Functional domain of caldesmon

Adam Szpacenko and Renata Dąbrowska*

Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur Str., 02-093 Warsaw, Poland

Received 21 April 1986; revised version received 20 May 1986

Limted proteolysis of caldesmon has been used in studying the structure-function relationship of this protein. Digestion with α-chymotrypsin yields three major fragments of 110, 80 and 40 kDa. Only the 40 kDa fragment preserves functional properties of the parent molecule: it binds to F-actin, causes inhibition of actomyosin ATPase and binds to calmodulin in a Ca^{2+}-dependent manner. Its further degradation produces an 18 kDa polypeptide that also retains all these properties. Neither F-actin nor calmodulin binding induces dramatic changes in susceptibility to chymotryptic cleavage and the sites of cleavage of caldesmon.

(Chicken gizzard) *Caldesmon* *Chymotryptic cleavage* *Functional domain*

1. INTRODUCTION

Caldesmon is a major actin- and calmodulin-binding protein of smooth muscle [1]. It is located in thin filaments and seems to play a role of regulator of actin-myosin interaction, complementary to myosin phosphorylation [2]. In vitro, its binding to F-actin causes inhibition of actin-activated ATPase activity of smooth and skeletal muscle myosin that can be released by calmodulin in the presence of Ca^{2+} [3–5].

The aim of the present work was to provide some insight into the structural and functional relationships of caldesmon. With the use of limited proteolysis of caldesmon by α-chymotrypsin we have identified the functional domain of this protein responsible for actin and calmodulin binding and involved in the inhibition of actomyosin ATPase activity. Preliminary reports of this work have been presented [6,7].

2. MATERIALS AND METHODS

Caldesmon was prepared from fresh chicken gizzard muscle according to Bretscher [8]. Rabbit skeletal muscle actin and chicken gizzard tropomyosin were obtained as described in [9]. Rabbit skeletal muscle myosin was obtained according to [10]. Calmodulin was prepared from bovine brain as in [11]. The purity of all these proteins was checked by SDS-polyacrylamide gel electrophoresis.

Digestion of caldesmon with α-chymotrypsin was performed at 1 : 1000 weight ratio of enzyme to substrate in a buffer solution containing 75 mM KCl, 20 mM imidazole-HCl (pH 7.0), 1 mM β-mercaptoethanol and 10 mM NaN_3 at 30°C. The reaction was terminated by addition of phenylmethanesulfonyl fluoride (PMSF) (10 × the weight of chymotrypsin).

ATPase activity of rabbit skeletal muscle actomyosin (reconstituted from 30 μg/ml F-actin and 120 μg/ml myosin) was assayed in the presence of chicken gizzard tropomyosin (at a 2:7 molar ratio to actin monomer) and various amounts of intact caldesmon or its fragments in medium containing 50 mM KCl, 2 mM $MgCl_2$, 2 mM ATP, 0.1 mM EGTA and 10 mM imidazole-HCl (pH 7.0) at 30°C. The amount of P_i liberated was measured as in [12].

SDS-polyacrylamide gel electrophoresis was carried out on 7.5–20% gradient slab gel according to

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

Laemmli [13]. For calculation of molecular mass, the following marker proteins were used: rabbit skeletal muscle myosin heavy chain (205 kDa) and light chains (26, 18 and 16 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (66 kDa), actin (42 kDa), troponin I (21 kDa) and calmodulin (16.7 kDa).

Protein concentration was determined by measuring UV light absorbance with the following values of extinction coefficients and molecular mass: caldesmon, E_{276} = 0.30, 140 kDa [8]; G-actin, E_{290} = 0.63 [14], 42 kDa; myosin, E_{279} = 0.54, 470 kDa [15]; tropomyosin, E_{278} = 0.29, 72 kDa [16]; calmodulin, E_{278} = 0.20, 16.7 kDa [17]; or by the method of Lowry et al. [18].

3. RESULTS

In the initial experiments caldesmon was digested with various concentrations of α-chymotrypsin for different times. It was found that a ratio of α-chymotrypsin to caldesmon of 1:1000 (w/w) was the most suitable as this allowed the observation of intermediate polypeptides. A representative digestion pattern is shown in fig.1. After 1 min digestion three major fragments of 110, 80 and 40 kDa and several minor fragments of 54, 29, 23, 18 kDa and sometimes a 16 kDa fragment of caldesmon are seen. The latter seem to derive from the major fragments since their amounts gradually increase during further digestion with a parallel decrease in the amounts of the major fragments (fig.1). The 54 kDa fragment seems to be a degradation product of the 110 kDa polypeptide, and the 23 and 18 kDa fragments presumably derive from the 40 kDa polypeptide.

