

# Interaction of isoforms of S100 protein with smooth muscle caldesmon

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**Abstract** Interaction of S100a and S100b with duck gizzard caldesmon was investigated by means of native gel electrophoresis, fluorescent spectroscopy and disulfide crosslinking. Both isoforms of S100 interact with intact caldesmon and its C-terminal deletion mutant 606C (residues 606–756) with apparent  $K_d$  of 0.2–0.6  $\mu$ M thus indicating that the S100-binding site is located in the C-terminal domain of caldesmon. The single SH group of duck gizzard caldesmon can be crosslinked to Cys-84 of the  $\beta$ -chain or to Cys-85 of the  $\alpha$ -chain of S100. Crosslinking of S100 reduces the inhibitory action of caldesmon on actomyosin ATPase activity. S100 reverses the inhibitory action of intact caldesmon and its deletion mutants 606C (residues 606–756) and H9 (residues 669–737) as effectively as calmodulin. S100a has higher affinity to caldesmon and is more effective than S100b in reversing caldesmon-induced inhibition of actomyosin ATPase activity. Although monomeric (calmodulin, troponin C) and dimeric (S100) Ca-binding proteins have different sizes and structures they interact with caldesmon in a very similar fashion.

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**Key words:** Caldesmon; S100; Calmodulin; EF-hand protein; Actomyosin

## 1. Introduction

Caldesmon is a ubiquitous actin-binding protein involved in the regulation of smooth muscle contraction, non-muscle motility and cytoskeleton organization [1,2]. Caldesmon functioning is regulated by  $\text{Ca}^{2+}$  via Ca-binding proteins. Calmodulin and troponin C containing four Ca-binding sites in a single polypeptide chain interact with the C-terminal part of caldesmon and reverse its inhibitory action on actomyosin ATPase activity [3–5].

Dimeric S100 proteins consist of two identical or different polypeptide chains each containing two Ca-binding sites [6]. The unfractionated mixture of brain-specific S100 proteins consisting of  $\alpha\alpha$  (S100a<sub>o</sub>) and  $\beta\beta$  (S100b) homodimers and  $\alpha\beta$  (S100a) heterodimers interacts with caldesmon and reverses the inhibitory action of caldesmon on actomyosin ATPase activity [7–9]. Two other members of the S100 family, smooth muscle-specific caltropin [10] and calcyclin (or S100A6) [11], also interact with caldesmon. Both caltropin and calcyclin seem to interact with the C-terminal part of caldesmon [12,13]. Although these two proteins are very sim-

ilar, caltropin reverses the inhibitory action of caldesmon on actomyosin ATPase activity [10], whereas calcyclin is completely ineffective [13].

The three-dimensional structure of monomeric (calmodulin and troponin C) [14] and dimeric (calcyclin, S100b) [15–17] Ca-binding proteins is significantly different. In order to understand the mechanism of interaction of caldesmon with monomeric and dimeric Ca-binding proteins we compared the interaction of smooth muscle caldesmon with homodimeric ( $\beta\beta$ ) S100b, heterodimeric ( $\alpha\beta$ ) S100a and monomeric calmodulin. We mapped the sites of caldesmon involved in the interaction with these Ca-binding proteins and compared their ability to reverse caldesmon-induced inhibition of actomyosin ATPase activity.

## 2. Materials and methods

Duck gizzard caldesmon and duck gizzard tropomyosin were purified as described earlier [18]. Caldesmon mutants 606C and H9 were obtained by bacterial expression in the pMW172 plasmid/BL21(DE3) cell system [19,20]. Bovine brain calmodulin was isolated according to Gopalakrishna and Anderson [21]. Unfractionated mixture of S100 proteins was purified from bovine brain [22] and separation of S100a and S100b was achieved by ion-exchange chromatography on DEAE-Sephadex A-25. The purity of isoforms was checked by a discontinuous native gel electrophoresis which was run in the presence of 1 mM EGTA [23] and by recording the UV spectra [22]. Rabbit skeletal actin and myosin were obtained as described earlier [24] and were kindly provided by Dr. D.I. Levitsky (A.N. Belosersky Institute of Physicochemical Biology, Moscow State University, Moscow, Russia). The purity of all proteins was determined by SDS-PAGE [25]. The protein concentration was determined spectrophotometrically using  $\epsilon_{\text{cm}^{280}}^{0.1\%}$  values equal to 0.33 for caldesmon, 0.20 for calmodulin, 0.53 for S100a and 0.19 for S100b. The concentration of caldesmon mutants was determined using fluorescamine [26].

