Product Release is Not the Rate-Limiting Step during Cytochalasin B-Induced ATPase Activity of Monomeric Actin

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Under conditions where cytochalasin B induces ATPase activity of monomeric actin (0.3 mM $MgCl_2$, 1 mM EGTA, 30 μM cytochalasin B, 1 mM ATP) the rate constant of the exchange of actin-bound ε-ATP for free ATP is about 4–6 times faster than steady state ATPase activity. When a stoichiometric ATP-actin complex is extracted with PCA (single turnover experiment) the apparent rate constant of P_i generation is not faster than steady state ATPase activity. – The experiments suggest that the hydrolysis of actin-bound ATP and not the subsequent release of hydrolysis products is rate-limiting during cytochalasin-induced ATPase activity of actin.

Introduction

Cytochalasins are fungal metabolites which strongly influence the behavior of actin which is a major protein of the motile and cytoskeletal apparatus of eukaryotic cells. The most frequently used cytochalasins are cytochalasin B and D. They have the following three main effects: (1) At low concentrations they retard the growth of actin filaments by slowing down monomer addition to the fast-growing end (the "barbed" end) of filaments [1–5]. These findings suggest that cytochalasins bind to filament ends and thus mimic the action of "capping" proteins (*cf.* Cooper and Pollard [6], Stossel *et al.* [7] for general reviews). (2) At concentrations which are more or less stoichiometric to those of actin cytochalasin B and D accelerate actin polymerization appreciably [8–12]. This is most probably due to accelerating the rate-limiting nucleation step of actin polymerization [13, 14]. (3) At the same stoichiometric concentrations cytochalasins B and D induce an ATPase activity of non-filamentous actin [8, 11]. Usually, actin catalyzes ATP hydrolysis only in association with polymerization. ATP which is originally bound to each monomer is hydrolyzed shortly after incorporation into the growing filament. The hydrolysis product ADP is normally not released at all and the product phosphate is released in a delayed manner (see Korn *et al.* [15], Carlier [16] for recent reviews). Hence, during polymerization there is only one turnover of ATP hydrolysis possible. Cytochalasin B and D, however, induce a steady ATPase activity uncoupled from polymerization. The conditions under which this ATPase activity is optimal (about 0.3 mM Mg^{2+}, absence of Ca^{2+}, about 30 μM of cytochalasin B) are similar to those under which cytochalasin B accelerates actin polymerization. This suggests that induction of ATPase activity and acceleration of polymerization (by facilitating nucleus formation) are possibly related. It is not yet clear whether dimers or monomers are the true ATPase. Goddette and Frieden [17] favor the first view whereas the strong correlation of monomer concentration and ATPase activity (Brenner and Korn [4], Dancker and Kliche [18]) suggest that ATPase activity is a property of monomers.

To date, relatively little is known about the mechanism of ATPase activity of cytochalasin-dependent ATPase activity. This communication ad-

Abbreviations: EDTA, Ethylenediamine tetraacetic acid; EGTA, Ethyleneglycol-bis-(2-aminoethylether)-N,N′-tetraacetic acid; ε-ATP, 1,N^6-ethenoadenosine triphosphate; P_i, inorganic phosphate; PCA, perchloric acid.

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dresses the question of the rate-limiting step by comparing ATP hydrolysis and nucleotide exchange under conditions of ATPase activity. We present evidence that product release is not the rate-limiting step of cytochalasin-induced ATPase activity.

Methods and Materials

Actin from rabbit skeletal muscle was prepared according to Pardee and Spudich [19]. Actin concentration was determined by optical absorption at 290 nm using an extinction coefficient $E_{1\,mg/ml} = 0.63$.

Preparation of actin free of unbound ATP. G-actin in G-buffer (2 mM Tris-HCl, pH 8.0, 0.1 mM ATP, 0.2 mM $CaCl_2$, 1.5 mM NaN_3) was filtered through a column (1 × 10 cm) containing the anion exchanger Dowex 1 × 8 (200–400 mesh) to remove unbound ATP and was collected in an ATP-free G-puffer.

Preparation of ε-ATP-actin. G-actin from an actin stock solution (about 5 mg/ml) in G-buffer was diluted to 1 or 0.5 mg/ml with ATP-free G-buffer. ε-ATP ($1,N^6$-ethenoadenosine triphosphate) was added to 20 μM at least 1 h before measuring nucleotide exchange.