Parallel investigation of the effects of intact and digested caldesmon on the actomyosin ATPase activity (in the presence of chicken gizzard tropomyosin) revealed that the extent of inhibition of the ATPase by the chymotryptic digest of caldesmon is comparable to that by the intact protein (fig.1). Thus, it is apparent that some of the fragments of caldesmon retain the ability of the parent molecule to inhibit the actomyosin ATPase.

F-Actin or calmodulin binding as well as the presence of tropomyosin did not significantly affect the susceptibility of caldesmon to chymotryptic attack and the sites of its cleavage (not shown). This indicates that these proteins do not induce extensive conformational changes in the structure of caldesmon. Digestion of F-actin–caldesmon complex with α-chymotrypsin and subsequent ultracentrifugation of the digest revealed that the 40, 23 and 18 kDa fragments of caldesmon co-sediment with F-actin (fig.2). The pellet fraction, but not the supernatant which contains mainly the high-molecular-mass fragments (110 and 80 kDa), caused the inhibition of actomyosin ATPase in the

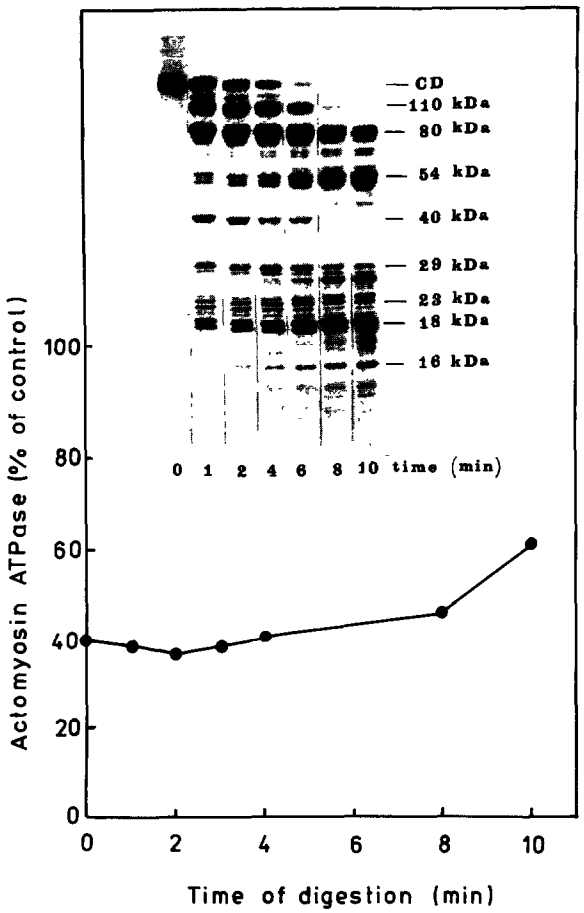

Fig.1. Effect of chymotryptic fragmentation of caldesmon on its ability to inhibit actomyosin ATPase activity. Digestion of caldesmon with α-chymotrypsin was performed at a weight ratio of protease to substrate of 1:1000 under the conditions described in section 2. Samples of the reaction mixture withdrawn during digestion were analysed by SDS-polyacrylamide gel electrophoresis and examined in ATPase assay. Actomyosin ATPase activity was assayed as described in section 2 at a 1:1.5 weight ratio of intact or digested caldesmon to actin. CD, caldesmon.

