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The Primary Structure of Troponin T and the Interaction with Tropomyosin

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1. Eight peptides were separated from the CNBr digest of troponin T from rabbit white skeletal muscle and characterized. 2. By study of the amino acid sequences of the methionine-containing peptides isolated after chymotryptic and tryptic digestion and of the *N*- and *C*-terminals of the CNBr peptides, six of the latter were shown to be arranged in the sequence CNB1-CNB2-CNB5-CNB6-CNB8-CNB7. The other two peptides, CNB1′ and CNB3, have been shown to be partial digestion products. 3. The CNBr peptides CNB1′ and CNB2 contained a common sequence and were the only peptides in CNBr digests of troponin T that formed a complex with tropomyosin as judged by viscometric and electrophoretic studies. 4. It is concluded that tropomyosin interacts with the *N*-terminal half of the troponin T molecule approximately in the region lying between residues 70 and 160. 5. Electrophoretic evidence indicates that tropomyosin and troponin C interact with troponin T. 6. None of the major CNBr peptides of troponin T isolated formed a complex with troponin C on electrophoresis at pH 8.6.

It is now well established that the system in vertebrate striated muscle by means of which contractile activity is regulated by Ca^{2+} consists of tropomyosin and the troponin complex. This complex consists of three well-defined proteins, two of which have been intensively studied and the amino acid sequences determined (Collins *et al.*, 1973; Collins, 1974; Wilkinson & Grand, 1975). Troponin C possesses a high affinity for Ca^{2+} and neutralizes the inhibitory action of troponin I on the Mg^{2+}-stimulated adenosine triphosphatase of actomyosin. The third component, troponin T (Potter & Gergely, 1972), has in the past been named variously by investigators, for example, '37000 component' (Wilkinson *et al.*, 1971, 1972), 'Troponin 3' (Greaser & Gergely, 1971) and 'troponin I' (Ebashi *et al.*, 1971). Its function is less well understood but it interacts with troponin C (Ebashi *et al.*, 1972; van Eerd & Kawasaki, 1973) and tropomyosin (Greaser *et al.*, 1972). The interaction is probably responsible for the marked increase in viscosity (Ebashi & Kodama, 1965) that occurs when solutions of tropomyosin and troponin are mixed.

Investigation of the effect of isolated troponin T on the viscosity of tropomyosin solutions is made difficult by the precipitation of the troponin T–tropomyosin complex. It is clear, however, that an enhancement of viscosity does occur when the two proteins are mixed (Yamamoto & Maruyama, 1973). Thus troponin T may have a role in linking the troponin complex with tropomyosin and hence extending Ca^{2+} regulation to the actin monomers that are not directly associated with the periodically localized troponin complex. Although there is evidence that troponin and troponin T interact with defined parts of the tropomyosin molecule (Greaser *et al.*, 1972; Margossian & Cohen, 1973; Yamaguchi *et al.*, 1974) there is no information as to the region of the troponin T molecule involved.

In the present work the interaction of tropomyosin with troponin T is studied and shown to be localized approximately one-third of the way from the *N*-terminal end of the troponin T polypeptide chain.

Methods

Preparation of muscle proteins

Tropomyosin was prepared from mixed skeletal muscle of New Zealand white rabbits as described by Cummins & Perry (1973) by a method based on the procedure of Bailey (1948, 1951). Alternate cycles of isoelectric precipitation at pH 4.6 by the addition of 1M-HCl and precipitation (usually three times) between 53 and 60% satd. $(NH_4)_2SO_4$ with the pH maintained at 7.6 by the addition of 2M-Tris was carried out on the tropomyosin solution (1mg/ml) until no bands other than those of the α and β components of tropomyosin (Cummins & Perry, 1973) could be detected on electrophoresis of 50μg on polyacrylamide gel in 0.1% sodium dodecyl sulphate, 6M-urea–82.5mM-Tris–400mM-sodium borate, pH 7.0 (Sender, 1971). Troponin complex was prepared from mixed skeletal muscle of New Zealand white rabbit as described by Ebashi *et al.* (1971). The absence of tropomyosin from these preparations was demonstrated by electrophoresis on polyacrylamide gel in

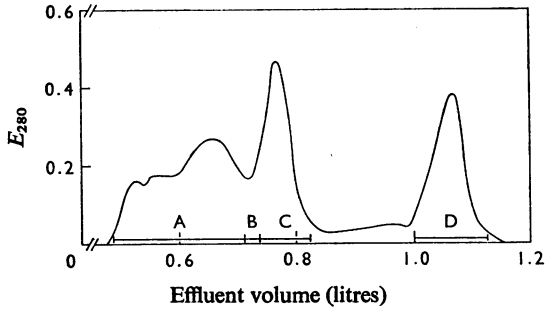


Fig. 1. *Gel filtration of the CNBr digest of troponin T on Sephadex G-50*

The CNBr digest from 250mg of troponin T dissolved in 6ml of 10% (v/v) formic acid was applied to a Sephadex G-50 column equilibrated with 10% (v/v) formic acid (fractionation system 1, see the Methods section) and 9ml fractions were collected. Fraction 1E is not shown; it consisted of all the fractions after 1D, effluent volume 1.13–1.8 litre, i.e. in excess of the column volume. Fraction 1E showed no absorbance at 280nm.

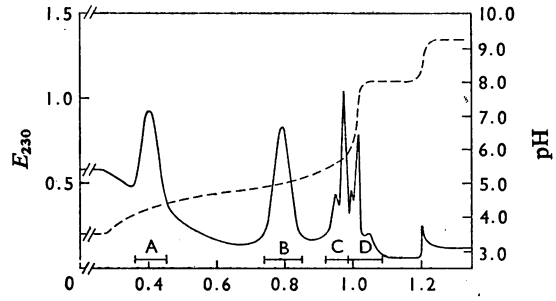


Fig. 2. *Chromatography of fraction 1A from gel filtration on CM-cellulose*

Fraction 1A (120mg) dissolved in 8.0ml of 0.2M-ammonium acetate buffer, pH3.5, was applied to a CM-cellulose column equilibrated against the same buffer. Elution was carried out as for fractionation system 2 (see the Methods section) and 5.0ml fractions were collected. ——, E_{230}; ----, pH.

0.1% sodium dodecyl sulphate, 6M-urea, 82.5mM-Tris–400mM-sodium borate (pH7.0). Unless otherwise stated, preparations of troponin T and troponin I were prepared from troponin B as described by Wilkinson (1974). On some occasions, troponin was fractionated into its components by chromatography on DEAE-cellulose as described by Perry & Cole (1974). Before use the purity of the isolated components was checked; for troponin C it was by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate at pH7.0 and in 6M-urea at pH8.6, and for troponin I and troponin T, sodium dodecyl sulphate at pH7.0 and 6M-urea at pH3.2 were used. Samples were acceptable only if they gave a single band when 50μg was electrophoresed in sodium dodecyl sulphate and in urea.

Digestion of troponin T with CNBr

Rabbit troponin T (250–300mg) was dissolved in 10ml of 70% (v/v) formic acid solution, and CNBr (approx. 500mg, i.e. about 100-fold excess over methionine residues) was added and dissolved with shaking. The mixture was left for 24h at room temperature, a similar amount of CNBr was then added and the solution left for a further 24h at room temperature. After the addition of 100ml of water the solution was freeze-dried.

Separation of the CNBr peptides

Unless otherwise stated elution of peptides was followed by measuring absorbance at 230nm.

Fractionation system 1. Partial separation of the CNBr peptides was achieved by gel filtration on a column (3.0cm×195cm) of Sephadex G-50 and elution with aq. formic acid solution (10%, v/v). Fractions were collected and the elution of peptides was followed by measuring E_{280}.