Measurement of nucleotide exchange. Since the discovery of Thames *et al.* [20] that the fluorescent ATP analog ε-ATP has a higher fluorescence intensity when bound to actin than when free, ε-ATP is widely used in studies of nucleotide exchange of actin. We measured nucleotide exchange by adding ATP to a cuvette containing ε-ATP-actin and which was in a Shimadzu RF 520 double beam fluorescence spectrophotometer with excitation and emission wavelength at 340 nm and 410 nm, respectively. Temperature was 20 °C.

Measurement of ATP hydrolysis. Determination of steady state ATPase activity has been described in [18]. "Single turnover experiments" are performed as follows: The assays contained 0.5 mg/ml G-actin free of unbound ATP in 0.3 mM $MgCl_2$, 5 mM Tris-HCl, pH 8, 1 mM EGTA. The reaction (at 22 °C) was initiated by the addition of actin to the other components. At different times after the onset of reaction 1 ml aliquots were added to 1 ml of ice-cold PCA (0.6 M). After centrifugation the P_i content was determined with the malachite green assay (Kodama *et al.* [21]): To 1 ml of the supernatant of the PCA extract 1 ml of malachite green reagent (see below) was added. After 40 min absorption was measured at 650 nm. The phosphate standards contained the same components as the enzymatic assay. For preparation of the malachite green reagent 0.3 g of malachite green oxalate and 0.5 g Triton X-100 were dissolved in 1 l of 0.7 M HCl.

Data analysis. The time course of fluorescence decay or P_i generation during "single turnover" experiments was fitted to a single exponential with a non-linear regression program in order to obtain the involved rate constants.

Materials. ATP, Dowex and cytochalasin B were from Serva, Heidelberg, malachite green oxalate and ε-ATP were purchased from Sigma, Deisenhofen. All other chemicals were from Merck, Darmstadt or Roth, Karlsruhe.

Results

During ATPase activity, release of the hydrolysis product ADP is an essential step. The kinetics of nucleotide release can be inferred from experiments on nucleotide exchange of G-actin where actin-bound nucleotide is displaced by exogenous nucleotide. Much work has been devoted to this issue (Kuehl and Gergely [22], Neidl and Engel [23], Waechter and Engel [24], Frieden [25], Nowak *et al.* [26], Novak and Goody [27], Frieden and Patane [28]). Most of this work has been done with the fluorescent ATP analog ε-ATP. The work of these authors has shown that nucleotide exchange obeys rather complex kinetics and is affected by divalent cations as well as by the concentration of the displacing nucleotide (in most cases ATP). It is still a matter of controversy whether nucleotides are bound as metal ion complexes or whether nucleotides and metal ions are bound at different though interacting sites. The latter view is supported by the work of Barden and dos Remedios [29], the former view is supported by the work of Loscalzo and Reed [30], Miki and Wahl [31], Brauer and Sykes [32], Nowak and Goody [27].

The experiment of Fig. 1 shows that even in the presence of EDTA (which complexes both Ca and Mg) there is rapid binding of ε-ATP (indicated by a rapid rise in fluorescent intensity) to G-actin which is too fast for the time resolution of the registrations. This initial binding is followed by a

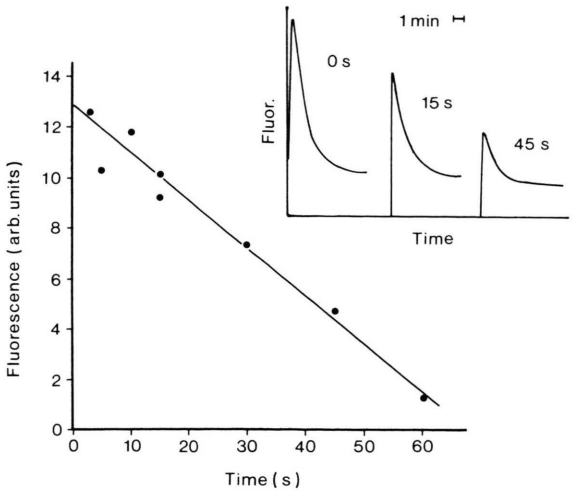

Fig. 1. Fluorescence of G-actin after addition of ε-ATP in the presence of EDTA. – 20 μM of ε-ATP were added to G-actin (0.5 mg/ml, 5 μM Tris-HCl, pH 8.0, 10 μM free ATP) either simultaneously with 1 mM EDTA or at various times after the addition of EDTA. The fluorescence intensity which is reached after the addition of ε-ATP (= peaks of the single registrations, examples of which are shown in the inset) are plotted against the time which elapsed between EDTA addition and ε-ATP addition.

