The Relationship between Biological Activity and Primary Structure of Troponin I from White Skeletal Muscle of the Rabbit

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1. A series of defined peptides which span the complete sequence were produced from troponin I isolated from white skeletal muscle of the rabbit. 2. Two peptides, CF1 (residues 64–133) and CN4 (residues 96–117) inhibited the Mg^{2+} -stimulated adenosine triphosphatase of desensitized actomyosin. This inhibition was potentiated by tropomyosin and neutralized by troponin C. 3. Basic proteins, salmine, lysozyme and cytochrome *c* inhibited the Mg^{2+} -stimulated adenosine triphosphatase of desensitized actomyosin. This inhibition, unlike that of troponin I and peptides derived from it, was not potentiated by tropomyosin. 4. The most active inhibitor, peptide CN4, was 45–75% as effective as troponin I when compared on a molar basis. The inhibitory peptide, CN4, and also whole troponin I were shown by affinity chromatography to interact specifically with actin. 5. A strong interaction with troponin C was demonstrated with peptide CF2 (residues 1–47), from the *N*-terminal region of troponin I. Somewhat weaker interactions were shown with peptides CN5 (residues 1–21) and with the inhibitory peptide CN4. 6. The significance of these interactions for the mechanisms of action of troponin I is discussed.

Troponin I was first isolated from rabbit muscle as an impure preparation, known as the inhibitory factor, by direct extraction of myofibrils (Hartshorne *et al.*, 1966, 1967). It is now known that troponin I is present in muscle as part of the troponin complex, in which it is associated with troponin C and troponin T. This complex is localized on the I filament at intervals of 38.5 nm (385 Å) and, together with tropomyosin, forms the protein system that regulates the hydrolysis of $MgATP^{2-}$ by the actomyosin system in response to changes in the Ca^{2+} concentration of the sarcoplasm.

Troponin I exhibits a number of properties that are relevant to its role in regulating contractile activity in muscle. It inhibits the Mg^{2+} -stimulated ATPase* activity of actomyosin, a property that is much potentiated by tropomyosin, but which is independent of Ca^{2+} concentration (Perry *et al.*, 1972). In the presence of troponin C, with which it forms a complex stabilized by Ca^{2+} (Head & Perry, 1974), the inhibitory activity of troponin I is neutralized. Troponin I can also be phosphorylated at specific sites by phosphorylase kinase and 3':5'-cyclic AMP-dependent protein kinase (Perry & Cole, 1974; Moir *et al.*, 1974; Huang *et al.*, 1974).

The reports from a number of laboratories (Wilkinson *et al.*, 1971, 1972; Greaser & Gergely, 1971; Drabikowski *et al.*, 1971; Shigekawa & Tonomura, 1972) indicating that troponin I can be

degraded by proteolysis to fragments of lower molecular weight that possess inhibitory activity, suggest that only part of the troponin I molecule is essential for its inhibitory activity. The present work is a systematic investigation of the relation between primary structure and biological function making use of defined peptide fragments prepared in the light of the knowledge of the primary sequence of troponin I of skeletal muscle determined in this laboratory (Wilkinson & Grand, 1975a).

Two regions of the troponin I molecule have been identified as of special significance for the function of troponin I. A peptide consisting of residues 96–117 inhibits the Mg^{2+} -stimulated ATPase of actomyosin in a similar way to that of the original molecule. This region probably represents the site of interaction with actin. An *N*-terminal peptide, consisting of residues 1–47, interacts with troponin C as does the peptide obtained by CNBr digestion that possesses inhibitory activity. Preliminary accounts of some aspects of this work have been reported (Wilkinson & Grand, 1975b; Perry *et al.*, 1975).

Materials and Methods

Materials

Lysozyme (egg-white; grade 1), bovine pancreatic ribonuclease (type IIIA) and cytochrome *c* were supplied by Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K. Salmine was

* Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethanedioxybis(ethylamine)tetra-acetic acid.

obtained from BDH Chemicals Ltd., Poole, Dorset BH12 4NN, U.K.

Methods

Preparation of muscle proteins. Troponin I was prepared by the method of Wilkinson (1974a), troponin C as described by Perry & Cole (1974) and actin by the method of Spudich & Watt (1971). All proteins were prepared from combined back and leg muscles of New Zealand White rabbits.

CNBr cleavage. The CNBr-cleaved peptides of troponin I were prepared as described by Wilkinson (1974b). Desalting and removal of partial digestion products from CNBr digests used for enzymic studies and affinity chromatography were carried out by chromatography on a Sephadex G-50 column (2.2 cm × 10 cm) equilibrated and eluted with 10 mM-HCl.

