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Chemical modification of the sole histidine residue of smooth muscle caldesmon

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Caldesmon was stoichiometrically *N*-carbethoxylated specifically at the only histidine residue (His-610) with diethylpyrocarbonate. Carbethoxylation of a 1:1 molar complex of caldesmon and calmodulin in the presence of Ca^{2+} resulted in the stoichiometric *N*-carbethoxylation of His-610 of caldesmon and His-107 of calmodulin. Carbethoxy-caldesmon, like the unmodified protein, bound to immobilized calmodulin (in the presence of Ca^{2+}) and to immobilized tropomyosin (at low ionic strength). The affinity of F-actin for carbethoxy-caldesmon ($K_d = 1.29 \times 10^{-6}$ M) was similar to that for unmodified caldesmon ($K_d = 0.88 \times 10^{-6}$ M), and the modified protein was as effective as control caldesmon in the inhibition of the actin-activated MgATPase of skeletal muscle myosin. We conclude that the predicted basic amphiphilic α-helical sequence (Arg-593–His-610) does not represent the calmodulin-binding site of caldesmon. Furthermore, His-610 does not play a major role in the interaction of caldesmon with F-actin or tropomyosin.

Caldesmon; Calmodulin; Chemical modification; Smooth muscle

1. INTRODUCTION

Caldesmon is a thin filament-associated protein which has been implicated in the regulation of smooth muscle contraction [1]. The isolated protein is capable of interaction in vitro with actin [2], myosin [3], tropomyosin [4] and calmodulin [2]. The calmodulin interaction is Ca^{2+}-dependent and has been suggested to be important in regulating the ability of caldesmon to inhibit smooth muscle actin-activated myosin MgATPase by dissociating caldesmon from actin [5]. Chicken gizzard caldesmon cDNA clones have been sequenced in two laboratories [6,7]. The deduced amino acid sequences probably represent the two isoforms of chicken gizzard caldesmon [8]. The dopmain structure of caldesmon has been extensively studied [9,10]. The myosin-binding domain is located in the N-terminal region of caldesmon, whereas the actin-, tropomyosin- and calmodulin-binding domains are located near the opposite end of the molecule. Calmodulin-binding sites have been identified in several known calmodulin-binding proteins and generally consist of ~18 amino acids having a basic amphilic α-helical structure [11]. Examination of the amino acid sequence of calmodulin-binding fragments of caldesmon reveals one such sequence: Arg-593–His-610 [6,12]. However, Bartegi et al. [13] recently concluded that this does not represent the calmodulin-binding site since a 10 kDa peptide corresponding to the C-terminus of caldesmon (Trp-659–Pro-756) binds calmodulin but does not contain this sequence. CNBr peptides corresponding to the remainder of the caldesmon molecule did not bind to immobilized calmodulin. We have pursued this surprising conclusion by examining the effects of specific chemical modification of His-610 located at the C-terminal end of the basic amphiphilic α-helix and conclude that this indeed is not the calmodulin-binding site.

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2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (20–40 Ci/mmol) was purchased from Amersham (Oakville, Ontario, Canada) and diethylpyrocarbonate and hydroxylamine from Sigma (St. Louis, MO). Dithiothreitol was purchased from Boehringer Mannheim (Dorval, Quebec, Canada) and electrophoresis reagents from Bio-Rad (Mississauga, Ontario, Canada). General laboratory reagents used were of analytical grade or better and were purchased from Fisher Scientific (Calgary, Alberta, Canada). Proteins were purified by methods published earlier: chicken gizzard caldesmon [14], tropomyosin [14] and actin [15], rabbit skeletal muscle actin [16] and myosin [17], and bovine brain calmodulin [18]. Calmodulin and tropomyosin were coupled to CNBr-activated Sepharose 4B (Pharmacia, Baie d'Urfe, Quebec, Canada) according to the manufacturer's instructions.