slower release of ε-ATP. The curves resemble the "Bateman function" of pharmacology which describes the sum of an initial "invasion" and a consecutive "evasion" of a pharmacon. Since the dissociation of CaATP or MgATP into its components is fast, this experiment shows that ATP free of metal ions is able to bind to actin albeit only transiently. The longer the time period between EDTA addition and ATP addition, the less ATP is bound. This reflects the long-known fact (*cf.* [33]) that actin devoid of bound divalent cations undergoes some kind of denaturation and loses its nucleotides. This denaturation is also reflected by the decreasing amount of ε-ATP which can initially be bound. This amount was the less the more time elapsed between addition of EDTA and addition of displacing ATP (Fig. 1). Although this decrease cannot be fitted by an exponential as expected, the general tendency is quite clear. This experiment shows that as long as actin has still Mg and/or Ca bound it is able to bind cation-free nucleotides. There is no Ca- or Mg-complex of nucleotides necessary for nucleotide binding to occur.

The dependence of the stability of the actin-nucleotide complex on cytochalasin B and on the concentration of free Mg^{2+} is shown in Fig. 2. The rate constant of the spontaneous loss of ε-ATP was the less the higher the concentration of free Mg^{2+}. Cytochalasin B accelerated ATP release

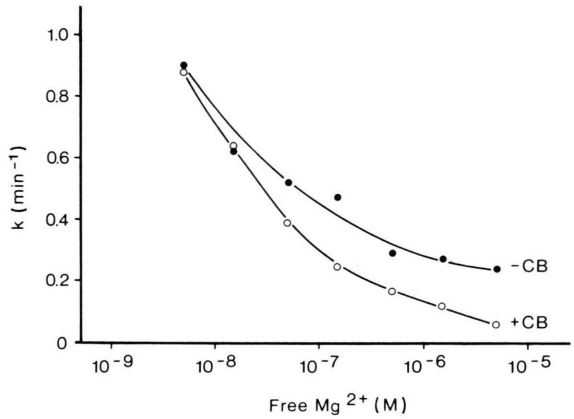

Fig. 2. Rate constants of the spontaneous exponential loss of ε-ATP bound to G-actin as dependent on concentration of free Mg^{2+}. – After treatment with anion exchanger actin was incubated with 20 μM ε-ATP for 1 h and was passed once more through the anion exchanger. This ε-ATP-actin free of unbound nucleotide was added to cuvettes containing 5 mM Tris-HCl, pH 8, 1 mM EGTA, 1 mM EDTA and various concentrations of $MgCl_2$ to give the desired concentrations of free Mg^{2+}. Cytochalasin B (CB) was either absent or 30 μM. An association constant of 2.69×10^8 M^{-1} for the MgEDTA complex was assumed. Final actin concentration was 0.5 mg/ml, temperature was 30 °C.

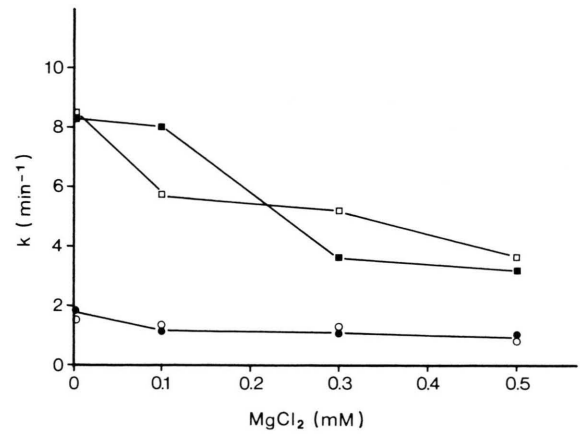

Fig. 3. Release of ε-ATP from actin after addition of 1 mM ATP at different concentrations of $MgCl_2$. – The cuvettes contained 0.5 mg/ml ε-ATP-G-actin, 5 mM Tris-HCl, pH 8 and, if desired, 1 mM EGTA and/or 30 μM cytochalasin B. ●: no CB, no EGTA; ○: + CB, no EGTA; ■: + EGTA, no CB; □: + CB, + EGTA.

only at higher Mg^{2+} concentrations where induction of ATPase activity can be expected to occur demonstrating that ADP-actin is less stable than ATP-actin.

