Biological activities of the peptides obtained by digestion of troponin C and calmodulin with thrombin

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1. Troponin C and calmodulin were not digested by thrombin at a significant rate in the presence of Ca^{2+} . 2. In the presence of EGTA, troponin C was digested by thrombin to yield three peptides, TH1 (residues 1–120), TH3 (residues 1–100) and TH2 (residues 121–159). 3. In the presence of EGTA calmodulin was digested by thrombin giving two peptides, TM1 (residues 1–106) and TM2 (residues 107–148). 4. The electrophoretic mobilities of peptides TH1 and TM1 were increased at pH 8.6 by Ca^{2+} both in the presence and absence of urea. The mobilities of peptides TH2 and TM2 were unaltered under these conditions. 5. Peptides TH1, TH3 and TM1 formed complexes with troponin I on polyacrylamide gels at pH 8.6 in the presence of Ca^{2+} . 6. The phosphorylation of troponin I by cyclic AMP-dependent protein kinase was significantly inhibited by peptides TH1 and TH3 and to a lesser extent by peptide TM1. 7. The calmodulin peptide TM1 activated myosin light-chain kinase when present in large molar excess. Peptide TM2 did not activate the enzyme.

The study of the properties of peptides obtained by selective enzymic and chemical cleavage has thrown considerable light on the location of the biologically active sites of the proteins of the troponin complex, troponin I (Syska *et al.*, 1976), troponin T (Jackson *et al.*, 1975; Pearlstone & Smillie, 1978) and troponin C (Weeks & Perry, 1977, 1978; Drabikowski *et al.*, 1977; Nagy *et al.*, 1978; Leavis *et al.*, 1978a). In the case of troponin C, study of the fragments by several techniques has enabled the sites of interaction with troponin I and the different types of Ca^{2+} -binding site to be assigned to regions of the primary sequence with some precision. More limited studies of this kind carried out on calmodulin have confirmed the underlying structural similarities between this protein and troponin C (Drabikowski, *et al.*, 1977; Vanaman & Perry, 1978; Nairn *et al.*, 1980; Grand *et al.*, 1980) that were indicated by sequence determinations. The demonstration (Leavis *et al.*, 1978b) that troponin C could be cleaved by thrombin at residue 120 into two fragments containing three and one Ca^{2+} -binding sites respectively has extended the methods available for selective cleavage of the Ca^{2+} -binding proteins. In the present investigation troponin C and calmodulin have been digested with thrombin under controlled conditions of Ca^{2+} concentration. Study of the properties of the defined peptides obtained has thrown further light on the sites of interaction of both proteins with troponin I and indicated the difference in structural require-

ments for calmodulin interaction with the catalytic subunit of myosin light-chain kinase and troponin I.

Materials and methods

Materials

Phenylmethanesulphonyl fluoride, bovine cardiac 3':5'-cyclic AMP-dependent protein kinase, highly purified freeze-dried bovine plasma thrombin and carboxypeptidase A and B were obtained from Sigma (London) Chemical Co., Poole, Dorset BH17 7NH, U.K. 1-Chloro-4-phenyl-3-L-tosylamido-butan-2-one-treated trypsin was obtained from Worthington Chemical Co., Cambrian Chemicals, Croydon, Surrey CR0 4XB, U.K.

Preparation of proteins

Troponin was prepared from rabbit fast skeletal muscle by the method of Ebashi *et al.* (1971) and fractionated into troponin I and troponin C by the method of Perry & Cole (1974). Calmodulin was prepared from bovine brain by the organic-solvent method (Method 2) of Grand *et al.* (1979).

Digestion of Ca^{2+} -binding proteins by thrombin

Time course. [^{14}C]Carbamoylmethylated troponin C or calmodulin (100 μg) was incubated at 30°C with 6 NIH units of bovine plasma thrombin in 200 μl of 50 mM-Tris brought to pH 8.0 with 1 M-

HCl/15 mM-2-mercaptoethanol. CaCl_2 , MgCl_2 or EGTA was added to the digest as indicated. Portions (25 μl) were removed and pipetted into 50 μl of 9 M-urea/7.5 mM-EGTA adjusted to pH 8.6 with 5 M-NaOH. The samples were analysed by polyacrylamide-gel electrophoresis, pH 8.6, in 6 M-urea (see below).

