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12-Fold difference between the critical monomer concentrations of the two ends of actin filaments in physiological salt conditions

(actin/capping protein/fluorescence)

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ABSTRACT **We determined the critical monomer concentrations at which association and dissociation reactions are balanced at the two ends of actin filaments. For measurement of the critical concentration of the pointed end, interference with the high dynamics of the barbed end was excluded by capping the barbed ends with an actin filament capping protein isolated from bovine brain. The critical concentration of the pointed end (1.5 μM) was found to be 12- to 15-fold higher than the critical concentration of the barbed end (0.10–0.12 μM) at a temperature of 37°C and physiological salt concentrations (100 mM KCl/1–2 mM $MgCl_2$/0.3 mM EGTA or 0.2 mM $CaCl_2$, pH 7.5).**

Actin filaments have been shown *in vitro* to lengthen at the barbed end and to shorten simultaneously at the pointed end under steady-state conditions (1–3). This treadmilling process is driven by a continuous ATP hydrolysis occurring during the association of actin monomers with a filament end (1, 4). At the barbed end of actin filaments, a lower critical monomer concentration is required to balance association and dissociation reactions as compared with the pointed end. The critical concentration of the pointed end could not be measured directly because association and dissociation at the barbed end occurs much faster than at the pointed end. Therefore, events occurring at the pointed end of filaments are difficult to observe without interfering with the dynamics of the barbed end (5, 6).

In this study this problem was overcome by capping the barbed filament end with an actin filament capping protein from bovine brain (7, 8). Capped filaments labeled with 7-chloro-4-nitrobenz-2-oxa-1,3-diazole were mixed with various concentrations of unlabeled monomeric actin. The monomer concentration range in which filaments can release subunits from the pointed ends was determined by a fluorescence change accompanying the monomer–polymer transition of labeled actin molecules. The critical concentration of the barbed end was measured by the reverse assay. Unlabeled filaments were mixed with various concentrations of labeled actin monomers. The monomer concentration range in which the barbed ends consume monomers was again determined by a fluorescence change.

MATERIAL AND METHODS

Preparation of the Proteins. Actin was prepared from rabbit skeletal muscle as described (9), including gel filtration on a Bio-Gel P-150 column. Part of the protein was modified with *N*-ethylmaleimide at cysteine-374 and subsequently with a fluorescent label (7-chloro-4-nitrobenz-2-oxa-1,3-diazole) at lysine-373 (10). Actin filament capping protein was extracted from bovine brain and purified by several ammonium sulfate fractionations and column chromatography steps according to a published method (8). The concentration of actin was determined photometrically at 290 nm by using an extinction coefficient of 24,900 M^{-1} cm^{-1} (1). The concentration of labeled actin was measured by the method of Lowry *et al.* (11), and the concentration of capping protein was determined as described by Bradford (12).

Fluorescence Assay. The monomer–polymer transition of actin was followed by the 2.2-fold fluorescence increase of the label on aggregation of actin (6, 10). Five percent of labeled actin was added to unmodified actin and copolymerized. These low proportions of labeled actin do not significantly alter the polymerization behavior of actin (6). The molar fluorescence intensities of monomeric and polymeric labeled actin were determined by measuring the fluorescence intensities of dilution series (0–3 μM monomeric or polymeric labeled actin). The measurements were performed by using a Jobin Yvon three-dimensional fluorimeter equipped with a mercury/xenon lamp. The excitation wavelength was 436 nm, and the emission wavelength was 530 nm. Physiologically relevant salt concentrations were used for the assays: 1 mM $MgCl_2$, 100 mM KCl, 5 mM triethanolamine·HCl (pH 7.5), 0.3 mM EGTA, 0.5 mM ATP, 1 mM dithiothreitol, and 200 mg of NaN_3 per liter. In some experiments 2 mM $MgCl_2$ was used or EGTA was replaced by 0.2 mM $CaCl_2$ in order to investigate the effect of different ions. The temperature was kept at 37°C. The results depicted in Figs. 1–4 were obtained in the presence of 1 mM $MgCl_2$ and 0.3 mM EGTA.

