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Characterization of a Region of the Primary Sequence of Troponin C involved in Calcium Ion-Dependent Interaction with Troponin I

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1. The CNBr digest of troponin C from rabbit fast skeletal muscle was shown to possess many of the functional properties of the whole troponin C molecule. 2. A peptide corresponding to residues 83–134 was isolated, which forms a Ca^{2+}-dependent complex with troponin I and neutralizes the inhibition by troponin I of the Mg^{2+}-stimulated adenosine triphosphatase of desensitized actomyosin. 3. The peptide inhibits the phosphorylation of fast-skeletal-muscle, but not cardiac-muscle, troponin I, by 3′:5′-cyclic AMP-dependent protein kinase. In this property it was as effective as whole skeletal-muscle troponin C when compared on a molar basis. 4. Biological activity was also present in other fractions obtained from the CNBr digest. 5. By gel filtration and affinity chromatography of the whole CNBr digest of troponin C, two peptides, one of which was identified as representing residues 83–134, were shown to form Ca^{2+}-dependent complexes with troponin I. 6. The significance of these findings for the mechanism of interaction of troponin C and troponin I is discussed.

Troponin C is now recognized as the component of the troponin complex in muscle that binds Ca^{2+} with high affinity. As a result it undergoes a conformational change that initiates events in the I filament, leading to the interaction of actin and myosin, the hydrolysis of ATP and the contraction of the muscle. Collins *et al.* (1973) have identified four regions of high density of acidic amino acids in the primary sequence of troponin C that, from their apparent similarity to the Ca^{2+}-binding sites on the protein parvalbumin, are presumed to be the binding sites for this cation. The precise function of each of these sites is not known, but they appear to fall into two categories. Two sites possess a high binding constant for Ca^{2+} but are also capable of binding Mg^{2+}, whereas the other two sites are specific for Ca^{2+} but have a slightly lower affinity for Ca^{2+} cation (Potter & Gergely, 1975; Potter *et al.*, 1976).

Troponin C also possesses a number of properties that reflect other aspects of its function as a component of the troponin complex. It is able: (1) to neutralize the inhibition of the Mg^{2+}-stimulated ATPase of actomyosin produced by troponin I (Hartshorne & Mueller, 1968; Schaub & Perry, 1969) by a mechanism that is not reversed by EGTA (Perry *et al.*, 1972); (2) to inhibit the phosphorylation of fast-skeletal-muscle troponin I by phosphorylase kinase and 3′:5′-cyclic AMP-dependent protein kinase (Perry & Cole, 1974), but not the phosphorylation of cardiac-muscle troponin I catalysed by 3′:5′-cyclic AMP-dependent protein kinase (Cole & Perry, 1975); (3) to form a complex with troponin I that is stable in 6–8M-urea and that requires Ca^{2+} for formation under these dissociating conditions (Perry *et al.*, 1972; Head & Perry, 1974); (4) to form a complex with troponin T that can be identified by gel electrophoresis (Van Eerd & Kawasaki, 1973; Jackson *et al.*, 1975).

Studies of the biological activity of CNBr peptides of troponin I (Syska *et al.*, 1976) have shown that troponin C can interact with the regions of the primary sequence of troponin I consisting of residues 1–47 and residues 96–117. As both sites on troponin I are highly basic regions it has been suggested that the interaction with troponin C may be largely electrostatic in nature and may possibly involve the acidic regions of troponin C (Perry *et al.*, 1975, 1976). The investigations on troponin I (Syska *et al.*, 1976) and similar studies on troponin T (Jackson *et al.*, 1975) indicate that certain of the functional activities of these proteins are associated with defined regions of the primary sequence that preserve their biological activity when they are isolated as relatively small peptide fragments. It is to be expected, therefore, that similar defined regions of the troponin C molecule may possess at least some of the biological properties listed above. To explore this possibility and examine further the hypothesis that troponin I may interact with acidic regions of troponin C, the peptides produced by CNBr digestion of troponin C have been studied for their ability to carry out the biological functions of the intact molecule. On the basis of this

Abbreviation used: ATPase, adenosine triphosphatase.

study a region of troponin C represented by residues 83–134 is suggested as the site involved in interaction at, or close to, the actin-binding site of fast-skeletal-muscle troponin I. Some aspects of this work have been briefly reported (Weeks & Perry, 1977).

Methods

Troponin and its components

The troponin complex was prepared by the method of Ebashi *et al.* (1971), with the modification that one rather than two washings with 0.4M-LiCl was used. Troponin I and troponin C were isolated from the complex by methods previously described (Perry & Cole, 1974; Head & Perry, 1974). Some preparations of troponin I were obtained from troponin B as described by Wilkinson (1974).

Preparation of desensitized actomyosin

Desensitized actomyosin was prepared from actomyosin extracted from myofibrils prepared from the longissimus dorsi muscles of New Zealand White rabbits as described by Schaub & Perry (1969). The turbid actomyosin extract was diluted with 9vol. of cold water, the precipitate separated by centrifugation and redissolved by the addition of solid KCl to 0.6M (Cummins & Perry, 1973). The solution of actomyosin was reprecipitated by the slow addition of 12vol. of cold water. The suspension was left for 30min, centrifuged and then washed by repeated suspension in water in a hand-operated homogenizer, followed by centrifugation at 55000*g* for 30min. The washing was continued with water adjusted to pH7.0 with 2M-Tris until the Mg^{2+}-stimulated ATPase activity of actomyosin was no longer sensitive to EGTA over the range 1–5mM (usually five washes were required). The precipitate was finally washed four times with 1M-Tris/HCl buffer, pH7.6. The densensitized actomyosin was stored at 0°C in the presence of trace amounts of toluene at a protein concentration of 3–5mg/ml and could be used for up to 2 weeks without significant loss of activity.

