The Components of Troponin from Chicken Fast Skeletal Muscle

A COMPARISON OF TROPONIN T AND TROPONIN I FROM BREAST AND LEG MUSCLE

By J. MICHAEL WILKINSON

*Department of Biochemistry, University of Birmingham, P.O. Box 363,
Birmingham B15 2TT, U.K.*

(Received 6 July 1977)

The three components of troponin were prepared from chicken breast and leg muscle. The troponin I and T components were separated by chromatography on DEAE-cellulose after citraconylation and without the use of urea-containing buffers. The troponin I and C components were similar to their counterparts from rabbit fast skeletal muscle, and a comparison of the troponin I components from breast and leg muscle by amino acid analysis, gel electrophoresis and peptide 'mapping' provides strong evidence for the identity of these proteins. The molecular weights of the troponin T components from breast and leg muscle were 33 500 and 30 500 respectively, determined by gel filtration. A comparison of these two proteins by methods similar to those used for the troponin I components suggested that they differed only in the *N*-terminal region of the sequence, the breast-muscle troponin T having an extra length of polypeptide chain of approx. 24 residues that is rich in histidine and alanine. The *N*-terminal hexapeptide sequence, however, is the same in both proteins and is (Ser,Asx,Glx)Thr-Glu-Glu. The genetic implications of these findings are considered.

Control of contractile activity in striated muscle by Ca^{2+} is mediated by the proteins of the troponin complex and by tropomyosin. In rabbit fast skeletal muscle the three components of troponin, troponin C, troponin I and troponin T, have been well characterized and their amino acid sequences determined (Collins *et al.*, 1973; Wilkinson & Grand, 1975; Pearlstone *et al.*, 1976). Studies of troponin from the other types of striated muscle have shown that although the components are similar in function they differ considerably between muscle types. Thus in cardiac muscle the amino acid sequences of troponin C (van Eerd & Takahashi, 1975) and troponin I (Grand *et al.*, 1976) differ markedly from their fast-skeletal-muscle counterparts, and the molecular weight of the troponin T components has been shown to be greater (Greaser *et al.*, 1972; Tsukui & Ebashi, 1973). In slow skeletal muscle the troponin I component has been shown to differ by a number of criteria from the analogous proteins from fast and cardiac muscle (Syska *et al.*, 1974), and the troponin C component appears to be quite similar to that from fast muscle (Head *et al.*, 1977). So far no studies of troponin T from slow muscle have been made.

The present study was undertaken to characterize the troponin components from the fast skeletal muscle from another species to assess the rate of evolution of the components within a single muscle type. Chicken muscle was chosen because of its ready availability

Abbreviations used: AcSer, *N*-acetyls erine; Dns, 5-dimethylaminonaphthalene-1-sulphonyl.

and because, with the rabbit, it has been extensively used in the study of muscle structure. Several workers have reported that the components of chicken troponin are similar to those of the rabbit (Hirabayashi & Perry, 1974; Perry & Cole, 1974; Hitchcock, 1975), and Perry & Cole (1974) have shown that troponin T isolated from breast and leg muscle differs in molecular weight as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, that from the leg being similar to rabbit troponin T, whereas that from the breast has a mol.wt. of approx. 44 000. Hirabayashi & Perry (1974) reported that troponin C from a mixed breast- and leg-muscle preparation was a single antigen, and thus is probably the same protein in both muscles. However, troponin I contained two antigens, one of which was a minor component, and it is not clear if this represents the presence of a small amount of slow-muscle troponin I or if there are different forms of troponin I in breast and leg muscles.

In the light of these results troponin T and troponin I were prepared separately from breast and leg muscle, and troponin C was prepared from a mixture of the two muscles. A comparison of the troponin I components has shown that they are in all probability identical, and it seems likely that the troponin T components differ only at the *N*-terminal end.

Materials and Methods

Troponin B, the mixture of troponins I and T, was prepared from both breast and leg muscle of chicken

by extraction of troponin with 1.2M-KCl/0.1M-HCl as described previously (Wilkinson, 1974). A troponin C-rich fraction was prepared from the precipitate obtained from the 1.2M-KCl/0.1M-HCl extract by re-extraction with 10mM-Tris/HCl, pH 8.0. The supernatant from this extraction was dialysed against water and freeze-dried. This material was used for the preparation of troponin C.

Troponins I and T were purified by chromatography of troponin B on DEAE-cellulose as follows. Troponin B (1 g) was dissolved in 30 ml of 10mM-HCl and the pH was adjusted to 8 with 5M-NaOH, which caused the precipitation of the protein. Citraconic anhydride (0.5 ml) was added and the pH maintained at 8 with 5M-NaOH until a clear solution was obtained and the pH was stable. The mixture of citraconylated proteins was then dialysed against 0.2M-Tris/0.1M-HCl, pH 8.2, to remove excess of reagents. The solution of citraconylated proteins was applied to a column (3cm×10cm) of DEAE-cellulose (Whatman DE52) equilibrated with 0.2M-Tris/0.1M-HCl, pH 8.2, and the column eluted with a gradient of the same buffer containing 0–0.4M-NaCl. Protein peaks were pooled and the citraconyl blocking groups removed by dialysis against 10mM-HCl.

