Utilization of troponin C as a model calcium-binding protein for mapping of the calmodulin-binding sites of caldesmon

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Troponin C, a structural analogue of calmodulin, was used for mapping the calmodulin-binding sites of caldesmon. The apparent K_d values for the formation of the caldesmon–calcium-binding-protein complex as determined by native gel electrophoresis were 0.5, 1.2 and 3.9 μM for calmodulin, rabbit skeletal muscle troponin C and bovine cardiac troponin C respectively. Troponin C induced a 4–6 nm blue shift of the Trp fluorescence of caldesmon without affecting the amplitude of fluorescence. In the presence of Ca^{2+} , troponin C induced partial displacement of caldesmon from actin–tropomyosin complexes. Addition of 5,5'-dithiobis(nitrobenzoic) acid to an equimolar complex of

caldesmon and troponin C induced disulphide cross-linking between Cys-98 of rabbit skeletal muscle troponin C and the single Cys residue of duck gizzard caldesmon, located in a position analogous to Cys-580 of the chicken gizzard protein. The cross-linked caldesmon–troponin C complex was ineffective in inhibiting actomyosin ATPase activity. It is concluded that Cys-580 of caldesmon can be located close to both the central helix of calcium-binding proteins and the C-terminal domain of actin. This may be important for the regulation of actomyosin ATPase activity by caldesmon.

INTRODUCTION

Caldesmon is actin-bound protein that is involved in regulation of the contractile activity of smooth muscle and non-muscle cells [1–3]. The inhibitory action of caldesmon on actomyosin ATPase activity is regulated by a calcium-binding protein that has not yet been identified, and calmodulin is a candidate for fulfilling this function [1–3]. Three calmodulin-binding sites have been identified in the C-terminal regulatory domain of caldesmon [4,5], and a model of caldesmon–calmodulin interaction has been proposed in the literature [3,6]. Some aspects of this model were tested by using deletion fragments of caldesmon and calmodulin mutants [6–8]. At the same time, many questions regarding calmodulin–caldesmon binding remain unanswered.

The regulatory mechanism of calmodulin–caldesmon action is similar to that of troponin complex [9], and the calcium-binding subunit of the troponin complex (troponin C) is highly similar to calmodulin [10]. Under *in vitro* conditions, troponin C interacts with caldesmon [11] and effectively reverses the inhibitory action of caldesmon on actomyosin ATPase activity [12]. Both skeletal and cardiac muscle troponin C contains Cys residues that are absent from calmodulin from animal tissues [10]. All of these features make troponin C a valuable model calcium-binding protein that is useful for the mapping of calmodulin-binding sites in the caldesmon structure. The present paper investigates the caldesmon–troponin C interaction and provides further experimental verification of the proposed model of calmodulin binding to caldesmon [3,6].

MATERIALS AND METHODS

Protein preparation

Bovine heart and rabbit skeletal muscle troponin C and bovine brain calmodulin were isolated as described previously [13–15]. Duck and chicken gizzard caldesmon and duck gizzard tropomyosin were purified as described by Vorotnikov and Gusev [16]. Rabbit skeletal muscle actin and myosin were obtained as described in [12], and were kindly provided by Dr. D. I. Levitsky

(A. N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow, Russia). The purity of proteins was determined by SDS/PAGE [17], and the protein concentration was determined spectrophotometrically using molar absorption coefficients published in the literature.

Interactions of caldesmon with calcium-binding proteins

Quantitative native gel electrophoresis under non-denaturing conditions [18] was used to investigate the binding of calmodulin and troponin C to caldesmon. Electrophoresis was performed in 80 mM Tris/glycine, pH 8.3. Calcium-binding proteins were titrated by caldesmon in the presence of 0.2 mM CaCl_2 or 2 mM EGTA, and the intensity of the band of free calcium-binding protein was plotted against the total concentration of caldesmon [11,18].

