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Spectrofluorimetric studies on C-terminal 34 kDa fragment of caldesmon

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Analysis of the tryptophan fluorescence emission spectra of caldesmon and its 34 kDa C-terminal fragment indicates that all tryptophan residues are located on the surface of the molecule, accessible to solvent. All three tryptophan residues of the 34 kDa fragment and four of the five tryptophan residues of intact potein are accessible to free water, whereas one located in the *N*-terminal region of molecule is accessible only to bound water molecules. The temperature dependence of the fluorescence parameters indicates higher thermal stability of the 34 kDa fragment than the whole caldesmon molecule. The interaction of the 34 kDa fragment of caldesmon (like that of the intact molecule) with calmodulin is accompanied by a blue shift of the fluorescence emission maximum and an increase in the relative quantum yield. Computer-calculated binding constants show that the binding of calmodulin to the 34 kDa fragment ($K = 2.5 \cdot 10^5\ M^{-1}$) is of two orders of magnitude weaker than that to intact caldesmon ($K = 1.4 \cdot 10^7\ M^{-1}$). The interaction with tropomyosin results in a blue shift of the spectrum of the 34 kDa fragment, yet there is no effect on the spectrum of intact caldesmon. Binding constants of tropomyosin to caldesmon ($K = 3.8 \cdot 10^5\ M^{-1}$) and its 34 kDa fragment ($K = 2.3 \cdot 10^5\ M^{-1}$) are similar. Binding of calmodulin to caldesmon and to the 34 kDa fragment affects their interaction with tropomyosin.

1. Introduction

Caldesmon is a thin-filament 89-kDa protein [1] implicated in the actin-linked regulation of the smooth muscle and non-muscle actomyosin system [2–5]. The binding of caldesmon to F-actin, which is regulated by Ca^{2+} and calmodulin, results in the tropomyosin-potentiated inhibition of actin-activated Mg^{2+}-ATPase activity of myosin [6–10].

Chymotryptic cleavage experiments have localized the actin- and calmodulin-binding sites to a C-terminal third of the caldesmon molecule [11–15]. The 34 kDa fragment (35–40 kDa as determined by SDS–PAGE [11,13,15]) containing these sites was shown to inhibit actomyosin ATPase to roughly the same extent as did intact caldesmon [11]. Recently, it was found that the amino acid sequence of the 34 kDa fragment resembles that of the tropomyosin binding component of troponin—troponin T [16]. This sequence is a potential binding site for tropomyosin, and is located in the *N*-terminal 15 kDa portion of the 35 kDa (M_r determined by SDS–PAGE) C-terminal fragment of caldesmon, obtained by thrombic digestion [17,18].

The interaction of intact caldesmon with actin, tropomyosin and calmodulin has been extensively studied utilizing various techniques including cosedimentation, gel-filtration, affinity chromatography and spectrofluorimetry with various fluorescent probes [2,8,9,19–24]. Some features of the interaction of the C-terminal chymotryptic 34 kDa fragment with actin and calmodulin were also determined [12,14,15]. This paper presents further

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characterization of the interaction of the fragment with calmodulin and tropomyosin by using spectrofluorimetry. By taking advantage of the absence of tryptophan residues in calmodulin and tropomyosin, we were able to study the effect of these two proteins on the intrinsic tryptophan fluorescence in the 34 kDa fragment, and to relate these results to the perturbation of tryptophan in the intact molecule.

2. Materials and methods

2.1 Preparations and reagents

Caldesmon from chicken gizzards was prepared according to the procedure described by Bretscher [19] with some modifications. The most relevant was the incorporation of ion-exchange chromatography on DE-52 cellulose before gel-filtration chromatography on a Bio-Gel A-0.5m column (1.5 × 120 cm) was equilibrated with 20 m*M* Tris–HCl pH 7.5 buffer containing 50 m*M* NaCl, 1 m*M* EGTA, 1 m*M* β-mercaptoethanol and 0.05% NaN_3). Chicken gizzard tropomyosin was prepared as described earlier by Dąbrowska et al. [25]. Bovine brain calmodulin was prepared according to the procedure reported by Brzeska et al. [26].