Phosphorylation experiments

Phosphorylations catalysed by phosphorylase kinase and protein kinase were carried out by using [γ-^{32}P]ATP by the methods described by Perry & Cole (1974). Phosphorylase kinase was prepared by the method of Cohen (1973); bovine cardiac 3′:5′-cyclic AMP-dependent protein kinase was obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. The controls contained all additions except the troponin substrates for phosphorylation, which were added after precipitation with 5% (w/v) trichloroacetic acid. When the amount of protein to be assayed for ^{32}P incorporation was less than 500μg, 0.1ml of a 1% solution of bovine serum albumin was added after addition of trichloroacetic acid to facilitate handling of the precipitate.

Determination of radioactivity

^{32}P was determined by the Čerenkov method (Gould *et al.*, 1972) at an efficiency of 25% or 33% by using a Philips model PW4510 liquid-scintillation analyser.

CNBr cleavage

CNBr cleavage was carried out by dissolving the protein in 70% (v/v) formic acid (Steers *et al.*, 1965) to a concentration of 5–10mg/ml, and adding 25mg of CNBr/ml of sample. The reaction was allowed to continue for 20h at room temperature (21°C), after which time the mixture was diluted with 20vol. of water and freeze-dried.

Carbamoylmethylation

This was carried out on the troponin C after digestion with CNBr. The freeze-dried digest (10mg) was dissolved in 1ml of 6M-guanidine/0.4M-Tris (adjusted to pH8.0 with 1.0M-HCl)/10mM-dithiothreitol and incubated at 30°C for 3h. The reaction was then started by addition of 10μl of iodo[1-^{14}C]-acetamide (1μCi/μl). After 15min a 10% molar excess of unlabelled iodoacetamide over the total protein plus dithiothreitol thiol groups was added. The reaction was stopped after 30min by the addition of the sample to a Sephadex G-50 column (2.5cm × 110cm) in 50mM-NH_4HCO_3, pH7.8.

Affinity chromatography

Troponin I linked to Sepharose 4B was prepared by coupling rabbit fast-skeletal-muscle troponin I to Sepharose by the method of March *et al.* (1974) as described by Head *et al.* (1977). The CNBr digest (5mg in 0.5ml) of troponin C in 50mM-NH_4HCO_3/1mM-$CaCl_2$ were applied to a column (10cm × 1.5cm) previously equilibrated against the solution. The column was washed with 8M-urea/75mM-Tris (adjusted to pH8.0 with 1M-HCl)/15mM-2-mercaptoethanol before re-use. The elution profile was determined by measuring the A_{215} and the radioactivity of the fractions. The urea interfered with measurement of A_{215}, and therefore non-radioactive samples of material eluted with urea were pooled and urea was removed by gel filtration on a Sephadex G-50 column (2.5cm × 110cm) with 50mM-NH_4HCO_3, pH7.8, as eluent before identification.

Gel electrophoresis

This was carried out as described by Head & Perry (1974) by using 15% acrylamide gels in 25mM-Tris/

80mM-glycine (pH8.6) with or without 6M-urea as indicated.

Electrophoresis in the presence of 0.1% sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969), in 100mM-sodium phosphate buffer, pH7.0.

ATPase assays

Determinations of ATPase activity were usually carried out by incubation for 5min at 25°C. Unless otherwise stated the incubation medium contained 0.2–0.6mg of desensitized actomyosin, 2.5mM-$MgCl_2$/2.5mM-Tris ATP/25mM-Tris, adjusted to pH 7.6 with 1M-HCl, in a total volume of 2ml. Where indicated in the text 1mM-EGTA was added to the incubation medium. The reaction was started by the addition of ATP and stopped with 1ml of 15% (w/v) trichloroacetic acid. The P_i released was determined by the method of Fiske & SubbaRow (1925).

Amino acid analysis

This was carried out by the method described by Wilkinson *et al.* (1972).

Protein and peptide determinations

Protein was measured by total-N determination by the Nesslerization procedure described by Strauch (1965), assuming that the protein samples contained 16% N. With peptide fractions prepared in 50mM-NH_4HCO_3, which was difficult to remove completely before analysis, the peptide concentrations were calculated from the amino acid analysis of the samples.

N-Terminal analysis

The *N*-terminus of peptide CB9 was determined by using the dansyl method of Hartley (1970), on polyamide plates (5cm×5cm).

Materials

Bovine serum albumin and calf thymus histone (type III-S, lysine-rich), were supplied by Sigma (London) Chemical Co. ATP was obtained as the disodium salt from Kyowo Makko Co., Tokyo, Japan. [γ-^{32}P]ATP and iodo[1-^{14}C]acetamide were supplied by The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Biological activity of the CNBr digests of troponin C

The digest obtained after treatment of troponin C from rabbit white muscle with CNBr was found to possess appreciable activity in neutralizing the inhibitory activity of troponin I on the Mg^{2+}-stimulated ATPase of actomyosin and in inhibiting the phosphorylation of troponin I catalysed by 3′:5′-cyclic AMP-dependent protein kinase and by phosphorylase kinase. These activities were not due to undigested troponin C, for the biological activity of the digests was much greater than would be expected from the small amount of undigested troponin C present (about 10%). Further, if the undigested troponin C was removed by gel filtration on Sephadex G-50 (fraction I, Fig. 1), the remaining mixture of CNBr peptides still possessed biological activity.

CNBr digests from which undigested troponin C had been removed were about 20–25% as effective as an equal weight of troponin C in neutralizing 50% of the inhibition of actomyosin ATPase obtained with a given amount of troponin I (Fig. 2). With excess of digest the inhibition of the ATPase activity was usually neutralized to about 90% of that obtained with troponin C itself. A slight inhibition of the Mg^{2+}-stimulated ATPase was obtained with the digest in the presence of EGTA (Ca^{2+} sensitivity) similar to that obtained with the whole troponin C (Fig. 2).