Effect of troponin C on caldesmon binding to actin–tropomyosin

Rabbit skeletal muscle F-actin (5 μM) and duck gizzard tropomyosin (1 μM) in 15 mM imidazole (pH 7.0) containing 5 mM MgCl_2 , 2 mM ATP, 50 mM NaCl and 20 mM β -mercaptoethanol were mixed with duck gizzard caldesmon (0.5 μM) and incubated at room temperature in the presence of rabbit skeletal muscle troponin C (0–6 μM). The incubation mixture contained either 0.1 mM CaCl_2 or 2 mM EGTA. Protein samples were subjected to ultracentrifugation (130 000 g, 1 h) and the compositions of the supernatant and pellet were determined by quantitative SDS/PAGE. By plotting the quantity of caldesmon detected in the supernatant against the total concentration of troponin C added, we could determine the ability of troponin C to displace caldesmon from the actin–tropomyosin complex.

Fluorescence spectroscopy

The effect of calcium-binding proteins on the intrinsic Trp fluorescence of caldesmon was used to analyse the interactions of

Abbreviation used: DTNB, 5,5'-dithiobis(nitrobenzoic) acid.

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caldesmon with troponin C and calmodulin [7,19]. Caldesmon (200 nM) in 10 mM Tris/HCl, pH 7.4, containing 100 mM KCl, 0.1 mM CaCl_2 and 4.2 mM β -mercaptoethanol was titrated with increasing quantities of calcium-binding proteins, and the Trp fluorescence of caldesmon was excited at 290 nm and measured at 300–400 nm on a Hitachi F-3000 spectrofluorimeter [7].

Disulphide cross-linking of caldesmon and troponin C

Two methods were used to cross-link caldesmon and troponin C. In method 1, reduced duck or chicken gizzard caldesmon in 10 mM Hepes/KOH, pH 7.0, was mixed with 5,5'-dithiobis(nitrobenzoic) acid (DTNB) dissolved in 100 mM Tris/HCl buffer, pH 7.3. The final concentrations of caldesmon and DTNB were 80 and 100 μM respectively. The mixture was incubated for 30 min at 30 °C. Reduced troponin C in quantities equimolar to that of caldesmon was added to the mixture (the final concentration of both troponin C and caldesmon was 40 μM) and the incubation was continued for another 30 min at 30 °C. The remaining free SH groups were blocked by addition of excess DTNB. In method 2, reduced caldesmon and troponin C in 10 mM Hepes/KOH (pH 7.0) containing 0.2 mM CaCl_2 were mixed in equimolar quantities (the final concentration of both proteins was 20 μM). DTNB was added to a final concentration of 20 μM and the mixture was incubated for 2 h at 20 °C. The yield of the cross-linked product (about 15–20 %) was comparable with the two methods, and was slightly diminished (up to 10–12 %) when the incubation mixtures were supplemented with 0.15 M NaCl.

The cross-linked complex was separated from free caldesmon and troponin C by ion-exchange chromatography on Q-Sepharose, equilibrated with 20 mM Tris/HCl, pH 7.5, containing 30 mM NaCl and 2 mM EGTA. The incubation mixture obtained after cross-linking by method 1 was loaded on the Q-Sepharose column (1.2 cm $\times$ 7 cm) and eluted by a linear (30–530 mM) gradient of NaCl. Under the conditions used, free caldesmon was eluted at 170 mM NaCl, the cross-linked complex appeared at 300 mM NaCl and free troponin C, being the most tightly bound, was eluted at 500 mM NaCl.

Cross-linking of isolated caldesmon

Two approaches were used for cross-linking of isolated duck or chicken gizzard caldesmon. Caldesmon (0.7 mg/ml) in 100 mM Tris/HCl, pH 7.4, containing 1 mM CaCl_2 was mixed with copper(II) *o*-phenanthroline (final concentration 0.5 mM) and incubated for 3 h at room temperature. The reaction was stopped by the addition of 5 mM EGTA. A similar approach was used previously for disulphide cross-linking of chicken gizzard caldesmon [20]. Alternatively, caldesmon (final concentration 20 μM) in 20 mM Tris/HCl, pH 7.5, was mixed with DTNB (final concentration 10 μM) and incubated for 3 h at 30 °C.

The formation of cross-linked caldesmon–troponin C complexes and oligomers of caldesmon was determined by gradient (5–18 %) PAGE in the presence of SDS. The apparent molecular mass was determined by using protein standards with molecular masses in the range 14–205 kDa.

