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Salt dependent dimerisation of caldesmon

R.A. Cross, K.E. Cross and J.V. Small

Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria

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Using analytical gel filtration (FPLC) we show here that avian gizzard caldesmon (chain molecular mass 150 kDa) self-associates to form end-to-end dimers. Increasing salt concentration promotes dimerisation: at 150 mM KCl, about 40% of the caldesmon was dimeric. Freshly gel filtered caldesmon had an actin gelating activity which decreased with increasing ionic strength. At 150 mM KCl, caldesmon at a 1:90 molar ratio to actin doubled the low shear viscosity of F-actin. Sixfold less filamin was required to produce the same effect.

Caldesmon; Muscle contraction; Actin binding protein; (Smooth muscle)

1. INTRODUCTION

Caldesmon is a heat stable 150 kDa actin-binding protein first isolated from smooth muscle [1], but present in a wide variety of cells [2]. Both its molecular conformation and its function are currently controversial. On the one hand it has been suggested to be a regulatory protein partly analogous to the troponin complex of skeletal muscle [3]. Caldesmon effects, in vitro and in the absence of calcium, an approx. 3-fold inhibition of the tropomyosin-actin-activated ATPase of myosin [4]. The binding of Ca-calmodulin to caldesmon has been found to relieve this inhibition [4], although probably not [3] in the simple flip-flop manner originally proposed [4]. On the other hand, caldesmon has been suggested to be an F-actin crosslinking protein. This proposal was first made by the discoverer of caldesmon [5], but later retracted on his finding [6] that freeze-thawed material had greater gelation activity than freshly prepared caldesmon. Correspondingly, a report that caldesmon is an F-actin bundling protein [7] has been qualified by its author after it was found that bundling appeared due to artefactual thiol-crosslinking of caldesmon [8]. Finally, there are reports that phosphorylation of caldesmon can reduce its inhibitory activity [9], and that phosphorylated caldesmon inhibits myosin light chain kinase [10].

Resolution of caldesmon's true structure and function depends heavily on reliable determination of the conformational state of the molecule in solution. Caldesmon was first thought to be a 300 kDa globular dimer [1]. Combined gel filtration and sucrose gradient centrifugation indicated an elongated monomer of Stokes radius 90 nm [7]. Our own low-angle rotary shadowing data suggested an elongated monomer which could form dimers in the 50% glycerol buffers used for electron microscopy [11]. Here we establish, using analytical gel filtration (FPLC), that caldesmon monomers associate to end-to-end dimers in solution at physiological ionic strength, and that the gel-filtered material does have a substantial effect on the low shear viscosity of F-actin. The data are consistent with caldesmon being an elongated, dimeric protein which has the capacity to crosslink F-actin.

Correspondence (present) address: R.A. Cross, MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 5AN, England

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2. MATERIALS AND METHODS

2.1. *Preparation of caldesmon*

We used a method slightly modified from that of Bretscher [7] to prepare turkey gizzard caldesmon. About 500 g finely minced muscle was washed twice at 4°C in 1 l of 40 mM KCl, 10 mM imidazole, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.2 mM EGTA, pH 6.5, using a Sorvall Omnimixer for resuspension and 10000 × *g* centrifugations for 30 min. The washed muscle was then similarly extracted with 25 mM $MgCl_2$, 40 mM KCl, 20 mM imidazole, 0.5 mM DTT, 0.5 mM EGTA, pH 7.5. The supernatant following centrifugation was brought rapidly to boiling point using a microwave oven and again centrifuged. The 30–55% ammonium sulphate cut of the resulting supernatant was dissolved in and dialysed against the wash buffer, and then applied to a 150 ml column of Q Sepharose in wash buffer. Elution was with a 2 × 500 ml gradient of 40–500 mM KCl in wash buffer at a flow rate of 250 ml/h. Electrophoretically pure caldesmon eluted ahead of tropomyosin, and was used without further purification.

2.2. *Preparation of actin*

Rabbit skeletal muscle F-actin was prepared according to [12], with a final gel filtration over Sephadex G-25.

2.3. *Preparation of filamin*

Turkey gizzard filamin was prepared from the residue remaining after caldesmon extraction. This residue was extracted with LAMES [11] and the supernatant following centrifugation made to 25 mM $MgCl_2$ and left overnight at 4°C. The resulting actomyosin precipitate was pelleted, and the 20–40% ammonium sulphate cut of the supernatant dialysed into 5 mM Tris-acetate, 0.1% 2-mercaptoethanol, pH 7.6. The dialysate was clarified (18000 × *g*, 30 min), and applied to a column of DEAE-Sepharose 6B-Cl equilibrated in the same buffer. The column was eluted with a 0–0.3 M KCl gradient, the filamin-containing fractions were pooled, concentrated by precipitation at 55% ammonium sulphate, dialysed into 30 mM KCl, 0.15 mM DTE, 1 mM $MgCl_2$, 5 mM imidazole, pH 7.3, and applied to a Sephacryl S-300 column equilibrated in the same buffer. Filamin-containing fractions were used directly, or further purified as necessary over a hydroxyapatite column.