RESULTS

Effect of the Capping Protein on Actin Polymerization and Depolymerization. Various concentrations of capping protein (5–50 nM) were added to polymerized actin. The capping protein induced depolymerization of actin in a concentration-dependent manner (Fig. 1). At 5 nM no depolymerization was detectable, whereas at 20 or 50 nM capping protein, 0.4 or 0.8 μM actin depolymerized, respectively. When monomeric actin was polymerized in the presence of various amounts of capping protein, the final concentrations of polymeric actin were lower as more capping protein was present. Different monomer concentrations were reached when the reaction was started from monomeric or polymeric actin (Fig. 1). Probably, the differences in the final monomer concentrations are caused by variations in the amount of capped filaments. A relatively small proportion of uncapped filaments can decrease the monomer concentration considerably. Because the assembly reaction at the barbed end is fast, a few uncapped filaments are able to consume the monomers released from the pointed ends efficiently (4, 5). These experiments confirm the previously reported depolymerizing effect of molecules capping the barbed end (2, 8, 13, 14). However, they also show that a quantitative statement about the critical concentration of the pointed end

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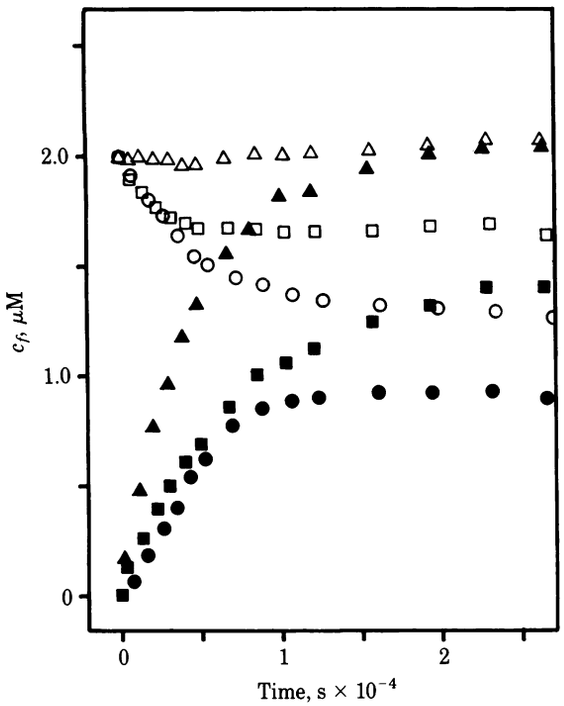

FIG. 1. Effect of various concentrations of capping protein on actin polymerization and depolymerization. Monomeric actin (2 μM) was mixed with 5 nM (▲), 20 nM (■), or 50 nM (●) capping protein. Polymeric actin (2 μM) was depolymerized by addition of 5 nM (△), 20 nM (□), or 50 nM (○) capping protein. When actin was polymerized in the absence of capping protein, the final filament subunit concentration was the same as in the presence of 5 nM capping protein. c_f is the concentration of actin filament subunits.

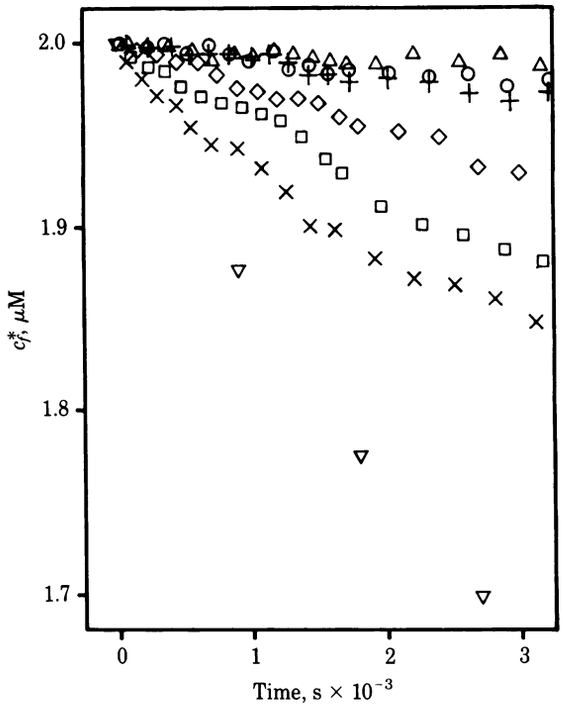

FIG. 2. Labeled filaments (2 μM) incubated with 50 nM capping protein were mixed with monomeric unlabeled actin. The time course of disassembly at the pointed end was determined by the fluorescence change. c_f^* is the concentration of labeled actin filament subunits. Monomer concentrations were derived from Fig. 3 [or Fig. 2 (▽)]: ▽, 0.0–0.3 μM; ×, 1.0–1.1 μM; □, 1.20–1.25 μM; ◇, 1.4–1.5 μM; △, 1.5–1.75 μM; ○, 1.7–2.0 μM; and +, 3 μM initial concentration.

may be difficult to obtain by this assay because of the divergence of the final monomer concentrations (Fig. 1).