The series of unresolved peaks of the most rapidly eluted peptides and the slowest moving peak were divided into four fractions, 1A, 1B, 1C and 1D (Fig. 1), and freeze-dried.

Fractionation system 2. The peptides of fraction 1A obtained as described above were further purified on a column (1.8cm×40cm) of CM-cellulose (Whatman CM-52) equilibrated with ammonium acetate buffer, pH3.5. All ammonium acetate buffers were made by adding approx. 5M-NH_3 to approx. 0.25M-acetic acid to bring it to the required pH and diluting to 0.2M. A pH gradient was applied to the column by running 500ml of 0.2M-ammonium acetate buffer, pH8.2, into a mixing chamber containing 500ml of 0.2M-ammonium acetate buffer, pH3.5. The gradient-making apparatus was such that there was a linear increase in the proportion of the buffer at the higher pH value, in the outflow from the mixing chamber. When the gradient was finished, elution was continued with the pH8.2 buffer alone (approx. 200ml) and then with 0.2M-ammonium acetate buffer, pH9.8 (approx. 100ml). Fractions 2A, 2B, 2C and 2D were collected as indicated in Fig. 2, and freeze-dried. Any remaining ammonium acetate was removed by dissolving in water and freeze-drying again.

Fractionation system 3. Fraction 2D was chromatographed on a column (1.2cm×40cm) of CM-cellulose with an ammonium acetate gradient system as for

fractionation system 2, modified as follows. Starting buffers in the mixing chambers were 0.2M-ammonium acetate, pH4.9 (300ml), and 0.2M-ammonium acetate, pH8.2 (300ml). After the gradient, elution was completed with the pH8.2 buffer (100ml). Fractions 3A, 3B and 3C (Fig. 3) were freeze-dried and any remaining ammonium acetate was removed as in fractionation system 2.

Fractionation system 4. The peptides of fraction 1D, obtained as described above, were further purified on a column (1.4cm×35cm) of CM-cellulose (Whatman CM 52), equilibrated with 0.025M-sodium phosphate buffer, pH7.48; 0.5M-sodium phosphate buffer, pH7.3, was made by adjusting the pH of 0.6M-sodium dihydrogen orthophosphate to pH7.3 with 10M-NaOH and diluting to a final concentration of 0.5M.

A pH gradient was applied to the column by running 250ml of 0.35M-sodium phosphate buffer, pH7.3 (made by diluting the 0.5M-sodium phosphate buffer, pH7.3, described above), into a mixing chamber containing 250ml of 0.025M-sodium phosphate buffer, pH7.48 (made by diluting the 0.5M-sodium phosphate buffer, pH7.3, described above), and designed to give a linear gradient.

Elution of peptides was followed by measuring the E_{215}, and fractions 4A and 4B (Fig. 4) were collected. The fractions containing peptides were pooled, freeze-dried and salt was removed by gel-filtration chromatography on Sephadex G-15.

Fractionation system 5. Fraction 1E was chromatographed on a column (2.0cm×110cm) of Sephadex G-25 with 0.005M-HCl as eluent, and the elution of peptides was followed by measuring the E_{215}. The large rapidly eluted peak was dried by rotary evaporation at 40°C and subjected to high-voltage paper electrophoresis at pH6.5, pH2.0 and 3.5, after which pure peptide CNB8 was eluted from the paper by descending chromatography in 5% (v/v) acetic acid. Peptides were detected by the ninhydrin–cadmium stain of Barrolier as described by Smith (1969).

Enzymic digestion of the purified CNBr peptides

Peptide CNB1 was digested with thermolysin by dissolving 8mg of peptide CNB1 in 2.0ml of 0.1M-NH_4HCO_3 and adding 0.08mg of thermolysin (1mg/ml; Sigma protease X, Sigma Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.) in 0.1M-ammonium acetate–2mM-$CaCl_2$. The mixture was incubated for 7h at 37°C and then freeze-dried.

Peptides CNB1′, CNB2, CNB5 and CNB6 were digested with trypsin (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated trypsin; Cambrian Chemicals, Croydon CR0 4XB, Surrey, U.K.) by dissolving 1–2μmol of each in 2.0ml of 1% (w/v) NH_4HCO_3 and adding 1% of the peptide weight of trypsin in an aqueous solution (1mg/ml). The mixtures were incubated for 5h at 37°C and then freeze-dried.

Isolation of the C-terminal peptides of the purified CNBr peptides

The proteolytic digests of all the CNBr peptides (except peptides CNB7 and CNB8) obtained as described above were subjected to high-voltage paper electrophoresis at pH6.5, 2.0 and 3.5, and peptide bands were stained with ninhydrin. After the pH3.5 step the peptides were eluted by descending chromatography in either 5% (v/v) acetic acid or 20mM-NH_3 and subjected to amino acid analysis. Peptides containing homoserine were taken to be *C*-terminal.

Preparation and purification of radioactively labelled methionine-containing peptides from troponin T

Troponin T (50mg) was labelled with iodo[2-^{14}C]-acetic acid (51mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) by the method of Wilkinson (1969). The labelled protein was exhaustively dialysed against water, freeze-dried and suspended in 1% (w/v) NH_4HCO_3 (8ml). To this suspension was added 2% (by weight) of trypsin (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated trypsin; Cambrian Chemicals) and 2% (by weight) chymotrypsin (Sigma Chemical Co.) The mixture was incubated for 6h at 37°C, freeze-dried and samples were dissolved and subjected to high-voltage paper electrophoresis at pH6.5.

The positions of the radioactive peptides on the paper were determined by radioautography by using Kodak Blue Brand medical X-ray film, BB54, with an 18–20h exposure time. The labelled peptides were cut out and subjected to high-voltage electrophoresis at pH2.0 and finally at pH3.5, after which they were eluted from the paper by descending chromatography with 5% (v/v) acetic acid or 20mM-NH_3. Where necessary purification was completed by gel-filtration chromatography in 5.0mM-HCl on Sephadex G-10 or G-15 columns.

Sequence determination

Amino acid residue sequences in the peptides obtained were determined by the dansyl–Edman technique (Hartley, 1970).

Amino acid analysis

Peptides were digested in 6M-HCl containing 10μl/ml of 0.1M-phenol solution for the protection of tyrosine residues (Sanger & Thompson, 1963) at 110°C for 20h. The HCl was then removed by rotary evaporation and the peptides were heated on a boiling-water bath for 1h in pyridine–acetic acid–water

(100:4:900, by vol.), pH6.5, to convert all homoserine lactone into homoserine (Ambler, 1965). Analysis was carried out on a Beckman 120B amino acid analyser. *N*-Terminal amino acid analysis was carried out by the dansyl chloride method (Hartley, 1970). Tryptophan was assayed by the method of Beavan & Holiday (1952) as modified by Wetlaufer (1962).

Viscometry

Viscosities were determined on 4ml samples in an Ostwald-type viscometer at 4°C with a flow-time for distilled water at this temperature of 35.4s. All protein solutions were dialysed overnight against the appropriate buffer before use.

Protein determination

Protein concentrations were determined by a micro method involving nesslerization after digestion (Strauch, 1965) assuming an N content of 16%.

Polyacrylamide-gel electrophoresis

Electrophoresis was carried out in 0.1% sodium dodecyl sulphate either in 100mM-sodium phosphate buffer (pH7.0) or 82.5mM-Tris–400mM-boric acid, pH7.0, as described by Perrie *et al.* (1973).