Gels stained with Coomassie Blue R250 were scanned with a Helena Quick Scan, using a white light course. Densitometric scanning of bands obtained on electrophoresis of known amounts of bovine brain calmodulin (1–10 μg) indicated a linear response.

Radioactivity was determined by cutting out Coomassie Blue-stained bands from the gel, incubating with 1 ml of 100-volume H_2O_2 at 80°C for 2 h and counting in scintillation fluid made up from 1 vol. of Triton X-100 and 2 vol. of a solution of 40 g of 2,5-diphenyloxazole and 1.2 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 10 litres of toluene.

Preparation of cleavage fragments by thrombin treatment. Troponin C (16 mg) was incubated with bovine plasma thrombin (4 NIH units/mg) in 4 ml of 50 mM-Tris adjusted to pH 8.0 with 1 M-HCl/2.5 mM-dithiothreitol/5 mM-EGTA, pH 8.0, at 30°C for 16 h. Calmodulin (11 mg) was incubated with thrombin (10 NIH units/mg) in 2.0 ml of 50 mM-Tris adjusted to pH 8.0 with 1 mM-HCl/1 mM-2-mercaptoethanol/5 mM-EGTA, pH 8.0, at 30°C for 2 h. 'Thrombin digests' (i.e. the products from digestion with thrombin) were applied directly to a column (2.4 cm $\times$ 240 cm) of Sephadex G-50 equilibrated in 50 mM- NH_4HCO_3 , pH 7.9, and eluted with the same buffer. Peptides were detected by measurement of A_{217} or A_{215} and by polyacrylamide-gel electrophoresis.

Protein analytical procedures

Peptide and protein determination. Protein was assayed by a micro-method involving precipitation by tannin (Mejbaum-Katzenellenbogen & Dobryszczyka, 1959) or by amino acid analysis. Peptide concentrations were determined by amino acid analysis.

Amino acid analysis. Duplicate samples of peptides were hydrolysed in 6 M-HCl containing 10 μl of 0.1 M-phenol solution/ml for the protection of tyrosine residues (Sanger & Thompson, 1963) for 16 h as described by Wilkinson *et al.* (1972). Peptides were analysed for trimethyl-lysine by using 'system C' of Kuehl & Adelstein (1969).

Carbamoylmethylation. Rabbit skeletal-muscle troponin C (10 mg/ml) was labelled with iodo- ^{14}C acetamide by the method described by Wilkinson *et al.* (1972).

C-Terminal determination. C-Terminal residues of peptides were determined by digestion with carboxypeptidase followed by dansylation (5-di-

methylaminonaphthalene-1-sulphonylation) (Wilkinson, 1978). Carboxypeptidase A (2 μl of a 1000 units/ml suspension) was washed with 300 μl of water, then dissolved by the sequential addition of 400 μl of 2 M-N-methylmorpholine/acetate pH 8.5, 5 μl of 1 M-NaOH and 5 μl of 1 M-HCl. Carboxypeptidase B (10 μl of a solution containing 1140 units/ml) was diluted with 4 ml of 0.2 M-N-methylmorpholine/acetate, pH 8.5. Portions (25 μl) of the enzyme solution were used for a time course, up to 1 h at 37°C, involving 5 nmol of peptide.

N-Terminal and sequence determination. The N-terminal amino acids of peptides were determined as described by Hartley (1970) and amino acid sequences by the rapid Edman procedure of Gray & Smith (1970). Dansylated amino acids were identified after chromatography on polyamide plates (5 cm $\times$ 5 cm) (BDH Chemicals, Poole, Dorset BH12 4NN, U.K.) by the method of Hartley (1970).

Phosphorylation of troponin I

Bovine cardiac 3':5'-cyclic AMP-dependent protein kinase (20 $\mu\text{g}/\text{ml}$) was incubated with troponin I (120 $\mu\text{g}/\text{ml}$) under the conditions specified by Cole & Perry (1975). The reaction was stopped by the addition of an equal volume of cold 24% (w/v) trichloroacetic acid and then 100 μl of bovine serum albumin solution (20 mg/ml) was added as carrier. The precipitated protein was washed three times with 5 ml of cold 8% (w/v) trichloroacetic acid. The pellet was dissolved in 10 ml of 0.1 M-NaOH and ^{32}P was determined by the Čerenkov method (Gould *et al.*, 1972).

