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Studies of the interaction of troponin I with proteins of the I-filament and calmodulin

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1. All lysine residues in native troponin I from rabbit fast-twitch skeletal muscle reacted with methyl acetimidate and ethyl acetimidate. 2. The reactivity of lysine-18 of troponin I to acetimidate was much diminished when the troponin I was complexed in the presence of Ca^{2+} with troponin C alone or in the whole troponin complex. 3. In the presence of EGTA, lysine-18 of troponin I in the troponin I–troponin C complex was more reactive to acetimidate than it was in the presence of Ca^{2+}. 4. No masking of lysine residues could be detected when troponin I interacted with calmodulin or actin. 5. Sedimentation-equilibrium studies indicated that the complex of troponin I with calmodulin was more readily dissociated in the absence of Ca^{2+} than was its complex with troponin C under otherwise identical conditions. 6. These studies suggest that the nature of the involvement of the *N*-terminal region of troponin I is a major difference between its modes of interaction with calmodulin and with troponin C.

A description in molecular terms of the interaction of two components of the troponin complex, troponin I and troponin C, and its modification by Ca^{2+}, is of fundamental importance for the understanding of the mechanism of the regulation of contraction in striated muscle mediated through the I-filament system. One of the outstanding problems is the nature of the mechanism by which the binding of Ca^{2+} to a molecule of troponin C located on every seventh actin monomer enables all or most of the actin molecules to change from an inactive or 'switched-off' form to one that interacts with myosin in a manner that leads to a rapid hydrolysis of MgATP and thus initiates the contractile cycle. In some way this process must involve troponin I, one molecule of which (or possibly two; see Sperling *et al.*, 1979) is complexed with the troponin C in the troponin complex. As troponin I is the only component of the myofibril with clearly defined properties as an inhibitor of the interaction of actin and myosin, it would be reasonable to suppose that it is directly involved in inhibiting the actomyosin interaction in resting muscle. The 'steric' hypothesis (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973; Squire, 1981), however, delegates this role to tropomyosin, but not all the biochemical evidence can be explained by such a mechanism (Eaton, 1976; Chalovitch *et al.*, 1981; Johnson *et al.*, 1981; Chalovitch & Eisenberg, 1982).

There is substantial evidence that two distinct sites on troponin I are involved in interaction with troponin C. This conclusion follows from the observation that troponin C is able to inhibit phosphorylation (Perry & Cole, 1974) at the two major phosphorylation sites, threonine-11 and serine-117, in troponin I from fast-twitch skeletal muscle of the rabbit (Moir *et al.*, 1974; Huang *et al.*, 1974), and from affinity-chromatographic (Syska *et al.*, 1976) and p.m.r. studies (Grand *et al.*, 1982) on the isolated peptides obtained by CNBr digestion. The latter studies in particular imply that Ca^{2+} is essential for the interaction of troponin C with both sites, namely the *N*-terminal region, residues 1–21, and the region represented by residues 97–116.

Calmodulin, which shows many homologies to troponin C, forms a similar complex with troponin I and this is also Ca^{2+}-dependent, but electrophoretic (Grand *et al.*, 1979) and enzymic studies (Amphlett *et al.*, 1976) suggest that some differences exist in the stability of the complex and the effect of Ca^{2+} in the interaction. In view of the fact that calmodulin and troponin C have probably evolved from the same gene (Barker *et al.*, 1977), the special features of the latter protein have no doubt been selected during evolution for its specific role in the regulation

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of contraction in striated muscle. We have therefore attempted to study the differences in the interaction of calmodulin and troponin C with troponin I to throw light on the aspects of the interactions that are unique to troponin C. The reactivity of lysine residues of troponin I on interaction with other I-filament proteins and in the absence and in the presence of Ca^{2+} has also been investigated. For this purpose we have used modification by methyl acetimidate and ethyl acetimidate, reagents that are particularly suitable for such a study as they are small highly reactive molecules that do not produce a change in charge after reacting with lysine side chains of proteins (Ludwig & Hunter, 1967). These reagents have been used by a number of authors for other studies involving protein interaction (Coggins, 1978; Makoff & Malcolm, 1980).

The present study has shown that lysine-18 of troponin I is masked on interaction with troponin C in the presence of $CaCl_2$. Decreased reactivity was also observed when the two proteins interacted in the presence of EGTA. In contrast, no significant evidence of masking of lysine-18 occurred when calmodulin formed a complex with troponin I. Some aspects of this work have been briefly described (Moir *et al.*, 1980).

Materials and methods

Preparation of proteins

Troponin was prepared from rabbit white skeletal muscle by the method of Ebashi *et al.* (1971), and troponin I and troponin C were isolated by chromatography on DEAE-cellulose (Perry & Cole, 1974). Calmodulin was prepared from bovine brain by the method of Watterson *et al.* (1976) or by the organic-solvent method of Grand *et al.* (1979).