ATPase measurements

Actomyosin ATPase activity was determined in 10 mM Pipes/KOH (pH 7.0) containing 5 mM MgCl_2 and 30 mM KCl. As a rule the incubation mixture contained 10 μM rabbit skeletal muscle actin, 2 μM duck gizzard tropomyosin, 1.6 μM rabbit skeletal muscle myosin and the desired quantity of cross-linked caldesmon–troponin C complex. Half of the samples contained

caldesmon–troponin C complex which was not reduced by β -mercaptoethanol, and the other half contained the same complex that had been incubated with 500 mM β -mercaptoethanol for 30 min at 30 °C. The reaction was started by the addition of ATP (final concentration 3 mM) and terminated by the addition of trichloroacetic acid after a 5 min incubation at 30 °C. P_i liberated during the incubation was determined by the method of Taussky and Schorr [21]. Under the conditions used the ATPase activity of actomyosin in the absence of caldesmon was 350–400 nmol of P_i /min per mg of myosin; this value was taken to represent 100 % activity.

RESULTS

Interaction of troponin C with caldesmon

We used three approaches for the investigation of troponin C binding to caldesmon. The first method consisted of titration of troponin C by increasing quantities of caldesmon and concomitant analysis of the composition of the incubation mixture by native gel electrophoresis. In the presence of Ca^{2+} , the addition of caldesmon was accompanied by a decrease in the intensity of the band representing free troponin C, due to the formation of caldesmon–troponin C complexes. In the absence of Ca^{2+} , caldesmon induced only a very small decrease in the intensity of the troponin C band (Figure 1, curve 4). The data in Figure 1 indicate that the apparent affinity of rabbit skeletal muscle troponin C for caldesmon is comparable with that of calmodulin, whereas bovine cardiac troponin C interacts with caldesmon with a slightly lower affinity. The apparent dissociation constants determined by this method were 0.5 ± 0.2 μM for calmodulin and 1.2 ± 0.4 and 3.9 ± 0.6 μM for skeletal muscle and cardiac troponin C respectively. These data agree with our earlier results [18] and indicate that, in sharp contrast with its interactions with many other calmodulin-binding proteins, caldesmon interacts with skeletal muscle troponin C with an affinity comparable with that for calmodulin. The lower affinity of cardiac troponin C for

Figure 1 Titration of calmodulin (curve 1), rabbit skeletal muscle troponin C (curve 2) and bovine cardiac troponin C (curves 3 and 4) with duck gizzard caldesmon

The calcium-binding proteins were present at 9.5 μM (5 μg per track). Titration was performed either in the presence of 0.2 mM CaCl_2 (curves 1–3) or in the presence of 2 mM EGTA (curve 4). The percentage of the calcium-binding protein remaining unbound is plotted against the total concentration of caldesmon added. Solid lines represent the best-fit curves. K_d values were determined as described in [18], and were 0.5, 1.2 and 3.9 μM for calmodulin, skeletal muscle troponin C and cardiac troponin C respectively.

Figure 2 Titration of caldesmon by calmodulin (curve 1) and rabbit skeletal muscle troponin C (curve 2)

The relative fluorescence (F/F_0) was measured at 320 nm. The smooth curve through the data points was calculated for the binding of 1 mol of calmodulin per mol of caldesmon. The K_d was determined as described in [7], and was 90 nM.

caldesmon may result from some peculiarities of its structure. The N-terminal α -helix of cardiac troponin C is different from that of its skeletal muscle counterpart, and the first calcium-binding site of cardiac troponin C is defective and cannot bind bivalent cations. We have shown previously that calmodulin mutants with defective first or second calcium-binding sites interact with caldesmon with significantly decreased affinity [7].

Characterization of the caldesmon–troponin C complex was continued by investigation of the effect of troponin C on the intrinsic Trp fluorescence of caldesmon. It is known that, upon binding, calmodulin increases the Trp fluorescence of caldesmon [7,19]. Indeed, addition of calmodulin induced a 1.6–1.8-fold increase in the Trp fluorescence of caldesmon at 320 nm (Figure 2), and the fluorescence maximum was shifted from 355 to 345 nm. Addition of small quantities of rabbit skeletal muscle (or bovine cardiac) troponin C was accompanied by a very small decrease in the caldesmon Trp fluorescence at 320 nm; this later returned to the initial level observed in the absence of calcium-binding proteins (Figure 2). At the same time, troponin C also induced a blue shift of the peak Trp fluorescence from 355 to 349–351 nm (Figure 3). The peak of fluorescence of free caldesmon or its complex with troponin C was rather broad, and the shift in the fluorescence maximum was very small (Figure 3). Therefore we were unable to detect any significant changes in the fluorescence of caldesmon upon its titration with troponin C in the range 320–350 nm.