Limited trypsin cleavage. Citraconic anhydride (BDH Chemicals Ltd.) was used to block the lysine residues of troponin I (Dixon & Perham, 1968). After alkylation of the cysteine residues with iodo[¹⁴C]-acetic acid (Wilkinson *et al.*, 1972), a solution of troponin I (4 ml, 15 mg/ml) in 10 mM-HCl was adjusted to pH 8.0 with 1 M-NaOH and maintained at that pH by the addition of 5 M-NaOH during the addition of 0.5 ml of citraconic anhydride. When the pH was stable the protein solution was dialysed against 1% NH₄HCO₃. After dialysis the citraconylated protein was digested with 2% (by wt.) 1-chloro-4-phenyl-3-tosylamidobutan-2-one - treated trypsin (Cambrian Chemicals Ltd., Croydon CR0 4XB, U.K.) for 3 h at 37°C. The digest was freeze-dried to remove NH₄HCO₃ and then dissolved in a buffer containing 6 M-urea, 0.2 M-sodium formate, pH 3.5. After incubation overnight to remove the blocking groups, the digest was applied to a column (2.2 cm × 110 cm) of Sephadex G-50 equilibrated with the same buffer, to separate the peptides.

Cleavage at cysteine residues. The method used was essentially that described by Jacobson *et al.* (1973). Troponin I (200 mg) was dissolved in 100 ml of 6 M-guanidine hydrochloride/0.4 M-Tris/0.2 M-acetic acid, pH 8.0, in the presence of a slight excess of dithiothreitol (0.2 mM) over thiol groups. After 30 min, 5,5'-dithiobis-(2-nitrobenzoic acid) was added to a concentration of 7 mM and after a further 15 min, solid KCN was added to make the concentration 70 mM. In some cases the modified cysteine residues were labelled radioactively by use of K¹⁴CN. The modification reaction was allowed to proceed for 30 min at room temperature and then the mixture was acidified by the addition of 100 ml of acetic acid. The protein was dialysed against water to remove excess of reagent and freeze-dried. The yellow colour of the freeze-dried material was removed by a desalting step on a column (90 cm × 2.6 cm) of Sephadex G-15 in 10 mM-HCl.

The modified troponin I was cleaved in 50 ml of 6 M-guanidine/50 mM-sodium borate, pH 9.0, at 37°C for 72 h, and then desalted on a column (2.5 cm × 90 cm) of Sephadex G-15 in 10 mM-HCl, and freeze-dried.

Phosphorylation of peptides. The N-terminal peptides, CN5 and CF2, were phosphorylated at threonine-11 by using [γ -³²P]ATP and phosphorylase kinase by the method of Perry & Cole (1974). Phosphorylase kinase was prepared from white skeletal muscle of the rabbit by the method of Cohen (1973). Phosphorylation was allowed to proceed at 30°C for about 16 h, and then the peptides were separated from phosphorylase kinase and ATP by application of the incubation mixture to a column (2.2 cm × 110 cm) of Sephadex G-50, equilibrated with 10 mM-HCl, and by elution with 10 mM-HCl. Fractions containing peptides CN5 and CF2 were freeze-dried before further use.

Affinity chromatography. Troponin C affinity columns were prepared by linking troponin C from rabbit white skeletal muscle to Sepharose 4B as described by Syska *et al.* (1974).

F-actin was dialysed for 72 h against a large volume of 2 mM-Tris (adjusted to pH 8.0 with 1 M-HCl)/0.2 mM-ATP / 0.5 mM- β -mercaptoethanol / 0.2 mM-CaCl₂, and centrifuged for 3 h at 80000g to remove any F-actin from the G-actin solution. A portion of the G-actin solution was dialysed for 24 h against 10 mM-triethanolamine (adjusted to pH 8.0 by the addition of 1 M-HCl), and then coupled to Sepharose 4B activated by the procedure of Porath *et al.* (1967), as described by Syska *et al.* (1974), or by the method of March *et al.* (1974) by using 10 mM-triethanolamine/HCl, pH 8.0, as the coupling buffer. Coupling was allowed to proceed for 20–40 h at 4°C.

Essentially the same technique was used to test all interactions between the peptides and troponin C-Sepharose and actin-Sepharose columns. The column (3 cm × 10 cm) was equilibrated with 50 mM-NH₄HCO₃, pH 7.8, and the sample (approx. 5 mg in 5 ml) applied in the same buffer. After washing with buffer until the extinction of the eluate at 215 nm returned to its original value, a buffer of 1 M-NaCl/50 mM-NH₄HCO₃ in the case of troponin C columns, or 0.6 M-NaCl/50 mM-NH₄HCO₃ in the case of actin columns, was applied and the column again washed until the extinction returned to its original value. The troponin C column was washed finally with 9 M-urea/50 mM-Tris/50 mM-HCl (pH 8.0)/15 mM-2-mercaptoethanol.

Detection of radioactively labelled peptides on a column was not a problem, but with unlabelled samples some difficulty was encountered, as elution with the urea buffer prohibited monitoring at 215 nm. Therefore with non-radioactive samples, any material eluted with urea was desalted on a Sephadex G-15 column (2.5 cm × 90 cm) (eluted with 10 mM-HCl),

freeze-dried and rechromatographed on Sephadex G-50. Material not bound to the column, or eluted with NaCl, was as a routine concentrated by rotary evaporation and rechromatographed on Sephadex G-50.