Determination of protein concentration

Protein concentrations were determined by amino acid analysis with a Locarte or LKB 4400 amino acid analyser as described by Wilkinson *et al.* (1972). They were subsequently checked by the micro turbidimetric method of Mejbaum-Katzenellenbogen & Dobryszycka (1959) and, in the case of troponin C and troponin I, by using the absorption coefficients given by Wilkinson (1974*a*).

Synthesis of methyl acetimidate and ethyl acetimidate

Unlabelled methyl acetimidate and ethyl acetimidate were synthesized from methanol and ethanol respectively (Bates *et al.*, 1975) and stored desiccated at −20°C. Two samples of methyl [1-^{14}C]-acetimidate were synthesized by this procedure with [1-^{14}C]acetonitrile (supplied by Amersham International, Amersham, Bucks., U.K.) and stored at −20°C. The specific radioactivities of the products were 7.4×10^4 d.p.m./μmol and 1.2×10^5 d.p.m./μmol. Ethyl [1-^{14}C]acetimidate was purchased from Amersham International immediately before use, and was diluted with unlabelled ethyl acetimidate and used for modification within 1 min of the vial being opened.

Acetimidation of proteins

Equimolar complexes of troponin I (20–30 mg) with troponin C and calmodulin were formed by mixing the proteins in 9 M-urea/50 mM-triethanolamine/15 mM-2-mercaptoethanol/1 mM-EGTA, adjusted to pH 8.0 with 11.4 M-HCl, with a final protein concentration of 5–10 mg/ml. The proteins were dialysed at 4°C into 50 mM-triethanolamine/15 mM-2-mercaptoethanol/1 mM-$MgCl_2$, adjusted to pH 8.2 with 11.4 M-HCl, containing either $CaCl_2$ (0.1 mM) or EGTA (1 mM). Unlabelled methyl acetimidate (90 mg) was dissolved in 1.5 ml of a solution consisting of equal volumes of 1 M-NaOH and 1 M-triethanolamine/HCl buffer, pH 8.2, and the pH adjusted to pH 8.2 with 1 M-NaOH where necessary. This reagent solution was added to the protein solution and modification proceeded at 4°C for 14 h, and the reagent was then removed by dialysis against the reaction buffer. The troponin I was isolated from the complex by chromatography on a DEAE-cellulose column (1.5 cm × 12 cm) in 9 M-urea / 50 mM - triethanolamine / 15 mM - 2 - mercapto-ethanol adjusted to pH 8.2 with 11.4 M-HCl. Under these conditions it passed through the column unretarded whereas the troponin C remained held. The combined fractions from the column containing troponin I were treated with methyl [1-^{14}C]-acetimidate or ethyl [1-^{14}C]acetimidate (30 mg) directly as described above for modification with unlabelled acetimidate. The labelled protein was recovered by gel filtration on a Sephadex G-25 column (3.5 cm × 40 cm) equilibrated and eluted with 10 mM-HCl.

Troponin I, troponin complex (30–90 mg) and equimolar complexes of actin and troponin I (total protein 30–50 mg) were dissolved in 50 mM-triethanolamine / 15 mM - 2 - mercaptoethanol / 0.5 mM-$CaCl_2$ adjusted to pH 8.2 with 11.4 M-HCl. In experiments with troponin I alone, it was necessary to add 0.2 M-NaCl to the buffer to increase its solubility. Each sample was modified with unlabelled methyl acetimidate, and the troponin I samples were recovered by chromatography on a DEAE-cellulose column as detailed above and then incubated with methyl [1-^{14}C]acetimidate or ethyl [1-^{14}C]-acetimidate in 9 M-urea / 50 mM-triethanolamine / 15 mM-2-mercaptoethanol adjusted to pH 8.2 with 11.4 M-HCl. The labelled troponin I was recovered, either by dialysis against 10 mM-HCl or by desalting on a Sephadex G-25 column equilibrated and eluted with 10 mM-HCl.

The mobilities of troponin C and of the troponin C–troponin I complex were not changed on polyacrylamide-gel electrophoresis in 6 M-urea (Head & Perry, 1974) as a result of modification of both proteins individually with methyl acetimidate. Formation of complex from the modified proteins required the presence of Ca^{2+}, as is the case with the unmodified proteins.

Isolation and purification of peptides

^{14}C-labelled troponin I was digested with CNBr in 70% (w/v) formic acid, and the resulting peptides were carboxymethylated and separated by gel filtration as described by Wilkinson (1974*b*). ^{14}C-containing peptides were further purified by chromatography on columns of phosphocellulose (Wilkinson, 1974*b*) and were identified by amino acid analysis. They were digested with thermolysin (Ambler & Meadway, 1968), and the peptides produced were separated by high-voltage electrophoresis (Moir & Perry, 1977, 1980). ^{14}C-labelled peptides were located by radioautography or by determination of the radioactivity by liquid scintillation in sections cut from a stained side strip of the electrophoretograms.