2.4. *Preparation of calmodulin*

Bovine brain calmodulin was prepared according to [14] with slight modifications [11].

3. RESULTS

3.1. *Solution structure of caldesmon*

The available hydrodynamic data [7] indicate that caldesmon is an elongated monomer of Stokes radius 90 nm. Analytical gel filtration (FPLC, Superose 6 column) of caldesmon confirmed that this was the case at 60 mM KCl concentration (fig.1). However as the salt concentration was raised a leading shoulder and then a distinct peak appeared on the chromatogram ahead of the major peak, in a position corresponding to a doubling of the monomer Stokes radius (fig.1). Additionally the major peak was shifted so as to indicate an increase in the Stokes radius of the monomer at higher salt concentrations. The results demonstrate that caldesmon monomers can self-associate into dimers having twice the monomer Stokes radius. These must correspond to the end-to-end dimers visualised by electron microscopy [11]. Dimer and monomer appeared as quite distinct peaks, rather than as a broadened peak of intermediate mobility. This suggests that caldesmon did not transit rapidly between monomer and dimer states on the time scale of the experiments (40 min for one FPLC run). Interestingly, the mobility of the caldesmon dimer species on the columns corresponded closely to that of filamin, and the caldesmon monomer species to that of heavy merofilamin [16] (filamin 'monomer'). Caldesmon and filamin thus appear to have similar Stokes radii. They appear very similar in low-angle Pt rotary shadowing [11], in spite of their substantially different chain molecular masses (filamin is 250 kDa), consistent with caldesmon having a lower mass/unit length than filamin.

Note that the FPLC runs were performed by applying identical samples of gizzard caldesmon to columns preequilibrated at the various different salt concentrations. Following the high salt runs in which dimers were observed, application of a further aliquot of sample to a low salt column

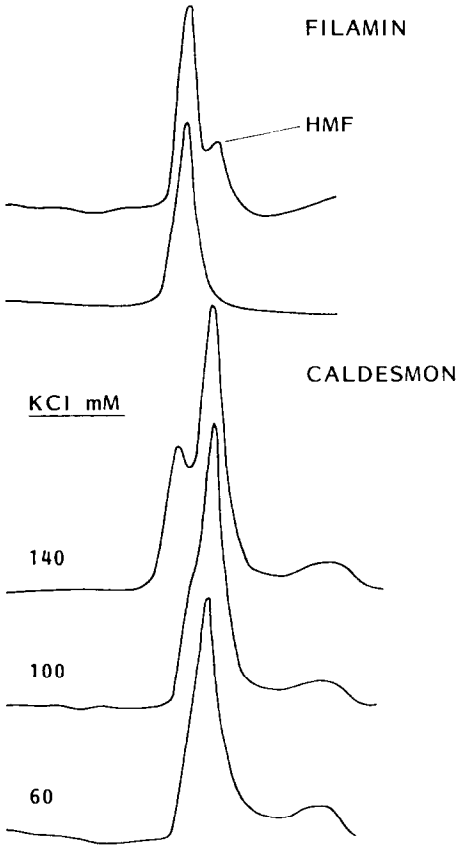

Fig.1. Analytical gel filtration. Caldesmon (lower 3 traces) was stored at a concentration of 1 mg/ml in 150 mM KCl Q-Sepharose buffer and was freshly filtered through 0.22 μM millipore membranes before application of 200 μl aliquots to HR 10/30 columns of Superose 6, preequilibrated at the salt concentrations indicated. Other buffer constituents were held constant. Elution was at 0.5 ml/min, corresponding to an operating pressure of about 0.5 MPa. The column was calibrated using Sigma globular protein standards, and a plot of K_d vs log M_r was linear at the flow rate used. The void volume was determined with Blue dextran, and the included volume with acetone. Filamin (upper traces) was run similarly.

reproduced the original single peak (fig.1a), excluding thereby that the observed dimerisation was a result of time-dependent formation of S-S bridges [8] between monomers. Addition of excess bovine brain calmodulin to the sample (5 mol calmodulin/mol caldesmon, 150 mM salt buffer) did not detectably affect the dimerisation in either the presence or absence of 100 μM Ca.

3.2. *Effects of caldesmon on F-actin viscosity*

The finding that caldesmon can reversibly dimerise raises the possibility that a functional caldesmon dimer could interact equivalently with two different actin filaments. There have been previous reports of an effect of caldesmon on the viscosity of F-actin, but their significance has been confused by the tendency of caldesmon toward aggregation. The experiments described above provided material of defined conformation, which was immediately tested for its effect on F-actin viscosity.