Electrophoresis was also carried out in 6M-urea, 5% acetic acid, pH3.2, as described by Panyim & Chalkley (1969) and in 25mM-Tris–80mM-glycine, pH8.6, as described by Perrie & Perry (1970) both in the presence and absence of 6M-urea. Gels run at pH8.6 and not containing sodium dodecyl sulphate were stained in Coomassie Brilliant Blue R (George T. Gurr, Division of Baird and Tatlock, Romford RM1 1HA, Essex, U.K.), 2.8g/litre in a 50% (w/v) solution of trichloroacetic acid. Sodium dodecyl sulphate-containing gels and those run in 6M-urea at pH3.2 were stained in Coomassie Blue, 2.8g/litre in methanol–acetic acid–water (5:1:14, by vol.). Excess of stain was washed from the gel in methanol–acetic acid–water (5:1:14, by vol.).

Affinity chromatography

The procedure of Porath *et al.* (1967) was used to couple tropomyosin to Sepharose 4B. Sepharose 4B (15g wet wt.) was washed and suspended in 15ml of deionized water. The pH of the suspension was adjusted to 11–11.5 with 6M-NaOH and the suspension was stirred slowly. Powdered CNBr (1g) was then added, the pH again adjusted to 11–11.5 with 6M-NaOH and the temperature kept below 25°C by the addition of chipped ice. After 15min the Sepharose was washed with a large volume of cold deionized water, then with cold 1M-KCl–0.1M-$NaHCO_3$ and finally suspended in 15ml of this solution. Tropomyosin (15mg) was dissolved in 2ml of 1M-KCl–0.1M-$NaHCO_3$ and added to the Sepharose suspension, which was then stirred gently overnight at 4°C. Remaining reactive groups on the Sepharose were blocked by suspending the gel for 2h in ethanolamine solution obtained by adjusting approx. 2M-ethanolamine to pH8.0 with 1M-HCl and diluting to 1M. Finally, uncoupled protein was removed by alternate washes with 1M-NaCl–18mM-sodium acetate–82mM-acetic acid, pH4.0, and 1M-NaCl–11.25mM-sodium borate–55mM-boric acid, pH8.5. The extent of coupling was estimated by comparing the total extinction at 280nm of the washes with the extinction of the applied protein solution. Usually about 80% of the tropomyosin added was coupled to the Sepharose. The method of March *et al.* (1974) for coupling protein to Sepharose was also used with similar results.

High-voltage paper electrophoresis

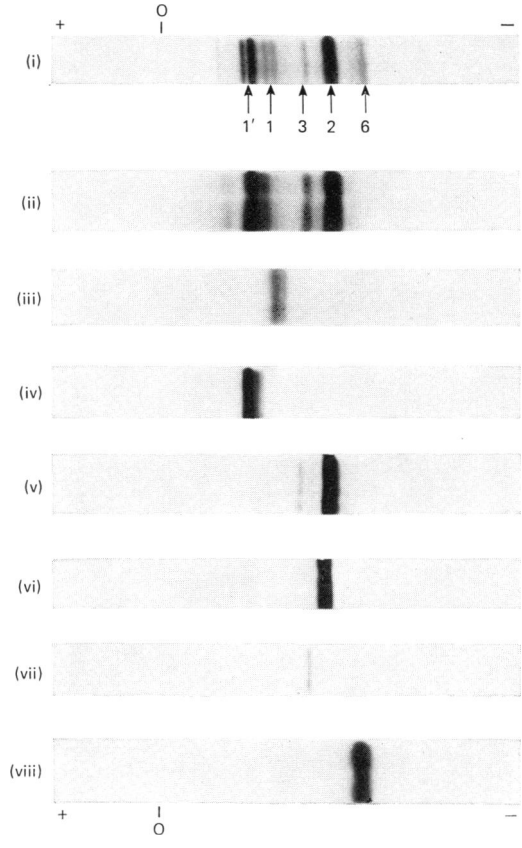
High-voltage paper electrophoresis of peptides obtained by enzymic digestion of either the CNBr peptides or of the iodo[2-^{14}C]acetic acid-labelled troponin T was carried out on Whatman 3MM chromatography paper (Fisons Scientific Co., Bishops Meadow Road, Loughborough, Leics., U.K.) with a potential gradient of 50V/cm at pH6.5, 3.5 and 2.0. The buffer solutions were constituted as follows: pH6.5, pyridine–acetic acid–water (100:4:900, by vol.); pH3.5, pyridine–acetic acid–water (10:100:900, by vol.); pH2.0, formic acid–acetic acid–water (20:80:900, by vol.).

Results

Primary structural studies on Troponin T

Purification of the CNBr peptides. Polyacrylamide-gel electrophoresis in 6M-urea at pH3.2 showed (Plate 1, gel ii) that fraction 1A from the Sephadex G-50 column (fractionation system 1, Fig. 1) contained, except for one peptide (CNB6) which was present in very small amounts, all of the most densely staining bands observed on electrophoresis of the CNBr digest of troponin T. Fraction 1B contained all of the most densely staining peptides present in the digest but little peptide CNB1′ and was not further investigated, whereas fraction 1C contained only one peptide, CNB6. Chromatography of fraction 1A on CM-cellulose by fractionation system 2 (see the Methods section) yielded three more pure peptides, namely CNB1 (fraction 2A), CNB1′ (fraction 2B) and CNB2 (fraction 2C). Fraction 2D contained peptide CNB2 and two additional peptides, CNB3 and a little CNB6.

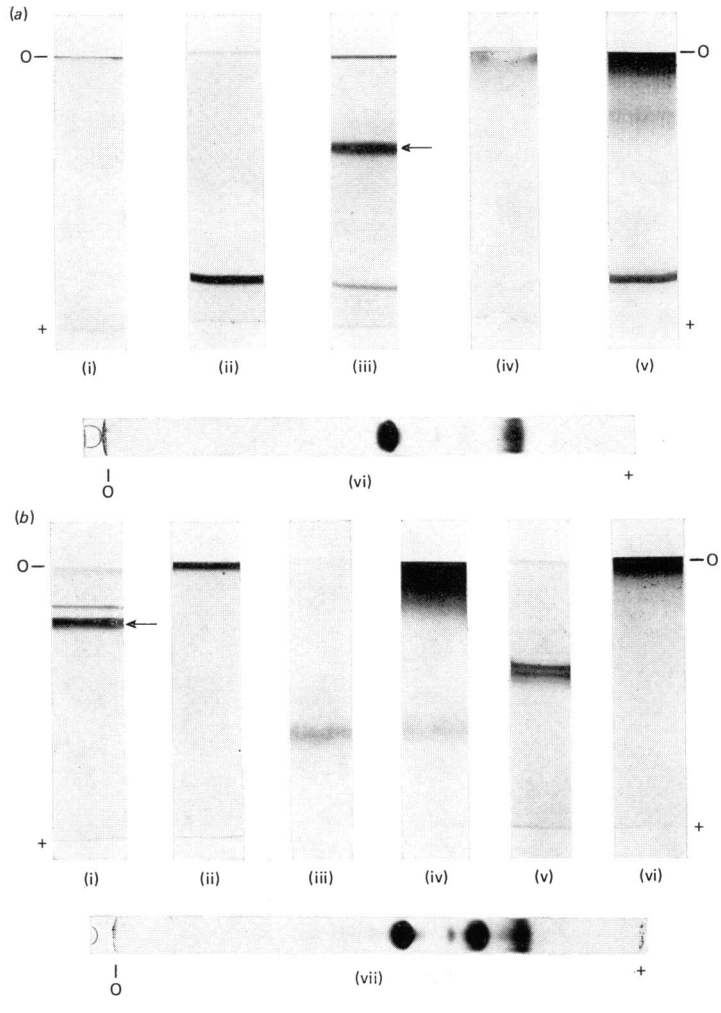
Chromatography of fraction 2D on CM-cellulose by fractionation system 3 (see the Methods section)



EXPLANATION OF PLATE 1

Electrophoresis of CNBr digest of troponin T and isolated peptides

Electrophoresis carried out on 15% (w/v) polyacrylamide gel, 6M-urea, 5% (v/v) acetic acid, pH 3.2. Troponin T was isolated from rabbit mixed skeletal muscle. O, Origin. Gels: (i) 40 μg of whole CNBr digest. Different CNBr peptides are indicated by numbers; (ii) 30 μg of fraction 1A; (iii) 20 μg of peptide CNB1 (fraction 2A) ; (iv) 20 μg of peptide CNB1′ (fraction 2B); (v) 20 μg of peptide CNB2 (fraction 2C); (vi) 15 μg of peptide CNB2 (fraction 3A); (vii) 5 μg of peptide CNB3 (fraction 3C); (viii) 20 μg of peptide CNB6 (fraction 1C).