Myosin light-chain kinase

The catalytic subunit of myosin light-chain kinase was prepared as a homogeneous protein from rabbit white skeletal muscle as described by Pires & Perry (1977) and assayed as described by Nairn & Perry (1979). Assays were carried out at 25°C in 50 mM-Tris adjusted to pH 7.6 with 1 M-HCl/12.5 mM-magnesium acetate/0.1 mM- CaCl_2 /1 mM-dithiothreitol/5.5 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (20 $\mu\text{Ci}/\text{ml}$)/myosin P light chain (2 mg/ml)/catalytic subunit of myosin light-chain kinase (1.9 μM) in a total volume of 0.1 ml.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in 25 mM-Tris/80 mM-glycine, pH 8.6, was carried out in the presence or absence of 6 M-urea by the method described by Head & Perry (1974). Electrophoresis in sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969) in 0.1 M-Tris/0.1 M-Bicine [N,N'-bis-(2-hydroxyethyl)glycine] buffer, pH 8.3, using a 1:10 ratio of (w/w) N,N'-methylenebisacrylamide to acrylamide as recommended by Swank & Munkres (1971).

Results

Effect of bivalent cations on the digestion of calmodulin and troponin C by thrombin

When the action of thrombin on calmodulin and troponin C at pH 8.0 was followed by the electrophoresis of portions of the digest, it was apparent that the course of the digestion was profoundly affected by the presence of bivalent cations. In the presence of 10 mM- CaCl_2 , little or no significant breakdown of either protein occurred as judged by the absence of bands other than those corresponding to the Ca^{2+} -binding proteins themselves after 30–45 min incubation with thrombin (Figs. 1*d* and 1*h*). If Ca^{2+} was removed by including 5 mM-EGTA in the incubation medium calmodulin was rapidly and completely transformed into two major bands of greater electrophoretic mobility in 6 M-urea, pH 8.6 (Figs. 1*f* and 1*g*). Troponin C was digested more slowly under these conditions and gave a more complex band pattern than calmodulin (Figs. 1*b* and 1*c*). Furthermore the digestion of troponin C was less complete, for after 45 min material with the same mobility as troponin C was still present in the digest. The faster moving peptides tended to diffuse out of the polyacrylamide gel during staining after electrophoreses of digests of calmodulin and troponin C and often appeared as fainter staining bands.

Preliminary experiments indicated that the progress of digestion by thrombin was not significantly changed after carbamoylmethylation of troponin C. This property was therefore used to study the effects

Fig. 1. Electrophoresis of digests of troponin C and calmodulin with thrombin

Ca^{2+} -binding proteins were digested and portions for electrophoresis in 6 M-urea, pH 8.6, were prepared as described in the Materials and methods section. Tracks (a)–(d), approx. 10 μg of rabbit fast-skeletal-muscle troponin C; tracks (e)–(h), approx. 10 μg of bovine brain calmodulin. EGTA (5 mM) was present in all cases except (d) and (h). (a), zero time; (b), 15 min; (c), 45 min; (d), 45 min + 5 mM- CaCl_2 ; (e), zero time; (f), 15 min; (g), 45 min; (h), 45 min + 5 mM- CaCl_2 not incubated at same time as (a)–(g). O, origin.

of bivalent cations on the digestion of the latter protein on a more quantitative basis. Calmodulin and [^{14}C]carbamoylmethylated troponin C were digested as described in the legend to Fig. 2, and the amount of undigested Ca^{2+} -binding protein was

Fig. 2. Effect of Ca^{2+} and Mg^{2+} on the digestion of troponin C and calmodulin by thrombin

