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LOCALIZATION OF HYDROPHOBIC SITES IN CALMODULIN AND SKELETAL MUSCLE TROPONIN C STUDIED USING TRYPTIC FRAGMENTS A SIMPLE METHOD OF THEIR PREPARATION

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The exposure of hydrophobic sites on calmodulin, skeletal muscle troponin C and their tryptic fragments was investigated using Phenyl-Sepharose chromatography. A strong binding of both proteins and their fragments corresponding to the NH_2-terminal halves of polypeptide chain of respective proteins in the presence of calcium ions was observed. Only a weak interaction with Phenyl-Sepharose or its lack was observed under these conditions for fragments corresponding to the COOH-terminal halves of calmodulin and troponin C, respectively. The elution of the samples from Phenyl-Sepharose column with ethylene glycol gradient allowed to compare relative hydrophobicity of both proteins and their fragments. The results show that hydrophobic properties of calmodulin and troponin C are virtually preserved in their fragments obtained as a result of their cleavage by trypsin in half. They also indicated that the exposure of hydrophobic residues caused by the binding of calcium ions takes place mainly in the NH_2-terminal halves of polypeptide chains of both proteins.

A simple method of purification of tryptic fragments of both proteins based on the difference in the strength of their interactions with Phenyl-Sepharose is described.

Calmodulin and troponin C belong to a family of homologous Ca^{2+}--binding proteins each of which contains four Ca^{2+}-binding domains. Calmodulin is a multifunctional protein which modulates the activity of various Ca^{2+}-dependent enzymes and cellular processes. Skeletal muscle troponin C has a unique function. It is the Ca^{2+}-binding subunit of the troponin complex which, together with tropomyosin, constitutes the thin filament-linked regulatory system of skeletal muscle.

We have previously described a procedure of limited tryptic digestion of both Ca^{2+}-binding proteins (1-3). The tryptic fragments originate from various parts of the protein and have proved to be very useful in studies on the localization of Ca^{2+}-dependent conformational changes in the molecule (4-7) and sites of interaction with target proteins (8,9). In our original procedure protein fragments were separated after digestion by preparative polyacryla-

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mide gel electrophoresis. The yield of this procedure was, however, rather low, owing to the contamination of the fragments with some UV-absorbing material that coeluted from the polyacrylamide gel and required further purification steps for its removal.

Recently Gopalakrishna and Anderson (10) published a simple method for calmodulin preparation using Phenyl-Sepharose CL-4B chromatography. This method is based on the presence of Ca^{2+}--induced hydrophobic region(s) in the calmodulin molecule. Preliminary studies in our laboratory using fluorescence probes have indicated that the NH_2- and COOH- terminal fragments of both calmodulin and troponin C differ in their hydrophobicity in the presence of calcium ions (11). In accordance with these observations we have studied hydrophobic interaction of tryptic fragments of calmodulin and troponin C with Phenyl-Sepharose and we have developed a simple procedure of the purification of those fragments based on the difference in their hydrophobicity.

MATERIALS AND METHODS

Preparation of proteins. In the earlier part of this work calmodulin was prepared from bovine brain using the procedure of Watterson et al. (12). Subsequently the method was developed in which the extract from alcohol-ether powder of brain homogenate was subjected to ammonium sulfate fractionation, isoelectric precipitation and heating at 95°C before final purification with the use of Phenyl-Sepharose chromatography. This procedure of preparation of calmodulin in which the bulk of contaminating proteins was removed prior to hydrophobic interaction chromatography was found superior over the original method of Gopalakrishna and Anderson (10).

Troponin C was obtained from rabbit skeletal muscle as previously described (13).

Preparation of tryptic fragments. Calmodulin and troponin C were digested with trypsin in 20 mM NH_4HCO_3 under conditions described previously (1,2,5). Depending on the conditions of digestion, two kinds of fragments were formed:those obtained by cleavage in the presence of 2 mM $CaCl_2$ called TR-C fragments, and those obtained in the presence of 2 mM EDTA called TR-E fragments (Fig. 1).

After digestion the fragments were separated by hydrophobic interaction chromatography carried out at room temperature using a Phenyl-Sepharose column (15,5 ml volume, 1 cm x 20 cm), initially equilibrated with solution I (20 mM NH_4HCO_3, 2 mM $CaCl_2$). A 15 ml sample of the digestion mixture (1 mg protein/ml) was applied to the column (in case of TR-E fragments, when the digestion was carried out in the presence of 2 mM EDTA, 4 mM $CaCl_2$ was added prior to chromatography). Fragments were eluted from the column with 60 ml of solution I followed by 75 ml of solution II (20 mM NH_4HCO_3, 2 mM EDTA). In the fractions of eluate fragments were detected by polyacrylamide gel electrophoresis in the presence of urea (1). Fractions containing individual fragments were combined and to those eluted with the solution II 2 mM $CaCl_2$ was added. $CaCl_2$ or Ca-EDTA present in the combined eluate fractions was removed using G-25 Sephadex column equilibrated with 2 mM NH_4HCO_3.