Interaction of caldesmon and its deletion mutant 606C with Ca-binding proteins was analyzed by means of native gel electrophoresis [27]. Fixed amounts of S100 or calmodulin were titrated by caldesmon (or its deletion mutant 606C) in the presence of 0.2 mM  $\text{CaCl}_2$  or 2 mM EGTA and the intensity of the band of free calcium-binding protein was determined by densitometry.

Fluorescent spectroscopy was also used for monitoring the interaction of caldesmon with calmodulin and S100. In this case we analyzed the effect of Ca-binding proteins on the intrinsic Trp fluorescence of caldesmon [3,4,28] or their effect on the fluorescence of bimeane attached to the single Cys residue of caldesmon. Caldesmon (420 nM) in 10 mM Tris-HCl (pH 7.4) containing 100 mM KCl, 4.2 mM  $\beta$ -mercaptoethanol was titrated by S100b protein or calmodulin either in the presence of 0.1 mM  $\text{CaCl}_2$  or in the presence of 2 mM EGTA. The fluorescence was excited at 290 nm and recorded at 322 nm. Fluorescence spectra were recorded in the range of 320–420 nm on a Hitachi F-3000 spectrofluorimeter.

Duck gizzard caldesmon containing a single SH group located in a position analogous to that of Cys-580 of chicken gizzard caldesmon [5,18] was modified by monobromobimane (0.7 mol of label per mol of caldesmon). Caldesmon with a modified SH group (460 nM) in 50 mM Tris-HCl, pH 7.5, was titrated either by calmodulin or by S100 protein. The fluorescence was excited at 395 nm and recorded at 450 nm. Fluorescence spectra of modified caldesmon in the absence

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**Abbreviations:** DTNB, 5,5'-dithiobis(nitrobenzoic) acid; EGTA, ethyleneglycoltetraacetate; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate

and in the presence of excess calcium-binding proteins were recorded in the range of 440–540 nm on a Hitachi F-3000 spectrofluorimeter.

Oxidation of SH groups of S100b (final concentration 70  $\mu$ M) was performed in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM  $\text{CaCl}_2$  by mixing with copper (II)-*o*-phenanthroline complex (final concentration of copper (II) 1 mM). After incubation for 2 h at 30°C the reaction was stopped with 5 mM EGTA and the incubation mixture was dialyzed against 20 mM Tris-HCl (pH 7.5). Protein composition of the oxidized sample was analyzed by SDS-PAGE run in the absence of  $\beta$ -mercaptoethanol.

Formation of the disulfide-crosslinked S100-caldesmon complex was performed as follows. S100 (final concentration 2.4 mg/ml) was dissolved in 10 mM HEPES/KOH (pH 7.0), containing 6 M urea and 56 mM  $\beta$ -mercaptoethanol, and incubated for 1.5 h at 30°C. Afterwards the protein sample was dialyzed against 10 mM HEPES/KOH (pH 7.0). SH groups of duck gizzard caldesmon were activated by incubation of the protein (final concentration 60  $\mu$ M) with an equimolar concentration of DTNB in 10 mM HEPES/KOH (pH 7.0) for 1 h at 30°C. Activated caldesmon was mixed with reduced S100 protein (final concentration of both proteins 25  $\mu$ M) and incubated for 30 min at 30°C. The incubation mixture contained 0.5 mM  $\text{CaCl}_2$  or 2 mM EGTA and in some experiments was supplemented by addition of 100 mM NaCl. The protein composition of the crosslinked mixture was analyzed by SDS-PAGE run both in the presence and in the absence of  $\beta$ -mercaptoethanol.

