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CYTOCHALASIN B MAY SHORTEN ACTIN FILAMENTS BY A MECHANISM INDEPENDENT OF BARBED END CAPPING

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Abstract—It is generally accepted that cytochalasin B (CB), as well as other cytochalasins, shorten actin filaments by blocking monomer addition at the fast-growing ("barbed") end of these polymers. Despite the predominance of this mechanism, recent evidence suggests that other interactions may also occur between CB and F-actin. To investigate this possibility further we have employed an actin derivative, prepared by substitution at Cys^{374} by a glutathionyl residue. We demonstrate here that CB did not significantly bind to glutathionyl F-actin under several ionic conditions. We further show that in the presence of CB the glutathionyl-F-actin exhibits a significantly higher ATPase activity than the non-modified F-actin. These data argue that the incorporation of glutathionyl groups prevents the high-affinity binding of CB to the barbed end of actin filaments, probably due to a decreased hydrophobicity of the CB binding site by the introduction of the hydrophylic glutathionyl residue. Despite the lack of substantial binding at equilibrium, we have found that the addition of CB to glutathionyl-F-actin results in extensive fragmentation of the filaments, as demonstrated by electron microscopy and by a significant reduction of the relative viscosity of actin solutions. These results are consistent with the idea that CB shortens glutathionyl-actin filaments by a mechanism distinct from barbed end capping. Glutathionyl F-actin offers an interesting model to study the complex mechanism of interaction of actin filaments with cytochalasins and with the physiologically important actin capping/severing proteins.

Key words: actin; glutathionyl-actin; cytochalasin B; barbed end binding; severing activity

Actin filaments are polar structures with fast growing ("barbed") and slow growing ("pointed") ends. Cytochalasins, a class of fungal compounds with well-established cytotoxic activities, have profound effects on actin polymerization including shortening of actin filaments *in vitro* [1–6]. It has been suggested that this action on actin filaments involves a high affinity binding at and capping of the fast growing end of the filaments, thus preventing further addition of actin monomers [2–5, 7, 8]. It is believed that this mode of action represents the predominant mechanism by which cytochalasins interact with F-actin [2–5]. However, a number of previous studies suggest that these substances may have additional modes of action on actin filaments including a severing activity [6, 9–12]. Based on these reports it can be assumed that additional interactions of cytochalasins with actin filaments may be masked by their predominant action on the barbed end of the filaments. To study the latter further, we have attempted to limit the interaction of cytochalasins to the barbed end of actin filaments and thus unmask any other type of shortening effect. For this we have employed a glutathione substituted actin derivative at Cys^{374} (glutathionyl-actin). This actin derivative was found to be useful in studying filament stabilization [13]. We demonstrate here that glutathionyl-actin shows undetectable binding for CB¶. We have evaluated the potential effect of CB on Glu-F-actin, performing viscometry, electron microscopy and ATPase activity measurements. As shown below, our data provide evidence that CB provokes a fragmentation of glutathionyl-actin filaments independent of its binding at the barbed ends of the polymer.

MATERIALS AND METHODS

ATP was purchased from Boehringer (Mannheim, F.R.G.), CB from the Sigma Chemical Co. (St Louis, MO, U.S.A.), and [^{3}H]CB from New England Nuclear (Wilmington, DE, U.S.A.). Sephadex G-25 and Sephacryl S-200 were from Pharmacia-LKB (Bromma, Sweden). DNPSSG was prepared as previously described [14]. All other reagents were purchased from various commercial sources in the purest grade available.

Actin preparations. Actin from rabbit skeletal muscle was prepared by two cycles of polymerization-depolymerization, as previously described [14]. For the preparation of G-actin, F-actin was depolymerized by homogenization at 4° in 1 mM

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¶ Abbreviations: DNPSSG, 2-4-dinitrophenyl glutathionyl disulfide; CB, cytochalasin B; DCC, dextran-coated charcoal; ED, equilibrium dialysis; Glu-F-actin, glutathionyl-F-actin.