Determination of the Critical Concentration of the Pointed End. An attempt was made to overcome the described difficulties by a more sophisticated experimental design. Various concentrations of unlabeled actin monomers (final concentrations, 0–3 μM) were mixed with capping protein (final concentration, 50 nM). Subsequently labeled actin filaments (final concentration, 2 μM filament subunits) were added. In Fig. 2 the time course of the concentration of labeled filament subunits is represented. Above a monomer concentration of 1.5 μM, subunits are not released from filaments. Below this monomer concentration, actin filaments release subunits as indicated by the initial decrease of the concentration of labeled filament subunits. Thus, association and dissociation at the pointed ends are balanced at a critical concentration of 1.5 μM. Increase of the magnesium concentration (2 mM) or replacement of EGTA by calcium (0.2 mM) had no effect on the critical concentration of the pointed end.

The monomer concentrations change during the time course of the above described experiment due to release of subunits or consumption of monomers at the pointed ends or at barbed ends of filaments that may have remained uncapped. Therefore, controls were performed by repeating the experiment, with the only modification being that both monomeric and polymeric actin were labeled. Consumption of monomers and release of subunits were measured by fluorescence change. Between 1.0 and 1.5 μM initial actin monomers, almost no change in the monomer concentration (<0.1 μM) was observed (Fig. 3). Below or above this concentration range, a considerable increase or decrease of the monomer concentration was measured, respectively (Fig. 3).

Determination of the Critical Concentration of the Barbed End. The critical concentration of the barbed end was measured by the inverse assay (6). Various concentrations of labeled monomeric actin (0.06–0.16 μM) were mixed with unlabeled filaments. The incorporation of monomers at the barbed end can be detected as the monomers are labeled. The disassembly of subunits from the pointed ends was not observed at early time points because the filament subunits were unlabeled. Capping the pointed ends was not necessary as the dissociation of subunits from the pointed ends was slow (see Fig. 2). The time course of actin monomer incorporation at the barbed filament ends is depicted in Fig. 4. Below a monomer concentration of 0.10–0.12 μM, no monomers were incorporated at the barbed end; above this concentration, filaments incorporate monomers at the barbed end as revealed by the initial decrease of the monomer concentration (Fig. 4). At the later stage, incorporation of labeled monomers into filaments was observed even if the initial monomer concentration was below the critical concentration of the barbed end. Presumably, the monomer concentration increases above the critical concentration of the barbed end by release of subunits from the two filament ends. The critical concentration of the barbed end (0.10–0.12 μM) appears to be 12- to 15-fold lower than the critical concentration of the pointed end (1.5 μM).

DISCUSSION

The depolymerizing effect of molecules capping the barbed actin filament ends has been reported in a number of studies (2, 8, 13, 14). Under conditions similar to the experiments described in this study, villin or severin has been found to increase the actin monomer concentration to 1 or 2 μM, respectively (13, 14). These values are quite consistent with the measured critical concentration of the pointed end of actin fil-

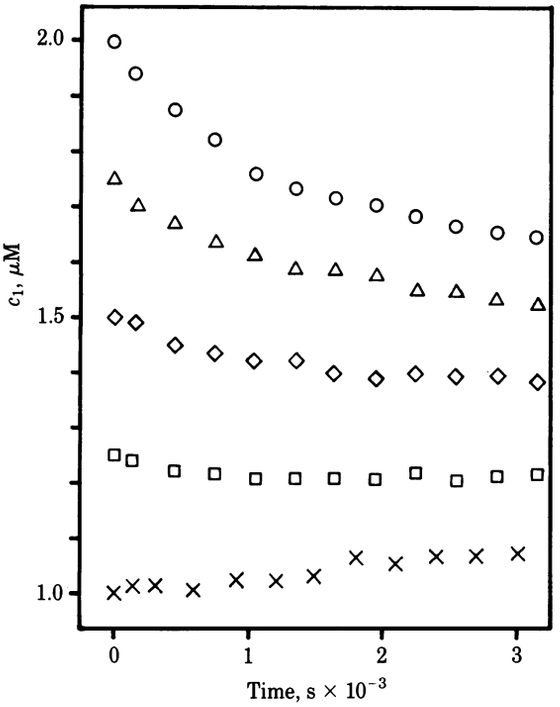

FIG. 3. The time course of the total actin monomer concentration during the experiment described in Fig. 2 was determined by mixing labeled filaments (2 μM) and capping protein (50 nM) with various concentrations of labeled monomeric actin. Initial monomer concentrations: ×, 1.00 μM; □, 1.25 μM; ◇, 1.50 μM; △, 1.75 μM; and ○, 2.00 μM. c_1 is the monomer concentration.