Actomyosin ATPase measurements were performed in 10 mM PIPES/KOH (pH 7.0), containing 5 mM  $\text{MgCl}_2$  and 10 or 70 mM KCl. Unmodified caldesmon or caldesmon crosslinked to S100 was added to the incubation mixture containing rabbit skeletal actin and myosin and duck gizzard tropomyosin. After 5 min preincubation the reaction was started by addition of ATP to a final concentration of 5 mM. Incubation was continued for 5–15 min, the reaction was stopped by addition of 10% TCA and inorganic phosphate was determined by the method of Taussky and Shorr [29]. Under conditions used the ATPase activity of actomyosin measured in the presence of 70 mM KCl at 37°C was equal to 1–1.2  $\mu$ mol of  $\text{P}_i$ /min per mg of myosin; this value was taken to represent 100% activity.

### 3. Results

The method of native gel electrophoresis was used for analyzing the interaction of two isoforms of S100 protein with intact caldesmon. In the presence of calcium addition of caldesmon to S100 resulted in a diminution of the intensity of the band of free S100 (Fig. 1). In the absence of calcium addition of caldesmon had practically no effect on the intensity of the band of S100 (Fig. 1). Plotting the intensity of the band of the free S100 against the total concentration of caldesmon added we determined the apparent  $K_d$  values for the caldesmon–S100a and caldesmon–S100b complexes. These values were  $0.17 \pm 0.02$   $\mu$ M for S100a and  $0.50 \pm 0.04$   $\mu$ M for S100b. Both these values are comparable to the corresponding values determined for calmodulin (0.2–0.5  $\mu$ M) [5,27] and troponin C (1–3  $\mu$ M) [5]. Thus, dimeric Ca-binding proteins (S100a and S100b) interact with caldesmon with affinity comparable to that of monomeric Ca-binding proteins (calmodulin and troponin C).

Calmodulin-binding sites are located in the C-terminal part of caldesmon [1–3], and caltropin also interacts with the C-terminal 27 kDa fragment of caldesmon [12]. Therefore we supposed that the S100-binding sites are located in the C-terminal domain of caldesmon. Using the method of native gel electrophoresis we found that in the presence of  $\text{Ca}^{2+}$  titration of S100 with the deletion mutant 606C (residues 606–756 of caldesmon) is accompanied by a decrease in the intensity of the band of free calcium-binding protein. The apparent  $K_d$  values of the complexes of S100a and S100b with 606C were  $0.077 \pm 0.038$   $\mu$ M and  $1.11 \pm 0.41$   $\mu$ M respec-

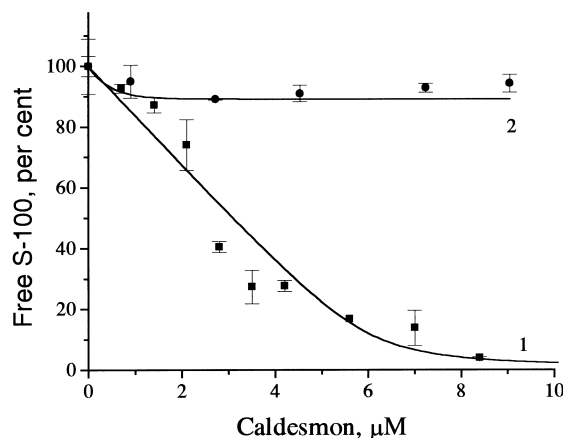


Fig. 1. Titration of S100a (9.1  $\mu$ M) by caldesmon. Titration was performed either in the presence of 1 mM  $\text{CaCl}_2$  (1) or in the presence of 2 mM EGTA (2). The percentage of the calcium-binding protein remaining unbound is plotted against the total concentration of caldesmon added.

tively. These values are comparable with those for intact caldesmon and indicate that the main S100-binding site is located in the C-terminal part (residues 606–756) of caldesmon. S100a interacts with caldesmon (or its deletion mutant) with higher affinity than S100b.