2.2. *Carbethoxylation of caldesmon*

The sole imidazole function of caldesmon was carbethoxylated with diethylpyrocarbonate using the method described by Miles [19]. Caldesmon (1.0 mg/ml) was incubated at 22°C in 0.1 M sodium phosphate buffer (pH 6.0) in the sample and reference cuvettes of a Pye Unicam PU8800 UV/visible double-beam spectrophotometer. The reaction was initiated by the addition to the sample cell of a fresh, concentrated solution of diethylpyrocarbonate in ethanol to give a final reagent concentration of 50 μM (a 4.4-fold molar excess over

caldesmon). An equal volume of absolute ethanol was added to the reference cell and the formation of carbethoxy-caldesmon was monitored by continuous recording of the increase in A_{242nm}. The extent of histidine modification was calculated from the absorbance using a value of 3200 $M^{-1} \cdot cm^{-1}$ for the molar extinction coefficient, at 242 nm, of *N*-carbethoxyhistidine residues in proteins [20]. For modification of the caldesmon-calmodulin complex, 10 μM caldesmon and 10 μM calmodulin were incubated at 22°C in 0.1 M sodium phosphate (pH 6.0), 1 mM $CaCl_2$ in the sample and reference cuvettes of the spectrophotometer. The reaction was started by addition to the sample cuvette of diethylpyrocarbonate (115 μM) and to the reference cuvette of an equal volume of absolute ethanol. At the time indicated, EGTA was added to each cuvette to a final concentration of 4 mM.

2.3. *Actin binding*

Smooth muscle actin (9 μM) and untreated or carbethoxylated caldesmons (0.57, 1.14, 1.72, 2.28, 2.85, 4.31 and 5.75 μM) were incubated for 30 min at 25°C in 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 1 mM ATP. Samples (0.2 ml) were then centrifuged at 100 000 × *g* in a Beckman TL100 centrifuge for 1 h at 2°C in order to sediment F-actin and bind caldesmon. Pellets and supernatants were subjected to SDS-PAGE. The distribution of caldesmon between the pellets and supernatants was quantified by densitometric scanning of the Coomassie blue-stained gels using an LKB model 2202 Ultroscan laser densitometer equipped with a Hewlett-Packard model 3390A integrator. The K_a values of actin for control and carbethoxy-caldesmons were calculated by Scatchard analysis of the sedimentation data.

2.4. *Actin-activated myosin MgATPase assay*

ATPase activities were measured as previously described [21] under the following conditions: 25 mM Tris-HCl (pH 7.5), 50 mM KCl, 3.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mM EGTA, 1 mM [γ-^{32}P]ATP (~9000 cpm/nmol), 3.6 μM skeletal actin, 0.57 μM skeletal myosin, in the absence or presence of control or carbethoxy-caldesmon (1.2 and 2.0 μM), in reaction volumes of 0.3 ml at 30°C. Reactions were started by addition of ATP. Samples (50 μl) of reaction mixtures were withdrawn at 0.5-min intervals up to 2.5 min for quantification of $^{32}P_i$ release [21]. Rates of ATP hydrolysis were calculated by linear regression analysis of the linear time-course data.

2.5. *Other procedures*

Protein concentrations were determined by spectrophotometric measurements in a Beckman DU-8B UV/visible spectrophotometer using the following extinction coefficients: calmodulin, $E^{1\%}_{277nm} = 1.9$ [22]; caldesmon, $E^{1\%}_{280nm} = 3.3$ [23]; tropomyosin, $E^{1\%}_{278nm} = 2.9$ [24]; actin, $E^{1\%}_{290nm} = 6.5$ [25]; and myosin, $E^{1\%}_{280nm} = 5.3$ [26]. SDS-PAGE was performed in 0.1% SDS/10% acrylamide mini-slab gels to analyse column elution profiles, or in full-size 0.1% SDS/7.5–20% polyacrylamide gradient slab gels with a 5% acrylamide stacking gel to analyse caldesmon binding to actin. In each case the Laemmli [27] buffer system was used.

3. RESULTS AND DISCUSSION

The time course of carbethoxylation of caldesmon with diethylpyrocarbonate is illustrated in Fig. 1. At the plateau, ~1 mol *N*-carbethoxyhistidine was detected/mol caldesmon. This modification was completely reversed by incubation of carbethoxylated caldesmon overnight with 0.1 M hydroxylamine at pH 7 and 22°C, indicating that modification of lysyl or sulfhydryl residues did not occur (data not shown) [19]. The spectra in Fig. 2A demonstrate very little change in A_{278nm} following incubation of caldesmon with diethylpyrocarbonate, indicating that little or no modification of