Troponin C was purified by chromatography on DEAE-cellulose by the method of Perry & Cole (1974).

Analytical methods

Amino acid analysis, cysteine and tryptophan determinations, determination of absorption coefficients and molecular-weight determination by chromatography on Sepharose 6B in the presence of 6M-guanidine hydrochloride were performed as described by Grand *et al.* (1976). The isolation of peptides with a blocked *N*-terminus was as described by Wilkinson (1974). Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate and in urea at pH 3.2 and 8.6 were performed as described by Syska *et al.* (1976).

Amino acid sequence determination

Peptides were purified by high-voltage paper electrophoresis at pH 6.5, 3.5 and 2.0 as described by Wilkinson (1974). Peptides were further digested with thermolysin (Calbiochem, San Diego, CA, U.S.A.) or the proteinase from the V8 strain of *Staphylococcus aureus* (Miles Laboratories, Stoke Poges, Bucks., U.K.) in 1% NH₄HCO₃, or with pepsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) in 10mM-HCl, all at an enzyme/substrate ratio of 1:50 (w/w) at 37°C. Thermolysin digests were allowed to proceed for 3 h and pepsin and V8-proteinase digests for 16 h. The methods for the use of the dansyl-Edman technique and the assignment of amide groups have been described previously (Wilkinson & Grand, 1975).

Carboxypeptidase B digestion

Troponin T (0.5mg) was dissolved in 250 µl of 10mM-HCl, precipitated by the addition of 50 µl of 100% (w/v) trichloroacetic acid and then washed with 300 µl of 20% (w/v) trichloroacetic acid and twice with 250 µl of acetone. The protein was then dissolved in 100 µl of 0.2M-*N*-ethylmorpholine adjusted to pH 8.5 with 1M-acetic acid, and 25 µl of a solution (10 µl in 4 ml of the *N*-ethylmorpholine buffer) of carboxypeptidase B (Worthington) was added and the mixture incubated at 37°C. Samples (25 µl) were taken at intervals of 0, 15, 30 and 60 min, frozen immediately and freeze-dried. The samples, after digestion, were dansylated as usual and the free amino acids present were identified as their dansyl derivatives by polyamide-layer chromatography (Wilkinson & Grand, 1975).

Peptide 'mapping'

Peptide 'maps' of the tryptic peptides of proteins were prepared as follows. Troponin T and troponin I, in which the methionine residues had been alkylated with iodo[¹⁴C]acetamide (Wilkinson, 1968), were dissolved in 1% NH₄HCO₃ and digested with 2% (w/w) of trypsin [treated with 1-chloro-4-phenyl-3-toluene-*p*-sulphonylamidobutan-2-one ('TPCK')] for 3 h at 37°C. The digest was then chromatographed on a column (1.8cm×140cm) of either Sephadex G-50 for troponin T, or Sephadex G-25 for troponin I, in 50mM-NH₄HCO₃. Samples (100 µl) were removed from alternate tubes, evaporated to dryness, redissolved in 5 µl of water and applied to Whatman no. 1 chromatography paper for high-voltage electrophoresis at pH 6.5. The resulting peptide 'maps' were radioautographed and subsequently stained with ninhydrin (Wilkinson, 1968).

Results

Troponin B was prepared from chicken breast and leg muscle by the method previously described for rabbit muscle (Wilkinson, 1974). The method gave results comparable with those obtained from rabbit muscle and with the same overall yield. The use of citraconylation (Dixon & Perham, 1968) as a reversible blocking group for troponin B gave a preparation that was soluble without the use of high concentrations of urea, and the components were separated by chromatography on DEAE-cellulose in dilute aqueous buffer by the use of a salt gradient. Such a separation is shown in Fig. 1. Removal of the citraconyl groups was accomplished by dialysis against 10mM-HCl. Gel electrophoresis showed that the first peak was troponin I and the second peak was troponin T. There was no observable difference between the chromatography of the breast and leg

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of chicken troponin components

(a) Troponin B from breast muscle; (b) troponin T from breast muscle; (c) troponin I from breast muscle; (d) troponin B from leg muscle; (e) troponin T from leg muscle; (f) troponin I from leg muscle; (g) troponin C from mixed breast and leg muscle.

