Calcium-dependent hydrophobic interaction chromatography of calmodulin, troponin C and their proteolytic fragments

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The homologous calcium-binding proteins calmodulin and skeletal and cardiac troponin C bind in the presence of 1 mM Ca^{2+} to phenyl-Sepharose and can be eluted in buffers containing chelators. Results obtained with a series of proteolytic fragments, which were prepared by limited tryptic or thrombic degradation, showed that this Ca^{2+}-dependent hydrophobic interaction chromatography provides a convenient method for the large scale purification of some of these peptides. Moreover, it was found that in troponin C only one Ca^{2+}-induced hydrophobic site is located in the amino-terminal half of the protein, but that calmodulin contained such sites in both the amino- and carboxy-terminal halves of the molecule. The probable location of the latter site is discussed.

Ca^{2+}/Ca^{2+}-dependence *Calmodulin* *Hydrophobic interaction chromatography* *Proteolytic-fragment* *Troponin C*

1. INTRODUCTION

Calmodulin (CaM) is the acidic heat-stable intracellular modulator protein that confers Ca^{2+}-based regulation to a large variety of important enzyme systems in eukaryotic cells (for review see [1] and references therein). After the original demonstration [2] that binding of phenothiazine drugs, like trifluoperazine (TFP), to CaM requires the presence of Ca^{2+}, various reports have appeared showing that the same applies for binding of several other drugs [3–5], as well as for a series of small proteins [6–8]. Although it is certainly not confirmed at present that the physiological mode of action of these drugs is mediated via their binding to CaM [9,10], these studies have provided a convenient method for the purification of CaM by using Ca^{2+}-dependent affinity chromatography with drugs covalently linked to Sepharose beads [11–13]. At the same time it was shown by studying fluorescent enhancement of hydrophobic probes that hydrophobic regions were exposed on the surface of CaM upon addition of Ca^{2+} [14,15]. As well, cadmium-113 NMR [16] and calcium-43 NMR [17] indicated that the exchange rate of Ca^{2+} bound to the two weak Ca^{2+}-binding sites [18] of CaM was markedly reduced by addition of TFP or other drugs, hence suggesting that conformational changes occur upon drug binding, a notion further substantiated by recent proton NMR studies [19].

Recently, it has become apparent that the homologous calcium binding proteins S100 and skeletal troponin C (sTnC) [13,20], as well as squid proteins [21], can also be purified using similar affinity chromatography. Here, we report that CaM and TnC can be purified using Ca^{2+}-dependent hydrophobic interaction chromatography on commercially available phenyl-Sepharose. Moreover, results obtained with a series of CaM [18,22–25] and sTnC [25–28] proteolytic fragments have provided some insight into the location of the Ca^{2+}-induced hydrophobic regions on the protein.

2. MATERIALS AND METHODS

Phenyl-Sepharose was a product of Pharmacia Fine Chemicals (Sweden). 2-Chloro-10-(3-aminopropyl)-phenothiazine maleate (CAPP) was

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obtained from Rhone-Poulenc (France). All other chemicals used were highest grade commercial products and were as in [18].

Bovine testis CaM and rabbit muscle sTnC were purified as in [12,29], whereas cTnC was purified from beef-heart using an unpublished method of Stepkowski and Drabikowski. Thrombic fragments TM_1 (1–106) and TM_2 (107–148) and the carboxy-terminal tryptic fragment TR_2C (78–148) from CaM were prepared and purified as in [18]. The amino-terminal fragment TR_1C (1–77) was purified in the laboratory of Dr C.B. Klee (National Institutes of Health, Bethesda MD) on a Waters Bondapak Phenyl column using reverse-phase high-pressure liquid chromatography essentially as in [30]. All fragments were judged pure by proton NMR, sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis [18], non-denaturing agarose gel electrophoresis [31] and amino acid composition determinations and sequence studies.