Other methods. The methods of amino acid analysis, *N*-terminal amino acid determination and high-voltage electrophoresis have been described by Wilkinson (1974a), and those for gel electrophoresis in sodium dodecyl sulphate and in urea at pH 3.2 by Wilkinson *et al.* (1972). Gel electrophoresis at pH 8.6 was performed by the method of Perrie & Perry (1970). Chromatography on phosphocellulose was performed as described by Chin & Wold (1972).

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 9 vol. of cold water, the precipitate separated by centrifugation and made up to 0.6M by the addition of solid KCl (Cummins & Perry, 1973). The solution of actomyosin was reprecipitated by the slow addition of 12 vol. of cold water. The suspension was left for 30 min, centrifuged and then washed by repeated suspension in water in a hand-operated homogenizer, followed by centrifugation at 55000g for 30 min. The washing was continued with water until the Mg²⁺-stimulated ATPase activity of the actomyosin was no longer sensitive to EGTA over the range 1–5 mM (usually five washes were required). The precipitate was finally washed twice with 1 mM-Tris/HCl buffer, pH 7.6. The desensitized actomyosin was stored at 0°C in the presence of trace amounts of toluene at a protein concentration of 3–5 mg/ml, and could be used for up to 2 weeks without significant loss of activity.

ATPase assays. Determinations of ATPase activity were usually carried out by incubation for 5 min at 25°C. Unless otherwise stated the incubation medium contained 0.2–0.6 mg of desensitized actomyosin, 2.5 mM-MgCl₂, 1 mM-EGTA, 2.5 mM-Tris/ATP, 25 mM-Tris/16 mM-HCl, pH 7.6, in a total volume of 2 ml. The reaction was started by the addition of ATP and stopped by 1 ml of 15% (w/v) trichloroacetic acid.

Results

Peptide isolation

Isolation of tryptic peptides of troponin I. Tryptic digestion of troponin I that had been citraconylated after [¹⁴C]carboxymethylation would be expected to give rise to a large peptide from residues 37–103 that accounts for more than one-third of the molecule and contains two of the cysteine and both the tyrosine residues. In addition there should be two peptides of

22 residues, one containing the third cysteine residue (see Fig. 1) and a number of smaller peptides down to free arginine.

As expected, chromatography of the digest on Sephadex G-50 gave rise to two radioactive peaks (Fig. 2). The first peak (A) contained all the tyrosine and approximately twice the radioactivity of peak C. After desalting it was found to have the composition shown in Table 1 for the peptide TF1. The composition agreed well with that expected, except that it contained no arginine. It appears most likely that trypsin has cleaved at phenylalanine-100 with the loss of the tripeptide Asp-Leu-Arg.

Analysis of peak C (Fig. 2) showed that it was probably a mixture of three peptides, TF2 from residues 14–35, TF3 from residues 117–138, and TF4 from residues 139–153 (see Fig. 1). Separation of these peptides was attempted by chromatography on phosphocellulose, as shown in Fig. 3. Some separation was obtained, but the radioactivity corresponding to the [¹⁴C]carboxymethylation of cysteine-134, which was taken as an indicator of peptide TF3, was distributed across all the peaks. Analysis of the fractions showed that none of them were pure.

Peptide TF3 was purified from peak 1 in rather poor yield by high-voltage paper electrophoresis at pH 6.5. Peak 3 contained peptide TF4 in an impure state, and peak 4 probably contained peptide TF2, but no further attempt was made to purify these fragments.

Fractions D, E and F from Sephadex G-50 chromatography (Fig. 2) of the tryptic digest of troponin I contained the smaller peptides. They were not examined in detail, except for peptide TF6, from residues 104–112, which was isolated from fraction E by paper electrophoresis at pH 6.5. The compositions of the peptides are shown in Table 1. The *N*-terminal amino acid composition of all three peptides was that expected from the sequence of troponin I (Wilkinson & Grand, 1975a).

Isolation of peptides from cleavage of troponin I at cysteine residues. Cleavage of troponin I at the cysteine residues would be expected to give rise to four peptides (see Fig. 1), one large peptide, CF1 from residues 64–133, two peptides of almost identical size, CF2 and CF3 from the *N*- and *C*-terminal ends of the molecule respectively, and one small peptide, CF4 from residues 48–63.

An initial separation of the cleavage products was obtained by chromatography on Sephadex G-50 in 10 mM-HCl as shown in Fig. 4; fractions were pooled as indicated. Fractions 1 and 2 appeared to contain uncleaved troponin I and partially cleaved material respectively and they were not investigated further.

Chromatography of fraction 3 (Fig. 4) on Sephadex G-50 in 6 M-urea/0.2 M-sodium formate, pH 4.0, gave a partial separation into two fractions. Rechromatography of the lower-molecular-weight material on

Fig. 1. Location in the polypeptide chain of the peptide fragments isolated from troponin I of rabbit white skeletal muscle

Peptides were selected on the basis of the primary sequence (Wilkinson & Grand, 1975b) so that they form an overlapping series covering the whole molecule. Peptides CN5, CN1, CN4 and CN2 were obtained by CNBr cleavage (Wilkinson, 1974a), peptides CF2, CF4, CF1 and CF3 by cysteine cleavage and peptides TF1, TF6 and TF3 by tryptic digestion of citraconylated troponin I. Threonine-11 and serine-118 are the major sites of phosphorylation by phosphorylase kinase and 3':5'-cyclic AMP-dependent protein kinase (Molr *et al.*, 1974; Huang *et al.*, 1974).