Ultracentrifugation

Sedimentation-equilibrium studies were conducted with a Beckman model E ultracentrifuge by the Chervenka meniscus-depletion method at 20°C and at two different speeds. Protein samples (1.1–1.7 mg/ml) were dialysed at 4°C for 24 h against 50 mM-succinic acid adjusted to pH 7.0 with 1 M-NaOH/0.1 M-KCl/1 mM-$CaCl_2$ or 5 mM-EGTA as appropriate. The concentration of dithiothreitol was varied between 0.25 mM and 5.0 mM.

Results

Effect of Ca^{2+}-binding proteins on the modification of troponin I with acetimidates in the presence of $CaCl_2$

Troponin C. When troponin I was treated with [^{12}C]acetimidate in the absence of urea and subsequently with [^{14}C]acetimidate in the presence of 9 M-urea (see the Materials and methods section), no significant amount of radioactivity was incorporated into the protein (Table 1). This indicated that urea treatment of the protein did not increase the number of lysine residues that could be labelled with the reagent. If, however, the troponin I was complexed with troponin C in the presence of Ca^{2+} before treatment with [^{12}C]acetimidate under non-denaturing conditions and subsequently treated with [^{14}C]acetimidate in 9 M-urea after isolation from the complex, significant radioactivity was incorporated in the protein. Some labelling with [^{14}C]acetimidate was obtained if the initial [^{12}C]acetimidate treatment was performed on the troponin I–troponin C complex in the presence of EGTA. The extent of labelling with [^{14}C]acetimidate was, however, greater when the initial treatment of the complex was with [^{12}C]acetimidate in the presence of $CaCl_2$ (Table 1).

Table 1. *Acetimidation of the N-terminal CNBr-cleavage peptide (residues 1–21) of troponin I in the presence of other myofibrillar proteins and calmodulin*

Troponin I from rabbit fast-twitch skeletal muscle was incubated in the presence of the proteins indicated with methyl [^{12}C]acetimidate. After isolation and denaturation it was treated with methyl [1-^{14}C]acetimidate or ethyl [1-^{14}C]acetimidate as indicated in the Materials and methods section. The *N*-terminal peptide CN5 was isolated as illustrated in Figs. 1 and 2. Where indicated, 0.1 mM-$CaCl_2$ or 1 mM-EGTA was present. The S.E.M. values are given and the numbers of experiments indicated in parentheses when the determination was performed more than once.

	Specific activity (mol/mol)	
Complex	Methyl acetimidate	Ethyl acetimidate
Troponin I	0.04	0.05
Troponin I, troponin C, Ca^{2+}	0.98 ± 0.07 (4)	0.54 ± 0.06 (4)
Troponin I, troponin C, EGTA	0.26	0.33 ± 0.08 (4)
Troponin complex, Ca^{2+}	0.90	0.74
Troponin I, calmodulin, Ca^{2+}	0.12 (2)	—
Troponin I, actin	0.07	0.09

The difference in specific radioactivities between the methyl [^{14}C]acetimidate-labelled and ethyl [^{14}C]-acetimidate-labelled troponin I, isolated from the troponin I–troponin C complex labelled in the presence of Ca^{2+}, is not thought to be significant. Similar differences have been obtained in experiments with actin (A. J. G. Moir & S. V. Perry, unpublished work) and are probably due to ^{14}C-labelled impurities in the ethyl [1-^{14}C]acetimidate that do not react with the protein. Specific radioactivities were estimated on the assumption that all the radioactivity of the reagent was located at C-1, and hence the presence of any non-reacting labelled impurities in the reagent would result in lower values being obtained for the labelling of lysine-18.

Calmodulin. If troponin I was combined with calmodulin rather than troponin C, and the complex and the isolated troponin I were treated with [^{12}C]acetimidate and [^{14}C]acetimidate respectively, no significant amounts of radioactivity were incorporated into troponin I. This implies that lysine-18 is not masked when troponin I is complexed with calmodulin.

Isolation of labelled peptides

When the [^{14}C]acetimidate-labelled troponin I isolated after the initial labelling of the troponin I–troponin C complex in the presence of 0.1mM-Ca^{2+} was digested with CNBr and subjected to gel filtration on Sephadex G-50, two peaks of radioactivity were located in the eluate. The fractions containing radioactivity were pooled, freeze-dried and named fractions A and B (Fig. 1*a*).

When fraction B was applied to a column of phosphocellulose equilibrated in 8.3mM-H_3PO_4, most of the radioactivity (70–80%) was eluted in a single peptide (peak B2, Fig. 2), obtained by application of a gradient of NaCl to 1.0M in the same solution. This radioactive peptide was desalted by gel filtration on a column of Sephadex G-25 equilibrated and eluted with 10mM-HCl.