Tris–HCl, 0.2 mM ATP, pH 7.4 (buffer G) and further purified by gel filtration on Sephacryl S-200 [15]. The concentration of actin was determined spectrophotometrically using $E_{290} = 26{,}460\ M^{-1}\ cm^{-1}$. Glutathionyl-Cys^{374}-actin was prepared from G-actin, as previously described [13, 16] In order to eliminate any contamination with unsubstituted actin the reaction of G-actin with DNPSSG was monitored in parallel spectrophotometrically at 405 nm. Only preparations showing the release of exactly 1 equiv. 2,4-dinitrothiophenolate ($E_{408} = 12{,}700\ M^{-1}\ cm^{-1}$) were used. The final product was purified by passing the mixture over Sephadex G-25, or Sephacryl S-200 [13]. Glu-F-actin was depolymerized as described for normal F-actin. In all experiments the protein concentrations were 20 μM, unless noted otherwise. Polymerization of all actin and glutathionyl-actin preparations was induced by the addition of $MgCl_2$ (final concentration 2 mM) or $MgCl_2$ and KCl (final concentration 2 and 100 mM, respectively). In all polymerization experiments the ATP concentration was 0.2 mM.

[^{3}H]*CB binding assays.* Half a mililitre of both F-actin and Glu-F-actin (final concentration 20 μM) in 1 mM Tris–HCl, 2 mM $MgCl_2$ pH 7.4 or in 1 mM Tris–HCl, 2 mM $MgCl_2$, 100 mM KCl, pH 7.4 or in 1 mM Tris–HCl, 2 mM $MgCl_2$, 20% glycerol pH 7.4 were incubated at 25° for different periods of time (5–60 min) with increasing concentrations of [^{3}H]CB (sp. act. 15 Ci/mmol), ranging from 0.5 nM to 50 μM. At the end of incubation, free CB was separated from bound CB by two different methods: (A) according to the first, free CB was adsorbed to DCC: 400 μL of each incubation mixture were treated with an equal volume of DCC (0.025–0.25% w/v) in 1 mM Tris–HCl, containing 2 mM $MgCl_2$, or 2 mM $MgCl_2$ plus 100 mM KCl, pH 7.4 at 25° for 20 min. After centrifugation at 1500 *g* for 15 min, the supernatants were removed and the bound radioactivity was counted in 0.25 mL aliquots in a Packard "Tri-carb" 4640 β-Counter. (B) As an alternative approach, free CB was dialysed at equilibrium (ED): 800 μL of each incubation mixture were dialysed against 100 mL of 1 mM Tris–HCl, 2 mM $MgCl_2$ pH 7.4 for 20 hr at 4° in Spectra Pore membranes. Bound radioactivity was determined in 0.25 mL of the dialysed material as described before. The number of binding sites (n) and the apparent dissociation constants (K_d) were determined by Scatchard analysis, with Rosenthal correction for the non-specific binding [17], using the Ligand computer program of Munson and Rodbard [18].

Finally, to assess the binding of CB to normal and modified G-actin, the actin preparations, both in buffer G in the absence or presence of non-polymerizing amounts of $MgCl_2$ or $CaCl_2$ (both at 0.2 mM final concentration), were incubated for 30 min. with varying concentrations of [^{3}H]CB (0.5 nM–50 μM). The free CB was separated from the bound by the DCC method, exactly as described above. Alternatively, 0.5 mL of G-actin or glutathionyl-G-actin were incubated at 25° for 15, 30, 60 and 120 min with 30 nM [^{3}H]CB (sp. act. 15 Ci/mmol). At the end of the incubation, the free CB was separated from the bound by gel filtration through a Sephadex G-25 column equilibrated with buffer G, containing 0.2 mM $MgCl_2$ or $CaCl_2$, respectively. In the collected fractions (1 mL) the protein content was estimated by measuring the optical density at 280 nm, and radioactivity was counted in 0.5 mL aliquots.

Viscosity measurements. These measurements were performed using an Ostwald capillary viscometer. A CB stock solution in absolute ethanol was used. The effects of CB were quantified by monitoring the relative viscosity after addition of 10 μL of the appropriate concentrations of this agent to normal and Glu-F-actin maintained into the viscometer. In control experiments the relative viscosities of normal- and Glu-F-actin, polymerized upon the addition of Mg^{2+} or Mg^{2+} and K^+ ions as described above, were checked for possible variations after addition of equivalent amounts (10 μL) of the CB solvent solution in the viscometer. Relative viscosity $\eta = t/t_o$ was plotted as in Fig. 2. where t_o is the outflow time of water (29 sec at 20°).

Electron microscopy. Samples were collected from the viscometer and placed on plastic/carbon-coated copper grids. The specimens were negatively stained with 2% uranyl acetate for 1 min. Micrographs were taken in a Jeol 100C electron microscope, operated at 100K, and recorded on Kodak electron microscope film 4489 (6.5 × 9 cm plates).

