The Amino Acid Sequence of Rabbit Slow-Muscle Troponin I

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Troponin I was isolated from six red muscles in the hind leg of the rabbit. Soleus, semi-tendinosus, vastus intermedius and adductor longus muscles contained primarily slow-muscle troponin I, vastus lateralis contained fast-muscle troponin I and quadratus femoris contained a mixture of the two. The complete amino acid sequence of the troponin I from slow muscle was determined. Seven CNBr fragments were isolated and sequenced by using the dansyl–Edman technique after digestion with proteolytic enzymes. The CNBr fragments were ordered by isolation of tryptic peptides containing carboxy[^{14}C]methyl-methionine. Direct evidence for the conjunction of residues 8 and 9 has not been obtained, and one of the carboxyl groups between residues 71 and 79 may carry an amide group. Slow-muscle troponin I is a single polypeptide chain of 184 residues with a mol.wt. of 21 146. It has a net overall positive charge of 18 at pH 7, and an absorption coefficient, $A_{280}^{1\%1\text{cm}}$, of 5.43. The protein was isolated with both a blocked and an unblocked *N*-terminus, although the nature of the blocking group was not determined. Proline was found to be the *N*-terminal amino acid. Two forms of the protein could also be distinguished by the presence of an extra two residues at the *C*-terminus. Comparison of sequences of troponin I from rabbit slow, fast and cardiac muscle shows that homology is most marked in the *C*-terminal half of the molecules. Towards the *N*-terminus the homology becomes much less marked. Detailed evidence on which the sequence is based has been deposited as Supplementary Publication SUP 50 079 (32 pages) at the British Library (Lending Division), Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies may be obtained in the terms given in *Biochem. J.* (1977), 161, 1.

The actomyosin ATPase* system of rabbit fast skeletal muscle has been shown by a number of workers to be regulated by the troponin complex in combination with tropomyosin (reviewed by Weber & Murray, 1973; Ebashi, 1974). A similar system of control is present in cardiac muscle, although the analogous troponin components have different primary structures. Syska *et al.* (1974) have shown that troponin I from the slow soleus and crureus muscles, although similar in size and electrophoretic mobility to the fast-muscle protein, is in fact not identical in amino acid sequence or inhibitory activity. It seems probable therefore that all of the constituents of the troponin complex in slow muscle will differ from their fast-muscle counterparts.

Syska *et al.* (1976) have localized the areas of interaction of the fast-muscle troponin-I molecule with troponin C and actin. Sequence studies of rabbit cardiac troponin I (Grand *et al.*, 1976) have shown the analogous regions in this protein to be similar. The actin-binding site, located towards the *C*-terminus of the molecule, differs only in three residues (two of which are very conservative replacements). The troponin-C-binding site, however, at the *N*-terminus is less similar in the cardiac and

skeletal-muscle troponin-I species probably reflecting differences in the troponin-C species from these tissues.

In view of these facts the primary structure of slow-muscle troponin I was investigated, specifically to determine the degree of similarity of the actin and troponin-C interaction sites and more generally to assess the extent of homology in the fast-muscle, cardiac and slow-muscle troponin-I components from the rabbit. This is of particular interest, as Head *et al.* (1977) have shown that rabbit slow-muscle troponin C is very similar indeed to the analogous protein from the fast skeletal muscle.

Materials and Methods

For routine preparations of slow-muscle troponin I, four slow muscles (soleus, vastus intermedius, semitendinosus and adductor longus) were dissected from the hind legs of New Zealand White rabbits (purchased in a frozen condition from the Buxted Rabbit Co., Buxted, Uckfield, East Sussex, U.K.). Troponin I was prepared from these by the affinity-chromatography method of Syska *et al.* (1974) by using a troponin C–Sepharose 4B column, as described previously (Grand *et al.*, 1976). The yield of troponin I was approx 30 mg from 50 g of muscle,

* Abbreviation: ATPase, adenosine triphosphatase.

with an 80 g Sepharose column to which about 200 mg of rabbit fast-muscle troponin C had been coupled.

Troponin C-Sepharose

Troponin C was prepared from rabbit fast skeletal muscle by the method of Perry & Cole (1974) and was coupled to Sepharose 4B as described by Syska *et al.* (1974).

CNBr cleavage

Slow-muscle troponin I was dissolved in 70% (v/v) formic acid (12 mg/ml) and 100-fold molar excess of CNBr over methionine residues was added. The digest was diluted 10-fold with water after 24 h at room temperature (20°C), and freeze-dried.

Radioactive labelling

Cysteine residues were carboxymethylated with iodo[¹⁴C]acetic acid in 6M-guanidine hydrochloride/0.4M-Tris/HCl/2mM-dithiothreitol, pH 8.2, as described by Wilkinson *et al.* (1972). CNBr peptides were alkylated after cleavage. Jacobson *et al.* (1973) have shown that, under these conditions, cleavage of the peptide chain at *S*-cyanocysteine residues does not occur and that treatment with excess of thiol reconverts such residues into cysteine.