It is long known (*cf.* [22, 23]) that Ca^{2+} retards the displacement of bound by free nucleotide. Since cytochalasin B-induced ATPase activity is inhibited by Ca^{2+} [8, 18] it is possible that these effects are interrelated. We therefore investigated the displacement of ε-ATP by ATP under conditions which are similar to those used in the ATPase experiments.

Fig. 3 shows that 1 mM ATP displaces bound ε-ATP much faster in the presence of EGTA than in its absence. The fact increasing Mg^{2+} concentrations lowered the apparent rate constants of displacement reflects the stabilizing effect of Mg^{2+} on nucleotide binding.

Cytochalasin B had no effect on the displacement rate. In the presence of 0.3 mM $MgCl_2$, 1 mM EGTA and 1 mM ATP (the standard conditions of the ATPase experiments) the rate constant of the exchange of bound for free ATP was 3.6 min^{-1} in this particular experiment, which was conducted at 30 °C. Many experiments (not documented in the paper) gave also at 20 °C rate constants in the range of 2 to 4 min^{-1}. Cytochalasin-induced steady-state ATPase activity, however, is much lower. It was under these conditions about 8 nmol $P_i \times$ mg actin$^{-1} \times$ min^{-1} corresponding to a k_{cat} of about 0.3 min^{-1} [18]. This is about 6 times slower than an exchange rate of about 2 min^{-1} (see above). In other words: an ATP molecule has a sixfold greater chance to become replaced by another ATP than to become hydrolyzed. But still the release of ADP could be rate-limiting if ADP would be released much slower than ATP. Although much evidence is against this possibility we addressed the problem of the rate-limiting step by performing a "single turnover" experiment. Free ATP was removed from a G-actin solution by treatment with an anion exchanger so that only actin-bound ATP remained and only one enzymatic turnover was possible. We quenched the reaction with acid and measured either the disappearance of ATP by bioluminescence [34] or the appearance of P_i (by the malachite green method). Both approaches gave the same results. We show here only experiments with P_i determination.

If ATP hydrolysis proper were much faster than release of P_i, then in a single turnover experiment P_i should be liberated much faster than during steady state (with many turnovers) since acid would release also the phosphate which is still bound at the enzyme. In other words, there should be a fast phosphate burst the velocity of which should exceed the steady state ATPase rate. If ATP hydrolysis were rate-limiting, the rate of P_i release and steady state ATPase activity should be the same. From Fig. 4 it can be seen that in this particular experiment P_i release was 0.44 min^{-1}. The Table summarizes the single turnover experi-

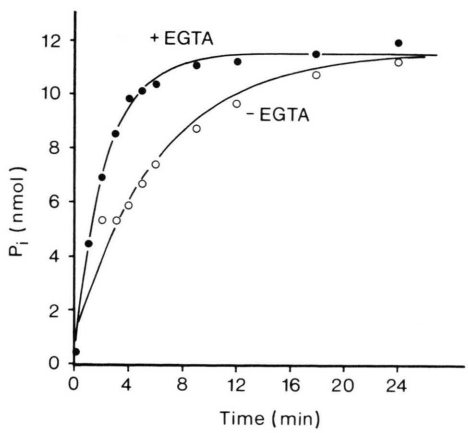

Fig. 4. Generation of inorganic phosphate (P_i) induced by cytochalasin B. – The assays contained 0.5 mg/ml (12 nmol/ml) of Dowex-treated G-actin, 5 mM Tris-HCl, pH 8, 0.3 mM $MgCl_2$ 30 μM Cytochalasin B and either no or 1 mM EGTA. The rate constants for P_i generation were 0.16 min^{-1} (no EGTA) and 0.44 min^{-1} (1 mM EGTA present). The calculated end values are 10.6 nmol·ml^{-1} (no EGTA) and 11.0 nmol·ml^{-1} (EGTA), respectively; ●: 1 mM EGTA present; ○: no EGTA present.