The C-terminal domain of caldesmon contains three Trp residues and binding of calmodulin affects the fluorescent properties of caldesmon [3,4,26,28]. Since S100 binds to the C-terminal domain of caldesmon we may expect that this

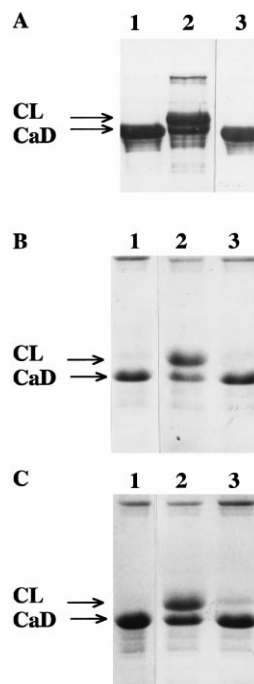


Fig. 2. Disulfide crosslinking of S100 to caldesmon. A: Crosslinking of unfractionated S100 to duck gizzard caldesmon. Caldesmon with DTNB-activated SH groups (1) or its equimolar mixture with unfractionated S100 (2, 3) before (2) or after (3) reduction with excess mercaptoethanol. Crosslinking of S100a (B) or S100b (C) to duck gizzard caldesmon. Crosslinking of activated caldesmon (1) with S100 was performed in the presence of either 0.5 mM  $\text{CaCl}_2$  (2) or 2 mM EGTA (3). Arrows indicate the positions of the cross-linked complex (CL) and free caldesmon (CaD).

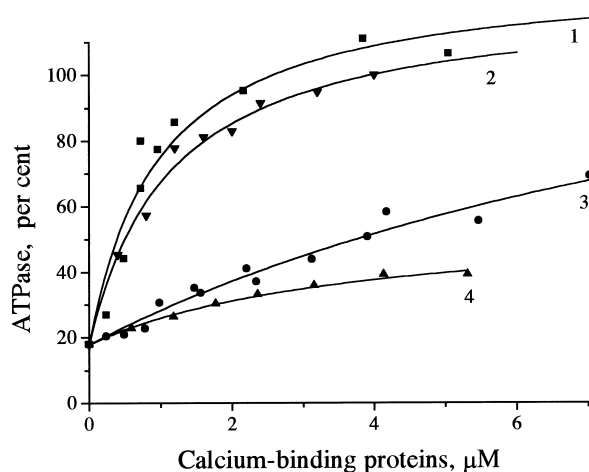


Fig. 3. Reversion of caldesmon-induced inhibition of actomyosin ATPase by S100a (1), calmodulin (2), S100b (3) and oxidized S100b (4). Conditions: 10 mM HEPES/KOH, pH 7.0, 70 mM KCl, 5 mM  $\text{MgCl}_2$ , 5 mM ATP, 0.5 mM  $\text{CaCl}_2$ , 37°C. Protein concentrations ( $\mu\text{M}$ ): actin, 3.1; tropomyosin 0.6; myosin, 0.7; caldesmon 0.35. Caldesmon inhibits ATPase activity of actomyosin up to 18%; 100% corresponds to the activity in the absence of caldesmon.

interaction will also influence the intrinsic Trp fluorescence of caldesmon. Of the two isoforms of S100 only S100b does not contain Trp residues and is suitable for fluorescence titration of caldesmon. Addition of about 5 molar excess S-100b was not accompanied by any significant change in the Trp fluorescence of caldesmon, whereas addition of calmodulin resulted in a 10–15 nm blue shift of caldesmon fluorescence and a 1.6–1.8-fold increase of the intensity of fluorescence at 320 nm. Thus, although S100 interacts with the C-terminal domain of caldesmon it does not affect the fluorescence of three Trp residues of caldesmon located in this domain. In this respect S100 is similar to troponin C [5] and caltropin [12] which also bind to the C-terminal domain of caldesmon without affecting its fluorescent properties.

