# Crosslinking of actin filaments is caused by caldesmon aggregates, but not by its dimers

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A recent report by Bretscher [(1984) J. Biol. Chem. 259, 12873–12880] showed that caldesmon prepared by his method crosslinks actin filaments to form thick bundles. This is in contrast to the results of previous work that caldesmon binds to F-actin but does not cause any gelation [(1981) Proc. Natl. Acad. Sci. USA 78, 5652–5655]. The present work clearly showed that caldesmon purified according to Bretscher does not cause any gelation of F-actin. However, caldesmon aggregates formed by concentration or by freeze-thawing gelated F-actin to form bundles.

*Caldesmon* *Actin-associated protein* *Calmodulin-binding protein* *Actin-crosslinker* *Chicken gizzard*

## 1. INTRODUCTION

Caldesmon is an actin-associated protein that binds to calmodulin in a $Ca^{2+}$-dependent manner first discovered in chicken gizzard [1–4]. Caldesmon binds to actin filaments irrespective of $Ca^{2+}$ concentrations, but in the presence of calmodulin it binds to F-actin only in the absence of $Ca^{2+}$, since caldesmon is released from actin filaments by forming a complex with calmodulin in the presence of micromolar $Ca^{2+}$ [1,2]. This manner of regulation is called the flip-flop mechanism [5]. We have shown that actin filaments form bundles in a $Ca^{2+}$-dependent way in the system of caldesmon–calmodulin–actin binding protein (ABP, filamin [6,7])–F-actin [4]. Caldesmon itself does not cause the bundle formation of actin filaments [3].

Recently, Bretscher [8] has developed a simple method of purifying caldesmon from chicken gizzard, and claimed that caldesmon thus prepared crosslinked actin filaments to form microscopic bundles. We have confirmed Bretscher's preparation method of caldesmon, but reached the conclusion that the purified caldesmon, largely consisting of a heterodimer of 150 and 145 kDa, does not cause actin bundles. However, the aggregates of caldesmon formed by concentration or by freeze-thawing crosslinked actin filaments.

## 2. MATERIALS AND METHODS

Caldesmon was purified from chicken gizzard smooth muscle by two different procedures as in [1] and [8], respectively. Calmodulin was prepared from bovine brain as in [9]. Preparation of rabbit skeletal muscle actin was essentially the same as in [10].

$M_r$ values of caldesmon in solution were estimated by gel filtration in a Sepharose 4B column using thyroglobulin, ABP, ferritin, catalase and aldolase as $M_r$ markers.

Viscosity was measured by the falling ball method as in [11]. Samples negatively stained by

[+] Deceased September 23, 1984

*Abbreviation:* DTT, dithiothreitol

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1% uranylacetate were observed under a JEM 100S electron microscope at an accelerating voltage of 75 kV.

The protein concentration was determined as in [12] and for column monitoring as in [13].

## 3. RESULTS

Sobue et al. [1] purified caldesmon by calmodulin-Sepharose 4B column chromatography, whereas Bretscher separated caldesmon by Sephacryl S 400 column chromatography starting from heated gizzard suspensions [8]. The latter method is simpler than the former, and proteolytic cleavage of caldesmon during the preparation can be avoided. The final products of caldesmon purified by the two procedures were subjected to gel filtration using a Sepharose 4B column. Fig.1 shows the elution profiles which were essentially similar to each other.

The results in fig.1 suggested that the molecular masses of caldesmon in solution are the same independent of preparative method. However, while Sobue et al. [1] estimated it to be approx. 310 kDa, Bretscher described that caldesmon exists in solution as a monomer of approx. 120 kDa based on a sedimentation coefficient of 2.7 S and a Stokes radius of about 9.1 nm. Therefore, gel filtration was repeated with caldesmon prepared according to Bretscher's procedure together with markers. Fig.2 shows the result, confirming the previous suggestion that caldesmon is present as dimer in solution [1].

As already reported [1,3], the binding of caldesmon to F-actin results in only slight elevation of viscosity values or of degrees of flow birefringence. On the other hand, Bretscher demonstrated a remarkable gelation of the caldesmon–F-actin complex in solution and bundle formation detectable even under a light microscope [8]. This was not confirmed here, so far as freshly gel-filtered caldesmon preparations were tested. During the trials to elucidate the discrepancy on the important property of caldesmon between the two groups, reproducible procedures to confer the actin gelating action on caldesmon have been worked

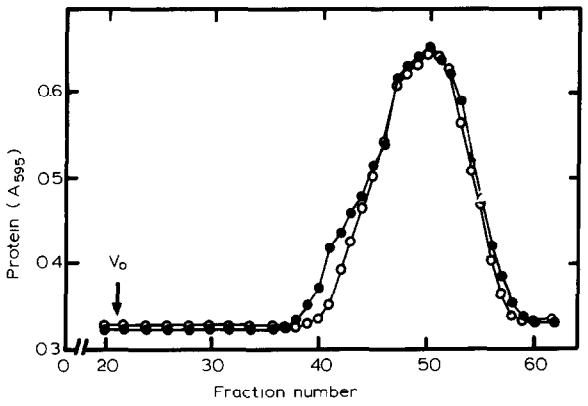


Fig.1. Gel filtration profile of caldesmon samples prepared by different methods. Purified samples were subjected to Sepharose 4B column (1.2 × 64 cm) equilibrated with a solution containing 0.1 M KCl, 0.1 mM EGTA, 0.1 mM DTT and 20 mM Tris-HCl (pH 7.5). Each fraction, 1 ml. ○, Prepared by the method of Sobue et al. [1]. ●, Prepared according to Bretscher [8].