Caldesmon was cleaved by α-chymotrypsin (Boehringer Mannheim GmbH.) under conditions described by Szpacenko and Dąbrowska [11]. The C-terminal 34 kDa fragment was purified from the chymotryptic digest of caldesmon, first on a calmodulin affinity column, than on a DEAE-Sephacel column (1 × 15 cm) equilibrated with 20 m*M* imidazole buffer pH 7.0, 50 m*M* NaCl. The flow-through fraction was collected and chromatographed on a SP-Sephadex H 50 column 2.5 × 25 cm), which was eluted with 50 m*M* (Na/K) phosphate, pH 6.0 and a linear gradient of NaCl from 0 to 300 m*M*.

Calmodulin was coupled to CNBr-activated Sepharose 4B (Pharmacia) following the procedure outlined by Pharmacia.

The digestion reaction and the homogeneity of proteins were monitored by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [27], except calmodulin, for which urea-gel electrophoresis was used [28].

The concentration of proteins was determined using the following UV absorption coefficients for 0.1% solutions and M_r: chicken gizzard tropomyosin $E_{278} = 0.19$, 68,000 [29]; bovine brain calmodulin $E_{280} = 0.20$, 16,700 [30]; chicken gizzard caldesmon $E_{278} = 0.38$ (our data), 89,000 [1].

CNBr-activated Sepharose was obtained from Pharmacia, Bio-Gel A-0.5m from Bio-Rad, and cellulose DE-52 from Whatman. Buffer salts were supplied by Sigma Chemical Co. Buffers were prepared by using bidistilled deionised water. Reagent-grade or best available grade chemicals were employed throughout the experiments.

2.2 Fluorescence measurements

Steady-state fluorescence measurements were carried out on a home-built spectrofluorimeter described earlier [31]. Fluorescence light was collected from the front surface of the quartz cell. All spectra were corrected for instrumental spectral sensitivity of the apparatus. Intensities in the corrected spectra were proportional to the number of photons emitted per unit wavelength interval. If it was required, fluorescence spectra were corrected for inner filter effects of screening and reabsorption [32]. The position of the middle of a chord drawn at the 80% level of the maximal intensity ($\tilde{\lambda}$) was taken as a measure of the spectral position.

The temperature in the thermostatically controlled cell of the spectrofluorimeter was measured by means of a copper–constantan thermocouple with an accuracy of approximately 0.1 K. The heating rate was about 1 K per minute.

Ultraviolet absorption spectra were measured with a Specord UV-VIS spectrophotometer (Karl Zeiss, Jena).

Fitting of the experimental data with theoretical curves was carried out with a computer using a non-linear regression scheme [33]. The accuracy of the evaluation of binding constants by the fitting procedure was about half an order of magnitude.

3. Results

3.1 Intrinsic tryptophan fluorescence spectra

It is known that smooth muscle caldesmon contains five tryptophan residues per molecule and its 34 kDa fragment contains three of these [1,16,34]. Figure 1 shows the tryptophan fluorescence spectra of both intact caldesmon and 34 kDa fragment. It is clearly seen that the spectra are very similar to each other. The spectra were analysed in terms of the model of discrete states of tryptophan residues in proteins described by Burstein [35,36]. This statistical model can distinguish the five most probable physical states of tryptophan residues termed states A, S, I, II, and III with UV absorption maxima at 307, 316, 330, 340 and 353 nm, respectively. States A, S, and I correspond to buried tryptophan residues. In state A they are unperturbed by any interactions, whereas in states S and I complexes can form with polar groups, 1 : 1 and 2 : 1, respectively. In states II and III tryptophan residues are located at the protein surface in contact with bound or free water molecules, respectively.