Measurements of ATPase activity. These assays were performed in solutions of normal F-actin and Glu-F-actin in buffer G containing 2 mM $MgCl_2$ and [γ-^{32}P]ATP (150 nCi/mg protein, 0.2% of total ATP). The samples were kept at 25° in the absence or presence of 30 μM CB (final concentration). Aliquots were taken at several time intervals and analysed for ^{32}Pi as described previously [16].

RESULTS

Binding of CB to modified and non-modified actin

Binding of [^{3}H]CB to F-actin and Glu-F-actin were performed in buffers containing $MgCl_2$ or

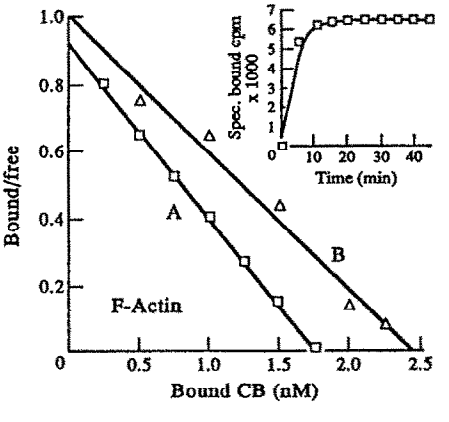

Fig. 1. Specific binding of [^{3}H]CB to F-actin. Half a mililitre of F-actin (20 μM) was incubated at 25° for 30 min with [^{3}H]CB, ranging from 0.5 nM to 500 nM. Free radioactivity was isolated with DCC (A) and ED (B). Data are expressed as Scatchard plots. Inset: saturation curve.

Table 1. Dissociation constants (K_d) and number of binding sites (n) of CB binding to F-actin and Glu-F-actin after 30 min incubation in the presence of $MgCl_2$ (2 mM) or $MgCl_2$ (2 mM) plus KCl (100 mM)

Conditions			K_d(nM)		n (nM)	
			F-actin	Glu-F-actin	F-actin	Glu-F-actin
Mg^{2+}	DCC	(N = 6)	2.5 ± 0.8	ND	4.6 ± 1.6	ND
Mg^{2+}	ED	(N = 3)	2.2 ± 0.7	ND	2.3 ± 0.9	ND
Mg^{2+}/K^+	DCC	(N = 6)	1.4 ± 0.2	ND	2.5 ± 0.7	ND
Control experiments +20% glycerol						
Mg^{2+}	DCC	(N = 4)	2.7 ± 0.4	1.1 ± 0.6	5.5 ± 1.3	0.17 ± 0.08

In control experiments K_d and n were determined in the presence of 20% glycerol.

Binding parameters were determined following DCC treatment or following ED, as described in Materials and Methods.

Values are the means ± SD from N = 6, N = 4 or N = 3 distinct experiments. ND = not detectable.

$MgCl_2$ plus KCl. The equilibrium for the [^{3}H]CB binding to F-actin was achieved within 15 min (Fig. 1, insert). The estimated K_ds of CB binding to F-actin in the presence of Mg^{2+} ions with both methods DCC and ED, were very similar: 2.5 ± 0.8 nM (N = 6) with the DCC method and 2.2 ± 0.7 nM (N = 3) with the ED method (Fig. 1A and B, and Table 1, respectively). Additionally, the number of binding sites, n, in the presence of Mg^{2+} ions was found to be 4.6 ± 1.6 nM (N = 6) with the DCC method and 2.3 ± 0.9 nM (N = 3) with the ED method, indicating that 1 mol CB bound to approximately 3400 mol of normal actin molecules. This value derives from a set of experiments using the DCC method. Very similar binding parameters [K_d = 1.4 ± 0.2 nM (N = 6) and n = 2.5 ± 0.7 nM (N = 6)], were also obtained for CB binding to F-actin in the presence of Mg^{2+} and K^+ ions (Table 1). Both the K_d and the number of binding sites (n) of CB available on F-actin were fully in line with previously reported data [11].

The binding assays of CB (at concentrations ranging between 0.5 nM and 50 μM) to Glu-F-actin were performed under the same experimental conditions as those for F-actin. The incubation times used were 5, 15, 30 and 60 min. Employing both the DCC and ED methods, the specific binding of CB to Glu-F-actin was undetectable in the presence of Mg^{2+}-ions or Mg^{2+} plus K^+-ions (Table 1).

