin-bound P_i did not rotate before the resynthesis of ATP (because the ^{16}O introduced during cleavage would be removed upon resynthesis). This possibility does not affect assays of the incorporation of $^{32}P_i$ into ATP. A total of 6 experiments, 4 with Mg-F-actin and 2 with Ca-F-actin, were performed as described in section 2.4.

In control experiments without actin, the ATP fraction contained 180–1600 cpm; the experimental samples varied between -100 and 700 cpm relative to their respective controls. If 100% of the F-ADP-$^{32}P_i$-actin had been converted to F-ATP-actin, there would have been about 2–8×10^4 cpm in the ATP fraction. Thus, these experiments failed to detect significant reversal of ATP hydrolysis (less than 0.001% in incubations as long as 20 h). In one experiment, incubation was carried out with continual sonication for 20 min to fragment the filaments and increase the number of filament ends that would participate in exchange reactions with G-ATP-actin, thereby possibly trapping any synthesized [γ-^{32}P]ATP. Again, there was no detectable incorporation of $^{32}P_i$ into ATP.

3.3. Medium exchange

The most sensitive measure of the reversal of ATP hydrolysis is the incorporation of ^{16}O from water into [$^{18}O_4$]P_i. In 9 experiments with Mg-actin, the exchange varied between 0.11 and 1.01% for an average of 0.53% and, in 4 experiments with Ca-actin, the exchange of ^{16}O for ^{18}O was -0.01, 0.02, 0.08 and 0.67% for an average of 0.19%. No significant difference was found between the experiments at pH 7.0 and 7.8 or with ADP or ATP (see section 3.3). Thus, in these incubations, which lasted for 20 h, there appeared to be measurable, but very low, reversal of the hydrolysis of ATP by F-actin. In one experiment, 46 μM Mg-F-actin was fragmented by sonication for 40 min (sonifier alternately on for 0.2 s and off for 2 s); only 0.6% exchange was detected although 40 μM ATP had been hydrolyzed during the course of the experiment.

4. DISCUSSION

Three independent techniques have been used to quantify the reversibility of the ATP hydrolysis associated with actin polymerization. Intermediate oxygen exchange measurements failed to detect any reversibility during the polymerization of Mg-actin or Ca-actin. These experiments would fail to detect reversal only if actin-bound P_i were not free to rotate. Measurement of the incorporation of $^{32}P_i$ into ATP by F-actin at steady state also failed to detect reversibility of ATP hydrolysis. These experiments would not have been affected by failure of actin-bound P_i to rotate, but would have given a misleadingly low estimate if medium P_i were bound to a different site on the F-actin than P_i produced by hydrolysis of ATP at the catalytic site. Very slight reversal of ATP hydrolysis during 20 h of incubation was detected by the medium oxygen exchange experiments. These measurements would also have been affected if medium P_i were bound to a different site on the actin than product P_i, i.e. not to the catalytic site.

Therefore, unless actin-bound P_i formed from ATP is not free to rotate and medium P_i binds to actin at a different site than product P_i, these experiments show that the hydrolysis of ATP by F-actin is practically irreversible. Thus, a large negative free energy change accompanies the cleavage of ATP on F-actin (scheme 1). In this respect, actin differs from myosin [19,20], dynein [21–23] and the coupling factors in oxidative phosphorylation [11], all of which can synthesize ATP from tightly bound ADP and P_i (because the steps from ATP cleavage to P_i release are reversible), but is similar to the DNA-dependent recA protein [24] and nitrogenase ATPases [25], which also hydrolyze ATP irreversibly. Similarly, hydrolysis of GTP by several of the guanine nucleotide-binding proteins, elongation factors Tu and G [26] and p21ras (Neal, S. et al., unpublished) has also been found to be irreversible. It is interesting that the hydrolysis of nucleoside triphosphates by both guanine nucleotide-binding proteins and actin (and microtubules) regulates protein-protein interactions and that in both cases the interactions are destablized by the release of P_i.

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