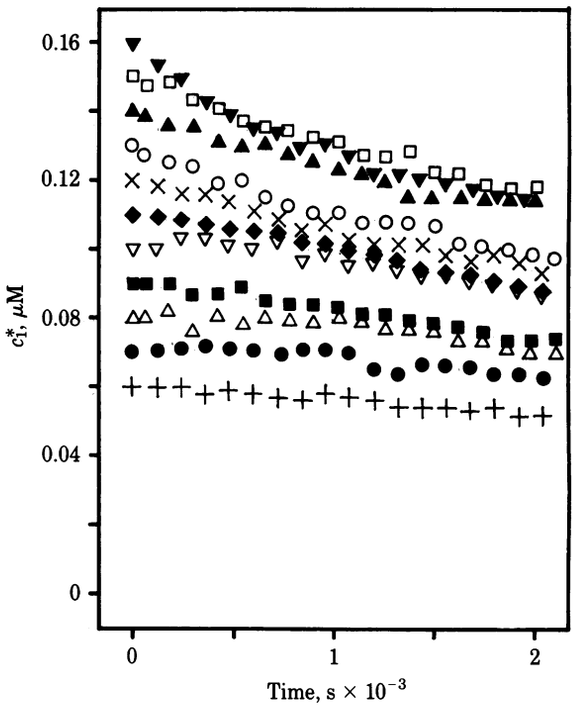

FIG. 4. Determination of the critical concentration of the barbed end. Various concentrations of labeled monomeric actin (0.06–0.16 μM) were mixed with 0.24 μM unlabeled polymeric actin. Initial labeled monomer concentrations: +, 0.06 μM; ●, 0.07 μM; △, 0.08 μM; ■, 0.09 μM; ▽, 0.10 μM; ◆, 0.11 μM; ×, 0.12 μM; ○, 0.13 μM; ▲, 0.14 μM; □, 0.15 μM; and ▼, 0.16 μM. The initial slope indicates that the critical concentration of the barbed end is between 0.10 and 0.12 μM. c_1^* is the concentration of labeled actin monomers.

aments (1.5 μM). The results of this study agree with the previous finding that, for pure muscle actin, the critical concentration of the pointed end is above 0.5 μM (6). The investigation on pure actin (6) demonstrates that the difference between the critical concentrations of the two ends is an inherent property of actin filaments and excludes the possibility that the capping molecules induce a disparity of the critical concentrations. The difference between the critical monomer concentrations provides additional evidence that actin filaments can treadmill at physiological salt concentrations (6). If the monomer concentration is between 0.12 and 1.5 μM, subunits are released from the pointed ends and monomers are incorporated at the barbed ends.

Treadmilling may be one of the molecular mechanisms that play a role in the turnover and organization of actin filaments in living cells. The dynamics of actin filament assembly and disassembly has been demonstrated by recent studies on the recovery of photobleached segments of fluorescently labeled stress fibers (15, 16). Also, it is known from *in vivo* experiments that capping protein microinjected into tissue culture cells leads to a loss of focal contact sides and a disintegration of stress fiber bundles (17). The same capping protein has no effect on Triton X-100-extracted cells. This suggests that the observed depolymerization of stress fibers is due to capping individual actin filaments from a bundle that is in a dynamic steady state with the pool of unpolymerized actin.

In nonmuscle cells, large amounts of actin (>10 μM) can occur in an unpolymerized state (18) because actin monomers form a stable complex with proteins that inhibit actin assembly [e.g., profilin (19)]. It has been suggested that the depolymerizing effect of profilin-like proteins might be amplified if the critical monomeric actin concentration is increased by capping of the barbed filament ends (20). The concentration of polymerized actin, c_f, has been shown to depend on the critical monomeric actin concentration, c_1, and on the total concentration of profilin, p, in the following way (20):

$$c_f = c_{tot} - c_1 - p\cdot\frac{c_1}{c_1 + K_d}, \qquad [1]$$

where c_{tot} is the total actin concentration and K_d is the dissociation equilibrium constant of the actin–profilin complex. The last term of Eq. 1 represents the concentration of the actin–profilin complex. The total concentrations of actin and profilin in Acanthamoeba are in the range of 100 μM (21). Unfortunately, the dissociation constant of the actin–profilin complex under the conditions of this study is unknown. At lower salt concentrations, a dissociation constant of 4–12 × 10^{-6} M has been reported (21). The critical monomer concentration of uncapped filaments is similar to the critical concentration of the barbed filament end. The barbed filament ends predominantly determine the critical concentration of uncapped filaments because most of the association and dissociation reactions occur at the barbed filament ends (3, 5, 6). In similar salt conditions, the critical concentration of uncapped filaments has been found to be 0.16 μM (6). If we assume this value and 4 μM for the dissociation constant of the actin–profilin complex, uncapped filaments would coexist with 4.0 μM unpolymerized actin (see Eq. 1). If, due to complete capping of barbed filament ends, the monomer concentration c_1 increases to 1.5 μM, the concentration of unpolymerized actin would increase to a value of 28.8 μM. These calculations demonstrate that the measured differences between the critical concentrations of uncapped and capped filaments is of considerable potential for regulating the distribution of polymerized and unpolymerized actin in living cells.

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