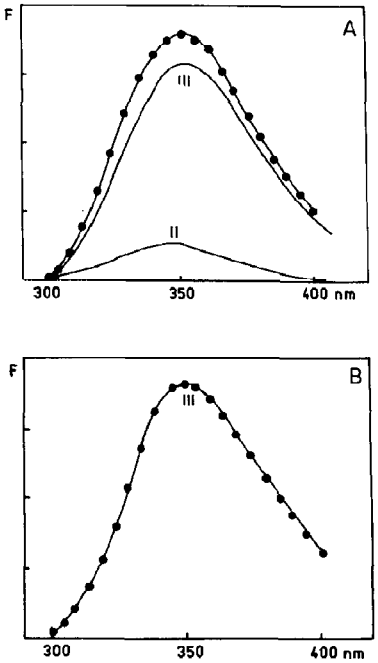

Fig. 1. Intrinsic tryptophan fluorescence spectra of intact caldesmon (A) and the 34 kD fragment (B). Points are the experimental data, and curves are drawn from the theoretical analysis based on the model of discrete states of tryptophans in proteins [35]. The curves were fitted to the experimental points by variation of the contributions from each spectral component. The spectral components which give the best fit are shown in the figure. Excitation at 296.7 nm. Conditions: 50 m*M* cacodilate buffer, pH 7.0.

Since caldesmon is a thin, extended molecule with regularly disposed polar side chains, it can not form a rigid hydrophobic core. The recently published hydrophobic profile of caldesmon [1] supports this view. Thus, we did not take into account participation of state A of tryptophans in the caldesmon emission spectrum. The experimental spectra of caldesmon and its 34 kDa fragment were fitted by the tryptophan spectra of states S, I, II, and III. In both cases we have not found components S and I.

The spectrum of intact caldesmon was best fitted by the components corresponding to states II and III (Fig. 1A) whereas, the spectrum of the 34 kDa fragment was best fitted by a single component corresponding to state III (Fig. 1B). These results indicate that all three tryptophan residues in 34 kDa fragment are accessible to free water molecules i.e. they are located on the surface of the polypeptide, while the intact protein contains tryptophan residues in contact with bound and free water molecules. We conclude that some of the tryptophan residues of intact caldesmon are located in a hydrophobic region, which contains bound water molecules (Fig. 1A, component III).

3.2 Thermal unfolding

Figure 2 shows the temperature dependence of fluorescence parameters of intact caldesmon and its 34 kDa fragment. The thermal unfolding of the intact protein is reflected by a very small shift of the fluorescence spectrum towards shorter and then towards longer wavelengths, and by a deflection of the temperature dependence of relative fluorescence quantum yield from the common thermal quenching curve. In the case of the caldesmon 34 kDa fragment similar spectral changes take place at higher temperatures (the shift is at approximately 10 °C higher). Thus, the

caldesmon fragment is more stable towards thermal unfolding in comparison with the intact protein.

3.3 The binding of calmodulin

Spectrofluorimetric titrations of intact caldesmon and its C-terminal 34 kDa fragment by bovine brain calmodulin in the presence of calcium ions are shown in Fig. 3. The binding of calmodulin to both intact caldesmon and its fragment results in a shift of the fluorescence spectrum towards shorter wavelengths which reflects transfer of the tryptophan residues into more hydrophobic environment and in an increase in the fluorescence quantum yield. However, in the case of the intact protein the effects are significantly more pronounced. The experimental data for fluorescence quantum yield was fitted by a theoretical curve computed according to the simplest one-site binding scheme:

$$\mathrm{CaD} + n \cdot \mathrm{CaM} \overset{K}{\leftrightharpoons} \mathrm{CaD} \cdot \mathrm{CaM}_n \qquad (1)$$