Fig. 2. Gel filtration of a tryptic digest of citraconylated troponin I

The tryptic digest of citraconylated troponin I from rabbit skeletal muscle (60mg) was dissolved in 6M-urea/0.2M-sodium formate, pH3.5, and applied to a column (2.2cm $\times$ 110cm) of Sephadex G-50 equilibrated against the same buffer. Fractions (5ml) were collected and radioactivity was measured in 2 μ l samples. —, E_{280} ; ----, radioactivity. For further details of peaks A-F see the text.

Fig. 3. Chromatography of fraction C from the tryptic digest of citraconylated troponin I

The whole fraction C (see Fig. 2) was dissolved in 8 mM- H_3PO_4 , and applied to a column (1.0 cm $\times$ 22 cm) of phosphocellulose equilibrated with the same solution. The column was eluted with a linear gradient of NaCl from 0 to 1.5 M in 8 mM- H_3PO_4 , the total volume being 800 ml. Fractions (10 ml) were collected and radioactivity was measured on 10 μl samples. —, E_{215} ; ····, radioactivity, ----, conductivity.

Sephadex G-50 in 10 mM-HCl gave a single peak that was shown by analysis to be peptide CF1 (see Table 1). The higher-molecular-weight material appeared to be a partial-cleavage product containing peptides CF1 and CF3, but was not investigated further.

Amino acid analysis suggested that the peptides CF2 and CF3 were present in fraction 4, but attempts to separate them by gel filtration or ion-exchange chromatography were unsuccessful. It was, however, possible to separate them by affinity chromatography on a troponin C-Sepharose column.

Fraction 4 was phosphorylated with [γ - ^{32}P]ATP by using phosphorylase kinase (see under 'Methods') and passed through a column (3 cm $\times$ 10 cm) of troponin C-Sepharose containing about 40 g of adsorbent. The column was eluted with 50 mM- NH_4HCO_3 , pH 7.8, until the extinction of the eluate returned to the base line (Fig. 5). The column was then washed with the buffer 9 M-urea/50 mM-Tris/50 mM-HCl, pH 8.0, which eluted a peak of radioactivity. Peak 1 (Fig. 5), containing peptide CF3, was re-run on the column to ensure complete removal of peptide CF2.

Amino acid analysis of peaks 1 and 2 confirmed that they were peptides CF3 and CF2 respectively. In addition, the N-terminal fragment, peptide CF2, was digested with trypsin giving rise to the expected peptides. Peptide CF4 was purified from fraction 5 of the digest produced by cleavage at cysteine residues by high-voltage electrophoresis at pH 6.5. The amino acid composition of the peptides is shown in Table 1.

As indicated above, in addition to the expected fragments, other peptides in which cleavage had not occurred at one or another of the cysteine residues were obtained. For example, in the cleavage reaction in which K^{14}CN was used, peptide CF4 was not obtained and the major large component was the partial-cleavage product comprising peptides CF4 and CF1 from residues 48–133. This has been termed CF1' and was used for interaction studies with actin-Sepharose columns because it was radioactive.

Enzymic studies

Inhibitory activity of CNBr-cleaved peptides of troponin I. The whole CNBr digest of troponin I from rabbit white muscle that had been carefully desalted by gel filtration (see under 'Methods') inhibited the Mg^{2+} -stimulated ATPase activity of desensitized actomyosin (Fig. 6). The inhibition was much more effective in the presence of tropomyosin, but in both the absence and the presence of the latter protein the digest was about 50% as effective as the original troponin I, on the basis of the molar amounts required to produce 50% of maximum inhibition. The inhibition obtained was not due to partial digestion, for the unretarded fraction obtained by gel filtration on Sephadex G-50 was not inhibitory, whereas the material retarded on gel filtration, particularly that in the more strongly retarded fractions, had inhibitory activity.

Table 1. *Amino acid composition of tryptic and cysteine peptides from troponin I of rabbit white skeletal muscle*

The first column of values for each peptide gives the composition calculated from the amino acid sequence (Wilkinson & Grand, 1975b). The second column gives the composition as determined by analysis in mol per mol of peptide. + indicates tryptophan and cysteine identified by u.v. absorption and radioactivity respectively.