Tryptic cleavage of sTnC was performed slightly differently, as in [28]. In a typical experiment 100 mg sTnC was dissolved in 10 ml 50 mM NH_4HCO_3, 50 mM NaCl, 2 mM $CaCl_2$ (pH 7.8). Proteolytic cleavage was initiated by addition of 250 μl of a 1 mg/ml TPCK-trypsin (Worthington, USA) stock solution in 1 mM HCl. After 6 h at 30°C, 500 μl of a 1 mg/ml stock solution of soybean trypsin inhibitor (Sigma) in 1 mM HCl was added. This mixture was immediately applied to a 1.4 × 200 cm Sephadex G-50 column which was eluted with 25 mM NH_4HCO_3 (pH 8.0). The first peak that eluted contained undegraded sTnC as well as trypsin and its inhibitor, the second peak, containing the proteolytic fragments, was pooled, freeze dried and dissolved in 50 mM Tris–HCl, 1 mM $CaCl_2$ (pH 7.5). The different fragments were purified using phenyl-Sepharose (see below). The yield of this preparation was 35 mg fragment 89–159 and 14 mg fragment 9–84.

Ca^{2+}-dependent hydrophobic interaction and affinity chromatography assays with proteolytic fragments (1–2 mg) were performed at room temperature on columns with a total volume of 3.2 ml of phenyl-Sepharose or CAPP-Sepharose [12], respectively. The buffer used was 50 mM Tris–HCl (pH 7.5) containing either 1 mM $CaCl_2$ or 1 mM EDTA (or EGTA) for phenyl-Sepharose and for CAPP-Sepharose, the buffer was 50 mM Tris–HCl, 5 mM $CaCl_2$ or 5 mM EDTA, 0.3 M NaCl, 1 mM β-mercaptoethanol (pH 7.0). Column-profiles were assayed spectrophotometrically at 280, 258 or 215 nm wavelengths and with non-denaturing agarose gel electrophoresis. After each determination the column was washed extensively with distilled water whereas an additional wash with buffer containing 8 M urea was performed after every change of protein.

Amino acid compositions were determined using a standard Beckman 119 CL single column automatic analyser with a conventional ninhydrin detection system. Sequence analysis was performed on an automated Beckman sequencer (1.0 M Quadrol), followed by high-pressure liquid chromatography of the derivatised amino acids.

3. RESULTS

3.1. *Preparation of calmodulin fragment 78–106*

Fig.1 indicates the position at which the proteolytic enzymes thrombin and trypsin cleave intact CaM in the presence of EDTA and Ca^{2+}, respectively [18,22–25]. Treatment with trypsin results in cleavage of the molecule into two halves. Only the carboxy-terminal peptide TR_2C (78–148) encompassing the two high affinity Ca^{2+} binding sites is normally obtained in good yield [18]. Fig.2 shows the results obtained upon gel filtration of TR_2C (78–148) which had been digested for 50 min with thrombin. From the electrophoresis data (insert fig.1) it is obvious that the first peak contains both undegraded TR_2C (78–148) and TM_2 (107–148), whereas the second peak contains another peptide. Subsequent amino acid analysis (see table 1) identifies this fragment as TT (78–106). Thus, as with intact CaM, cleavage occurs specifically at residue Arg-106. However, TM_1 (1–106) and TM_2 (107–148) are relatively stable to further degradation by thrombin [18,25], but TT (78–106) is not since it completely disappears if the digestion of TR_2C is extended to 2 h (not shown).

3.2. *Calcium dependent chromatography of calmodulin and its proteolytic fragments*

Since CaM can bind in a Ca^{2+}-dependent manner to phenothiazine columns [11,12,29] and phenyl-Sepharose columns [32] (see fig.3A), we decided to compare the binding characteristics of a series of proteolytic fragments of CaM to both

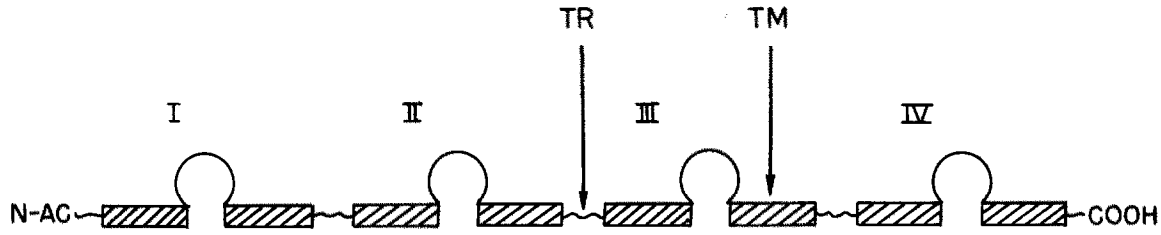

Fig.1. Schematic representation of calmodulin indicating the 4 calcium-binding domains each comprising a helix–loop–helix segment and the sites of proteolytic cleavage by trypsin (TR) in the presence of Ca^{2+} and thrombin (TM) in the presence of EDTA.