Recently published models suggest that the folded domains 3 and 4 of caldesmon are involved in formation of the calmodulin-binding site [2,5,26,30]. If the mode of binding of S100 is similar to that of calmodulin we may suppose that Cys-580 located in domain 3 of caldesmon will be close to S100-binding sites. Using duck gizzard caldesmon containing a single Cys in a position analogous to that of Cys-580 of chicken gizzard caldesmon [5,18] we analyzed the effect of S100 on the fluorescence of bimeane attached to the Cys residue and the probability of crosslinking of S100 to caldesmon. When duck gizzard caldesmon modified by monobromobimane was titrated with calmodulin or an unfractionated mixture of S100 protein we observed a 15–20% increase of the intensity and 10–15 nm blue shift of the fluorescence maximum. Under the conditions used S100 induced larger changes in the spectrum of fluorescence of bimeane-labeled caldesmon than calmodulin. These data indicate that the binding of S100 as well as calmodulin affects the environment of the Cys residue located in domain 3 of caldesmon.

In order to obtain more direct proof of this suggestion we tried to crosslink caldesmon and S100 via a disulfide bridge. Caldesmon with a DTNB-activated SH group was incubated with an equimolar quantity of preliminary reduced S100 (see Section 2). Modified caldesmon moved as a band with  $M_r$  140

kDa (Fig. 2A, lane 1). Addition of the unfractionated mixture of S100 protein leads to the formation of an additional band with apparent  $M_r$  155 kDa (Fig. 2A, lane 2). If before electrophoresis this sample was reduced by excess mercaptoethanol the band of 155 kDa completely disappeared with a simultaneous increase in the intensity of the band of uncrosslinked caldesmon (Fig. 2A, lane 3). The molecular weight of the complex is close to that expected for a 1:1 crosslinked complex of caldesmon ( $M_r$  140 kDa) and a single chain of S100 ( $M_r$  10 kDa). All these data mean that the single Cys residue of caldesmon may interact and form a disulfide bond with either Cys-84 (and/or Cys-68) of the  $\beta$ -chain or Cys-85 of the  $\alpha$ -chain of S100. To determine residues of S100 involved in the binding with caldesmon we analyzed the crosslinking of two isoforms of S100 and caldesmon in more detail.

Crosslinking of caldesmon with S100a and S100b was performed at high (10 mM HEPES/KOH plus 100 mM NaCl) ionic strength in the presence and in the absence of  $\text{Ca}^{2+}$ . Under the conditions used the crosslinked caldesmon–S100 complex was formed only in the presence of  $\text{Ca}^{2+}$  (Fig. 2B,C). The portion of caldesmon ( $\sim 60\%$  of total) crosslinked to S100a was slightly higher than that crosslinked to S100b ( $\sim 40\%$ ). S100b is a homodimer of two  $\beta$ -chains and contains four SH groups (two Cys-68+two Cys-84). S100a is a  $\alpha\beta$  heterodimer and contains three SH groups (Cys-68 and Cys-84 of the  $\beta$ -chain+Cys-85 of the  $\alpha$ -chain). Since the probability of crosslinking of S100a was larger than that of S100b we may conclude that Cys-84 (or 85) rather than Cys-68 is involved in the crosslinking with caldesmon.

It was important to determine whether the binding of S100 affects the ability of caldesmon to inhibit actomyosin ATPase activity. Under the conditions used addition of caldesmon up to a molar ratio of actin:caldesmon equal to 10:1 to 8:1 reduced the ATPase activity by 75–80%. The inhibitory effect of caldesmon was reversed by addition of calcium-binding proteins. In the presence of 1 mM  $\text{CaCl}_2$  addition of a 5-fold molar excess of calmodulin or S100a over caldesmon completely reversed the inhibitory action of caldesmon (Fig. 3). S100b was less effective than S100a and was unable to reverse the inhibitory action even after addition of 18 molar