Methionine residues were alkylated with iodo[¹⁴C]acetic acid by the method of Wilkinson (1969) after carboxymethylation of the cysteine residues at pH 8.2 with iodoacetic acid.

Determination of molecular weight

The molecular weight of slow-muscle troponin I was determined by using gel filtration on a column of Sepharose 6B in the presence of 6M-guanidine hydrochloride, as described for cardiac troponin I (Grand *et al.*, 1976).

Analytical methods

Amino acid analysis was by the method described by Wilkinson *et al.* (1972), tryptophan was determined spectrophotometrically by the method of Crumpton & Wilkinson (1963) and cysteine was determined as cysteic acid by the method of Moore (1963). Absorption coefficients were determined by analytical ultracentrifugation by the method of Babul & Stellwagen (1969). Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate at pH 7.0 was performed by the method of Weber & Osborn (1969), in urea at pH 3.2 as described by Wilkinson *et al.* (1972), and in urea at pH 8.6 as described by Syska *et al.* (1974).

Enzymic digests

Slow-muscle troponin I (50 mg), carboxymethylated with iodo[¹⁴C]acetic acid at the methionine residues, was dissolved in water and 10% (w/v) NH₄HCO₃ added to give a final concentration of 1%; 1 mg of 1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated trypsin (Worthington, Freehold, NJ, U.S.A.) was added and the digest incubated at 37°C for 4 h. The digest was then chromatographed on Sephadex G-50 (2.2 cm × 110 cm) eluted with 50 mM-NH₄HCO₃.

Peptides were digested with trypsin and thermolysin (Calbiochem, San Diego, CA, U.S.A.) for 4 h, and with the proteinase from the V8 strain of *Staphylococcus aureus* (Houmard & Drapeau, 1972) (Miles Laboratories, Stoke Poges, Slough, Berks., U.K.) overnight, all at an enzyme/substrate ratio of 1:50 (w/w) in 1% NH₄HCO₃ at 37°C.

Citraconylation of CNBr peptide SCN2

Approx. 15 mg of the CNBr fragment SCN2, at pH 8, was citraconylated with 2 × 50 μl of citraconic anhydride (BDH Chemicals Ltd., Poole, Dorset, U.K.), the pH being maintained at 8 by the addition of 5M-NaOH. After completion of the reaction the peptide was desalted on a column (2 cm × 100 cm) of Sephadex G-25 eluted with 50 mM-NH₄HCO₃, and freeze dried. Citraconylated peptide SCN2 was dissolved in 4 ml of 1% NH₄HCO₃ and digested for 3 h at 37°C with 1-chloro-4-phenyl-3-tosylamidobutan-2-one-treated trypsin (2%, w/w). After freeze-drying, the material was redissolved in 2 ml of 0.1M-HCl, left at room temperature overnight, and then chromatographed on a column (2 cm × 140 cm) of Sephadex G-50 eluted with 10 mM-HCl.

Preparation of cysteine cleavage fragment CF1

Cleavage at the cysteine residues of slow-muscle troponin I was by a modification of the method of Jacobson *et al.* (1973). For this, 80 mg of slow-muscle troponin I was dissolved in 6M-guanidine hydrochloride/0.4M-Tris/0.2M-acetic acid, pH 8.0, in the presence of a slight excess of dithiothreitol over thiol groups. After 30 min a 10-fold molar excess of 5,5'-dithiobis-(2-nitrobenzoic acid), over thiol groups, was added and, after a further 15 min, a 10-fold excess of KCN over 5,5'-dithiobis-(2-nitrobenzoic acid) was added. The protein was acidified, desalted on a column (2 cm × 110 cm) of Sephadex G-25 eluted with 10 mM-HCl and freeze-dried. The modified troponin I was cleaved in 6M-guanidine hydrochloride/50 mM-sodium borate, pH 9.0, at 37°C for 48 h and then chromatographed on a column (2.2 cm × 110 cm) of Sephadex G-50 eluted with 10 mM-HCl. The fraction that was believed to contain the *N*-terminal fragment of the molecule was freeze-

dried, dissolved in 5 ml of 50mM-NH₄HCO₃, pH 8, and chromatographed on the troponin C-Sepharose 4B affinity column that had been equilibrated with the same buffer. Material bound to the column was eluted with 8M-urea/75mM-Tris/HCl(pH 8.0)/15mM-2-mercaptoethanol. The eluate was desalted on a column (2cm×110cm) of Sephadex G-25 eluted with 10mM-HCl.