where CaD is caldesmon, CaM is calmodulin, K

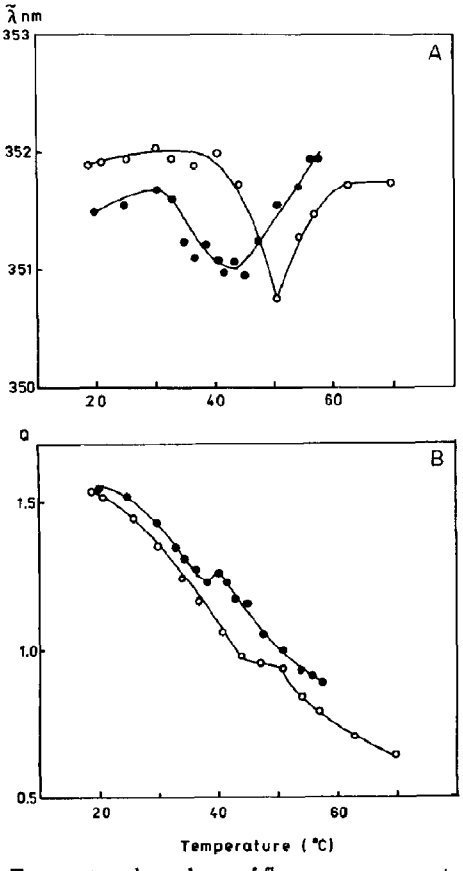

Fig. 2. Temperature dependence of fluorescence parameters for intact caldesmon (●) and the 34 kD fragment (○), A–fluorescence spectrum position ($\tilde{\lambda}$), B–relative fluorescence quantum yield (Q). Excitation at 296.7 nm. Conditions: 2 μM caldesmon and 4 μM 34 kDa fragment in 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EGTA, 1 mM β-mercaptoethanol, and 0.05% NaN_3.

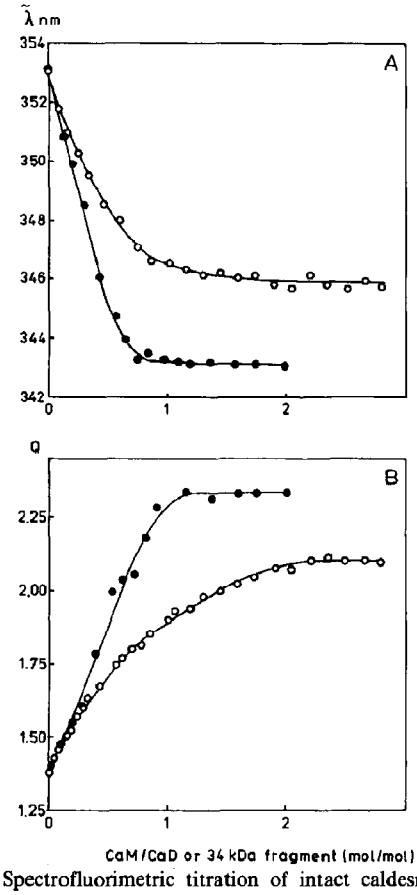

Fig. 3. Spectrofluorimetric titration of intact caldesmon (●) and the 34 kD fragment (○) with bovine brain calmodulin. A–fluorescence spectrum position ($\tilde{\lambda}$); B–relative fluorescence quantum yield (Q). Excitation at 296.7 nm. Conditions as given in the legend to Fig. 2 except that 1 mM EGTA was replaced by 5 mM $CaCl_2$. (CaD–caldesmon, CaM–calmodulin.)

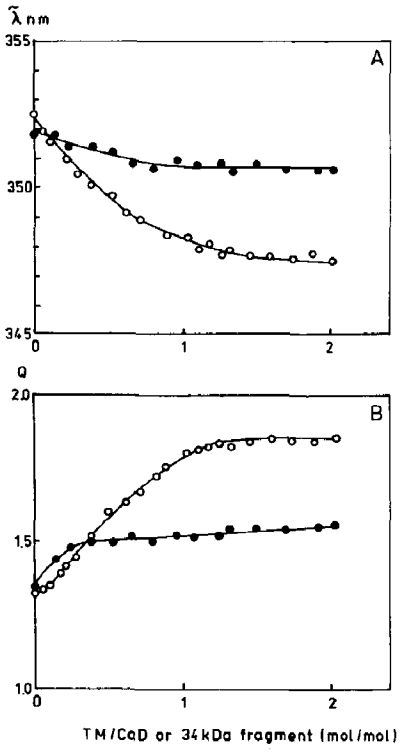

Fig. 4. Spectrofluorimetric titration of intact caldesmon (●) and the 34 kD fragment (○) with gizzard tropomyosin. A–fluorescence spectrum position ($\tilde{\lambda}$); B–relative fluorescence quantum yield (Q). Excitation at 296.7 nm. Conditions as given in the legend to Fig. 2. (CaD–caldesmon, TM–tropomyosin.)