Ca^{2+} -binding protein (100 μg) was incubated at 30°C in 200 μl of 50 mM-Tris adjusted to pH 8.0 with 1 M-HCl/15 mM-2-mercaptoethanol/thrombin (6 NIH units) and CaCl_2 , MgCl_2 or EGTA as indicated. Portions (25 μl) were removed at intervals as indicated, pipetted into 50 μl of 9 M-urea/7.5 mM-EGTA and examined by polyacrylamide-gel electrophoresis in 6 M-urea/25 mM-Tris/80 mM-glycine, pH 8.6. The amounts of undigested Ca^{2+} -binding proteins were determined as described in the Materials and methods section. (a) Shows the results for [^{14}C]carbamoylmethylated troponin C. O, No additions; ▼, 0.5 mM-EGTA/2.5 mM- MgCl_2 ; ▽, 1.0 mM- CaCl_2 ; ●, 5 mM-EGTA. (b) shows the results for calmodulin. O, 1 mM- CaCl_2 ; ●, 2 mM-EGTA; □, 0.5 mM-EGTA/1 mM- MgCl_2 ; ■, 0.5 mM-EGTA/5.0 mM- MgCl_2 .

determined either by densitometry or from the radioactivity present in the band of the undigested protein obtained on electrophoresis. The results illustrated in Figs. 2(a) and 2(b) confirm the conclusions about the course of the digestion suggested from inspection of the gels after staining for protein. Both proteins were undigested in the presence of Ca^{2+} and rapidly digested in the presence of EGTA. Digestion of calmodulin was much faster than troponin C, which took three to four times as long to disappear from the digest.

MgCl_2 was less effective than CaCl_2 in preventing digestion, particularly in the case of calmodulin. Whereas inhibition of digestion of calmodulin was complete at 1 mM- CaCl_2 , in the presence of 1 mM- MgCl_2 and 0.5 mM-EGTA the rate of digestion was very similar to that in the presence of 2 mM-EGTA. Some inhibition of digestion was apparent in 5 mM- MgCl_2 . The digestion of both proteins was inhibited completely, irrespective of the presence or absence of bivalent cations, if 0.5 mM-phenylmethanesulphonyl fluoride was added to the incubation mixture before the thrombin.

Isolation and identification of the peptides obtained by cleavage of troponin C by thrombin

On gel filtration of the digests obtained after prolonged digestion of [^{14}C]carbamoylmethylated troponin C with thrombin two main peaks were obtained (Fig. 3). Three fractions A, B and C were pooled as indicated by the bars in Fig. 3 and freeze-dried. Fractions A and B each contained a single peptide that migrated with very similar mobilities on polyacrylamide-gel electrophoresis in 6 M-urea at pH 8.6 in the presence of either 5 mM- CaCl_2 or 5 mM-EGTA. The peptides were not identical, however, for on electrophoresis at pH 8.6 in the absence of urea the peptide in fraction A migrated with a lower mobility towards the anode in the absence of Ca^{2+} [Fig. 4a, (i) and (ii)]. In the presence of Ca^{2+} the mobility of one peptide increased and that of the other decreased so that a mixture migrated as a single band at pH 8.6 [Fig. 4a, (ii)]. The peptides were designated peptide TH1 (fraction A) and peptide TH3 (fraction B), and were further purified by repeating the gel filtration of the fractions using the same column and conditions. Polyacrylamide-gel electrophoresis in 6 M-urea at pH 8.6 in the absence and the presence of Ca^{2+} indicated that fraction C consisted of a single peptide, which was designated peptide TH2.

No *N*-terminal amino acids could be detected in peptide TH1 or peptide TH3 using the dansyl procedure. It was therefore assumed that they were both derived from the *N*-terminal part of the molecule. The C-terminal sequence of peptide TH1 was (Ile, Phe)Arg. In view of this observation, the known sequence of rabbit fast troponin C (Collins

Fig. 3. Gel filtration of digests of carbamoylmethylated troponin C from rabbit fast-skeletal muscle with thrombin. [^{14}C]Carbamoylmethylated troponin C (16 mg) was incubated in 4 ml of 50 mM-Tris adjusted to pH 8.0 with 1 M-HCl/5 mM-EGTA/2.5 mM-dithiothreitol containing 64 NIH units of bovine plasma thrombin at 30°C. After incubation for 16 h the whole digest was applied to a column (2.4 cm $\times$ 240 cm) of Sephadex G-50 equilibrated with 50 mM- NH_4HCO_3 , pH 7.9, and eluted with the same solution. Fractions (10 ml) were collected. Fractions indicated by the bars A, B and C were pooled. —, A_{217} ; ----, radioactivity.

et al., 1973), the amino acid analysis of the peptide (Table 1) and the fact that it was radioactively labelled, it was considered that this peptide spanned residues 1–120 (Fig. 5).