These data indicate that the local environment of Trp residues or their mobility in caldesmon–calmodulin and caldesmon–troponin C complexes is slightly different. Fluorescence spectroscopy is very sensitive to even minor changes in the structure of calcium-binding proteins. Therefore some point mutations of calmodulin significantly affect the sign and amplitude of calmodulin-induced changes in caldesmon Trp fluorescence [6,7]. In this respect, troponin C seems to be similar to calmodulin mutant F92A, which also induces a small blue shift in the Trp fluorescence maximum and has practically no effect on the amplitude of fluorescence of a C-terminal deletion mutant of caldesmon, 658C [6]. Although the fluorescence changes induced by troponin C were not completely identical to those induced by calmodulin, the data from native gel electrophoresis indicate that

Figure 3 Fluorescence spectra of incubation buffer (curve 1), rabbit skeletal muscle troponin C (2), duck gizzard caldesmon (3) and a mixture of troponin C and caldesmon (4)

The protein concentration was 200 nM for caldesmon and 3 μ M for troponin C. Fluorescence was excited at 290 nm (slit width 5 nm) and recorded in the range 310–390 nm (slit width 1.5 nm).

Figure 4 Release of caldesmon from actin–tropomyosin complexes by skeletal muscle troponin C

The mixture of actin (5 μ M), tropomyosin (1 μ M) and caldesmon (0.5 μ M) was titrated with rabbit skeletal muscle troponin C in the presence of 1 mM EGTA (curve 1) or 0.2 mM CaCl_2 (curve 2), and the quantity of caldesmon remaining in the supernatant after ultracentrifugation was determined by quantitative SDS/PAGE and expressed as a percentage of the total added.

the two calcium-binding proteins possess a similar affinity for caldesmon.

Next, we analysed the effect of troponin C on caldesmon binding to actin–tropomyosin complexes. It is well known that, under certain conditions, the addition of rather a large excess of calmodulin or S-100 protein results in partial dissociation of caldesmon from actin or actin–tropomyosin complexes [22–24]. We analysed the effect of rabbit skeletal muscle troponin C on the interaction of caldesmon with the actin–tropomyosin complex. The mixture of actin, tropomyosin and caldesmon was titrated by rabbit skeletal muscle troponin C in both the presence and the absence of CaCl_2 . The protein mixture was subjected to ultracentrifugation and the quantity of caldesmon remaining in the supernatant was plotted against the total concentration of troponin C. In the absence of troponin C only 15% of the caldesmon was not bound to actin–tropomyosin (Figure 4).

Addition of 2–6 μ M troponin C resulted in a greater than 2-fold increase in the quantity of caldesmon remaining in the supernatant after ultracentrifugation (Figure 4). This effect of troponin C was observed only in the presence of CaCl_2 . Thus troponin C, like calmodulin [22,23] and S-100 protein [24], can induce the partial displacement of caldesmon from actin–tropomyosin complexes. The similarity in the behaviour of calmodulin and troponin C makes possible the utilization of troponin C as a model calcium-binding protein for the mapping of contact sites in caldesmon–calmodulin complexes.

Cross-linking of caldesmon and troponin C

The recently published model of the caldesmon–calmodulin complex [3,6] predicts that the central helix of calmodulin is located in the vicinity of Cys-580 of caldesmon. It is difficult to check this suggestion using wild-type calmodulin, which does not contain Cys residues. However, rabbit skeletal muscle troponin C contains Cys-98 located in the central α -helix, and bovine cardiac troponin C contains Cys-35 in the first defective calcium-binding loop and Cys-84 in the central helix. This makes both isoforms of troponin C valuable calmodulin analogues that can be used for verification of the model of caldesmon–calmodulin interaction.