is the binding constant, and n depicts the number of calmodulin molecules bound to caldesmon. The results of fitting of the experimental data shows that the calmodulin association constant is equal to $1.4 \cdot 10^7\ M^{-1}$ for intact caldesmon and $2.5 \cdot 10^5\ M^{-1}$ for the 34 kDa fragment, and in both cases $n = 1$. Thus, the intact protein possesses much higher affinity for calmodulin in comparison to its C-terminal fragment.

3.4 The binding of tropomyosin

Figure 4 shows the spectrofluorimetric titration of intact caldesmon and its 34 kDa fragment with gizzard tropomyosin. The titration of tropomyosin causes enhancement of relative fluorescence quantum yield and a shift of the fluorescence spectrum position ($\tilde{\lambda}$) towards shorter wavelengths which indicates transfer of tryptophan residues to a less polar environment. These effects are more pronounced in the case of caldesmon C-terminal fragment. The data for relative fluorescence quantum yield were fitted by theoretical curves computed according to the one site binding scheme (Scheme (1) with CaM replaced by TM—tropomyosin). The tropomyosin association constants are $2.3 \cdot 10^5\ M^{-1}$ and $3.8 \cdot 10^5\ M^{-1}$ for intact caldesmon and its fragment, respectively, with $n = 1$ for both cases.

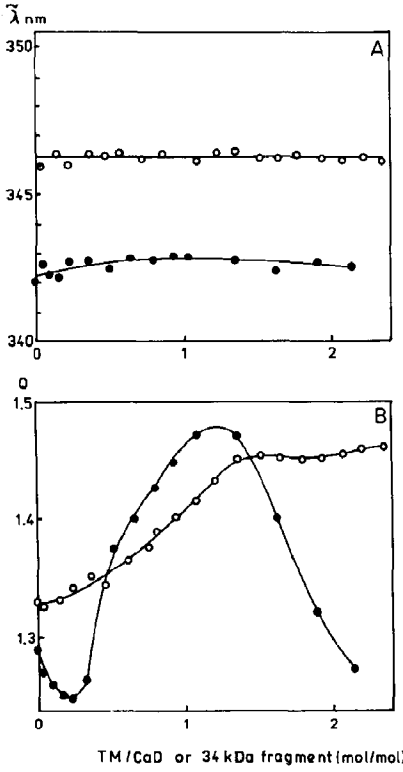

Fig. 5. Spectrofluorimetric titration of the complexes of bovine brain calmodulin and intact caldesmon (●) or the 34 kD fragment (○) with gizzard tropomyosin. A–fluorescence spectrum position ($\tilde{\lambda}$); B–relative fluorescence quantum yield (Q). Excitation at 296.7 nm. Condition as given in the legend to Fig. 2 except that 1 mM EGTA was replaced by 5 mM $CaCl_2$. Preformed complexes of 2 μM caldesmon with 6 μM calmodulin or 4 μM 34 kDa fragment with 12 μM calmodulin were incubated 30 min at room temperature and than titrated with tropomyosin. (CaD–caldesmon, TM–tropomyosin.)

Results of spectrofluorimetric titration of the complexes of calmodulin and intact caldesmon or its 34 kDa fragment, with tropomyosin, are shown in Fig. 5. The titration with tropomyosin, induces a rise in the relative fluorescence quantum yield, with practically no spectral shifts. The curves for fluorescence yield are rather complex and have a sigmoidal (cooperative) character. The rather small changes in fluorescence yield, and hence, the poor accuracy of these data, do not permit a quantitative evaluation of the binding parameters in the case of intact caldesmon. Estimation of an effective association constant for the fragment gives the value $4.0 \cdot 10^5\ M^{-1}$. In both cases, however, there is more than one binding site of tropomyosin and the binding appears to be cooperative.