For complete inhibition of the phosphorylation of rabbit skeletal-muscle troponin I by 3′:5′-cyclic AMP-dependent protein kinase the molar amount of the troponin C-free digest required was 4–5 times that of troponin I (Fig. 3*a*). With troponin C inhibition was complete with a 1:1 molar ratio. Phosphorylation of troponin I catalysed by phosphorylase kinase was also inhibited by the digest, but higher molar amounts were likewise required per mol of troponin I to achieve effects comparable with those obtained with whole troponin C (Fig. 3*b*).

These results suggested that a peptide, or mixture of peptides, in the digest was interacting with troponin I in a manner that neutralized its inhibitory effect on actomyosin ATPase and prevented the

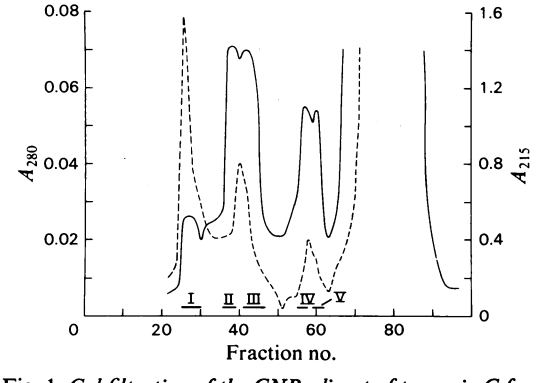


Fig. 1. *Gel filtration of the CNBr digest of troponin C from rabbit white skeletal muscle*

Digest (80mg) in 3ml of 50mM-NH_4HCO_3, pH7.8, was applied to a column of Sephadex G-50 (2.5cm×110cm) equilibrated against the same solution: 5ml fractions were collected. The solid line represents A_{215} and the broken line A_{280}.

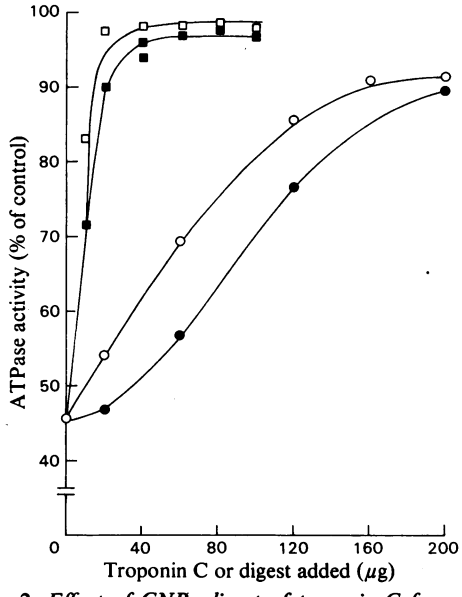


Fig. 2. *Effect of CNBr digest of troponin C from rabbit white muscle on the Mg^{2+}-stimulated ATPase of desensitized actomyosin inhibited by troponin I*

The digest was chromatographed on Sephadex G-50 to remove undigested material (see the text and Fig. 1). Assays were carried out in 2ml under conditions described in the Materials and Methods section with 600μg of desensitized actomyosin, 100μg of tropomyosin and 20μg of rabbit fast-skeletal-muscle troponin I. Controls were carried out with all additions except troponin I or troponin C. Symbols: □, troponin C+0.2mM-$CaCl_2$; ■, troponin C+1mM-EGTA; ○, CNBr digest of troponin C+0.1mM-$CaCl_2$; ●, CNBr digest of troponin C+1.0mM-EGTA.

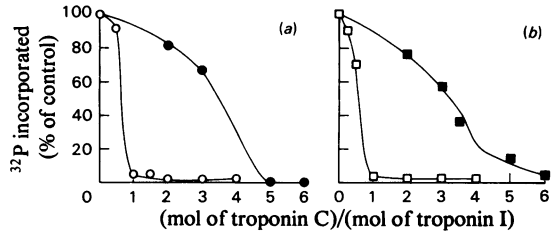


Fig. 3. *Effect of CNBr digests of troponin C on the phosphorylation of troponin I from rabbit white muscle catalysed by 3′:5′-cyclic AMP-dependent protein kinase and phosphorylase kinase*