Peptide ...	TF1		TF3		TF6		CF1		CF2		CF3		CF4	
	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found	Expected	Found
Asp	6	5.2	2	2.0	—	—	6	6.9	3	3.1	6	6.0	—	—
Thr	—	—	—	—	—	—	—	—	2	1.9	1	1.0	—	—
Ser	4	3.6	2	1.6	—	—	4	3.8	1	1.1	3	2.3	2	1.8
Glu	16	15.4	—	—	—	—	9	11.7	12	10.8	9	9.0	3	3.1
Pro	3	3.9	—	—	2	1.7	2	2.7	—	—	—	—	3	2.6
Gly	1	1.4	1	1.1	1	1.1	2	3.2	2	2.3	3	3.0	1	1.1
Ala	6	6.4	3	2.9	—	—	6	5.1	6	5.8	1	2.3	1	1.1
Val	2	2.4	1	1.0	—	—	3	3.0	1	1.1	2	1.9	1	1.1
Met	3	2.2	3	2.4	—	—	4	3.0	1	1.1	3	1.5	1	0.9
Ile	2	2.0	—	—	—	—	2	1.7	2	2.0	1	1.2	—	—
Leu	8	7.2	4	3.9	1	1.2	8	7.6	4	4.2	2	3.5	3	3.3
Tyr	2	2.0	—	—	—	—	1	0.7	1	0.9	—	—	—	—
Phe	1	1.1	—	—	1	1.0	2	1.6	—	—	1	0.6	—	—
His	2	2.2	1	0.9	—	—	2	1.4	2	1.9	—	0.5	—	—
Lys	8	8.3	3	2.9	2	2.1	12	10.3	4	3.8	8	7.0	—	—
Arg	1	—	1	1.1	2	2.6	6	5.5	6	5.6	4	4.0	—	—
Trp	—	—	—	—	—	—	—	—	—	—	1	+	—	—
Cys	2	1.8	1	+	—	—	1	—	—	—	1	—	1	—

Fig. 4. Gel filtration of the products of cysteine cleavage of troponin I

Cleavage products from 200mg of troponin I were dissolved in 10mM-HCl and applied to a column (2.2cm×110cm) of Sephadex G-50 equilibrated with 10mM-HCl. ----, E_{280} ; —, E_{215} .

The peptides listed in Fig. 1 were tested in turn for inhibitory activity on the Mg^{2+} -stimulated ATPase of actomyosin. Of these, only four, namely peptides CF1, CF1', CN4 and CN5, showed any inhibition (Table 2). The first three of these peptides possessed highly significant activity that was increased by the addition of tropomyosin. The activity of peptide CN5 was low and relatively large amounts were required to produce an effect, i.e. it was about 40 times less effective than peptide CN4 when compared on a molar basis. Examination of the homogeneity of preparations of peptide CN5 by electrophoresis in 6M-urea/5% (v/v) acetic acid, pH 3.2, indicated the presence of trace amounts of peptide CN4, which were very difficult to remove completely from preparations of peptide CN5. It was therefore considered that the inhibitory activity of peptide CN5 was due to contamination with traces of peptide CN4.

Properties of the active peptides. Comparison of the inhibitory activities of peptides CF1 and CN4 with troponin I on the Mg^{2+} -stimulated ATPase of desensitized actomyosin indicated that on a molar basis their effectiveness both in the presence and the absence of tropomyosin was in the order troponin I > peptide CN4 > peptide CF1. In the presence of tropomyosin both peptides produced the same maximum inhibition (about 90%) as troponin I (Fig. 7). Under these conditions peptide CN4 was 75–45% and peptide CF1 50–20% as active as troponin I when calculated on a molar basis. The inhibitory activity of the whole CNBr digest was very similar to that of peptide CN4 when both were tested on the same desensitized actomyosin preparation. These results, taken with those described above, strongly suggested that peptide CN4 was the peptide responsible for activity of CNBr digest of troponin I.

Fig. 5. Affinity chromatography of fraction 4 obtained on fractionation of the products of cleavage at cysteine

Fraction 4 (10 mg) (see Fig. 4) was phosphorylated with [γ - 32 P]ATP and phosphorylase kinase as described in the Materials and Methods section, dissolved in 50 mM- NH_4HCO_3 , pH 7.8, and applied to a column (3 cm $\times$ 10 cm) of troponin C-Sepharose equilibrated with the same buffer. The column was eluted with 50 mM- NH_4HCO_3 , followed by 9 M-urea/50 mM-Tris/50 mM-HCl/15 mM-2-mercaptoethanol; 5 ml fractions were collected. $\cdots$, E_{280} ; —, E_{215} ; ---, radioactivity.

As for troponin I (Perry *et al.*, 1972), troponin C neutralized the inhibitory activity of peptides CF1 and CN4 in the absence of tropomyosin, in the presence of either endogenous Ca^{2+} or 1.0 mM-EGTA. Under these conditions approximately one molecule of troponin C was required to neutralize the activity of one molecule of peptide CN4. In this respect troponin C was slightly less effective in neutralizing the activity of peptide CN4 than that of troponin I. Extrapolation of the linear portion of the graph in Fig. 8, representing neutralization of the inhibitory activity, suggested that neutralization occurred with less than 1 mol of troponin C per mol of troponin I (cf. Perry *et al.*, 1972). Usually the neutralization of inhibitory activity of the peptides in the presence of tropomyosin was independent of Ca^{2+} concentration. Occasionally, as has been reported for troponin I (Perry *et al.*, 1972), EGTA did have a slight effect. Under these circumstances the Ca^{2+} sensitivity was about 15–25% of that obtained with the complete

troponin system, and the effect was observed with both peptide CN4 and troponin I.