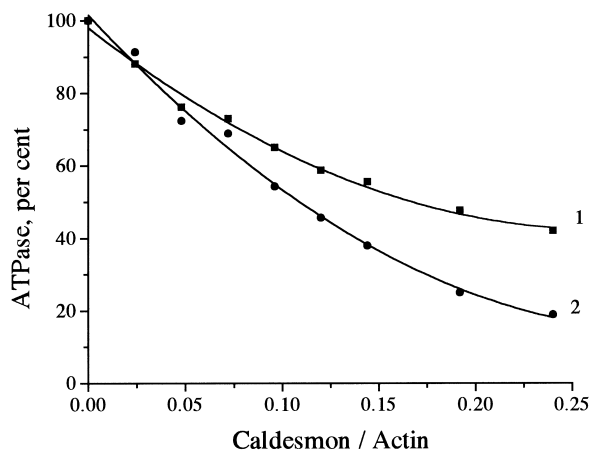


Fig. 4. Effect of non-reduced (1) and reduced (2) crosslinked caldesmon–S100 complex on the ATPase activity of actomyosin. Conditions: 10 mM HEPES/KOH, pH 7.0, 10 mM KCl, 5 mM  $\text{MgCl}_2$ , 5 mM ATP, 0.5 mM  $\text{CaCl}_2$ , 30°C. Protein concentrations ( $\mu\text{M}$ ): actin, 0.68; tropomyosin, 0.6; myosin, 0.68. ATPase is expressed as a percentage of that in the absence of caldesmon.

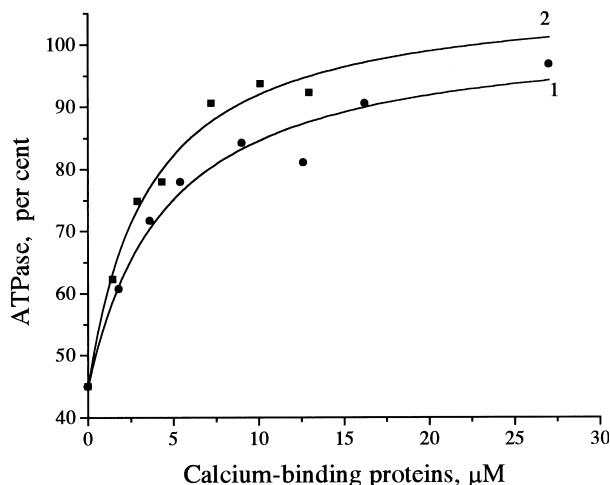


Fig. 5. Reversion of inhibition induced by H9 by unfractionated S100 (1) and calmodulin (2). Conditions: 10 mM HEPES/KOH, pH 7.0, 10 mM KCl, 5 mM  $\text{MgCl}_2$ , 5 mM ATP, 0.5 mM  $\text{CaCl}_2$ , 25°C. Protein concentrations ( $\mu\text{M}$ ): actin, 5.1; tropomyosin, 1.33; myosin, 1.3; H9, 6.2. H9 inhibits the ATPase activity up to 45%; 100% corresponds to the activity in the absence of H9.

excess over caldesmon (Fig. 3). In the presence of 1 mM EGTA neither calmodulin nor two isoforms of S100 reversed caldesmon-induced inhibition of actomyosin ATPase. The lower effectivity of S100b can be at least partly due to the lower affinity of this isoform to caldesmon.

S100b undergoes oxidation and forms oligomers both *in vivo* [31] and *in vitro* [32]. Under the conditions used in our experiments oxidation results in the formation of a number of oligomers. According to the data of native gel electrophoresis oxidized monomers and dimers comprising more than half of the protein in the oxidized sample practically do not interact with caldesmon (data not shown). Therefore the oxidized preparation of S100b was less effective than S100b in reversing the inhibitory effect of caldesmon on actomyosin ATPase (Fig. 3).

The data presented indicate that the reversible Ca-dependent interaction with S100 prevents the inhibition induced by caldesmon. As shown earlier (Fig. 2) caldesmon can be cross-linked to S100 via a disulfide bond. The crosslinked complex was purified by ion-exchange chromatography on Q-Sepharose [5] and was analyzed for its ability to inhibit actomyosin ATPase before and after reduction by excess mercaptoethanol. The non-reduced cross-linked complex was less effective than the reduced complex in inhibiting actomyosin ATPase (Fig. 4). This means that covalent crosslinking of S100 to Cys located in domain 3 of caldesmon decreases the inhibitory action of caldesmon on actomyosin ATPase activity.