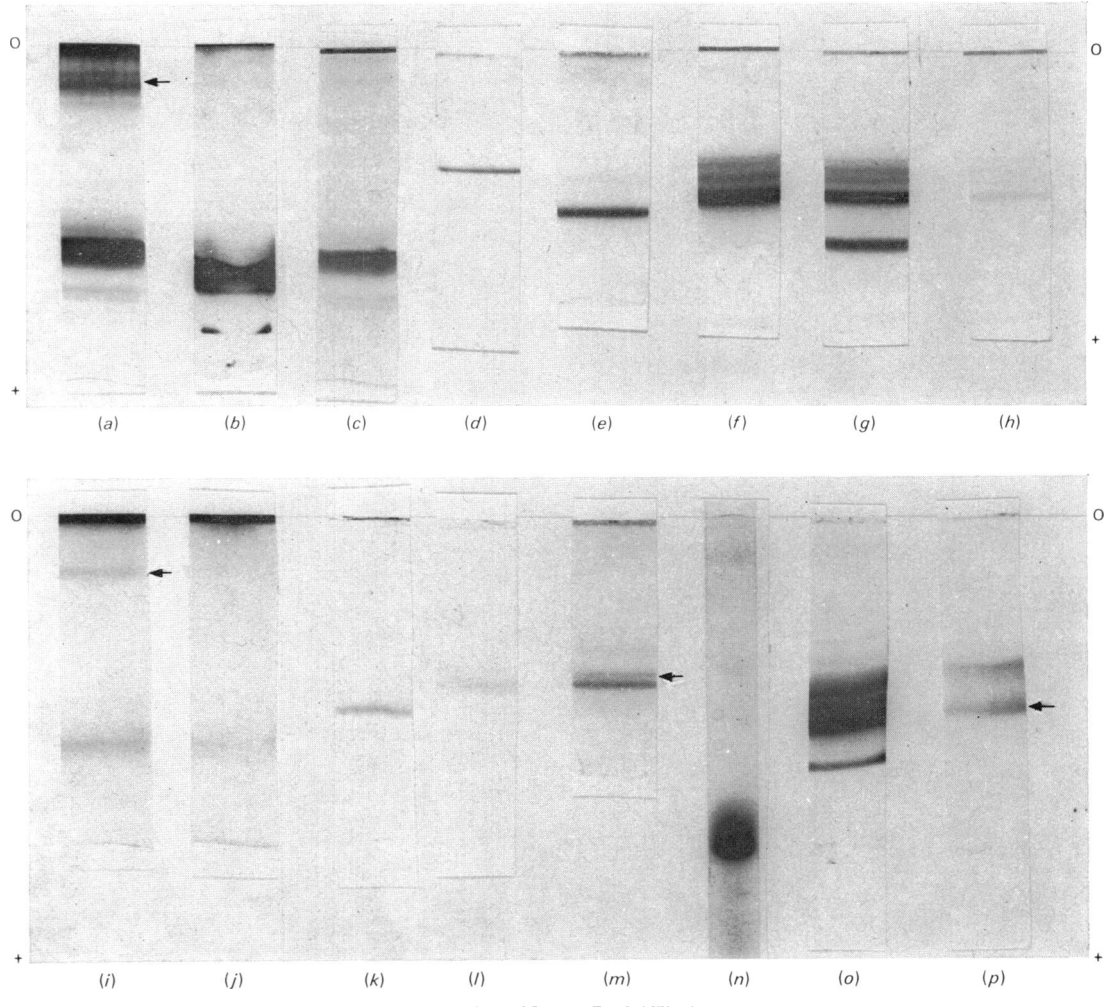
Digest was prepared as described in Fig. 2. Phosphorylation conditions were described in the Methods section. Activities are expressed as the percentage of ^{32}P incorporated in troponin I incubated with enzyme in the absence of troponin C or digest. The radioactivity incorporated into the enzyme under the same conditions without troponin components has been deducted from the results presented. Controls were carried out with all additions except troponin C or CNBr digest. (*a*) 3′:5′-Cyclic AMP-dependent protein kinase (50μg/ml). Symbols: ○, troponin C; ●, CNBr digest of troponin C. (*b*) Phosphorylase kinase (50μg/ml). Symbols: □, troponin C; ■, CNBr digest of troponin C.

phosphorylation of the site specific for 3′:5′-cyclic AMP-dependent protein kinase in particular. Confirmation that interaction was occurring with a peptide fraction was obtained by polyacrylamide-gel electrophoresis at pH8.6 of the digest in the presence of troponin I. When electrophoresed alone troponin I did not migrate into the gel, but in the presence of Ca^{2+} and the CNBr digest of troponin C it migrated towards the cathode at pH8.6 as a new band not observed in the digest alone. In the presence of EGTA this band disappeared (Plate 1, gels *a* and *b*). This observation suggested that a Ca^{2+}-sensitive complex, similar to that obtained with troponin I and troponin C (Head & Perry, 1974), was formed between some component or components of the digest and troponin I. Complex-formation with troponin I could not be demonstrated on electrophoresis of the digest at pH8.6 in the presence of 6M-urea. Under these conditions intact troponin C forms a complex with troponin I.

Isolation of active fragments from the CNBr digest of troponin C

On gel filtration of the CNBr digest of troponin C on Sephadex G-50 a number of peaks could be identified by following the A_{215} of the eluate. Those fractions containing the larger peptide components were numbered I–V (Fig. 1, Plate 1). Fraction I contained undigested troponin C and large partial-cleavage products. Fractions II and III were apparently eluted as a single peak when the A_{280} of the eluate was measured. From examination of the bands obtained on polyacrylamide-gel electrophoresis at pH8.6 of fractions II, III and IV, the following conclusion could be drawn. The material represented by the leading half of fraction II was essentially pure and consisted of a single peptide fragment that migrated with an electrophoretic mobility to the anode at pH8.6 greater than that of troponin C itself (Plate 1*e*). Fraction III contained components giving rise to three main bands, whereas fraction IV contained two bands of similar electrophoretic mobilities to those of components present in fraction III (Plate 1). Fraction V material did not stain very well with Coomassie Blue, probably owing to the relatively low molecular weights of the peptides present in the fraction.

Ability to neutralize the inhibitory activity of troponin I on actomyosin ATPase was shown by material from peaks II, III and IV. No activity was



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoresis of CNBr peptides of troponin C from rabbit fast skeletal muscle

With the exception of gel (*n*), which was carried out in 0.1% sodium dodecyl sulphate/0.1 M-sodium phosphate buffer, pH 7.00, electrophoresis was carried out in 25 mM-Tris/80 mM-glycine buffer, pH 8.6. In gels (*d*), (*e*), (*f*), (*g*), (*h*) and (*m*), 6 M-urea was present in addition. The troponin I was from rabbit fast skeletal muscle. Fractions were obtained as illustrated in Fig. 1. Abbreviation: O, origin. Gels were loaded with the following: (*a*) whole CNBr digest of troponin C (100 μg), plus troponin I (50 μg) and 1 mM-$CaCl_2$. Band of complex is indicated by the arrow; (*b*) as for (*a*), but $CaCl_2$ replaced with 5 mM-EGTA; (*c*) whole CNBr digest of troponin C (100 μg); (*d*) fraction I (50 μg); (*e*) fraction II (peptide CB9) (50 μg); (*f*) fraction III (50 μg); (*g*) fraction IV (50 μg); (*h*) fraction V (50 μg); (*i*) peptide CB9 (50 μg), troponin I (20 μg) and 1 mM-$CaCl_2$. Complex of peptide CB9 and troponin I is indicated by an arrow; (*j*) as for (*i*), but with $CaCl_2$ replaced with 5 mM-EGTA; (*k*) peptide CB9 (50 μg)+1 mM-$CaCl_2$; (*l*) peptide CB9 (50 μg)+5 mM-EGTA; (*m*) more retarded peak of eluate obtained by gel filtration in Sephadex G-50 in 50 mM-NH_4HCO_3/1 mM-$CaCl_2$ (100 μg). Radioactive band is marked with an arrow. (*n*) as for (*m*), but electrophoresis (100 μg) was carried out in sodium dodecyl sulphate, under which condition the troponin I migrates as the main band, which it does not on electrophoresis in urea at pH 8.6; (*o*) fraction I, Fig. 9 (100 μg); (*p*) fraction II, Fig. 9 (100 μg). Radioactive peptide CB9 is indicated by an arrow.