The primary structure of duck gizzard caldesmon is unknown, but a previous study indicated that this protein contains a single Cys residue located in the C-terminal region, in a position analogous to that of Cys-580 of chicken gizzard caldesmon [16]. Indeed, chemical cleavage of duck gizzard caldesmon at Cys residues results in the production of only two peptides, a large N-terminal peptide of 115 kDa and a small C-terminal peptide of 21 kDa [25]. Complete chemical cleavage of chicken gizzard caldesmon at Cys residues produces three peptides with molecular masses of 24, 85 and 22 kDa, corresponding to the N-terminal, central and C-terminal fragments respectively of the protein [25]. Since the sizes of the C-terminal peptides of duck and chicken gizzard caldesmon are practically identical, we may conclude that the single Cys of duck gizzard caldesmon is located in a position analogous to Cys-580 of chicken gizzard caldesmon. The presence of a single Cys residue simplifies the interpretation of the results obtained on cross-linking of caldesmon and troponin C through Cys residues.

If the proposed model of the caldesmon–calmodulin complex is correct, and if troponin C interacts with caldesmon in a manner similar to that of calmodulin, then we can expect that Cys-580 of caldesmon and Cys-98 of troponin C will be located close to each other. In this case mild oxidation of Cys residues will lead to the formation of a cross-linked disulphide complex. When duck gizzard caldesmon having its single Cys residue activated by DTNB was mixed with reduced skeletal muscle troponin C, we observed the formation of a new band with a molecular mass of 170 kDa (Figure 5A, track 2). This band disappeared after reduction with excess β -mercaptoethanol (Figure 5A, track 6). Similar results were obtained when DTNB was added directly to caldesmon–troponin C complexes formed in the presence of Ca^{2+} (method 2 in the Materials and methods section). We failed to observe the formation of a cross-linked complex when troponin C modified at Cys-98 by DTNB was mixed with reduced caldesmon. Similar results were obtained with bovine cardiac troponin C.

It is known that chicken gizzard caldesmon tends to aggregate and is able to form intra- and inter-molecular disulphide cross-links upon mild oxidation [20,26]. In order to check the possibility that the 170 kDa band consists of cross-linked caldesmon, we subjected duck and chicken gizzard caldesmon to mild oxidation.

Figure 5 Disulphide cross-linking of duck (A) or chicken (B) gizzard caldesmon with rabbit skeletal muscle troponin C

Samples were: free caldesmon (track 1), cross-linked mixture of caldesmon and troponin C (track 2), free caldesmon oxidized by copper(II) *o*-phenanthroline (track 3) or DTNB (track 4), cross-linked caldesmon–troponin C complex (track 5), cross-linked mixture of caldesmon and troponin C after reduction by β -mercaptoethanol (track 6), and caldesmon oxidized by copper(II) *o*-phenanthroline or DTNB after reduction by β -mercaptoethanol (tracks 7 and 8 respectively). Arrows indicate the positions of cross-linked complex (C), free caldesmon (CaD) and free troponin C (TnC).

Oxidation by copper(II) *o*-phenanthroline or DTNB had no effect on the electrophoretic mobility of duck gizzard caldesmon (Figure 5A, tracks 3 and 4), and resulted in the production of a number of high-molecular-mass bands in the case of chicken gizzard caldesmon (Figure 5B, tracks 3 and 4). The difference in behaviour of the duck and chicken gizzard caldesmons seems to be due to the fact that the chicken protein contains two Cys residues, whereas duck caldesmon contains only one. Since upon oxidation duck gizzard caldesmon is unable to form high-molecular-mass complexes, we concluded that the 170 kDa protein detected after incubation of duck gizzard caldesmon with troponin C corresponds to the cross-linked caldesmon–troponin C complex. It is worth noting that the mild oxidation of a mixture of chicken gizzard caldesmon and troponin C by DTNB results in the formation of the same protein band of 170 kDa (located just above the band of free caldesmon) (Figure 5B, track 2) in addition to caldesmon oligomers (Figure 5B, tracks 3 and 4). Since the protein composition of a mixture of oxidized chicken gizzard caldesmon and troponin C was complicated, we used duck gizzard caldesmon for the purification of the cross-linked complex of molecular mass 170 kDa.

