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AMINO ACID SEQUENCE OF A 15 KILODALTON ACTIN-BINDING FRAGMENT OF TURKEY GIZZARD CALDESMON: SIMILARITY WITH DYSTROPHIN, TROPOMYOSIN AND THE TROPOMYOSIN-BINDING REGION OF TROPONIN T

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SUMMARY: We have determined the amino acid sequence of a 15 kDa actin-binding fragment of turkey gizzard caldesmon. The 96-residue fragment contains 29 acidic and 29 basic residues, and is predicted to have an extended helical conformation stabilized by numerous internal salt bridges. CaD15 bears some resemblance to dystrophin, tropomyosin and several other proteins, but is most strikingly similar to the tropomyosin-binding segment of troponin T.

Caldesmon is a 120 kDa, asymmetrical, elongated, actin- and calmodulin-binding protein abundant in smooth muscle (1). It is thought to play a regulatory role similar to that of troponin in striated muscle (2,3). Limited proteolysis of caldesmon yields a C-terminal 35 kDa fragment which binds to both F-actin and calmodulin, and a 90 kDa N-terminal fragment which does not (4,5). Recently, Mornet et al. (6) found that further proteolysis of the 35 kDa fragment yields a 15 kDa N-terminal subfragment, CaD15, which interacts with F-actin but not calmodulin. Crosslinking studies indicated that CaD15 may induce a conformational change in the F-actin filament during smooth muscle contraction. In the present study we have determined, by protein chemistry methods, the amino acid sequence of CaD15 from turkey gizzard caldesmon. Analysis of the sequence reveals structural similarities to troponin T which may be functionally significant.

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EXPERIMENTAL PROCEDURES

Materials. All chemicals were of the highest grade commercially available. Sequencer reagents were from Applied Biosystems. Phenylthiohydantoin amino acid standards were from either Pierce or Applied Biosystems. Amino acid standards, phenylisothiocyanate (for non-sequencer use), constant boiling HCl and cyanogen bromide (CNBr) were from Pierce. Pepsin was from Worthington. HPLC-grade water and trifluoroacetic acid were from Fisher. HPLC-grade acetonitrile was from Burdick and Jackson. All other chemicals were from Fisher.

Amino acid compositions and sequence analysis. Amino acid compositions were determined by the Waters "PICO-Tag" method, as described previously (7). Cysteine was measured as pyridylethylcysteine, following derivatization of CaD15 with 4-vinylpyridine. Amino acid sequences were determined with an Applied Biosystems Model 477A gas phase Protein Sequencer, equipped with an on-line model 120A PTH amino acid analyzer. Computer analyses of the CaD15 sequence were done with the aid of GENEPRO and PC/GENE programs.

Preparative HPLC. All peptide mixtures were separated by reverse-phase HPLC using either a 4.6mm x 25cm Vydac 218TP54 (C-18) or 214TP54 (C-4) column. Two Waters M510 pumps, a Waters M680 controller, a Waters M480 variable wavelength absorbance detector, a Linear dual channel recorder, and a Glenco SV-3 injector were used as our HPLC system. Solvent A was 0.1% TFA in acetonitrile:water (5:95, v/v), and solvent B was 0.1% TFA in acetonitrile:water (95:5, v/v). All separations were carried out using a linear gradient from 0%B to 40% or 50%B over 60 minutes at a flow rate of 1 ml/min. The eluent was monitored at 220nm.

Peptide purification: CaD15 was prepared as described previously (6), following limited proteolysis of turkey gizzard caldesmon with submaxillarus arginine-C protease. In order to obtain material of sufficient purity for sequence determination, a final step of purification by HPLC was carried out, using the C-4 column (Figure 1).

For CNBr digestion, 5 nmol of CaD15 was dissolved in 200 ul of 70% formic acid. To this was added 1 mg of CNBr freshly dissolved in 70% formic acid. The digest was stirred in a closed screw-cap tube for 19 hours at room temperature. Digestion was stopped by addition of 2 mL of water and drying under nitrogen. The sample was redissolved in 200 ul of 70% formic acid and applied to the C-18 HPLC column (Figure 2).