present in peak V (Fig. 4). Activities determined from the weight of peptide required to produce 50% neutralization were in the order peak II > peak III > peak IV, with peak II about half as effective as troponin C. Although attempts were made to remove all NH_4HCO_3 from the fractions by repeated freeze-drying, some contamination from this ammonium salt may have been present in the peptide fraction. The activities of the peptides illustrated in Fig. 4 were calculated from the total N values obtained by Nesslerization of freeze-dried fractions after wet-ashing. Peptide amounts determined in this way will tend to be over-estimated and the specific activities calculated are therefore minimum values.

Fractions II, III and IV also inhibited the phosphorylation of troponin I catalysed by 3′:5′-cyclic AMP-dependent protein kinase. Fraction II was clearly most effective in producing 95% inhibition at a weight ratio of peptide to troponin I of 0.2:1. The biological activity present in fractions III and IV could have been due to a common peptide, for electrophoresis indicated that bands of similar mobility were present in both fractions (Plate 1, gels *f* and *g*). It was concluded from the electrophoretic and gel-filtration evidence that the active component(s) in fractions III and IV was not identical with the peptide that was present in fraction II.

Properties of the biologically active peptide in fraction II

Identification. The CNBr peptide present in fraction II was clearly one of the largest of the fragments obtained on CNBr digestion of troponin C from its elution behaviour and the fact that it could be detected as a discrete band on staining with Coomassie Blue after polyacrylamide-gel electrophoresis in 6M-urea at pH 8.6.

The sequence studies of Collins *et al.* (1973) have shown that the largest peptides present in CNBr digests of troponin C are, with these authors' nomenclature, peptide CB9, corresponding to residues 83–134, and peptide CB8 (Fig. 5), corresponding to residues 44–78. These peptides would be expected to be eluted on gel filtration immediately after any undigested material. Therefore if the active component present in fraction II was not a partial-digestion product it was likely that it corresponded to either peptide CB9 or CB8. The amino acid composition of the peptide in fraction II (Table 1) very closely resembled that of peptide CB9 (Collins *et al.*, 1973).

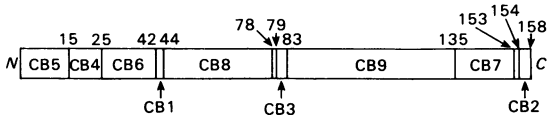


Fig. 5. *Schematic representation of the arrangements of the peptides obtained on CNBr digestion of troponin C from rabbit fast skeletal muscle*

The peptide nomenclature and arrangement are from Collins *et al.* (1973). Numbers indicate the position of the methionine residues cleaved by CNBr.

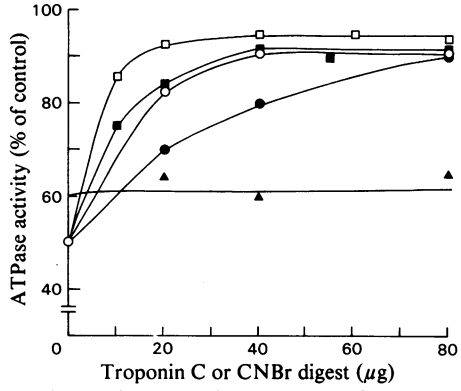


Fig. 4. *Effect of fractions obtained by gel filtration of the CNBr digest of troponin C on the neutralization of the inhibition of the ATPase of desensitized actomysin obtained in the presence of troponin I*

Fractions II, III, IV and V were obtained as indicated in Fig. 1. Enzymic assays were carried out as indicated in Fig. 2. Controls were carried out with all additions except troponin C or peptide fractions. Symbols: □, troponin C; ■, fraction II; ○, fraction III; ●, fraction IV; ▲, fraction V.

Table 1. *Amino acid analysis of peptide CB9*

Analysis was carried out by the procedure described by Wilkinson *et al.* (1972) for duplicate preparations 1 and 2. The peptide was isolated by gel filtration as described in the text.

	Composition of peptide CB9 (mol/mol of peptide)		
	Preparation 1	Preparation 2	From sequence (Collins *et al.*, 1973)
Asp	7.2	7.4	6
Thr	0.5	0.7	1
Ser	2.3	1.8	3
Glu	12.9	12.4	12
Gly	3.9	4.8	3
Ala	4.5	4.7	6
Val	2.0	2.0	2
Ile	4.0	4.0	4
Leu	3.3	3.3	3
Tyr	1.2	1.1	1
Phe	2.6	2.6	3
His	1.0	1.3	1
Lys	3.2	3.1	2
Arg	3.3	3.4	4

The *N*-terminal residue was shown to be lysine, which is the *N*-terminal residue of peptide CB9. Further, if troponin C was treated with iodo-[^{14}C]acetamide before digestion with CNBr, fraction II obtained on gel filtration contained virtually all the radioactivity present in the eluate, other than that in the undigested material present in peak I (Fig. 6). As the single cysteine residue present in rabbit troponin C is located at residue 98 in peptide CB9, this confirmed that the active peptide in fraction II did indeed consist of residues 83–134.