For the regeneration the Phenyl-Sepharose column was washed with two volumes of urea, one volume of water, one volume of ethanol, two volumes of butanol, one volume of ethanol, three volumes of water and then equilibrated again with the solution I. Phenyl-Sepharose CL-4B was the product of Pharmacia.

RESULTS AND DISCUSSION

In the presence of calcium ions trypsin cleaves both calmodulin and troponin C essentially in half (Fig. 1). The NH_2-terminal half of each protein is reffered to as TR_1-C and the COOH-terminal half as TR_2-C. When the digestion mixture in solution I is applied to the Phenyl-Sepharose column the TR_2-C fragment of troponin C is eluted immediately in the void volume, and the TR_2-C fragment of calmodulin after washing with 3 volumes of solution I indicating that both TR_2-C fragments interact weakly if at all with Phenyl-Sepharose. In contrast the TR_1-C fragments remain bound to the column being eluted only by subsequent washing with 2 mM EDTA solution (Fig. 2). These results clearly show that hydrophobic interaction chromatography can be used for separation of TR-C fragments of calmodulin and skeletal muscle troponin C. They also indicate that virtually all of the hydrophobic sites that are exposed in the presence of calcium ions are present in the NH_2-terminal part of both proteins studied.

As a measure of the relative hydrophobicity of the proteins and their TR-C fragments, concentration of ethylene glycol necessary for their elution from Phenyl-Sepharose in the presence of calcium ions has been determined. Intact calmodulin is dissociated

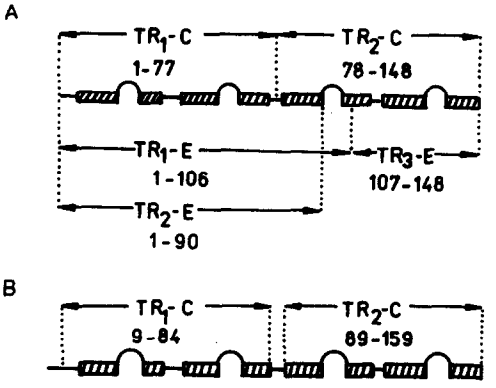

Figure 1. Tryptic fragments of calmodulin (A) and skeletal muscle troponin C (B). Digestion was carried out under the conditions described previously (1-3). Numbers refer to amino acid residues present in the particular fragment.

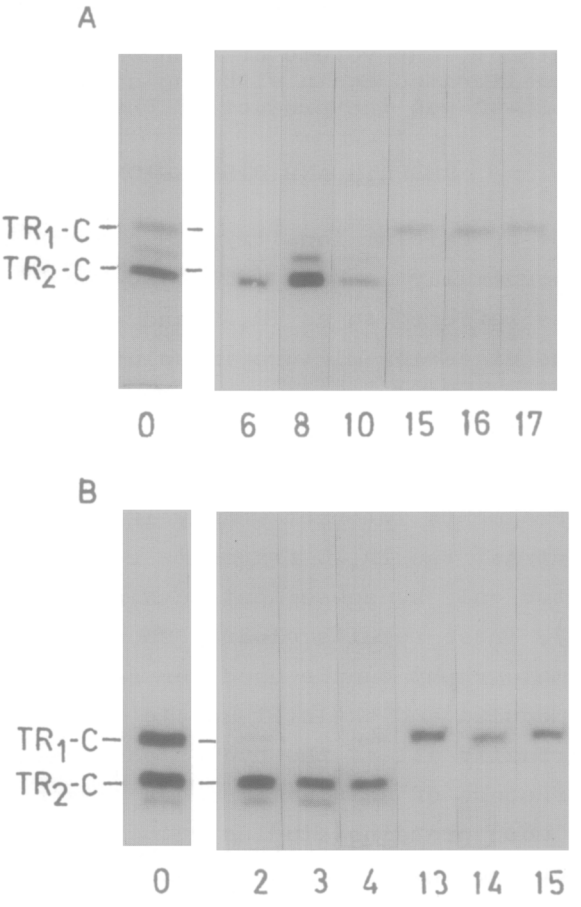

Figure 2. Phenyl-Sepharose chromatography of TR-C fragments of calmodulin (A) and troponin C (B). Tryptic digest was applied to the Phenyl-Sepharose column and eluted with the solution I and subsequently with the solution II (for details see Materials and Methods). Figure shows the protein pattern in the eluate detected by polyacrylamide gel electrophoresis in the presence of urea (1), stained with Coomasie Blue. As observed earlier (2) calmodulin TR_1-C band is stained weaker than TR_2-C one. During digestion of calmodulin and troponin C in the presence of Ca^{2+} in addition to TR_1-C and TR_2-C fragments a small amount of another fragment appeared. Its localization in the protein molecule is under investigation. Fig. 2A: 0-original digestion mixture, 6,8,10-selected samples eluted with the solution I, 15,16,17-samples eluted with the solution II. Fig. 2B: 0-original digestion mixture, 2,3,4-selected samples eluted with the solution I, 13,14,15-samples eluted with the solution II.

from the column at 60-75% concentration of ethylene glycol, its TR_2-C fragment already at 14-24% and TR_1-C at 45-60% ethylene glycol concentration. Skeletal muscle troponin C is also bound to Phenyl-Sepharose in a calcium-dependent manner. It is somewhat less hydrophobic than calmodulin and it is eluted at 45-60% ethylene glycol and its TR_1-C fragment at 50-75%. TR_2-C fragment of troponin C is not bound to the Phenyl-Sepharose at all (Table I).