EXPLANATION OF PLATE 2

Polyacrylamide-gel electrophoresis at pH 3.2 of the troponin T and I components and their CNBr peptides

Gel conditions were 7.5% (w/v) polyacrylamide, 6.25M-urea and 0.9M-acetic acid. (a) Breast-muscle troponin I; (b) CNBr digest of breast-muscle troponin I; (c) leg-muscle troponin I; (d) CNBr digest of leg-muscle troponin I; (e) breast-muscle troponin T; (f) CNBr digest of breast-muscle troponin T; (g) leg-muscle troponin T; (h) CNBr digest of leg-muscle troponin T.

Fig. 1. *Chromatography of citraconylated troponin B* Citraconylated troponin B was applied to a column of DEAE-cellulose in 0.2M-Tris/0.1M-HCl, pH8.2, and eluted with a linear gradient of 0–0.4M-NaCl. The total volume of the gradient was 400ml. Horizontal bars indicate the fractions that were pooled.

Table 1. *Absorption coefficients and molecular weights of troponin components*
Abbreviation: ND, not determined.

	$A_{280}^{1\%}$, 1 cm	Mol.wt.
Breast-muscle troponin T	6.23	33 500
Leg-muscle troponin T	7.66	30 500
Breast-muscle troponin I	4.97	ND
Leg-muscle troponin I	5.60	ND

37000 obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Perry & Cole, 1974), but that for leg-muscle troponin T is in good agreement with the value of 30500 determined for rabbit troponin T by Pearlstone *et al.* (1976) from the amino acid sequence.

The amino acid composition of each of the proteins is shown in Table 2. The values obtained for troponin C are in good agreement with those obtained previously by Hirabyashi & Perry (1974) and confirm the absence of tyrosine and tryptophan from this protein. The compositions of the two troponin I components are very similar, the greatest difference being in the values for alanine. Chicken troponin I bears a close resemblance to rabbit troponin I (Wilkinson, 1974); however, it only appears to contain one cysteine residue. The overall compositions of the troponin T components are also similar, both to each other and to rabbit troponin T. The most striking difference between the breast- and leg-muscle proteins is in the value for histidine, which in the breast-muscle protein is more than twice that in the leg-muscle protein; there is also a significant difference between the alanine values. The difference between the methionine values is probably not significant, as this residue is susceptible to oxidation. The amounts of cysteic acid found in the troponin T preparations after performic acid oxidation were less than unity and it is not clear if either protein contains any cysteine.

Absorption coefficients were determined for both troponins T and I, and the values are shown in Table 1.

Comparison of troponin I from breast and leg muscle

As the amino acid compositions of troponin I from breast and leg muscle suggest that they are very similar, if not the same protein, other comparisons were made to investigate their relationship.

Complex-formation with troponin C was studied by using gel electrophoresis in 6M-urea at pH8.6 by the method of Head & Perry (1974). Complexes were formed, as expected, by both the troponin I components with chicken troponin C. The electrophoretic mobility of the troponin I–troponin C complex was the same in each case.

preparations. The u.v. spectra of the unblocked proteins showed no evidence of irreversible blocking.

Both troponin I components possessed inhibitory activity when tested in a system containing desensitized actomyosin from rabbit fast muscle (H. Syska, personal communication) and this activity was very similar to that obtained with rabbit fast-muscle troponin I.

Re-extraction of the precipitate from the troponin B preparation with 10mM-Tris/HCl, pH8.0, gave a fraction that was rich in troponin C but also contained some troponin T and troponin I. The troponin C was purified from this material by chromatography on DEAE-cellulose in a buffer containing 8M-urea.

Polyacrylamide-gel electrophoresis of the proteins in sodium dodecyl sulphate (Plate 1) and in 6M-urea at pH3.2 (Plate 2) showed that each of them was substantially a single component. The relative molecular weights were those expected from previous studies, and as the troponin I and C components correspond to their counterparts from rabbit troponin, their molecular weights were taken as 20 500 and 18 000 respectively, these being the molecular weights of the rabbit proteins obtained from their amino acid sequences. As expected, the troponin T from breast muscle was apparently larger than that from leg muscle, and their molecular weights were determined by chromatography on Sepharose 6B in 6M-guanidine hydrochloride. The values obtained were 33 500 and 30 500 for breast- and leg-muscle troponin T respectively (Table 1). These values are considerably lower than the previous determinations of 44 000 and

Table 2. *Amino acid composition of troponin components*

		Composition (residues/molecule)				
		Breast-muscle troponin T	Leg-muscle troponin T	Breast-muscle troponin I	Leg-muscle troponin I	Troponin C
		33 500	30 500	20 500	20 500	18 000
Mol.wt. ...						
Asp		24.2	23.2	16.3	16.5	24.8
Thr		8.3	7.2	7.9	7.1	7.0
Ser		8.4	8.8	8.4	9.5	5.8
Glu		54.2	53.3	26.6	27.1	28.3
Pro		8.2	6.5	8.0	6.8	1.4
Gly		12.5	10.7	9.7	8.8	13.7
Ala		32.5	28.1	17.2	15.4	13.5
Val		9.2	8.6	9.9	9.0	6.3
Met		4.5	2.8	5.2	5.6	10.7
Ile		11.8	11.7	5.3	4.8	10.7
Leu		23.3	21.4	18.3	19.2	11.4
Tyr		4.9	5.1	2.7	2.5	—
Phe		7.2	4.9	4.0	3.5	10.0
His		13.9	6.2	4.6	4.6	0.9
Lys		38.5	38.3	22.8	23.9	9.9
Arg		22.2	22.2	11.8	12.9	5.6
Cys		0.9	0.5	1.2	1.0	1.3
Trp		2.6	2.8	1.2	1.2	—
Total		287.3	262.3	181.1	179.4	161.3