EXPLANATION OF PLATE 2

Electrophoresis of tropomyosin, troponin components and CNBr peptides of troponin T

Electrophoresis was carried out in all cases except (*a*) gel (vi) and (*b*) gel (vii) on 8% (w/v) polyacrylamide gel, 0.11% bisacrylamide, 25mM-Tris–80mM-glycine, pH 8.6. For (*a*) gel (vi) and (*b*) gel (vii), electrophoresis was carried out in 10% (w/v) polyacrylamide gel, 0.12% bisacrylamide, 100mM-sodium phosphate buffer, pH 7.0, 0.1% sodium dodecyl sulphate. All proteins were isolated from rabbit mixed skeletal muscle. (*a*) Gels: (i) 15μg of troponin T; (ii) 10μg of troponin C; (iii) 15μg of troponin T+10μg of troponin C (complex marked with arrow); (iv) 25μg of tropomyosin; (v) 25μg of tropomyosin, 15μg of troponin T and 10μg of troponin C; (vi) troponin T–troponin C complex eluted from (*a*) gel (iii). (*b*) gel (i) 15μg of troponin; (ii) 25μg of tropomyosin+15μg of troponin; (iii) 10μg of peptide CNB1; (iv) 10μg of CNB1+80μg of tropomyosin; (v) 10μg of peptide CNB1′; (vi) 10μg of peptide CNB1′+50μg of tropomyosin; (vii) band of troponin complex from (*b*) gel (i) (marked with arrow).

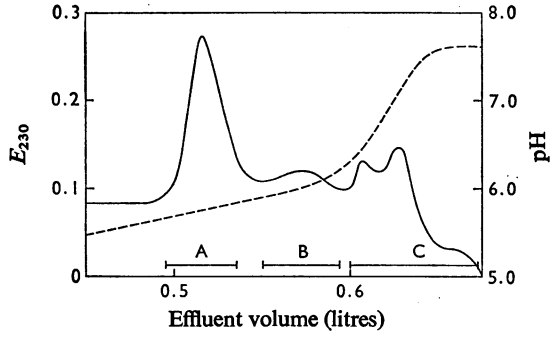


Fig. 3. *Chromatography of fraction 2D on CM-cellulose*

Fraction 2D (10mg) from fractionation system 2 was dissolved in 4.0ml of 0.20M-ammonium acetate buffer, pH4.9, and applied to a CM-cellulose column equilibrated against the same buffer. Elution was carried out as described for fractionation system 3 (see the Methods section) and 5.0ml fractions were collected. ——, E_{230}; ----, pH.

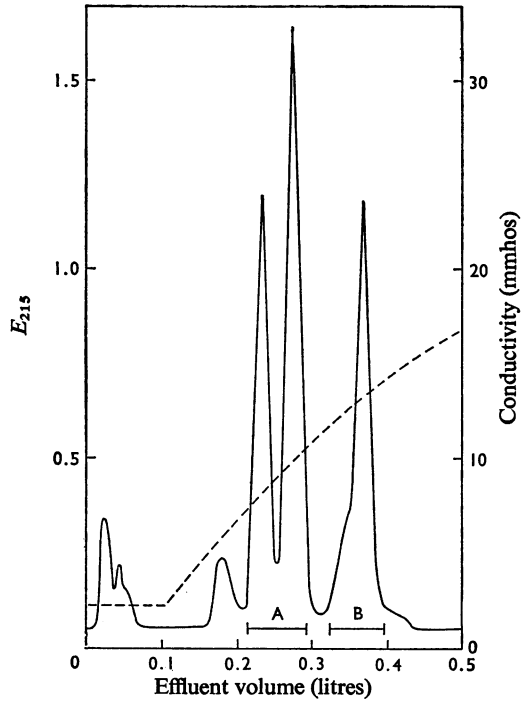


Fig. 4. *Chromatography of fraction 1D on CM-cellulose*

Fraction 1D (approx. 30mg) from fractionation system 1 dissolved in 4ml of 25mM-sodium phosphate buffer, pH7.48, was applied to a CM-cellulose column, equilibrated against the same buffer. Elution was carried out as for fractionation system 4 (see the Methods section) and 7.5ml fractions were collected. ——, E_{215}; ----, conductivity.

yielded three small peaks (Fig. 3). Fraction 3A contained peptide CNB2, fraction 3B, peptides CNB2 and CNB3 and fraction 3C, peptides CNB3 and negligible amounts of peptides CNB2 and CNB6. Peptides from fraction 1D obtained from the Sephadex G-50 column were separated with a pH gradient on CM-cellulose as described in the Methods section (fractionation system 4, Fig. 4). Fraction 4A contained peptide CNB5, and fraction 4B contained peptide CNB7. The purity of peptides CNB1, CNB1′, CNB2, CNB3 and CNB6 was checked by polyacrylamide-gel electrophoresis at pH3.2 (Plate 1, gels iii–viii). Peptides CNB5, CNB7 and CNB8 were too small to be satisfactorily examined by this technique. Dansyl *N*-terminal analysis confirmed the purity of all the CNBr peptides except for peptides CNB1 and CNB1′, which gave negative results.

The amino acid analyses of the purified CNBr peptides are shown in Table 1. The sum of the residues of each amino acid present in peptides CNB1′, CNB5, CNB6, CNB7 and CNB8 were similar to those reported for whole troponin T (Wilkinson, 1974) but corresponded to a molecular weight of about 33000. The total number of residues of each amino acid in peptides CNB1 and CNB2 was the same as or very close to the value for that amino acid present in peptide CNB1′. The presence of small quantities of methionine in peptides CNB1′ and CNB3 indicated that these peptides were products of incomplete cleavage of methionyl peptide bonds, a phenomenon reported by Piggott & Press (1967) when digesting immunoglobulins with CNBr.

All the CNBr peptides were isolated in reasonable yield except for peptide CNB3, which was present in very small amounts. From 250mg of freeze-dried troponin T the following yields were obtained: peptide CNB1, 25mg; peptide CNB1′, 22mg; peptide CNB2, 27mg; peptide CNB3, 4mg; peptides CNB5+CNB7, 27mg; peptide CNB6, 22mg.

N-terminal and C-Terminal sequences of the CNBr peptides. No *N*-terminal amino acid could be detected when the dansyl–Edman procedure was applied to peptides CNB1 and CNB1′. In all the other peptides isolated, positive *N*-terminal identifications were made, and the sequence was determined as far as was possible (Table 2).