Inhibitory activity of other proteins on the Mg^{2+} -stimulated ATPase of desensitized actomyosin. In view of the strongly basic nature of the inhibitory peptide from troponin I a number of basic proteins (salmine, lysozyme, ribonuclease and cytochrome *c*) were tested for inhibitory activity on the actomyosin ATPase. Three of the four proteins tested had inhibitory activities which were in the order salmine > lysozyme > cytochrome *c* which were in the same order as their isoelectric points (pI 12.1, 11.1 and 9.8 respectively). Ribonuclease, with an isoelectric point of 9.1, had no inhibitory activity. The isoelectric point of troponin I is 9.3 (Wilkinson, 1974a).

The effects obtained with salmine and lysozyme are illustrated in Fig. 9, which shows that tropomyosin has no potentiating effect. On the contrary, some neutralization of inhibition was obtained with these proteins on the addition of tropomyosin. The most

active of the proteins, salmine, was, on a molar basis, about one-fifth as effective as troponin I in the presence of tropomyosin (Fig. 9). In view of these facts

and the observation that in no case was the inhibition obtained by these basic proteins neutralized by troponin C it was considered that the effects were non-specific. The results do suggest, however, that by interacting with actin the basic proteins prevent the interaction with myosin required for a high rate of $MgATP^{2-}$ hydrolysis.

Fig. 6. Inhibitory activity of CNBr digest of troponin I from rabbit white skeletal muscle on the Mg^{2+} -stimulated ATPase of desensitized actomyosin

Incubation conditions: 450 μ g of desensitized actomyosin; 2.5mM- $MgCl_2$; 2.5mM-ATP; 1.0mM-EGTA; 25mM-Tris/16mM-HCl, (pH7.6). ○, CNBr digest of troponin I; ●, CNBr digest of troponin I+tropomyosin (150 μ g); □, troponin I; ■, troponin I+tropomyosin (150 μ g).

Fig. 7. Comparison of inhibitory activities of peptides CF1 and CN4 on the Mg^{2+} -stimulated ATPase of desensitized actomyosin

Conditions were as for Fig. 6. See Fig. 1 for details of peptides CF1 and CN4. ○, Peptide CF1; ●, peptide CF1+tropomyosin (150 μ g); □, peptide CN4; ■, peptide CN4+tropomyosin (150 μ g); Δ, troponin I; ▲, troponin I+tropomyosin (150 μ g).

Table 2. Inhibitory activity of peptide fragments of troponin I on the Mg^{2+} -stimulated ATPase of desensitized actomyosin
The apparent inhibitor constant is the amount of actomyosin required to produce 50% of the maximum inhibition of the ATPase obtained with an excess of peptide or protein. The conditions of the ATPase assays were as in the Materials and Methods section, except that tropomyosin (150 μ g) was present.

Peptide	Position in molecule (residue numbers)	Amount tested (nmol/mg of actomyosin)	Maximum inhibition obtained (%)	Apparent inhibitor constant (nmol/mg of actomyosin)
CN5	1-21	58	22	—
CF2	1-50	45	0	—
CN1	22-57	64	0	—
TF1	37-100	35	0	—
CF1	64-133	12	90	6.4
CN4	96-117	5	90	2.3
TF6	104-112	45	0	—
TF3	117-138	33	0	—
CN2	136-168	42	0	—
CF3	134-179	38	0	—
Troponin I		1.5	90	1.2

Fig. 8. Effect of troponin C on the inhibitory activity of troponin I and active peptides derived from it on the Mg^{2+} -stimulated ATPase of desensitized actomyosin

Conditions were as for Fig. 6, with 150 μg of tropomyosin present per incubation. ●, Troponin I (23 μg ; 1.1 nmol); ▲, peptide CF1 (28.6 μg ; 3.5 nmol); ■, peptide CN4 (3.3 μg ; 1.2 nmol).

Interaction studies

G-actin-Sepharose columns. When troponin I (0.5–1.0 mg/ml) dissolved in 75 mM-Tris/48 mM-HCl (pH 7.6)/15 mM-2-mercaptoethanol was applied to a column (8 cm $\times$ 2 cm) of G-actin-Sepharose containing approx. 0.5 μ mol of actin, approx. 0.1 μ mol of troponin I was bound. On application of a gradient of 0–0.5 M-KCl to the column, bound troponin I was eluted as a broad peak with a maximum at 0.3 M-KCl/75 mM-Tris/48 mM-HCl (pH 7.6)/15 mM-2-mercaptoethanol. The eluted material was shown to be troponin I by electrophoresis on sodium dodecyl sulphate, pH 7.0, and complex-formation with troponin C in 6 M-urea, pH 8.6.