Table. Generation of P_i during "single turnover" experiments. Concentrations: Dowex-treated actin 0.5 mg/ml (= 12 μM); $MgCl_2$: 0.3 mM; EGTA: 1 mM; Tris-HCl, pH 8.0: 5 mM, cytochalasin: 30 μM, no ATP was added. k is the rate constant of the exponential time course of P_i generation.

k [min^{-1}]	Amount generated [nmol·ml^{-1}]
0.44	11.6
0.46	14.5
0.40	8.34
0.30	6.9
0.29	6.9

ments and shows that the rate constants are between 0.3 and 0.4 min^{-1}. In other words, steady state ATPase activity and generation of P_i are of equal rates demonstrating that ATP hydrolysis and not product release is the rate-limiting step during cytochalasin-induced ATPase activity of monomeric actin.

Discussion

We have shown that under conditions where cytochalasin B accelerates ATPase activity of G-actin it does not accelerate nucleotide exchange (see also Frieden [25]). This was the first hint that during cytochalasin-induced ATPase activity product release is not rate-limiting. This conclusion is substantiated by the observation that exchange of ε-ATP for exogenous ATP was much faster than ATPase activity. Further, during single turnover experiments, acid quenches revealed that generation of phosphate is not faster than steady state ATPase activity showing that ATP hydrolysis itself and not product release or a transition of an ADP-P_i-actin complex as Brenner and Korn [35] supposed, is rate-limiting during cytochalasin-induced ATPase activity. (That not in all experiments the theoretical amount of P_i (12 nmol) was produced indicates that Dowex treated actin loses part of its ATP already before the onset of ATPase activity.) This is remarkable since other ATPase activities have been shown to be rate-limited by product release (*e.g.* myosin ATPase, Lymn and Taylor [36], dynein ATPase, Holzbaur and Johnson [37]). ATPase activities of actin which are supposed to be rate-limited by product release are the ATPase activity associated with monomer addition to filament ends in the presence of cytochalasin D (Estes *et al.* [38]) and the ATPase activity of monomeric actin induced by protamine (Ferri *et al.* [39]).

The fact that ATP hydrolysis and not product release is the rate-limiting step of cytochalasin B-induced ATPase activity implies that under steady state conditions (excess of ATP) ATP-actin remains the prevailing species. Consider a simple kinetic scheme of cytochalasin-induced ATPase activity:

$$A + T \xrightarrow{1} A{-}T \xrightarrow{2} A{-}D{-}P_i \xrightarrow{3}$$
$$A{-}D + P_i \xrightarrow{4} A + D + P_i$$

with A = actin, T = ATP, D = ADP. Step 2 is rate-limiting. When we neglect back reactions ATPase activity during steady state equals the product of the concentration of any intermediate and its forward rate constant. Therefore ATPase activity v is also

$$v = [A{-}D]\, k_4.$$

Since fluorescence does not discriminate between ε-ATP and ε-ADP, the exchange rate E is

$$E = [A{-}T]\, k_{-1} + [A{-}D]\, k_4.$$

If E is n times faster than v:

$$n\,[A{-}D]\, k_4 = [A{-}T]\, k_{-1} + [A{-}D]\, k_4$$

or

$$[A{-}D]/[A{-}T] = k_{-1}/(k_4(n{-}1)).$$

If the rate constants of ADP release and of ATP release are more or less equal as Neidl and Engel [23] assume then

$$[A{-}D]/[A{-}T] = 1/(n{-}1).$$

If ADP dissociates about 100 times faster than ATP, as Nowak and Goody [27] assume ($k_4 \approx 100\, k_{-1}$) then

$$[A{-}D]/[A{-}T] = 1/(100(n{-}1)).$$

Since n is about 4 to 6 there is in any case $[A{-}T] > [A{-}D]$. Since step 2 is rate-limiting, the concentration of ATP-actin (*i.e.* the intermediate A−T) is higher than that of ADP-P_i-actin (the intermediate $A{-}T{-}P_i$), therefore, under the conditions of cytochalasin-induced ATPase activity the majority of actin exists as ATP-actin, ADP-actin is only very shortlived. Hence, effects of cytochalasin B on actin are not due to a conversion of ATP-actin to ADP-actin.

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