Pronase digestion of peptides CNB1 and CNB1′ liberated in each case a peptide that was not retained on sulphonated polystyrene resin Zeo-Karb 225 (Permutit Co., 632 London Road, Isleworth, Middx., U.K.) in the acid form developed with water. The acidic peptides not retained on Zeo-Karb 225 in each case had the composition Asp, Ser, Glx_3, Val (Wilkinson, 1974) and gave negative dansyl *N*-terminal analyses. When peptide CNB2 was digested with

Table 1. *Amino acid composition of the peptides obtained from CNBr digestion of rabbit troponin T*

The values are expressed as mol/mol of peptide or troponin T. Values underlined were obtained from single samples. Homoserine was sometimes not well separated from glutamic acid in our amino acid analysis system. The values for the methionine present in small amounts in peptides CNB1′ and CNB3 have not been included in the Table. Values in parentheses are nearest whole numbers of residues. The molecular weights of the peptides were determined by assuming that each contained one homoserine residue, or, where the quantity of homoserine could not be determined accurately, by assuming an integral number of residues of leucine, isoleucine or valine that gave a homoserine value approximating to unity. For peptide CNB7 it was assumed that there was 1 mol of valine/mol of peptide. This gave a tryptophan value closest to unity, which would be expected since there are two tryptophan residues in whole troponin T (Wilkinson, 1974). Peptide CNB7 moved with CNB5 on Sephadex G-50 (Fig. 1) indicating that the two peptides were of similar size.

Peptide ...	CNB1	CNB1′	CNB2	CNB3	CNB5	CNB6	CNB7	CNB8	Total of each* amino acid	Amino acid analysis (mol/37000g) (Wilkinson, 1974)
Asp	6.4 (6)	11.3 (11)	4.3 (4)	4.7 (5)	1.9 (2)	7.5 (8)	0.0 (0)	1.0 (1)	22	24.5
Thr	1.0 (1)	1.4 (1)	0.0 (0)	0.8 (1)	1.1 (1)	2.1 (2)	1.8 (2)	0.0 (0)	6	7.1
Ser	1.8 (2)	4.7 (5)	2.6 (3)	3.1 (3)	1.6 (2)	1.5 (2)	1.1 (1)	0.0 (0)	10	10.9
Glu	21.9 (22)	46.9 (47)	23.1 (23)	19.9 (20)	3.0 (3)	10.7 (11)	0.0 (0)	1.0 (1)	62	67.6
Pro	6.6 (7)	8.6 (9)	0.0 (0)	0.0 (0)	0.0 (0)	1.2 (1)	0.0 (0)	0.0 (0)	10	11.3
Gly	1.4 (1)	1.6 (2)	0.0 (0)	1.7 (2)	2.1 (2)	2.1 (2)	3.9 (4)	0.0 (0)	10	9.4
Ala	4.8 (5)	17.2 (17)	10.8 (11)	11.0 (1)	3.9 (4)	3.3 (3)	3.0 (3)	1.0 (1)	28	29.2
Val	7.3 (7)	7.8 (8)	1.2 (1)	1.0 (1)	0.0 (0)	1.1 (1)	1.0 (1)	1.7 (2)	12	10.3
Met	0.0 (0)	0.3	0.0 (0)	0.2	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	5	5.2
Ile	1.9 (2)	5.3 (5)	2.7 (3)	2.3 (2)	0.0 (0)	3.0 (3)	0.0 (0)	0.0 (0)	8	10.0
Leu	2.0 (2)	10.0 (10)	7.0 (7)	6.1 (6)	1.0 (1)	8.4 (8)	1.1 (1)	0.0 (0)	20	23.4
Tyr	0.0 (0)	0.0 (0)	0.0 (0)	1.0 (1)	1.7 (2)	1.9 (2)	0.0 (0)	0.0 (0)	4	5.1
Phe	1.1 (1)	2.3 (2)	1.1 (1)	0.8 (1)	0.0 (0)	1.8 (2)	1.0 (1)	0.0 (0)	5	5.8
His	3.7 (4)	4.2 (4)	1.0 (1)	1.0 (1)	0.0 (0)	1.4 (1)	0.0 (0)	0.0 (0)	5	7.4
Lys	7.4 (7)	19.7 (20)	10.8 (11)	14.2 (14)	3.6 (4)	12.0 (12)	5.3 (5)	0.0 (0)	41	53.4
Arg	2.0 (2)	16.8 (17)	12.7 (13)	11.0 (11)	1.9 (2)	5.8 (6)	0.9 (1)	1.8 (2)	28	29.8
Trp	0.0 (0)	0.0 (0)	0.0 (0)	nd (nd)	0.0 (0)	0.8 (1)	0.7 (1)	0.0 (0)	2	2.1
Hse	1.0 (1)	nd (1)	1.0 (1)	1.0 (1)	0.8 (1)	1.0 (1)	0.0 (0)	1.0 (1)		
Totals	70	159	79	80	24	66	20	8	278	312.5
No. of samples	4	4	5	2	2	2	2	1		

* Sum of the amino acid contents of peptides CNB1′, CNB5, CNB6, CNB7 and CNB8.

Table 2. *N- and C-terminal amino acid sequences of the CNBr peptides of rabbit troponin T*

The symbol → represents the identification of the dansyl-amino acid at the appropriate step of the Edman degradation. No *C*-terminal peptide was isolated from peptide CNB8, the sequence of which was determined by complete Edman degradation. Except for peptides CNB7 and CNB8, *C*-terminal peptides were isolated from their respective CNBr peptides, after digestion with trypsin or thermolysin, by high-voltage paper electrophoresis at pH 6.5, 2.0 and 3.5. Mobilities at pH 6.5 were determined relative to free aspartic acid ($m = -1$) and the charge values were calculated as described by Offord (1966), from which the presence of asparagine or glutamine was deduced.

Peptides	*N*-Terminal sequences	*C*-Terminal sequences	*C*-Terminal peptides: Mobility at pH 6.5	*C*-Terminal peptides: Calculated net charge
CNB1	Blocked	Leu-Hse	0	0
CNB1′	Blocked	Ala-Leu-Ser-Ser-Hse	0	0
CNB2	Glx-Leu	Ala-Leu-Ser-Ser-Hse	0	0
CNB5	Gly-Ala-Asx-Tyr-Ser	Glu-Hse	−0.62	−1
CNB6	Lys	Tyr-Asp-Ile-Hse	−0.40	−1
CNB7	Leu-Ala	Not determined	–	–
CNB8*	Asx-Val-Arg	Val-Glx-Hse	–	–

*The complete sequence of peptide CNB8 is Asx-Val-Arg-Ala-Arg-Val-Glx-Hse.

Pronase under similar conditions all peptides were retained on the resin. From these results and the fact that peptides CNB1 and CNB1′ both contained almost all of the total proline present in troponin T (Table 1), it was concluded that both peptides were derived from the *N*-terminal region of troponin T. It followed that peptide CNB1′ was a partial digestion product that included peptides CNB1 and CNB2, a conclusion that was compatible with the amino acid analyses (Table 1) of the three peptides.

The *C*-terminal sequences of the CNBr peptides were determined by digesting each peptide, except peptides CNB1 and CNB7, with trypsin and isolating the homoserine-containing peptides by high-voltage electrophoresis. The sequence of these peptides which represent the *C*-terminal regions of the CNBr peptides are given in Table 2. The *C*-terminal peptide of CNB1 was obtained by digestion with thermolysin, and sequencing the peptide that contained homoserine. Peptide CNB7 was not digested because it lacked homoserine and was therefore assumed to be the *C*-terminal peptide of troponin T.