Isolation of the blocked N-terminal peptide from slow-muscle troponin I

Troponin I (10mg) was dissolved in 1 ml of water and 0.1 ml of 10% NH₄HCO₃ was added. A mixture of trypsin and V8 proteinase was added (enzyme/substrate ratio 1:50, w/w, for each enzyme) and incubated overnight at 37°C. The digest was then dried, and redissolved in 10mM-HCl; the A₂₈₀ was measured and from this the concentration of protein estimated by using the absorption coefficient-given below. Zeo-Karb 225 (0.5g) (H⁺ form) was added, left for 10min and removed by centrifugation (at 1500 rev./min (*r*_{av} 11cm) for 10min. The resin was washed with water and the supernatants were freeze-dried. The peptide was redissolved in 100 μl of water and subjected to amino acid analysis.

Amino acid-sequence determination

CNBr fragments of slow-muscle troponin I were sequenced after digestion with trypsin, thermolysin or

V8 proteinase. The CNBr fragments were ordered by the isolation and sequencing of tryptic peptides containing [¹⁴C]carboxymethylmethionine. Peptides were isolated, after proteolytic digestion, by chromatography on columns of Sephadex G-50 (1.9cm×140cm or 2.2cm×110cm) or G-25 (2cm×110cm), eluted with 50mM-NH₄HCO₃ or 10mM-HCl, followed by high-voltage paper electrophoresis by using the method described previously (Wilkinson, 1974), or directly by high-voltage paper electrophoresis with Whatman no. 1 or 3MM paper. Radioactive peptides were detected by radioautography by using Kodak Blue Brand X-ray film.

Peptides containing arginine, histidine, tryptophan and tyrosine residues were detected by the method of Easley (1965). Peptides with a blocked N-terminus were detected by the method of Pan & Deutcher (1956). Amide groups were assigned by the method of Offord (1966). Peptides were sequenced by the dansyl-Edman method as described by Gray (1967a) or by the micro Edman procedure of Gray & Smith (1970). The N-terminal residue of troponin I was determined by the method of Gray (1967b) as modified by Wilkinson (1974).

Results

Properties of slow-muscle troponin I

Troponin I was prepared by the single-step affinity-chromatography procedure of Syska *et al.*

Table 1. Amino acid composition of troponin I from slow and fast skeletal muscle and cardiac muscle

Amino acid	Amino acid composition		
	Slow-skeletal-muscle troponin I (mol/21 000g)	Fast-skeletal-muscle troponin I (mol/21 000g)*	Cardiac-muscle troponin I (mol/24 000g)*
Asp	15.2	17.1	17.7
Thr	5.4	3.3	10.1
Ser	11.1	8.9	8.9
Glu	24.7	33.2	32.2
Pro	6.1	6.3	6.2
Gly	7.6	7.8	11.2
Ala	17.8	15.2	24.5
Val	13.2	7.1	8.5
Met	6.2	7.4	3.2
Ile	4.5	4.9	6.7
Leu	23.6	17.9	22.5
Tyr	2.3	2.0	3.3
Phe	2.4	2.8	4.1
His	4.1	3.6	3.1
Lys	22.9	25.8	23.5
Arg	17.6	14.3	25.0
Cys	4.7	2.7	2.0
Trp	1.4	0.9	1.0
Total	189.6	181.2	213.7

* Values from Grand *et al.* (1976)

(1974) and was shown to be homogeneous by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate at pH 7.0 and in 6M-urea at pH 3.2.

Six red muscles (soleus, vastus intermedius, vastus lateralis, semitendinosus, adductor longus and quadratus femoris) in the hind leg of the rabbit were chosen for investigation, and troponin I was prepared from each of these. Although slow- and fast-muscle troponin I migrate together on electrophoresis in the presence of sodium dodecyl sulphate and in the presence of urea at pH 3.2, they may be distinguished by electrophoresis at pH 8.6 in urea in the presence of fast-muscle troponin C. The complexes formed, in the presence of Ca^{2+} , between troponin C and the two types of troponin I have different mobilities (Syska *et al.*, 1974). Four muscles could, on this basis, be seen to contain predominantly slow troponin I (soleus, vastus intermedius, semitendinosus and adductor longus), one contained wholly fast-muscle troponin I (vastus lateralis), and the other (quadratus femoris), a mixture of slow- and fast-muscle troponin-I species in approximately equimolar amounts. Amino acid analysis of these proteins confirmed this designation. The four slow-muscle troponin-I components had similar analyses, which were different from fast-muscle troponin I (Table 1). The amount of fast-muscle troponin I in the preparations from these muscles was about 10%, judged by electrophoresis at pH 8.6 in the presence of troponin C.

After the initial investigation of the separate muscles to determine which contained slow-muscle and which contained fast-muscle troponin I, the

four slow muscles were homogenized together for routine preparations. The legs from 15 animals were dissected at one time and the muscles frozen until required. Approx. 15 g of slow muscle was obtained from each animal.