For Peptic digestion, 10 nmol of CaD15 was dissolved in 200 ul of 5% formic acid. To this was added 20 ug of pepsin freshly dissolved in 5% formic acid. Digestion took place in a capped tube with constant stirring at room temperature for 2 hours. The sample was then applied directly to the C-4 HPLC column (Figure 3).

RESULTS AND DISCUSSION

The CaD15 used in the present study was prepared by ion exchange chromatography (6). Our initial N-terminal sequence analysis of this material partially confirmed earlier results (6), but also revealed the presence of low-yield impurities which would have interfered with further sequence determination. HPLC purification of this sample (Figure 1) yielded a single major peak of CaD15 and numerous minor peaks which were not further

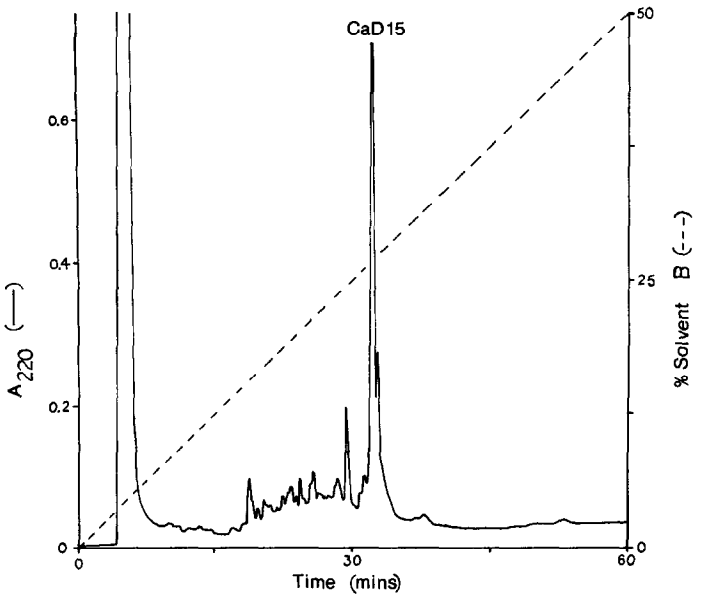

Figure 1. HPLC purification of CaD15.

studied. N-terminal sequence analysis of HPLC-purified CaD15 showed that this preparation was essentially homogeneous, and therefore it was used for all subsequent studies. We identified residues 1-37, confirming and extending previous results (6). We also reproduced the earlier observation (6) of an apparently sequence-specific drop in repetitive yield following cycle 15, and in addition noticed a further drop after cycle 31.

Figure 4 summarizes the strategy and results of our sequence determination of CaD15. The sequence is in complete agreement with amino acid analyses, which revealed the absence of Cys, His, Tyr and Thr residues, and an abundance of Ala, Arg, Glu and Lys. CaD15 also contains two Met, and HPLC of the CNBr digest of CaD15 (Figure 2) yielded the expected three peptides CB1, CB2 and CB3, plus a small amount of the partial cleavage product CB2-CB3. The complete sequences of CB2 (residues 67-84) and CB3 (residues 85-96) were determined. For sequence analysis of CB1 (residues 1-66), we anticipated a drop in repetitive yield at cycle 15. To obtain an extended sequencer run (59 cycles) on CB1, the normal sequencer program was modified to include double coupling and cleavage at cycles 14-16. HPLC of the peptic digest of CaD15 is shown in Figure 3. Sequence analysis of the