The 170 kDa complex was separated from free caldesmon and troponin C by means of ion-exchange chromatography on Q-Sepharose. The pI values of free caldesmon and troponin C are 5.4 and 3.7 respectively, and we expected the cross-linked caldesmon–troponin C complex to have an intermediate pI value. Indeed, the 170 kDa protein was eluted from Q-Sepharose immediately after caldesmon and before troponin C. Although

Figure 6 Effects of non-reduced (curve 1) and reduced (curve 2) cross-linked caldesmon–troponin C complexes on the ATPase activity of skeletal muscle actomyosin

Protein concentrations: skeletal muscle myosin, 1.6 μ M; skeletal muscle actin, 9.6 μ M; smooth muscle tropomyosin, 2.1 μ M. All measurements were performed in the presence of 0.1 mM CaCl_2 . ATPase activity is expressed as a percentage of that in the absence of caldesmon–troponin C complexes.

we were unable to resolve the peaks of caldesmon and the 170 kDa protein completely, the preparation obtained after ion-exchange chromatography was significantly enriched in the 170 kDa complex (Figure 5A, track 5). When this protein was reduced by an excess of β -mercaptoethanol, the intensity of the caldesmon band was increased and a very faint band of troponin C appeared at the end of the gel (Figure 5A, track 6). These results indicate that the 170 kDa complex is indeed formed by caldesmon and troponin C cross-linked via Cys residues.

Effect of the cross-linked caldesmon–troponin C complex on the ATPase activity of actomyosin

Caldesmon inhibits actomyosin ATPase activity by direct interaction with actin and tropomyosin [1,3,9]. One of the putative tropomyosin-binding sites is located close to Cys-580 of caldesmon [1,3], and this residue can be cross-linked to Cys-374 of actin in actin–tropomyosin–caldesmon complexes [20]. Therefore we expected that cross-linking of troponin C to Cys-580 of caldesmon would affect the ability of the latter to inhibit the ATPase activity of actomyosin. Addition of cross-linked caldesmon–troponin C complex up to 1 mol of caldesmon/7 mol of actin produced a rather weak inhibition of ATPase activity (Figure 6). Reduction of this complex by excess β -mercaptoethanol significantly increased the inhibitory action of caldesmon on actomyosin ATPase activity (Figure 6).

DISCUSSION

The data presented here, together with our earlier results, indicate that troponin C interacts effectively with caldesmon and reverses the caldesmon-induced inhibition of actomyosin ATPase [11,12]. The affinity of troponin C for caldesmon is only 3–5 times less than that of calmodulin. This is unexpected since, as a rule, troponin C is ineffective in the activation of calmodulin-dependent enzymes [27,28]. It is supposed that this difference is due to a more rigid structure of the central helix of troponin C. The long central α -helix of troponin C is stabilized by an N-terminal helix arm which is absent from the calmodulin structure [28,29].

Being more flexible, calmodulin can adopt many different conformations suitable for the activation of a number of different target proteins. Similarities in the interactions of calmodulin and troponin C with caldesmon may indicate that, in this particular case, the calcium-binding proteins interact with their target in the extended (not the bent) conformation. This agrees with the recently published experimental data of Mabuchi et al. [8].

Since troponin C is a good analogue of calmodulin, we used it to map the contact sites in the caldesmon–calmodulin complex. We found that Cys-98 of troponin C is easily cross-linked to Cys-580 of caldesmon. In the primary structure, Cys-580 is located far from three putative calmodulin-binding sites of caldesmon [3–5]. At the same time, according to the recently published two-dimensional model of the caldesmon–calmodulin complex [3,6], Cys-580 may be close to the central helix of calmodulin. Our experimental data agree with this model, and indicate that the polypeptide chain of domains 3 and 4 of caldesmon folds into a comparatively compact structure, and several segments of caldesmon are close to or interact directly with calmodulin. Domain 4 is involved in the regulation of actomyosin ATPase activity [1–3], and Cys-580 of caldesmon can be cross-linked to Cys-374 located in the C-terminal domain of actin [20]. This means that, in the three-dimensional structure, Cys-580 of caldesmon is close to both Cys-374 of actin and the central helix of calmodulin. Therefore actin and calcium-binding proteins may compete for binding at this site. This suggestion agrees with our finding that the cross-linked caldesmon–troponin C complex is ineffective in inhibiting actomyosin ATPase activity (Figure 6), and with the data of Graceffa and Jancso [20] indicating that addition of calmodulin prevents the disulphide cross-linking of Cys-580 of caldesmon with Cys-374 of actin.

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