In an effort to estimate the binding capacity of monomeric glutathionyl actin for CB, we incubated both, G-actin and glutathionyl G-actin with [^{3}H]CB under non-polymerizing conditions in the absence or presence of Mg^{2+} or Ca^{2+} ions as described in Materials and Methods. Then we separated the free from the bound CB using gel filtration chromatography or the DCC method. We found that under these experimental conditions no detectable binding of CB to both, G-actin and glutathionyl G-actin was observed (not shown), even in the presence of high CB concentrations (up to 50 μM), thus, excluding the possibility of low affinity binding capacity of CB to monomeric glutathionyl-actin.

Finally, in an effort to elucidate the mechanism responsible for the lack of detectable binding of CB to glutathionyl-F-actin we performed binding experiments in the presence of 20% glycerol. As has been previously described for other receptor systems [19], glycerol increases the hydrophobicity of the binding envelope by driving out water molecules. As shown in Table 1, under these experimental conditions, the binding capacity of Glu-F-actin for CB was partly restored, since the K_d was 1.1 ± 0.6 nM (N = 4) (i.e. very similar to that estimated for F-actin) (Table 1). However the number of binding sites thus obtained was significantly lower (Table 1), since 1 mol of CB bound to approximately 92,000 mol of glutathionyl actin molecules, indicating that under these experimental conditions only a diminished number of binding sites could be detected.

Effects of CB on normal F-actin and Glu-F-actin

Figure 2A depicts the effect of CB on the viscosity of Glu-F-actin and normal F-actin. Both types of actin were polymerized by addition of $MgCl_2$. The kinetics of polymerization were in agreement with previously published data [16]. Addition of CB (30 μM) directly into the viscometer clearly decreased the relative viscosity of the polymerized glutathionyl-actin from $\eta = 1.55$ to $\eta = 1.2$ (Fig. 2A).

Figure 2B shows the polymerization kinetics in the presence of K^+ ions. In this experiment normal actin was polymerized in the presence of $MgCl_2$ plus KCl, and glutathionyl-actin in the presence of $MgCl_2$, followed by addition of KCl at the time indicated by the arrow. As previously reported [16], both types of actin finally reached similar maximum relative viscosity values ($\eta = 1.8$), indicating similar filament stabilities in the presence of K^+ ions. Upon the addition of CB the decline of the relative viscosity of Glu-F-actin ($\eta = 1.55$) was also clear (Fig. 2B).

In control experiments, addition of the solvent in which CB was dissolved to Mg^{2+} or Mg^{2+}/K^+ polymerized glutathionyl actin directly into the viscometer did not affect their relative viscosity (not shown).

From these data, it becomes apparent that while they do not significantly bind CB, the glutathionyl-

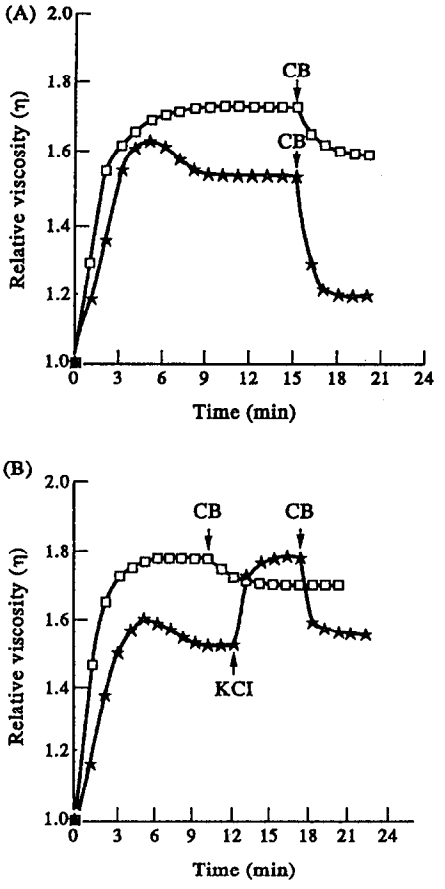

Fig. 2. (A) Effects of CB on the relative viscosity of $MgCl_2$ polymerized Glu-F-actin. CB (30 μM) was added at the time indicated by the arrows to glutathionyl-actin (☆—☆) and normal actin (□—□) solutions, both 20 μM, polymerized in an Ostwald viscometer by addition of 2 mM $MgCl_2$. (B) Effects of CB on the relative viscosities of Glu-F-actin polymerized in the presence of stabilizing K^+ ions. CB (30 μM) was added at the time indicated by the arrows to glutathionyl-actin (☆—☆) and normal actin (□—□) solutions, both 20 μM, prepolymerized in an Ostwald viscometer by addition of 2 mM $MgCl_2$, followed by 100 mM KCl (arrow) for glutathionyl-actin and by addition of 2 mM $MgCl_2$ plus 100 mM KCl for normal actin.