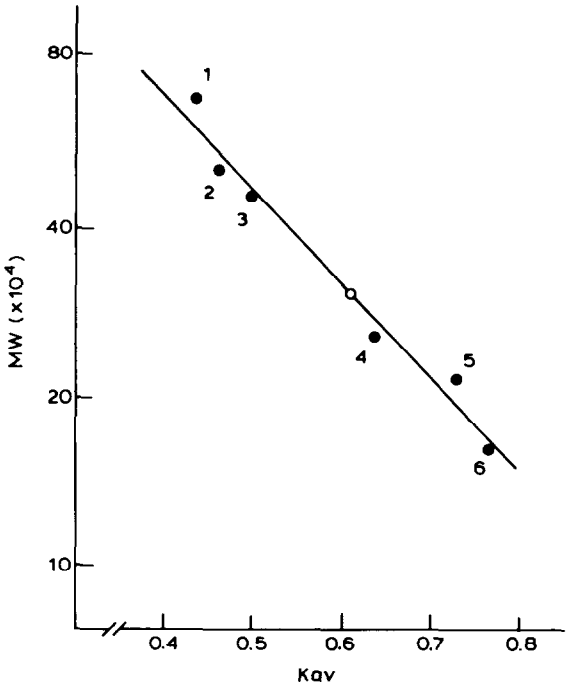


Fig.2. Determination of apparent molecular mass of caldesmon in solution by gel filtration. Conditions as in fig.1. ○, Caldesmon purified by Bretscher's procedure [8]. Molecular mass markers: 1, thyroglobulin (669 kDa); 2, ABP (filamin) dimer (500 kDa); 3, ferritin (440 kDa); 4, ABP monomer (250 kDa); 5, catalase (210 kDa); 6, aldolase (158 kDa).

out: concentration and freeze-thawing of fresh caldesmon preparations. Fig.3 summarizes the results with a caldesmon sample prepared by Bretscher's procedure which was concentrated to 4.5 mg/ml from 0.8 mg/ml. The original caldesmon solution did not cause gelation of F-actin even in the amount of 30% of F-actin by weight, whereas the concentrated sample gelated F-actin in the amount of 20% of F-actin. When this sample was clarified by centrifugation for 4 h at 150000 × *g*, the gelating action was markedly reduced. A sample recovered by suspending the sediments formed during centrifugation resulted in a complete gelation of F-actin at a concentration of 7% of F-actin. As shown in the inserts, the main band of the resuspended sample was the same as that of the original caldesmon solution in an SDS-gel electrophoresis [14]. The same result was obtained using caldesmon prepared according to the method

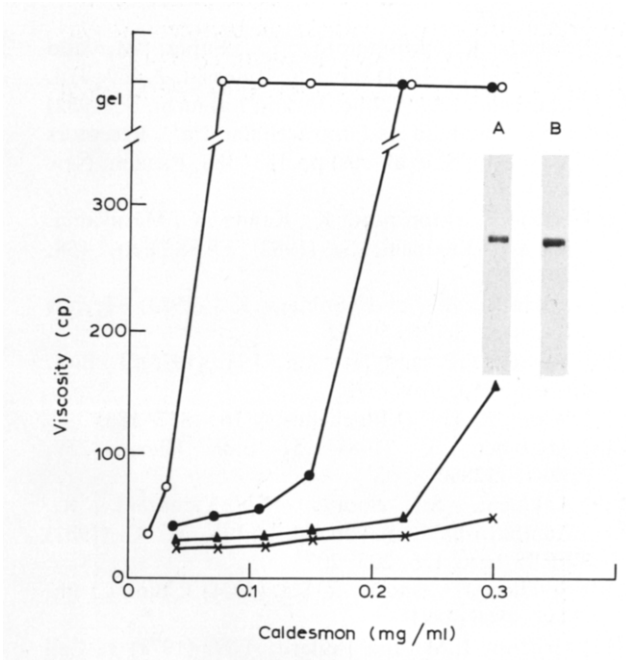


Fig.3. Effects of various preparations of caldesmon on the viscosity of an F-actin solution, 1.1 mg/ml, containing 0.1 M KCl, 0.5 mM EGTA, 0.1 mM DTT and 20 mM imidazole-HCl (pH 7.2). Viscosity was measured at 23°C by the falling-ball method [11]. (×) Sepharose 4B chromatographed caldesmon sample prepared by the Bretscher's method [8]; (●) concentrated sample; (▲) caldesmon sample clarified for 4 h at 150000 × *g* after concentration; (○) the sediment after clarification was vigorously suspended by a teflon-glass homogenizer, and centrifuged for 5 min at 1000 × *g*. The supernatant was used. Inserts: SDS-gel electrophoresis patterns of caldesmon samples. A, ○; B, ×.