The C-terminal sequence of peptide TH3 was Phe-Arg but unlike the results with peptide TH1 no isoleucine could be detected. On the basis of this observation and the amino acid content (Table 1) it was assumed that peptide TH3 was the *N*-terminal peptide resulting from the cleavage of the bond between arginine-100 and isoleucine-101 in peptide TH1 (Fig. 5). Based on this assumption the radioactivity per mole of peptide TH1 and peptide TH3 isolated from [^{14}C]carbamoylmethylated troponin C would be expected to be the same and this was found to be the case. Confirmation of the non-identity of peptide TH1 and peptide TH3 was

Fig. 4. Electrophoretic studies on the peptides of troponin C and calmodulin in the presence of Ca^{2+} and troponin I from fast skeletal muscle

(a) shows the effect of Ca^{2+} on the mobilities of peptides from troponin C and calmodulin. Electrophoresis was carried out in 25 mM-Tris/80 mM-glycine, pH 8.6. Approx. $10\mu\text{g}$ of protein was applied. Peptides TH1 and TH3 are indicated by arrows. O, origin. (i), Pool B of thrombin digests of troponin C (see Fig. 3) + 5 mM-EGTA; (ii), pool B (troponin C) + 5 mM- CaCl_2 ; (iii), peptide TH2 + 5 mM-EGTA; (iv), peptide TH2 + 5 mM- CaCl_2 ; (v), peptide TM1 + 5 mM-EGTA; (vi), peptide TM1 + 5 mM- CaCl_2 ; (vii), peptide TM2 + 5 mM-EGTA; (viii), peptide TM2 + 5 mM- CaCl_2 . (b) shows complex-formation with troponin I from rabbit fast skeletal muscle. Electrophoresis was in 25 mM-Tris/80 mM-glycine (pH 8.6)/15 mM- CaCl_2 /6 M-urea. About $10\mu\text{g}$ of peptide or protein was applied in each case. (i), Troponin C; (ii), troponin C + troponin I; (iii), peptide TH1; (iv), peptide TH1 + troponin I; (v), peptide TH3; (vi), peptide TH3 + troponin I; (vii), peptide TM1; (viii), peptide TM1 + troponin I.

provided by electrophoresing the peptides on polyacrylamide gels in the presence of sodium dodecyl sulphate (see the Materials and methods section). Under these conditions peptide TH3 migrated slightly further than peptide TH1, indicating that it was of slightly lower molecular weight.

Peptide TH2 was not radioactive and had an N-terminal sequence of Ala-Ser. From the known sequence of troponin C and the amino acid analysis (Table 1) it was concluded that peptide TH2 spanned the sequence from alanine-121 to the C-terminal residue glutamine-159.

Isolation and identification of peptides obtained by cleavage of bovine brain calmodulin with thrombin

Gel filtration of the digest obtained after digestion

of bovine brain calmodulin with thrombin yielded an elution profile consisting of two discrete peaks. The fractions representing each of these peaks were pooled and designated fractions A and B respectively, as indicated in Fig. 6. Electrophoresis indicated that each of the fractions consisted of a single peptide and these were designated peptide TM1 (fraction A) and peptide TM2 (fraction B). The absence of other peptides in the eluate was consistent with the results of the electrophoretic studies on the digest and suggested that thrombin had cleaved calmodulin at one peptide bond to produce two fragments. No N-terminal amino acid could be detected in peptide TM1, which had Leu-Arg as the C-terminal sequence. This information, taken with the amino acid analysis data (Table 2), indicated

Table 1. *Amino acid composition of peptides obtained by digestion of rabbit skeletal-muscle troponin C with thrombin*
Results are averages of one analysis carried out on each of three different preparations of peptide in the case of peptides TH1 and TH2; peptide TH3 was a single preparation. Carbamoylmethylcysteine (CM-Cys) was determined by the measurement of radioactivity (see the Materials and methods section).