actin filaments are very sensitive to CB action. Thus, it would appear that binding of CB to the barbed end of actin filaments is not a prerequisite for filament shortening.

By electron microscopy, there was no qualitative difference between normal- and glutathionyl-actin filaments assembled in the presence of $MgCl_2$ or $MgCl_2$ plus KCl (Fig. 3, panels: a, c, e and g). The addition of CB to normal F-actin in the presence of $MgCl_2$ or $MgCl_2$ and KCl, resulted in shortening of the filaments (Fig. 3f and h), while it had a similar or even more pronounced effect on glutathionyl actin filaments (Fig. 3b and d). Very similar results were also obtained by electron microscopy of normal- and Glu-F-actin samples, which were not previously subjected to viscometric analysis (not shown). Although, as it is generally accepted, the assessment of filament fragmentation by electron microscopy is not a quantitative method, these results show a clear cut glutathionyl actin filament shortening induced by CB and corroborate with the viscosity experiments described above.

ATPase measurements in CB-treated glutathionyl-actin filaments

Our data showed that while CB does not bind significantly to Glu-F-actin, its ability to shorten those filaments is actually maintained. As a consequence, a higher reassembly rate of glutathionyl-actin subunits could be expected to occur at the expense of ATP, thus causing an increased ATPase activity. To investigate this possibility, we measured the ATPase activity in normal and glutathionyl-actin preparations in the presence of 30 μM CB. These data are depicted in Fig. 4. As it would be expected from previously reported values [13, 16], the ATPase activity of Glu-F-actin at room temperature was enhanced when compared with normal F-actin. However, in the presence of CB a significant increase in the ATPase activity was found (Fig. 4). Under the same experimental conditions and in line with earlier observations [20], normal F-actin showed a lower ATPase activity, which was slightly increased in the presence of CB (Fig. 4). These results indicate the presence of a higher rate of subunit reassembly in the CB-treated glutathionyl-actin filaments, most probably due to the existence of a higher number of uncapped barbed ends.

DISCUSSION

The aim of this study was to find out if cytochalasins can shorten actin filaments by a mechanism independent of barbed end binding and capping. Although this possibility has been discussed in the past, the experimental approaches used did not offer a conclusive answer to this question. Thus, by measuring elongation rate constants and critical concentrations it was reported that CB does not prevent monomer addition at the barbed end of actin filaments although it does slow down the rate of elongation [9]. Using fluorescence photobleaching recovery technique, a recent work suggested that CB binds to protomers within the filament rather than to the barbed end [21]. It has been also suggested that CB may have two competing activities, one for the barbed end and the other with lateral binding sites along the filament [10]. However, assuming that barbed end binding and capping is a predominant mechanism by which cytochalasins shorten F-actin [2–5], it is possible that this effect may mask any other mode of cytochalasin action, for example a possible "severing" effect, as has recently been proposed [6, 10–12]. To address this question, we searched for alternative methods which "quench" the cytochalasin interaction with the barbed end. This was finally accomplished by the introduction of a glutathione residue at Cys^{374}, thus creating the glutathionyl-Cys^{374}-actin.

We performed binding experiments of normal and substituted actin with a series of [^{3}H]CB concentrations, varying the ionic milieu. In the