A comparison was also made between the CNBr fragments from each protein by electrophoresis in 6M-urea at pH 3.2 (Plate 2). The parent proteins had the same mobility in this system, but although the overall pattern of the CNBr fragments (which gave a somewhat diffuse set of bands) was similar, there were some differences. The diffuse nature of the bands may be due to the small size of the fragments, and the differences may be caused either by differences in primary structure or by the existence in one or other digest of partial-cleavage products, but it is not possible to draw a firm conclusion from this result.

The final method of comparison was by peptide 'mapping'. The methionine residues in a sample of each protein were alkylated with iodo[^{14}C]acetamide, the proteins were digested with trypsin and chromatographed on a column of Sephadex G-25 in 50mM-NH₄HCO₃. The elution profiles of each protein are shown in Fig. 2. The pattern of u.v. absorption at both 215 and 280nm and also of radioactivity differ only in the relative sizes of the peaks, whereas the position of the peaks is identical in the two chromatograms. Samples were taken from alternate tubes across each of the two chromatograms and were submitted to electrophoresis at pH 6.5. The resulting peptide 'maps' were stained with ninhydrin and radioautograms were prepared. No difference could be detected either in the pattern of ninhydrin-positive peptides or in the radioactive methionine-containing peptides.

Comparison of troponin T from breast and leg muscle

In view of the differences in the amino acid compositions of the two troponin T components referred to above, experiments similar to those used to investigate the troponin I components were made with the breast- and leg-muscle troponin T components.

A small amount of each protein was digested with CNBr and the fragments were separated by polyacrylamide-gel electrophoresis in 6M-urea at pH 3.5. The results obtained are shown in Plate 2. The two proteins had different mobilities in this system, as did some of their respective CNBr fragments. Apart from a small amount of undigested protein in each digest, the breast-muscle troponin T gave two major bands whereas the leg-muscle troponin T gave three, two of these being a closely spaced doublet. The major band with the highest mobility appeared to be the same in each protein. In addition, there were some minor bands that again appeared to be common to the two proteins. An electrophoretic analysis of the CNBr fragments of rabbit troponin T has been carried out under the same conditions by Jackson *et al.* (1975). The pattern that they obtained was similar to that reported here for chicken troponin T, and consisted of two major and a number of minor bands. They showed that the major band of higher mobility is the second fragment, CNB2, from the *N*-terminal end, and the slower band was due to the partial-digestion product, CNB1', consisting of the first two CNBr fragments, the other fragments only appearing as

Fig. 2. Chromatography of tryptic digests of troponin I

A tryptic digest of troponin I, with the methionine residues alkylated with iodo[^{14}C]acetamide, was applied to a column (1.8 cm $\times$ 140 cm) of Sephadex G-50 in 50 mM- NH_4HCO_3 . (a) Breast-muscle troponin I; (b) leg-muscle troponin I. —, A_{215} ; $\cdots$, A_{280} ; ----, radioactivity.

minor bands on the gel. If the distribution of methionine residues and the pattern of CNBr cleavage is similar in the chicken proteins, the gels of the CNBr digests may be interpreted in a similar way, i.e. that the second CNBr fragment is similar in the breast- and leg-muscle proteins but they differ in the *N*-terminal fragment.

Peptide 'maps' were prepared in the same way as for the troponin I components. The methionine residues in samples of the two troponin T components were alkylated with iodo[^{14}C]acetamide. The proteins

Fig. 3. Chromatography of tryptic digests of troponin T

A tryptic digest of troponin T, with the methionine residues alkylated with iodo[^{14}C]acetamide, was applied to a column (1.8 cm $\times$ 140 cm) of Sephadex G-50 in 50 mM- NH_4HCO_3 . (a) Breast-muscle troponin T; (b) leg-muscle troponin T. —, A_{215} ; $\cdots$, A_{280} ; ----, radioactivity. Horizontal bars indicate the elution positions of the *N*-terminal tryptic peptides.

were digested with trypsin and the tryptic peptides separated by chromatography on Sephadex G-50 in 50 mM- NH_4HCO_3 . The elution profiles for the two proteins are shown in Fig. 3, and are very similar to one another, with the exception of the first peak eluted from each column. Samples from alternate tubes were taken, starting at an elution volume of 210 ml. The peptides in these samples were 'mapped' by electrophoresis at pH 6.5 in the same manner as for the troponin I peptides. The 'maps' were stained with ninhydrin and for tyrosine-containing peptides and were also radioautographed. No obvious differences in the pattern of peptides were observed, and in particular the pattern of radioactive peptides containing methionine was the same. It therefore seems probable that the two proteins possess a considerable amount of sequence in common and that they contain the

same number of methionine residues, the sequence around these residues being the same.