types of columns. Table 2 provides an overview of the results we obtained: the outcome with both different columns is identical. Moreover, the table shows that although Ca^{2+}-dependent chromatography on phenyl-Sepharose provides a convenient method for the purification of the thrombic fragments TM_1 (1–106) and TM_2 (107–148) (see fig.3B), the tryptic fragments TR_1C (1–77) and TR_2C (78–148) both bind and can thus not be purified using this ionic strength. Possibly purification at lower ionic strength or temperature will be possible, since we noticed that TR_1C (1–77) bound tighter than TR_2C (78–148).

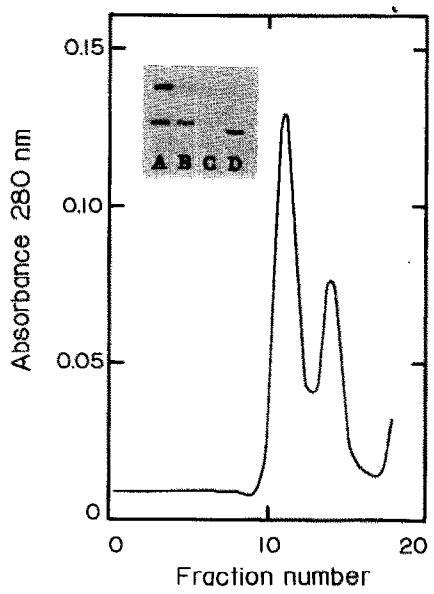

Fig.2. Column profile obtained for a 2.5 mg sample of TR_2C (78–148) degraded for 50 min by thrombin (25 I.E.) on a Sephadex G-50 superfine column of 1.0 × 36 cm. The column was eluted with 50 mM NH_4HCO_3 buffer (pH 8.0). The fraction size was about 1.0 ml. The insert shows an agarose gel of fractions 11–14 (A–D). Fractions 11 and 12 contain both TR_2C (78–148) and TM_2 (107–148) fraction 14 contains pure TT (78–106).

3.3. *Calcium-dependent chromatography of cardiac and skeletal troponin C and tryptic fragments*

Since CaM and TnC are highly homologous pro-

Table 1

Amino acid composition of the proteolytic fragments of CaM and sTnC[a]

	CaM (78–106)	TnC (9–84)	TnC (89–159)
Asx	4.3 (4)[b]	8.4 (8)[c]	11.9 (12)[c]
Thr	0.8 (1)	3.8 (4)	1.8 (1)
Ser	1.5 (2)	3.4 (4)	2.6 (3)
Glx	4.5 (5)	13.5 (13)	14.9 (13)
Pro	0.1 (0)	0.8 (1)	0.2 (0)
Gly	1.8 (2)	6.9 (6)	6.3 (7)
Ala	2.4 (3)	4.7 (5)	4.6 (5)
Val	1.1 (1)	5.0 (5)	2.1 (2)
Met	0.1 (1)	6.7 (7)	3.2 (3)
Ile	1.7 (2)	4.5 (5)	5.0 (5)
Leu	1.0 (1)	5.0 (5)	4.6 (4)
Tyr	0.9 (1)	0.6 (1)	0.5 (1)
Phe	1.7 (2)	4.9 (5)	4.8 (5)
Lys	1.2 (1)	4.2 (4)	3.8 (4)
His	0.4 (0)	0.3 (0)	1.0 (1)
Arg	2.4 (3)	2.0 (2)	3.5 (4)

[a] Hydrolysis of peptides was performed for 24 h in 6 M HCl at 110°C

[b] The number between brackets indicates the expected number of amino acids [1,28]

[c] Amino acid sequencing of the first 8 amino acids gave exactly the predicted sequence [28]