On application of the CNBr digest of troponin I in 75 mM- NH_4HCO_3 , pH 7.9, to a G-actin-Sepharose column equilibrated against the same buffer, only peptide CN4 was bound to the column after washing with buffer. Peptide CN4 could be eluted by application of 0.5 M- NH_4HCO_3 , pH 7.9. After removal of NH_4HCO_3 in the eluted fraction by repeated rotary evaporation, peptide CN4 was identified by poly-

Fig. 9. Effect of troponin I and basic proteins on the Mg^{2+} -stimulated ATPase of desensitized actomyosin

Standard conditions for ATPase assay (see the Materials and Methods section) with 740 μg of desensitized actomyosin were used. ----, Inhibition obtained with troponin I from rabbit white muscle (mol. wt. 21 000) in the presence of 150 μg of tropomyosin. ●, Lysozyme (mol. wt. 14 300); ○, lysozyme+150 μg of tropomyosin; ▲, salmine (mol. wt. 4200); Δ, salmine+150 μg of tropomyosin.

acrylamide-gel electrophoresis in 6 M-urea/5% (v/v) acetic acid, pH 3.2. It co-electrophoresed with peptide CN4 isolated directly by conventional procedures (Wilkinson, 1974b) and possessed the expected amino acid analysis. This peptide inhibited the Mg^{2+} -stimulated ATPase activity of desensitized actomyosin (see under 'Enzymic studies'); this inhibition was much increased by tropomyosin. The molar yield of peptide from the G-actin-Sepharose column appeared to be less than that expected from the binding capacity for troponin I (see above). Peptides CN4 and CF1' were bound by a G-actin-Sepharose column when applied singly in 50 mM- NH_4HCO_3 , pH 7.8. They were subsequently eluted by 0.6 M-NaCl.

Interaction with troponin C. Of the peptides obtained by cyanilation, only peptide CF2 was bound by a troponin C-Sepharose column when applied in 50 mM- NH_4HCO_3 , pH 7.8. The interaction was strong, as the peptide was not eluted with 1 M-NaCl or 10 mM-HCl, pH 2.3, but with 9 M-urea.

When the whole CNBr digest of unphosphorylated troponin I was applied to a troponin C-Sepharose

EXPLANATION OF PLATE I

Electrophoresis of peptides CN5 and CF2 from troponin I in the presence and absence of troponin C

Conditions: 8% (w/v) polyacrylamide gel; 20 mM-Tris/124 mM-glycine, pH 8.6. Complex indicated by an arrow in each case. O, Origin. (a) Peptide CN5 (4 μ g); (b) peptide CN5 (4 μ g)+troponin C (10 μ g); (c) troponin C (10 μ g); (d) peptide CF2 (4 μ g)+troponin C (10 μ g); (e) peptide CF2 (4 μ g).

column in 50mM-NH₄HCO₃, peptide material was selectively bound and eluted with 1M-NaCl. Gel electrophoresis at pH3.2 showed that the bound material was a mixture of peptides CN4 and CN5, and that the material eluted in the void volume of the column was lacking in these components. When the bound peptides were eluted with either a NaCl gradient or a series of steps of increasing NaCl concentration, peptide CN4 was bound more tightly to the column than was peptide CN5 (A. J. G. Moir, personal communication).

The interaction of the fragments with troponin C was studied by gel electrophoresis in 20mM-Tris/124mM-glycine, pH8.6. Each fragment was run with and without troponin C and the presence of an extra band on the addition of troponin C was taken as an indication of interaction. The formation of extra bands was observed with peptides CN5 and CF1 as shown in Plate 1. Some interaction was also observed with peptide CN4, although CN4 itself does not run into the alkaline gel. The two bands observed with peptide CN5 alone may be due to the presence of forms containing homoserine and homoserine lactone. The multiple band pattern of peptide CF2 is more difficult to explain, but may be due to deamidation (or phosphorylation). None of the interactions observed could be disrupted by the presence of EGTA in the gels or samples.

Discussion

The combined evidence obtained from enzymic, electrophoretic and affinity-chromatographic studies strongly suggests that two regions of the primary sequence of troponin I from rabbit skeletal muscle, namely the *N*-terminal peptide CF2 (residues 1-47) and the peptide CN4 (residues 96-117) are involved in interactions with troponin C and actin. The amino acid sequences of these regions are shown in Fig. 10.

The inhibitory activity obtained with peptides CF1 and CN4 is qualitatively identical with that obtained with troponin I itself in that the effect is much poten-

tiated by tropomyosin and neutralized by troponin C. In this way it is distinguished from the non-specific inhibitory activity of proteins such as salmine, lysozyme and cytochrome *c*, which is probably due to their net basic character and may result from binding to those regions on the actin molecule that normally interact with troponin I or the active peptides derived from it. This explanation may account for the activity of polylysine and protamine in inhibiting the interaction of actin and myosin that has been reported by Barany & Barany (1960).

The fact that the large peptide CF1 is less active on a molar basis than peptide CN4, although it contains the sequence of the latter peptide, requires some comment. A possible explanation of the result is that peptide CF1 adopts a conformation different from that which it has in the intact molecule and which renders the inhibitory region (residues 96-117) less effective. It is difficult to define precisely the extent of the active site, but the two regions of the primary sequence on either side of peptide CN4, namely those corresponding to peptide TF1 (residues 37-100) and peptide TF3 (residues 117-138) (Fig. 1), are completely without activity. From these observations and the fact that the small peptide TF6 (residues 104-112), which comes from the centre of peptide CN4, is also without activity, it seems likely that peptide CN4 corresponds fairly closely to the optimum length of polypeptide required for activity.