By far the largest tryptic peptide obtained from rabbit troponin T comes from the *N*-terminal end of the molecule and is 42 residues long (Pearlstone *et al.*, 1976). If a tryptic digest of rabbit troponin T is chromatographed on Sephadex G-50 under conditions similar to those used here, the *N*-terminal peptide is eluted near the front of the column and is well separated from the rest of the peptides (Moir *et al.*, 1977). It is likely that the peaks eluted near the front of the Sephadex G-50 column from the tryptic digests of the chicken troponin T components are the *N*-terminal peptides of these proteins and that the larger size of the breast-muscle troponin T accounts for the earlier elution of the first peak from the digest of this protein.

High-voltage electrophoresis at pH 6.5 showed that the material in each peak was a single component, the peptides from breast- and leg-muscle troponin T having mobilities of -0.27 and -0.67 respectively, relative to aspartic acid = -1.0 . Dansylation showed that neither peptide contained a free *N*-terminal amino acid. The amino acid compositions of the peptides are shown in Table 3 and are compared with that of the *N*-terminal tryptic peptide from rabbit troponin T taken from the sequence of Pearlstone *et al.* (1976). There is a strong similarity between the composition of the peptide from leg-muscle troponin T and that from rabbit troponin T. The peptide from breast-muscle troponin T is approx. 28 residues longer than that from the leg-muscle protein, and this agrees quite well with the difference in size found be-

tween the two complete proteins. The differences in composition are almost wholly in the amounts of glutamic acid, alanine and histidine, those in the breast-muscle troponin T peptide being greatly in excess of those in the leg-muscle peptide. The amount of histidine in the breast-muscle troponin T peptide is greater than that found in the whole protein. The reason for this is not clear, but it seems unlikely that this is due to contamination with a small peptide, as only a single component was found by electrophoresis. The breast-muscle peptide, unlike that from the leg muscle, contains no tyrosine. Although these differences are similar to those noted above between the compositions of the whole proteins, they are somewhat greater than would be expected from that comparison.

N- and C-Terminal sequences of troponin T

For investigation of the *N*-terminal amino acid sequences of the breast- and leg-muscle troponin T components, samples of each protein were digested with Pronase and the digest was passed through a column (0.6 cm $\times$ 8.0 cm) of Zeo-Karb 225 (H^+ form). Peptides having no free amino group are not retarded by such a column, and a peptide of composition (Asx,Thr,Ser,Glx₃) was isolated in good yield from both proteins. These peptides had an electrophoretic mobility of -1.24 at pH 6.5, relative to aspartic acid = -1.0 . Redigestion of these peptides with pepsin gave rise to a ninhydrin-negative peptide of composition (Asx,Ser,Glx) and a ninhydrin-positive peptide of composition (Thr,Glx₂) and mobility -0.85 at pH 6.5. Edman degradation of the second peptide showed it to have the sequence Thr-Glu-Glu. The *N*-terminal sequence of both proteins is thus (Ser,Asx,Glx)Thr-Glu-Glu. This is very similar to the *N*-terminal sequence of rabbit troponin T, which is AcSer-Asp-Glu-Glu-Val-Gln (Wilkinson, 1974; Pearlstone *et al.*, 1976). It has not been possible to determine the sequence of the first three residues, but they may well have the same sequence as in the rabbit protein. The *N*-terminal serine residue in the rabbit protein is phosphorylated (Moir *et al.*, 1977), but insufficient material was available to check whether this was also the case in the chicken proteins.

The C-terminal sequence of each protein was determined by digestion with carboxypeptidase B. The breast and leg proteins were digested in parallel with a sample of rabbit troponin T as a control. The results from all three proteins were the same. After digestion for 15 min, lysine was the only amino acid released, after 30 min some tryptophan was also released and after 60 min strong spots of bis-Dns-lysine and Dns-tryptophan were the only derivatives visible. The two chicken troponin T proteins thus have the same C-terminal sequence, which is the same as that for rabbit troponin T, namely Trp-Lys.