Biological activity. In neutralizing the inhibition of actomyosin ATPase by troponin I (Fig. 7) the purified peptide CB9 was about 50% as active on a molar basis as the troponin C from which it was derived. For the maximum neutralization effect about 2mol of peptide CB9 was required per mol of troponin I present. Some slight Ca^{2+}-sensitivity of the actomyosin ATPase (i.e. inhibition in the presence of EGTA) was observed at concentrations of peptide CB9 that were less than was required for the maximum neutralization of inhibition of troponin I.

The phosphorylation of troponin I from rabbit fast skeletal muscle catalysed by 3′:5′-cyclic AMP-dependent protein kinase was also sharply inhibited

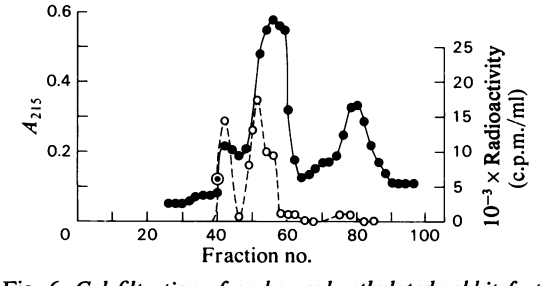


Fig. 6. *Gel filtration of carbamoylmethylated rabbit fast-skeletal-muscle troponin C*

CNBr digest (10mg) was labelled with iodo[1-^{14}C]-acetamide, as described in the Methods section, then dissolved in 50mM-NH_4HCO_3 (pH7.8) and applied to a Sephadex G-50 column (138cm×3cm) equilibrated against the same solution; 5ml fractions were collected. Symbols: ●, A_{215}; ○, radioactivity.

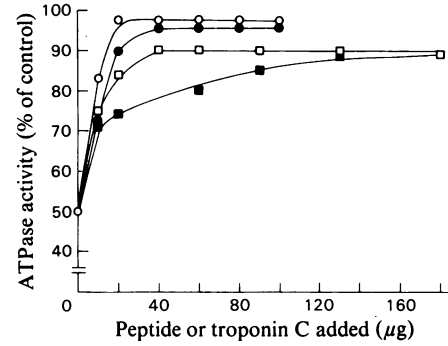


Fig. 7. *Effect of peptide CB9 from the CNBr digest of troponin C on the inhibition of the Mg^{2+}-stimulated ATPase of desensitized actomyosin by troponin I*

Enzymic assays were carried out as indicated in Fig. 2. Controls were carried out with all additions except troponin C or peptide CB9. Symbols: ○, troponin C; ●, troponin C+1mM-EGTA; □, peptide CB9; ■, peptide CB9+1mM-EGTA.

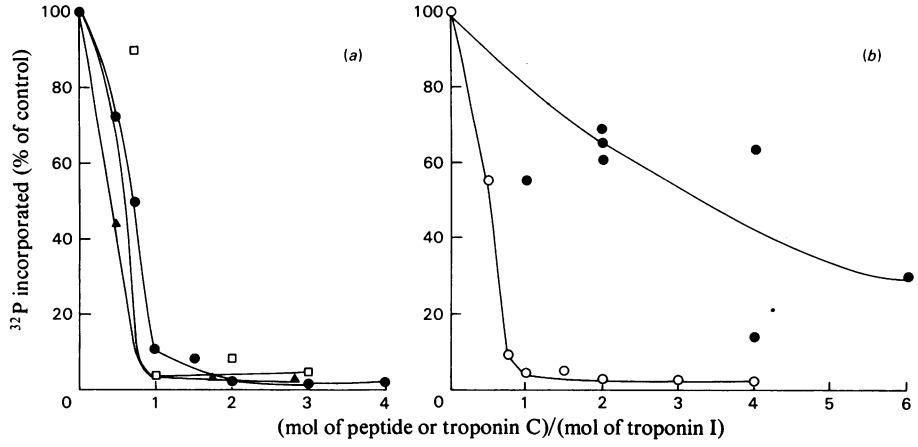


Fig. 8. *Inhibition of phosphorylation of troponin I from rabbit white muscle by CNBr peptides from rabbit and chicken white muscle*

Experimental conditions were as for Fig. 3. Controls were carried out with all additions except troponin C or peptide fractions. (*a*) 3′:5′-Cyclic AMP-dependent protein kinase (50μg/ml). Symbols: □, rabbit troponin C; ●, peptide CB9 from rabbit troponin C; ▲, peptide from chicken white skeletal muscle corresponding to peptide CB9 of rabbit fast-skeletal-muscle troponin C. (*b*) Phosphorylase kinase (50μg/ml). Symbols: ○, rabbit troponin C; ●, peptide CB9.

by peptide CB9. In this respect it was almost as effective as troponin C itself, and virtually complete inhibition of phosphorylation was obtained when 1 mol of peptide/mol of troponin C was present (Fig. 8). Similar results were obtained with a peptide consisting of residues 86–138 (Wilkinson, 1976) that was isolated from a CNBr digest of troponin C of chicken breast muscle and analogous to peptide CB9.

The peptide CB9 was much less effective in inhibiting the phosphorylase kinase-catalysed phosphorylation of troponin I from rabbit white skeletal muscle. Even with a large molar excess complete inhibition of phosphorylation by phosphorylase kinase could not be obtained. The rapid phosphorylation of rabbit cardiac-muscle troponin I catalysed by 3′:5′-cyclic AMP-dependent protein kinase is not inhibited by troponin C from skeletal or cardiac muscle (Cole & Perry, 1975). Peptide CB9 did not significantly inhibit the phosphorylation of rabbit cardiac-muscle troponin I under these conditions. The phosphorylation of calf thymus histone by 3′:5′-cyclic AMP-dependent protein kinase was not inhibited by peptide CB9, indicating, as did the results with cardiac troponin I (Cole & Perry, 1975), that its effect was specific for skeletal-muscle troponin I and not a non-specific effect on the activity of the protein kinase.