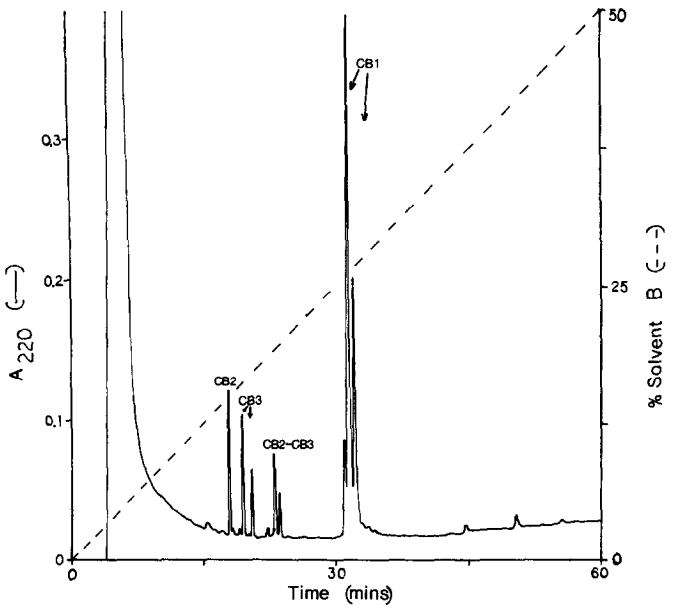

Figure 2. HPLC of CNBr digest of CaD15.

peptic fragment (28-90) was successful through residue 86 and confirmed the alignment of the CNBr peptides. This information was sufficient to complete the sequence determination of CaD15. The calculated molecular weight is 11,479, considerably lower than the previously estimated value of 15,000. This difference is consistent with recent sedimentation equilibrium studies

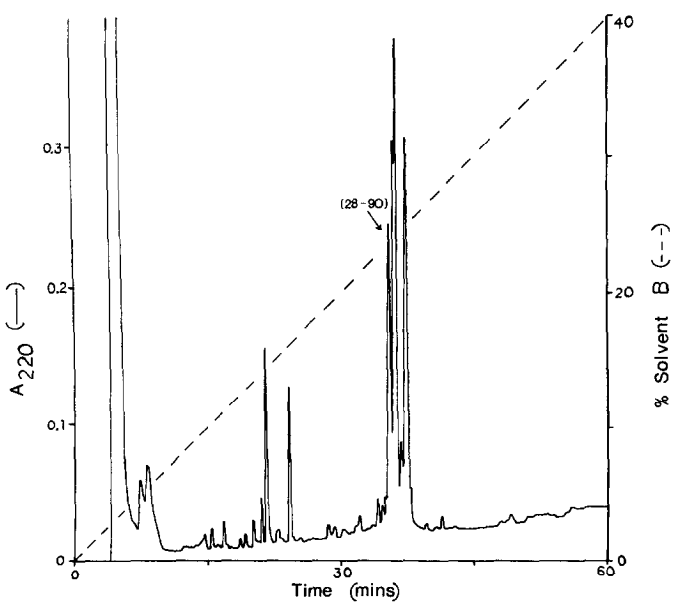

Figure 3. HPLC of peptic digest of CaD15.

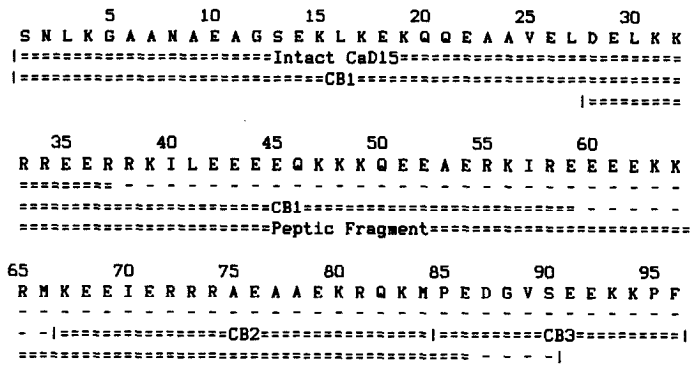

Figure 4. Sequence determination of CaD15. Double lines (=) indicate residues identified by sequence analysis.

(8) which yield molecular weights of 93,000 for whole caldesmon and 25,000 for the "35 kDa" fragment.