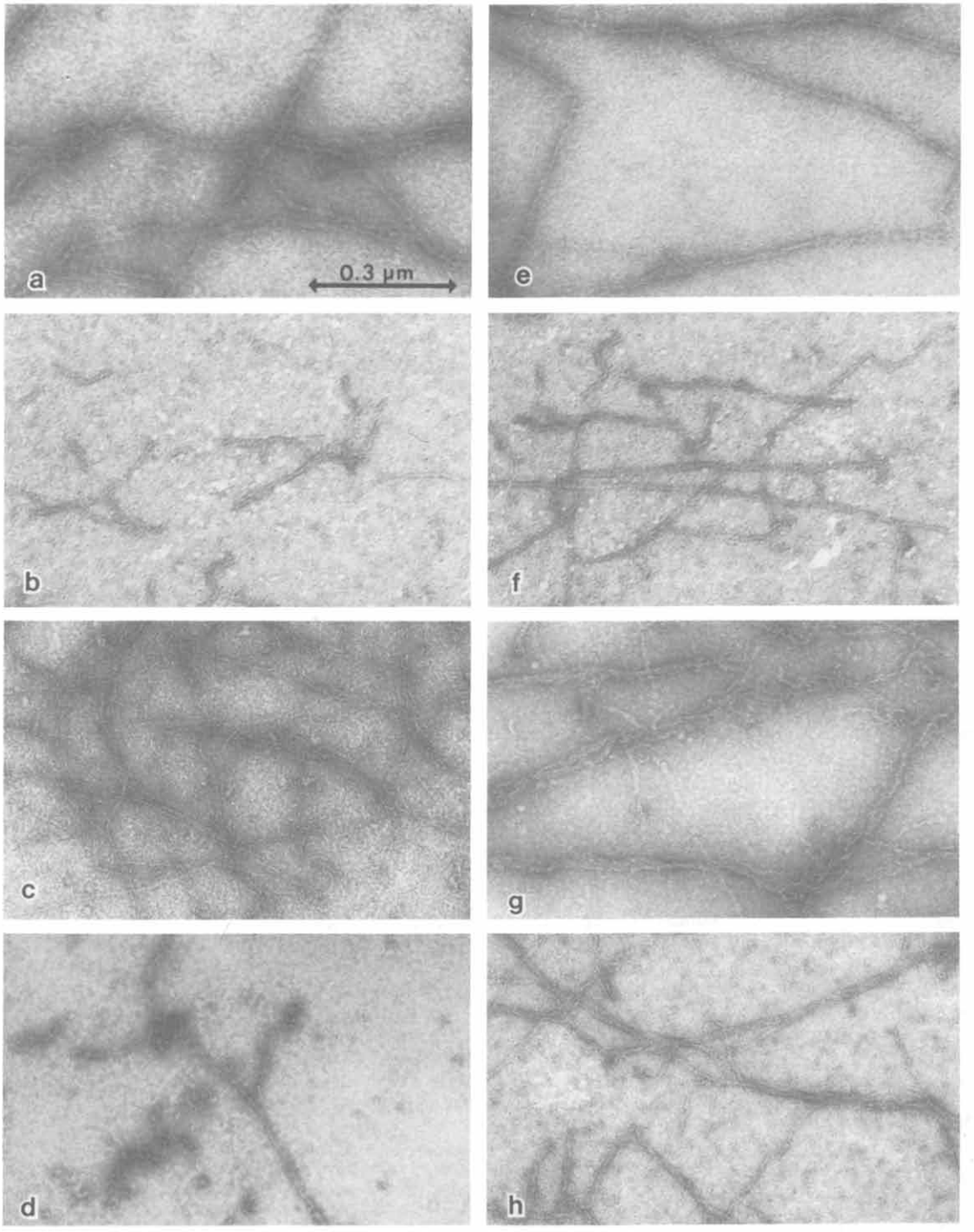

Fig. 3. Electron microscopy showing the effects of CB treatment on glutathionyl-actin filaments. Probes of Glu-F-actin (a,b,c,d) and F-actin (e,f,g,h) were obtained from the Ostwald viscometer corresponding to the experiments of Fig. 2A and B, as described in Materials and Methods. (a,e) Control polymerized in 2 mM $MgCl_2$. (b,f) Probes obtained after CB (30 μM) treatment, from samples polymerized in 2 mM $MgCl_2$. (c,g) Control polymerized in 2 mM $MgCl_2$–100 mM KCl. (d,h) Probes obtained after CB (30 μM) treatment from samples polymerized in 2 mM $MgCl_2$–100 mM KCl. Magnification × 70,000.