The amino acid composition of the major radioactive peptide from fraction B, given in Table 2, is identical with that of the *N*-terminal 21-residue peptide isolated from the CNBr digest of troponin I from rabbit fast-twitch skeletal muscle by Wilkinson & Grand (1975). The major radioactive peptide in fraction B corresponds to peptide CN5 in the nomenclature of the latter authors.

Two other minor peaks of radioactivity (peaks B1 and B3, Fig. 2) were obtained on chromatography on phosphocellulose. The larger of these (peak B3), representing in all about 10% of the total radioactivity in the original fraction B, was further purified and desalted by a similar method to that used for peptide CN5. It was shown from the amino acid analysis to correspond to the CNBr-cleavage fragment represented by residues 96–116, peptide CN4 (Wilkinson & Grand, 1975). The amounts of radioactivity associated with peptide CN4 were too small to enable an unambiguous identification of the labelled lysine residues.

Chromatography of fraction A (Fig. 1) on CM-cellulose indicated that the radioactivity in the peak was associated with a single peptide (Fig. 3). This had an amino acid composition similar to that

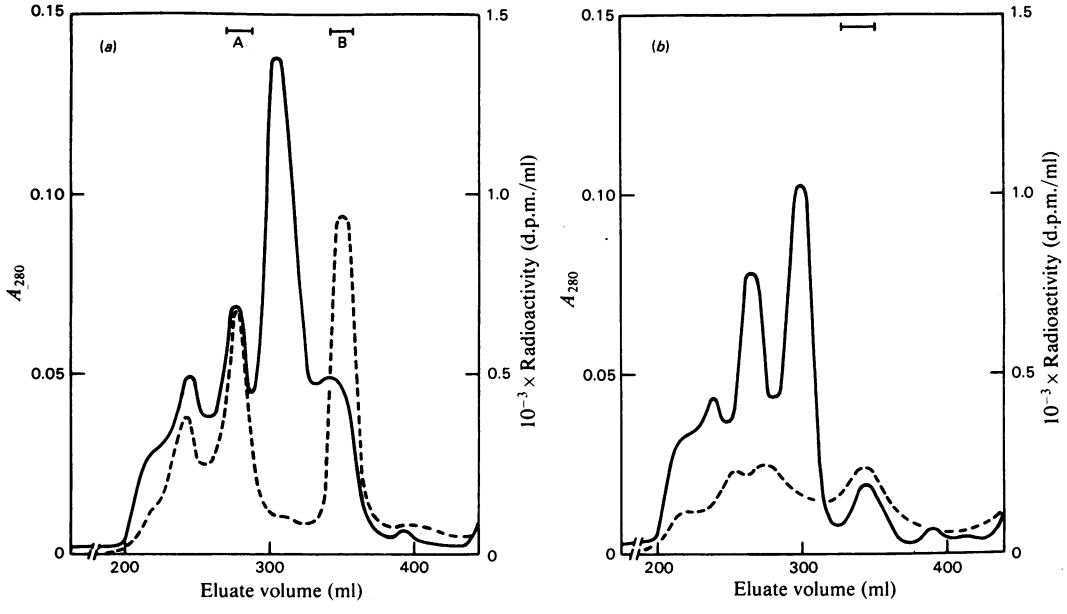

Fig. 1. *Gel filtration of the products of CNBr digestion of* [^{14}C]*acetimidate-labelled troponin I*

Troponin I (approx. 30mg) was modified with methyl [^{12}C]acetimidate as the complex with troponin C or calmodulin as described in the Materials and methods section. The isolated troponin I was incubated with methyl [^{14}C]acetimidate (specific radioactivity 1.17×10^5 d.p.m./μmol) in the presence of 9M-urea (see the Materials and methods section). The labelled proteins were digested with CNBr in 70% formic acid, and the samples were dried and then carboxymethylated (Moir *et al.*, 1974). The digests were applied to a column (2.2cm × 110cm) of Sephadex G-50, equilibrated and eluted with 0.01M-HCl. Fractions (5ml) were collected and pooled and freeze-dried for chromatography on phosphocellulose (see Fig. 2) as indicated by bars. ——, A_{280}; – – – –, radioactivity. (*a*) Troponin I from a troponin I–troponin C complex in 0.1mM-$CaCl_2$. (*b*) Troponin I from a troponin I–calmodulin complex in 0.1mM-$CaCl_2$.