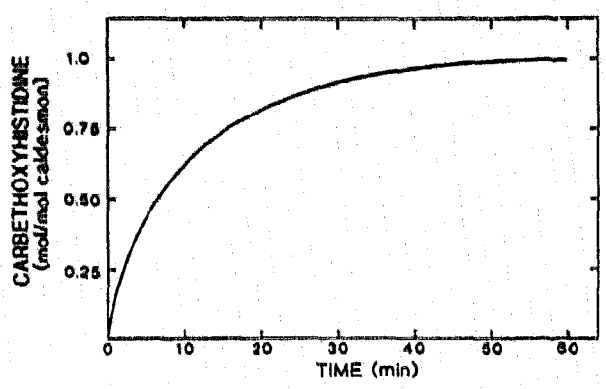

Fig. 1. Chemical modification of caldesmon with diethylpyrocarbonate. Caldesmon was incubated with diethylpyrocarbonate as described in section 2. *N*-carbethoxylation was followed by continuous recording of A_{242nm}. The stoichiometry of modification was calculated from the absorbance values.

tyrosine residues has occurred. The spectra in Fig. 2B illustrate the time-dependent increase in A_{242nm} (histidine modification) with no significant change in A_{278nm}, indicative of no chemical modification of tyrosine residues. Treatment of caldesmon with a 4.4-fold molar excess of diethylpyrocarbonate therefore results in specific and stoichiometric modification of the sole histidine residue.

Carbethoxylation of a 1:1 molar mixture of caldesmon and calmodulin in the presence of Ca^{2+} resulted in the incorporation of 0.92 mol *N*-carbethoxyhistidine/mol protein (Fig. 3). Calmodulin, like caldesmon, contains one histidine residue [28]

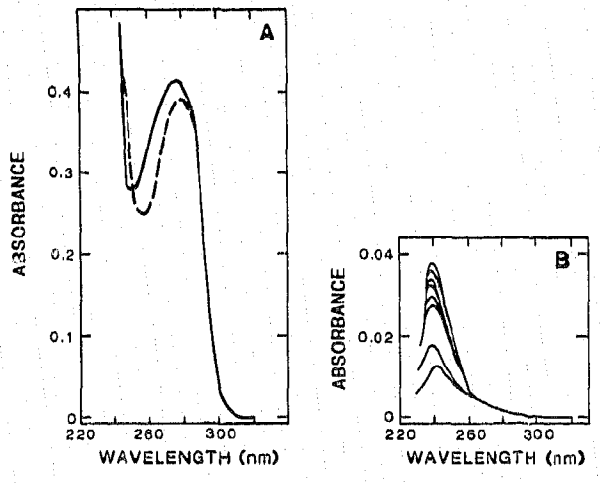

Fig. 2. Spectral analysis of the carbethoxylation reaction. A. Spectra were recorded before (——) and after (– – –) the reaction shown in Fig. 1 with reaction buffer in the reference cell of the Pye Unicam spectrophotometer. B. During the course of a reaction identical to that shown in Fig. 1, spectra were recorded at selected times (10, 15, 17, 20, 25, 30, 40 and 50 min). The reference cell contained an identical mixture except for the omission of diethylpyrocarbonate which was replaced by an equal volume of vehicle (ethanol).

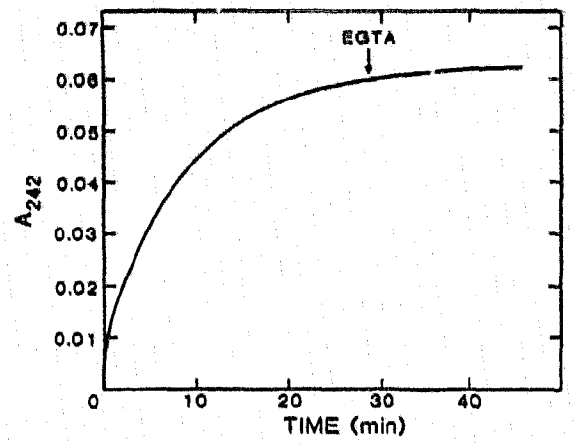

Fig. 3. Chemical modification of the caldesmon-calmodulin complex with diethylpyrocarbonate. A 1:1 molar complex of caldesmon and calmodulin in the presence of Ca^{2+} was incubated with diethylpyrocarbonate as described in section 2. *N*-carbethoxylation was followed by continuous recording of A_{242nm}. At the time indicated, an excess of EGTA was added to dissociate caldesmon and calmodulin.

which can be carbethoxylated in the presence or absence of Ca^{2+} [29]. Removal of Ca^{2+} with EGTA, causing the dissociation of calmodulin and caldesmon, did not result in any spectroscopic change. We can conclude therefore that His-610 of caldesmon is accessible to diethylpyrocarbonate when complexed with calmodulin, suggesting that this residue is not part of the calmodulin-binding site. In support of this conclusion, carbethoxylated caldesmon, like untreated caldesmon, bound to a calmodulin-Sepharose affinity column in the presence of Ca^{2+} and was eluted with EGTA (data not shown).