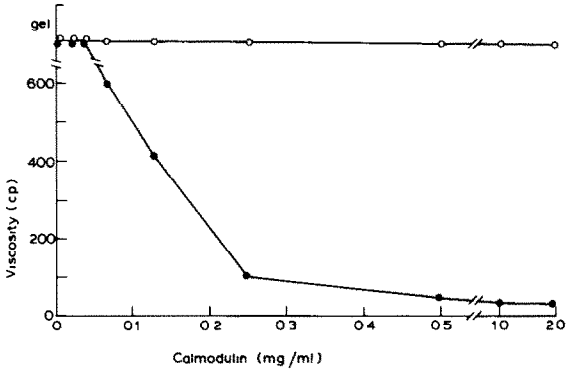


Fig.4. $Ca^{2+}$-dependent interactions of freeze-thawed caldesmon and F-actin in the presence of various amounts of calmodulin. Caldesmon prepared by the method of Sobue et al. [1] was frozen at −80°C and thawed. An F-actin solution, 1.1 mg/ml, contained 0.1 M KCl, 0.1 mM DTT, 20 mM imidazole-HCl (pH 7.2). Freeze-thawed caldesmon, 0.13 mg/ml. Viscosity was measured at 23°C by the falling-ball method [11]. (○) 0.5 mM EGTA; (●) 0.1 mM $CaCl_2$.

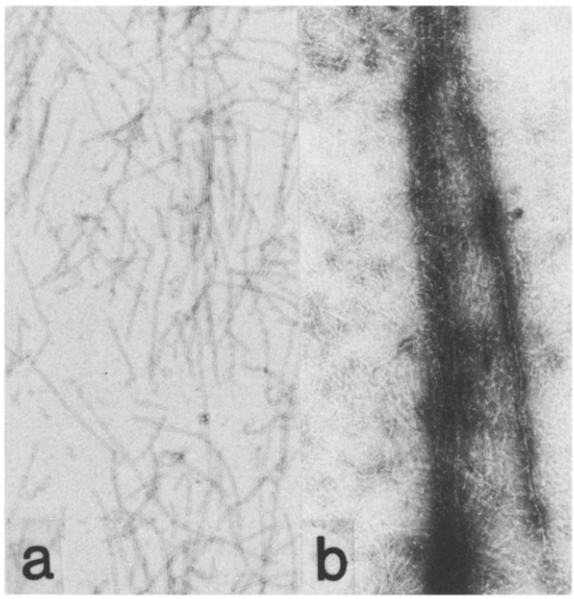


Fig.5. Electron micrographs of actin filaments in the presence of caldesmon. (a) Gel filtered caldesmon (20% F-actin by wt); (b) freeze-thawed caldesmon (20% F-actin). Bar, 0.5 μm.

of Sobue et al. As seen in fig.4, a freeze-thawed caldesmon sample also caused gelation of F-actin. Bundles of actin filaments formed by freeze-thawed caldesmon samples were observed under an electron microscope (fig.5). Therefore, it is very likely that the aggregates of caldesmon are responsible for gelation of F-actin.

Fig.4 shows the $Ca^{2+}$-dependent binding of freeze-thawed caldesmon to F-actin in the presence of various amounts of calmodulin. The extent of binding was estimated by the increase in viscosity of F-actin solution. The decrease in viscosity in the presence of $Ca^{2+}$ and calmodulin was to the same extent as intact caldesmon [1]. This result was very similar to that of Bretscher [8].

## 4. DISCUSSION

Bretscher's new procedure [8] to isolate caldesmon from chicken gizzard has an advantage in denaturing proteins other than caldesmon and tropomyosin by heat treatment to begin with. This study has confirmed that the method is useful and reproducible. The main product was the same as that purified by the original procedure of Sobue et al. [1]. As for an apparent discrepancy on the molecular mass of caldesmon in solution, since both determinations depended on rather limited techniques, it is highly desirable to settle this problem by a systematic investigation of the molecular size and shape of intact caldesmon molecule, e.g., sedimentation equilibrium, low-angle shadowed electron microscopy, etc.

Bretscher called caldesmon an actin-crosslinker [8]. This is true of the aggregates of caldesmon formed by concentration or by freeze-thawing. The extent of caldesmon aggregation was unclear, although largely pelleted by centrifugation for 4 h at 150000 × *g*. The aggregates were dissociated into monomers under the conditions for an SDS-gel electrophoresis. On the other hand, caldesmon freshly prepared by gel filtration did not cause any sign of gelation. Therefore, it is concluded that caldesmon in solution, very probably dimer, binds only to an actin filament but does not crosslink several actin filaments.

## ACKNOWLEDGEMENT

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