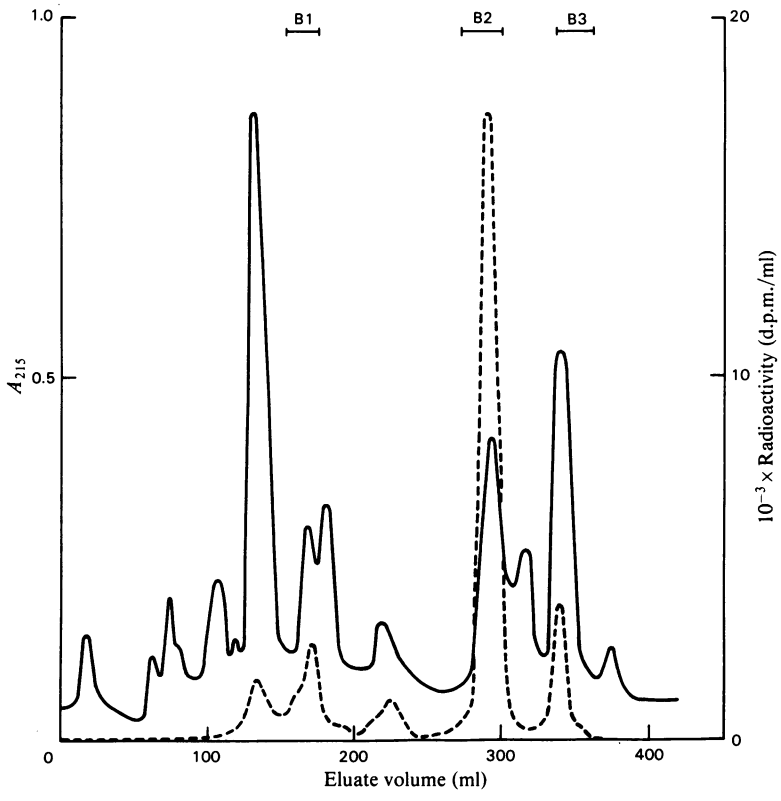

Fig. 2. *Chromatography on phosphocellulose of peptides in fraction B obtained by gel-filtration of CNBr digests of [^{14}C]acetimidate-labelled troponin I*

Fraction B obtained as described in Fig. 1(*a*) was freeze-dried, redissolved in 8.3 mM-H_3PO_4 and applied to a column (1.5 cm × 20 cm) of phosphocellulose P-11 equilibrated in 8.3 mM-H_3PO_4. The column was developed with 8.3 mM-H_3PO_4 (approx. 50 ml) followed by a gradient of 0–1.0 M-NaCl in 8.3 mM-H_3PO_4 (total volume of gradient 400–500 ml). Fractions (5 ml) were collected and pooled as indicated. ——, A_{215}; – – – –, radioactivity.

expected for the *N*-terminal peptide obtained from cleavage of the methionine residue at position 57, i.e. the partial cleavage product consisting of peptide CN5 and peptide CN1 joined by a methionyl bond at residue 21 that had not been cleaved.

Identification of residues labelled with [^{14}C]acetimidate

After digestion with thermolysin, peptide CN5 was separated by high-voltage electrophoresis at pH 6.5 and pH 2.0 into 11 peptides, several of which stained very faintly with ninhydrin. Each peptide was eluted and portions were taken for amino acid analysis and determination of ^{14}C. Radioactivity was detected in one peptide only, designated CN5A, the amino acid composition of which is presented in Table 2. As this peptide consisted of approximately equimolar amounts of serine, leucine and ε-acetimidolysine, it could only be derived from residues 17–19, with the modified lysine residue at position 18. A further peptide isolated from the digest, designated CN5B in Table 2, consisted of residues 1–7 of the molecule and contained the only other lysine residue in peptide CN5, which is located at residue 5. No radioactivity was associated with peptide CN5B.

Confirmation that all the radioactivity was located at lysine-18 was obtained by digestion with thermolysin of the peptide from fraction A (Fig. 1) that consisted of residues 1–57 and similar digestion of [^{14}C]acetimidate-labelled troponin I isolated from the [^{12}C]acetimidate-treated troponin I–troponin C complex. On electrophoresis at pH 6.5 of the products of both digests, a major band of radioactivity that migrated with the same mobility as peptide CN5A was obtained. This was designated

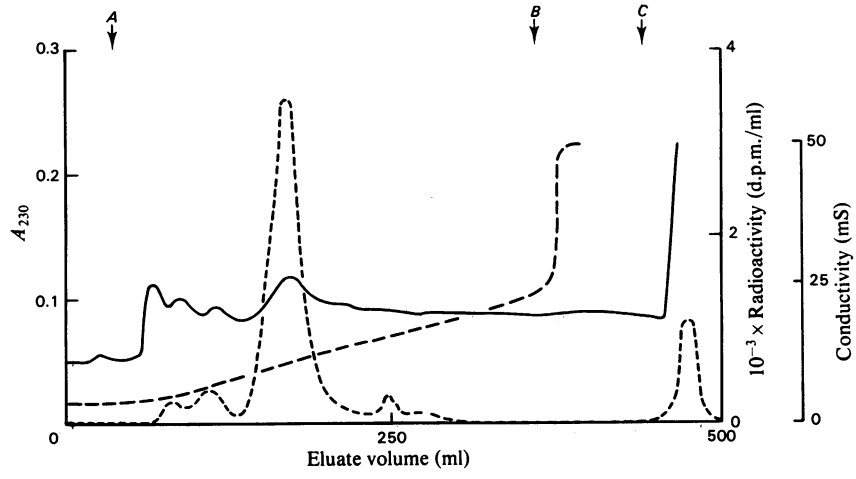

Fig. 3. *Chromatography on CM-cellulose of peptides in fraction A obtained by gel filtration of CNBr digests of [^{14}C]acetimidate-labelled troponin I*