CaD15 is a very hydrophilic, highly charged peptide which contains an equal number (29 of each type) of acidic and basic residues. Secondary structure analysis by the method of Garnier, et al. (9) predicts that CaD15 is 100% alpha-helical. Identical results were obtained with the Chou and Fasman method (10), except that a beta turn is predicted near the C-terminus (residues 85-88, Pro-Glu-Asp-Gly). If CaD were completely helical, it would contain approximately 27 turns and have a length of about 140 A^{O}, accounting for a significant fraction of the 740 A^{O} (8) overall length of the very assymetric caldesmon molecule. The CaD15 sequence does not show the strong 4-3-4 spacing of nonpolar residues found in amphipathic helices or in the two-chain coiled coils of tropomyosin (11). On the other hand, the distribution of acidic and basic residues in CaD15 bears a striking resemblance to an unusual 8.7-residue periodicity found in the sequence of a tropomyosin-binding fragment of troponin T (12). The extended helices of both CaD15 and the troponin T fragment would be strengthened by numerous intrahelical salt bridges. (See discussion in ref. 13).

A search of the Protein Identification Resource database (release 18) failed to identify any proteins which were clearly homologous (i.e., having evolved from a common ancestor) to CaD15. The strongest resemblance was with the above-mentioned tropomyosin-binding segment of troponin T. There

was a lesser degree of similarity with tropomyosin, light meromyosin, and several non-muscle structural proteins. There was no notable similarity with troponin I, myosin subfragment-1, or other actin-binding proteins. CaD15 was also compared with dystrophin (14), and found to resemble one of 25 repeating segments which are thought to be similar to segments of actinin and spectrin. CaD15 was not particularly similar to either actinin or spectrin. Figure 5 aligns the sequence of CaD15 with segments of troponin T, tropomyosin and dystrophin.

The structural similarity of CaD15 and the tropomyosin-binding segment of troponin T raises intriguing questions about their functional properties and gives impetus for further investigations. CaD15 binds to actin, but troponin T does not. CaD15 bears some resemblance to tropomyosin, and could mimic the latter's actin-binding properties. The interaction of CaD15 with actin is rather weak (6), but the parental 35 kDa caldesmon fragment contains additional amino acid residues which strengthen the binding (17). It has recently been shown that caldesmon interacts directly with purified tropomyosin (18,19), and also with tropomyosin bound to actin filaments (20). It seems reasonable to suggest that, in light of their sequence similarity, the CaD15 region of caldesmon binds to tropomyosin and plays a role analogous to that of troponin T in striated muscle. A further

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                5       10        15        20        25        30
CaD15    s N L K g a a n a E A g s E k l K E K Q Q e a A v E L d e L K K R R
TnT      r q n K d l m e l q A l i d s h f E a r k k e e e E L v A L K e R i
TM         m e a i k k k m q m l k l d K E n a i d r A e q a e A d K K q a
Dystr    d N L q q r i t d E r k r E e i K i K Q Q l l q t k h n A L K d l R

         35        40        45        50        55        60
CaD15     E e R R - K i L E E E e Q k - - - K k Q e E a E R K i R e E E E K K
TnT       E k R R - a e r a E q Q r i - - - r a e K E r E R q n R l a E E K a
TM        E d R c - K q L E E E Q Q g - - - l q k K l k g t e d e v E k y s e
Dystr     s q R R k K a L E i s h Q w y q y K r Q a d d l l K c l d d i E K K

         65        70        75        80        85        90        95
CaD15     R - - m K E E i E r R R A e a A E K r q K m p E d g V s e e k k p f
TnT       R - - r e E E d a k R R A E E d l K K k K - A l s s m g a n y s s y
TM        s - - v K E a q E - - K l E q A E K K a t d A E a E V a s l n R r i
Dystr     l a s l p E p r d e R K i k E i d r e l q k k k e E l n a v r R q a
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Figure 5. Sequence alignment of CaD15 with residues #64-128 of rabbit skeletal muscle troponin T (15), #1-94 of chicken smooth muscle tropomyosin 1 (16), and #1832-1933 of human dystrophin (14). Identical residue are capitalized.

investigation of the behavior of CaD15 may therefore lead to both a clarification of caldesmon function in smooth muscle and a better understanding of the role of troponin T in striated muscle.

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