Only slight inhibition of the phosphorylation of troponin T by phosphorylase kinase was obtained, e.g. 4:1 molar ratio of peptide CB9 to troponin T gave 15% inhibition of phosphorylation.

Ca^{2+}-dependent binding of peptide CB9 to troponin I. Peptide CB9 could not be examined by electrophoresis in sodium dodecyl sulphate, for under these conditions a stainable band was not obtained. On polyacrylamide-gel electrophoresis under non-dissociating conditions, e.g. 80mM-glycine/25mM-Tris, pH8.6, it migrated as a single blurred band that sharpened up to a single clear band on addition of 6M-urea to the electrophoresis system (Plate 1, gel *e*). In 80mM-glycine/25mM-Tris, pH8.6, a complex was formed with troponin I that migrated with about 25% of the mobility of the peptide alone (Plate 1, gels *i* and *j*) and similar to that observed in the whole digest (Plate 1*a*). The complex required Ca^{2+} for formation, as it dissociated in the presence of EGTA.

In the presence of $CaCl_2$ at pH8.6 the mobility of peptide CB9 to the anode was slightly greater than when EGTA was present (Plate 1, gels *k* and *l*). This is the opposite to what would be expected from the charge effect, and the behaviour was very similar to that of troponin C itself in this respect. It probably indicates that the change in conformation that occurs on binding Ca^{2+} has a greater effect on mobility than does the modification of charge. Unlike the case with troponin C, Ca^{2+} had little effect on the mobility of the peptide in 6M-urea.

Complex-formation between peptide CB9 and troponin I could also be demonstrated by mixing troponin I with a CNBr digest of ^{14}C-labelled carbamoylmethylated troponin C and subjecting the mixture to gel filtration on Sephadex G-50. On elution with 50mM-NH_4HCO_3/1mM-$CaCl_2$ most of the radioactive peptide CB9, which was retarded on gel filtration in the absence of troponin I, was eluted with non-retarded troponin I peak. This could be identified as a stainable band on electrophoresis of the troponin I peak in urea at pH8.6 (Plate 1, gel *m*). Another fainter band of slower mobility was also observed. Under these conditions of electrophoresis the troponin I remains at the origin; however, on electrophoresis of the fraction in sodium dodecyl sulphate at pH7.0 the CNBr peptides of troponin C do not stain, but troponin I does (Plate 1, gel *n*). If the gel filtration of the mixture was carried out in the presence of 10mM-EGTA all the radioactive peptide was eluted in the normal position and none was associated with the non-retarded troponin-I peak.

Further confirmation of Ca^{2+}-dependent binding of peptide CB9 to troponin I was obtained by applying a sample of [1-^{14}C]carbamoylmethylated CNBr digest dissolved in 50mM-NH_4HCO_3 to a troponin I–Sepharose affinity column (Fig. 9). When the column was washed with 50mM-NH_4HCO_3 most of the peptide material passed through the column unretarded (Plate 1, gel *o*). On application of 10mM-

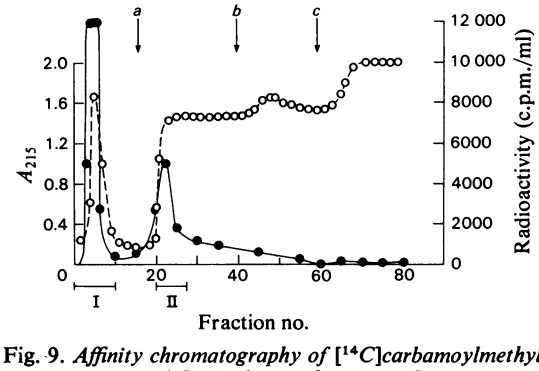


Fig. 9. *Affinity chromatography of* [^{14}C]*carbamoylmethylated CNBr digest of troponin C*

CNBr digest (0.5mg in 1ml) was labelled with iodo-[1-^{14}C]acetamide as described in Fig. 6, then dissolved in 50mM-NH_4HCO_3, pH7.8, and applied to a column of Sepharose–troponin I equilibrated against the same solution. Fractions (3ml) were collected. Fractions contained in peaks I and II were pooled separately. Elution solutions changed as follows: (*a*) 50mM-NH_4HCO_3/10mM-EGTA; (*b*) 1M-NH_4HCO_3; (*c*) 8M-urea/75mM-Tris adjusted to pH8.0 with 1-M-HCl. The solid line represents radioactivity, and the broken line represents A_{215}.

EGTA/50mM-NH_4HCO_3 to the column when no further peptide material could be eluted by 50mM-NH_4HCO_3, a peak of radioactive material was eluted. Examination of this fraction by electrophoresis in 6M-urea/25mM-Tris/80mM-glycine, pH 8.6, after concentration by freeze-drying and desalting by gel filtration, indicated the presence of two bands of peptide material (Plate 1, gel *p*). The peptide with the highest mobility was radioactive and could be separated from the slower peptide by gel filtration on Sephadex G-50 equilibrated with 50mM-NH_4HCO_3. It was concluded that this peptide was CB9, which is the only radioactive peptide in the CNBr digest of troponin C treated with iodo[1-^{14}C]-acetamide. This conclusion was confirmed by amino acid analysis. Amino acid analysis of a single preparation of the peptide of lower mobility indicates that it was similar to that of peptide CB8 (Collins *et al.*, 1973).