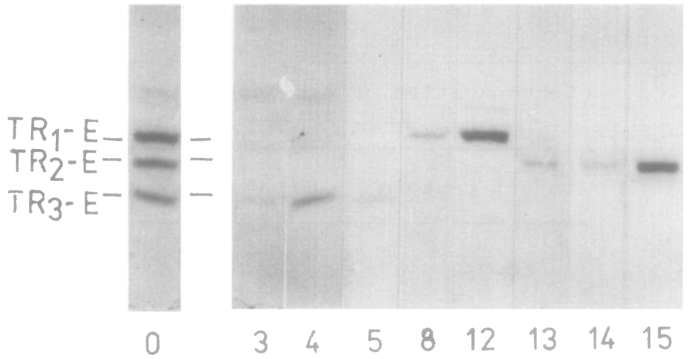

Figure 3. Phenyl-Sepharose chromatography of TR-E fragments of calmodulin. For conditions see legend to the Fig. 2. 0-original digestion mixture, 3,4,5,8,12-selected samples eluted with the solution I. 13,14,15-samples eluted with the solution II.

Limited tryptic digestion of calmodulin in the absence of calcium yields three fragments: TR_1-E containing calcium binding domains I, II and a part of the domain III, a somewhat smaller fragment TR_2-E arising from the rapid degradation of TR_1-E, and TR_3-E consisting of domain IV and a part of domain III (Fig. 1). Separation of the fragments using hydrophobic interaction chromatography is easily achieved. TR_3-E does not bind to Phenyl-Sepharose column in the presence of 2 mM $CaCl_2$ and is eluted in the void volume. The affinity of TR_1-E is also low and this fragment can be eluted from the column by prolonged washing with solution I. Subsequent addition of 2 mM EDTA releases TR_2-E (Fig. 3).

Previous studies (4-7) have shown that the NH_2- and COOH-terminal halves of both calmodulin and skeletal muscle troponin C differ in the extent of their Ca^{2+}-dependent conformational changes. The α-helix content in the presence of calcium is higher in the NH_2-terminal parts of both molecules than in their COOH-terminal parts. Upon removal of Ca^{2+} the decrease in helicity in the NH_2-terminal part is small compared to that in COOH-terminal half. This is particularly evident in troponin C which exhibits large decrease of ordered structure under these conditions.

The results of this work as well as preliminary data using fluorescence probes to measure hydrophobicity (11) clearly indicate that NH_2-, and COOH-terminal halves of the two Ca^{2+}-binding proteins differ considerably not only with respect to the Ca^{2+}-dependent changes in their secondary structures but also with respect to the extent to which these changes result in the exposure of hydro-

Table I. Elution of calmodulin, troponin C and their TR-C fragments from a Phenyl-Sepharose column using ethylene glycol gradient.

	Concentration of ethylene glycol (%) necessary for elution of:		
	intact protein	TR_1-C fragment	TR_2-C fragment
calmodulin	60-75	45-60	14-24
troponin C	45-60	50-75	0-4

3 ml samples containing calmodulin, troponin C or their purified TR-C fragments dissolved in the solution I (protein concentration 1 mg/ml) were applied to Phenyl-Sepharose column (0,5 cm x 15 cm, 3 ml volume) and eluted with 20 ml of 0-80% ethylene glycol gradient in solution I in case of intact proteins and their TR_1-C fragments, 0-20% for TR_2-C fragment of troponin C and 0-40% for TR_2-C fragment of calmodulin. Proteins were detected in the eluate by polyacrylamide gel electrophoresis (1).

phobic residues. Virtually all of the hydrophobic groups become exposed in the NH_2-terminal part of both proteins.

Studies on the secondary structure of calmodulin indicate that as a result of tryptic cleavage there is no appreciable loss of α-helix content measured both in the presence and in the absence of Ca^{2+}. The comparison of the relative hydrophobicity of the intact molecule with those of the fragments (see Table I) shows that only a small loss of hydrophobicity occurs as a result of cleavage of both molecules. This observation is in agreement with previous studies (6) indicating a lack of significant interactions between the halves of calcium binding proteins.

When this paper was ready to be submitted for publication it came to our knowledge that Vogel and Lindahl (14) also used recently Phenyl-Sepharose chromatography for purification of Ca^{2+}-binding proteins and their fragments.

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