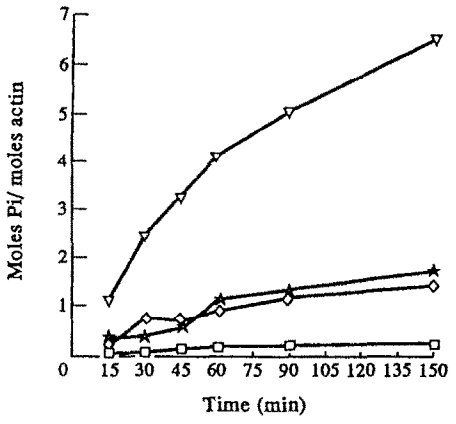

Fig. 4. Release of ^{32}Pi from [γ-^{32}P]ATP of polymerized F-actin and Glu-F-actin in the absence and presence of CB. Both actin preparations (20 μM) were polymerized in Buffer G containing 2 mM $MgCl_2$ and were kept at 25°. The release of ^{32}Pi from [γ-^{32}P]ATP was measured for F-actin in the absence (□—□) and presence of 30 μM CB (◇—◇) and for Glu-F-actin in the absence (☆—☆) and presence of 30 μM CB (△—△).

presence of Mg^{2+} or Mg^{2+} plus K^+ ions we failed to detect any high or low affinity binding of CB to Glu-F-actin as estimated by two different binding assays (DCC, ED). We concluded that the binding capacity of the substituted Glu-F-actin for CB under all experimental conditions used is practically undetectable. A possible explanation of this phenomenon could be the decrease in the hydrophobicity of the cytochalasin binding site which is located near the region where the hydrophilic glutathionyl residue was introduced [22]. This possibility was elucidated by binding experiments with Glu-F-actin in buffers containing glycerol, which increases the hydrophobicity of the binding envelope of receptors by driving out water molecules [19]. Thus, in the presence of 20% glycerol, CB bound to the substituted F-actin to a significant degree, which, however, is still lower than its binding to the unsubstituted F-actin as far as the number of binding sites but comparable as far as K_d. These data indicate that a possible interpretation of the observed lack of binding of CB to the modified F-actin is the decreased hydrophobicity at the CB binding site of the molecule due to the incorporation of the negatively charged glutathionyl residue.

Surprisingly, the Glu-F-actin appeared to be very sensitive to CB. Thus, following exposure to CB, the relative viscosity of the Glu-F-actin was clearly reduced indicating extensive filament fragmentation. This event was confirmed by electron microscopy, which also showed fragmentation of the glutathionyl-actin filaments. The CB effect to the modified actin was also shown in the presence of stabilizing K^+ ions, indicating that the filament fragmentation is not the result of the simple shearing stress of the viscometer. This conclusion is supported by control experiments which demonstrated the absence of any alterations in relative viscosities of the polymerized glutathionyl actin preparations after addition of the solvent solution of CB. This fragmentation resembled the one described for the filament shortening by severing, attributed to actin capping/severing proteins [23, 24].

The binding data were further supported by ATPase activity measurements. A significant increase of ATPase activity was found in glutathionyl-actin filaments, indicating the existence of a high proportion of barbed ends available for monomer addition.

While binding of CB to monomeric actin has not been demonstrated [2, 4, 25], cytochalasin D was found to bind to G-actin in the presence of low concentrations of Mg^{2+} or Ca^{2+} [26, 27]. Thus, a possible explanation of our surprising effect is that CB could bind preferably to the monomeric form of the substituted actin, thus affecting the association–dissociation rate at the ends of the polymer. However, our binding assays, showing a lack of significant high or low affinity binding of CB to glutathionyl-G-actin contradict such a hypothesis. In our view, CB seems to have a poisoning effect on glutathionyl actin filaments with characteristics of a "hit and run"/catalytic mode of action, characterized by a fast "off-rate". Such a mechanism, including catalytic poisoning of nucleation with weak filament capping, has also been recently suggested for profilin interaction with actin [28].

Although the mechanism of CB and Glu-F-actin interaction is not yet fully understood, our data clearly showed that the effect of CB is not solely due to capping the barbed end of actin filaments. Since cytochalasins have the ability to mimic the effects of actin binding proteins and were proposed as a possible model for the action of the physiologically important capping proteins [29], we think that the observed CB mode of action on Glu-F-actin may not be a peculiarity of this toxin, but it can also be attributed to actin capping/severing proteins. Thus, it could be very interesting to focus on the effects of actin capping proteins to the polymerization of glutathionyl actin, as well as to the binding kinetics and the effects of these proteins to the above polymer.

Thus, the glutathionyl actin filaments offer an interesting model to study not only the apparently complex mechanism of CB interaction with actin filaments, but also the association mechanism of the filaments with actin binding proteins.

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