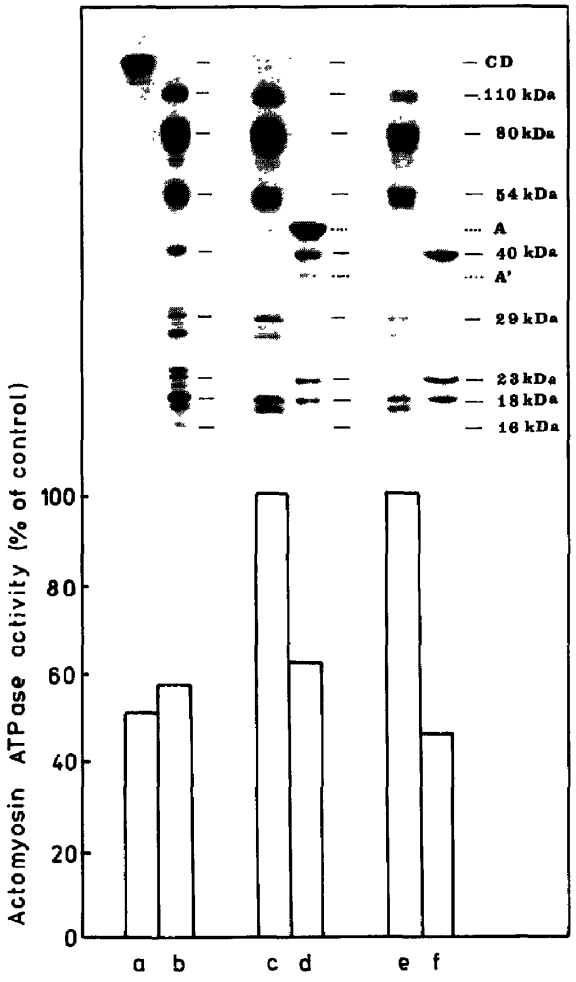

Fig.2. Binding of caldesmon fragments to F-actin and calmodulin and their effects on actomyosin ATPase activity. To identify F-actin-binding fragments of caldesmon an excess of intact caldesmon was mixed with F-actin (at 1 mg/ml) in a buffer containing 100 mM KCl, 10 mM imidazole (pH 7.0), 0.1 mM $CaCl_2$, 5 mM NaN_3 and 1 mM β-mercaptoethanol and ultracentrifuged for 3 h at 100000 × g. Pellet resuspended in the same buffer was treated with α-chymotrypsin (6 min at a 1:1000 weight ratio of protease to substrate). The digest was ultracentrifuged for 3 h at 100000 × g. Dissolved pellet and supernatant were analysed by SDS-polyacrylamide gel electrophoresis and examined in ATPase assay at a weight ratio of each fraction to actin of 1:1.5. To identify calmodulin-binding fragments caldesmon was digested with α-chymotrypsin (6 min at 1:1000 weight ratio of protease to substrate). The digest was applied on a calmodulin-Sepharose 4B column (1.5 × 26 cm) equilibrated with buffer containing 0.2 mM $CaCl_2$, 50 mM NaCl, 40 mM Tris-HCl (pH 7.5), 10 mM NaN_3 and 1 mM β-mercaptoethanol. The non-retarded and EGTA (2 mM)-eluted fractions were analysed by SDS-polyacrylamide gel electrophoresis and examined in ATPase assay at a 1:1.5 (w/w) ratio of each fraction or intact caldesmon to actin. (a) Intact caldesmon, (b) digested caldesmon, (c) fragments of caldesmon that do not bind to F-actin, (d) F-actin-binding fragments of caldesmon, (e) caldesmon fragments that do not bind to calmodulin, (f) calmodulin-binding fragments of caldesmon. CD, caldesmon; A, actin; A', actin degradation product.

presence of chicken gizzard tropomyosin indicating that only those caldesmon fragments which bind to F-actin can affect the actomyosin ATPase. There was no evidence of significant degradation of F-actin alone under identical conditions of chymotryptic digestion, only a thin band below actin derived from this protein (fig.2).

To identify calmodulin-binding fragment(s) of caldesmon, the chymotryptic digest of the latter protein was applied on an affinity column of calmodulin-Sepharose 4B. SDS-polyacrylamide gel electrophoresis showed that the fraction of polypeptides specifically bound to the calmodulin column and eluted with 2 mM EGTA contained the 40, 23 and 18 kDa polypeptides, the same as those binding to F-actin. The nonretarded fraction contained the high-molecular-mass polypeptides 110 and 80 kDa, and several minor fragments. This fraction did not affect the ATPase activity of actomyosin (fig.2).

The fraction containing calmodulin-binding polypeptides of caldesmon was applied to an Affi-Gel blue column and eluted with a linear 0–1.8 M NaCl gradient. The electrophoretically pure 18 and 40 kDa fragments of caldesmon were selected from the leading and trailing part of the protein peak, respectively (fig.3). As shown in fig.4 both fragments had an ability to inhibit the actomyosin ATPase activity in the presence of smooth muscle tropomyosin. The extent of inhibition was roughly the same as that by intact caldesmon. Digestion of the isolated 40 kDa polypeptide with chymotrypsin confirmed our presumption that the 18 kDa (as well as the 23 kDa) fragment is its degradation