The molecular weight of slow-muscle troponin I was found to be 21800 by chromatography in the presence of 6M-guanidine hydrochloride. The protein co-chromatographed with fast-muscle troponin I.

The amino acid composition is shown in Table 1, and was determined after hydrolysis of duplicate samples for 24 and 72 h. The absorption coefficient of slow-muscle troponin I, $A_{280}^{1\%, 1\text{cm}}$, was found to be 5.43.

The *N*-terminal amino acid of the troponin I from each of the four muscles used was proline. The amount of protein with a blocked *N*-terminus was approx. 25% of the whole, estimated by isolation of the peptide X(Glu,Pro) after digestion of the whole protein with V8 proteinase and trypsin.

Isolation of CNBr fragments

After radioactive labelling of cysteine residues, the CNBr digest of slow-muscle troponin was chromatographed on a column (2.2 cm $\times$ 150 cm) of Sephadex G-75, eluted with 6M-urea/0.4M-sodium formate, pH 3.8 (Fig. 1). Fractions were pooled as indicated. Fractions F1–F8 were desalted on columns of Sephadex G-25, G-15 or G-10 eluted with 10 mM-HCl and freeze-dried. Fraction F1 contained the CNBr fragment SCN1, which was shown to be a partial cleavage product (see below). Fraction F2 was

Fig. 1. Chromatography of a CNBr digest of troponin I

The digest, after alkylation of cysteine residues with iodo[^{14}C]acetic acid, was applied to a column (2.2 cm $\times$ 150 cm) of Sephadex G-75 in 6M-urea/0.4M-sodium formate, pH 3.5. —, A_{280} ; ----, radioactivity. Horizontal bars indicate the fractions that were pooled.

Table 2. Amino acid composition of CNBr fragments from slow-skeletal-muscle troponin I

The first column of values for each fragment gives the composition as determined by analysis in mol/mol of peptide. The second column gives the composition calculated from the amino acid sequence. The final column gives the total composition of the protein calculated from the sequence. + indicates the presence of tryptophan or homoserine (Hse) in a peptide identified by u.v. absorption or by partial resolution on the amino acid analyser respectively.

Peptide ...	Composition																Analysis of slow skeletal-muscle troponin I (mol/21000g)	
	SCN1	SCN2		SCN3		SCN4		SCN5		SCN6*		SCN7		SCN8		CF1		
		Ana-lysis	Se-quence	Ana-lysis	Se-quence	Ana-lysis	Se-quence	Ana-lysis	Se-quence	Ana-lysis	Se-quence	Ana-lysis	Se-quence	Ana-lysis	Se-quence	Ana-lysis		Se-quence
Amino acid											6a	6b						
Asp	9.2	7.9	6		4.3	3	1.6	—	—	—	1.1	1.0	1	—	—	—	10	
Asn			1	2	1.2	1	1.0	1	—	—	1.0	0.8	1	—	—	1.0	3	
Thr	3.0	2.5	2		1.7	1	2.3	3	1.9	2	1.8	1.0	2	—	—	2.8	5	
Ser	5.5	3.5	3		5	5	4.0	2	—	—	0.9	—	1	—	—	3.1	12	
Glu	17.5	16.6	10		5.7	—	—	—	—	—	—	—	—	+	—	—	18	
Gln			6			—	—	—	—	—	—	—	—	—	—	—	7	
Pro	6.4	3.4	3		0.8	1	0.7	1	—	—	—	—	1	—	—	0.7	6	
Gly	3.3	2.8	2		1.6	1	0.9	—	1.0	1	1.1	1.1	—	1.0	1	—	6	
Ala	10.8	9.6	11		2.1	2	2.0	1	1.1	1	2.0	1.8	2	—	—	3.4	17	
Val	7.5	6.1	7		3.1	4	1.5	1	1.1	1	—	—	—	—	—	1.3	13	
Ile	3.4	3.2	3		—	—	1.0	1	—	—	—	—	—	—	—	1.0	4	
Leu	16.5	14.5	14		2.8	2	4.0	3	3.0	3	—	—	—	—	—	5.1	22	
Tyr	2.0	2.1	2		—	—	—	—	—	—	—	—	—	—	—	—	2	
Phe	1.0	1.1	1		—	—	—	—	—	—	0.9	1.1	1	—	—	—	2	
His	3.3	3.2	3		—	—	—	—	0.8	1	—	—	—	—	—	—	2	
Lys	14.3	9.5	10		4.2	5	4.1	4	2.2	2	1.0	1.0	1	2.0	2	5.9	4	
Arg	13.2	11.1	12		3.4	3	2.9	2	1.4	1	—	—	—	0.9	1	1.9	24	
Cys	3.0†	3.0	3		—	—	—	—	—	—	—	—	—	—	—	—	19	
Trp	—	—	—		+	+	—	—	—	—	—	—	—	—	—	—	3	
Hse	+	+	1		+	1	+	1	+	1	—	—	—	+	1	—	1	
																	6	