Fraction A obtained as described in Fig. 1(*a*) was dissolved in 50mM-NaOH adjusted to pH4.0 with formic acid and applied to a column (2.5cm × 12cm) of CM-cellulose CM-52 equilibrated in this buffer. A linear gradient of NaCl (0–0.5M) in this buffer was applied at *A*, 1.0M-NaCl in 50mM-sodium formate (pH4.0) applied at *B* and 8M-urea/50mM-Tris (adjusted to pH8.0 with 11.4M-HCl)/15mM-2-mercaptoethanol applied at *C*. Fractions (5ml) were collected, and the A_{230} and radioactivity were measured in alternate fractions. ——, A_{230}; – – –, radioactivity; ——, conductivity.

Table 2. *Amino acid compositions of peptides isolated from [1-^{14}C]acetimidate-labelled troponin I from rabbit fast-twitch skeletal muscle*

In calculating the amount of ε-acetyliminolysine (ε-AcN-Lys) the colour factor for arginine was used. For peptide nomenclature, see the text. Values for residues other than lysine present at less than 0.2mol/mol are excluded. Numbers in parentheses are the numbers of residues predicted from the sequence (Wilkinson & Grand, 1975). N.D., Present but not determined.

	Composition (mol of residue/mol of peptide)			
Peptide ...	CN5	CN5A	CN5B	Th1
Asp	2.2 (2)	—	1.9 (2)	—
Thr	1.0 (1)	—	—	—
Ser	1.0 (1)	1.0 (1)	—	1.0 (1)
Glu	3.3 (3)	—	2.3 (2)	—
Pro	—	—	—	—
Gly	1.1 (1)	—	1.3 (1)	—
Ala	1.7 (2)	—	—	—
Val	1.0 (1)	—	—	—
Ile	0.9 (1)	—	—	—
Leu	1.1 (1)	0.8 (1)	—	1.1 (1)
Tyr	—	—	—	—
Phe	—	—	—	—
His	1.1 (1)	—	—	—
Lys	0.3 (2)	0.18 (1)	0.1 (1)	0.3 (1)
ε-AcN-Lys	2.0	1.1	0.6	N.D.
Arg	3.4 (4)	—	0.8 (1)	—
Hsr	N.D. (1)	—	—	—
No. of analyses	10	3	1	2

peptide Th1, and in each case the amino acid analysis of the radioactive peptide was identical with that of peptide CN5A.

Acetimidation of troponin I–troponin C complex in the presence of EGTA

If the labelling procedure as described above was carried out on the complex in the presence of EGTA, lysine-18 of the troponin I was still labelled, but the results were somewhat more variable. The extent of labelling was significantly lower, particularly when methyl [^{14}C]acetimidate was used, than that obtained with the complex in the presence of Ca^{2+} (Table 1).

Acetimidation of the troponin complex

The effect of troponin C on the reactivity of lysine-18 of troponin I was also evident when the troponin complex was treated with [^{12}C]acetimidate and the troponin I isolated from it was subsequently labelled with [^{14}C]acetimidate (Table 1). On gel filtration of CNBr digests of this [^{14}C]acetimidate-labelled troponin I (Fig. 4), the major peak of radioactivity was eluted in the same position as peptide CN5 obtained from digests of [^{14}C]-acetimidate-labelled troponin I isolated from the troponin I–troponin C complex. Further examination indicated that the radioactivity in this peak was due to peptide CN5. The elution profile obtained on gel filtration differed from that given by digests of