4. Discussion

Caldesmon is an extended molecule, 76 nm long [37] composed of 771 amino acid residues, and its sequence has been deduced from cDNA library [1]. The 306 C-terminal residues comprise the 34 kDa fragment produced by chymotryptic digestion [16]. Tryptophan residues occur at positions 170, 465, 674, 707, and 737.

Tryptophan fluorescence emission spectra recorded for caldesmon and its 34 kDa fragment reveal that all three tryptophan residues of the 34 kDa fragment (Trp^{674}, Trp^{707}, Trp^{737}) are on the surface of polypeptide chain and accesible to free water molecules. Since some of the tryptophan residues of intact caldesmon also interact with free water molecules, one can assume that these are the tryptophans which are situated in C-terminal portion of the molecule. The remaining tryptophan(s) accesible to structured water is (are) probably located in the hydrophobic region of caldesmon.

The secondary structure prediction based on the cDNA sequence [1] places the C-terminal tryptophan residues in the longest, most unstructured part of protein chain, either on the edge of helical segments (Trp^{674} and Trp^{737}) or on the edge of a β-strand (Trp^{707}). This is consistent with their being accessible to unstructured water molecules. This leaves only residues Trp^{170} and Trp^{465} as the candidates for producing the component II contributions to the fluorescence spectrum of caldesmon (Fig. 1A). However, since residue Asp^{466} is hydrolysed by chymotrypsin during generation of 34 kDa fragment, the polypeptide chain in this region should be unstructured, thus Trp^{465} should be in contact with unstructured water. This leaves only Trp^{170} as the source of state II tryptophan fluorescence, and it would therefore appear that it occurs in a hydrophobic region of the protein.

Existence of a long, poorly structured region between residues 680–740 [1], which is preserved in 34 kDa fragment, suggests that the stability of the fragment should be lower than that of the parent molecule. However, the thermal unfolding experiment shows that the 34 kDa peptide unfolds at about 10 °C higher temperature than intact caldesmon (Fig. 2). This implies that there is another part of the polypeptide chain that has a more compact tertiary structure (with greater thermal stability) in the fragment, than that in the intact protein.

In agreement with previous observations [2,23,24], interaction of calmodulin with caldesmon was accompanied by a blue shift of the tryptophan emission spectrum of caldesmon and an increase in the relative quantum yield. Similar changes were observed for the 34 kDa fragment upon interaction with calmodulin (Fig. 3). The predicted by Bryan et al. [34] calmodulin binding site is at the C-terminal domain of caldesmon, located between Val^{629} and Asn^{700} (Val^{644} and Asn^{715} in the sequence given by Hayashi et al. [1]) i.e. in the region where the three C-terminal tryptophans are located. Thus, it is not surprising that the local environment of these tryptophans changes upon binding of calmodulin to either the intact protein or 34 kDa fragment. Another evidence for the direct involvement of tryptophan residues at the calmodulin binding site was previously obtained from measurements of fluorescence anisotropy excitation spectra that show an increase in the average local rigidity of tryptophan residues upon complex formation [24]. Hayashi et al. [16], on the basis of the sequence homology of 34 kDa fragment and troponin I, predicted that the calmodulin binding sites occur between Asp^{466}–Glu^{526} or Thr^{686}–Ala^{765}. Our results sug-

gest that the latter segment is much more probable candidate. Small differences in the shifts of fluorescence spectrum position and in the relative quantum yields between the 34 kDa fragment (7 nm) and intact protein (10 nm) are probably due to the differences in the C-terminal tertiary structures.