* Yield of 6a/6b is 4:1 (w/w).
† Contains 0.8 mol of methionine/mol.
‡ Determined as carboxymethyl cysteine.

rechromatographed on a column (2cm×115cm) of Sephadex G-75 eluted with 10mM-HCl, and contained the CNBr fragment SCN2. Fraction F3 was found to contain very little peptide material and was discarded. Fractions F4 and F5 were used without further purification and corresponded to peptides SCN3 and SCN4 respectively. However, peptide SCN4 was found, after enzymic digestion, to be contaminated with peptide SCN3. Fractions F6, F7 and F8 were subjected to high-voltage paper electrophoresis and contained peptides SCN5 (in fraction F6), SCN6a, SCN6b and SCN7 (in fraction F7) and SCN8 (in fraction F8). Amino acid analysis and high-voltage electrophoresis of the peptides SCN6a and SCN6b (Table 2) showed them to be similar, except that the former had an extra glutamine and serine residue. The relative yields of peptides SCN6a/SCN6b were approx. 4:1.

Amino acid sequence of CNBr fragments

The amino acid sequence of slow-muscle troponin I is shown in Fig. 2. The positions of the CNBr fragments are also indicated.

Peptide SCN2 was citraconylated to block lysine residues, digested with trypsin and chromatographed on a column (1.9cm×140cm) of Sephadex G50 eluted with 10mM-HCl. The peptides were then purified by high-voltage electrophoresis, and re-digested with V8 proteinase and trypsin. Arginine-containing thermolysin peptides were isolated and used to identify the peptides overlapping the citraconylated tryptic peptides.

Peptides SCN3 and SCN4 were digested with trypsin and thermolysin and peptides SCN5 and SCN6 with trypsin. Peptides SCN7 and SCN8 were sequenced without digestion. The sequences of peptides SCN6a and SCN6b were similar, except that peptide SCN6a was two residues longer.

Tryptic digestion of peptide SCN1 gave rise to a number of peptides characteristic of peptides SCN2 and SCN4, including the *N*-terminal peptide Pro-Glu-Val-Glu-Arg, in both its blocked and unblocked form. Peptide SCN1 is therefore a partial cleavage product, comprising peptides SCN4 and SCN2 in which cleavage at methionine-20 has not occurred. This is analogous to the situation in rabbit cardiac troponin I, in which cleavage at the corresponding methionine has not occurred in the peptide CCN1 (Grand *et al.*, 1976).

Cysteine-containing sequences

The elution pattern of the CNBr digest shown in Fig. 1 shows that the radioactivity is located in peptides SCN1 and SCN2. Since peptide SCN1 is a partial cleavage product containing peptide SCN2, the latter fragment is the only CNBr fragment

Fig. 2. Amino acid sequence of rabbit slow-muscle troponin I. The position of the CNBr fragments is shown.

containing cysteine. The elution profile for the tryptic digest of citraconylated peptide SCN2 (Fig. 3), and high-voltage paper electrophoresis of these peptides shows that three cysteine residues are present. These have been located at positions 27, 63 and 84.

Isolation of methionine-containing peptides

Slow-muscle troponin I was carboxymethylated at the methionine residues with iodo[^{14}C]acetic acid and then digested with trypsin. The digest was chromatographed on a column of Sephadex G-50 (2.2 cm $\times$ 110 cm) eluted with 50 mM- NH_4HCO_3 . The methionine-containing peptides were purified by high-voltage electrophoresis and sequenced and this permitted the CNBr fragments to be ordered in the sequence SCN4-SCN2-SCN5-SCN3-SCN8-SCN7-SCN6a. One of the overlap peptides, Tr2g, had twice the specific radioactivity of the others, and was shown by analysis to contain twice as much methionine. This peptide overlapped the fragments SCN3-SCN8-SCN7.

Supplementary publication

The detailed data used for the elucidation of the sequence of slow-muscle troponin I has been deposited as Supplementary Publication SUP 50079 at the British Library (Lending Division). Details of the chromatography of the tryptic digests of citraconylated peptide SCN2 and of troponin I, carboxymethylated at methionine residues with iodo[^{14}C]acetic acid, are given. The composition and electrophoretic mobility of the peptides isolated from enzymic digests of the CNBr fragments as well as the overlap peptides containing carboxy[^{14}C]methylmethionine are given. Results of the dansyl-Edman method are described.