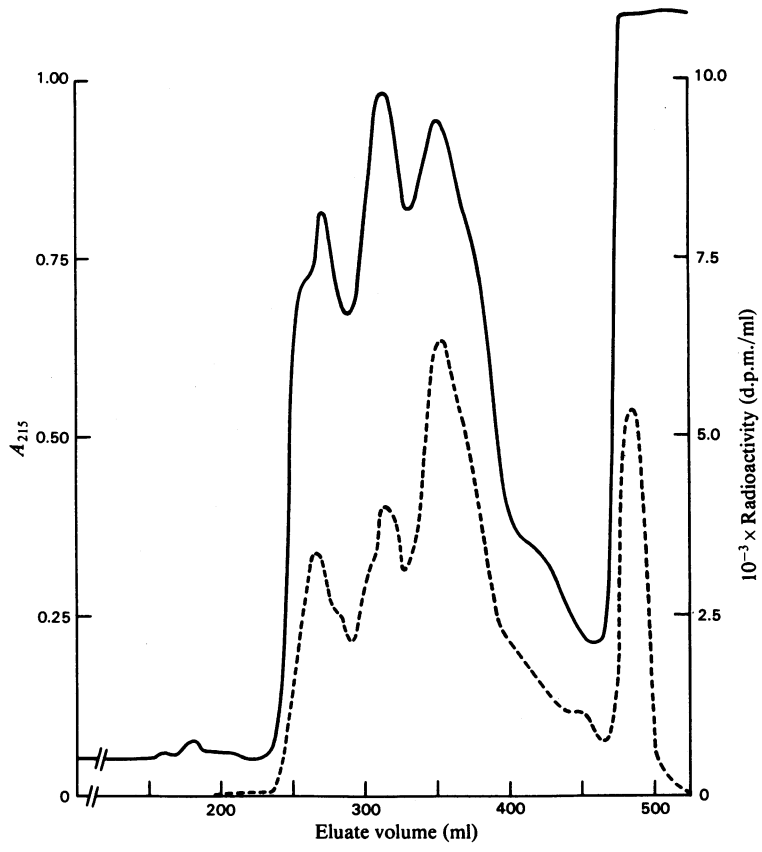

Fig. 4. *Gel filtration of the products of CNBr digestion of troponin I labelled with [^{14}C]acetimidate after isolation from the troponin complex*

Troponin (approx. 80mg) was modified with methyl [^{12}C]acetimidate as described in the Materials and methods section. The troponin I was isolated from the complex and labelled with ethyl [^{14}C]acetimidate. After digestion with CNBr, the fragments were separated by gel filtration on Sephadex G-50 as described in Fig. 1. ——, A_{215}; – – –, radioactivity.

troponin I labelled after isolation from the troponin I–troponin C complex in that it contained an additional [^{14}C]acetimidate-labelled peptide, which was eluted just in front of the peak containing peptide CN5. Although not analysed in detail, this peak was obtained on both occasions when the troponin complex was treated with [^{12}C]acetimidate and was presumed to contain a labelled peptide representing an additional site that was masked in the whole troponin but not in the troponin I–troponin C complex.

Effects of actin on the modification of troponin I with acetimidate

Actin is known to form a complex with troponin I, involving the region consisting of residues 96–116, although the complex is much less stable and p.m.r. evidence implies that the interaction is not modified by Ca^{2+} (Grand *et al.*, 1982). When the troponin I was labelled in the presence and in the absence of F-actin under similar conditions to those used for labelling when complexed with troponin C (see the Materials and methods section), no significant amounts of radioactivity were incorporated into the troponin I.

Sedimentation-equilibrium analysis of the interaction of troponin I with troponin C and with calmodulin

Although it is well established that troponin I forms a complex with calmodulin similar to that obtained with troponin C (Grand *et al.*, 1979), the results obtained above suggest that there are

Table 3. *Molecular weights of complexes of troponin I with troponin C determined by sedimentation equilibrium in the presence of Ca^{2+} and EGTA*

Sedimentation was conducted in 50mM-succinic acid adjusted to pH 7.0 with 0.1M-NaOH/0.1M-KCl/5mM-dithiothreitol, with the exception of the experiment indicated by an asterisk (*), which was conducted in 50mM-sodium succinate (pH 7.0)/0.1M-KCl/0.25mM-dithiothreitol. $CaCl_2$ (1.0mM) and EGTA (5mM) were present as indicated. Experiments were performed as indicated in the Materials and methods section.

System	Speed (rev./min)	10^{-3} × Mol.wt. Min.	Max.
Troponin I, troponin C, Ca^{2+}	25885	35.9	46.4
	21643	35.5	42.7
Troponin I/troponin C, EGTA	25952	26.8	39.0
	21740	36.1	43.2
Troponin I/calmodulin, Ca^{2+}	25880	37.6	37.6
	21351	39.1	39.1
Troponin I/calmodulin, EGTA	35553	21.0	21.0
	31323	22.4	22.4
*Troponin I/calmodulin, Ca^{2+}	25980	44.6	60.8
	21740	52.8	71.8

differences in the reactivity of lysine-18 of troponin I in the two complexes.

The results of earlier studies on the effect of the two complexes on the ATPase of desensitized actomyosin (Amphlett *et al.*, 1976) could be explained if in the absence of Ca^{2+} the troponin I–calmodulin complex was dissociated whereas the troponin I–troponin C complex was not.