Amino acid content (residues/mol)						
	Peptide TH1		Peptide TH2		Peptide TH3	
	Sequence (1–120)*	Analysis	Sequence (121–159)*	Analysis	Sequence (1–100)*	Analysis
CM-Cys	1	†	—	—	1	†
Asp	14	14.4	8	7.4	10	10.5
Thr	5	4.9	1	1.0	5	5.1
Ser	5	4.9	2	1.8	5	5.1
Glu	24	24.4	7	7.0	21	22.0
Pro	1	†	—	—	1	†
Gly	9	9.2	4	4.1	8	8.9
Ala	12	11.9	1	1.3	9	8.7
Val	5	5.2	2	2.0	5	5.3
Met	7	5.4	3	2.5	7	2.1
Ile	8	7.3	2	2.1	5	4.3
Leu	7	7.9	2	2.2	6	6.5
Tyr	2	2.0	—	—	1	1.0
Phe	8	8.0	2	2.0	6	6.0
His	—	—	1	1.1	—	—
Lys	6	6.2	3	3.1	6	5.8
Arg	6	6.3	1	1.0	4	4.0

* Based on sequence determination by Collins *et al.* (1973).

† Detected but not measurable.

Fig. 5. *Schematic representation of the primary sequences of troponin C from rabbit fast skeletal muscle and calmodulin from bovine brain*

The shaded regions represent the presumptive Ca^{2+} -binding sites I to IV. Numbers represent the position of residues in the sequences and position of the peptides isolated are indicated by brackets. Ca^{2+} -binding sites are as described by Collins (1976) and Kretzinger & Barry (1975). Arrows indicate sites of cleavage by thrombin. N, N-Terminus; C, C-terminus.

that peptide TM1 spanned residues 1–106 (Fig. 5). The N-terminal amino acid of peptide TM2 could not be identified unambiguously but the second, third and fourth residues were valine, methionine

and threonine respectively. From these results and the amino acid analysis of peptide TM2 it was concluded that the N-terminal sequence was His-Val-Met-Thr and the peptide was the C-terminal

Fig. 6. Gel filtration of thrombin digest of bovine brain calmodulin

Bovine brain calmodulin (11 mg) dissolved in 2 ml of 50 mM-Tris adjusted to pH 8.0 with 1 mM-HCl/1 mM-2-mercaptoethanol/5 mM-EGTA was incubated at 30°C with 110 NIH units of bovine plasma thrombin. After 2 h the digest was chromatographed on a column (2.4 cm × 250 cm) of Sephadex G-50 equilibrated and eluted with 50 mM-NH₄HCO₃, pH 7.9. Fractions (10 ml) were collected. Fractions indicated by bars A and B were pooled.

fragment resulting from cleavage of calmodulin by thrombin at the carboxy side of arginine-106 (Fig. 5).

Electrophoretic studies on the peptides of troponin C and calmodulin from digestion with thrombin

All the purified peptides obtained from troponin C and calmodulin migrated as single bands, both in the presence or absence of Ca²⁺ whether the electrophoresis was carried out in the absence or presence of 3 M- and 6 M-urea. The electrophoretic behaviour at low ionic strength is illustrated in Fig. 4. Similar mobility changes in the presence of Ca²⁺ were observed in 3 M-urea. From these results it can be seen that with the exception of peptide TH3, the large N-terminal fragments obtained from both proteins retain the property shown by the intact molecule to increase in electrophoretic mobility to the anode in the presence of Ca²⁺ ± urea when on normal charge considerations the mobility would be expected to decrease. Of particular interest is the

Fig. 7. Effect of thrombin cleavage peptides of troponin C (a) and calmodulin (b) on the phosphorylation of troponin I by cyclic AMP-dependent protein kinase

Phosphorylation experiments were carried out as described in the Materials and methods section with the added amounts of Ca²⁺-binding proteins or peptides derived from them as indicated. Phosphorylation is expressed as a percentage of incorporation in the absence of peptides or Ca²⁺-binding protein. (a) ○, Peptide TH1; ●, peptide TH2; △, peptide TH3; □, troponin C; ■, carbamoylmethylated peptide TH1. (b) ○, Peptide TM1; ●, peptide TM2; □, calmodulin.

observation that peptide TH3 increased its mobility in the presence of urea but decreased in mobility in the absence of urea on binding Ca²⁺ (see above). In all cases the mobilities of the C-terminal fragments were unaffected by Ca²⁺.