Discussion

In the past it has been considered that there is a reasonably good correlation between the 'redness' of a muscle and whether it is 'fast' or 'slow'. Two slow, red muscles which have been studied are the soleus and vastus intermedius (also known as the crureus muscle) from rabbit leg. Syska *et al.* (1974) showed that the troponin I from these muscles was not the same as that from fast skeletal muscles. The two proteins could be distinguished on the basis of the mobility of the troponin-I-troponin-C complex on polyacrylamide gels and the electrophoretic pattern of the CNBr peptides.

To obtain sufficient protein for the present study, however, it was necessary to obtain larger amounts of slow muscle than were present in the soleus and vastus intermedius muscles. Therefore an investi-

gation was made of the six 'red' muscles in the hind leg of the rabbit. The troponin I from four of these was primarily of the slow-muscle type (approx. 90%), one yielded only fast-muscle troponin I and one a mixture of slow- and fast-muscle troponin I. Preliminary investigation of the myosin light chains present in the six muscles (N. Frearson, unpublished work) has confirmed the designation of 'slow' and 'fast' based on the troponin-I results. Polyacrylamide-gel electrophoresis at pH 8.6 in 8 M-urea showed that myosin prepared from soleus, vastus intermedius, semitendinosus and adductor longus muscles contained predominantly the light chains characteristic of slow muscle, whereas preparations from vastus lateralis contained predominantly those of fast muscle, and quadratus femoris contained a mixture.

It is probable that the soleus, vastus intermedius, semitendinosus and adductor longus, although primarily slow muscles, contain a small proportion of fast-muscle fibres. However, Amphlett *et al.* (1975) found considerable amounts of fast-muscle troponin I in rabbit soleus muscle; this was judged to account for about 30% of the protein. Conversely Weeds (1976) found no cross-contamination of fast muscle fibres in rabbit and cat soleus and semitendinosus muscles on the basis of light chains of myosin present. It is difficult to explain these inconsistencies without further investigation, but it seems probable that the age and the species of the animals used could have considerable effect on the composition of the red muscles.

The troponin I was prepared from the slow muscles by the single-step affinity-chromatography procedure used previously (Grand *et al.*, 1976). Although the yields were relatively low (approx. 25 mg) the protein was of very high purity, except for the fast-muscle troponin I contamination mentioned above. However, no peptides characteristic only of the fast-muscle protein were detected at any time.

Results of the amino acid analysis of slow-muscle troponin I (Table 1) are in good agreement with the composition derived from the sequence data (Table 2). The protein has an overall net positive charge of 18 at pH 7 (compared with 14 and 9 for the cardiac and fast-muscle proteins respectively). Slow-muscle troponin I had a calculated mol.wt. of 21 146, which is in good agreement with the value of 21 800 determined by gel filtration, and was composed of 184 amino acid residues. Rabbit fast-skeletal-muscle troponin I comprised 178 residues and had a calculated mol.wt. of 20 708 (Wilkinson & Grand, 1975). Rabbit cardiac troponin I comprised 206 residues and had a mol.wt. of 23 550 (Grand *et al.*, 1976).

The sequence of the slow-muscle troponin-I CNBr fragments has been determined after subdigestion with trypsin, thermolysin and V8 proteinase. The CNBr fragments have been ordered unequivocally after isolation of the tryptic peptides con-

		10	20	30
Slow				Pro-Glu-Val
Fast				X-Asp Glu
Cardiac	X-Ala-Asp-Glu-Ser-Arg-Asp-Ala-Ala-Gly-Glu-Ala-Arg-Pro-Ala-Val-Arg-Arg-Ser-Asp-Arg-Ala-Tyr-Ala-Thr-Glu			His-Ala
		40	50	60
Slow	Glu-Arg-Lys-Ser-Lys-Ile-Thr-Ala-Ser-Arg-Lys-Leu —			Gln-Gln-Glu-His-Glu-
Fast	Lys Asn-Arg-Ala —			Ala-Thr-Glu-Leu-Glu-Lys-Glu
Cardiac	Lys-Ser Lys Ser			Gln-Ile Gln-Ile Gln-Glu-Leu-Glu-Arg-Glu-Ala
		70	80	90
Slow	Ala —			Arg-Glu-Ala-Glu-Lys-Val-Arg-Tyr-Leu-Ala-Glu-Arg-Ile-Pro-Ala-Leu-Gln-Thr-Arg-Gly-Leu-Ser-Leu-Ser-Ala-Leu-Gln-Asp-
Fast	Gly-Arg Gln-Asn			His-Cys Pro Ser-Leu-Pro — Met-Ala-Glu-Val Glu
Cardiac	Glu-Glu Arg-Gly Gly Ala Ser-Thr Cys-Gln-Pro Glu-Leu-Ala			Gly-Phe-Ala-Glu
		100	110	120
Slow	Leu-Cys-Arg-Gln-Leu-His-Ala-Lys-Val-Glu-Val-Val-Asp-Glu-Glu-Arg-Tyr-Asp-Ile-Glu-Ala-Lys-Cys-Leu-His-Asn-Thr-Arg-Glu-Ile-			Leu
Fast	Lys			Val-Gln-Lys-Ser-Ser-Lys
Cardiac	Arg Asp-Lys			Val-Thr-Lys Ile-Thr
		130	140	150
Slow	Lys-Asp-Leu-Lys-Leu-Lys-Val-Leu-Asp-Leu-Arg-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg —			Arg-Val-Arg-Val-Ser-Ala-Asp-Ala-Met-
Fast	Glu Met-Asn-Gln Leu-Phe			Met
Cardiac	Ala Thr-Gln Ile-Phe			Ile
		160	170	180
Slow	Leu-Arg-Ala-Leu-Leu-Gly-Ser-Lys-His-Lys-Val-Ser-Met-Asp-Leu-Arg-Ala-Asn-Leu-Lys-Ser-Val-Lys-Lys-Glu-Asp-Thr-Glu-Lys-Glu-			
Fast	Lys			
Cardiac	Met-Gln Thr-Arg-Ala Glu-Thr-Leu Cys			
		190	200	210
Slow	— Arg-Pro-Val-Glu-Val-Gly-Asp-Trp-Arg-Lys-Asn-Val-Glu-Ala-Met-Ser-Gly-Met-Glu-Gly-Arg-Lys-Lys-Met-Phe-Asp-Ala-Ala-Lys-			
Fast	— — — Asp			Glu-Ser-Glu-Ser
Cardiac	Asn — —			Lys Glu-Gly — —
Slow	Ser-Pro-Thr-Ser-Gln			
Fast	— — — —			
Cardiac	— — — —			