Amino acid sequences of the methionine peptides of troponin T. After prolonged digestion with trypsin and chymotrypsin of troponin T that had previously been treated with iodo[2-^{14}C]acetic acid at pH 3.5, seven radioactively labelled peptides were isolated from the digest. The sequence data on these peptides, isolated as described in the Methods section, are summarized in Table 3. By comparing the sequences of the methionine peptides with those of the *N*- and *C*-terminals of the CNBr peptides as listed in Table 2, the overlaps between the CNBr peptides could be deduced. These are listed in Table 3.

Methionine-containing peptides 5, 6 and 7 (Table 3) appeared to arise from the same region in troponin T, i.e. that linking peptides CNB5 and CNB6. In each case glutamic acid was the *N*-terminal amino acid, homoserine was obtained after the first Edman cycle (a usual breakdown product of dansyl-alkylated methionine residues) and all the radioactivity was removed during the second Edman cycle, which revealed, in low yield, α- or ε-dansyl-lysine. These isomers cannot be separated easily. [α-Danysl-lysine is obtained from lysine residues becoming *N*-terminal as a result of an Edman-degradation cycle and ε-dansyl-lysine is produced in low yield from lysine residues not at the *N*-terminal (Gray, 1972).] A similar result was obtained from the first two Edman-degradation cycles of peptide CNB6. It was therefore concluded that ^{14}C-labelled peptides 6 and 7 were produced by partial tryptic cleavage of the Lys-Lys bonds. The sequences assigned to these peptides fit well with their mobilities found on paper electrophoresis. One

Table 3. *Amino acid sequences of peptides of rabbit troponin T containing methionine*

The peptides were isolated from rabbit troponin T that had been labelled with iodo[2-^{14}C]acetic acid and digested with a mixture of trypsin and chymotrypsin. Mobilities at pH6.5 and 2.0 were determined relative to free aspartic acid ($m = -1$) and free serine ($m = +1$) respectively. The peptide charge values were calculated as described by Offord (1966). The presence and position of asparagine and glutamine were deduced from the charge values in conjunction with those obtained for the *C*-terminal peptides isolated from the CNBr peptides (see Table 2) and that for peptide CNB8 (at pH6.5, $m = 0.26$, charge $= +1$). The symbol → represents the identification of the dansyl-amino acid at the appropriate step of the Edman degradation. (Dansylcarboxymethylmethionine was identified by its breakdown products, dansylmethionine and dansylhomoserine.)

Peptide number	Peptide sequence	pH 6.5 Mobility	pH 6.5 Calculated net charge	pH 2.0 Mobility	pH 2.0 Calculated net charge	10^{-6} x Radioactivity recovered (d.p.m.)	Specific radioactivity (d.p.m./nmol)	CNBr peptides bridged
1	Glu-Leu-Met-Glu-Leu	−0.63	−2	+0.61	+1	6.00	12 000	CNB1–CNB2
2	Leu-Ser-Ser-Met-Gly-Ala-Asn-Tyr	0	0	+0.060	+1	5.94	13 500	CNB2–CNB5
3	Asp-Ile-Met-Asn-Val-Arg	0	0	+1.1	+2	4.50	9 600	CNB6–CNB8
4	Val-Glu-Met-Leu-Ala-Lys	0	0	+1.0	+2	3.57	8 800	CNB8–CNB7
5	Glu-Met-Lys	0	0	+1.37	+2	2.48	7 700	CNB5–CNB6
6	Glu-Met-Lys-Lys	+0.46	+1	+1.72	+3	1.70	10 200	CNB5–CNB6
7	Glu-Met-Lys-Lys-Lys	+0.69	+2	+1.93	+4	0.55	13 300	CNB5–CNB6

of the ^{14}C-labelled peptides, peptide 5 (Table 3), was not obtained pure but was contaminated with a dipeptide, Ala-Asn. The latter was distinguished from peptide 5 and its structure deduced after considering the dansyl *N*-terminal and amino acid analyses and the Edman degradations of fractions containing the two peptides. The total yield of radioactivity measured in all labelled peptides isolated was 42% of the total radioactivity associated with troponin T before enzymic digestion.

At this stage of the investigation six peptides CNB1, CNB1′, CNB2, CNB5, CNB6 and CNB7 had been isolated from the CNBr digest of troponin T. Peptide CNB7 was the only peptide that did not contain homoserine and was presumed to be the *C*-terminus. This fact, coupled with the information summarized in Table 3, indicated that the probable order of the peptides in troponin T was:

CNB1-CNB2-CNB5-CNB6—CNB7

Of the five unique methionine peptides that would be expected from the amino acid analysis (Wilkinson, 1974), only three were identified as bridging the CNBr peptides CNB1, CNB2, CNB5 and CNB7. As the remaining two methionine peptides could not bridge peptides CNB6 and CNB7 it seemed probable that an as yet unidentified peptide was present in the CNBr digests. This information and the report that a peptide in addition to those listed above was present in CNBr digests of troponin T (L. B. Smillie, personal communication) prompted a search for an additional peptide. Peptide CNB8 was isolated by paper electrophoresis of fraction 1E (fractionation system 5, see the Methods section). At pH6.5 peptide CNB8 moved as two bands, with mobilities of +0.55 and +0.26, relative to free aspartic acid ($m = -1$). Both bands stained yellow with the ninhydrin–cadmium reagent of Barrolier (see Offord, 1966) and on electrophoresis at pH2.0 both had a mobility of +1.30 relative to free serine ($m = +1$). The peptides purified from both bands had the same composition. When material, with a mobility of +0.55 on electrophoresis at pH6.5, was heated on a boiling-water bath for 20min in pyridine–acetic acid–water (100:4:900, by vol.), pH6.5, and then subjected to paper electrophoresis at pH6.5, two bands staining yellow with the ninhydrin–cadmium reagent were obtained. One of these bands migrated with the mobility of the original material, +0.55, and the other with a mobility of +0.26. It was therefore concluded that the peptide band that moved with a mobility of +0.55 at pH6.5 was the homoserine lactone form of peptide CNB8 and that with a mobility of 0.26 was the free acid form.

The properties and amino acid composition of peptide CNB8 indicated that it was different from the peptides identified. The sequence of CNB8 was found to be Asn-Val-Arg-Ala-Arg-Val-Glu-Hse which indicated that this peptide linked peptide CNB6 to CNB7 (Table 3). Therefore the arrangement of the CNBr peptides in troponin T was concluded to be:

CNB1-CNB2-CNB5-CNB6-CNB8-CNB7

Interaction of troponin T with tropomyosin

Viscometric studies. (*a*) Interaction between troponin components and tropomyosin. The increase in viscosity obtained on addition of the whole troponin complex to tropomyosin was sensitive to ionic strength (Fig. 5). It was quite marked in 0.3M-KCl–2.25mM-sodium borate–11mM-boric acid (20mM-borate), pH8.5, under which conditions the viscosity of tropomyosin was very low at the concentrations used. In 0.06M-KCl–20mM- borate, pH8.5, the viscosity effect was greater, less easy to determine with reproducible accuracy, and the viscosity of tropomyosin itself was quite high. Once isolated from the troponin complex, both troponin I and troponin T are only slightly soluble at pH8.5, particularly at low ionic strength. A higher ionic strength was therefore chosen for studying the interactions, as up to about 1.5mg of troponin T/ml (prepared from troponin B) could be obtained in solution in 0.3M-KCl–20mM-borate, pH8.5. For reasons that are not clear, troponin T isolated by DEAE-cellulose chromatography was usually less soluble than that isolated from troponin B by the method of Wilkinson (1974) and could not be used over the whole concentration range. Nevertheless, once the troponin T was in solution no difference between the two preparations could be detected in the viscometric experiments. Addition of

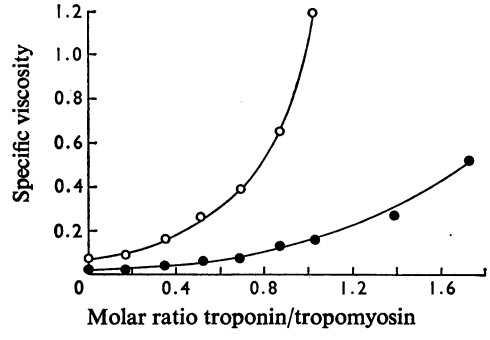


Fig. 5. *Effect of ionic strength and molar ratio on the viscosity of the troponin–tropomyosin complex*

Tropomyosin (0.5mg/ml) was mixed with troponin (0–1.2mg/ml) in 20mM-borate, pH8.5, containing 0.3 or 0.06M-KCl, left at room temperature for 3h and the viscosity determined at 4°C. The viscosity of troponin in the absence of tropomyosin was neglibible at all the concentrations used. ●, 0.3M-KCl; ○, 0.06M-KCl.