Both troponin C (Head & Perry, 1974) and calmodulin form complexes with troponin I that are stable in urea and that are Ca²⁺-dependent. This property was shown by the cleavage peptides of troponin C, peptides TH1 and TH3, both in the presence and absence of 6 M-urea [Fig. 4b, (iii)–(vi)]. Peptide TH2, the C-terminal peptide of troponin C, did not possess this property. Under these conditions the N-terminal peptide of calmodulin, peptide TM1, did not migrate as a complex band in the presence of Ca²⁺, although the peptide band was decreased in intensity and some 'streaking' between it and the origin was observed. Peptide TM2 did not interact with troponin I.

Effect of cleavage peptides of troponin C and calmodulin on the phosphorylation of troponin I catalysed by 3':5'-cyclic AMP-dependent protein kinase

The interaction of troponin C and calmodulin

Table 2. *Amino acid composition of peptides obtained by digestion of bovine brain calmodulin with thrombin*
Results are averages of one analysis carried out on each of two different preparations of peptide in each case.
Abbreviation used: Tml, trimethyl-lysine.

Amino acid content (residues/mol)				
	Peptide TM1		Peptide TM2	
	Sequence (1-106)*	Analysis	Sequence (107-148)*	Analysis
Asp	16	16.0	7	7.0
Thr	9	8.8	3	2.9
Ser	4	4.2	—	—
Glu	18	18.1	9	9.0
Pro	2	1.6	—	—
Gly	8	7.9	3	3.0
Ala	9	8.9	2	2.0
Val	3	3.3	4	3.9
Met	5	3.6	4	2.3
Ile	6	6.1	2	2.0
Leu	7	7.8	2	2.2
Tyr	1	0.9	1	1.1
Phe	7	7.8	1	1.1
His	—	—	1	0.9
Lys	6	6.4	1	1.0
Arg	5	4.1	1	1.1
Tml	—	—	1	1.0

* Based on the sequence determined by Watterson *et al.* (1980).

with troponin I results in blocking of the sites of phosphorylation by cyclic AMP-dependent protein kinase on fast-skeletal-muscle troponin I at serine-117 (Perry & Cole, 1974; Vanaman & Perry, 1978). Studies to date (Weeks & Perry, 1978; Leavis *et al.*, 1978a) have indicated that a region of troponin C represented by residues 84-135 and the corresponding region on calmodulin (Vanaman & Perry, 1978) are concerned with this interaction. Both troponin C peptides TH1 and TH3 were almost as effective as troponin C in inhibiting the phosphorylation of fast-skeletal-muscle troponin I catalysed by cyclic AMP-dependent protein kinase (Fig. 7a). The equivalent calmodulin peptide TM1 also inhibited phosphorylation but was significantly less effective than calmodulin itself in this respect (Fig. 7b). No inhibition was obtained with the C-terminal peptides from either protein.

Effect of thrombin peptides of calmodulin on the activity of myosin light-chain kinase catalytic subunit

In the presence of equimolar concentrations of preparations of calmodulin, myosin light-chain kinase catalytic subunit consisting of a single component of mol.wt. 77 000 is activated to 70-80% of the activity obtained in the presence of a large molar excess of calmodulin (Nairn & Perry, 1979; Fig. 8). With a molar ratio of calmodulin to the

catalytic subunit of 0.4:1, 50% of the maximum activation is obtained. With a very large molar excess of calmodulin peptide TM1, activation equal to that given by the undigested protein was obtained. To produce 50% of maximal activation, however, a molar ratio of peptide TM1 to catalytic subunit of about 200:1 was required (Fig. 8). The C-terminal peptide TM2 did not significantly activate the kinase when present in 200 times molar excess.