Fig. 3. Comparison of the amino acid sequences of rabbit slow-, fast- and cardiac-muscle troponin I

The sequence of slow-muscle troponin I is given on the top line; where the fast- and cardiac-muscle sequences differ, the alternative amino acid is given; where the sequences are the same, no amino acid is given. — denotes a deletion. X denotes an unknown blocking group.

taining carboxy[^{14}C]methylmethionine residues. There is one point in the sequence, between residues 8 and 9, at which overlap evidence has not been obtained; digestion with both thermolysin and trypsin results in cleavage at residue 8, giving peptides with C-terminal lysine. The situation was further complicated by the fact that all preparations of the N-terminal CNBr fragment (SCN4) were impure. The analysis of peptide SCN4 (Table 2) shows that appreciable amounts of aspartic acid and glycine were present, but no tryptic or thermolysin peptides, which were not characteristic of other CNBr fragments, containing aspartic acid or glycine could be found. In an attempt to resolve this point, the N-terminal cysteine fragment was isolated in pure form by using affinity binding to a troponin C-Sepharose column. (The analysis of this fragment is given in Table 2.) This experiment also locates the troponin-C-binding site in the area on the N-terminal side of residue 26 (55 in the numbering of cardiac troponin I). The analysis of the peptide CF1 shows that it contains no aspartic acid or glycine and is in very good agreement with the peptides already isolated from peptide SCN4. It is assumed, on the basis of this evidence, that the sequence is as shown in Fig. 2 and that no other amino acids are present between the lysine and isoleucine residues. Analysis of the whole protein shows a slight excess of aspartic acid and glycine over the contents shown in the sequence (Table 2) but it seems probable, in view of the other evidence, that this discrepancy is not significant. On the basis of peptide mobility however, it is possible that one of the carboxyl residues between 71 and 79 carries an amide group.

Slow-muscle troponin I seems to exist in at least two forms, with and without a blocking group present on the N-terminal proline residue, and possibly with variation at the C-terminus. A determination of the N-terminal amino acid of the whole protein and of the CNBr fragments SCN1 and SCN4 gave proline. However, digestion of these with trypsin allowed the isolation of two forms of the peptide Pro-Glu-Val-Glu-Arg, one having a blocked N-terminus. The relative proportions of blocked/unblocked protein was approx. 3:1 (mol/mol) as determined by the isolation of the peptide X(Glu, Pro). This is the only example of a myofibrillar protein without a blocked N-terminus so far isolated.

Two forms of the C-terminal CNBr peptide, SCN6, were also isolated, one being two residues longer than the other. Whether this is simply a result of proteolytic degradation or reflects the situation present *in vivo* is not clear. No explanation can be given at present of the polymorphism of slow-muscle troponin I. The variation at the N-terminus is not coincident with variation in muscle, as the troponin I from all the slow muscles examined had N-terminal proline. It is not clear whether the two

types coincide with different fibre types or coexist within the same fibre.

A number of deletions must be introduced into the troponin-I sequences, and these are shown in Fig. 3 (the numbering of residues begins at the N-terminus of cardiac troponin I). The area from residue 181 to 184 is markedly different in the three proteins, each having at least one deletion. No explanation can be offered for this, but it is particularly remarkable, as the sequences on either side of this area are homologous.