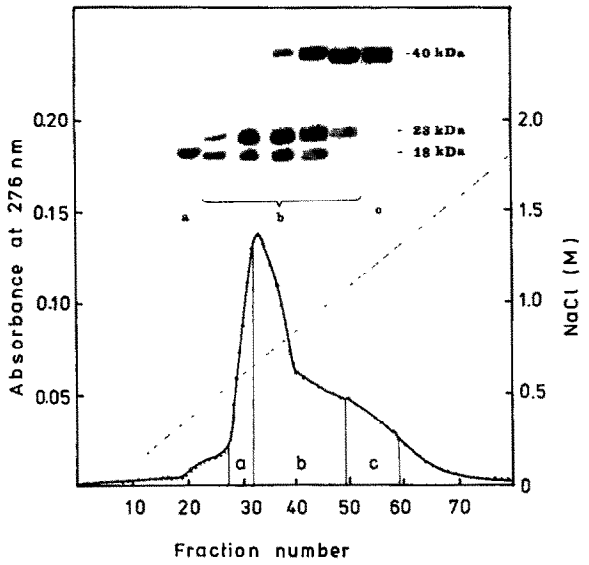

Fig.3. Purification of the 40 and 19 kDa fragments of caldesmon on Affi-Gel blue column. The fraction of caldesmon fragments eluted from calmodulin-Sepharose 4B column with EGTA (see legend to fig.2) was applied to an Affi-Gel blue column (2 × 20 cm) equilibrated with 20 mM K_2HPO_4, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.02% NaN_3. The proteins were eluted with a 0–1.8 M NaCl gradient. Pure 40 and 18 kDa fragments were selected by SDS-polyacrylamide gel electrophoresis (see inset) and pooled as indicated.

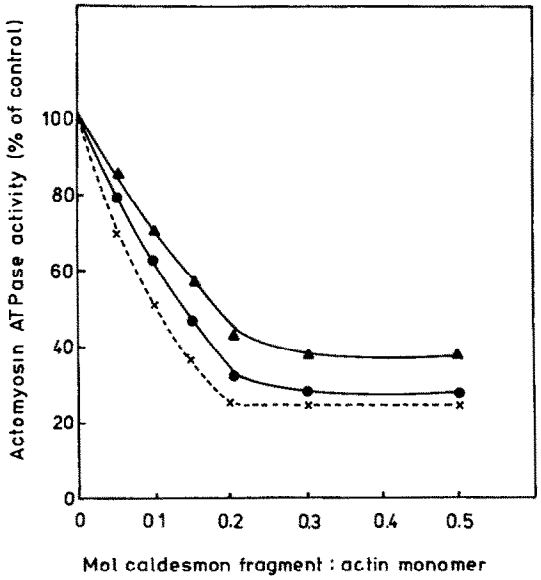

Fig.4. Effect of 40 and 18 kDa fragments of caldesmon on actomyosin ATPase activity. ATPase activity was assayed as described in section 2. Amounts of the 40 kDa (●), 18 kDa (▲) fragment and intact caldesmon (×) as indicated on the abscissa.

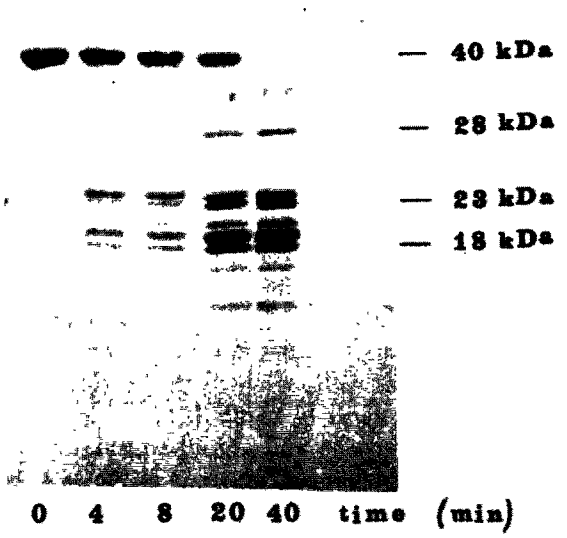

Fig.5. Chymotryptic digestion of the 40 kDa fragment of caldesmon. Digestion was performed at a weight ratio of protease to substrate of 1:1000 under the conditions described in section 2. The digestion pattern was examined by SDS-polyacrylamide gel electrophoresis.

product (fig.5). Thus, it can be concluded that caldesmon contains a functional domain that is present in the 18 kDa fragment of its polypeptide chain (representing less than 13% of the molecular mass of the parent molecule). This domain is responsible for the binding of caldesmon to F-actin that induces the inhibition of actomyosin ATPase activity, and for the Ca^{2+}-dependent binding to calmodulin.

4. DISCUSSION

Caldesmon seems to play an important role in regulation of smooth muscle and probably non-muscle motile processes [19]. The inhibition of actin-myosin interaction by this actin-binding protein can be regulated by Ca^{2+} either via calcium-dependent binding to calmodulin [2,3] or/and by its Ca^{2+}- and calmodulin-dependent phosphorylation [4,20].