Freshly gel-filtered caldesmon from either the leading or trailing component of the double peak (fig.1c) increased the low-shear viscosity of F-actin (fig.2). The two fractions had closely similar effects, with an increase in potency as the salt concentration was lowered. At physiological ionic strength (150 mM KCl buffer), a molar ratio of 1

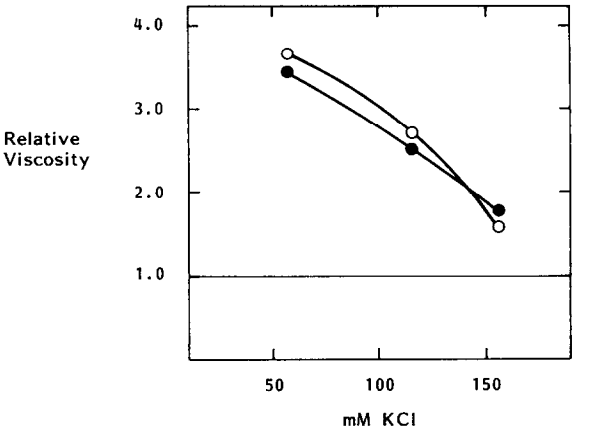

Fig.2. Low-shear viscometry. Gel-filtered G-actin in a buffer of 2 mM Tris, 0.2 mM ATP, 0.5 mM 2-MCE, 0.2 mM $CaCl_2$, 0.005% NaN_3, pH 8.0, was polymerised in 100 μl capillaries at 25°C by the addition of $MgCl_2$ to 5 mM and varying amounts of 3 M KCl, in either the presence or absence of freshly gel-filtered caldesmon. The molar ratio of caldesmon to actin monomer was 1:90, assuming a molecular mass for caldesmon of 300 kDa. After 1 h at 25°C, triplicate capillaries were clamped at 60° to the vertical, and the descent rate for 0.64 mm stainless steel balls rolling under gravity at 25°C was recorded. Relative viscosity is the ratio of the descent rates for caldesmon-actin to those of appropriate actin-only controls. (○) Caldesmon from the trailing (monomer) peak obtained by FPLC gel filtration at 150 mM KCl buffer. (●) Caldesmon from the corresponding leading (dimer) peak.

caldesmon dimer to 90 actin monomers was enough to double the low-shear viscosity of F-actin (fig.2). For comparison, filamin produced under the same conditions an equivalent effect at a six-fold lower molar ratio to actin.

4. DISCUSSION

Here we have established that in solution at physiological ionic strength caldesmon forms non-covalently linked dimers, that this material does increase F-actin low-shear viscosity, but that the activity is highly salt dependent and hence easy to overlook. Marston and Smith [3] have emphasised the strong influence of temperature and ionic conditions on caldesmon-actin interactions. Combination of their data with our own suggests that increasing salt concentration to physiological levels has the dual effect of weakening the binding of caldesmon to actin and strengthening caldesmon's end-to-end self-association. The net effect is apparently to produce a slight negative dependence of the actin-gelating activity on salt concentration (fig.2). The present work thus reemphasises the need to concentrate attention on caldesmon's behaviour in the physiological range of ionic conditions. That dimerisation becomes stronger with increasing ionic strength suggests an involvement of hydrophobic forces in stabilising the dimers. Calmodulin however had no detectable effect on the dimerisation.

Concerning the function of caldesmon, we wish to suggest here a speculative model by which the present and much of the existing data can be reconciled. Important points are as follows. Firstly, the specific end-to-end dimerisation of caldesmon indicates that the two ends of the caldesmon monomer are nonequivalent. Secondly, chymotryptic cleavage of the caldesmon molecule generates an actin, tropomyosin and calmodulin-binding 40 kDa N-terminal fragment and a 100 kDa C-terminal species [17,18], of unknown function, indicating that one end only of each caldesmon chain binds actin. Lastly, freshly gel filtered caldesmon increases F-actin low-shear (this work) and high-shear [11] viscosity. Taken together, these data suggest the scheme outlined in fig.3, in which caldesmon is proposed to operate similarly to filamin [16] in making flexible crosslinks between adjacent actin filaments. Note however that in comparison to filamin, both caldesmon's actin-binding and its dimerisation appear relatively weak around physiological ionic strength, perhaps providing for slippage between linked actin filaments. Such a property might be consistent with an involvement of caldesmon in the observed slow relaxation of smooth muscle. In the scheme shown, the N-terminal domain of each 150 kDa caldesmon chain would act under relaxing conditions to inhibit the actin-tropomyosin-activated myosin ATPase as described [17], whilst its C-terminal domain would associate with a partner on an adjacent actin filament and resist relaxation.

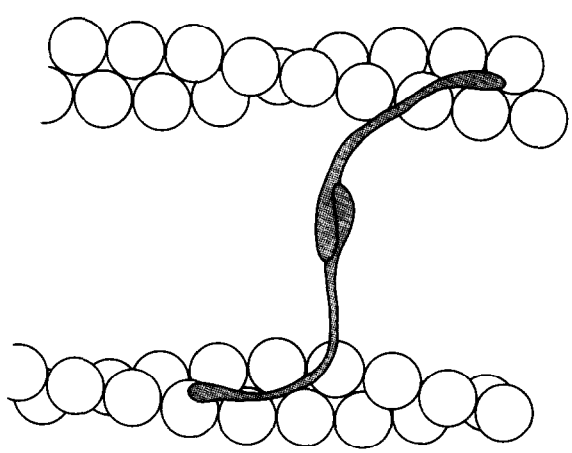

Fig.3. A notional model for the caldesmon-actin interaction.

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