Table 3. Amino acid composition of *N*-terminal peptides from troponin T

	Composition (residues/molecule)		
	Chicken troponin T		Rabbit troponin T*
	Breast-muscle	Leg-muscle	
Asp	0.8	1.3	1
Thr	0.8	1.0	—
Ser	0.8	1.0	2
Glu	23.3	18.3	17
Pro	5.0	4.8	6
Gly	1.3	1.6	—
Ala	14.1	1.6	4
Val	2.5	2.8	6
Tyr	—	0.8	—
His	16.5	3.3	4
Lys	1.1	1.1	1
Arg	0.8	0.9	1
Total	67	38.5	42

* Taken from sequence of Pearlstone *et al.* (1976).

Sequence of tryptic peptides

The *N*-terminal tryptic peptides from each protein were digested with pepsin and the *N*-terminal peptides from these digests were isolated in the same manner as those from the whole proteins. A peptide of composition (Asx,Thr,Ser,Glx₂), with no free α -amino group, was obtained in good yield from each digest, confirming the fact that the two proteins have an identical *N*-terminal sequence. Apart from these peptides a large number of others were obtained, many in poor yield, and although they were clearly related to the sequence of rabbit troponin T it was not possible to order them into a sequence.

The only other peptide from the leg-muscle tryptic peptide that could be positioned with confidence had the sequence Glu-Glu-Lys-Pro-Arg. It is identical with the *C*-terminal pentapeptide from the rabbit troponin T *N*-terminal tryptic peptide. Attempts to isolate a similar peptide from the breast-muscle troponin T peptide were unsuccessful, but a larger peptide was isolated in poor yield, which also contained lysine, proline and arginine and may be related to it.

The sequences of the two troponin T components in the remainder of the molecules were investigated by isolating a certain number of tryptic peptides from the remaining fractions of the tryptic digests (Fig. 3). No attempt was made to isolate all the tryptic peptides, as the number and distribution of lysine and arginine residues in rabbit troponin T suggested that there would be a large number of small peptides, some of very similar composition, and that there would also be a number of points of partial cleavage. Attention

was therefore concentrated on those peptides that were present in good yield and were clearly homologous with peptides from rabbit troponin T. Five such peptides were isolated from both digests, and their composition is shown in Table 4. These peptides are in all respects identical when isolated from either protein. Of the other peptides isolated or partially purified, there was no indication of differences between the two proteins.

The amino acid sequence of each of the five peptides was determined, either directly by Edman degradation or, for peptides A2, A3 and A5, by further digestion with either thermolysin or the V8 proteinase. Amides were assigned either from the mobility of the peptide at pH 6.5 or, for peptides A2Th4 and A5Th1, from the mobility of the remaining peptide after three and two steps respectively of the Edman degradation.

The sequences of the five peptides are shown in Fig. 4, together with differences from the sequence of rabbit troponin T. In addition to these peptides the dipeptide Trp-Lys, from the *C*-terminus of each protein, was isolated from the two digests. All these peptides can be positioned by homology with the rabbit sequence, and indeed three are identical and all of the four replacements are of a highly conservative nature. Assuming the chicken and rabbit sequences to be of equal length from residue 43 to the *C*-terminal end, a total of 48 out of 217 residues, i.e. 22%, have been placed in sequence. On this basis it seems likely that the two sequences are identical, at least from residue 43 to the *C*-terminal end, and are certainly identical from residues 44–62, which follow the *N*-terminal tryptic peptides immediately.

Table 4. *Amino acid composition of tryptic peptides from chicken breast- and leg-muscle troponin T*
The values are expressed as mol/mol of peptide. Mobilities were measured relative to aspartic acid = -1.0.

Peptide ...	A2		A3		A5		BN4		B7	
	Breast	Leg	Breast	Leg	Breast	Leg	Breast	Leg	Breast	Leg
Asp	3.0	3.0	—	—	4.0	3.7	—	—	—	—
Thr	—	—	—	—	—	—	—	—	0.8	1.0
Glu	3.0	3.3	4.0	4.0	1.3	1.1	1.2	1.2	—	—
Pro	1.2	1.1	—	—	0.9	1.0	—	—	1.1	1.0
Gly	1.0	1.1	—	—	—	—	—	—	—	—
Ala	—	—	1.2	1.1	—	—	1.1	1.1	1.0	1.2
Val	1.0	1.1	1.1	1.1	—	—	0.9	0.9	—	—
Ile	2.1	1.8	—	—	0.8	0.8	—	—	—	—
Leu	—	—	1.9	1.9	2.0	1.8	0.9	0.9	0.8	1.0
Phe	1.0	1.0	—	—	—	—	—	—	—	—
His	—	—	—	—	0.6	0.7	—	—	—	—
Lys	1.8	1.9	1.7	2.0	2.3	2.0	—	—	1.0	1.0
Arg	—	—	—	—	—	—	0.9	0.9	—	—
Mobility	-0.41	-0.40	-0.36	-0.36	0.19	0.20	0	0	0.40	0.41
Mol.wt.	1634	1634	1187	1187	1438	1438	587	587	529	529
No. of amide groups	1	1	0	0	2	2	0	0	0	0
<i>N</i> -Terminus	Ile	Ile	Lys	Lys	Lys	Lys	Val	Val	Leu	Leu

Fig. 4. Amino acid sequence of tryptic peptides from chicken breast- and leg-muscle troponin T

The numbering of the residues is that from the homologous peptides from rabbit troponin T. Residues in *italics* differ from the rabbit sequence, this being shown in the upper line. All other residues are identical. Th and V8 indicate peptides from thermolysin and V8-proteinase digests respectively. → indicates one step of the dansyl-Edman procedure.