We report here the results of studies on limited

proteolysis of chicken gizzard caldesmon as a probe of the structure-function relations of this protein. This approach allowed us to identify a domain of caldesmon containing the actin-binding site, the Ca^{2+}-dependent calmodulin-binding site as well as the site responsible for the inhibition of actomyosin ATPase activity. It is located in the 18 kDa polypeptide which is a product of further digestion of the 40 kDa fragment of caldesmon. The appearance of the latter fragment, in parallel with the high-molecular-mass fragments of 110 and 80 kDa that do not affect actomyosin ATPase activity, after 1 min digestion suggests that it originates from a terminal part of caldesmon molecule. The 18 kDa polypeptide has a highly basic character since it does not bind to an anion-exchange column. Although the whole molecule of caldesmon is moderately acidic, it has a relatively high content of lysine and arginine residues [20] which are presumably concentrated in the 18 kDa fragment. Both the 40 and 18 kDa fragments possess the ATP-binding site as indicated by their binding to the Affi-Gel blue column as does the intact molecule. Hence, one can suppose that the phosphorylation site of caldesmon, important from the point of view of its function [20], is also located in the 18 kDa fragment.

It has been shown that caldesmon is an integral constituent of smooth muscle thin filaments [21]. Its distribution on F-actin filaments is not known. It can be speculated that having elongated molecules with a tendency to form dimers [19,22], caldesmon can extend along actin filaments similarly to tropomyosin. The location of its actin-binding site in a relatively small domain (possibly at a terminal part of the molecule) could suggest that each caldesmon molecule binds to an actin filament at a specific point, possibly at a site where the ends of four tropomyosin molecules meet. This model would agree very well with the stoichiometry of actin monomer:tropomyosin:caldesmon of 1:1/7:1/28 in smooth muscle thin filaments [21]. However, it does not explain how the effect of binding of one caldesmon molecule is transmitted along 28 actin monomers, especially in view of the present observation that inhibition of actomyosin ATPase by intact caldesmon and its small fragment is nearly equal.

REFERENCES

[1] Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981) Proc. Natl. Acad. Sci. USA 78, 5652–5655.
[2] Marston, S.B. and Smith, C.W.J. (1985) J. Muscle Res. Cell Motility 6, 669–706.
[3] Sobue, K., Morimoto, K., Inui, M., Kanda, K. and Kakiuchi, S. (1982) Biomed. Res. 3, 188–196.
[4] Ngai, P.K. and Walsh, M.P. (1984) J. Biol. Chem. 259, 13656–13659.
[5] Dąbrowska, R., Goch, A., Gałązkiewicz, B. and Osińska, H. (1985) Biochim. Biophys. Acta 842, 70–75.
[6] Dąbrowska, R., Szpacenko, A. and Gałązkiewicz, B. (1986) 14th European Conference on Muscle and Motility, Ulm, FRG, Sept.11–15, Abstr. p.8.
[7] Szpacenko, A., Makuch, R. and Dąbrowska, R. (1986) EMBO Workshop on Contractile Mechanism of Smooth Muscle, Marian Alm, Austria, March 11–15, Abstr. p.24.
[8] Bretscher, A. (1984) J. Biol. Chem. 259, 12873–12880.
[9] Dąbrowska, R., Nowak, E. and Drabikowski, W. (1980) Comp. Biochem. Physiol. 65B, 75–83.
[10] Perry, S.V. (1955) Methods Enzymol. 2, 582–588.
[11] Gopalakrishna, R. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[12] Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Houk, T. and Ue, K. (1974) Anal. Biochem. 62, 66–74.
[15] Wagner, P.D. and Weeds, A.G. (1977) J. Mol. Biol. 109, 455–473.
[16] Hartshorne, D.J. and Mueller, H. (1969) Biochem. Biophys. Res. Commun. 175, 301–319.
[17] Klee, C.B. (1977) Biochemistry 16, 1017–1024.
[18] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[19] Dąbrowska, R. and Gałązkiewicz, B. (1986) Biomed. Biochim. Acta 45, S153–S158.
[20] Ngai, P.K. and Walsh, M.P. (1985) Biochem. J. 230, 695–707.
[21] Marston, S.B. and Lehman, W. (1985) Biochem. J. 231, 517–522.
[22] Fürst, D.O., Cross, R.A., De Mey, J. and Small, J.V. (1986) EMBO J. 5, 251–257.