The similarities in the three sequences are particularly marked in the C-terminal half of the molecules, whereas variation is much more apparent towards the N-terminus, although the presumed troponin-C-binding site, from about residue 44 to 54, is rather more homologous than the surrounding area. The proposed actin-binding sites from residues 125 to 145 (Syska *et al.*, 1976) in the three molecules are very similar indeed and lend support to the view that the actin molecules present in slow, fast and cardiac muscle are very similar. However, it seems unlikely that the slight variations in actin-binding sites between the three forms of troponin I could be responsible for the large variations in inhibitory activity noted by Syska *et al.* (1974).

Although the binding site for troponin C has not been located as closely as that for actin, it seems to occupy the area approximately from residue 40 to residue 60. The similarities between the three troponin I components are not particularly marked, and this may be due to variations in the troponin C species in fast, slow and cardiac muscle. Consideration of the troponin-C sequences determined for rabbit fast skeletal muscle, ox cardiac muscle and chicken fast skeletal muscle by Collins *et al.* (1973), Van Eerd & Takahashi (1976) and Wilkinson (1976) respectively indicates that most of the variations in the cardiac and fast-muscle proteins are due to tissue rather than species differences. This probably accounts for the variation in the troponin-C-binding sites in these troponin-I species. Head *et al.* (1977), however, have shown that troponin C from rabbit soleus muscle is very similar to the fast-skeletal-muscle protein. On the basis of the present sequence data for slow-muscle troponin I it is probable that differences between the slow- and fast-muscle troponin C species would be as marked as those between cardiac and fast-muscle proteins. This point will remain unresolved until the sequence of the slow-muscle troponin C is determined.

Note Added in Proof (Received 27 July 1977)

Residue 12 of the amino acid sequence of rabbit cardiac troponin I (Grand *et al.*, 1976) has been shown to be arginine, and not lysine as has been reported previously.

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References

- Amphlett, G. W., Perry, S. V., Syska, H., Brown, M. D. & Vrbova, G. (1975) *Nature (London)* **257**, 602–604
- Babul, J. & Stellwagen, E. (1969) *Anal. Biochem.* **28**, 216–221
- Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G. & Jackman, N. (1973) *FEBS Lett.* **36**, 268–272
- Crompton, M. J. & Wilkinson, J. M. (1963) *Biochem. J.* **88**, 228–234
- Easley, C. W. (1965) *Biochim. Biophys. Acta* **107**, 386–388
- Ebashi, S. (1974) *Essays Biochem.* **10**, 1–36
- Grand, R. J. A., Wilkinson, J. M. & Mole, L. E. (1976) *Biochem. J.* **159**, 633–641
- Gray, W. R. (1967a) *Methods Enzymol.* **11**, 469–475
- Gray, W. R. (1967b) *Methods Enzymol.* **11**, 139–151
- Gray, W. R. & Smith, J. F. (1970) *Anal. Biochem.* **33**, 36–42
- Head, J. F., Weeks, R. A. & Perry, S. V. (1977) *Biochem. J.* **161**, 465–471
- Houmard, J. & Drapeau, G. R. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3506–3509
- Jacobson, G. R., Schaffer, M. H., Stark, G. R. & Vanaman, T. C. (1973) *J. Biol. Chem.* **248**, 6583–6591
- Moore, S. (1963) *J. Biol. Chem.* **238**, 235–237
- Offord, R. E. (1966) *Nature (London)* **211**, 591–593
- Pan, S. C. & Deutcher, J. D. (1956) *Anal. Chem.* **28**, 836–838
- Perry, S. V. & Cole, H. A. (1974) *Biochem. J.* **141**, 733–743
- Syska, H., Perry, S. V. & Trayer, I. P. (1974) *FEBS Lett.* **40**, 253–257
- Syska, H., Wilkinson, J. M., Grand, R. J. A. & Perry, S. V. (1976) *Biochem. J.* **153**, 375–387
- van Eerd, J. P. & Takahashi, K. (1976) *Biochemistry* **15**, 1171–1180
- Weber, A. & Murry, J. M. (1973) *Physiol. Rev.* **53**, 612–673
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Weeds, A. G. (1976) *Eur. J. Biochem.* **66**, 157–173
- Wilkinson, J. M. (1969) *FEBS Lett.* **4**, 170–172
- Wilkinson, J. M. (1974) *Biochim. Biophys. Acta* **359**, 379–388
- Wilkinson, J. M. (1976) *FEBS Lett.* **70**, 254–256
- Wilkinson, J. M. & Grand, R. J. A. (1975) *Biochem. J.* **149**, 493–496
- Wilkinson, J. M., Perry, S. V., Cole, H. A. & Trayer, I. P. (1972) *Biochem. J.* **127**, 215–228