Discussion

In general, vertebrate skeletal muscle may be classified as being composed of either fast or slow fibres [for reviews see Peachey (1968), Hess (1970) and Close (1972)]. The original classification relied on a correlation of the contractile response with morphological and histochemical differences (Hess, 1970) and with the specific adenosine triphosphatase activity of myosin (Barany *et al.*, 1965). More recent work has shown a correlation with differences in the myosin light chains (Sarkar *et al.*, 1971) and with

different forms of troponin I (Syska *et al.*, 1974). In the chicken most comparative work has been performed on the anterior latissimus dorsi and the posterior latissimus dorsi muscles, these being composed almost exclusively of slow and fast fibres respectively. Differences have been shown in mechanical properties and heat production (Canfield, 1971), in the myosin composition and Ca^{2+} -activated myosin adenosine triphosphatase activity (Syrový, 1973; Hoh *et al.*, 1976) and in the myosin light chains (Lowey & Risby, 1971). Differences have also been

shown between the Ca^{2+} -activated myosin adenosine triphosphatase of the red muscles of chicken leg, namely the adductor longus, quadriceps femoris and flexor digitus, and the breast muscle (Wu, 1969), but this difference was much less marked than that found between the two latissimus dorsi muscles by Syrový (1973); indeed, if myosin was prepared from whole leg muscles, no such distinction could be observed. This is in agreement with the observation of Lowey & Risby (1971) that myosin from breast and leg muscles has the same electrophoretic pattern of light chains as for the fast posterior latissimus dorsi muscle. The conclusion may be drawn from these results that the breast muscle and the majority of the leg muscles contain fast fibres, whereas a small percentage of leg muscles contain a mixture of fast and slow fibres.

The results in the present paper for troponins I and C suggest that these components are identical in both breast and leg muscle, and this view is confirmed by the amino acid sequence of the two components (Wilkinson, 1976; Wilkinson & Grand, 1977). It is difficult to prove complete identity, but working with material from a mixture of both types of muscle a coherent sequence has been obtained for each protein, there being no positions at which more than one amino acid residue has been found. The sequences of the proteins are very similar to their counterparts from rabbit fast skeletal muscle, and this confirms the view, expressed above, that both breast and leg muscles are fast and are homologous to rabbit fast muscles. The minor precipitin band reported by Hirabayashi & Perry (1974) in their troponin I preparation may be due to a small amount of slow-muscle troponin I from the mixed and slow muscles of the leg, which would not be picked up by the techniques used in the present paper or by amino acid sequence determination, but which would be found by the more sensitive immunological techniques used by them.

The results of Perry & Cole (1974), showing that there are two forms of troponin T present in breast and leg muscle, have been confirmed. The localization of these two forms appears to be quite clear-cut, the purified preparations each containing only a single component. The two forms of troponin T differ in their amino acid sequence, the leg troponin T being similar in size and amino acid composition to rabbit troponin T, whereas the breast troponin T is larger.

The evidence presented here shows that the two forms of troponin T are in part identical and that those parts which have been sequenced are very similar to rabbit troponin T. The peptides that have been isolated are those which are particularly similar to the rabbit protein and therefore indicate the maximum homology, but it is probable that the chicken and rabbit troponin T components are at least as similar as are the other troponin components from the two

species. With the rabbit troponin T numbering system, the *N*-terminal tryptic peptide extends to residue 42. The sequence immediately *C*-terminal to this (residues 44–62) is identical in the two chicken proteins. The remaining peptides are distributed throughout the rest of the sequence and the *C*-terminal ends of the proteins have been shown to be identical. In addition to this, it has been shown that the sequence of the first six residues at the *N*-terminal ends are identical.

It will only be possible to draw definite conclusions about the relationship of the proteins when the complete sequences are known, but it seems probable that they are identical except for part of the region covered by the *N*-terminal tryptic peptides. The breast-muscle troponin T has an extra length of polypeptide chain of approx. 25 residues in this region, which contains a high proportion of histidine and alanine. In addition, the leg-muscle peptide contains one residue of tyrosine and the breast-muscle peptide does not. The two proteins must thus be products of different genes and are not merely formed by different routes of post-synthetic modification. The fact that both proteins have the same *N*-terminal sequence suggests that either an insertion or a deletion has occurred in the gene coding for troponin T, but the presence of tyrosine in the peptide from leg troponin T implies that if this is so then further mutations must have taken place since this event.