The affinities of smooth muscle actin for control and carbethoxylated caldesmons were compared using a sedimentation assay as described in section 2. The K_d for control caldesmon was determined to be 0.88×10^{-6} M and for carbethoxylated caldesmon, 1.29×10^{-6} M. Histidine modification therefore caused a slight, possibly insignificant, reduction in the affinity of caldesmon for actin. Consistent with this observation, carbethoxylated caldesmon was as effective as the unmodified protein in inhibition of the actin-activated MgATPase activity of skeletal myosin (Table I).

Table I

Comparison of the effects of control and carbethoxylated caldesmons on the skeletal actin-activated myosin MgATPase

Caldesmon	ATPase rate (nmol P_i/min·mg myosin)
None	693.9±14.7 ($n=2$)
Control, 1.2 μM	371.6±11.1 ($n=4$)
Control, 2.0 μM	387.8± 4.7 ($n=2$)
Carbethoxylated, 1.2 μM	383.7±11.5 ($n=4$)
Carbethoxylated, 2.0 μM	332.1±11.2 ($n=2$)

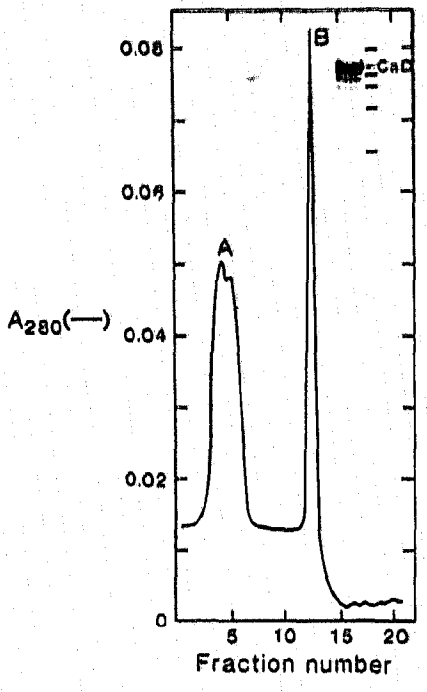

Fig. 4. Binding of carbethoxy-caldesmon to immobilized tropomyosin. Carbethoxylated caldesmon (3 mg; see Fig. 1) was dialyzed vs 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM dithiothreitol and applied, at a flow rate of 10 ml/h, to a column (1 × 10 cm) of tropomyosin-Spharose 4B. The column was washed with the same buffer and bound protein was eluted with this buffer containing 0.4 M NaCl. Fractions of 2.5 ml were collected. The inset shows SDS-PAGE analysis of the unbound material (peak A) and bound protein (peak B). The positions of M_r markers are shown.

Carbethoxylated caldesmon, like the untreated protein, was found to bind to a tropomyosin-Sepharose affinity column at low ionic strength and was eluted with a buffer containing 0.4 M NaCl (Fig. 4). The flow-through peak from this column contained polypeptides of M_r 110 and 90 kDa which probably represent proteolytic fragments of caldesmon (see gel inset of Fig. 4).

Specific modification of His-610 of caldesmon did not affect its interaction with calmodulin suggesting that the basic amphiphilic α-helical sequence (Arg-593–His-610) does not represent the calmodulin-binding site. These results support the conclusion of Bartegi et al. [13] that this site is located in a different part of the molecule (towards the C-terminus). Carbethoxylation of His-610 of caldesmon had little, if any, effect on its affinity for actin or ability to inhibit the actin-activated MgATPase activity of skeletal muscle myosin suggesting that this residue does not play an important role in the interaction of caldesmon with actin. Finally, carbethoxy-caldesmon bound to tropomyosin-Sepharose at low ionic strength and could be eluted with NaCl, as for the untreated protein [30]. On the basis of sequence homologies with the tropomyosin-binding domains of skeletal muscle troponin T, two regions within the caldesmon molecule

(Glu-508-Lys-565 and Lys-607-Arg-621) have been implicated as tropomyosin-binding regions [31]. The latter sequence contains His-610 but carbethoxylation clearly did not prevent the binding of caldesmon to tropomyosin. Either this residue is not involved in tropomyosin binding or it plays a relatively minor role in the interaction of caldesmon with tropomyosin.