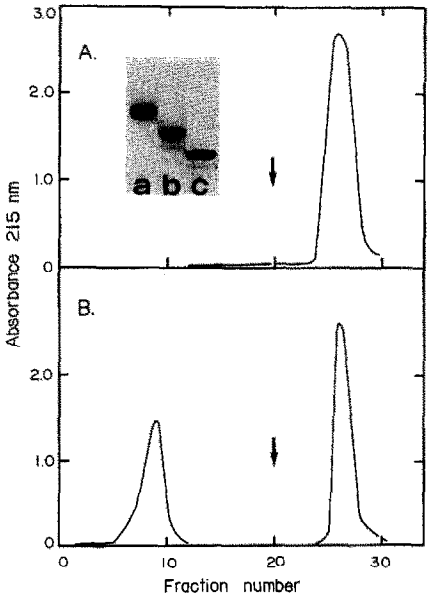

Fig.3. (A) Ca^{2+}-dependent chromatography of CaM (1.1 mg) on a phenyl-Sepharose column (0.7 × 20 cm). The arrow indicates the time when the 1 mM Ca^{2+} in the buffer was replaced by 1 mM EGTA. (B) Chromatography (as in fig.3A) of a mixture (1.8 mg) of the thrombic fragments TM_1 (1–106) and TM_2 (107–148). The insert shows an SDS–polyacrylamide gel [18] of phenyl-Sepharose-purified CaM (a), TM_1 (b) and TM_2 (c).

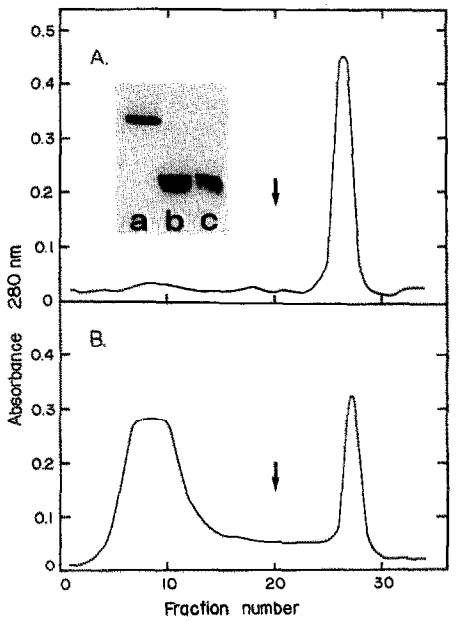

Fig.4. (A) Ca^{2+}-dependent chromatography of sTnC (25 mg) on a phenyl-Sepharose column (1.5 × 15 cm). The arrow indicates the time when the 1 mM Ca^{2+} in the buffer was replaced by 1 mM EDTA. (B) Chromatography (identically as in fig.4A) of a mixture (55 mg) of the tryptic fragments of sTnC. The insert shows an SDS–polyacrylamide gel [18] of phenyl-Sepharose-purified sTnC (a), fragment 9–84 (b) and fragment 84–159 (c).

teins it seemed worthwhile to investigate Ca^{2+}-dependent binding of TnC to phenyl-Sepharose, especially since it was recently shown that sTnC binds similarly to immobilised phenothiazines [20]. Our results (fig.4A) indicate that both sTnC and cTnC (not shown) bind to the phenyl-Sepharose in the presence of 1 mM Ca^{2+}. sTnC did not bind in the presence of 10 mM Mg^{2+}.

Table 2

Ca^{2+}-dependent binding of calmodulin proteolytic fragments[a]

Peptide	Amino acids	Phenyl-Sepharose	CAPP-Sepharose
TR_1C	1– 77	+	+
TR_2C	78–148	+	+
TM_1	1–106	+	+
TM_2	107–148	–	–[b]
TT	78–106	–	–[b]

[a] Conditions as described in section 2

[b] About 85% of electrophoretically pure peptide did not bind, but 15% did

Tryptic cleavage of sTnC in the presence of Ca^{2+} is reported to give rise to two fragments, 9–84 and 89–159, each containing two complete calcium-binding domains [26–28]. When we applied the mixture of proteolytic peptides (section 2) we obtained the column profile as shown in fig.4B. Thus only one of the fragments binds in a Ca^{2+}-dependent manner to phenyl-Sepharose. This one was identified by amino acid analysis as the fragment 9–84 (see table 1), whereas the other peptide is the proteolytic fragment 89–159 (see table 1). Thus Ca^{2+}-dependent chromatography on phenyl-Sepharose provides an attractive large-scale alternative for the purification of sTnC tryptic fragments, compared to the cumbersome preparative gel electrophoresis method usually employed [26–28].