To throw further light on the differences in the nature of the interaction of the two Ca^{2+}-binding proteins with troponin I, equimolar complexes were examined by ultracentrifugation, by using the long-column meniscus-depletion sedimentation technique of Chervenka (1970). The molecular weights, which were determined in 5mM-dithiothreitol, are presented in Table 3. At lower dithiothreitol concentrations, e.g. 0.25mM, higher-molecular-weight aggregates were formed. For complexes of troponin I with troponin C, plots of $\ln y$ against r^2 showed a slight upward curvature, and the maximum and minimum molecular weights given were determined by taking tangents at the meniscus and cell bottom respectively. Plots of $\ln y$ against r^2 for the troponin I–calmodulin complex were linear.

In the presence of Ca^{2+}, both troponin C and calmodulin formed complexes with troponin I of molecular weights in the region of 36000–40000. When troponin I plus calmodulin were examined in the presence of EGTA, molecular weights of about 21000 were obtained, implying no interaction between the two proteins. Although there was evidence for formation of a complex between troponin I and troponin C in the presence of EGTA, the more pronounced curvature of the plot of $\ln y$ against r^2 detected at both speeds (seen in Table 3 as a greater variation in the maximum and minimum molecular weights) suggests a greater tendency for the complex to dissociate in EGTA than in the presence of Ca^{2+}.

Discussion

The observation that denaturation did not change the reactivity of any of the 24 lysine residues in troponin I indicates that in the native molecule all of these residues are available to acetimidate. It follows therefore from the change in reactivity of lysine-18 observed in the presence of troponin C that the side chain of this residue is masked in the complex. Lysine-18 also exhibits diminished reactivity in the whole troponin complex, suggesting that the interaction between the isolated proteins is very similar to that which occurs when troponin T is also present.

The changed chemical reactivity of lysine-18 is a little difficult to reconcile with the results of recent p.m.r. studies on the complex (Grand *et al.*, 1982). These have shown that, although arginine side chains were perturbed in a Ca^{2+}-dependent manner when the *N*-terminal CNBr-cleavage peptide of troponin I, consisting of residues 1–21, interacted with troponin C, there was no evidence of perturbation of the side chains of lysine-5 or lysine-18. It would be expected that the changed environment of lysine-18 indicated by its changed reactivity would be reflected in the p.m.r. studies. The p.m.r. investigation, however, was conducted on the *N*-terminal peptide, whereas whole troponin I was used for chemical modification. It is possible that the change in the environment of lysine-18 is much more pronounced when troponin C interacts with the whole troponin I molecule rather than with the peptide consisting of residues 1–21. Certainly the investigations by Syska *et al.* (1976) indicated that the peptide consisting of residues 1–47 interacts more strongly with troponin C than does the peptide consisting of residues 1–21.

The nature of the interaction with troponin C at the other site on troponin I, however, may be different, for there is evidence that the lysine side chains in the region represented by residues 96–107 are perturbed, as judged from the p.m.r. study (Grand *et al.*, 1982), and also change in reactivity in the presence of troponin C. It should be noted that there are three lysine residues in this region of the molecule, and in neither study have the precise residues involved been identified.

The results obtained with acetimidate labelling

with intact proteins confirm the earlier findings (Syska *et al.*, 1976) indicating that one of the sites of interaction between troponin I and troponin C is close to the *N*-terminus of troponin I.

The presence of additional lysine residues that are masked in whole troponin but not in the troponin I–troponin C complex possibly either reflects a slightly different mode of interaction in the two systems or suggests that the presence of troponin T leads to the masking of other regions of the surface of troponin I. This might be expected on the basis of the chemical cross-linking experiments reported by Hitchcock (1975).

The acetimidate-labelling experiments and the ultracentrifuge studies both show that differences exist between the interaction of troponin I with calmodulin and troponin C. The involvement of the *N*-terminal region of troponin I in the interaction with the two Ca^{2+}-binding proteins clearly differs, for the reactivity of lysine-18 in troponin I is diminished in the presence of troponin C but not in the presence of calmodulin. This observation indicates that calmodulin does not block access to this residue, and is in good agreement with the enzymic studies showing that calmodulin, unlike troponin C, does not block phosphorylation of the nearby threonine-11 (Vanaman & Perry, 1978; Perry, 1980). It suggests that the interaction of the *N*-terminal region of troponin I is weaker with calmodulin than with troponin C, and confirms similar conclusions suggested by electrophoretic studies (Grand *et al.*, 1979).

A weaker interaction with calmodulin would explain the ready dissociation of the troponin I–calmodulin complex in the absence of Ca^{2+} and the difference in the ability of the two Ca^{2+}-binding proteins in conferring Ca^{2+}-sensitivity to the inhibition by troponin I of the actomyosin ATPase system (Amphlett *et al.*, 1976).

Evidence from other studies indicates that the interactions of troponin C and calmodulin with the site on troponin I represented by peptide CN4 are very similar (Vanaman & Perry, 1978; Perry, 1980). Thus the unique aspect of complex-formation with troponin C is in the nature of the interaction that occurs with the *N*-terminal region of troponin I, and which is presumably of special significance for the function of troponin C in the regulation of contraction in striated muscle.

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