The specific inhibition of actomyosin ATPase implies that troponin I and the active fragments derived from it prevent the interaction of actin with myosin, which effects the stimulation of the rate of hydrolysis of MgATP²⁻. The evidence from the enzymic and affinity-chromatographic studies implies that this occurs by interaction with actin, as does the observation that troponin I will precipitate actin from solution at low ionic strength (Wilkinson *et al.*, 1972). The experiments with G-actin-Sepharose columns clearly indicate that the region containing residues 96-117 has a special affinity for actin, which is not simply of an electrostatic nature caused by the high

Fig. 10. Amino acid sequences of peptides CF2 and CN4 (from Wilkinson & Grand, 1975b)

The major site phosphorylated by phosphorylase kinase (Moir *et al.*, 1974; Huang *et al.*, 1974) and the minor site phosphorylated by skeletal-muscle 3':5'-cyclic AMP-dependent protein kinase (Huang *et al.*, 1974) are indicated by a bold and normal P respectively.

Peptide CF2

¹ AcGly-Asp-Glu-Glu-Lys-Arg-Asn-Arg-Ala-Ile-Thr-Ala-Arg-Arg-Gln-His-Leu-Lys-Ser-Val-Met-Leu-Gln-Ile-Ala-⁴⁷
³⁰ Ala-Thr-Glu-Leu-Glu-Lys-Glu-Glu-Gly-Arg-Arg-Glu-Ala-Glu-Lys-Gln-Asn-Tyr-Leu-Ala-Glu-His⁴⁷

Peptide CN4

⁹⁶ Asn-Gln-Lys-Leu-Phe-Asp-Leu-Arg-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-Hse¹¹⁷

basic amino acid content of peptide CN4. Peptide CN4 was the only peptide from the CNBr digest of troponin I that was retained on the column in 50 mM-NH₄HCO₃, pH 7.9, whereas the *N*-terminal peptide CN5, with a similar content of basic amino acids to that of peptide CN4, was not retained under these conditions.

In contrast with the results with actin, where evidence of two types indicates one specific site of interaction on the troponin I molecule, interaction with troponin C appears to involve two sites. The electrophoretic and affinity-chromatographic studies indicate that troponin C can interact with peptides from the *N*-terminal and inhibitory peptide regions of the troponin I molecule. These findings confirm similar conclusions made as a result of enzymic studies which indicated that phosphorylation of troponin I from rabbit skeletal muscle by phosphorylase kinase, which occurs principally at threonine-11, and phosphorylation by 3':5'-cyclic AMP-dependent protein kinase, which is largely confined to serine-118 (Moir *et al.*, 1974), are both inhibited by troponin C (Cole & Perry, 1975). Judged by the conditions required for elution from a troponin C-Sepharose column, peptide CN4 is more strongly bound to troponin C than is the *N*-terminal CNBr-cleaved peptide CN5. Nevertheless, the larger *N*-terminal peptide CF2 is more strongly bound to the troponin C-Sepharose column than is peptide CN5, indicating that residues in troponin I in addition to those corresponding to residues 1-21 may also be involved in the interaction with troponin C. As troponin C is a strongly negatively charged protein, the possibility of purely electrostatic effects determining relative binding properties must always be borne in mind in the interpretation of these experiments, especially those involving peptides with a net positive charge.

Despite these reservations, the observations must be of significance for the role of troponin C, which on interaction with troponin I neutralizes its inhibitory activity. This latter function is not Ca²⁺-dependent in isolated systems, but becomes so when tropomyosin and the whole troponin complex is present (Perry *et al.*, 1972). This implies that interaction of troponin C either directly or indirectly changes the ability of troponin I to interact with actin.

Two possible mechanisms suggest themselves for the manner in which troponin C neutralizes the inhibitory activity of troponin I (Perry *et al.*, 1975). In the first scheme troponin C interacts with troponin I in the *N*-terminal region in a way that does not physically block the region of the inhibitory peptide. By a conformational change, induced as a result of the interaction, the conformation of the inhibitory peptide region becomes modified so that it can no longer interact with actin, nor can the adjacent serine residue at position 118 be phosphorylated by 3':5'-cyclic AMP-dependent protein kinase. The alterna-

tive scheme does not rely on a conformational change to render the inhibitory site ineffective. In this case the folding of the polypeptide chain in troponin I is such that the *N*-terminal region and that of the inhibitory peptide are adjacent on the surface of the protein. Thus interaction of troponin I with troponin C would effectively render the inhibitory peptide region unavailable for phosphorylation or interaction with actin. At the moment it is not possible to decide which of these mechanisms is the correct one, but there is some evidence in favour of the second, in that the interaction of troponin C with the inhibitory peptide region is not readily explained by the first scheme.

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