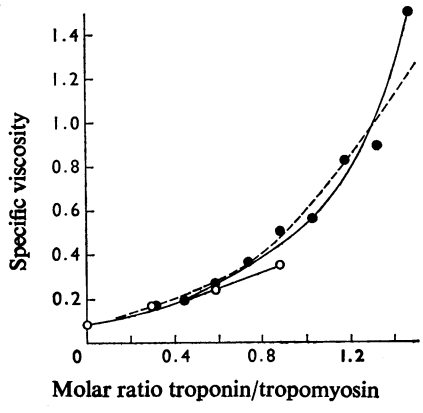


Fig. 6. *Effect of troponin T on the viscosity of tropomyosin solutions*

Tropomyosin (0.5 mg/ml) was mixed with troponin T (0–0.5 mg/ml) in 0.3 M-KCl–20 mM-borate, pH 8.5. Conditions for viscosity measurement were otherwise as in Fig. 1. The viscosities of troponin T and the troponin T–troponin C mixtures were negligible at the concentrations used. ----, Effect of the whole troponin complex (from Fig. 1). ○, Troponin T alone; ●, troponin T in the presence of an equal weight of troponin C.

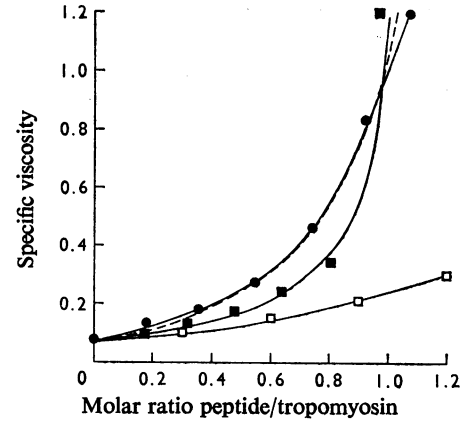


Fig. 7. *Effect of peptides obtained by CNBr digestion of troponin T on the viscosity of tropomyosin solutions*

Troponin T peptides, as indicated below, were added to tropomyosin (final concentration 0.5 mg/ml) in 0.06 M-KCl–20 mM-sodium borate, pH 8.5, and viscosities determined as for Fig. 1. ----, Effect of the whole troponin complex (from Fig. 1). The viscosity of the peptides in the absence of tropomyosin was negligible at all the concentrations used. ●, Whole CNBr digest; ■, peptide CNB1′; □, peptide CNB2.

troponin T to tropomyosin produced an increase in viscosity as the molar ratio of the troponin T to tropomyosin increased (Fig. 6). Above a molar ratio of about 0.8 some precipitation of troponin T and tropomyosin occurred. Up to this point there was no significant difference between the viscometric effect caused by troponin T and that caused by an equimolar amount of whole troponin (assuming molecular weights of 37000 and 80000 respectively). To maintain troponin T in solution (up to 0.5 mg/ml), particularly in the presence of tropomyosin, a molar ratio of troponin C/troponin T of slightly greater than 1 was required. When troponin T was solubilized by using an equal weight of troponin C the effect on the viscosity of tropomyosin was identical with that of whole troponin, at relative concentrations of up to 1.5 mol of troponin T/mole of tropomyosin. Neither troponin C nor troponin I had any significant effect on the viscosity of tropomyosin when added alone or together.

(*b*) Interaction between the CNBr peptides of troponin T and tropomyosin. The addition of the unfractionated CNBr digest of troponin T to tropomyosin dissolved in 0.3 M-KCl–20 mM-borate, pH 8.5, caused a small, but significant, increase in viscosity. The whole digest was less effective on a molar basis than undigested troponin T and the addition of troponin C did not further increase the viscosity. One peptide isolated from the digest, CNB1′, caused a similar viscosity increase, on a molar basis, to the unfractionated digest. The measurement of such small viscosity changes, however, could not be carried out accurately and no definite conclusions could be drawn from the magnitude of the changes.

When viscosities were determined in 0.06 M-KCl–20 mM-borate, pH 8.5, the unfractionated digest was found to be as effective in enhancing the viscosity of tropomyosin as either whole troponin or a mixture of equal weights of troponin T and troponin C (Fig. 7). The effect of troponin T alone could not be investigated under these conditions because of its extreme insolubility even in the presence of solubilizing agents such as 10 mM-ATP. Two peptides, CNB1′ and CNB2, had a marked effect on viscosity at this salt concentration (Fig. 7). Peptide CNB1′ had a similar effect on a molar basis to the unfractionated digest, whereas peptide CNB2, which comprises approx. 50% of peptide CNB1′, had a much smaller effect. An equimolar mixture of peptides CNB2 and CNB1 (the other half of peptide CNB1′) was no more effective than peptide CNB2. Peptides CNB1, CNB3, CNB5, CNB6, CNB7 and CNB8 produced no measurable viscometric effect when added to tropomyosin.

Electrophoretic study of the interactions of troponin T and peptides with tropomyosin and troponin C. Study of the complex-formation between the troponin components by electrophoresis is made difficult by

the fact that not all of the components of the regulatory system migrate to the same electrode under a given set of conditions. This is either because of the very different net charge (in the case of troponin C compared with troponin I and troponin T) or the size and shape of the polymerized aggregate under the conditions of low ionic strength required for electrophoresis (in the case of tropomyosin). Nevertheless, the appearance of new bands or the retardation of components can be interpreted as evidence of interaction.

Clear evidence of a troponin T–troponin C complex was obtained by studying the electrophoretic behaviour of mixtures of these two proteins in gels prepared from 8% (w/v) acrylamide, 0.11% bisacrylamide, 25mM-Tris–80mM-glycine, pH8.6. Under these conditions troponin T remained at the origin, troponin C migrated rapidly and the complex migrated at about 40% of the mobility of troponin C (Plate 2*a*, gels i–iii). The presence of approximately equimolar amounts of troponin T and troponin C in the complex was shown by elution of the band due to the complex and electrophoresis in sodium dodecyl sulphate (Plate 2*a*, gel vi). The relative amounts of protein in the two bands was evaluated by densitometric measurement. The stoicheiometry of the complex was confirmed by carrying out electrophoresis of mixtures containing different molar proportions of the two proteins in 25mM-Tris–80mM-glycine, pH8.6. A troponin C band was only apparent when the molar proportions of the latter protein in the mixture exceeded those of troponin T. In the presence of tropomyosin, which does not migrate under these conditions, the band corresponding to the troponin T–troponin C complex disappeared (Plate 2*a*, gels iv and v).