Discussion

Because of its acidic nature, peptide CB9 produced by CNBr digestion of troponin C would be expected to interact non-specifically with troponin I, a protein with a net positive charge at physiological pH values, and possibly modify its properties in this way. Evidence has been presented (Hartshorne, 1970) that compounds with a net negative charge such as heparin can mimic certain effects of troponin C on the biological activity of troponin I. It would therefore not be suprising if the acidic CNBr peptides had effects on the biological activity of troponin I. Although electrostatic forces no doubt play an important part in the formation of its complex with troponin I, the interaction is considered to be specific in that peptide CB9 possessed most of the properties of troponin C listed in the introduction. It is therefore very probable that this peptide is derived from a region of the primary sequence of troponin C that interacts with troponin I. In particular the demonstration that it is able to form a complex in the presence of Ca^{2+} with troponin I and has different effects on the phosphorylation of fast-skeletal- and cardiac-muscle troponin I catalysed by 3′:5′-cyclic AMP-dependent protein kinase, as with troponin C itself, are strong indications that it represents a specific interacting region. In view of its more effective inhibition of the phosphorylation catalysed by 3′:5′-cyclic AMP-dependent protein kinase, i.e. principally at serine-117 [originally serine-118 (Moir *et al.*, 1974), but see correction to the primary sequence of rabbit fast-skeletal-muscle troponin I (Grand & Wilkinson, 1977)], it is postulated that peptide CB9 preferentially binds close to serine-117 in fast-skeletal-muscle troponin I, i.e. close to the actin-binding site, which includes residues 98–116 (Syska *et al.*, 1976). As might be expected, peptide CB9 was usually less effective on a molar basis than troponin C, but its activity was remarkably high for a fragment consisting of about 25% of the whole molecule, particularly in inhibiting the phosphorylation of troponin I catalysed by 3′:5′-cyclic AMP-dependent protein kinase.

The investigation has also shown that the CNBr digest contained another peptide (or peptides) that was able to neutralize inhibition of the Mg^{2+}-stimulated ATPase of desensitized actomyosin and inhibit the phosphorylation of fast-skeletal-muscle troponin I. The preliminary results reported suggest that, in addition to peptide CB9, another CNBr peptide can also form a Ca^{2+}-dependent complex with troponin I. Further work is required to establish firmly whether this peptide was responsible for the biological activity in fractions of the CNBr digest of troponin C that did not contain peptide CB9.

The findings suggest that two regions of the troponin C molecule can interact with troponin I. These results complement our earlier findings that two regions of rabbit fast-skeletal-muscle troponin I, namely those represented by the first 30–40 residues at the *N*-terminus and residues 98–116, form complexes with troponin C (Syska *et al.*, 1976). Both these regions possess a high density of positive charges and would be expected to interact with net negatively charged regions of the troponin C molecule, but for peptide CB9, as discussed above, the interaction appears to be at a specific site.

Direct evidence that troponin C may contain more than one site that can interact with troponin I is provided by immunological studies on the troponin I–troponin C complex with antibodies to troponin C (Hirabayashi & Perry, 1974). Similar conclusions also follow from the fact that under some circumstances phosphorylation of troponin I (Perry & Cole, 1974) and inhibition of Mg^{2+}-stimulated actomyosin ATPase are neutralized by molar ratios of troponin C to troponin I of less than 1 (Perry *et al.*, 1972; Amphlett *et al.*, 1976). In contrast the molar ratio for the combination of troponin I with troponin C to form a complex that is Ca^{2+}-sensitive in urea is 1:1 (Head & Perry, 1974). Elsewhere we have implied that troponin T can also interact with one of the acidic sites on troponin C (Perry *et al.*, 1975, 1976). It is possible that under some conditions in the absence of troponin T, troponin I (which carries a similar net charge to troponin T) interacts with the troponin T-specific site on troponin C. Kretzinger & Barry (1975) have also made similar suggestions.

In view of the uncertainty as to how many Ca^{2+} cations are bound to each molecule of troponin C when contraction occurs, it is difficult to define precisely the role of Ca^{2+} binding at the peptide-CB9 site in the regulation of the contractile process. There is evidence (Potter *et al.*, 1976) that peptide CB9

undergoes conformational change in the presence of Ca^{2+}, suggesting that it preserves the ability to bind Ca^{2+}. This conclusion is supported by our finding that peptide CB9 forms a Ca^{2+}-dependent complex with troponin I. The cysteine residue is not essential for interaction with troponin I or Ca^{2+} binding for neutralization of troponin I-inhibitory activity; inhibition of phosphorylation of troponin I and the formation of a Ca^{2+}-dependent complex are exhibited by carbamoylmethylated peptide CB9.

The demonstration that the peptide requires Ca^{2+} to form a stable complex indicates that Ca^{2+} must be bound either at or close to the site on the peptide (and presumably on troponin C itself) involved in interaction with troponin I. In view of these properties of the peptide CB9 it is not necessary to postulate a mechanism in which Ca^{2+} binds at one site on the troponin C molecule and thus produces a conformational change that enables another site some distance away in the primary sequence to interact with troponin I. Simple binding of Ca^{2+} at the acidic region would be expected to decrease the net charge in that area and therefore decrease the electrostatic force available for interaction with the positively charged site on troponin I. Ca^{2+} clearly has the reverse effect, and presumably when Ca^{2+} is bound some constraint is imposed on the peptide so that the groups at the adjacent interacting region are brought into a more favourable conformation for interaction with troponin I.

The identification of a CNBr peptide of troponin C that preserves much of the biological activity of the original molecule is further evidence indicating that the functions of the components of the troponin complex are associated with relatively small discrete regions of the primary sequence of each component of the complex. The findings and the comparable results obtained with troponin I (Syska *et al.*, 1976) and with troponin T (Jackson *et al.*, 1975) suggest that this type of precise localization of function in specific regions of the primary sequence may be a special feature of interacting protein systems such as the troponin complex.

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