Frank & Weeds (1974) have shown that the two alkali light chains of rabbit fast-muscle myosin are related to each other in a somewhat similar way, in that the A1 light chain is 41 residues longer than the A2 light chain and that apart from the eight *N*-terminal residues of the A2 light chain the sequences are identical. They have shown that the two proteins are products of different genes, but the situation is different from the troponin T components in that the *N*-terminal sequences of the light chains are different. The two alkali light chains are present in the same muscle fibres in the rabbit, and the results of Lowey & Risby (1971) would indicate that this is also the situation in the chicken, whereas the two forms of troponin T in the chicken are clearly located in different muscles.

The only other species in which multiple forms of troponin T have been shown to occur in skeletal muscle is ox. Clarke *et al.* (1976) have demonstrated the occurrence of two forms that appear superficially to be similar to those found in the chicken. They suggested that the two forms might be correlated with fast- and slow-fibre types, but were not able to demonstrate this. Whether this is so or not, the two types appear to be present in the same muscle.

In chicken, fast-skeletal-muscle troponin I and troponin C are both the products of single genes, whereas there are clearly two genes, which are closely related, that can code for troponin T, and these two

genes are expressed in different fast-muscle tissues. Troponin T interacts with tropomyosin to localize the troponin complex on the thin filament and with troponin C to modify the Ca^{2+} -sensitivity of the contractile apparatus. The question of the functional difference between the two proteins, however, remains open, and it is not possible to say whether the presence of the larger protein in the flight muscle modifies either of these interactions in any way.

Dr. Peter Jackson is thanked for advice on the use of his method for carboxypeptidase digestion of proteins. I also thank Miss Susan Brewer for excellent technical assistance and the Medical Research Council for financial support.

References

- Barany, M., Barany, K., Reckard, T. & Volpe, A. (1965) *Arch. Biochem. Biophys.* **109**, 185–191
- Canfield, S. P. (1971) *J. Physiol. (London)* **219**, 281–302
- Clarke, F. M., Lovell, S. J., Masters, C. J. & Winzor, D. J. (1976) *Biochim. Biophys. Acta* **427**, 617–626
- Close, R. I. (1972) *Physiol. Rev.* **52**, 129–197
- Collins, J. H., Potter, J. D., Horn, M. J., Wilshire, G. & Jackman, N. (1973) *FEBS Lett.* **36**, 268–272
- Dixon, H. B. F. & Perham, R. N. (1968) *Biochem. J.* **109**, 312–314
- Frank, G. & Weeds, A. G. (1974) *Eur. J. Biochem.* **44**, 317–334
- Grand, R. J. A., Wilkinson, J. M. & Mole, L. E. (1976) *Biochem. J.* **159**, 633–641
- Greaser, M. L., Yamaguchi, M., Brekke, C., Potter, J. D. & Gergely, J. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 235–244
- Head, J. F. & Perry, S. V. (1974) *Biochem. J.* **137**, 145–154
- Head, J. F., Weeks, R. A. & Perry, S. V. (1977) *Biochem. J.* **161**, 465–471
- Hess, A. (1970) *Physiol. Rev.* **50**, 40–62
- Hirabayashi, T. & Perry, S. V. (1974) *Biochim. Biophys. Acta* **351**, 273–289
- Hitchcock, S. E. (1975) *Eur. J. Biochem.* **52**, 255–263
- Hoh, J. F. Y., McGrath, P. A. & White, R. I. (1976) *Biochem. J.* **157**, 87–95
- Jackson, P., Amphlett, G. W. & Perry, S. V. (1975) *Biochem. J.* **151**, 85–97
- Lowey, S. & Risby, D. (1971) *Nature (London)* **234**, 81–85
- Moir, A. J. G., Cole, H. A. & Perry, S. V. (1977) *Biochem. J.* **161**, 371–382
- Peachey, L. D. (1968) *Annu. Rev. Physiol.* **30**, 401–440
- Pearlstone, J. R., Carpenter, M. R., Johnson, P. & Smillie, L. B. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1902–1906
- Perry, S. V. & Cole, H. A. (1974) *Biochem. J.* **141**, 733–743
- Sarkar, S., Sreter, F. A. & Gergely, J. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 946–950
- Syrovy, I. (1973) *Int. J. Biochem.* **4**, 35–41
- 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
- Tsukui, R. & Ebashi, S. (1973) *J. Biochem. (Tokyo)* **73**, 1119–1121
- van Eerd, J.-P. & Takahashi, K. (1975) *Biochem. Biophys. Res. Commun.* **64**, 122–127
- Wilkinson, J. M. (1968) *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. & Grand, R. J. A. (1977) *Eur. J. Biochem.* in the press
- Wu, C.-S. C. (1969) *Biochemistry* **8**, 39–48