The formation of the troponin–tropomyosin complex could also be demonstrated by electrophoresis at pH8.6. Unfractionated troponin moved as a relatively slowly migrating band (Plate 2*b*, gel i), which, when eluted and examined by electrophoresis in sodium dodecyl sulphate, contained all three components in roughly equimolar proportions (Plate 2*b*, gel vii). On addition of tropomyosin this band disappeared and the troponin–tropomyosin complex remained at the origin (Plate 2*b*, gel ii).

Most of the peptides present in CNBr digests of troponin T are positively charged at pH8.6; only peptides CNB1 and CNB1′ migrated from the origin to the anode on polyacrylamide-gel electrophoresis at pH8.6. Peptide CNB1 stained as a doublet but rapidly diffused out of the gel on staining and usually appeared as a diffuse band (Plate 2*b*, gel iii). Peptide CNB1′ migrated more slowly and also stained as a doublet (Plate 2*b*, gel v). The appearance of peptides CNB1 and CNB1′ as doublets on electrophoresis in 6M-urea at pH3.2 was thought to be due to partial phosphorylation, for both these peptides contain the *N*-terminal site reported to be phosphorylated in troponin T (A. J. G. Moir & S. V. Perry, unpublished work; Perry *et al.*, 1975). The possibility of heterogeneity of the amino acid sequence in this region of the molecule cannot, however, be excluded. All peptides (except CNB5, CNB7 and CNB8, which were not readily detected by staining with Coomassie Blue), gave single sharp bands on electrophoresis in the Tris–boric acid–sodium dodecyl sulphate system. Peptide CNB1 did not interact with tropomyosin for, on the addition of tropomyosin, its electrophoretic mobility was unchanged (Plate 2*b*, gel iv). In contrast, peptide CNB1′ no longer migrated from the origin when tropomyosin was present (Plate 2*b*, gel vi).

Affinity chromatography of tropomyosin and troponin T. Tropomyosin–Sepharose affinity columns were found to be of limited value for studying the interaction of tropomyosin with troponin T. This was due to the relative insolubility of troponin T and particularly of the tropomyosin–troponin T complex. Columns were run in 1M-urea to improve the solubility of troponin T, with 10mM-glycine included in the buffer to prevent carbamoylation. The running buffer was made by adjusting approx. 2M-urea–20mM-glycine–100mM-Tris to pH8.5 with 1M-HCl and diluting to 50mM-Tris. When chromatography was carried out in this buffer, 1.5mg of troponin T bound to a 5g freshly made column of Sepharose–tropomyosin (1mg of tropomyosin/g of Sepharose) and was subsequently eluted by 0.25M-HCl. On further application of troponin T in the urea buffer to the same column, however, no binding occurred. Effects of urea on tropomyosin were also noted in the viscosity experiments. After treating tropomyosin (10mg/ml) with 8M-urea–10mM-glycine–50mM-Tris adjusted to pH8.5 with 1M-HCl, and removing the urea by exhaustive dialysis, the viscosity of the tropomyosin–troponin complex was lower than that obtained with untreated tropomyosin.

When the CNBr digest of troponin T was applied to the tropomyosin–Sepharose column in a buffer made by adjusting 100mM-Tris to pH8.5 with 1M-HCl and diluting to 50mM, all peptides except peptide CNB1 were held. All the other peptides were eluted at about 0.4M-KCl on application of a KCl gradient to 0.5M to the column. As judged by electrophoresis peptide CNB1′ was the only peptide in addition to peptide CNB1 present in the CNBr digest that possessed a net negative charge at pH8.6. Therefore unlike the case with the other peptides bound under these conditions the binding of peptide CNB1′ to the column could not be solely of an electrostatic nature.

Discussion

Troponin T produced an increase in viscosity on interaction with tropomyosin at higher ionic strengths

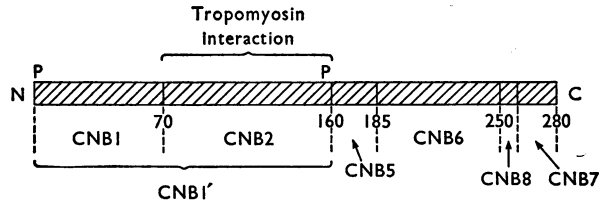


Fig. 8. *Relation of biological activity to the primary structure of troponin T from white skeletal muscle of the rabbit*

The scheme indicates the relative sizes and positions of the CNBr peptides in the primary sequence of troponin T. The values indicating residue numbers should be taken as approximate. P represents the approximate positions of the serine residues that can be phosphorylated by phosphorylase kinase. The serine residue close to the *N*-terminus is usually phosphorylated in isolated troponin.

that was similar to that obtained with the whole troponin complex. Troponin T also interacts with troponin C but clearly, from the absence of measurable viscometric effects, this complex has different properties from that obtained with tropomyosin. The evidence suggests that troponin C is not directly involved in the viscometric changes resulting from the troponin T–tropomyosin interaction and its role in the experiments described may have been simply to facilitate interaction by solubilization of the troponin T. The fact that the complete CNBr digest of troponin T produced a similar viscometric effect with tropomyosin to troponin itself supports this view.

The viscometric and electrophoretic studies indicate that two of the peptides obtained on CNBr digestion of troponin T, namely peptides CNB1′ and CNB2, interact with tropomyosin. Nevertheless, it is probable that only one region of the molecule is involved, for the CNB2 peptide sequence is common to both peptides. This suggests that interaction with tropomyosin is confined to the *N*-terminal half of troponin T, with the region lying between residues 70 and 160 being principally involved (Fig 8). Unlike troponin T itself, none of the major CNBr peptides isolated formed a complex with troponin C on electrophoresis. It is likely that the interaction site on troponin T for troponin C is different from that involved in interaction with tropomyosin. This follows from the electrophoretic evidence that suggests that troponin T forms a complex with both troponin C and tropomyosin simultaneously.

From the amino acid analyses of the CNBr peptides the troponin T molecule is estimated to contain about 280 residues. This value is slightly lower than that deduced from the analytical data of Wilkinson (1974) and indicates a molecular weight of approx. 33000. As for troponin I (Wilkinson & Grand, 1975), the actual molecular weight of troponin T from skeletal muscle appears to be significantly smaller than the apparent molecular weight of 37000 determined by electrophoresis in sodium dodecyl sulphate. The limited sequence data do not permit any firm conclusions about the conformation of troponin T. Nevertheless, the *N*-terminal half of the molecule contains a high proportion of hydrophilic residues, 90% of the total proline and therefore probably less α-helix than the *C*-terminal half. Much of this region must be exposed for it also contains two phosphorylation sites and the site of interaction with tropomyosin.

Two sites on troponin T that can be phosphorylated by phosphorylase kinase have been identified (A. J. G. Moir & S. V. Perry, unpublished results; Perry *et al.*, 1975). A peptide of composition (Asx, Glx_2, Ser P) with a blocked *N*-terminal and which is presumably the *N*-terminus of troponin T, was shown to be the site that is normally phosphorylated when troponin is isolated from skeletal muscle. The other site, which was identified as Ala-Leu(Ser, Ser P)Met-Gly-Ala-Asn-Tyr, can now be localized as the bridge peptide between peptides CNB2 and CNB5 (Table 3, Fig. 8). It may be significant that both phosphorylation sites are in the half of the molecule involved in interaction with tropomyosin, the second site being adjacent to the peptide shown to be involved. This makes the third example of a phosphorylation site in a component of the troponin complex that is localized close to a region of the polypeptide chain involved in interaction with another component (see Perry *et al.*, 1975, for review). The findings with troponin T give further support to the view (Perry *et al.*, 1975; Cole & Perry, 1975) that phosphorylation on the troponin components may modify the function of the complex by changing the net charge on regions of the polypeptide chain involved in protein–protein interactions.

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