Discussion

The nature of the major peptides obtained on digestion of the Ca²⁺-binding proteins with thrombin accord well with the known specificity of this enzyme for arginyl bonds (Blomback *et al.*, 1977). Bonds other than those split that might be susceptible to cleavage are present in troponin C at arginine-8, -44, -104, -120 and -145 and in calmodulin at arginine-37 and -90. In the original study of the action of thrombin on troponin C by Leavis *et al.* (1978b) cleavage at arginine-100 was not observed. It is possible that these authors failed to identify the appropriate cleavage product by not strictly controlling the Ca²⁺ concentrations during electrophoresis in non-dissociating conditions. Under the conditions described the peptides produced

Fig. 8. Effect of calmodulin and its peptides on the activity of myosin light-chain kinase

Assays were carried out as described in the Materials and methods section in 50 mM-Tris/HCl (pH 7.6)/12.5 mM-magnesium acetate/0.1 mM- CaCl_2 /1 mM-dithiothreitol/5.5 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (20 $\mu\text{Ci/ml}$)/myosin P light chain (2 mg/ml)/myosin light-chain kinase catalytic subunit (1.9 nM)/bovine brain calmodulin or its peptides at the concentrations indicated. Activity is expressed as a percentage of that obtained with 130 nM-bovine brain calmodulin. O, Calmodulin; ●, peptide TM1.

by cleavage at the other sites were either absent or present in amounts too low to be detected by the technique employed. Presumably these bonds are rendered unavailable by the conformation of the proteins.

In the presence of Ca^{2+} the sites of cleavage in both proteins were unavailable to thrombin and much less available in the presence of Mg^{2+} compared with the situation in the presence of EGTA (Figs. 2a and 2b). So far as the effect of Ca^{2+} is concerned, the results fit in well with other studies that imply that the Ca^{2+} -binding proteins undergo marked conformational change to a much more compact structure on binding Ca^{2+} (Head & Perry,

1974). The observation that the effect of Mg^{2+} corresponds more closely to Ca^{2+} in modifying the susceptibility of troponin C to thrombin than is the case with calmodulin is of interest. It may relate to the fact that the Ca^{2+} -binding sites III and IV of troponin C have affinities for Mg^{2+} (Potter & Gergely, 1975), whereas the corresponding sites on calmodulin have not yet been so clearly defined as Ca^{2+} - and Mg^{2+} -binding sites (Potter *et al.*, 1977). Indeed this difference in binding properties of the sites may reflect the specialization that has occurred in troponin C in adaptation to its particular regulatory role in muscle.

The studies indicate clearly that the environment in the regions represented by residues 100–120 in troponin C and that which includes residue 106 in calmodulin undergo marked changes on binding Ca^{2+} . It can also be concluded that the sequence isoleucine-101–arginine-120, i.e. that which contains Ca^{2+} -binding site III of troponin C, must remain intact for the mobility of the peptide fragments on electrophoresis in urea at pH 8.6 to be such that they move faster to the anode in the presence than in the absence of Ca^{2+} . This implies that the effect of the change in the hydrated volume of the fragment is greater than the increased positive charge on binding Ca^{2+} .

The interaction studies confirm the finding from studies in the CNBr peptides that the region on the C-terminal side of Ca^{2+} -binding site II and up to and possibly including site III is of particular significance for both troponin C and calmodulin in interaction with troponin I. Both CNBr peptide CB9 (residue 84–135; Weeks & Perry, 1978) and peptide TH3 (residues 1–100) of troponin C interact with troponin I as indicated by electrophoretic and phosphorylation studies. This indicates that the region common to both peptides, residues 84–100, has particular importance and that Ca^{2+} -binding site III is not essential for the interaction. The situation with calmodulin is not quite so clear. Peptide TM1 (residues 1–106) is not as effective in inhibiting phosphorylation of troponin I as the corresponding troponin C peptides and the electrophoretic evidence for interaction is much less impressive. In view of the clear evidence of interaction obtained with the CNBr peptide consisting of residues 77–124 of calmodulin (Vanaman & Perry, 1978) the results may suggest that the region incorporating Ca^{2+} -binding site III has more significance in the interaction of calmodulin with troponin I than is the case with troponin C.

The study does, however, bring out differences in the interaction of myosin light-chain kinase and troponin I with calmodulin. Of the two peptides studied, only that spanning residues 1–106 activated the enzyme. The latter peptide was much less effective on a molar basis than calmodulin itself, but

in very large excess activated the enzyme maximally. The possibility that the activation obtained was due to a trace of undigested calmodulin in the preparation of peptide TM1 cannot, however, be completely excluded. Less than 0.5% contamination of the peptide by calmodulin would be sufficient to produce the results obtained. Nevertheless it can be concluded that, although a relatively well-defined region of calmodulin is essential for interaction with troponin I, this is not the case for activation of myosin light-chain kinase.

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