Fluorescence titrations of caldesmon or the 34 kDa fragment with calmodulin show a 1 : 1 stoichiometry of binding. According to the recent publication of Wang et al. [38], caldesmon is capable of binding a second molecule of calmodulin in the *N*-terminal region. If this binding site is located far from Trp^{170}, binding of calmodulin (at this site) may not affect its fluorescence spectrum. The binding constant determined for intact caldesmon ($K = 1.4 \cdot 10^7\ M^{-1}$) was similar to that recently reported by Malncik et al. [24], and is one order of magnitude higher than that reported earlier by Shirinsky et al. [23] and by Marston's group [9,22,39]. The latter difference can be due to the difference in experimental condition, since it is known that the interaction between calmodulin and caldesmon is strongly ionic strength and temperature dependent [23,24]. The binding constant of calmodulin to the 34 kDa fragment is two orders of magnitude lower than that for intact caldesmon. We attribute this to the structural alterations in C-terminal portion of protein as mentioned above.

Binding of tropomyosin to the 34 kDa fragment also appears to cause subtle changes in the conformation of the region containing the three tryptophans as indicated by the 5 nm blue shift of the maximum of emission spectrum and the increase in relative quantum yield (Fig. 4). However, it appears that tryptophan residues seem not to be directly involved in the binding. Hayashi et al. [16] suggest that the two C-terminal regions of caldesmon (residues Leu^{524}–Val^{581} and Pro^{623}–Leu^{637}), which show homology with troponin T sequence, have tropomyosin-binding ability. Neither of these segments contain tryptophan residues. Since interaction of tropomyosin with intact caldesmon practically does not affect the position of the fluorescence maximum, one can assume that the distance between tryptophan residue(s) and the tropomyosin binding site in the intact protein and in the 34 kDa fragment, is different, i.e. caldesmon and its C-terminal fragment have somewhat different tertiary structures. Computer aided analysis of the relative quantum yield vs. ratio of tropomyosin to caldesmon or 34 kDa fragment enabled us to determine binding constants, $3.8 \cdot 10^5\ M^{-1}$ and $2.3 \cdot 10^5\ M^{-1}$, respectively.

Recently, the interaction of tropomyosin with caldesmon [14,21], as well as with its 34 kDa fragment [14,15] was the subject of a study by other methods including a fluorescence study of labeled proteins. Pyrene-labeled caldesmon binds to tropomyosin with a binding constant of $3 \cdot 10^5\ M^{-1}$, and in the presence of F-actin the stoichiometric ratio was 1 : 1 [15]. These results are in agreement with ours. Fujii et al. [14] studied the interaction of caldesmon with dansyl-labeled tropomyosin (in the presence of 10 m*M* KCl), and have found the stoichiometric ratio to be 2 : 1, with a binding constant of $4 \cdot 10^7\ M^{-1}$. However, elution of caldesmon bound to tropomyosin–Sepharose column with 150 m*M* NaCl seems to be inconsistent with the high binding constant.

According to Fujii et al. [14] calmodulin, in the presence of Ca^{2+} weakens the caldesmon–tropomyosin interaction, so that caldesmon can be eluted from a tropomyosin affinity column. In our experiments, calmodulin appears to potentiate the binding of tropomyosin to caldesmon (Fig. 5). Moreover, in the presence of calmodulin there appears to be more than one binding site for tropomyosin.

Using affinity chromatography Katayama et al. [15] found a weak interaction between the 34 kDa fragment and tropomyosin in the presence of 50 m*M* NaCl, and no interaction in the presence of 100 m*M* NaCl. Fujii et al. [14] reported the interaction of the 34 kDa fragment with a tropomyosin affinity column in the absence of salt, and found that the fragment can be eluted by calmodulin. Our data obtained in the presence of 50 mM NaCl, demonstrate the interaction of the 34 kDa fragment with tropomyosin in the absence (Fig. 4) and in the presence of calmodulin (Fig. 5). Based on our results it is possible to postulate that the 34 kDa fragment–calmodulin complex can exhibit a cooperative binding process, with bind-

ing of more than one molecule of tropomyosin per caldesmon fragment.

Acknowledgements

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