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REFERENCES

[1] Walsh, M.P. (1990) Prog. Clin. Biol. Res. 327, 127–140.
[2] Sobue, K., Muramoto, Y., Fujita, M. and Kakiuchi, S. (1981) Proc. Natl. Acad. Sci. USA 78, 5652–5655.
[3] Ikebe, M. and Riordan, S. (1988) J. Biol. Chem. 263, 3055–3058.
[4] Graceffa, P. (1987) FEBS Lett. 218, 139–142.
[5] Sobue, K., Morimoto, K., Inui, M., Kanda, K. and Kakiuchi, S. (1982) Biomed. Res. 3, 188–196.
[6] Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R.G. and Lin, W.-G. (1989) J. Biol. Chem. 264, 13873–13879.
[7] Hayashi, K., Kanda, K., Kimizuka, F., Kato, I. and Sobue, K. (1989) Biochem. Biophys. Res. Commun. 164, 503–511.
[8] Riseman, V.M., Lynch, W.P., Nefsky, B. and Bretscher, A. (1989) J. Biol. Chem. 264, 2869–2875.
[9] Szpacenko, A. and Dabrowska, R. (1986) FEBS Lett. 202, 182–186.
[10] Fujii, T., Imai, M., Rosenfeld, G.C. and Bryan, J. (1987) J. Biol. Chem. 262, 2757–2763.
[11] O'Neil, K.T. and DeGrado, W.F. (1990) Trends Biochem. Sci. 15, 59–64.
[12] Lenzyk, J., Mornet, D., Audemard, E. and Collins, J.H. (1989) Biochem. Biophys. Res. Commun. 160, 1371–1378.
[13] Bartegi, A., Fattoum, A., Derancourt, J. and Kassab, R. (1990) J. Biol. Chem. 265, 15231–15238.
[14] Bretscher, A. (1984) J. Biol. Chem. 259, 12873–12880.
[15] Ngai, P.K., Gröschel-Stewart, U. and Walsh, M.P. (1986) Biochem. Int. 12, 89–93.
[16] Zot, H.G. and Potter, J.D. (1981) Prep. Biochem. 11, 381–395.
[17] Persechini, A. and Rowe, A.J. (1984) J. Mol. Biol. 172, 23–39.
[18] Walsh, M.P., Valentine, K.A., Ngai, P.K., Carruthers, C.A. and Hollenberg, M.D. (1984) Biochem. J. 224, 117–137.
[19] Miles, E.W. (1977) Methods Enzymol. 47, 431–442.
[20] Ovádi, J., Libor, S. and Elödi, P. (1967) Biochim. Biophys. Acta 2, 455–458.
[21] Ikebe, M. and Hartshorne, D.J. (1985) Biochemistry 24, 2380–2387.
[22] Klee, C.B. (1977) Biochemistry 16, 1017–1024.
[23] Graceffa, P., Wang, C.-L.A. and Stafford, W.F. (1988) J. Biol. Chem. 263, 14196–14202.
[24] Eisenberg, E. and Kielley, W.W. (1974) J. Biol. Chem. 249, 4742–4748.
[25] Rees, M.R. and Young, M. (1967) J. Biol. Chem. 242, 4449–4458.
[26] Margossian, S.S. and Lowey, S. (1982) Methods Enzymol. 85, 55–71.
[27] Laemmli, U.K. (1970) Nature 227, 680–685.
[28] Watterson, D.M., Sharief, F. and Vanaman, T.C. (1980) J. Biol. Chem. 255, 962–975.
[29] Walsh, M. and Stevens, F.C. (1977) Biochemistry 16, 2742–2749.
[30] Fujii, T., Ozawa, J., Ogoma, Y. and Kondo, Y. (1988) J. Biol. Chem. (Tokyo) 104, 734–737.
[31] Hayashi, K., Yamada, S., Kanda, K., Kimizuka, F., Kato, I. and Sobue, K. (1989) Biochem. Biophys. Res. Commun. 161, 38–45.