4. DISCUSSION

Although the original discoveries that antipsychotic drugs [2] and smooth muscle relaxing agents [3,4] bound to CaM led to the suggestion that specific binding sites for these agents may exist, our studies show that identical results are obtained with phenyl- and CAPP-Sepharose, thus indicating that rather non-specific hydrophobic forces must be involved in the interaction between the drugs and the Ca^{2+}-dependent domains on the surface of CaM. Similar conclusions, based on different types of experiments, have been put forward [9,14,15]. Be that as it may, since the binding of such agents often prevents the interaction between CaM and its receptor proteins [1–3], they provide a useful tool for studying the molecular mechanisms of Ca^{2+}-regulatory events.

CaM is reported to contain two high affinity binding sites for TFP [2,16,19,33]. Since both fragments TR_1C (1–77) and TR_2C (78–148) bind in a Ca^{2+}-dependent manner to phenyl- and CAPP-Sepharose (see table 2) it appears that each half of the molecule contains one high affinity binding site for phenothiazines. Since the fragment TM_2 (107–148), which still has considerable affinity for metal ions [18], does not bind, it is unlikely that one of the TFP binding sites is not solely located on the carboxy-terminal α-helix of calmodulin as had been suggested [33]. Rather since TR_2C (78–148) does bind but not TT (78–106) and TM_2 (107–148) (see table 2), it appears that the carboxy-terminal α-helix of calcium binding domain III could comprise one binding site, and that proteolytic cleavage at position 106 destroys it. An alternative explanation of these results would be that the hydrophobic site is located on the α-helix preceding site III and that the affinity of the fragment TT for Ca^{2+} is too low to assure proper folding. Finally, the possibility that the site is composed of different stretches of amino acids that are brought in proximity due to protein folding cannot be excluded. Interestingly, a cyanogen bromide fragment 77–124 was also retained in a Ca^{2+}-dependent manner on a CAPP-Sepharose column [34] and TR_2C (78–148), but not TM_2 (107–148) activated phosphorylase kinase [23]. It is not clear at present what specific amino acids would contribute to the hydrophobic site, although proton NMR [19,33] data suggested that phenylalanine, methionine and methyl groups are involved.

Our observation that only the amino-terminal half of sTnC exposes a hydrophobic surface upon Ca^{2+} binding (fig.4A) is somewhat surprising, since recently evidence was provided for binding of TFP to the amino-terminal α-helix of calcium binding domain III [35]. However, this observation is consistent with the finding that the presence of 10 mM Mg^{2+} does not induce binding of sTnC to phenyl-Sepharose. This cation would bind only to the high affinity calcium binding sites III and IV [26]. Thus binding of Ca^{2+} to the two low affinity Ca^{2+}-specific sites I and II is apparently a necessity for the exposure of the hydrophobic surface on the amino-terminal half of the protein molecule. This particular hydrophobic domain is probably involved in the interaction between TnC and troponin I (TnI), since it has been reported that this fragment and TnI are capable of forming a Ca^{2+}-dependent complex in solution [26,27].

Our studies have shown that Ca^{2+}-dependent chromatography on phenyl-Sepharose provides a convenient method for purification of CaM [32], cTnC, sTnC as well as some of their proteolytic fragments (fig.3,4). In addition, metal ion-dependent hydrophobic interaction chromatography has recently been used for purification of other calcium-binding proteins like S100 [36] and α-lactalbumin [37]. The latter binds, in contrast to all other calcium binding proteins studied to date, in the presence of EDTA and is eluted upon addition of Ca^{2+} [37]. One note of caution should be extended, however. When phenyl-Sepharose was used for the purification of sTnC immediately after the first ion-exchange step [29] we noticed proteolytic breakdown which was probably caused by co-purification of Ca^{2+}-dependent proteases [38]. Furthermore, we observed that sTnC purified using phenyl-Sepharose as a last chromatography step was somewhat less stable upon storage than normal preparations. These problems are currently under investigation in our laboratory.

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