S100 interacts with the 606C deletion mutant of caldesmon with affinity comparable to that of intact caldesmon. Therefore we supposed that S100 will reverse the inhibitory action of 606C on actomyosin ATPase activity. Indeed addition of unfractionated S100 completely reversed the inhibitory action of 606C on actomyosin ATPase and half-maximal reversion was observed at similar concentrations of S100 and calmodulin.

The deletion mutant of caldesmon H9 (residues 669–737) contains fewer than 70 C-terminal residues of caldesmon (including two calmodulin-binding sites B and B') and inhibits

the ATPase activity of actomyosin [2,3]. Under the conditions used H9 induced 40–50% inhibition of actomyosin ATPase activity and this inhibition was completely reversed by addition of both calmodulin and unfractionated S100 (Fig. 5). The concentration of S100 needed for half-maximal reversal of ATPase inhibition was only slightly higher than that of calmodulin. These data mean that S100 interacts with a very short C-terminal caldesmon fragment and successively reverses its inhibitory action on actomyosin ATPase activity.

#### 4. Discussion

The data presented demonstrate that S-100 interacts with and reverses the inhibitory action of short C-terminal fragments of caldesmon (606C and H9) in a manner similar to that of intact caldesmon. Therefore we may conclude that the main S100-binding sites are located in the C-terminal part of caldesmon.

It has been found that calmodulin and S100 interact with sequences of different primary structure. The primary structure SSRINEWLTKT around Trp-692 of caldesmon resembles RLSLDSWLKR, KINLQDWLLL and KYLTLDLWLR which were recognized by Ivanenkov et al. [33] as potential S100-binding sites. S100 reverses the inhibitory action of H9 (containing Trp-692) (Fig. 5), thus indicating that the S100-binding sites may be located in the very C-terminal part of caldesmon (residues 669–737 of chicken gizzard caldesmon).

Cys-580 of domain 3 is within 1.5 nm of at least one of the Trps 659, 692 and 722 of domain 4 of caldesmon [3,34]. It has been shown that Cys-580 of caldesmon can be crosslinked to calmodulin [35] and troponin C [5]. S100 can also be cross-linked via its Cys-84 (or 85) to Cys-580 of caldesmon (Fig. 2) and covalent binding of S100 to caldesmon reduces its inhibitory action on the actomyosin ATPase (Fig. 4). These data indicate that the overall structure of the complex of caldesmon with monomeric (calmodulin, troponin C) and dimeric (S100) Ca-binding proteins is rather similar.

The three-dimensional structure of S100 is significantly different from that of calmodulin or troponin C. Recently published NMR data reveals that apo-S100b has a rather compact globular structure with overall dimensions of about  $38 \times 38 \times 30$  Å [16]. At the same time in the crystal form calmodulin has a dumbbell-like shape approximately 65 Å long with N- and C-terminal globular domains ( $25 \times 20 \times 20$  Å) separated by a long central helix [14,36]. In solution residues 78–81 (in the central helix) adopt a non-helical conformation and the maximal length of calmodulin is reduced by 10–12 Å [36]. Even under these conditions the length of the calmodulin (or the troponin C) molecule (50–55 Å) is significantly larger than that of S100 (38 Å).

Using a fluorescently labeled calmodulin mutant containing modified Cys-34 and Cys-110 Mabuchi et al. [37] concluded that in the complex with caldesmon calmodulin is in an extended conformation. If this suggestion is correct and caldesmon interacts with completely extended calmodulin with a long axis of 60–65 Å, then the structure of the caldesmon–calmodulin complex should be significantly different from that of caldesmon–S100 because the size of S100 is much smaller ( $38 \times 38 \times 30$  Å). At the same time S100 and calmodulin have similar affinities to caldesmon and both Ca-binding proteins are equally effective in reversing the inhibitory action of cal-

desmon on actomyosin ATPase activity. There are three possible explanations for this discrepancy. (1) Calmodulin interacts with caldesmon in at least a partly bent conformation. (2) The three-dimensional structure of Ca-saturated S100 (which is still not resolved) is different from that of apo-S100 and has significantly larger dimensions. (3) The C-terminal domain of caldesmon is very flexible and can adopt Ca-binding proteins of a very different size and different orientation of the Ca-binding sites.

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